Transpl Int (2000) 13: 241–246

ORIGINAL ARTICLE

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Improved efficiency of gene transfer to the transplanted lung by retrograde vascular gene delivery

Received: 14 June 1999
Revised: 22 February 2000
Accepted: 20 April 2000

Presented at the Eighteenth Annual Meeting of the International Society for Heart and Lung Transplantation, Chicago, USA, April 15–18, 1998

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Abstract Experiments were designed to evaluate the efficiency of antegrade compared to retrograde vascular gene transfection of donor lungs used for transplantation. Rat donor lungs (n = 5/group) were transduced with an adenoviral vector encoding for β-galactosidase (Adβ Gal), either antegrade in the pulmonary artery (Group A, 3×10^8 pfu, Group B, 3×10^9 pfu) or retrograde into the pulmonary vein (Group C, 3×10^8 pfu), immediately after pneumoplegia. After storage at 4 °C for 1 h, the transduced lungs were transplanted orthotopically in syngeneic animals. The lungs were assessed for transgene expression by ELISA and X-Gal-staining at day 7 after operation. Inflammation was graded based on the extent of inflammatory cell infiltration. Transgene expression was similar between Groups A (1.7 ± 0.7 ng/mg protein) and B (2.1 ± 1.0 ng/mg protein). With retrograde delivery, there was a four-fold (8.3 ± 2.6 ng/mg protein) increase ($P < 0.05$) in transgene expression compared to either group A or B. In all groups, pneumocytes were transduced most frequently. The degree of inflammation correlated positively with the extent of transgene expression ($r = 0.75$, $P < 0.01$). The efficiency of vascular gene delivery to transplanted lungs can be improved by retrograde delivery of the vector via the pulmonary vein. Transgene expression predominates in pneumocytes following both antegrade and retrograde delivery. The severity of inflammation in the transplanted lung appears to correlate with the extent of transgene expression.

Key words Gene transfer · Lung transplantation · β-galactosidase

Introduction

Lung transplantation is an accepted treatment for selected patients with end-stage pulmonary disease. However, major obstacles remain, including the high incidence of postoperative infection and acute and chronic rejection with subsequent development of obliterative bronchiolitis[5]. Gene therapy to the donor lung may of-

fer a therapeutic modality by which these processes can be prevented or treated. The transplant setting, with access to the organ during the time of explantation, offers a unique opportunity for gene transfer.

The feasibility of adenovirus-mediated gene transfer to pulmonary grafts has been demonstrated previously, both via the vasculature and airways [2, 3, 6]. Conditions by which the genes are delivered are critical to achieve efficient gene transfer to the transplanted lung. For example, transbronchial gene administration to explanted lungs resulted in effective gene transfer, although limited mainly to pneumocytes [6, 7]. In contrast, gene delivery via the pulmonary artery appears to be relatively inefficient, whereas the cellular distribution of transgene expression is similar compared to airway administration [2, 3]. Similar distribution and expression of transgene are observed with in vivo transfection of nontransplanted lungs [9, 10, 12]. Gene transfer via the pulmonary vasculature may be improved by using alternative delivery conditions such as increasing the dose of vector or infusion of the vector through the pulmonary vein instead of the pulmonary artery. Therefore, experiments were designed to evaluate and compare the effects of varying the concentration of virus and antegrade vs retrograde infusion of vector on transgene distribution and expression in transplanted lungs. Retrograde delivery of genes via the pulmonary vein could theoretically target the bronchial vasculature and, subsequently, bronchial and bronchiolar epithelial and smooth muscle cells, where obliterative bronchiolitis would occur.

The advantages of adenoviral vectors have been reviewed recently [4]. A major disadvantage of these vectors is the inflammatory response, which may in part limit the duration of transgene expression in transduced organs [4, 13, 16]. Thus, a second aim of the study was to evaluate the inflammatory response following adenovirus-mediated gene transfer and relate the degree of inflammation to the extent of transgene expression. Another potential disadvantage is that preceding immunity to adenovirus resulting from infection may preclude gene expression using adenoviral vectors.

Materials and methods

Animals

Thirty-six inbred Lewis rats (Harlan Sprague-Daley), weighing 250–320 g were used in the experiments. Animal care was conducted in accordance 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 Institutes of Health (NIH Publication, revised 1996).

Adenovirus vector

A replication-defective serotype 5 adenovirus encoding for β-galactosidase under the control of the CMV promoter (AdLacZ, a kind gift from James Wilson, Institute for Human Gene Therapy, University of Pennsylvania) was used as the reporter gene. This adenoviral vector is a first-generation, E1–E3 deleted vector [15]. The recombinant virus was propagated in 293 cells and then isolated and purified. Viral titers were determined by plaque assay. All viral transductions were done using 300 μl of the viral solution (diluted in 199 medium with 2 % fetal calf serum). Control animals received the same volume of medium.

Study groups

Donor lungs in Group A ($n = 5$) and Group B ($n = 5$) received a bolus injection of Adβ Gal of either 3×10^8 pfu (A) or 3×10^9 pfu (B) antegrade in the pulmonary artery before transplantation. Group C ($n = 5$) received a bolus dose of 3×10^8 pfu of Adβ Gal retrograde in the pulmonary vein before transplantation. In addition, three animals received diluent only (one antegrade and two retrograde), delivered exactly as in the study groups. These animals served as controls for endogenous β-galactosidase activity.

Gene transfer

After anesthesia (sodium pentobarbital, 50 mg/kg intraperitoneal), the donor rat was intubated and ventilated (Harvard Rodent Ventilator). A median sternotomy was performed to expose the lungs. Following dissection of the hilum, the rat was heparinized with 100 U of aqueous heparin injected into the inferior vena cava. Caval veins were ligated, the left atrium opened, and the aortic root divided, after which 20 ml of pneumoplegia (Euro-Collins) was infused in the main pulmonary artery.

In Groups A and B, 100 μl of viral solution was injected into the left pulmonary artery immediately after pneumoplegia. The left pulmonary vein was then occluded and the remaining 200 μl injected, after which the infusion catheter was removed from the left pulmonary artery. The pulmonary artery then was occluded to prevent back leakage. Delivery with clamping of the potential drainage sites was chosen to achieve increased intravascular pressure and distension of the vasculature to enhance the efficiency of gene transfer (Yap J, Pellegrini C et al.; submitted). In Group C, the same viral delivery technique was used with the exception that the viral solution was infused retrograde into the pulmonary vein rather than the pulmonary artery. After vector delivery, the lung was explanted and preserved for 60 min in cold (4 °C) Euro-Collins solution in order to mimic procurement conditions of lungs used for transplantation in humans.

Single lung transplantation

In the recipient, a left thoracotomy was performed and the left lung dissected and removed. The preserved, transduced donor left lung was then implanted orthotopically into the recipient by anastomosing the pulmonary vein and artery. The lung was reperfused, after which the bronchus was anastomosed. All anastomoses were performed with 10–0 monofilament sutures. The chest wall was closed with a small chest tube in situ, which was removed during recovery from anesthesia.

Assessment of transgene expression

Seven days after transplantation, transplanted and native lungs, hearts, and livers were harvested. Cross sections from the mid-portion of the organs were embedded in OCT compound (Miles, Elkhart, Ind.) and snap frozen in a liquid nitrogen-cooled 2-methylbutane bath. Adjacent segments were snap frozen in liquid nitrogen for ELISA.

A commercially available β-galactosidase ELISA kit (5 Prime → 3 Prime, Boulder, Colo.) was used to assess β-galactosidase expression. In brief, the snap-frozen transplanted and native lungs, heart, and livers were homogenized and centrifuged 10 min at 18,000 g. A rabbit polyclonal antibody specific to the Escherichia coli β-galactosidase protein was coated onto polystyrene microwells. The supernatant of the homogenized samples was incubated in the wells, and a biotinylated secondary antibody to β-galactosidase was added. The biotinylated antibody was quantitated colorimetrically by incubation with streptavidin-conjugated alkaline phophatase and color development substrate. Spectrophotometric analysis was performed with an automated analyzer, SPECTRAmaxTM 340 (Molecular Devices, Sunnyvale, Calif.). Total protein concentration was assessed by bicinchonicic acid protein assay (Pierce, Rockford, Ill.).

Histology

Five cryostat sections (5 μmm thick) were cut at 25 μm intervals of the midsection of the transplanted lung. The specimens were fixed in 1.25% glutaraldehyde for 10 min at 4 °C and rinsed three times with PBS (phosphate-buffered saline). The sections were then stained in a solution of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim, Indianapolis, Ind.) for 4 h at 37 °C. The X-Gal-stained specimens were rinsed in PBS and counterstained with eosin. Blue cells indicated expression of β-galactosidase. Adjacent sections were stained with hematoxylin and eosin for routine histopathologic examination. Inflammation was graded by an observer blinded to the origin of the slides, using a reported previously semiquantitative inflammation scale (from 1 to 12) for transplanted lungs [7], based on the extent (1 to 3) and severity (1 to 4) of inflammatory cell infiltration. The total score is given by the extent score multiplied by the severity score.

Statistical Analyses

All results are expressed as mean and standard error of the mean. The nonparametric Kruskal-Wallis test followed by Dunn's post test was used to compare transgene expression among groups. A P value of less than 0.05 was considered statistically significant.

Results

Surgical procedure

Cold ischemic time (60 min) and total operative time did not differ among the groups. Warm ischemic time was 53 ± 9 min in Group A, 47 ± 4 min in Group B, and 53 ± 10 min in Group C (n = 5/group). Two animals died related to the surgical procedure. All other animals survived to sacrifice without any evidence of respiratory distress.

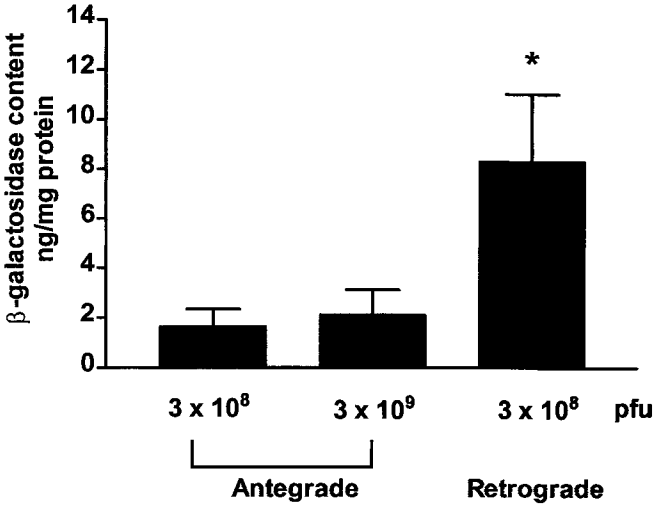

Fig. 1 Transgene expression in transplanted lungs transduced antegrade via the pulmonary artery with either 3×10^8 pfu or 3×10^9 pfu, or transduced retrograde via the pulmonary vein with 3×10^8 pfu. Data are shown as mean ± SEM. Asterisk denotes statistical significant difference from both groups with antegrade vector administration by analysis of variance, * = $P < 0.05$. $n = 5$ in each group

Transgene expression

Total transgene expression as measured by ELISA was the same in Groups A and B receiving different doses of vector by antegrade delivery (Fig. 1). Total transgene expression was significantly higher with retrograde delivery compared to both groups of antegrade delivery, even when the lower dose of virus was used (Fig. 1). No detectable transgene expression in the native lung, liver, or heart was observed in any of the transduced animals. Endogenous β-galactosidase content in the native organs of transduced animals was not different compared to the levels in the same organs in nontransduced control animals (0.05–0.15 ng/mg protein).

Cellular distribution of transgene expression

Pneumocytes were the most frequently stained cell type in all groups (Fig. 2). Occasional macrophages and endothelial cells stained positively, but no staining was observed in airway epithelial cells (Fig. 3).

Histology

Overall inflammation scores determined by histopathologic examination of hematoxylin and eosin-stained sections of lung cut immediately adjacent to the slides stained for β-galactosidase were: 0, Group A (antegrade

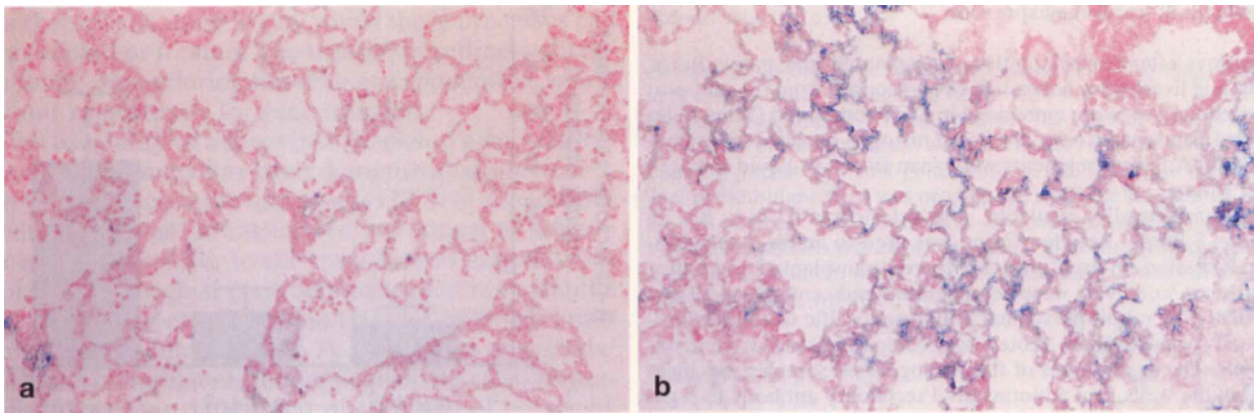

Fig. 2 Light photomicrographs of sections of transplanted lungs from the antegrade groups (*left*) and the retrograde group (*right*). The blue stained cells indicates β-galactosidase expression. No difference in transgene expression was observed between Groups A and B with antegrade administration of the vector. Pneumocytes were the most frequently stained cell type in all groups. Occasional macrophages stained positively (magnification × 50)

3×10^8 pfu); 1.6 ± 0.8, Group B (antegrade 3×10^9 pfu); and 4.2 ± 2.0, Group C (retrograde 3×10^8 pfu). In Group B, sections from three (60%) showed no inflammation, one (20%) showed mild inflammation, and one had moderate inflammation, characterized by focal perivascular and intra-alveolar collections of eosinophils and lymphocytes, combined with an acute and organizing pneumonia. In lungs transduced with retrograde infusion (group C), four of five (80%) lungs showed mild inflammation and one showed severe inflammation with marked perivascular lymphocyte infiltration similar to grade 3 rejection [16]. A statistically significant correlation ($r = 0.75$, $P = 0.0018$) between inflammation score and transgene expression was observed (Fig. 3). Inflammation score did not correlate with vector dose, cold and warm ischemic time or total operative time.

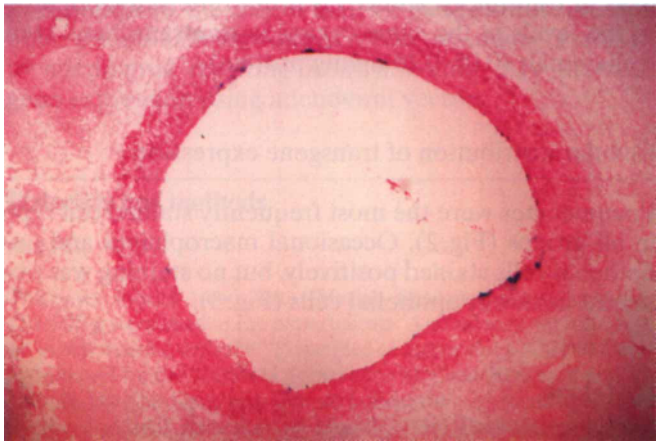

Fig. 3 Light photomicrograph of section of transplanted lung transduced after retrograde transduction with adenoviral vector encoding for β-galactosidase showing endothelial transgene expression (magnification × 10)

Discussion

Results of this study indicate:

1. Antegrade adenovirus-mediated gene transfer to the transplanted lung via pulmonary artery is relatively inefficient.
2. Retrograde delivery via the pulmonary vein improves transduction efficiency.
3. Transgene expression is limited predominantly to pneumocytes with both antegrade and retrograde vascular delivery.
4. The degree of inflammation correlates with the extent of transgene expression.

Viral concentrations for the present study were chosen to correspond with a previous study from our laboratory in which transbronchial gene transfer to the transplanted lung resulted in efficient gene transfer with approximately 30% of the pneumocytes staining [6]. In contrast, in the current study our aim was to assess the results of vascular administration of vector to the donor lung and examine the pattern of transgene expression. We observed that vascular administration via the pulmonary artery was markedly less effective than airway administration. While a direct comparison with the previous paper cannot be made since quantitative assessment by ELISA was not performed, review of multiple sections from both studies reveals increased efficiency with airway administration. Furthermore, we have quantified β-galactosidase 4 days after airway delivery in another paper [7], and the levels were 100 times greater than our current series with vascular delivery.

These results are consistent with previous reports from other groups [2, 3]. In addition, increasing the vector dose tenfold did not improve the effciency of gene transfer through the pulmonary artery. In contrast to gene delivery through the pulmonary artery, gene delivery through the pulmonary veins increased efficiency of gene transfer. The distribution of transgene expression from venous delivery was the same as for arterial delivery of the gene, that is, primarily pneumocytes.

The use of a variety of cold pneumoplegia solutions perfused in an antegrade fashion is the most common method of lung preservation used clinically at the present time. The advantages have been described elsewhere [8]. Usually an infusion of prostaglandin is administered during antegrade pneumoplegia perfusion to mitigate the potential for pulmonary arterial vasoconstriction during perfusion, thus allowing more optimal pulmonary distribution of perfusate. In our experiments, due to size and other constraints, this was not used and so may have resulted in some compromise of antegrade perfusate distribution in the lung. Retrograde perfusion of cold pneumoplegia solution via the pulmonary veins has an additional advantage of perfusing the bronchial circulation, which does not occur with antegrade perfusion [1, 14]. A significant proportion of the tracheal and bronchial blood supply comes from the bronchial arteries. The ability to perfuse the bronchial circulation by retrograde perfusion of the lungs may have enhanced the increased efficiency of vascular gene therapy delivery seen in the current experiments. Somewhat disappointingly, the distribution of gene delivery was principally to the pneumocytes rather than the bronchi, despite retrograde perfusion. The reason for lower transduction efficiency of cell types other than pneumocytes is unclear. Previous reports have indicated that pneumocytes are the only cell type susceptible to widespread adenovirus-mediated gene transfer in pulmonary grafts [2, 3, 6]. Airway administration delivers the vector directly to the pneumocytes. When the vector is delivered via the vasculature, as in the present study, the predominant cell type transduced is still the pneumocyte, but this time at a much lower efficiency compared to airway administration. This suggests that the number of pneumocytes directly exposed to the vector may determine the overall transduction efficiency. This may also explain why transgene expression was increased when the vector was delivered retrograde into the pulmonary vein. Increased postcapillary pressure and leakage out of the vasculature to the alveoli may occur when the vector is delivered by this route. It is not clear why transduction of vascular endothelial cells was observed infrequently. Density of viral receptors in different cell types or tissue susceptibility to factors which may enhance viral transduction, e.g., ischemia, may be important.

With airway administration, total transduction efficiency (assessed with X-Gal staining only) was not enhanced by increasing the dose of vector from 3×10^8 pfu to 3×10^9 pfu [6]. The same pattern was found with antegrade vascular administration, where no difference in transgene expression was observed between 3×10^8 and 3×10^9 pfu. Thus, transduction efficiency was not improved by increasing the dose tenfold.

Transgene expression was not observed in the native lung, heart, or liver of animals with transplanted transduced lungs using these doses of vector (3×10^8 and 3×10^9 pfu). This does not, however, exclude that low levels of transgene expression may have been present which were not detected by the method (immunoassay to β-galactosidase) used in this study. With amplification methods (polymerase chain reaction), Chapelier et al. detected transgene DNA in tissue from the heart, liver, and other organs 3 days after transplantation of lungs transduced with higher vector doses (2×10^{11} pfu) [3].

An interesting finding in the current study was the correlation of the extent of inflammation with transgene expression. As mentioned previously, a disadvantage of adenoviral vectors is an immune response, which probably limits the duration of transgene expression. The controls in the present study received medium without vector and did not show any signs of inflammation. This suggests that it is the vector construct (virus + gene) and not the cold ischemia and surgical procedure that causes the inflammation in the transduced, transplanted lungs. Inflammation after adenovirus-mediated gene delivery to nontransplanted lungs is correlated with the dose of vector [11]. In transplanted lungs, where cold ischemia and surgery make immediate comparisons to nontransplanted conditions uncertain, inflammation has previously not been correlated with either viral dose or transgene expression. The results of the current study do not differentiate between the effect of the adenovirus per se or the foreign insert. Indeed, both have been implicated in the inflammatory response after adenovirus-mediated gene transfer [17]. The increased inflammation in lungs with enhanced transgene expression may be an obstacle if widespread and prolonged gene expression is desired. It should be noted, however, that immunosuppression administered after allogenic transplantation may attenuate this effect [18].

In summary, adenovirus-mediated gene transfer to transplanted lungs can be improved by using retrograde delivery of vector through the pulmonary vein. Transgene expression in the transplanted lung is predominantly to pneumocytes independent of delivery through bronchi, pulmonary arteries, or pulmonary veins. The extent of transgene expression appears to correlate with the degree of inflammation.

Acknowledgements This work was supported by the Mayo Clinic and Foundation, Rochester, Minnesota and the Bruce and Ruth Rappaport Program in Vascular Biology. The skillful technical assistance from Sandra Severson and Sharon Guy is gratefully acknowledged. Dr. Anders Jeppsson was a visiting scientist supported by grants from Sahlgrenska University Hospital, University of Gothenburg, The Foundation for Medical Research and Education (SMFS), Assar Gabrielsson Foundation, Gunnar, Arvid and Elisabeth Nilsson Foundation, Swedish Medical Society, Swedish Medical Research Council and Gothenburg Medical Society.

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