CARDIAC AND PULMONARY REPLACEMENT

EX VIVO ADENOVIRUS-MEDIATED GENE TRANSFER TO THE ADULT RAT HEART

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Objective: The ability to transfer genes to adult myocardium may have therapeutic implications for cardiac transplantation. We investigated the feasibility of adenovirus-mediated transfer of marker genes *LacZ* and *Luciferase*, as well as the potentially therapeutic gene of the human β_2 -adrenergic receptor in a rat heterotopic heart transplant model. **Methods:** Donor hearts were flushed with 10^{12} total viral particles of one of three transgenes. Hearts were harvested at various time points after transplantation. *LacZ*-treated hearts were assessed by histologic staining and *Luciferase*-treated hearts were assayed for specific luminescence activity. Hearts treated with β_2 -adrenergic receptor underwent radioligand binding assays and immunohistochemistry with the use of an antibody specific for the human β_2 -adrenergic receptor. **Results:** *LacZ* hearts revealed diffuse myocyte staining as opposed to none within controls at 5 days. *Luciferase* hearts demonstrated a mean activity of $970,000 \pm 220,000$ arbitrary light units versus 500 ± 200 for the controls ($p = 0.001$). Total β_2 -adrenergic receptor densities (fmol/mg membrane protein) for hearts that received the β_2 -adrenergic receptor transgene at 3, 5, 7, 10, and 14 days after infection were as follows: right ventricle, 488.5 ± 126.8 , 519.4 ± 81.8 , 477.1 ± 51.8 , 183.0 ± 6.5 ,* and 82.7 ± 19.1 ; left ventricle, 511.0 ± 167.6 , 1206.4 ± 321.8 ,* 525.3 ± 188.7 , 183.5 ± 18.6 ,* and 75.9 ± 15.2 (* $p < 0.05$ vs control value of 75.6 ± 6.4). Immunohistochemical analysis revealed diffuse staining of varying intensity within myocardial sarcolemmal membranes. **Conclusions:** We conclude that global overexpression of different transgenes is possible during cardiac transplantation and, ultimately, adenovirus-mediated gene transfer may provide a unique opportunity for genetic manipulation of the donor organ, potentially enhancing its function. (J Thorac Cardiovasc Surg 1998;115:623-30)

Cardiac gene transfer refers to the alteration of the genetic content of cells within the heart. Typically this is facilitated by the vector-mediated

addition of a foreign gene. The further development of such technology might allow novel therapeutic approaches in the treatment of cardiovascular diseases. However, lack of effective and clinically applicable gene delivery systems resulting in high levels of transgene overexpression has been a major obstacle for successful cardiac gene transfer. To date, the adenovirus has proved to be a fairly reliable vector for cardiac gene transfer, because it has the characteristic of being able to infect nondividing cells, which is an absolute requirement for the terminally differentiated myocyte. Direct injection of plasmid deoxyribonucleic acid (DNA) results in localized gene delivery and is not clinically applicable.¹⁻⁴ Percutaneous in vivo delivery systems, such as coronary artery catheterization in rabbits^{5,6} and

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dogs,^{7,8} have had variable success, are difficult to reproduce, and infect a limited region of the heart. Ex vivo delivery systems, such as during cardiac transplantation, are clinically relevant and have been shown to allow gene transfer by several different vectors.⁹⁻¹³

The application of gene transfer to cardiac transplantation is especially appealing because of the direct access to the donor organ at the time of harvest. One could potentially transfer the foreign genetic material at that time, and expression of the transgene could be evident within 12 to 24 hours. This has been demonstrated in mice, rats, and rabbits.⁹⁻¹³ Most of these reports however, have focused on the use and overexpression of a marker gene. Because of differences in individual transgene factors such as transgene immunogenicity, the expression of one transgene does not necessarily correlate to the expression of others. Thus in this report we attempted to deliver three different transgenes to the myocardium, including the human β_2 -adrenergic receptor (β_2 -AR). If myocardium-targeted overexpression of β_2 -AR occurs, cardiac function might be enhanced, as has been shown in transgenic mice.¹⁴

Materials and methods

Construction of recombinant adenovirus. Construction of the cytoplasmic β -galactosidase expressing adenovirus (Ad.LacZ) has been described elsewhere.¹⁵ The *Luciferase* expressing adenovirus (Ad.Luc) was a kind gift from Dr. R. Gerard (University of Texas, Southwestern Medical Center). Construction of the human β_2 -adrenergic receptor (Ad. β_2 -AR), cell culture conditions, and virus preparation have been described in detail elsewhere.¹⁶

Animals. All procedures and protocols were approved by the Animal Care and Use Committee of Duke University. Adult male Long Evans rats (250 to 300 gm), obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were housed under standard conditions and fed a standard diet and water. 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. 85-23, revised 1985).

Heterotopic heart transplantation. Heterotopic intra-abdominal heart transplantation was performed using the technique as previously described by Ono and Lindsey.¹⁷ In brief, both donor and recipient underwent anesthesia with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) injected intraperitoneally. The donor was heparinized. A clamshell incision was created to expose the thoracic organs. Donor hearts were arrested with 5 ml of normothermic Roe's cardioplegic solution (20 mEq KCl, 27 mEq NaCl, 3 mEq MgSO_4 , 250 mg methylprednisolone

sodium succinate, and 2.25 mEq NaHCO_3 for a pH of 7.4). Cardiectomy was performed and 1 ml of adenoviral solution (10^{12} total viral particles) was rapidly injected into the aortic root.

At this point, attention was turned to the recipient. The infrarenal aorta and vena cava were exposed via a midline abdominal incision. An end-to-side anastomosis was performed between the donor and recipient aorta followed by an end-to-side anastomosis of the donor pulmonary artery to the recipient vena cava. Bleeding was controlled with direct pressure. All hearts resumed normal sinus rhythm within 5 minutes. Total ischemic time was between 30 and 50 minutes. During transplantation, the heart was wrapped in gauze and kept at approximately 4° C through use of topical iced saline solution, although myocardial temperature was not measured. After completion of each procedure, the rats recovered under a heat lamp. They were then returned to their cages and allowed free access to food and water. Abdomens were palpated daily to assure a functioning graft.

Experimental design. To assess gene transfer of the marker gene *LacZ*, we gave one group of rats ($n = 6$) the Ad.LacZ solution and another group ($n = 6$) the control solution containing an adenovirus that does not express a transgene (Ad.MT). Both groups of rats were put to death 5 days after transplantation. To assess successful transfer of the *Luciferase* marker gene, we injected one group of rats ($n = 6$) with Ad.Luc and the control group ($n = 6$) with Ad.MT. Both groups were put to death 5 days after transplantation. To determine gene transfer of the β_2 -AR, one group ($n = 6$) received Ad. β_2 -AR and the controls ($n = 6$) received Ad.MT. Both of these groups were put to death 5 days after transplantation and all hearts were divided into three samples: (1) left and right atria, (2) right ventricle, and (3) left ventricle. Furthermore, groups of three rats each were then infected with Ad. β_2 -AR and put to death 3, 5, 7, 10, and 14 days after transplantation. Likewise, all hearts were divided into the previously described three samples.

β -Galactosidase staining. Hearts were excised, rinsed in isotonic saline solution, frozen in a dry ice/isopentane solution, and stored at -80° C. Specimens were mounted on a freezing microtome and 10 μm sections were transferred to glass slides pretreated with aminoalkylsilane (Sigma Chemical Company, St. Louis, Mo.). Sections were fixed in 10% formalin for 2 minutes at room temperature and washed twice in isotonic saline solution. β -Galactosidase staining was carried out in 2 mmol/L $\text{K}_4\text{Fe}(\text{CN})_6$, 2 mmol/L $\text{K}_3\text{Fe}(\text{CN})_6$, 2 mmol/L MgCl_2 , 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside) in tromethamine-buffered saline solution, pH 7.4. After being stained (usually 30 minutes to 2 hours), the sections were rinsed in tromethamine-buffered saline solution and counterstained with eosin.

***Luciferase* assays.** Hearts were excised, rinsed in isotonic saline solution, and weighed. One milliliter per gram of tissue of 5 mmol/L tromethamine/HCl (pH 7.4), 132 mmol/L NaCl, and 0.5 mmol/L of ethylenediaminetetraacetic acid (EDTA) was added. Specimens were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.) and flash-frozen in liquid nitrogen. After one freeze/thaw cycle, the homogenate

Fig. 1. **A**, Cross section of a rat heart taken at the mid-ventricular level ($2.5\times$ magnification) 5 days after Ad.LacZ infection. **B**, Same view at higher magnification ($100\times$). Notice individual myocyte staining.

was centrifuged at $10,000g$ for 2 minutes and the supernatant was saved and assayed for *Luciferase* activity according to the manufacturer's instructions (Promega Corp., Madison, Wis.). In brief, in a 1.5 ml screw-cap Eppendorf tube (Sarsted, Inc., Newton, N.C.), $2\ \mu\text{l}$ of the extract was added to $18\ \mu\text{l}$ of 1x reporter lysis buffer (Promega). Twenty microliters of reconstituted *Luciferase* assay substrate (Promega) was added and incubated for 2 minutes at room temperature. Photon production was then measured in a scintillation counter in single photon counting mode (Beckman Instruments, Inc., Fullerton, Calif.).

β -AR binding assays. Membrane fractions were prepared from hearts and resuspended in binding buffer ($75\ \text{mmol/L}$ tromethamine-HCl, pH 7.4/ $12.5\ \text{mmol/L}$ MgCl_2 / $2\ \text{mmol/L}$ EDTA). Binding assays were performed on $25\ \mu\text{g}$ of membrane protein using saturating amounts of the β -AR-specific ligand [^{125}I]cyanopindolol ($300\ \text{pmol/L}$). Nonspecific binding was determined in the presence of $20\ \mu\text{mol/L}$ alprenolol. Reactions were conducted in $500\ \mu\text{l}$ of binding buffer at 37°C for 1 hour and terminated by vacuum filtration through glass-fiber filters. β_1 and β_2 subtype proportions were determined by competition with varying doses of the β_2 -selective ligand ICI 118,551. All

Fig. 2. β -AR density in native recipient hearts ($n = 6$), control transplanted hearts after Ad.LacZ delivery ($n = 6$), and in β_2 -AR-treated hearts ($n = 6$) 5 days after transplantation. * $p < 0.02$ versus controls.

assays were performed in triplicate, and receptor density (fmol) was normalized to milligrams of membrane protein.

Immunohistochemical labeling. Frozen sections were cut at 10 μ m for indirect immunofluorescence studies. Sections were rinsed three times for 3 minutes in phosphate-buffered saline solution (PBS) and 3 minutes in PBS with 0.05% octyphenoxypolyethoxyethanol (Triton X-100; Union Carbide Corp., Danbury, Conn.) (Triton-PBS), blocked with serum diluent (10% goat serum in PBS with 0.1% bovine serum albumin and 0.1% sodium azide), and then rinsed for 15 minutes in Triton-PBS before overnight incubation at 4°C with a primary rabbit antihuman β_2 -AR antiserum¹⁸ (1:500 dilution in serum diluent). The sections were then washed four times for 10 minutes in Triton-PBS at room temperature and incubated for 1 hour in fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G (1:50 dilution in serum diluent). After five 3-minute rinses in PBS, the sections were mounted with sodium iodide (25 gm/L) in 1:1 PBS/glycerol solution and photographed.

Statistical analysis. Quantitative data such as myocardial *Luciferase* activity and β -AR density after adenoviral transgene delivery is expressed as the mean $\pm$ standard error of the mean. The difference in the level of transgene expression between control and infected hearts was evaluated with Student's *t* test.

Results

Survival. Approximately 10% of all rats that underwent transplantation died within 24 hours of the operation. Deaths were equally distributed among the groups. No animals died if they survived the first 24 hours after transplantation. All hearts were in normal sinus rhythm at time of explantation. Histologic examination revealed no abnormalities and differences between the experimental and control

groups at all the various times when the animals were put to death.

LacZ overexpression. All hearts that underwent infection with Ad.LacZ demonstrated diffuse staining throughout both ventricles, as well as both atria. Staining was nonuniform and myocytes staining blue were found throughout the various layers of the myocardium, with little evidence of endothelial cell infection (Fig. 1). None of the control hearts stained positive for β -galactosidase.

Luciferase overexpression. All hearts that underwent infection with Ad.Luc demonstrated significant overexpression ($970,000 \pm 220,000$ arbitrary light units) as compared with the controls (500 ± 200 arbitrary light units; $p = 0.001$). Because whole hearts were homogenized for this assay, determination of the distribution of this transgene was not studied.

Human β_2 -AR time course of overexpression. Hearts injected with Ad. β_2 -AR revealed marked overexpression in both the right and left ventricles as compared with the control hearts (Fig. 2). This represents an approximate sixfold increase in the right ventricle and fourteenfold increase in the left ventricle. The fraction of β_2 -ARs increased to 90% in the left ventricle from a fraction of 30% in the control hearts, demonstrating that the increased β -AR density was due exclusively to β_2 -AR overexpression. Furthermore, immunohistochemical analysis for the human β_2 -AR revealed several areas of diffuse staining of myocardial sarcolemmal membranes of various intensities (Fig. 3).

In rats put to death 3 to 14 days after transplantation, peak overexpression occurred at 5 days, and β -AR density was back to control values by 14 days, although at 10 days overexpression was still significant. The atria exhibited the same temporal pattern of overexpression as the left ventricle, whereas overexpression in the right ventricle peaked at 3 days and remained at that level for the first 7 days before returning to control values by postoperative day 14 (Fig. 4).

Discussion

The milieu of the heart during transplantation is an ideal situation for the introduction of foreign genetic material into cardiac myocytes. Gene transfer to the donor organ, at the time of harvest, can easily be accomplished with an adenoviral vector. The role of gene transfer for the treatment of cardiac disorders is evolving and may potentially

Fig. 3. Representative immunohistochemical detection of expressed human β_2 -ARs 5 days after Ad. β_2 -AR delivery to transplanted rat hearts. **A**, Cross-sectioned myocytes at 25 $\times$ magnification. **B**, Longitudinally sectioned myocytes at 100 $\times$ magnification.

include applications for the treatment of congestive heart failure, transplant rejection and dysfunction, and ischemic heart disease. However, it remains to be seen whether different transgenes that would be required to treat these disorders can be directed to and overexpressed in the human myocardium under the conditions of current technology.

Recently, the adenovirus vector has been shown to have increased efficacy of gene transfer into cells both in vivo and in vitro.^{6, 11, 12, 16} Recombinant adenoviral vectors have a number of advantages over other viral and nonviral vectors. Adenoviral vectors provide efficient transfer into cells that are terminally differentiated. They can be made replication deficient, can be produced in high titer, and can

carry up to 7.5 kb of exogenous genetic material.¹⁹ Therefore this study took advantage of these characteristics to test delivery of three different transgenes, one with a potential future application of improving myocardial contractility.

The present study demonstrates that the time of cardiac transplantation is an ideal setting for the administration of an adenoviral solution to “genetically modulate” the organ. Several reports have shown that the expression of reporter genes directly injected into the myocardium is limited to a finite area around the site of injection and is further complicated by local inflammation.^{1, 3} Therefore this raises doubts about the clinical applicability of this method of gene delivery. In vivo intracoronary

Fig. 4. Time course of overexpression at 3, 5, 7, 10, and 14 days of β -ARs in atria, right ventricle (RV), and left ventricle (LV) after delivery of 10^{12} total viral particles of Ad. β_2 -AR ($n = 3$ per time point). * $p < 0.05$ compared with β -AR density of control native hearts (75.6 ± 6.4).

delivery has been demonstrated,^{5,6} but it is very difficult to reproduce consistently, probably because of insufficient contact time between the adenovirus and the beating myocardium, as well as a host of other reasons that have yet to be identified.

Unlike direct injection, infection through the coronary vasculature during cardiac transplantation results in robust and widespread overexpression of the transgene, as evidenced by the extensive β -galactosidase staining of the right and left ventricles on histologic examination, as well as the marked overexpression of the β_2 -AR, not only in both ventricles, but in both atria as well. The transplantation model is unique in that it provides the mechanism to accomplish global myocardial gene transfer. First, adenoviral solution is injected into the aortic root, presumably via both right and left coronary arteries, providing access to the entire myocardium. The solution is delivered immediately after removal of the heart at normothermia. No hypothermia is used in this model because this may reduce the efficacy of gene transfer by slowing the kinetics of the virus-receptor interaction. In fact, we have observed lower amounts of adenoviral gene transfer with hypothermia (data not shown). Furthermore, donor heart ischemia may facilitate viral transfection by making the endothelium more permeable. This, combined with the fact that the adenoviral solution is in

constant contact with the myocardium throughout the procedure, likely further enhances gene transfer across the endothelium and into the myocardium itself. This constant exposure probably saturates the viral receptors and must partially account for the robust overexpression of transgenes seen in this model. As opposed to other reports,^{9, 11, 13} we have not observed any significant expression in endothelial cells within the vasculature of the heart, but extensive gene transfer to myocytes was evidenced by the histologic pattern of *LacZ* staining, as well as the pattern of β_2 -AR overexpression detected by immunohistochemistry. The lack of endothelial expression was surprising, and further studies will be required to shed more light on the mechanism of myocyte/endothelial cell gene transfer in our model. Ultimately, however, enhancement of cardiac function through β -AR overexpression requires targeting myocytes rather than endothelial cells, which has been achieved in this report.

The analysis of the time course of gene expression in our model correlates fairly closely with other reports.^{11, 12} Peak β_2 -AR overexpression was reached at 5 days, and by 14 days expression was back to control values. However, we have no explanation for the apparent difference in the kinetics of transgene expression in the right ventricle (Fig. 4). More recently, we have looked at expression at 28

days and have found no evidence of significant overexpression. Some reports²⁰ describe stable gene expression up to months, but these are in different animal models with different delivery systems. Clearly, further work will have to be done to increase the stability of these transgenes. Probable advances will come in work done on the adenovirus itself by further deleting immunogenic regions, in essence "hiding" the adenovirus from the host's immune system. An immune response to adenovirus has been reported^{1, 21} and may be partly responsible for the transient overexpression seen with this model. Of note, immunosuppressive therapies have been shown to greatly extend the duration of transgene expression mediated by recombinant replication-deficient adenovirus.^{22, 23} Although we have not yet examined the role of immunosuppression in our model, it is possible that the immunosuppressive regimen used currently in the cardiac posttransplant setting might allow for extended transgene expression mediated by these viruses. However, this may also prove to make the recipient more vulnerable to potential adenoviral infections using the current vectors. The creation of advanced gene transfer vectors, which is the focus of several groups, should eventually eliminate this possibility. Importantly, in our model, there was no histologic evidence in the transplanted rat hearts of any direct injury caused by the adenovirus out to 28 days after transplantation.

In conclusion, we have shown that ex vivo adenovirus-mediated gene transfer is possible in the adult rat heterotopic heart transplant model. Not only have we demonstrated successful gene transfer of two marker genes, but we have also demonstrated for the first time robust and global overexpression of a gene encoding for an endogenous membrane protein (β_2 -AR), which has been shown to increase myocardial contractility in transgenic mice.¹⁴ Thus, in future studies, it will be critical to document the functional significance of β_2 -AR overexpression in this model to determine whether genetic modification can be successful at improving myocardial function in the posttransplantation setting.

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Discussion

Mr. Magdi H. Yacoub (*London, United Kingdom*). Have you looked at the effect of overexpression of β -receptors on function of these cells in any way, either in vitro or in any other way?

Dr. Alan P. Kypson (*Durham, N.C.*). Yes, we have just started our preliminary studies.

We transfected six hearts with the β_2 -receptor and showed overexpression. (Average β -receptor density was 400 fmol as compared our controls [80 fmol]). These same rat hearts were then mounted on a Langendorff apparatus 5 days after infection and there was almost a doubling of the baseline rate of pressure rise in these infected hearts

as compared with the control hearts. We have yet to do β -agonist dose/response curves as well as evaluate the amount of overexpression correlating with the functional effect. These are very preliminary data, but there does seem to be some sort of a functional enhancement.

Dr. Tirone E. David (*Toronto, Ontario, Canada*). These measurements are done at what stage of the experiment?

Dr. Kypson. At 5 days we remove the heart from the abdomen and perfuse it on a Langendorff apparatus.

Dr. David. Did you do any after 14 days?

Dr. Kypson. No. We have looked only at 5 days so far.

Mr. John H. Kennedy (*Cambridge, United Kingdom*). Is the life of the transfected cells shorter than the life of the host or longer? They are obviously better.

Dr. Kypson. There are some who believe that the cells that overexpress these transgenes undergo an immune response by the host. We have looked at the pathology of these hearts 28 days after transplantation and have seen normal histologic characteristics.

Dr. D. Glenn Pennington (*Winston-Salem, N.C.*). Is there a real difference in the right and the left ventricles, or is that just a matter of muscle mass?

Dr. Kypson. Actually, this probably relates to some sort of an increased transmural pressure wall gradient generated in the left ventricle, which enhances transduction of the viral particles across the endothelium into the myocardium.