Influence of temperature on adenovirus-mediated gene transfer

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Abstract

Objective: The transfer of recombinant genes to donor organs may allow for novel therapeutic approaches to the challenges of acute and chronic rejection. Adenoviral vectors are capable of efficient gene transfer, but use of these vectors during donor organ preservation may be less efficient due to the low temperature. This study was designed to examine the effect of temperature on the efficiency of adenovirus-mediated gene transfer.

Methods: Gene transfer to human endothelial cells, porcine vascular smooth muscle cells and cultured rat thoracic aortas was examined. Incubation with an adenoviral vector encoding for E. coli β-galactosidase was performed for 1 h at three different temperatures: 4 °C, 10 °C and 37 °C. Transgene expression was assessed by histochemical staining for β-galactosidase in transduced cells and by evaluation of β-galactosidase activity in organ cultures.

Results: Both in human endothelial cells and vascular smooth muscle cells the percentage of positively staining cells following transduction at 37 °C was significantly greater than at 4 °C and at 10 °C (30.55 ± 7.26% vs. 14.29 ± 3.79% and 12.43 ± 2.47%, respectively for endothelial cells, P < 0.01 vs. 4 °C and 10 °C; and 28.25 ± 4.52% vs. 17.91 ± 3.76% and 16.63 ± 3.92%, respectively for smooth muscle cells, P < 0.05 vs. 4 °C, P < 0.01 vs. 10 °C). β-galactosidase activity was significantly greater in aortas transduced at 37 °C than in vessels transduced at 4 °C and 10 °C (289 700 ± 113 300 vs. 149 600 ± 54 390 and 108 800 ± 23 140 relative chemiluminesce units/mg of total protein, respectively; P < 0.05 vs. 4 °C, P < 0.001 vs. 10 °C).

Conclusions: The present study demonstrates that the efficiency of adenovirus-mediated gene transfer is significantly reduced at lower temperatures. The need for cold preservation of donor organs may render efficient adenovirus-mediated gene transfer more difficult in the transplantation setting.

Keywords: Adenovirus; Gene transfer; Gene therapy; Endothelial cells; Vascular smooth muscle cells; Transplantation

1. Introduction

In heart transplantation, gene therapy may provide the potential for site-specific immunosuppression and induction of tolerance [1]. In addition, gene transfer may enable the study of the pathologic processes related to chronic rejection (graft arteriopathy) at a molecular level, thus allowing the development of rational therapeutic strategies to prevent this complication. A significant obstacle to attaining these goals is the difficulty of transferring genes to cells and tissues in vivo. Replication defective adenoviruses have been used to accomplish efficient gene transfer and expression of several recombinant genes in different transplant settings [2–4]. In contrast to in vivo vascular gene transfer, transplantation may represent an ideal setting for administration of the vector via the blood vessels as the inevitable period of donor organ ischemia following harvesting allows a prolonged dwell time within the target tissue. However, the efficiency of gene transfer during the period of cold ischemia may be influenced by the low temperature of the preservation solutions. This study was designed to examine the effect of temperature on the efficiency of adenovirus-mediated gene transfer.
1.1. Materials and methods

1.1.1. Adenovirus vector

A replication defective, E1a deleted serotype 5 adenoviral vector encoding for Escherichia coli β-galactosidase, under the control of the CMV promoter was used in this study (AdCMV-LacZ, which was a kind gift from James Wilson, Institute for Gene Therapy, University of Pennsylvania). The recombinant virus was propagated in transformed human embryonic kidney carcinoma cells (293 cells), isolated, purified by ultracentrifugation through two cesium chloride gradients, dialyzed against 140 mM NaCl, 0.5 M Hepes and 1 M MgCl₂ for 3.5 h at 4°C and stored at -70°C in 10% glycerol until use. Viral titers were determined by plaque assay and expressed as plaque forming units per ml (pfu/ml).

1.1.2. Transduction of cells in vitro

Endothelial cells were purchased from Clonetics Corporation (San Diego, CA). They were grown in endothelial cell basal medium without phenol red (Clonetics Corporation, San Diego, CA) supplemented with human recombinant epidermal growth factor (0.01 ng/ml), hydrocortisone (10 mg/ml), bovine brain extract (12 g/ml), gentamicin (50 μg/ml), amphotericin B (0.05 μg/ml), bovine brain extract (12 μg/ml) and 2% fetal bovine serum. Vascular smooth muscle cells were obtained from porcine coronary arteries, as previously described [5]. They were grown in modified 199 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD). Cell identity was confirmed by positive staining with a monoclonal antibody reactive with smooth muscle α-actin (Sigma, St. Louis, MO).

Endothelial cells and smooth muscle cells were incubated in a humidified, 5% carbon dioxide atmosphere at 37°C and routinely passaged just before reaching confluence by brief exposure to trypsin-EDTA solution (trypsin 0.05%, EDTA 0.53 mM; Gibco BRL, Gaithersburg, MD). Cells at passage 4-8 were used in all experiments. Cells (5 × 10⁴) were plated on 6-well plates and allowed to attach overnight.

After removal of medium, cells were washed twice with phosphate-buffered saline (Gibco BRL, Gaithersburg, MD) and then incubated for 1 h in 0.5 ml of phosphate-buffered saline + 0.1% bovine serum albumin containing 2 × 10⁶ pfu/ml, which was equivalent to a multiplicity of infection of 200. Endothelial cells and smooth muscle cells were incubated at three different temperatures: 4°C, 10°C and 37°C. At the end of incubation the cells were washed twice with phosphate-buffered saline and incubated for 24 h in fresh medium. In all experiments (n = 4), 12 wells for each cell type at each incubation temperature were used. Control cells were incubated using the same experimental conditions in virus-free phosphate-buffered saline + 0.1% bovine serum albumin.

Twenty four hours later cell viability was assessed by Trypan Blue exclusion. The viability of two wells in each group was evaluated using a standard hemocytometer following detachment of cells by trypsin-EDTA solution.

Prior to staining for β-galactosidase, endothelial cells and smooth muscle cells were washed twice with phosphate-buffered saline and fixed for 15 min in 1.25% glutaraldehyde at 4°C. After a further rinse in phosphate-buffered saline, the cells were stained in a solution of 500 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim, Indianapolis, IN) for 4.5 h at 37°C. Blue stained cells indicated the presence of β-galactosidase expression. For quantitative analysis, the total number of positively staining and negative cells were counted manually by an observer blinded to experimental groups using an inverted bright light microscope (magnification = 200x). Fourteen randomly selected fields in each well were evaluated. The percentage of transduced cells was calculated as the number of positively staining cells divided by the total number of counted cells × 100.

1.1.3. Transduction of rat thoracic aorta ex vivo

Lewis rats weighing 400-450 g (n = 7) were used. All animals received humane care in compliance with the European Convention on Animal Care.

The rats were anesthetized with an intraperitoneal injection of 100 mg/kg of sodium pentobarbital. The rats were then intubated and ventilated (Harvard Rodent Ventilator). A median sternotomy was performed and 200 units of aqueous heparin was injected into the inferior vena cava. Under aseptic conditions, thoracic aorta (from arch to diaphragm) was removed using an operating microscope, cut into 5 mm segments and placed in 12-well plates containing 1 ml of medium (Dulbecco’s modified Eagle’s medium supplemented with 0.5% bovine serum albumin, penicillin (120 units/ml), streptomycin (120 μg/ml) and 0.075% NaHCO₃. The vessels were incubated in a humidified, 5% CO₂ atmosphere for 6 h.

After removal of the medium the vessels were washed twice with phosphate-buffered saline and then incubated for 1 h in 0.5 ml of phosphate-buffered saline + 0.1% bovine serum albumin containing 2 × 10⁶ pfu/ml at three different temperatures: 4°C, 10°C and 37°C. One hour later, the vessels were washed twice with phosphate-buffered saline and incubated for 24 h in fresh medium. Control aortas (n = 3 from different animals) were incubated under the same experimental conditions in virus-free phosphate-buffered saline + 0.1% bovine serum albumin.

At the end of 24 h incubation, the vessels were washed twice with phosphate-buffered saline and then homogenized in ice-cold buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100 (Sigma, St. Louis, MO) and 200 mM phenylmethylsulfonyl fluoride). Homogenization was performed for 1 min at full speed using a tissue homogenizer (Tekmar, Cincinnati, OH). Homogenates were centrifuged at 18,000 × g for 10 min at 4°C to remove tissue debris. The supernatant was collected and frozen at -70°C. Aliquots were assayed for β-galactosidase activity (Galacto-Light...
Light emission was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). All measurements were performed in triplicate with 50-fold dilution of the supernatant. The results were subtracted from background counts and compared to a standard curve, generated by using purified *Escherichia coli* β-galactosidase (Sigma, St. Louis, MO), to determine the amount of transgene product present. Additional aliquots were used to assess total protein concentration using bicinechonic acid protein assay reagent (Pierce, Rockford, IL).

### 1.4. Statistical analysis
Results are expressed as mean ± standard deviation. Analysis of variance was performed to evaluate overall differences between the three groups. If overall significance was present, Bonferroni’s post hoc test was used for pair comparisons (GraphPad Prism). A *P* value of less than 0.05 was considered significant.

### 2. Results

#### 2.1. In vitro gene transfer to cells

##### 2.1.1. Toxicity
The viability assessed at the end of the 24 h incubation ranged from 75 to 80% in endothelial and smooth muscle cells transduced at 4°C and 10°C and from 80 to 85% in cells transduced at 37°C.

##### 2.1.2. Human umbilical vein endothelial cells
Following transduction at 4°C and 10°C, the percentage of cells positively staining for β-galactosidase was 14.29 ± 3.79% and 12.43 ± 2.47%, respectively. No significant difference was noted between these groups. In contrast, when the cells were transduced at 37°C, the positively stained cells were 30.55 ± 7.26% (*P*, 0.01 vs. 4°C and 10°C) (Fig. 1). Control endothelial cells, not exposed to the adenoviral vector, were negative for β-galactosidase staining.

##### 2.1.3. Porcine vascular smooth muscle cells
Following transduction at 4°C and 10°C, the percentage of cells positively staining for β-galactosidase was 17.91 ± 3.76% and 16.63 ± 3.92%, respectively. There was no significant difference between these groups. In contrast, when the cells were transduced at 37°C, 28.25 ± 4.52% of the cells expressed the transgene (*P*, 0.05 vs. 4°C, *P*, 0.01 vs. 10°C). (Fig. 2). Control smooth muscle cells, not exposed to the adenoviral vector, were negative for β-galactosidase staining.

##### 2.1.4. Ex vivo gene transfer to thoracic aortas
In fresh harvested aortas and in control vessels, not exposed to adenovirus, reporter gene activity was undetectable. In aortas transduced at 4°C and at 10°C, β-galactosidase activity was 149 600 ± 54 390 and 108 800 ± 23 140 relative chemiluminescence units (RLU)/mg of total protein, respectively. In vessels transduced at 37°C, the transgene protein activity was 289 700 ± 113 300 RLU/mg of total protein. The amount of transgene product was determined comparing these results to a standard curve generated by using purified β-galactosidase. In vessels transduced at 4°C and 10°C, the β-galactosidase content was 31.15 ± 16.63 ± 3.92%.

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**Fig. 1.** Effect of incubation temperature on gene transfer efficiency to human umbilical vein endothelial cells (HUVEC). Multiplicity of infection n = 200. Data represent mean ± SD of the percentage of cells positively staining for the reporter gene (wells no. = 10, experiment performed on four separate occasions). Asterisks denote significant difference compared to cells at 4°C and 10°C (ANOVA, Bonferroni’s post hoc test, *P* < 0.01).**

**Fig. 2.** Effect of incubation temperature on gene transfer efficiency to porcine vascular smooth muscle cells (PVSMC). Multiplicity of infection n = 200. Data represent mean ± SD of the percentage of cells positively staining for the reporter gene (wells no. = 10, experiment performed on four separate occasions). Asterisks denote significant difference compared to cells incubated at 4°C and 10°C (ANOVA, Bonferroni’s post hoc test, *P* < 0.05 vs. 4°C, *P* < 0.01 vs. 10°C).

Fig. 1. Effect of incubation temperature on gene transfer efficiency to human umbilical vein endothelial cells (HUVEC). Multiplicity of infection n = 200. Data represent mean ± SD of the percentage of cells positively staining for the reporter gene (wells no. = 10, experiment performed on four separate occasions). Asterisks denote significant difference compared to cells at 4°C and 10°C (ANOVA, Bonferroni’s post hoc test, *P* < 0.01).**
vs. 10°C transfer. Temperature may have significant effects on the efficiency of gene transfer. These observations suggest that temperature was accentuated in superficial and less well preserved regions of the heart. Invasiveness of the vector to other tissues when blood flow above a critical rate affects gene transfer to smooth muscle cells and cultured vascular smooth muscle cells in vitro and to rat thoracic aortas ex vivo is significantly influenced by temperature [8]. In that study, efficiency of gene transfer to vascular endothelial cells in vitro appeared to be dependent on the titer of the viral solution, whereas the endothelium in situ seemed to be resistant to adenoviral infection even at the highest viral titer (10^11 pfu/ml). In contrast, Chapelier et al. [4] have demonstrated that efficiency of adenoviral gene transfer to endothelial cells is significantly impaired at 10°C. Our results are consistent with those of Chapelier. In addition, we demonstrated that a comparable impairment affects gene transfer to smooth muscle cells and cultured thoracic aortas.

In clinical transplantation, efficient gene transfer to the donor organ will be necessary at 4°C. If inefficient gene transfer in this setting results in generation of low quantities of biologically active transgene, efforts will be needed to improve gene transfer efficiency at this temperature. As stated above, increasing the dose of the vector may enhance gene transfer efficiency. However, this may also augment the risk of vector-induced toxicity. As efficiency of adenoviral gene transfer is directly related to the length of exposure to the vector [9], improvement of donor organ preservation may allow more prolonged dwell time within the target organ and thus may afford enhanced gene transfer efficiency. Similarly, increasing the perfusion pressure during the administration of the viral vector may yield improved transduction efficiency. It is known that anatomic barriers may cause remarkable limitations to the penetration of adenoviral vectors into the vasculature and that the incorporation of particles in the size range of adenovirus (about 100 nm) is dependent on pressure [10]. Recently, Donahue et al. [11] have demonstrated that efficiency of adenovirus-mediated gene transfer to the heart by intracoronary infusion of the viral vector is dependent on the delivery flow rate. The authors hypothesize that flow above a critical rate opens precapillary sphincters and allows enhanced perfusion of myocardial capillaries, thus maximizing the surface area of virus delivery and reducing diffusion distances. Finally, the presence of the vector within the donor organ during the inevitable rewarming that occurs at the time of the transplant procedure may represent a helpful method to increase gene transfer efficiency. However, from a biosafety standpoint, the prolongation of the dwell time into the period of warm ischemia raises concerns related to the subsequent distribution of the virus to other tissues when blood circulation is restored.

In summary, this study demonstrates that efficiency of adenovirus-mediated gene transfer to endothelial cells, vas-
cular smooth muscle cells and cultured thoracic aortas is significantly reduced by low temperature. Requirement of donor organ cold preservation may undermine the effectiveness of gene therapy in the transplant setting.

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References


Appendix A. Conference discussion

Dr N. Mendler (Munich, Germany): In all instances and in all parameters you have shown the lowest transfection was obtained at 10°C and not what you would expect at 4°C. Do you have any explanation for that phenomenon?

Dr Pellegrini: Even if efficiency of gene transfer was lower at 10°C than at 4°C degrees, no statistical difference was observed between these groups. For this reason, we did not formulate any hypothesis in order to explain this phenomenon.