Immunosuppression prolongs adenoviral mediated transgene expression in cardiac allograft transplantation

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Abstract

Background: The immune response to adenoviral vectors used in gene transfer limits the duration of transgene expression and thus poses a potential limitation to their effectiveness for gene therapy. The need for immunosuppression in transplantation may modify this immune response and facilitate prolonged transgene expression. This study hypothesizes that in the setting of heart transplantation, the use of routine immunosuppression will prolong adenoviral-mediated transgene expression. Methods and results: In a model of rat heterotopic abdominal heart transplantation, 350 μl of viral solution (1 × 10⁷ pfu/ml) was infused into the coronary arteries of donor hearts at the time of procurement. The duration of transgene expression was examined following (a) syngeneic transplantation in non-immunosuppressed animals (group A); (b) syngeneic transplantation in immunosuppressed animals (group B); and (c) allogeneic transplantation in immunosuppressed animals (group C). After transplantation donor hearts were studied at: 1, 4, 8 and 12 weeks. Transgene expression was assessed by histochemical staining of tissue cross sections for β-galactosidase activity. In the non-immunosuppressed syngeneic group group A, transgene expression had largely disappeared by 4 weeks, whereas in both the immunosuppressed syngeneic (group A) and immunosuppressed allogeneic (group C) animals expression of the reporter gene persisted for the 12 weeks of the study, although the level of expression decreased significantly over time. Conclusions: This study demonstrates that transgene expression using adenoviral vectors is prolonged by immunosuppression in the heart transplantation setting.

Keywords: Gene therapy; Heart transplantation; Adenovirus; Immunosuppression

I. Introduction

Gene transfer offers a potential approach to modifying pathologic processes in heart transplantation. Various strategies have been used to deliver genes to tissues. Adenoviral vectors have been shown to efficiently transduce myocytes in vivo [1] and in vitro [2]. In addition to gene transfer efficiency, the principle advantage of these vectors for the cardiovascular system is the ability to transduce quiescent cells [3]. However, one major limitation of first generation adenoviral vectors has been the transient nature of transgene expression. In previous studies using adenoviral vectors, transgene expression was lost or greatly diminished after 30 days [4,5]. Although the reason for this is not entirely clear, cell mediated immune responses appear to play an important role in the initial loss of transgene expression and the development of neutralizing antibodies by activated B cells reduces the effectiveness of subsequent adenoviral transduction [6,7]. The prolongation of transgene expression in transduced immunologically immature neonatal animal models [8] and with the use of immunosuppression [9] further supports the role of the immune system in this process. This immune response may also prevent second administration of an adenoviral vector due to an increased immunological reaction to such a second dose of virus [10]. In the heart transplantation setting, the necessary use of immunosuppression and the possibility of a prolonged viral dwell time during the period of donor organ ischemia may be a more ideal setting for efficient gene transfer and prolonged transgene expression. This study, therefore, hypothesizes...
that in the setting of heart transplantation, the routine use of immunosuppression prolongs transgene expression in adenoviral-mediated gene transfer.

2. Methods

2.1. Adenovirus vector

A replication defective adenovirus vector encoding for β-galactosidase under the control of the CMV promoter (AdCMVLacZ, a kind gift from James Wilson, Institute for Gene Therapy, University of Pennsylvania) was used as reporter gene. The recombinant virus was propagated in 293 cells and then isolated and purified as previously described [11]. Viral titers were determined by plaque assay and expressed as plaque forming unit per ml (pfu/ml).

2.2. Animals

A total of 152 rats (donors and recipients) were used in these experiments. Inbred male Lewis (RT-1) rats were used as syngeneic donors and Brown Norway (RT-1) rats were used as allogeneic donors. All recipients were Lewis rats (RT-1). 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).

2.3. Operation and gene transfer

Rat heterotopic abdominal 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 Laboratory cannula. The donor heart was arrested with an infusion of 10/0 monofilament sutures. The total period of incubation with the virus was 90 min (60 min cold ischemia during the cold storage period plus a further 30 min of warm ischemia during the transplant procedure when inevitable rewarming of the heart occurs). All rats received analgesia post-operatively and recovered with oxygen in a warm environment. Function of the grafts was checked daily by palpation of the beating transplanted heart.

2.4. Experimental groups

The duration of transgene expression was examined following (a) syngeneic transplantation in non-immunosuppressed animals (group A); (b) syngeneic transplantation in immunosuppressed animals (group B); and (c) allogeneic transplantation in immunosuppressed animals (group C). Immunosuppressed animals were given cyclosporine by daily oral-gastric injections from the day of surgery. The cyclosporine dose was 10 mg/kg/day for the first 2 weeks after transplant and 5 mg/kg/day thereafter until harvest [13]. This dose was chosen as a balance between effective immunosuppression and drug toxicity. All animals were weighed weekly for dose adjustment. After transplantation, animals were studied at 1, 4, 8 and 12 weeks (n = 6 in each group for each time point giving a total number of 72 transplants).

2.5. Heart excision, histochemical analysis and rejection grading

At the termination of the experiment, transplanted hearts were removed and flushed with saline. A midventricular cross section was embedded in OCT compound (Miles, Elkhart, Ind.) and snap frozen in a liquid nitrogen-cooled isopentane bath. Five 5 μm thick cryostat sections were then cut at 25 μm intervals. Specimens were fixed in 1.25% glutaraldehyde for 10 min at 4°C and rinsed three times with phosphate buffered saline (PBS). Sections were then stained in a solution of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) for 4 h at 37°C. The specimens 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 positively staining cells was counted manually for each 5 μm section under magnification (×100).

Fig. 1. Transgene expression by histochemical staining for β-galactosidase at 1, 4, 8 and 12 weeks. The blue cells are positively stained. Panels A–D demonstrate β-galactosidase expression in syngeneic non-immunosuppressed animals at 1, 4, 8 and 12 weeks, respectively. Panels E–H demonstrate β-galactosidase expression in immunosuppressed syngeneic transplants at 1, 4, 8 and 12 weeks, respectively. Panels I–L demonstrate β-galactosidase expression in immunosuppressed allogeneic animals at 1, 4, 8 and 12 weeks, respectively. Note that the greatest transgene expression is in the immunosuppressed allogeneic animals (panels I–L) and that in all three groups the level of expression decreases with time. (All magnifications ×100).
and the mean value was calculated from five sections. The overall mean values were calculated for each group and the results expressed as mean and median number of positively stained cells per section were then analyzed. For assessment of rejection, formalin preserved heart sections were embedded in paraffin, cut, stained with hematoxylin and eosin and then scored according to the guidelines set by the Heart Rejection Working Group [14]. All slides were read by an experienced cardiac pathologist blinded to the identification of the slides.

2.6. Statistical analysis

Results were expressed as mean ± standard deviation, and median (range) of the number of positively staining cells per cross section. As the data did not follow a Gaussian distribution and variances were unequal, a non-parametric test (Kruskal–Wallis) of analysis of variance was performed to evaluate overall group differences for more than two groups. If overall significance was present, Dunn’s post hoc test was used for pair comparisons (Prism, GraphPad, San Diego, CA). A p value of <0.05 was considered significant.

3. Results

Two animals died early from post-operative bleeding and one animal at 8 and 12 weeks died giving an overall mortality of 5%. All transplanted hearts in the study were beating at the time of harvest.

Group A (Non-immunosuppressed syngeneic): The total number of positively stained cells for β-galactosidase activity was 240.6 ± 171.9 (mean ± standard deviation) with

![Graphs showing transgene expression at different time points](https://example.com/graphs)

Fig. 2. (a) Comparison of level of transgene expression in group A, B and C at 1 week. Data represents mean ± SD of the number of positively stained cells for β-galactosidase per section. (—) denotes median value. No significant differences among the groups (Kruskal–Wallis non parametric ANOVA, p > 0.05). (b) Comparison of level of transgene expression in group A, B and C at 4 weeks. Data represents mean ± SD of the number of positively stained cells for β-galactosidase per section. (—) denotes median value. Asterisk (*) denotes significant difference compared to Group A (Kruskal–Wallis non parametric ANOVA, Dunn’s post hoc test, p < 0.05). (c) Comparison of level of transgene expression in group A, B and C at 8 weeks. Data represents mean ± SD of the number of positively stained cells for β-galactosidase per section. (—) denotes median value. Asterisk (*) denotes significant difference compared to Group A (Kruskal–Wallis non parametric ANOVA, Dunn’s post hoc test, p < 0.05). (d) Comparison of level of transgene expression in group A, B and C at 12 weeks. Data represents mean ± SD of the number of positively stained cells for β-galactosidase per section. (—) denotes median value. No significant differences among the 3 groups (Kruskal–Wallis non parametric ANOVA, p > 0.05).
Fig. 3. Evidence of moderate rejection (grade 3A) in allogeneic immunosuppressed heart transplant. (Magnification ×100).

a median (range) of 254.3 (24–510.6) at 1 week, 0.1 ± 0.1 and 0.1 (0–0.2) at 4 weeks, 0.03 ± 0.08 and 0 (0–0.2) at 8 weeks and 0.7 ± 0.8 and 0.4 (0–2) at 12 weeks. (Kruskal–Wallis, non parametric ANOVA, p < 0.05 for all time-points comparisons). Transgene expression was almost gone by 4 weeks post transduction (Fig. 1A–D).

Group B (Immunosuppressed syngeneic): The total number of positively stained cells was 374.2 ± 488.2 and 218 (7.4–1307) at 1 week, 142.5 ± 135.2 and 80.5 (18.6–321.8) at 4 weeks, 44.2 ± 48.1 and 31.2 (0.4–121.4) at 8 weeks and 13.7 ± 23.7 and 4.8 (0–61.6) at 12 weeks (Kruskal–Wallis, non parametric ANOVA, p < 0.05 for all time-points comparisons). The use of immunosuppression in these syngeneic transplants resulted in prolongation of transgene expression (Fig. 1E–H).

Group C (Immunosuppressed allogeneic): The total number of positively stained cells was 697.1 ± 517.7 and 827 (32.4–1352) at 1 week, 250.8 ± 104.2 and 224.5 (142.6–386.6) at 4 weeks, 162.5 ± 201.4 and 45.2 (0–476.4) at 8 weeks and 75.9 ± 141 and 8.8 (0–326.8) at 12 weeks (Kruskal–Wallis, non parametric ANOVA, p > 0.05 for all time-points comparisons). As with group B, immunosuppression in allogeneic transplants resulted in prolongation of transgene expression (Fig. 1I–L).

In group A (non-immunosuppressed syngeneic group), transgene expression was virtually undetectable by 4 weeks, whereas in both groups B (the immunosuppressed syngeneic) and C (immunosuppressed allogeneic), expression of the reporter gene persisted until the termination of the experiment at 12 weeks. However, the number of positively staining cells per section in both groups decreased substantially with time. There were no significant differences in the number of positively staining cells between the three groups at 1 week although there was a trend towards more positively stained cells in group C (Fig. 2a). At 4 weeks, transgene expression in group C (immunosuppressed allogeneic) was significantly greater than group A (non-immunosuppressed syngeneic), (Fig. 2b) but not group B (immunosuppressed syngeneic). At 8 weeks, transgene expression in both group B (immunosuppressed syngeneic) and C (immunosuppressed allogeneic) was significantly greater than group A (non-immunosuppressed syngeneic), (Fig. 2c). At 12 weeks, there were no significant differences among the three groups (Fig. 2d).

Despite the use of immunosuppression, histologic evidence of rejection was present in many of the allogeneic animals (Fig. 3). At 1 week, 4 animals were completely free of rejection while 2 had evidence of moderate grade rejection (3A). At 4 weeks, 1 animal had low grade (1A) and 5 had moderate grade (3A) rejection. At 8 weeks, 2 animals had low grade (1A) and 4 had moderate grade (3A) rejection. At 12 weeks, 3 animals had no rejection and 3 had low grade (1A to 2) rejection. In the non-immunosuppressed syngeneic group, 2 animals at 4 weeks, 1 animal at 8 weeks and 2 animals at 12 weeks had patchy mononuclear cell infiltrates with rare foci of myocyte damage.

Although all cell types including myocytes, endothelial cells and fibroblasts stained positively for beta galactosidase, the myocyte was the most frequently transduced cell type. There was no consistent pattern of distribution of staining with the exception that staining appeared somewhat more accentuated in the subepicardial region.

4. Discussion

Adenoviral vectors can be used to achieve efficient gene transfer in different animal models and in a variety of cell types, including non-dividing cells. The advantages of these vectors have been previously outlined [15]. One of the major limitations of these vectors has been the tran-
sient nature of transgene expression. Previous studies using adeno-viral vectors have shown that transgene expression is lost or greatly diminished after 30 days [4,5]. This may render these vectors unsuitable for gene therapy approaches to chronic disorders. However, there may be situations when transient gene expression is sufficient to modify pathological processes. It is also possible that immunosuppressive agents may prolong transgene expression [9,16]. This may be of particular relevance in situations, such as heart transplantation, where these agents are routinely administered.

This study has demonstrated for the first time that immunosuppression prolongs transgene expression in the heart transplant setting. Transgene expression was significantly greater at 8 weeks in the immunosuppressed syngeneic and allogeneic animals than in the non-immunosuppressed animals. At 12 weeks, although there was no statistically significant difference in the number of positively staining cells per section detected between the 3 groups, transgene expression was still detectable in the immunosuppressed groups.

The limited duration of transgene expression after adeno-viral-mediated gene transfer may be due to immune or non-immune mediated mechanisms [6]. In support of immune mechanisms playing a role, prolonged transgene expression has been reported in immunologically immature neonatal animals [8], in immunodeficient animals [17] and with the use of immunosuppression [9]. Furthermore, while some authors report the absence of inflammatory infiltrates in the myocardium after catheter-mediated delivery of adeno-viral vectors to both non-transplanted and transplanted hearts [4,18], direct injection has been associated with inflammation around the injection site [19–21]. While some of the inflammation may be due to an effect of the needle, immune-mediated responses to the viral vectors may also be responsible. The latter possibility is supported by our results. In this study, transgene expression was barely detectable in the non-immunosuppressed syngeneic hearts by 4 weeks; but patchy lymphocytic infiltrates consistent with myocarditis were noted in 2 of 6 animals at 4 weeks, 1 of 6 animals at 8 weeks and 2 of 6 animals at 12 weeks. This may indicate an immune response to the vector or transgene even in the absence of detectable transgene expression in these animals. Additionally, we have demonstrated that transgene expression can be prolonged by immunosuppression with cyclosporine after heart transplantation also implying an important role for immunologic factors. It is probable that the broad range of positively staining cells per section is due to the yet unexplained biologic variability of adeno-viral-mediated transgene expression.

While transgene expression was prolonged to at least 8 weeks with the use of cyclosporine, in both the syngeneic and allogeneic immunosuppressed groups, there was a significant decline with time despite immunosuppression. This may have been due to a suboptimal cyclosporine dose used in the current study or non-immune mechanisms. In support of the former hypothesis, rejection was frequently detected by histologic examination in the allogeneic animals suggesting that the dose of immunosuppression used in this study was insufficient to prevent rejection. However, there was no correlation between the number of positively staining cells and cellular infiltrate or degree of rejection in any of the 3 groups. In a recent study, similar decrease in transgene expression was observed in myocardium of athymic rats transduced with an adeno-viral vector [17] suggesting that non-immune mediated mechanism may also limit the duration of transgene expression.

In addition to manipulating the immune response to adeno-viral vectors, as we have done in this study, the problem of transient transgene expression is also being addressed by modifications in vector design. Second generation adeno-viral vectors which have an E1a deletion and a temperature sensitive mutation in the E2a region have been shown to diminish viral protein expression [22]. In a mouse model [23], this has been shown to prolong transgene expression in the liver to at least 8 weeks. Recombinant adeno-viral vectors with all viral genes deleted and which require the use of helper virus for propagation have also been generated, but separation of recombinant and helper virus is a problem with the use of these vectors [24]. Further refinement in vector generation may ultimately lead to a vector system that is efficient, non toxic and is capable of long term transgene expression.

In conclusion, this study demonstrates that transgene expression using adeno-viral vectors is prolonged by immunosuppression in the heart transplantation setting. The decrease of transgene expression with time is probably due to a combination of immune and non-immune mechanisms. This study suggests that the routine use of immunosuppression in the heart transplant setting may prolong transgene expression but the level of expression decreases significantly with time. The effect of alternative immunosuppressive regimens on transgene expression in the heart transplant setting is worthy of further study.

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References