CARDIAC AND PULMONARY REPLACEMENT

LIPOSOME-MEDIATED GENE TRANSFER TO LUNG ISOGRAFTS

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Objectives: Our objectives were to determine the feasibility, efficacy, and safety of in vivo and ex vivo liposome-mediated gene transfer to lung isografts. **Methods:** Fischer rats were divided into three main groups: (1) *Nontransplant setting:* Liposome-chloramphenicol acetyl transferase cDNA was intravenously injected, and lungs were harvested at different time points: 2, 6, 12, and 24 hours; 2, 5, 8, and 21 days ($n = 3$). Chloramphenicol acetyl transferase activity was determined in lungs, hearts, livers, and kidneys. The distribution and type of transfected cells were evaluated by in situ hybridization. Lung toxicity was assessed by arterial oxygen tension, histology, and tumor necrosis factor- α levels. (2) *In vivo graft transfection:* Left lungs were transplanted 6 hours, 4 hours, and 15 minutes after intravenous injection and were assessed for chloramphenicol acetyl transferase activity and arterial oxygen tension on postoperative day 2. (3) *Ex vivo graft transfection:* Grafts were infused ex vivo with either 660 μg ($n = 3$) or 330 μg ($n = 3$) of DNA complexed to liposomes and stored at 10° C for 4 hours. Chloramphenicol acetyl transferase activity was assessed 44 hours after transplantation. **Results:** Transgene expression was detected in endothelial cells, macrophages, and interstitial cells. Chloramphenicol acetyl transferase activity was present as early as 2 hours, increased significantly between 6 hours and 8 days, and then decreased to minimal levels by 21 days. Chloramphenicol acetyl transferase activity was greatest in donor lungs and hearts and minimal in livers and kidneys. Arterial oxygen tension was normal in treated animals. Inflammation was minimal, and tumor necrosis factor- α levels increased only sevenfold in treated animals. **Conclusion:** In vivo and ex vivo liposome-mediated gene transfer to lung isografts allows significant transgene expression with minimal effects on graft function. (J Thorac Cardiovasc Surg 1997;114:783-92)

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Supported by the National Institutes of Health grants 1 R01 HL-41281 and HL-29594 and Alan A. and Edith Wolff Charitable Trust.

Read at the Seventy-seventh Annual Meeting of The American Association for Thoracic Surgery, Washington, D.C., May 4-7, 1997.

Received for publication March 25, 1997; revisions requested May 15, 1997; revisions received June 18, 1997; accepted for publication June 19, 1997.

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0022-5223/97 \$5.00 + 0 12/6/84276

Lung transplantation has become an effective therapeutic option for the treatment of a variety of end-stage pulmonary disorders. At present, ischemia-reperfusion injury and infection are the most common causes of early morbidity and mortality, whereas chronic rejection accounts for the majority of late mortality. Modifications of donated organs before implantation, by means of gene therapy techniques, may improve graft function and overall survival.

Several prerequisites must be met for gene therapy to be clinically useful in the setting of organ transplantation: (1) Efficient and reproducible transgene expression in the graft should be present; (2) graft toxicity should be avoided to minimize

further worsening of the transient functional impairment that is inherent to the transplant procedure itself; (3) transgene expression should be homogeneously distributed throughout the graft parenchyma; (4) organ specificity should be achieved because multiple cadaveric organ recovery is routine; and (5) for the prevention of ischemia-reperfusion injury, transgene expression should be present before reperfusion of the graft in the recipient. Although transient expression is regarded as one of the drawbacks of current gene delivery systems, this actually is desirable in the treatment of acute, self-limited conditions such as ischemia-reperfusion injury.

A variety of delivery systems, both viral and nonviral, have been used.¹ Retroviruses require active cell division to integrate the transferred DNA into the host genome and initiate transcription. Because this insertion occurs randomly in the genome, the potential for cancerous mutagenesis exists.² Further, retroviral vectors are not suitable for lung or heart tissues, because cell turnover is slow in these organs.

Adenoviruses, rendered replication deficient by deletion of the E1 region, can be produced in high titers and are capable of transfecting a wide variety of dividing and resting cells with good efficiency.³ They do not integrate into the host genome and therefore have little chance to activate a dormant oncogene or interrupt a tumor suppressor gene.⁴ The major limitation of current adenoviral vectors is the resultant host inflammatory and immune response, leading to the destruction of transduced cells.⁵⁻⁷ Another limitation is the potential for the production of replication-competent viral strains through homologous recombination, which can have serious consequences in the setting of immunosuppression and transplantation.

Because of limitations with viral vectors, attempts are underway to develop efficient nonviral alternative delivery systems. Cationic liposomes consist of a positively charged lipid mixed with a neutral lipid such as 1,3-dioleoyl phosphatidyl-ethanolamine (DOPE) that forms complexes with plasmid DNA and facilitates its transport into the cell. Such liposomes can introduce DNA into replicating and nonreplicating cells with little toxicity.⁷ Because they do not incite a significant host immune response, repeat administration is possible. The administered plasmid DNA remains extrachromosomal and results in transient gene expression, similar to adenoviral vectors.⁸ There are also no apparent restrictions on the size of the transferred genes.

The aim of this study was to achieve transgene

expression in transplanted lung grafts after in vivo and ex vivo administration of cationic lipids complexed to a reporter gene. The time course of gene expression, distribution, and type of transfected cells and the effects of the transplant procedure on transgene expression were examined.

Materials and methods

Plasmid expression vector. 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.⁹

Preparation of cationic lipid/DNA complexes. Lipid 67 (Genzyme) is an amphiphile consisting of a hydrophobic cholesterol lipid anchor linked to a spermine head group in a T-shaped configuration.⁹ Lipid 67 was mixed with DOPE in a 1:2 molar ratio as previously described.⁹ Before use, the dried lipid films were hydrated with sterile water, mixed with an equal volume of plasmid DNA, and allowed to sit at room temperature for a minimum of 15 minutes. Final concentrations were 1 mmol/L for the cationic lipid mixture and 4 mmol/L for the plasmid DNA. Immediately before injection in animals, the resulting complex was diluted with an equal volume of normal saline solution.

Animals. Inbred male Fischer rats weighing 270 to 300 gm (Charles River Laboratories, Wilmington, Mass.) were used in all experiments. All 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 Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Nontransplant setting: In vivo transfection of normal lungs. The first group of experiments aimed to determine (1) the feasibility of in vivo gene transfer to donor lungs by way of the intravenous route, (2) the rapidity of transgene expression, (3) the duration of transgene expression, (4) the degree of vector-related lung toxicity, and (5) the extent of extrapulmonary transgene expression.

Rats were premedicated with inhaled halothane and a subcutaneous injection of ketamine chloride (25 mg/kg) and atropine sulfate (0.25 mg/kg). After endotracheal intubation with a 14-gauge catheter, animals' lungs were mechanically ventilated with a small-animal Harvard ventilator (Harvard Apparatus Co., Inc., South Natick, Mass.) (tidal volume 3 ml, respiratory rate 60 breaths/min) with 0.5% halothane and 99.5% oxygen. A small incision was made in the left lower part of the neck and the left external jugular vein was mobilized. The liposome-CAT complex (1320 μ g of DNA) was injected intravenously over a period of 3 to 4 minutes. The incision was then closed and the animals allowed to recover from anesthesia.

Animals were divided into eight groups ($n = 3$) and killed at various times after intravenous injection: 2, 6, 12, and 24 hours and 2, 5, 8, and 21 days. In addition, four animals were killed at 6 months. Before they were put to death, arterial oxygenation was measured with both lungs ventilated at an inspired oxygen fraction of 100% for a minimum of 5 minutes. At the time of harvest, both lungs were flushed with normal saline solution via the main pulmonary artery. The right lung, heart, liver, and kidneys were harvested, snap-frozen in liquid nitrogen, and stored at -80°C for assessment of CAT activity and tumor necrosis factor- α (TNF- α) levels. The left lung was fixed in 10% buffered formalin for histologic examination.

Transplant setting: In vivo graft transfection. The second group of experiments studied the influence of the transplant procedure on transgene expression. Donor animals were treated with an intravenous injection of the liposome-CAT complex as described earlier and were divided into six groups. In groups I, II, and III, the left lungs were harvested 6 hours, 4 hours, and 15 minutes after intravenous administration, respectively, and then transplanted. Recipient animals were killed on postoperative day 2. After a normal saline solution flush, the harvested transplanted lung grafts were snap-frozen in liquid nitrogen and stored at -80°C for later assessment of CAT activity. In groups IV, V, and VI, lungs were not transplanted. These groups served as controls and consisted of transfected left lungs that were harvested 6 hours (group IV), 4 hours (group V), and 48 hours (group VI) after intravenous administration of liposome-DNA complexes.

Transplant setting: Ex vivo graft transfection. The third group of experiments studied the feasibility of ex vivo liposome-mediated gene transfer to lung isografts. Left lungs were harvested and divided into two groups. In group I ($n = 3$), 330 μg of CAT cDNA:lipid 67:DOPE (0.25 ml of the original preparation) was diluted in 10 ml of 0.9% saline solution and infused into the left lung via the left pulmonary vein over a 7- to 10-minute period. Grafts were stored at 10°C for 4 hours and then implanted. In group II ($n = 3$), grafts underwent the same procedure but received 660 μg of CAT cDNA:lipid 67:DOPE (0.5 ml of the original preparation).

Rat lung transplantation. A rat orthotopic left lung transplant model (Fischer to Fischer) was developed with the use of a modification of the cuff technique,¹⁰ as described elsewhere.¹¹ In brief, donors were anesthetized, intubated, and heparinized and then underwent a median sternotomy-laparotomy. The abdominal aorta, inferior vena cava, and the left atrial appendage were incised, and the lungs were flushed through the main pulmonary artery with cold low-potassium dextran/1% glucose solution. After the heart-lung block was excised with the lungs inflated at end-tidal volume, the left lung was dissected away. In the case of ex vivo transfection, a second flush of normal saline solution containing the CAT gene was performed at this point, as described earlier. After this, 14-gauge Teflon cuffs were inserted into the left pulmonary artery and vein. The left lung was then stored in low-potassium dextran/glucose solution at 4°C (or at 10°C in the case of ex vivo transfection) until implantation.

Recipient rats were anesthetized, intubated, and subjected to a left thoracotomy. The left main bronchus was ligated distally and the pulmonary vessels were cross-clamped proximally. The donor lung vessels were then anastomosed to the corresponding recipient vessels by means of the cuff technique. The bronchial anastomosis was performed with a running 9-0 Prolene suture (Ethicon, Inc., Somerville, N.J.). Ventilation and perfusion to the graft were restored and a chest tube was inserted temporarily and then removed after the return of spontaneous respirations.

CAT activity assay. Transgene expression was detected by a CAT activity assay as described elsewhere.¹² In brief, after tissue homogenization and dilution in Tris-ethylenediamine tetraacetic acid, three consecutive freeze/thaw cycles were performed. After incubation at 65°C , samples were centrifuged at 10,000 rpm, and then the supernatant was recovered and a quantitative spectrophotometric protein analysis was performed. Protein extract, 300 μg , was incubated overnight at 37°C with 40 μl of acetyl coenzyme A (5 mg/ml) and 8 μl of ^{14}C -chloramphenicol. Ethyl acetate was added, the samples were placed in a vortex, centrifuged at 14,000 rpm, and the organic supernatant was recovered. This was nitrogen-dried and then resuspended in ethyl acetate. Thin-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 nonacetylated chloramphenicol.

In situ hybridization and immunohistochemistry. In situ hybridization was performed with sense and antisense ^{35}S -radiolabeled cRNA for CAT as previously described.¹³

Immunohistochemistry with the ED1 antibody, which is specific to rat macrophages, was performed as previously described.¹⁴ A double-label study (immunohistochemistry-in situ hybridization) was performed to confirm that macrophages expressed the CAT gene.

Evaluation of pulmonary toxicity. The resulting host inflammatory response was evaluated by measurement of TNF- α levels in lung tissue from treated and untreated animals, using an enzyme-linked immunosorbent assay kit (Interstest, Genzyme).

The inflammatory infiltrate was also evaluated by examining thin sections of lung tissue stained with hematoxylin and eosin. In addition, macrophages (ED1-positive cells) were counted both in intravenously transfected lungs ($n = 3$) and in normal controls ($n = 3$). Ten high-power fields ($100\times$) for each sample were analyzed and averaged.

Lung gas exchange was determined by measurement of arterial oxygen tension (Pao_2). In brief, the right pulmonary artery and right main-stem bronchus of transplant recipients were clamped and the left lung was ventilated for 5 minutes at a 100% inspired oxygen fraction, a tidal volume of 2 ml, and a respiratory rate of 80 breaths/min. An arterial blood sample was obtained from the abdominal aorta. In the nontransplant setting, arterial oxygenation was determined while both lungs were ventilated.

Statistical analysis. Data were compared by one-way analysis of variance or unpaired t test, as applicable, by means of the software SYSTAT 6.0 for Windows

Fig. 1. Lung CAT activity at different times after intravenous administration. Each *column* corresponds to one animal. The *bottom row* represents nonacetylated chloramphenicol. The *middle* and *top rows* represent monoacetylated and diacetylated chloramphenicol, respectively. **A**, CAT activity is maximal as early as 6 hours after intravenous infusion. One animal in the 12-hour group lost the intravenous injection because of a technical failure. **B**, CAT activity is high for at least 8 days.

(SYSTAT Inc., Evanston, Ill.). Data were expressed as mean $\pm$ standard error, with a p value < 0.05 considered statistically significant.

Results

All animals survived the operative procedures. Transgene expression was present in all animals treated with the lipid 67:DOPE:pCF1-CAT complex, except in one animal of the 12-hour group in which the injection was missed because of a technical failure.

Onset and duration of transgene expression. In the nontransplant setting, transgene expression was detectable at low levels as early as 2 hours after intravenous administration. Higher expression was detected at 6 hours and continued until 8 days. By 21 days, transgene expression declined to minimal levels (Fig. 1, *A* and *B*).

Distribution of transfected cells. In situ hybridization revealed homogeneous and widespread distribution of transgene expression throughout the

lung parenchyma. The majority of transfected cells were in the pulmonary interstitium (Fig. 2). Cells lining the lumen of the larger pulmonary vessels, presumably endothelial cells based on their location, also expressed CAT mRNA (Fig. 2). Macrophages also displayed CAT expression, which was confirmed by double-label studies (immunohistochemistry-in situ hybridization). Transgene expression was not observed in airway epithelial cells lining bronchi and bronchioles.

Pulmonary toxicity of lipid 67:DOPE:pCF1-CAT.

In the nontransplant setting, hematoxylin-eosin staining revealed only a minimal inflammatory infiltrate present in the lungs of treated animals. An average of 4.3 ± 0.71 macrophages per high-power field was noted in control nontransfected lungs compared with 9.1 ± 1.18 macrophages per high-power field in transfected lungs harvested 48 hours after intravenous transfection ($p = 0.04$, unpaired t test).

Arterial oxygenation showed no differences between transfected and nontransfected animals as

Distribution of CAT Gene Expression

Fig. 2. In situ hybridization performed with an ^{35}S -radiolabeled CAT antisense cRNA probe. **A**, CAT expression occurred widely throughout the pulmonary interstitium, 48 hours after intravenous administration. **B** and **C**, CAT expression was also detected in cells lining the lumen of pulmonary vessels, presumably endothelial cells.

demonstrated by Pao_2 s obtained 2 days, 21 days, and 6 months after injection and from nontransfected normal controls ($p = 0.08$, one-way analysis of variance, Table I).

$\text{TNF-}\alpha$, a proinflammatory cytokine, increased sevenfold in transfected nontransplanted lungs compared with nontransfected normal lungs (74 ± 24 vs 10.7 ± 2 , respectively, $n = 3$ per group).

Extrapulmonary transgene expression. CAT activity was measured in several donor organs including the heart, liver, and kidneys in the setting of intravenous administration followed by organ harvest without transplantation. Heart tissue showed levels of CAT activity comparable with that in the lungs (Fig. 3). CAT gene expression was high in samples obtained separately from the free walls of the right and left ventricles. CAT activity was minimal in livers and negligible in kidneys (Fig. 3).

In vivo graft transfection. CAT activity in lung grafts transfected in vivo for 4 and 6 hours, transplanted, and assessed on postoperative day 2 was similar to CAT activity in nontransplanted lungs transfected in vivo for 48 hours (Fig. 4). Transgene expression was minimal in lungs transfected in vivo only 15 minutes before harvest, transplanted, and assessed on postoperative day 2.

Table I. Arterial oxygenation in transfected animals versus nontransfected normal controls

Time of assessment following liposome-DNA intravenous administration	Pao_2 (mm Hg)
2 days ($n = 10$)	545 ± 15.7
21 days ($n = 4$)	468.1 ± 36.3
6 months ($n = 4$)	572.8 ± 36.1
Normal controls ($n = 5$)	523.5 ± 18.4

p Value = 0.08, one-way analysis of variance.

There were no differences ($p = 0.99$, one-way analysis of variance) in arterial oxygenation at 48 hours in any of these groups ($\text{Pao}_2 = 523.5 \pm 18.4$ mm Hg, 521.1 ± 40.8 mm Hg, 529.3 ± 11 mm Hg, and 537.2 ± 71.2 mm Hg, obtained from nontransfected normal controls and animals transfected 6 hours, 4 hours, and 15 minutes before transplantation, respectively).

Ex vivo graft transfection. In this setting, all lung grafts in groups I and II showed transgene expression (Fig. 5). There was no impairment of graft function when the lipid-DNA dose was increased to $660 \mu\text{g}$ of DNA:lipid 67:DOPE (0.5 ml of the original solution), as demonstrated by the Pao_2 data (387.9 ± 6.8 mm Hg and 374.7 ± 24.5 mm Hg for

Fig. 3. CAT expression in lungs, livers, hearts, and kidneys 48 hours after intravenous administration ($n = 3$). Minimal CAT activity is present in livers and kidneys when compared with the CAT activity in lungs and hearts.

normal controls and transfected animals, respectively, $p = 0.6$, unpaired t test). However, dose-dependent toxicity was observed in lungs transfected with 660 μg of DNA:lipid 67:DOPE (0.5 ml) and stored at 23° C, then transplanted. The grafts in this setting demonstrated extensive hemorrhage and edema. This was not observed in nontransfected lungs preserved for 4 hours at 23° C and transplanted or lungs transfected ex vivo with the use of the same conditions but with 330 μg of DNA and 0.25 μmol of liposome.

Discussion

Viruses are considered the most efficient gene delivery systems. They have developed efficient mechanisms for cellular attachment, penetration, and avoidance of intracellular lysosomal degradation.³ Despite this, we¹⁵ and others^{16,17} have observed an unpredictable, nonhomogeneous, patchy pattern of transgene expression with low efficiency, when a first-generation adenovirus serotype 5 vector was used to introduce exogenous DNA into the pulmonary microvasculature, either in vivo or ex vivo.

Liposomes, on the other hand, are regarded as less efficient delivery systems.¹⁸ It has been demonstrated in vitro that large amounts of liposome-cDNA complexes that enter the cell are trapped in endosomes, precluding the cDNA from reaching the nucleus and initiating transcription.¹⁸ In addition, activation of the complement system by cationic lipids, depending on the chemistry of the individual compounds used, has been reported.¹⁹ Opsonization of DNA complexes by complement proteins can facilitate phagocytosis by the reticuloendothelial system, resulting in rapid clearance of these complexes from the bloodstream, thereby worsening the efficiency of intravenous administration.¹⁹

Despite these impediments to efficient transgene expression, we observed transgene expression in all treated animals in which we used a liposomal vector (lipid 67:DOPE) and a reporter gene (CAT), both in transplanted and in nontransplanted lungs. Transfected cells were homogeneously distributed throughout the lung parenchyma, as confirmed by in situ hybridization. CAT mRNA signal was localized mainly in the pulmonary interstitium, making it difficult to determine whether these were endothelial, macrophage, or interstitial cells. However, ED1 immunohistochemistry-in situ hybridization studies demonstrated CAT expression in macrophages. In addition, cells lining the lumen of larger pulmonary vessels, likely endothelial cells, were also transfected. Inasmuch as the donor endothelium is the first structure to have contact with recipient inflammatory cells, gene therapy targeted to donor endothelial cells is especially attractive and could address two important problems in organ transplantation: ischemia-reperfusion injury and rejection.

Several investigators²⁰⁻²⁴ have observed a significant inflammatory response when viral vectors were used. Vector modifications^{4, 5, 23} and administration of immunosuppressive drugs^{23, 24} have partially circumvented the immune and inflammatory responses raised by these systems. Nevertheless, viral protein expression still occurs,⁵ resulting in a decrease in the duration of transgene expression. Because of the humoral and cellular host immune response, repeat administration is not efficient, since transduced cells express viral proteins and are promptly destroyed by the host immune system.²¹

Using a liposomal vector, we observed a minimal inflammatory response in transfected lungs, consist-

Fig. 4. CAT activity measured in lung isografts. **A**, Lungs transfected in vivo for 48 hours, serving as controls for **B**. **B**, Lung isografts transfected in vivo for 4 hours followed by transplantation and assessed on postoperative day 2. **C**, Lung isografts transfected in vivo for 6 hours, transplanted, and then assessed on postoperative day 2. **D**, Lungs transfected in vivo for 48 hours and assessed for CAT activity. This group was used as a control for group C. CAT activity was not significantly different between transplanted and control lungs.

ing essentially of a mononuclear infiltrate. Although TNF- α levels in transfected lungs increased sevenfold compared with levels in nontreated normal lungs, arterial oxygenation showed no statistical differences between transfected and normal animals, in either the nontransplant or transplant settings. In the nontransplant setting, a decrease in arterial oxygenation was observed at 21 days, but the decrease was not statistically significant, possibly because of the small number of animals in this group ($n = 4$). This demonstrates that there was no significant functional impairment as a result of the transfection procedure when liposomal vectors were used. It is also consistent with studies in healthy human volunteers, revealing no acute adverse effects on pulmonary function when a liposome administered by aerosol inhalation was used.²⁵ In our study, the intravenous administration of liposome-DNA complexes did not result in chronic adverse functional effects in lungs, as demonstrated by gas exchange determined 6 months after intravenous transfection. This is of paramount importance in the transplant setting, where the delivery system must not be another source of injury to the transplanted organ.

The route of administration seems to influence the magnitude of the inflammatory response. We observed significantly higher levels of TNF- α when similar amounts of lipid 67:DOPE:pCF1-CAT complexes were administered intratracheally than when given intravenously (300- to 400-fold increase versus sevenfold increase, respectively, compared with nontransfected lungs; data not shown). One explanation may be that airway defense mechanisms are somehow more reactive as the airways are exposed directly to environmental insults on a regular basis.

This could lead to a prompt and more intense inflammatory response to external agents introduced by this route.

Liposomal systemic toxicity has not been found in previous studies.^{26, 27} No deleterious effects have been shown on hearts, kidneys, and livers according to electrocardiographic, histologic, serum enzyme, and blood chemistry studies. Studies with functional genes might clarify whether transgene expression in other organs would pose a problem in the setting of multiple cadaveric organ retrieval.

Transient expression is considered one of the drawbacks of current adenoviral and liposomal vectors. In this study, CAT activity was detected for a minimum of 8 days after intravenous administration, decreasing significantly by 21 days. Possible explanations for this include (1) loss of plasmid from transfected cells, (2) down-regulation of the cytomegalovirus promoter, and (3) loss of transfected cells owing to necrosis or apoptosis.⁹ However, inasmuch as ischemia-reperfusion injury is a self-limiting phenomenon, transient expression may be sufficient to ameliorate such injury. Furthermore, liposome-DNA complexes have been repeatedly administered without inducing an inflammatory response. Canonico and associates²⁸ have demonstrated that weekly intravenous or aerosol delivery of liposome: α_1 -antitrypsin cDNA complexes in rabbits resulted in sustained transgene expression and did not affect lung histology, mechanics, and oxygenation. Lee and coworkers⁹ have administered lipid 67:DOPE:CAT to mice previously treated with lipid 67:DOPE:cystic fibrosis transmembrane conductance regulator cDNA. The level of CAT activity was equivalent to that detected in mice that received a single administration of lipid 67:DOPE:CAT,

Fig. 5. CAT activity was present in all grafts transfected ex vivo at 10° C with either 660 µg or 330 µg of DNA. **A,** Lung grafts transfected with 660 µg of DNA and 0.5 µmol of lipid 67:DOPE diluted in 10 ml of normal saline solution ($n = 3$). **B,** Lung grafts transfected with 330 µg of DNA and 0.25 µmol of lipid 67 diluted in 10 ml of normal saline solution ($n = 3$).

demonstrating no immune response against the liposomal vector.

If the goal is a modification of ischemia-reperfusion injury, then transgene expression at the time of implantation is ideal. We have developed a strategy to transfect the grafts in vivo, before harvest, so that transgene expression would be present at the time of reperfusion. Our results demonstrate that gene expression, albeit low, was present as early as 2 hours after intravenous injection and increased significantly 4 to 6 hours after intravenous injection in the donor. In addition, we observed that the transplant procedure itself did not affect subsequent transgene expression.

Target specificity is also desirable in the transplant setting. In our study, donor intravenous treatment with liposome-CAT complexes before harvest led to transgene expression in donor hearts at levels comparable with those detected in lungs. Analysis of CAT activity in isolated right and left ventricles showed equivalent transgene expression, indicating that the lipid-CAT complex can cross the pulmonary microvasculature. On the other hand, CAT activity was minimal in livers and negligible in kidneys. It is not clear whether this transfection pattern is due to tissue affinity or to a first-passage phenomenon, inasmuch as hearts and lungs are the first organs to receive the liposome-DNA complex when the jugular route is used. A similar pattern of transgene expression has also been reported by other investigators using liposomes injected through the tail vein of mice.^{26, 29} In another study conducted in rabbits,²⁸ the intravenous administration of a cationic

lipid complexed to the human α_1 -antitrypsin cDNA resulted in transgene expression predominantly in lungs and livers, but not in kidneys. Surprisingly, in the same study transgene expression was also detected in livers when the liposome-DNA complexes were administered through the airways, demonstrating that this route of administration does not assure gene expression exclusively in lungs.

Although the in vivo approach offers the advantage of transgene expression at the time of implantation, it lacks target organ specificity. The ex vivo approach may address this issue. Recently, the feasibility of efficient ex vivo cationic lipid-mediated gene transfer to rabbit heart allografts at 4° C has been demonstrated.³⁰ In the lung transplant setting, the ex vivo approach offers the opportunity to specifically target the lungs. Our results demonstrate that reproducible transgene expression can be achieved with this approach, without significant injury to the graft. All lung isografts treated ex vivo showed transgene expression.

In summary, our data demonstrate that in vivo and ex vivo cationic lipid-mediated gene transfer to lung isografts allows reproducible and widespread transgene expression, without acute or chronic impairment of graft function. Whether these levels of transgene expression are sufficient to yield a functional effect requires further studies with functional genes. In addition, the ischemic insult imposed on the grafts by the transplant procedure did not alter transgene expression. Thus liposomal vectors are relatively safe gene delivery systems and may prove useful in lung transplantation.

We thank Richard Schuessler, PhD, for statistical consultation and Rebecca Bagley for performing the TNF- α assays.

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Discussion

Dr. Duane Davis (*Durham, N.C.*). I have a few questions regarding the actual percent of the cells that were transfected. You presented global assays of activity and some photo monographs. Do you have any idea of what percentage of cells were transfected?

Second, what kind of dose response did you obtain? I saw little difference between 330 and 660 μ g of DNA. Was expression actually better with higher doses? What is the effect of temperature or pressure or any of the other things that we would usually try to alter to enhance delivery?

Dr. Boasquevisque. We did not count the number of cells that were transfected. This information would be more interesting to have when we use functional genes, so that the number of transfected cells could be correlated with the presence or absence of a functional effect.

We cannot say whether there is a difference in the amount of transgene expression when we use 330 μ g compared with 660 μ g. Even in this assay, if more than 50% of the substrate is converted by the enzyme in the extract, it is not possible to detect any difference between the groups.

Regarding the influence of temperature on transgene efficiency, in some animals transfection was done at room temperature, and the expression was really much higher than at cold temperatures. In this study the transfection was done at 10° C, but we also have performed some experiments in which we transfected

the lungs at 4° C, and we noted a clear difference. We did not check the effect of pressure. All these perfusions in the ex vivo setting were done at 20 cm H₂O.

Dr. Larry R. Kaiser (*Philadelphia, Pa.*). You showed a couple of in situ hybridizations. Do you have any idea about the distribution in your in situ cases? Did you retrieve biopsy specimens of various areas of the lung, or did you just look at a random area to get some idea, at least qualitatively, of the percentage or the numbers of cells that are expressing your CAT gene?

Dr. Boasquevisque. We analyzed the whole lung, but we did not count the percentage of cells.