SUCCESSFUL IN VIVO AND EX VIVO TRANSFECTION OF PULMONARY ARTERY SEGMENTS IN LUNG ISOGRAFTS

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Objective: Gene transfer to lung grafts may be useful in ameliorating ischemia-reperfusion injury and rejection. Efficient gene transfection to the whole organ may prove problematic. Proximal pulmonary artery endothelial transfection might provide beneficial downstream effects on the whole graft. The aim of this study was to determine the feasibility of transfecting proximal pulmonary artery segments in lung isografts. **Methods:** Male Fischer rats were divided into six groups. **In vivo transfection:** In group I ($n = 7$), a proximal segment of the left pulmonary artery was isolated and injected with saline solution by means of a catheter inserted through the right ventricle. After an exposure period of 20 minutes, clamps were removed and blood flow was restored. In group II ($n = 7$), the isolated arterial segments were injected with adenovirus carrying the *Escherichia coli* LacZ gene encoding for β -galactosidase. **Ex vivo transfection:** In group III ($n = 5$), arterial segments were injected ex vivo with saline solution and in group IV ($n = 5$) with the adenovirus construct. In group V ($n = 6$), arteries were injected with saline solution and in group VI ($n = 11$) with liposome chloramphenicol acetyl transferase cDNA. In groups I to IV, animals were killed on postoperative day 3 and transgene expression was assessed by Blue-Gal staining. In groups V and VI, animals were killed on postoperative day 2 and transgene expression was assessed by chloramphenicol acetyl transferase activity assay. **Results:** Transgene expression was detected grossly and microscopically in endothelial and smooth muscle cells of pulmonary artery segments from all surviving animals of groups II and IV. In group VI, chloramphenicol acetyl transferase activity was significant in all assessed arterial segments. **Conclusion:** Significant transgene expression is observed in proximal pulmonary artery segments after both in vivo and ex vivo exposure. (J Thorac Cardiovasc Surg 1997;114:793-802)

Recent advances in gene therapy techniques have made possible the introduction of recombinant genes into mammalian somatic cells, opening the possibility of treating inherited and acquired dis-

eases at the genetic level. Currently, 107 human gene transfer clinical trials have been approved by the Recombinant DNA Advisory Committee of the National Institutes of Health.¹

Gene therapy has been tested in various transplant systems: heart,² liver,³ and kidney.⁴ Recently, we⁵ have demonstrated ex vivo gene transfer to whole lung isografts using an adenoviral vector. However, transgene expression was patchy and unpredictable. One possible explanation for this pattern of transgene expression is the huge surface area of the pulmonary microvasculature, which would alter the adenovirus/host cell ratio, an important factor for transduction efficiency. Thus we hypothesized that gene transfer to the proximal segment of the pulmonary artery could avoid this variable and allow a downstream beneficial effect to the whole graft. The purpose of the present study was to

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Table I. Total ischemic time and survival

Group	Total ischemic time (min)		Survival	
			Alive/total	(%)
I	27.1 ± 1.0	NS	5/7	71.4
II	28.9 ± 0.5		3/7	42.9
III	177.6 ± 4.6	NS	5/5	100.0
IV	193.4 ± 7.1		5/5	100.0
V	97.9 ± 5.6	NS	5/6	83.3
VI	110.0 ± 3.7		9/11	81.8

NS, Not significant.

demonstrate the feasibility of gene transfer to pulmonary artery segments using both adenoviral and liposomal vectors.

Materials and methods

Adenoviral vector. First-generation replication-deficient adenovirus serotype 5 carrying the *Escherichia coli* LacZ gene encoding for β -galactosidase and driven by the constitutive cytomegalovirus promoter (Ad5.CMV. β -gal) was used. The LacZ gene was chosen because β -galactosidase activity is easily measured in situ with reliable and sensitive histochemical assays that use chromogenic substrates. Ad5.CMV. β -gal, provided by Dr. Allan Schwartz (Children's Hospital, St. Louis, Mo.), was grown in the human embryonic kidney 293 cell line (American Type Culture Collection, Rockville, Md.). The virus was purified and the number of viral particles was assessed on the basis of the optical density at 260 nm ($1 \text{ OD}_{260} = 5 \times 10^{11}$ particles per milliliter). Purified virus aliquots were stored at -80°C in a buffered solution of 10% glycerol, $1 \times 10^{-3} \text{ mmol/L}$ MgCl_2 . The viability of adenoviral preparations was assayed by adenoviral transduction of 293 cells plated in six-well plates, using limiting dilution of adenovirus (10^{-3} to 10^{-12}) followed by in situ Blue-Gal staining 12 hours later. After an 18-hour staining period at 37°C , plates were examined with a light microscope for the presence of blue-stained cells, corresponding to transduced cells producing β -galactosidase. In the present study, the concentration of adenoviral vectors administered was 2 to 4×10^{12} viral particles per milliliter.

Cationic lipid. The plasmid pCF1-CAT (Genzyme Corporation, Framingham, Mass.) consists of the human cytomegalovirus immediate early gene promoter/enhancer, a hybrid intron, the chloramphenicol acetyl transferase (CAT) cDNA, the bovine growth hormone polyadenylation signal sequence, and the kanamycin resistance gene, as previously described.⁶ Lipid 67 (Genzyme Corporation) is an amphiphile consisting of a hydrophobic cholesterol lipid anchor linked to a spermine head group in a T-shape configuration and was used in a 1:2 molar ratio. Before use, dried lipid films were hydrated with sterile water, treated in a vortex, placed on ice for 10 minutes, then placed in a vortex again. Equal volumes of lipid 67/DOPE (L-dioleoyl phosphatidyl-ethanolamine)

and plasmid DNA 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.), weighing 250 to 290 gm, 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 "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).

In vivo gene transfer to pulmonary artery segments. Animals were divided into groups I ($n = 7$) and II ($n = 7$). All animals were anesthetized with an intramuscular injection of ketamine (30 mg/kg) and atropine sulfate (0.15 mg/kg). After intubation, the lungs of all animals were ventilated mechanically (model 683, Harvard Apparatus, Inc., South Natick, Mass.) (tidal volume 10 ml/kg, respiratory rate 60 breaths/min, positive end-expiratory pressure 1.0 cm H_2O), and anesthesia was maintained with 0.5% halothane. A left thoracotomy was performed through the third intercostal space. The left pulmonary artery was isolated from the hilum to the pulmonary trunk after excision of the left superior vena cava. A 24-gauge polyethylene catheter was inserted from the right ventricle into the left pulmonary artery, which was then ligated distally. The proximal end of the left pulmonary artery was ligated over the catheter just distal to the main pulmonary artery bifurcation, and blood was aspirated from the occluded segment of left pulmonary artery. In group I, 0.03 ml of 0.9% saline solution was injected into the isolated arterial segments. In group II, the arterial segment received 0.03 ml of the adenoviral solution (6 to 12×10^{10} viral particles). The catheter was removed immediately after injection, and right ventricular bleeding was controlled with compression. The isolated segment of pulmonary artery was distended by the injection and maintained this appearance during the 20-minute exposure period. This 20-minute exposure period was judged to be an acceptable period of warm ischemia. After this exposure period, clamps were removed and blood flow restored to the left lung. A thoracic tube was placed and the thorax closed. After spontaneous breathing resumed and the animal recovered from anesthesia, the thoracic tube and endotracheal tube were removed.

Ex vivo gene transfer to pulmonary artery segments. Orthotopic left lung transplantation was performed by means of a modification of the previously described "cuff technique."^{7,8} With 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 in groups III and IV. In groups V and VI, lungs were flushed with saline solution to avoid precipitation of cationic lipid. Heart-lung blocks were extracted and, with the use of the same procedure as in the in vivo study, 0.03 ml of saline solution in group III ($n = 5$), 0.03 ml of adenoviral solution (6 to 12×10^{10} viral particles) in group IV ($n = 5$), 0.03 ml of saline solution in group V ($n = 6$), and 0.03

*Tris dialysis buffer: 137 mmol/L NaCl; 5 mmol/L KCl; 25 mmol/L Tris; 0.7 mmol/L Na_2HPO_4 .

Fig. 1. Recombinant gene expression with Bluo-Gal staining on the endothelial surface. In vivo transfection (group II) (A) and ex vivo transfection (group IV) (B) are seen with blue spots through the microscope (original magnification $\times 100$).

ml of cationic lipid in group IV ($n = 11$) was injected into the isolated left pulmonary artery segments. After injection, lung grafts in groups III and IV were stored for 2 hours in 10°C low-potassium dextran/glucose solution. This exposure time and temperature were selected because our previous studies indicated that subsequent graft function would be acceptable and transgene expression would be achieved. The lung grafts in groups V and VI were stored for 1 hour in room temperature (23°C) saline solution. Room temperature was selected to avoid precipitation of cationic lipid. This 1-hour exposure period was set to avoid severe warm ischemia-reperfusion injury and to obtain efficient recombinant gene expression. Subsequently the ligature and distal clamp were removed from the pulmonary artery. A 14-gauge polyethylene catheter

cuff was attached to the proximal stump of the pulmonary artery, vein, and left bronchus. The donor left lung graft was implanted.

Bluo-Gal staining. All surviving animals in groups I to IV were killed 72 hours after reperfusion. Recombinant gene expression appeared 48 to 72 hours after exposure.^{9, 10} The native right and transplanted left lungs were harvested and stained with Bluo-Gal solution. Harvested heart-lung blocks were flushed through the pulmonary arterial trunk with 20 ml of phosphate-buffered saline solution (PBS), 20 ml of 2% paraformaldehyde solution with added 0.2% glutaraldehyde, and 0.02% nonionic detergent (Nonidet P-40 [octylphenol-ethylene oxide]) and stored in 2% paraformaldehyde solution for 20 minutes. After that, lungs were flushed with 20 ml of PBS,

Fig. 2. Recombinant gene expression with Blu-Gal staining on the pathologic specimens of a pulmonary artery segment. In vivo transfection (group II) (A) and ex vivo transfection (group IV) (B) are seen with blue spots on the endothelial cells and smooth muscle cells stained with nuclear fast red stain (original magnification $\times 400$).

followed by a flush of 10 ml of Blu-Gal, and finally were immersed in Blu-Gal buffer for 3 hours at 37° C. The Blu-Gal buffer was prepared by mixing 1 mg/ml Blu-Gal (5-bromo-indolyl- β -o-galactopyranoside; Gibco BRL, Gaithersburg, Md.), 5 mmol/L $K_3Fe^{III}(CN)_6$, 5 mmol/L $K_4Fe^{II}(CN)_6$, 2 mmol/L $MgCl_2$, 0.1% Nonidet P-40, and PBS. After another flushing with 20 ml PBS, lungs were fixed with 4% paraformaldehyde. Histologic sections were stained with nuclear fast red dye.

CAT activity assay. All survival animals in groups V ($n = 5$) and VI ($n = 9$) were killed 48 hours after reperfusion. In all surviving animals in group V and five animals in group VI, CAT activity was assessed. Trans-

gene expression was detected by a CAT activity assay as described elsewhere.¹¹ In brief, after tissue homogenization and dilution in tromethamine-ethylenediaminetetraacetic acid, three consecutive freeze/thaw cycles were performed. After incubation at 65° C, samples were centrifuged at 10,000 rpm; then the supernatant was recovered and a quantitative spectrophotometric analysis was performed. Protein extract, 300 μ g, was incubated overnight at 37° C with 40 μ l of acetyl coenzyme A and 8 μ l of ^{14}C chloramphenicol; then ethyl acetate was added. The samples were placed in a vortex, centrifuged at 14,000 rpm, and the supernatant was recovered. This was nitrogen-dried and then resuspended in ethyl acetate. Thin-

Fig. 3. CAT activity in groups V and VI. In the lipid group (VI), strong and consistent expression of CAT is apparent. *di-AC*, Diacetylated; *mono-AC*, monoacetylated; *non-AC*, nonacetylated.

layer chromatography was followed by overnight autoradiography. In the presence of functional CAT enzyme, both monoacetylated and diacetylated forms of chloramphenicol are produced, which are distinct from the non-acetylated chloramphenicol by thin-layer chromatography.

In situ hybridization. In remaining survival animals ($n = 4$) in group VI, in situ hybridization was performed with sense and antisense ^{35}S -radiolabeled cRNA for CAT as previously described.¹² In brief, ^{35}S -labeled RNA probes were transcribed in vitro from cDNA probes for the CAT gene with the use of ^{35}S -uridine triphosphate. Sections of paraffin-embedded lung tissue were pretreated with nuclease-free proteinase K and washed in triethanolamine buffer containing 0.25% acetic anhydride. Hybridization solution containing 2.5×10^5 cpm of ^{35}S -labeled probes was added and incubated with RNase and incubated overnight. This was washed extensively under stringent conditions and then incubated with RNase A to remove unhybridized probe. Washed slides then were processed by autoradiography.

Graft function. In groups III to VI, the right (contralateral) hilum was clamped immediately before the animal was killed. Isolated function of the left lung isograft 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 H_2O).

Histology of graft lungs. In all surviving animals of groups I to IV, after fixation with 2% paraformaldehyde solution, histologic sections of lung grafts were stained with hematoxylin and eosin in addition to nuclear fast red stain. In all surviving animals of groups V and VI, lung grafts were fixed with 10% formalin and stained with hematoxylin and eosin.

Statistical analysis. All values are presented as the mean $\pm$ standard error of the mean. Unpaired two-group t test and Mann-Whitney test were used to compare differences of total ischemic time and arterial oxygen tension between corresponding groups. Differences were considered significant when $p < 0.05$.

Results

The ischemic times and survivals for each group are shown in Table I. There were no significant

differences between control groups and corresponding transfection groups. Five of seven animals in group I and three of seven animals in group II survived until they were killed at 72 hours. In groups III and IV, all animals survived 72 hours. Five of six animals in group V and nine of 11 animals in group VI survived for assessment at 48 hours.

Recombinant gene expression. All surviving animals in group II and all animals in group IV showed multiple blue spots on the endothelial surface of the left pulmonary artery as recombinant gene expression (Fig. 1). No blue spots appeared on the endothelial surface of the contralateral right pulmonary artery in groups II and IV. In groups I and III, blue spots did not appear on the endothelial surface of either pulmonary artery.

Microscopically, transgene expression was observed as the blue granular spots in groups II and IV in endothelial cells and smooth muscle cells located near the lumen (Fig. 2). Transgene expression was not observed in either lung or the right pulmonary artery in any of the groups.

CAT activity. In all five group VI animals, transgene expression was detected in pulmonary artery segments submitted for CAT activity assay. Each pulmonary artery segment showed high levels of CAT activity (Fig. 3). The intensity of CAT activity was similar in all animals.

In situ hybridization. In the four group VI animals, pulmonary artery segments were submitted for in situ hybridization. CAT gene was detected with concentration of positive signals around endothelial cells (Fig. 4).

Arterial blood gas analysis. Mean arterial oxygen tension in groups III to VI is shown in Fig. 5. There were no significant differences between groups III and IV (348.1 ± 78.3 vs 367.6 ± 85.9 mm Hg) and between groups V and VI (317.1 ± 45.2 vs 266.0 ± 69.2 mm Hg).

Fig. 4. In situ hybridization in group VI. CAT gene was detected with concentration of positive signals around endothelial cells using both bright-field microscopy (A) and dark-field microscopy with reflected polarized light (B) (original magnifications $\times 200$).

Histology. In groups I and II, histologic specimens of the lung graft showed severe edema of alveolar wall and neutrophil infiltration. Similar infiltration was seen in specimens of groups V and VI. The infiltration in groups III and IV was much less significant than in other groups. There were no significant differences between control groups and corresponding transfection groups in the pathologic specimens of the lungs and pulmonary arteries.

Discussion

Ischemia-reperfusion injury and rejection remain major obstacles to successful transplantation.¹³ Gene transfer to the lung graft has the potential to reduce these major problems. We⁵ have previously demonstrated whole lung graft transduction using an adenoviral vector. However, the method was inefficient.

Transfection of a proximal segment of the pulmonary artery offers certain advantages. First, in the in

vivo setting, it is possible to confine the vector-DNA complexes to the isolated arterial segments and not expose other organs to a gene that is not necessary. In addition, in cases of functional gene transfection, the secreted protein may have beneficial downstream effects to the whole graft. Finally, with high construct to endothelial cell ratios and ideal storage times, transgene expression efficiency might be maximized.

In the present study, we tested both adenoviral and liposomal vectors. Adenoviral vectors have some advantages.^{1,14} They can accept relatively large pieces of exogenous DNA and do not require replicating cells to introduce their recombinant gene as retroviruses. Furthermore, they can be rendered replication defective by deletion of the E1 genomic region and can be produced at high titers. Nevertheless, viral vectors can trigger a host immune-inflammatory response, thereby limiting their utility. Using second-generation adenovirus vectors from which the E1 and E4 genomic regions have been deleted minimizes their pathogenicity.¹⁵ Liposomes have been regarded as less efficient delivery systems. Despite this perception, we¹⁶ have recently demonstrated consistent and reproducible transgene expression when a liposomal vector was used to transfect whole lung grafts in vivo and ex vivo. In addition, liposomes, unlike viral vectors, have no replication risk and do not activate the host immune-inflammatory response, as occurs with viral vectors.

In vivo adenovirus-mediated gene transfer to segments of pulmonary arteries was achieved in all animals that survived the operative-transfection procedure. However, the survivals of groups I and II were unexpectedly low. These could be explained by the warm ischemic injury to which lungs were subjected when the arterial segments were isolated for about 30 minutes. Also, bleeding from the insertion point of the right ventricular catheter might have contributed to the high mortality in these groups. Histologically, lungs in these groups showed severe edema in the alveolar wall and leukocyte infiltration into alveolar spaces characteristic of reperfusion injury. There were no histologic differences between the adenoviral transduced group and the control group that received only saline solution.

In the ex vivo adenovirus-mediated gene transfer group, recombinant gene expression was detected in all cases. There were no deaths. The ex vivo approach allows manipulation of gene transfer conditions

Fig. 5. Arterial oxygen tension in groups III to VI. There are no differences between corresponding groups. *NS*, Not significant.

such as temperature and exposure time. In these experiments the cold storage time was 2 hours. Nonetheless, there was no severe ischemia-reperfusion injury as was observed in the in vivo group. Arterial oxygenation levels were high in the ex vivo group. There were no differences between adenoviral transduced grafts and controls with respect to gas exchange or histologic appearance.

In lungs transduced with the adenoviral vector (groups II and IV), recombinant gene expression was assessed by Blue-Gal staining. Blue-Gal was selected because it is more stable than X-Gal (5-bromo-4-chloro-3-indolyl-d-galactosidase) in that the blue color of transduced cells does not fade during the staining process of paraffin-embedded histologic sections.¹⁷ Many blue spots were observed on the endothelial surface of the pulmonary artery segments. Histologically, transgene expression was detected in endothelial and smooth muscle cells located near the endothelial layer, demonstrating that the adenovirus could traverse the endothelium and transduce the smooth muscle cells. However, in comparison with the gross appearance of the endothelial surface before histologic preparation, the blue spots seemed to decrease. It is possible that during the histologic staining process some spots faded. Although recombinant gene expression was easily assessed by Blue-Gal staining, the staining was heterogeneous even in this study, and gene expression was not quantified. It is possible to count the stained cells microscopically per high-power field, but the number of blue-stained cells did not correspond to what was observed grossly.

Although it is difficult to compare in vivo and ex vivo studies, 20 minutes' incubation at body temperature in vivo nearly equaled 2 hours of ex vivo incubation at 10° C. The low temperature (10° C) did not seem to have much effect on adenoviral infectivity.

In the ex vivo liposome-mediated gene transfer group (group VI), significant and consistent recombinant gene expression was observed in all arterial segments assessed by either thin-layer chromatography or in situ hybridization. In this group, transfection was processed at room temperature to avoid precipitation of liposome. Histologically, the left lungs of groups V and VI showed severe neutrophil infiltration, perhaps because of the increased storage temperature (23° C). In the histologic sections assessed by in situ hybridization, many endothelial cells have been peeled off. It is difficult to define which process caused that. However, it might concern room temperature storage.

In the present study, complete segmental occlusion was possible in the in vivo and ex vivo studies. The pulmonary arteries easily expanded. This pressure seemed to result in satisfactory transfection even to smooth muscle cells. Inasmuch as the pulmonary artery endothelium is surrounded by a thick smooth muscle layer, leakage of the adenoviral vector to the systemic or lymphatic circulation does not seem to occur, contrary to what has been demonstrated when the adenoviral vector is infused into the pulmonary microvasculature.⁵

Our ultimate goal is to use gene therapy to target two important problems that occur after lung transplantation: ischemia-reperfusion injury and rejection. Gene transfer with plasmid DNA encoding transforming growth factor- β_1 prolonged allograft survival in a mouse cardiac transplantation model.¹⁸ Alloreactivity has been suppressed with adenovirus-mediated gene transfer expressing viral interleukin-10 using an in vivo rat liver transplantation model.¹⁹ In vitro, retrovirus-mediated superoxide dismutase cDNA transfer has been reported to prevent ischemia-reperfusion injury.²⁰ Likewise, inducible nitric oxide synthase gene transfer is possible²¹ and may improve graft function, as we have previously demonstrated the effects of inhaled nitric oxide^{22, 23} and nitroprusside²⁴ after canine lung transplantation. Gene transfer to segments of pulmonary artery by means of functional genes may be useful in reducing ischemia-reperfusion injury and rejection.

In conclusion, we have demonstrated the feasibility

of successful gene transfer to rat pulmonary arterial segments in vivo and ex vivo.

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Discussion

Dr. Larry R. Kaiser (*Philadelphia, Pa.*). Do you have an idea of the multiplicity of infection that you would have used with your adenoviral construct? It looked like a fairly high dose of adenovirus. Also, did you look at different doses of adenovirus to see whether you could influence the efficiency of gene transfer?

Can you offer some idea of the efficiency of gene transfer between liposomal versus adenoviral transfection? Also, would you comment on the effect of temperature on adenoviral uptake and protein synthesis? It seems to me that at these lower temperatures, especially with adenovirus, the uptake into cells might be affected, and then at those lower temperatures, there might be an effect on protein synthesis. This has an impact on the use of strategies like this for transplantation as you start to look at more therapeutic sorts of vectors when you need to have efficient protein synthesis.

In the in situ photograph, was that the sense construct control or was that just a histologic section? It looked like the histologic section. I assume you have the sense control also and have some idea about background levels.

This is an exciting strategy. It is clearly going to have an impact on the modification of transplant rejection and ischemia-reperfusion.

Dr. Yano. Thank you, Dr. Kaiser. We did not assess variable doses of viral vector. However, I am sure that at higher doses of adenoviral vector, transfection will increase. In comparing the effect of the adenoviral and liposomal vectors, my impression is that the adenoviral

vector is more effective. We did not assess variable temperature or time of exposure.

Dr. Kaiser. You think the adenoviral vector was more effective than your liposomes?

Dr. Yano. Yes, because if I used the adenoviral vector in my studies, not only endothelial cells but also smooth muscle cells were transfected.

Dr. Casey W. Daggett (*Durham, N.C.*). What are your thoughts about the mechanisms of the loss of expression of the gene product? Also, we have noticed in some of our models that a lot of the adenovirus is taken up in the livers of these animals. Have you found similar results?

Dr. Patterson. No. This was done as a preliminary series of experiments to see whether it was possible to achieve focal in vivo or ex vivo transfection.

Dr. Alan P. Kypson (*Durham, N.C.*). Was there a reason that you chose a 20-minute exposure time? Have you evaluated the length of time that the adenoviral or the liposomal solution sits within the pulmonary artery segment? Can you explain why you think there is a focal overexpression? Have you looked at any other segments of the pulmonary artery further distally?

Dr. Patterson. No, we just examined the pulmonary artery segments themselves. We did not conduct the same analysis of the pulmonary parenchyma. The administered dose is so small to that tiny segment of pulmonary artery that one would not expect to see any expression distally.

Dr. Yano. In the in vivo experiments we chose 20 minutes because we believed that would be the maximal tolerable time of warm ischemia. A 5-minute exposure time is not enough to get a good expression.

Dr. Kypson. Do you think there was a reason for the 50% mortality?

Dr. Yano. In the in vivo experiments, the survival is approximately 50%. This is presumably due to the technical difficulty including the cardiac puncture in addition to the effects of warm ischemic injury.

Dr. Kaiser. Dr. Patterson, do you have any comments about the effect of temperature in the system? I think it is going to become important as you start looking at ways of expanding this model, and you still want to maintain the cold ischemia, especially if you are using a viral product.

Dr. Patterson. We know that lungs can be preserved for several hours at 10° or 15° C and still have good graft function. In fact, some believe that better graft function can be obtained at modest hypothermia. Yet those temperatures are high enough to measure gene expression. Now, whether that is enough expression or efficient enough a transfer to get a functional effect, I do not know. It seems that the higher the temperature up to room temperature, the better the expression.

Dr. Jhngook Kim (*Seoul, Korea*). I want to ask about the strategy. If we apply the medication to the isograft, we can apply the medication directly. That is, if we want to have only increased angiogenicity, we can apply the vascular endothelial growth factor to the pulmonary artery directly. Otherwise, if we want to make a prolonged expression of vascular endothelial growth factor, we should use the regular different types of the vector, such as retrovirus. Otherwise, if we use the adenoviral vector system, there will be just a brief expression of that kind of expected expression. Therefore I prefer to

use the retroviral vector system for the prolonged production.

Our other method is to manage the endothelium in vitro and then make a patch with that endothelialized graft, the interposition graft, in the animal experiments. With that kind of strategy, we can make a prolonged expression of the expected gene and easily control the level of the expression.

Dr. Altorki. I believe the questioner wants to know why you want to use adenovirus versus retrovirus.

Dr. Yano. In this study, retrovirus is not suitable for use because the endothelium is not a rapidly dividing cell

population. Furthermore, short-term expression may be an advantage in this situation.

Dr. Kaiser. I would echo that point. This strategy might be very useful if one is looking for short-term gene expression. Retroviruses create a significant number of problems, and perhaps adeno-associated virus may even be the better strategy. For that matter, liposomal gene transfers also could be given repeatedly without any sort of immune response. Many different strategies are available.

Dr. Kim. If so, use of the end product of the gene, like the protein, would be an easier way to obtain that kind of effect.