Intratracheal adenovirus-mediated gene transfer is optimal in experimental lung transplantation

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Objective: Gene transfer to experimental lung grafts has been shown to reduce ischemia-reperfusion injury and acute rejection. The optimal delivery route should produce high lung expression with no inflammation and minimal systemic expression. The goal of this study was to determine the optimal gene transfer route for use in experimental lung transplantation.

Methods: F344 rats were injected with 2.9×10^{10} plaque-forming units of adenovirus vector encoding β -galactosidase through intratracheal, intravenous, intraperitoneal, or intramuscular delivery routes and killed 48 hours later. Gene expression was measured by means of enzyme-linked immunosorbent assay.

Results: Intratracheal delivery produces significantly greater gene expression in the lung ($75,350 \pm 47,288$ pg/100 μ g of protein, $P < .001$ vs intravenous, intraperitoneal, and intramuscular routes) and minimal systemic expression (nonsignificant in serum, kidney, liver, spleen, and muscle vs that seen in control animals, $P = .016$ for heart). Immunohistochemistry staining showed β -galactosidase expression in the bronchial epithelium of lungs transfected through the intratracheal route with mild inflammation.

Conclusions: Intratracheal gene transfer provides significant expression in the lung with mild to no inflammation and minimal systemic expression. This delivery strategy has tremendous potential in experimental lung transplant models to reduce ischemia-reperfusion injury and acute allograft rejection and should be investigated further.

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Gene therapy has emerged as a potential therapeutic modality for the treatment of a wide variety of diseases, including ischemia-reperfusion injury and acute allograft rejection in transplantation.^{1,2} In experimental donor lung grafts, gene transfection has been shown to reduce these acute lung injuries.^{3,4}

Gene therapy requires a vector to deliver therapeutic genetic material to the target cell.⁵ Adenoviral vectors possess high transfection efficiency and can be purified and concentrated to high titers.⁶ Unfortunately, adenovirus-

TABLE 1. Comparison of delivery route and tissue expression

Delivery route	Lung	Serum	Muscle	Liver	Kidney	Heart
Control	0 ± 0	1 ± 1	0 ± 0	0.05 ± 0.01	0.16 ± 0.11	0 ± 0
Intratracheal	75350 ± 47288*	5.6 ± 8.9	0.46 ± 0.45	0.18 ± 0.11	0.16 ± 0.03	170 ± 234*
Intravenous	0 ± 0	409 ± 507*	0.02 ± 0.04	4668 ± 3118*	0.14 ± 0.05	0 ± 0
Intraperitoneal	92 ± 35*	21.3 ± 31.5	0.73 ± 0.85	1.4 ± 1.4*	0.89 ± 0.88	0.18 ± 0.43
Intramuscular	0 ± 0	12.3 ± 6.1*	8.8 ± 2.5*	0.27 ± 0.30	0.09 ± 0.02	0 ± 0

Organ expression units are given in picograms per 100 µg of protein, and serum expression is given in picograms per milliliter.

**P* < .05 compared with control values by using Mann-Whitney nonparametric analysis.

mediated transfection causes mild-to-moderate host inflammation and elicits a strong host immune response, limiting the duration of expression and effective retransfection.^{7,8}

Several different delivery routes exist for gene therapy. However, the optimal delivery route in lung transplantation has not been determined. The preferred delivery strategy would produce high pulmonary expression with no inflammation and minimal systemic expression. Intravenous or intraperitoneal administration of adenoviral vectors leads to successful transfection, but systemic toxicity remains a concern.^{9,10} Other studies indicate that intratracheal and intramuscular transfection hold the most promise for reducing ischemia-reperfusion injury and acute rejection in lung transplantation.¹¹⁻¹³ Our laboratory has shown that transgene expression is effective in ameliorating acute lung injury with potentially fewer side effects.^{11,14,15} Furthermore, they possess future clinical potential.¹⁶ Intratracheal transfection could be performed in the intensive care unit before lung harvest, and a recipient could receive intramuscular transfection as soon as a donor is identified.

In this study different delivery routes were compared for use in experimental lung transplantation to identify which route provides maximal pulmonary expression with minimal pulmonary inflammation and minimal systemic expression.

Materials and Methods

Animals

Male Fischer 344 rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) weighing between 250 and 300 g were used in all experiments. All animal protocols were approved by the Animal Studies Committee at Washington University. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1996).

β-Galactosidase Vector Delivery

Animals were injected with 2.9×10^{10} plaque-forming units of adenovirus carrying the *Escherichia coli* LacZ gene encoding for β-galactosidase (β-gal; n = 6 for each group) by using the following routes: intravenous, intraperitoneal, intramuscular, or in-

tratracheal. The order of magnitude of 10^{10} of adenovirus was chosen to ensure a higher level of expression when comparing the different delivery routes. The adenovirus vector is replication deficient, serotype 5, and driven by the constitutive cytomegalovirus promoter purchased from the Gene Therapy Center at the University of North Carolina (Chapel Hill, NC).

Animals were anesthetized with halothane inhalation (1%-2%) and subcutaneous injection of ketamine (25 mg/kg) and atropine (30 mg/Kg) before injection. Intravenous injection was delivered into the penile vein, and intramuscular injection was administered to the gluteal muscle. Intratracheal administration was performed through a 21-gauge catheter after intubation and mechanical ventilation (model 683; Harvard Apparatus Co, South Natick, Mass) maintained at room air with a 2.5-mL tidal volume and respiratory rate of 65 breaths/min. A 21-gauge catheter was placed through the 14-gauge intubation catheter, and 250 µL of vector was instilled with the animal left-side down. The catheter was removed, and the animal was ventilated for 30 seconds. The process was then repeated with 250 µL of vector with the animal right-side down, and animals were recovered.

Animals were killed 48 hours later. This time point was chosen because prior work in our laboratory indicated that adenoviral β-gal expression peaked at 48 hours after administration.¹¹ An intraperitoneal injection of 0.3 mL of pentobarbital (30 mg/kg) was used for anesthesia. After intubation with a 14-gauge angiocatheter by means of tracheotomy, mechanical ventilation, and systemic heparinization (0.4 mL), a median laparosternotomy was performed. Lungs were flushed with 20 mL of cold (4°C) saline solution at 20 cm H₂O pressure through the main pulmonary artery, and then a systemic flush of 60 mL of cold saline solution was administered through the left ventricle. The lungs, heart, liver, spleen, kidneys, and gluteal muscle were harvested, frozen in liquid nitrogen, and preserved at -70°C. Blood was collected and centrifuged at 3200g for 15 minutes at 4°C. The plasma obtained was also stored at -70°C.

Enzyme-Linked Immunosorbent Assay Assessment

β-Gal tissue and serum expression was detected after the enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim GmbH, Mannheim, Germany) protocol. The optical density used was 405 nm (corrected optical density of 490 nm) and standardized to the total protein present in that sample. All tissues studied were homogenized in lysis solution containing 100 mmol/L potassium phosphate (pH 7.8), 0.2% triton X-100 with pepstatin A (5 µg/mL), and protease inhibitor (Complete mini-Tabs, Boehringer-Mannheim). The homogenate was stored for 15 minutes at room

Figure 1. Immunohistochemistry staining (blue) of lung tissue after intratracheal delivery of β -gal. (Original magnification 100 $\times$.)

temperature and then centrifuged at 15,000 rpm. The supernatant was used to quantify β -gal expression. Blood samples of 3 mL were collected in ethylenediamine tetraacetic acid tubes and centrifuged at 3200g for 10 minutes, and supernatants were frozen at -70°C until ELISA assessment. Each sample was standardized to its total protein concentration by using the BCA protein assay kit (Pierce, Rockford, Ill).

Histologic Assessment

Histologic Blueo-gal staining. Harvested organs were stained with Blueo-gal solution, as previously described.¹⁷ Briefly, the heart-lung blocks at harvest were flushed through the pulmonary arterial trunk with phosphate-buffered saline (PBS) solution and 2% paraformaldehyde/0.2% glutaraldehyde solution supplemented with 0.02% nonionic detergent (Nonidet P-40 [octylphenol-ethylene oxide]) and stored in 2% paraformaldehyde solution for 20 minutes. Systemic flush was through the left ventricle, with PBS and 4% paraformaldehyde. After that, lungs and systemic organs were flushed with PBS, followed by Blueo-gal. Finally, all organs were immersed in Blueo-gal buffer for 24 hours at 37°C . The Blueo-gal buffer consisted of 1 mg/mL Blueo-gal (5-bromo-indolyl- β -o-galactopyranoside; Gibco BRL, Gaithersburg, Md), 5 mmol/L $\text{K}_3\text{Fe}^{\text{III}}(\text{CN})_6$, 5 mmol/L $\text{K}_4\text{Fe}^{\text{II}}(\text{CN})_6$, 2 mmol/L MgCl_2 , 0.1% Nonidet P-40, and PBS. After a final flush with PBS, organs were fixed with 4% paraformaldehyde and paraffin embedded. Histologic sections were counterstained with nuclear fast red.

Immunohistochemistry. Organs were flushed, as described above, and then fixed with Histochoice (AMRESCO, Solon, Ohio) or Bouin (Ricca Chemical Co, Arlington, Tex). The immunohistochemistry was performed as previously described.¹⁸ Briefly, after blocking for nonspecific sites, slides were incubated overnight at 4°C with anti-*E Coli* β -gal polyclonal rabbit antibody (Polysciences, Warrington, Pa) at a 1:40 dilution in TNB buffer (blocking reagent in 100 mmol/L Tris and 500 mmol/L NaCl, pH 7.4)/0.1% saponin. Slides were washed in Tris-buffered saline solution/0.2% Triton X (100 mmol/L Tris and 500 mmol/L NaCl, pH 7.4)/0.1% saponin. Slides were incubated with biotinylated anti-rabbit IgG (Biogenex, San Roman, Calif). Finally, slides were

incubated for 30 minutes with streptavidin-alkaline phosphatase. Chromogenic detection for β -gal was performed with the Vector NBT/BCIP Substrate Kit (Vector Laboratories, Inc, Burlingame Calif) in 100 mmol/L Tris, pH 8.2, including 5 mmol/L levamisole and counterstained with nuclear fast red.

The pattern of protein expression and degree of inflammation were assessed by our pathologist (J.H.R.).

Statistical Analysis

One-way analysis of variance, Fisher least significant difference test, and Mann-Whitney *U* test statistical analyses were used. Data are expressed as means $\pm$ SD. The funding agency (National Institutes of Health) had no role in data interpretation.

Results

Intratracheal Route Provides Maximal Lung Expression With Minimal Systemic Expression

β -Gal gene expression in the lung was markedly increased with intratracheal delivery compared with all other routes and expression in control animals ($P < .001$ and $P < .04$, Table 1). The order of magnitude of lung expression was more than 1000 fold with intratracheal instillation compared with the next highest level found with intraperitoneal delivery (75,350 vs 92, Table 1). Importantly, expression in the serum, muscle, liver, kidney, heart, and spleen was negligible, and only heart expression was found to be of statistical significance ($P = .016$, Table 1). Histologically, staining of β -gal is found in both lung (Figure 1) and heart parenchyma (Figure 2, A). No staining was detected in the spleen, kidney, liver, or muscle. Intratracheal delivery produces very high pulmonary expression with minimal systemic expression.

Intramuscular Route Provides Significant Muscle and Serum Expression

Muscle and serum expression were significantly elevated with intramuscular injection compared with that seen in control animals ($P < .05$), whereas lung, liver, kidney, heart, and spleen expression were nearly identical to control values (Table 1). The serum expression confirms that intramuscular injection effectively transfects muscle (Figure 2, B) and produces its systemic effect through release into the bloodstream.

Intravenous Route Provides Significant Systemic Expression and Nondetectable Lung Expression

Liver, serum, and splenic expression were all significantly elevated with intravenous administration ($P < .05$, Table 1). Liver expression was most dramatic. Expression in lung, muscle, kidney, and heart was nondetectable and comparable with that seen in control animals. Injection through the penile vein results in liver and spleen transfection (Figure 2, C and D) because these are the major organ beds seen as the penile vein drains toward the heart. It is likely that the liver clears the rest of the vector administered intravenously, and

Figure 2. β -Gal expression (blue) by means of immunohistochemistry of heart tissue after intratracheal delivery (A), immunohistochemistry of muscle after intramuscular delivery (B), immunohistochemistry of liver after intravenous delivery (C), and Bluo-gal staining of spleen after intravenous delivery (D). (Original magnification 100 $\times$.)

therefore no expression was seen in the kidney, heart, and lung. Similar to the intramuscular route, the serum expression supports the idea that intravenous injection effectively transfects the liver (Figure 2, D) and produces its systemic effect through release into the bloodstream.

Intraperitoneal Route Provides Both Systemic and Lung Expression

Liver and lung expression, as determined by means of ELISA, were significantly increased with intraperitoneal injection versus control expression ($P < .05$, Table 1). Splenic expression was not statistically significant, but when examined histologically with Bluo-gal, there was marked staining throughout (Figure 3, A). This likely reflects splenic uptake of β -gal either hematogenously or through lymphatic channels. Histologic assessment of the liver shows only the surface of the liver staining for β -gal. No intraparenchymal staining is evident (Figure 3, B). In examining the peritoneal lining in the abdomen, very strong β -gal staining is found (Figure 3, C). For the lung, we found intraparenchymal β -gal staining, as well as staining lining the surface (Figure 3, D). Like the spleen, the intraparenchymal component probably represents either hematogenous or lymphatic uptake, whereas the surface staining is caused by direct exposure with the vector.

Discussion

Gene therapy has emerged as a new strategy for the treatment of ischemia-reperfusion injury and acute allograft rejection in experimental lung transplantation models.¹⁻⁴ The lung is a particularly attractive organ for *in vivo* gene therapy because it is directly accessible through the airway. Furthermore, adenoviral vectors can efficiently transfect nondividing or slowly dividing cells, such as the airway epithelium.^{19,20}

Gene transfer has used several different delivery routes, including intravenous, intraperitoneal, intramuscular, and intratracheal routes. Gene expression with adenoviral vectors has been demonstrated in rodent lungs by means of intratracheal instillation.²¹ A major advantage cited with this route is the high level of lung expression with low or undetectable levels in other organs.^{22,23} However, critical shortcomings are that intratracheal injection is invasive, leads to nonuniform distribution of instilled material, and is associated with alveolar inflammation.^{22,23}

The results of our study with intratracheal administration show significant transgene expression in pulmonary epithelial cells. A wide range in this expression exists (Table 1), and this might reflect differences in the site of uptake, rate of lung clearance, and variation in the ability of different lung cell types to express the transgene.²² With respect to

Figure 3. β -Gal expression in blue by means of Bluo-gal staining of spleen tissue after intraperitoneal delivery (A), Bluo-gal staining of liver after intraperitoneal delivery (B), Bluo-gal staining of peritoneum after intraperitoneal delivery (C), and immunohistochemistry of lung after intraperitoneal delivery (D). (Original magnification 100 $\times$.)

cell types transfected, Mastrangeli and colleagues²⁴ demonstrated that ciliated, secretory, basal, and undifferentiated airway epithelial cells were all transfected after intratracheal installation of an adenoviral vector expressing β -gal. The airway epithelium was transfected in our study, including type I and type II pneumocytes and a few ciliated cells. There was no vascular endothelial transfection, and only mild inflammation was found in the airways (Figure 1). Adenovirus-mediated gene delivery is known to cause inflammation, and it is likely that our findings of only mild inflammation 48 hours after transfection would show more pronounced inflammation if examined at later time points.¹⁴ In addition, strategies exist that decrease adenoviral vector-associated inflammation in the transplant setting, which could be used in future studies.²⁵

The first-generation adenoviral vector encoding β -gal did not transfect any pulmonary vascular endothelium through either the intratracheal or intravenous routes. In fact, no lung expression was found by using the intravenous route. It is possible that minimal expression occurred but that such levels were below the range of detection. In contrast, Canonico and associates²⁶ used plasmid-liposome complexes and detected their protein in both the pulmonary vascular endothelium and airway epithelium through intravenous injection. Similar to our findings, only the airway epithelium and not the vascular endothelium was trans-

fected with intratracheal delivery. Zhu and coworkers²⁷ also found that the majority of cells present in the lung were transfected after intravenous injection by using a chloramphenicol acetyltransferase-liposomal vector. This highlights the fact that different routes of gene delivery to the lung and different vectors affect which target cells are transfected.

The drawback with intravenous injection is the broad spectrum of organs that might be undesirably transfected. Expression has been detected in the spleen, liver, heart, kidney, lymph nodes, thymus, uterus, ovary, skeletal muscle, pancreas, bone marrow, stomach, small intestine, and colon.²⁷ We found substantial expression in the liver, spleen, and serum after intravenous injection (Table 1). However, expression in lung, muscle, kidney, and heart was comparable with that seen in control animals. We believe that penile vein injection results in liver and spleen transfection (Figures 2, C and D) because these are the major organ beds seen as the vein drains toward the heart. It is possible that the liver clears the rest of the adenoviral vector, and therefore no expression will be seen in the other organs. We chose the penile route over the jugular route for its ease of administration. However, in finding that the penile route resulted in preferential liver uptake, future studies might be directed at comparing whether lung and

liver expression are influenced by the route of vector delivery.

Intraperitoneal administration resulted in increased liver and lung expression (Table 1). Surprisingly, splenic expression was not statistically significant, but when examined histologically with Bluo-gal, there was marked staining throughout (Figure 3, A). This likely reflects splenic uptake of β -gal either hematogenously or through lymphatic channels. Similar to our findings, Thierry and colleagues²⁸ found splenic targeting after intraperitoneal plasmid injection. Lipshutz and associates²⁹ also contend that adenoviral particles are transported across the peritoneum into lymphatics and eventually enter the systemic circulation or enter directly into peritoneal blood vessels. This would support the liver and lung expression documented in this study. Intraparenchymal lung β -gal staining, as well as staining lining the surface, was found (Figure 3, D). The intraparenchymal component probably represents hematogenous or lymphatic uptake, whereas the surface staining is caused by direct exposure with the vector.

Finally, the intramuscular route with adenoviral vectors has been extensively studied by Acsadi and coworkers.³⁰ They found prolonged muscle expression and long-term stability with intramuscular delivery. The other theoretic advantage with intramuscular injection is that any vector-associated inflammation is distant from the transplanted lung. In our study muscle and serum expression were significantly elevated compared with that seen in control animals ($P < .05$), whereas lung, liver, kidney, heart, and spleen expression were nearly identical to control values (Table 1). As stated before, the serum expression supports the idea that intramuscular injection effectively transfects muscle and produces its systemic effect through release into the bloodstream. The intramuscular approach has been shown to be beneficial in affecting acute lung allograft rejection, and our results support these findings.^{4,11}

In conclusion, intratracheal gene transfer provides significant expression in the lung with mild inflammation and minimal systemic expression. It provides maximal lung expression for use with gene transfer in lung transplant models when compared with intramuscular, intravenous, or intraperitoneal administration. This delivery strategy has tremendous potential in experimental lung transplant models to reduce ischemia-reperfusion injury and acute allograft rejection and should be investigated further.

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