CARDIAC AND PULMONARY REPLACEMENT

GENE THERAPY FOR DONOR HEARTS: EX VIVO LIPOSOME-MEDIATED TRANSFECTION

Objective: Liposomes may be an appropriate transfection vehicle for transplanted hearts, avoiding the use of viruses in immunosuppressed hosts and allowing transfection of nondividing cells. To study whether liposome-mediated transfection could be accomplished during transplantation, we used a liposome-reporter gene system in a rabbit model of allograft cardiac transplantation. **Methods:** After aortic crossclamping, Staufland donor hearts were injected with 10 ml Stanford cardioplegic solution; then a 1.3 to 2.0 mg/kg dose of chloramphenicol acetyl transferase in 1:1 deoxyribonucleic acid-liposome complexes was injected proximal to the aortic cross-clamp for coronary artery perfusion. The hearts were transplanted into New Zealand White rabbit recipients in the heterotopic cervical position ($n = 11$ transplants). Recipients were sacrificed at 24 hours. Myocardial specimens (right and left ventricles) and vascular specimens (epicardial coronary artery, aortic root, and coronary sinus) from both the transplanted and native hearts were analyzed for chloramphenicol acetyl transferase protein by means of the enzymatic liquid scintillation assay (counts per minute per milligram of total protein). **Results:** In the recipient, myocardial chloramphenicol acetyl transferase activity was significantly greater in treated donor hearts (mean 4.6×10^5 cpm/mg $\pm 1.1 \times 10^5$ [standard error]) than in native hearts (mean 4.1×10^2 cpm/mg ± 72 [standard error], $p < 0.01$, Mann-Whitney U test). In treated donor hearts, right and left ventricular specimens, as well as apical and basal myocardial specimens, were transfected equally. Chloramphenicol acetyl transferase activity in vascular specimens also indicated transfection (mean 5.4×10^5 cpm/mg $\pm 2.5 \times 10^5$ [standard error]). Chloramphenicol acetyl transferase activity in the coronary sinus was comparable with that in the coronary arteries, which suggests that liposomes can traverse the coronary capillary beds. **Conclusions:** These findings demonstrate that ex vivo transfection of donor hearts with a liposome-reporter gene system results in significant in vivo expression of the transfected gene product after cardiac transplantation. Genetic alteration of myocardium and cardiac vasculature has potential clinical applications in the prevention of posttransplantation rejection, ischemia-reperfusion injury, and both transplant and nontransplant coronary artery disease. (J THORAC CARDIOVASC SURG 1996;111:416-22)

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Gene therapy during transplantation has widespread potential applications in clinical medicine and vascular biology. Transfection of donor hearts before cardiac transplantation might create a form of "organ-specific immunosuppression" by modifying immune or inflammatory pathways, such as cytokine, growth factor, or adhesion molecule expression in the graft. Posttransplantation rejection and arteriopathy might be reduced, as suggested by studies in which antibody blockade of these pathways has been used.¹⁻⁵ Requirements for immunosuppression might be decreased or obviated. Despite these benefits, the risks would be limited only to the donor receiving the transfection treatment, with the recipient remaining unaffected.

Transfection of vascular tissues in multiple animal models has been variably successful, but most previous attempts have used viral vectors. The requirement for viral vectors to introduce desired genes into donor organs has limited immediate clinical applications in transplantation, inasmuch as the risks of retroviruses and adenoviruses in immunosuppressed recipients remain unknown. The advent of a noninfectious method, liposome-mediated transfection, would allow gene therapy to be extended safely to transplanted organs. Early descriptions of efficient systemic transfection with reporter genes in mice after peripheral venous injection⁶ have prompted the investigation of liposome-mediated transfection in a rabbit model of cardiac transplantation.

Transplantation provides an opportunity to transfect the donor organ *ex vivo*. This *ex vivo* organ is isolated from the recipient circulation to localize and to concentrate the administered deoxyribonucleic acid (DNA). The recipient, then, is exposed to neither systemic transfection nor its attendant possible risks. The data regarding transfection during transplantation, however, remain scarce.⁷

This project was designed to determine the feasibility of transfecting donor hearts *ex vivo*, to assess the activity of a transfected gene after transplantation, and to examine regional differences in transfection within the myocardium. To meet these objectives, we investigated transfection of donor hearts in a standard rabbit model of cardiac transplantation. Liposome-mediated transfection with a reporter gene was selected as the mechanism of gene transfer.

Materials and methods

Heterotopic cardiac transplantation was performed in 11 treated rabbit transplants with the use of *ex vivo*

transfection of the donor heart. In each transplant, the untreated native heart served as the control for the transfected donor heart in the same recipient.

Animals. Stauffland rabbits were used as heart donors and New Zealand White rabbits as recipients (R & R Rabbitry, Stanwood, Wash.). The transplant pairs were matched in weight: donor mean 1.4 kg, range 1.1 to 1.7 kg; recipient mean 1.5 kg, range 1.3 to 1.6 kg. Humane animal care complied 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).

Liposomes. DNA-liposome complexes were made with cationic and neutral lipid (1:1) liposomes mixed with plasmid DNA to form a supercomplex (Megabios Corporation, Burlingame, Calif.). The chloramphenicol acetyl transferase (CAT) expression vector contains the human cytomegalovirus enhancer and promoter, as well as a 5' intron from the preproinsulin gene.⁸ The CAT gene is followed by the SV40 early region poly A site. These liposomes were administered in 5% dextrose in water solution as 1.5 mg DNA per milliliter water.

Animal preparation. Anesthesia was induced with a mixture of ketamine (35 mg/kg), xylazine (5 mg/kg), and atropine (0.032 mg/kg) given intramuscularly. After endotracheal intubation, general anesthesia was maintained with halothane in 100% oxygen by mechanical ventilation. Both donor and recipient rabbits were given heparin (1000 U and 500 U intravenously, respectively).

Donor heart excision. The donor venae cavae were ligated and transected; the pulmonary artery was transected. After the aorta was crossclamped, cold (4° C) Stanford cardioplegic solution (10 ml) was infused into the ascending aorta for coronary perfusion.

Liposome-mediated transfection. CAT-liposomes, 1.3 to 2.0 mg/kg DNA diluted in 6 ml of cold (4° C) 5% dextrose in water, were injected into the isolated donor heart proximal to the aortic crossclamp after cardioplegic arrest. After ligation of the pulmonary veins, the excised donor heart was stored in iced heparinized saline solution until its implantation.

Cardiac transplants. The transfected donor heart was transplanted into the recipient by anastomosing the donor ascending aorta to the recipient carotid artery and the donor pulmonary artery to the recipient jugular vein in an end-to-side fashion, a modification of the Carrel technique.^{1,9} The mean ischemic time for this series was 41 ± 6 minutes. The donor heart was resuscitated to spontaneous, continuous beating when blood flow was restored. The recipient rabbit was extubated and allowed to recover. Recipients were sacrificed 24 hours after the transplant operation with euthanasia solution (2.2 ml/kg intravenously) after anticoagulation (heparin, 500 U/kg, given intravenously).

Specimen preparation. All donor and native hearts were excised and sectioned transversely to obtain apical and basal transmural myocardial samples from both the right and left ventricles of each heart. Specimens of approximately 125 mm³ (5 × 5 × 5 mm) of left anterior

Fig. 1. Gene product expression in myocardial specimens. Mean CAT activity, measured by CAT assay, in CAT-liposome treated transplanted donor hearts compared with untreated native hearts excised 24 hours after transplantation; $n = 11$. Error bars represent the standard errors of the mean. The error bar for the left ventricular apical specimens has been interrupted to maintain scale.

descending epicardial coronary artery, a 5 mm length of aortic root including the coronary ostia, and the entire coronary sinus were also removed from the donor hearts. Specimens were frozen in liquid nitrogen.

CAT assay. All cardiac tissue samples were homogenized to cell extracts and aliquots of the supernatants were divided between the two assays described later. The supernatants were incubated with ¹⁴C-chloramphenicol and *n*-butyryl coenzyme A (Promega kit, Promega Corp., Madison, Wis.) for 2 hours at 37° C. A liquid scintillation CAT assay was performed as described by Gorman, Moffatt, and Howard.¹⁰ Total protein content was determined for each sample with BCA protein assay (Pierce kit, Pierce Chemical Company, Rockford, Ill.). Data are expressed as ratios of CAT activity, in counts per minute, to total protein weight, in milligrams, of the tissue sample (cpm/mg) and include the standard error of the mean (SE).

Statistics. Statistical analysis of the data was performed by an independent observer.

Myocardial specimens. Ranked analysis of the data from the myocardial specimens was used. Median CAT activities from the myocardial specimens were compared between corresponding donor and native heart sections by the Mann-Whitney U test. Also, for each heart, the median CAT activity of all right ventricular myocardial specimens was compared with the median from all left-sided specimens by means of Pearson's correlation. The mean CAT activities of apical and basal myocardial

specimens were compared by repeated-measures analysis of variance (ANOVA).

Vascular specimens. For specimens in each vascular location (epicardial coronary artery, aortic root, and coronary sinus), the mean CAT activity in all donor hearts was compared with the means of the other two vascular sections in donor hearts and with the donor myocardial samples by means of ANOVA.

Results

Myocardial transfection. CAT activity, assayed by liquid scintillation, was compared between the myocardial samples for treated donor and control native hearts (Fig. 1). For each myocardial section, CAT activity was significantly higher in the treated donor hearts than in the untreated native hearts, indicating transfection ($p < 0.01$, Mann-Whitney U test). Furthermore, the magnitude of this difference was remarkable: the mean CAT activity in donor hearts, 4.6×10^5 cpm/mg $\pm 1.1 \times 10^5$ (SE) was 1000-fold greater than the mean activity in native hearts, 4.1×10^2 cpm/mg ± 72 (SE). Transfection appeared to be well distributed throughout all regions of the donor heart. For each heart, no significant difference was demonstrated between right

Fig. 2. Gene product expression in vascular specimens. Mean CAT activity, measured by CAT assay, in CAT-liposome treated transplanted donor hearts excised 24 hours after transplantation; $n = 11$. Error bars represent the standard errors of the mean. Note that the scale in this figure is different from that in Fig. 1.

versus left ventricle ($p > 0.05$, Pearson's correlation). Also, mean myocardial CAT activities were not different between apical and basal sections ($p > 0.05$, ANOVA).

Vascular transfection. Fig. 2 illustrates the mean CAT activities recorded from the vascular specimens in treated transplanted donor hearts. The magnitude of CAT activity in vascular samples (mean 5.4×10^5 cpm/mg $\pm 2.5 \times 10^5$ [SE]) and myocardial samples was not different ($p > 0.05$, ANOVA), demonstrating that equivalent transfection occurred in these vascular samples. Mean CAT activities in the epicardial coronary artery, aortic root, and coronary sinus ($p > 0.05$, ANOVA) were not significantly different, which suggests that the liposomes crossed the coronary capillary beds.

Discussion

These experiments were designed to answer three questions: Is ex vivo transfection of donor hearts feasible? Will the transfected gene be expressed in the allograft after transplantation? Are there regional differences in the distribution of the transfected gene in transplanted donor hearts?

The results demonstrate effective expression in transplanted donor hearts of genes transfected ex

vivo. In the current study, the gene product of a transfected reporter gene was demonstrated at 24 hours after cardiac transplantation. Persistence of liposome-mediated transfection has been previously studied by other researchers in nontransplant models. Working with this CAT gene construct, in a slightly different liposome formulation by the same supplier (Megabios), Zhu and associates⁶ reported the presence of transfected DNA for up to 9 weeks after systemic intravenous delivery. With other liposome systems used in vivo by Nabel and coworkers,¹¹⁻¹³ introduced DNA has persisted in transfected arteries for at least 3 weeks. This time limit appears independent of the gene or animal model used. Although this study demonstrates that gene expression is intact after the ischemic insult of the operation, it is unclear whether similar long-term expression can be expected after transplantation; gene expression in the donor heart might also be affected by the transplant recipient's immunologic response to the donor organ and by cell turnover. Nonetheless, the magnitude of the reporter product expression at 24 hours in this study is an auspicious early result. Recent work in this laboratory with this rabbit cardiac transplant model has demonstrated graft infiltration by neutrophils, as well as some

T-lymphocytes, at 24 hours; expression of transfected genes even at 24 hours, then, might be useful in ameliorating ischemia-reperfusion phenomenon or early graft rejection.³ In transplantation, nonpermanent or transient gene expression of 3 to 9 weeks' duration may be sufficient to modify the recipient immune response on initial exposure to donor antigens until natural accommodation to the graft develops.

In this study, no regional differences were found in donor heart transfection. This global nature of transfection is probably the result of nonspecific binding of the cationic liposome-DNA complexes to any cell membrane to which they are exposed.^{14, 15} Also, CAT activities indicated similar transfection in the epicardial coronary arteries and the coronary sinus, suggesting that liposomes can cross the coronary capillary bed. Inasmuch as high endothelial cell-like venules are the site of lymphocyte emigration into the myocardium,¹ this feature of liposome-mediated transfection may prove important in gene therapy directed at the treatment or prevention of rejection.

Since their introduction by Felgner and associates¹⁴ in 1987, liposomes have been demonstrated to provide both a safe^{6, 16, 17} and efficient¹⁸ method of gene transfection. Liposome-mediated transfection does not require cell division, an advantage for cells, such as cardiac myocytes and coronary endothelial cells, with slow turnover. Because of the rapid uptake across cell membranes, highly localized transfection is possible,^{19, 20} thus decreasing or avoiding systemic genetic modification.

Other laboratories, using a different lipid formulation of CAT-liposomes by the same manufacturer (Megabios), have reported efficient transfection of viscera after intravenous administration,⁶ spleen and abdominal lymph nodes after intraperitoneal injection,¹⁸ and lung after endotracheal aerosolization in mice.¹⁹ Liposome-mediated transfection has also been used with success to transfer reporter genes into isolated peripheral and thoracic arteries in vivo^{11, 20-23} and into vascular smooth muscle cells in cell culture.^{22, 24} Recently, biologically functional genes have been transfected into rabbit aortic endothelial cells in organ culture²⁵ and into porcine peripheral arteries in situ.^{12, 13}

Viral vectors, usually retrovirus or adenovirus, are the more frequent current alternatives to liposomes for vascular transfection. Retrovirus has been used to introduce reporter genes into rat myocardium by direct ventricular injection.²⁶ Viral transduction of

peripheral arteries has been accomplished with reporter genes, both by direct instillation of retrovirus or adenovirus into isolated segments and by seeding of cultured cells transduced with retrovirus.^{20, 27-30}

Viral transduction, though, is plagued by various technical difficulties, and the biologic consequences of a viral vector in an immunosuppressed host are unknown. Additionally, antibodies to adenovirus may develop over time, which would limit further therapy.

This study shows that liposome-mediated transfection might provide a practical alternative to viral vectors as an efficient transfection agent for donor hearts. The ex vivo gene delivery technique could also serve as a model for transfection of hearts during routine nontransplant cardiac operations, because the heart is similarly isolated from the systemic circulation during instillation of cardioplegic solution with the aortic crossclamp in place. Either antegrade coronary arterial or retrograde coronary sinus perfusion might be used. Further work will be needed to determine the duration of transfected gene expression in both transplanted and nontransplanted hearts. Histologic identification of the specific cell types transfected will determine which gene targets are chosen for modification, for example, diffusable cytokines or growth factors versus cell-specific gene products.

Conclusions

This project demonstrates that ex vivo liposome-mediated transfection of donor hearts results in in vivo expression of the transfected gene in the recipient for at least 24 hours after transplantation. After aortic root injection, liposomes appear to be distributed equally to all myocardial segments of the heart and provide transfection in both coronary arterial and venous beds. In future applications, liposome-mediated transfection might allow localized genetic alteration of donor organs for amelioration of post-transplantation rejection, ischemia-reperfusion injury, and both transplant and nontransplant coronary arteriopathy.

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Discussion

Dr. Adnan Cobanoglu (Portland, Ore.). In the past decade major advances in molecular biology, gene transfer, and gene targeting techniques have resulted in dramatic expansion of the field of human gene therapy. This effort by the University of Washington group is one of the

few efforts to extend these advances in molecular biology to the transplantation arena and into heart transplantation. The application of gene transfer techniques to organ transplantation would offer the possibility of modification of antigen expression in the transplanted tissues. The major obstacle to therapeutic management of human disease has been the site-specific expression of genes in vivo. The objective of gene therapy is the efficient delivery of the desired genetic material to target cells, resulting in stable and localized production of biologically effective recombinant protein. Dr. Dalesandro has shown us that cationic liposome-mediated transfection is an efficient method of gene transfection and that when transfected ex vivo the gene is expressed in the transplanted heart 24 hours after transplantation. In their study the distribution of the DNA-liposome complexes was homogeneous in the myocardium, and there was no regional difference in donor heart transfection. A few other studies like this one have shown that circulating DNA-liposome complexes appear to readily extravasate across vascular endothelial barriers in all tissues.

Because of access to donor organ at the time of procurement, this technique is uniquely appealing for heart transplant surgeons. The donor organ antigenicity can be controlled by controlling human lymphocyte antigen expression via antisense oligonucleotide transfer. Also, through efforts like this one, genetic modification of nonhuman hearts may allow xenotransplantation.

I have a few questions for the authors, some of which were addressed as future areas of investigation. Will the expression of the transfected plasmid DNA modify biologic function of the arteries or myocardium?

Dr. Dalesandro. Modification of the function of coronary vasculature and myocardium is certainly a goal of this project. In part, gene selection will be determined by the transfection pattern on histologic features, but at this point our histologic system is not perfected. If histologic studies demonstrate that a few focal cells are transfected, we would target a gene encoding a diffusible protein. On the other hand, if histologic studies show widespread transfection of the majority of cells, including the endothelium, then we would try to affect endothelial expression of surface molecules.

Dr. Cobanoglu. The endothelium seems to have higher relative expression of the CAT antigen than myocytes. What do you think the duration of the gene expression in the transfected myocardium will be?

Dr. Dalesandro. We plan to study that in the future. Other work in which this type of liposome was used has demonstrated in both vascular and nonvascular tissues that the duration of the transfection can be up to 11 weeks. In published work, the longest transfection duration is 9 weeks. The experiment that we plan for the immediate future deals with the duration of the transfection in this cardiac transplantation system. We expect to see a similar duration, that is, about 3 months, but a

duration study using liposome-mediated transfection in a transplantation model has not been reported previously.

Dr. Cobanoglu. Were any histologic studies done in these experiments?

Dr. Dalesandro. We have attempted now with two separate monoclonal antibodies to demonstrate the site-specific localization of the CAT protein, but we have not yet evaluated those systems fully. I can discuss only the preliminary results. With the use of cell blocks from transfected donor hearts with the highest CAT assay values, immunocytochemical staining would indicate that only a few of the cells within the cell block have been transfected. These transfected cells, however, have been producing CAT protein vigorously.

Dr. Cobanoglu. Was there an acute cellular rejection or interstitial cellular infiltration?

Dr. Dalesandro. Yes, there was. At 24 hours the cellular reaction represents mostly ischemic damage, with neutrophils seen as the predominant cell type. This rabbit cardiac transplantation model produces full rejection, with lymphocytic and macrophage infiltrates, within 1 week. As we complete our duration studies, we will be seeing these other cellular infiltrates and we can evaluate how that inflammation is interfering or aiding in our transfection. Interestingly, one of our most consistent observations is that the hearts that grossly provide the most evidence of ischemic damage at 24 hours tend to have the most evidence of transfection represented by the highest levels of CAT activity.

Dr. Cobanoglu. Quite a few good investigative models and experiments have been developed in rabbits and mice. What is the next step?

Dr. Dalesandro. We believe that we have much more work to do with our system, so we will continue our work with the rabbit heart transplantation model and possibly a carotid transplantation model, which we have also attempted. Once we have those systems defined, particularly after we know the histologic features of the transfection, then we will be able to progress to work in nonhuman primates and then to human beings.

Dr. D. Craig Miller (Stanford, Calif.). Is the word *transfection* still semantically correct inasmuch as this is not a virally mediated taxicab?

Dr. Dalesandro. Yes, it is. The word *transduction* is specific to biologically active systems and particularly to viruses; however, when a nonbiologically active system is used, such as liposomes or a calcium phosphate system, then the word *transfection* is appropriate.

Dr. David A. Fullerton (Denver, Colo.). Have you compared the efficiency of your transfection with that of a viral carrier?

Dr. Dalesandro. No, we have not, and again the limitation has been histology. Currently we are looking for systems that have better histologic markers as end-product proteins. As soon as we develop such a system, we will be able to investigate the transfection efficiency.