Conditions of vector delivery improve efficiency of adenoviral-mediated gene transfer to the transplanted heart

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Abstract

Objectives: Conditions for ex vivo gene transfer to the transplanted heart were studied in a model of syngeneic abdominal heterotopic heart transplantation in the rat. Various methods of adenoviral-mediated gene transfer to the transplanted heart were compared. Methods: In the first experiment, a dose response study, an adenoviral vector encoding the β-galactosidase gene was infused into the donor heart with the pulmonary artery open and flushed out prior to performing the transplant. In the second experiment, the effects of clamping the pulmonary artery during vector infusion and not flushing out the viral solution, resulting in vector dwell during the warm ischemia, were examined. Results: In the first experiment, gene transfer was relatively inefficient; however, transgene expression improved with increases in the vector dose (range, $1 \times 10^7$–$1 \times 10^9$). The efficiency of gene transfer was significantly greater when the conditions of the second experiment were applied. In all models studied, cardiomyocytes and not vascular endothelial cells were the predominant cell type transduced. Conclusions: This study indicates that the conditions of adenoviral vector delivery are critical for optimizing gene transfer in the transplant setting. In addition, intravascular administration of adenoviral vector to the donor heart results predominantly in cardiomyocyte transgene expression.

Keywords: Adenovirus; Gene therapy; Gene transfer; Heart; Transplantation

1. Introduction

Heart transplantation is an accepted treatment for end-stage cardiac disease in selected patients [1,2]. However, limitations to clinical heart transplantation include acute and chronic rejection, infection, and the side-effects of immunosuppressive therapy. Gene therapy offers the potential to modify these processes at the molecular level. In previous in vivo studies of vascular gene transfer, one challenge has been the need to interrupt blood flow to obtain luminal administration of recombinant DNA [3,4]. This limits the time in which the vector is in contact with target cells. Heart transplantation may be a more ideal setting for gene transfer as a more prolonged dwell time can be achieved during the necessary period of ischemia. Vector delivery during aortic cross-clamping in general cardiac surgery may also be possible.

Adenoviral vectors have been used to achieve efficient transfer and expression of recombinant genes in different vascular beds, both ex vivo and in vivo [5,6]. One advantage of these vectors for gene transfer to the vasculature is the ability to transduce non-replicative cells. Adenoviral vectors may stimulate a host immune response directed at the transduced cells [7] which may limit the duration of gene expression and result in toxicity. However, the necessary use of immunosuppression in heart transplantation may reduce this effect and has been shown to prolong gene expression [8].

Earlier studies have demonstrated the feasibility of gene transfer to the transplanted heart with liposomal [9] and adenoviral vectors [10]. However, the conditions for optimal gene transfer to the transplanted heart using adenoviral vectors have not been defined. This series of experiments was designed to investigate the efficiency of gene transfer using varying conditions for gene transfer in the heart transplant setting. Specifically, we sought to examine the effect of viral dose, the presence of virus in the donor heart during warm ischemia and the effect of clamping the pulmonary artery during vector delivery.

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2. Materials and methods

2.1. Animals

Inbred male Lewis rats, weighing 250–320 g, were used as syngeneic donors and recipients. 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. 86-23, revised 1985). All post-operative rats received analgesia and were allowed to recover with oxygen in a warm environment.

2.2. Adenoviral vector

A first generation E1A deleted (replication-defective) adenovirus encoding for non-nuclear-targeted β-galactosidase under the control of the cytomegalovirus (CMV) promoter was used in these experiments (AdCMVLacZ was a kind gift from James Wilson, Institute for Gene Therapy, University of Pennsylvania). The recombinant virus was propagated in 293 cells (human embryonic kidney cells which provides the E1 gene in trans) [11], and then isolated and purified. Viral titers were determined by plaque assay and expressed as plaque forming units/ml (pfu/ml). For the experiments, viral doses are expressed as pfu/ml.

2.3. Adenovirus delivery

A volume of 350 μl of viral solution (diluted in 2% fetal calf serum in 199 medium) was used in all experiments. Sham animals injected with the same volume of medium were used as controls. The volume for coronary infusion in the rat was determined in an earlier experiment in which medium stained with Evans Blue was used. The stained medium was infused into the aortic root using the same technique as for all the experiments in this study and by the same operator in order to maintain consistency and to avoid differences in the infusion pressure. The volume of medium infused resulting in the effluent from the pulmonary artery becoming fully stained was determined in five animals and the mean volume was used in all further experiments (data not shown).

In addition, the hearts of five animals were weighed and the mean weight was 1 g. This was used in expressing the viral dose as pfu/g of heart tissue.

2.4. Operation and gene transfer

Abdominal heterotopic heart transplantation using standard microsurgical techniques was performed [12]. After anesthesia, the donor rat was intubated and ventilated (Harvard Rodent Ventilator). A median sternotomy was performed to expose the heart. The rat was heparinized with 200 units of aqueous heparin injected into the inferior vena cava. The innominate artery was cannulated with a 24-gauge cannula and the vena cavae and pulmonary veins were ligated en bloc with 4/0 silk. The pulmonary artery was divided and the ascending aorta tied distal to the cannula. The donor heart was arrested with an infusion of cold cardioplegic solution (Plegisol, Abbott Laboratories, Abbott Park, IL) into the aortic root at a rate of 0.44 ml/min for 3 min via the indwelling cannula. The donor heart was then excised and transferred to a cardioplegic solution at 4°C. A volume of 350 μl of viral solution was infused over 5 s into the coronary arteries via the aortic root. After 60 min of cold storage at 4°C, all donor hearts were heterotopically transplanted into the recipients by end-to-side anastomoses of the aorta and the pulmonary artery to the abdominal aorta and inferior vena cava, respectively using 10/0 monofilament sutures. The function of the grafts was assessed daily by palpation of the beating transplanted heart.

2.5. Experimental groups

In experiment A, the pulmonary artery was left open during viral infusion and at the end of 60 min, the virus was flushed out with repeat infusion of cold cardioplegic solution prior to transplantation. A dose response experiment was carried out to determine the optimal viral concentration for gene transfer under these conditions. Viral doses of $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$ pfu/ml, and medium only as the control, were used ($n = 5$ for control, $1 \times 10^7$ and $1 \times 10^8$ pfu/ml; $n = 6$ for $1 \times 10^9$ pfu/ml). When expressed as pfu/g of tissue, these doses correspond to $3.5 \times 10^6$, $3.5 \times 10^7$ and $3.5 \times 10^8$ pfu/g. The optimal viral dose was then used in subsequent experiments (below) to compare the efficiency of gene transfer under different conditions.

In experiment B, using the viral dose as determined in the initial experiment ($1 \times 10^9$ pfu/ml), the pulmonary artery was clamped during viral infusion and the virus was not flushed out with cold cardioplegic solution at the end of 60 min prior to performing heart transplantation ($n = 6$).

Having determined the above improved conditions for gene transfer, a further dose response study (experiment C) was carried out with two additional viral doses ($1 \times 10^8$ and $1 \times 10^10$ pfu/ml, $n = 4 >$ for each group) to determine the optimal dose for gene transfer under the conditions of experiment B.

2.6. Heart retrieval and histochemical analysis

Four days after surgery, the transplanted heart was removed and flushed with saline. A midventricular cross-section was embedded in OCT compound (Miles, Elkart, IN) and snap frozen in liquid nitrogen. Five 5 μm thick cryostat sections were cut at 25 μm intervals. The specimens were fixed in 1.25% glutaraldehyde for 10 min at 4°C and rinsed three times with phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD). The sections were then stained in a solution of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 4.5 h at 37°C. The speci-
mens were then rinsed in PBS and counterstained with eosin. Blue stained cells indicated the presence of β-galactosidase expression. For quantitative analysis, the total number of positive staining cells/section were counted under magnification (×100), and the mean value was calculated from five sections in each animal. Then, the median values were calculated for each group and the results analyzed.

2.7. Statistical analysis

The results are expressed as the median (range) of the number of positive staining cells/section in each group. As the data did not appear to follow a Gaussian distribution and the variances were unequal and could not be stabilized by transformation, a non-parametric test (Kruskal–Wallis) of analysis of variance was performed to evaluate overall group differences for more than two groups. If an overall significance was present, Dunn’s post hoc test was used for pair comparisons (Prism GraphPad, San Diego, CA). For comparison between two groups, Mann–Whitney test was used. A P value of less than 0.05 was considered significant.

3. Results

The operative mortality was 6%. All transplanted hearts were beating at the time of harvest. The mean transplant time (during implantation of the donor heart) was 29.7 min (range, 25–35 min). There were no differences in ischemic times between the groups. In the initial dose response study (experiment A), the median (range) number of positive staining cells for β-galactosidase/section was 0.2 (0–1.8) in the 1×10^7 pfu/ml group, 1.8 (0–12.6) in the 1×10^8 pfu/ml group and 17.8 (0.6–37.6) in the 1×10^9 pfu/ml group (P < 0.05, 1×10^7 vs. 1×10^9 pfu/ml). No sham transplanted hearts stained for β-galactosidase (Fig. 1).

In experiment B, a combination of clamping the pulmonary artery during viral infusion with a period of warm dwell was evaluated. The total number of positive staining cells (median (range)) for β-galactosidase was 238.1 (32–608.2) in the 1×10^9 pfu/ml group. This resulted in a significant increase in gene transfer (P < 0.005) compared with the 1×10^9 pfu/ml group in experiment A (Fig. 2).

Having determined that experiment B represented dramatically improved conditions for gene transfer, a further dose response evaluation was carried out with two additional viral concentrations (1×10^8 and 1×10^10 pfu/ml) under the same conditions as experiment B. The total number of positive staining cells for β-galactosidase (median (range)) was 0.4 (0–0.8) in the 1×10^8 pfu/ml group and 276 (109–564.2) in the 1×10^10 pfu/ml group (Fig. 3). There was no difference in the efficiency of gene transfer between 1×10^9 and 1×10^10 pfu/ml, but gene transfer was significantly less efficient when a dose of 1×10^8 pfu/ml was used (P < 0.05).

All cell types, including cardiomyocytes, endothelial cells and interstitial fibroblasts, stained positively for β-galactosidase (Fig. 4a–c). The transduction of cardiomyocytes was most efficient. There was no consistent pattern of distribution of staining, with the exception that staining appeared somewhat more accentuated around subepicardial zones of organizing ischemia. There was no difference in the extent of transgene expression through both right and left ventricles. None of the sham sections stained, including areas of ischemic damage. There were no inflammatory cells seen in either sham or β-galactosidase transduced transplanted hearts.

4. Discussion

This series of experiments was designed to determine the
conditions for optimizing gene transfer in the heart transplant setting. We sought to perform experiments with conditions relevant to clinical transplantation. In agreement with an earlier study using a mouse model [13], the results showed that gene transfer to the transplanted heart using adenoviral vectors is feasible. The efficiency of gene transfer, however, was markedly affected by the experimental conditions.

In the initial dose response experiments, gene transfer at 4°C, although dependent on vector dose, was inefficient. This level of gene transfer may well be inadequate for effective clinical gene therapy. The reason for the inefficiency was unclear, but potential causes include temperature and anatomical barriers to vector penetration. Adenoviral uptake into cells occurs via receptor-mediated endocytosis [5], and the rate of incorporation into the target cells is temperature dependent [13]. Recently, the steps involved in cellular attachment and internalization of the adenovirus vector have been defined [14,15]. The expression of αvβ5 integrin in the target cells is important for efficient adenovirus-mediated gene transfer [14]. In addition, it is also known that anatomical barriers may impose significant limitations on the penetration of adenoviral vectors [16]. The penetration of particles in the size range of adenoviral vectors in the arterial system is dependent on the distending pressure [16].

We, therefore, evaluated the potential benefits of further distending the coronary system and leaving the virus in the donor heart during warm ischemia by clamping the pulmonary artery during vector delivery and not flushing out the viral solution prior to transplantation. This resulted in a highly significant improvement in the efficiency of gene transfer. While the current set of experiments was performed in the syngeneic model, we believe that the results obtained are also relevant to the clinically applicable allogeneic setting.

While the experiments described in this study do not differentiate whether greater coronary distension or viral dwell during the transplant procedure is of most importance, the warm ischemic period may be the most important difference between experiments A and B. Indeed, transgene expression was somewhat more accentuated in the peri-ischemic regions. Areas that warm up may get ischemic first and if temperature is a key factor in improving gene...

![Fig. 3. Effect of viral dose on the efficiency of gene transfer in the transplanted heart in a syngeneic rat abdominal heart transplantation model under the conditions of experiment C, as defined in the text. The data are represented by a scatter diagram with the median and actual data of the number of positively stained cells for β-galactosidase/section. Asterisk denotes a significant difference compared with 1×10⁸ pfu/ml dose (Kruskal-Wallis, Dunn post hoc, P ≤ 0.05). n = 4 for groups 1×10⁸ and 1×10⁹ pfu/ml; n = 6 for group 1×10¹⁰ pfu/ml.](image)

![Fig. 4. (a) Myocardium from a control animal (experiment A), showing no blue staining, indicating a lack of endogenous β-galactosidase activity. (b) Myocardium from an animal in experiment B showing the overall staining intensity and distribution of expression of the β-galactosidase gene as indicated by the blue stained cells. (c) Cardiac myocytes and endothelial cells (arrow) showing expression of the β-galactosidase gene from an animal in experiment C.](image)
transfer [17], it is not unexpected that increased transgene expression would be present in the peri-ischemic areas.

Having identified that the conditions in experiment B resulted in more efficient gene transfer, the second dose response experiment showed that a viral concentration of $1 \times 10^9$ pfu/ml remained the most effective dose. The reason for this is unclear, but it appears that there is a threshold concentration before efficient gene transfer occurs. In addition, increasing the viral dose ten-fold to $1 \times 10^{10}$ pfu/ml did not increase the efficiency significantly. Higher doses of viral vector were not used due to the large amount of virus required and also to avoid the potential for cytotoxicity.

Several methods of gene transfer to the heart have been studied, including direct myocardial injection [18,19] and intracoronary infusion [10,20]. The effectiveness of direct injection was limited by the spatial expression and also the consistent intense inflammatory response seen around the needle track [19]. Our study demonstrated that intracoronary viral infusion resulted in widespread distribution of gene expression and the efficiency of gene transfer was enhanced by clamping the pulmonary artery during vector infusion and leaving the vector in the donor heart during the period of warm ischemia. Furthermore, the accentuation of positive staining in zones of organizing ischemia suggests the relevance of warm ischemia to gene transfer efficiency. There were no inflammatory cells seen in either sham or $\beta$-galactosidase transduced hearts. Therefore, under the conditions of the experiments described, there was no evidence of adenoviral-mediated cytotoxicity. Of interest, it appeared as though the efficiency of gene transfer was greater in cardiomyocytes compared with endothelial cells. This has also been observed recently in studies which have shown that the efficiency of gene transfer to the transplanted heart can be increased by perfusion of the vector through the donor organ using a pump [21]. Similar observations have been made in other animal models [10]. This finding may be of relevance if one is targeting graft vasculopathy. It is unclear whether cardiomyocyte expression, as demonstrated in our model, will have a therapeutic effect on a process which predominantly affects the vascular intima. If one were to over-express a gene with a diffusible product, it is conceivable that expression outside of the vessel wall may impact intimal processes. We have demonstrated this with adventitial over-expression of endothelial nitric oxide synthase (eNOS) [22,23]. The over-expression of non-diffusible gene products may require alternative means of vector delivery which would result in increased endothelial or vessel wall transgene expression. The reason for the increased transduction efficiency of cardiomyocytes versus endothelial cells is unclear, but may be related to structural differences between cell types or surface receptor expression. However, targeted expression to the vasculature would be advantageous if one is trying to treat or prevent cardiac allograft vasculopathy.

In summary, we have demonstrated a method of adenoviral vector administration and defined the optimal conditions for effective gene transfer to the transplanted rat heart using this system. This model results predominantly in cardiomyocyte transgene expression. The methodology used in these experiments is applicable in the clinical cardio-thoracic surgery setting. Additionally, this new technique may allow the introduction of biologically relevant genes that may over-express proteins that have immunosuppressive, cardioprotective or endothelial protective properties. Such genetic alteration of the transplanted heart may, in the future, lead to improved results in heart transplantation. In addition, the selective application of these techniques may be appropriate during aortic cross-clamping in general cardiac surgery.

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References


