Targeted Modification of Atrial Electrophysiology by Homogeneous Transmural Atrial Gene Transfer

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Background—Safe and effective myocardial gene transfer remains elusive. Heterogeneous ventricular gene delivery has been achieved in small mammals but generally with methods not readily transferable to the clinic. Atrium-specific gene transfer has not yet been reported. We hypothesized that homogeneous atrial gene transfer could be achieved by direct application of adenoviral vectors to the epicardial surface, use of poloxamer gel to increase virus contact time, and mild trypsinization to increase virus penetration.

Methods and Results—We “painted” recombinant adenovirus encoding the reporter gene Escherichia coli β-galactosidase directly onto porcine atria. Investigational variables included poloxamer use, trypsin concentration, and safety. Using the painting method, we modified the atrial phenotype with an adenovirus expressing HERG-G628S, a long-QT-syndrome mutant. Our results showed that application of virus with poloxamer alone resulted in diffuse epicardial gene transfer with negligible penetration into the myocardium. Dilute trypsin concentrations allowed complete transmural gene transfer. After trypsin exposure, echocardiographic left atrial diameter did not change. Left atrial function decreased on postoperative day 3 but returned to baseline by day 7. Tissue tensile strength was affected only in the 1% trypsin group. HERG-G628S gene transfer prolonged atrial action potential duration and refractory period without affecting ventricular electrophysiology.

Conclusions—We show complete transmural atrial gene transfer by this novel painting method. Adaptation of the method could allow application to other tissue targets. Use with functional proteins in the atria could cure or even prevent diseases such as atrial fibrillation or sinus node dysfunction. (Circulation. 2005;111:264-270.)

Key Words: arrhythmia ■ gene therapy ■ electrophysiology ■ atrium

The intense interest in new therapies for atrial fibrillation (AF) illustrates the need for improved treatment of this disease. Conventional antiarrhythmic drug therapy is often inadequate, with AF recurrence in ~50% of patients within 1 year of treatment.1,2 Atrium-specific drugs, radiofrequency ablation techniques, and ativoventricular nodal gene therapy are a few of the newer modalities in various stages of development for AF therapy.3,4 For patients with symptomatic AF, particularly those with underlying structural heart disease, the lack of proven, effective treatment options suggests the need for continued research into alternative forms of therapy.

We hypothesized that gene therapy could become a viable treatment for AF. Development of a gene-therapeutic option will require effective, homogeneous, and specific delivery to atrial myocytes and demonstration of an altered atrial phenotype after gene transfer. Previously reported delivery methods included perfusion through coronary arteries and injection directly into the myocardial wall.4-6 Neither approach would be viable in the atrium. The absence of an isolated blood supply would limit coronary perfusion options, and the wall thickness and geometric complexity would make injection unreliable. Pericardial administration has been reported, but this delivery method did not allow vector penetration beyond the epicardial layer or specific targeting to a particular region of the heart.7 We hypothesized that complete transmural atrial gene transfer could be achieved by epicardial “painting” of adenovirus vectors complexed to poloxamer gels to isolate and prolong contact with the atria and low concentrations of trypsin to allow penetration through the structure. In this proof-of-concept work, we report efficacy and safety parameters of this novel delivery method. In addition, we used the delivery method to transfer a dominant-negative potassium channel to demonstrate the ability of this delivery method to alter the atrial electrical phenotype without affecting ventricular electrophysiology.

Methods

Adenoviruses and Solutions
Recombinant E1,E3-deleted adenovirus expressing Escherichia coli β-galactosidase (Adβ-gal) was a gift from Dr Frank Graham (McMaster University, Montreal, Canada). A plasmid containing HERG-G628S was a gift from Dr Eduardo Marbán (Johns Hopkins University, Baltimore, Md). AdHERG-G628S was constructed by...
using the Cre-Lox system, as previously reported. The resulting virus was plaque-purified, expanded, and characterized as previously described. Virus titers were determined by the average of 2 plaque assays. Virus stocks were free of replication-competent adenovirus when tested by polymerase chain reaction analysis for the E1 gene.

Infection solutions were made by adding trypsin at the appropriate concentration to phosphate-buffered saline (PBS), chilling to 4°C, and adding slowly to 1 g polyoxyethylene/polyoxypropylene block copolymer F127 (also known as Pluronic or poloxamer F127 or P407; BASF Corp) for a final volume of 5 mL. After the poloxamer had dissolved into solution, the mixture was warmed at 37°C to achieve a gel-like consistency. Immediately before use, a total of 5×10^9 plaque-forming units (pfu) Adβgal from stock solution was added to the poloxamer/trypsin/PBS solution for a final virus concentration of 1×10^9 pfu/mL.

Gene Transfer Procedure

Thirty-five domestic pigs (20 to 30 kg) underwent a 3-day protocol that evaluated the effect of trypsin concentration and virus delivery. Five pigs each were included in the following experimental categories: sham-operated controls; Adβgal solution without trypsin/poloxamer; Adβgal solution with 20% poloxamer and 0%, 0.1%, 0.5%, or 1% trypsin; and AdHERG-G628S with 20% poloxamer and 0.5% trypsin. An additional 6 pigs were observed for 21 days after delivery of adenovirus (3 with Adβgal and 3 with AdHERG-G628S) in 20% poloxamer and 0.5% trypsin.

Pigs were sedated with ketamine (30 mg/kg) and anesthetized with sodium thiopental (2 to 5 mL of 5% solution). Anesthesia was maintained with 1% to 2% isoflurane. After sterile preparation, the chest was opened by median sternotomy. The pericardium was incised to expose both atria. The pleurae remained intact, and the lung fields were never exposed. The adeno viral solution was painted onto the atria with a round-bristle, flat paintbrush composed of camel hair. The heart was manipulated to expose all epicardial surfaces of the atria. The 5-mL total volume of 5×10^9 pfu virus/20% (wt/vol) poloxamer/trypsin was divided in half, so each atrium received 2.5 mL of solution. Each atrium was coated twice for 30 seconds each, and ~60 seconds elapsed between painting coats to allow adsorption. After being painted, the atria were left exposed to air for 10 minutes to allow virus penetration. After surgery, pain was managed with narcotics and nonsteroidal anti-inflammatory medications as needed. Postoperative monitoring included 30 seconds of 6-lead ECG recordings daily, as well as daily assessment of behavior and feeding habits.

The animals used in this study were maintained in accordance with the guiding principles of the American Physiological Society regarding experimental animals. The institutional animal care and use committee at Johns Hopkins University approved the experimental protocol.

MAP Recording and Electrophysiological Measurements

Immediately before and 3 or 21 days after gene transfer, monophasic action potential (MAP) and effective refractory period (ERP) were assessed from the epicardial wall under visual control to reproduce the locations. MAPs were acquired in digital format (Boston Scientific) with use of a 7F MAP/pacing catheter (Boston Scientific). The MAP catheter was positioned at the center of 10 predefined epicardial atrial regions and in the basal region of the ventricles, adjacent to atrial sites 4 and 9. The MAP duration was measured as the interval from the steepest part of the MAP upstroke to the level of 90% repolarization (MAPd90) during regular pacing with a drive train cycle length of 400 ms. ERPs were recorded from the center of atrial areas 4 and 9 and from the same basal ventricular regions by programmed stimulation with a drive train cycle length of 400 ms. During follow-up, dofetilide (Sequoia Research Products Ltd), a specific blocker of the rapid component of the delayed rectifier potassium current channel (IKr), was administered intravenously at a dose of 10 μg/kg, based on a previous report, and MAP and ERP recordings were repeated in the presence of intravenous dofetilide.

Tissue Processing

After data acquisition on day 3 or 21, the heart, lungs, and sections of liver, kidney, spleen, skeletal muscle, and gonads were removed and rinsed with PBS. Sections of each atrium were excised for tensile strength testing as described later. Gross specimens were fixed and stained with X-gal, as previously described. Microscopic sections of each organ were fixed; embedded in paraffin; cut to 7-μm thickness; and stained with X-gal, hematoxylin and eosin (HE), and Masson's trichrome, as previously reported. X-gal staining was performed at pH 8 to minimize nonspecific staining. The percentage of cells expressing β-galactosidase was determined by counting cells in 5 randomly selected microscopic sections for epicardial, midmyocardial, and endocardial layers (100 cells per field per layer; 500 cells per atrium for each layer). Histological analysis for inflammation, tissue architecture, and fibrosis was performed in a blinded manner, as previously described.

Measurement of Tissue Structure and Function

Echocardiographic examinations were performed 1 day before and 3, 7, 14, and 21 days after gene transfer. Ejection fraction of the left atrium was calculated from a right parasternal approach. Chamber volume was automatically calculated from the short axis of the left atrium during systole and diastole with software provided with the system (Agilent 5500).

Measurement of Tissue Tensile Strength

Structural integrity of the tissue was evaluated by the progressive addition of force to the tissue to define the breaking point. Strips of atria were cut to a uniform size (10 mm long, 2 mm wide, and 3.5-mm-thick wall) and attached to the tensile strength apparatus. Force on the nonfixed end of the tissue was progressively increased at a fixed rate (0.015 N/s for 5-second intervals followed by 10-second pauses) to define the breaking point of the strip of tissue.

Statistical Analysis

The data are presented as mean±SEM. Statistical differences were determined by Student's t test and repeated-measures ANOVA, where appropriate. A probability value ≤0.05 was considered statistically significant.

Results

Gene transfer efficiency was tested 3 days after painting the solutions containing 10^9 pfu/mL adenovirus (5×10^9 total pfu), 20% (wt/vol) poloxamer F127, and varying concentrations of trypsin in PBS onto the epicardial surfaces of pig atria. The 20% poloxamer concentration was chosen after trials with concentrations from 5% to 30% showed that 20% most effectively went into solution at 4°C and formed a gel at body temperature. In this proof-of-concept work, observations were not extended beyond 21 days to avoid confounding by the loss of transgene expression that has been well documented with first-generation adenovirus vectors (for a review, see Kovesdi et al). Initial studies on the application of 5 mL virus solution containing 1×10^9 pfu/mL Adβgal in the absence of poloxamer or trypsin showed scattered areas of epicardial gene transfer and no penetration into the tissue (data not shown). Addition of 20% poloxamer to the virus solution allowed homogeneous epicardial gene transfer, but penetration of the virus into the myocardium was still negligible. Trypsin at a concentration of 0.1% allowed marginally better penetration, and concentrations of 0.5% and 1% allowed complete transmural gene transfer. Tissues from control animals that underwent the same procedures without addition of virus to the
solution or with addition of AdHERG-G628S failed to show any blue staining.

Microscopic evaluation of tissue sections confirmed the observations from the gross examination. The blue coloration was contained within the myocytes of the Ad β-gal animals, further verifying that gene transfer and not inflammation was responsible for the color change (Figure 1A). The sham and AdHERG-G628S control groups had no microscopic blue coloration, indicating that the blue coloration in the active treatment group was specific for Ad β-galactosidase gene transfer (Figure 1B). The 0% trypsin group had negligible gene transfer beyond the epicardium. The 0.1% trypsin group had negligible gene transfer beyond the epicardium. The 0.1% trypsin sections had detectable β-galactosidase activity in approximately half of the midmyocardial and endocardial cells, and both 0.5% and 1% trypsin treatments had gene transfer to 100% of cells in all layers (Figure 1C). Microscopic evaluation of the atria from the 21-day poloxamer/0.5% trypsin experiments demonstrated persistence of 100% transmural gene expression at that time point.

Gene transfer safety was assessed by clinical evaluation, histological analyses, serial echocardiography, and tensile strength testing. Clinical evaluation included daily ECGs and assessment of behavior and feeding habits. All animals survived the procedure. There were no appreciable differences in behavior or feeding habits between treatment groups. Compared with human responses to median sternotomy, the porcine recovery time was much faster: all animals were behaving normally within 1 to 2 days of the procedure, and pain and tenderness were minimal. Over the observation period, all animals had sinus rhythm on every ECG recording. At the time of euthanization, no spontaneous arrhythmias were seen, and no new arrhythmias were provoked in any animal from the pacing protocols associated with MAP and ERP measurement. Some animals were induced into nonsustained AF with burst pacing during the baseline (prepainting) study, and these animals remained inducible on follow-up. All other animals were uninducible at baseline and during follow-up. No sustained atrial arrhythmias and no ventricular arrhythmias were seen in this study.

Histological analyses included X-gal staining for nontarget gene transfer, HE staining for inflammation and structural changes, and Masson’s trichrome staining for fibrosis. In addition to the atrial results reported earlier, X-gal staining was performed on the ventricles (including the basal ventricular regions touching the atria); lungs; and sections of liver, spleen, kidney, skeletal muscle, and gonads. No intracellular blue coloration was observed on gross or microscopic analysis in any of these nontarget organs, indicating that the painting procedure was target-specific.

Extensive pericardial adhesions were noted in all animals at the time of euthanization. Consistent with these observations, HE staining revealed pericardial and epicardial mononuclear cell infiltrates in all animals (Figure 1D), and Masson’s trichrome staining revealed epicardial fibrosis. Each sample was graded on a scale of 1 to 5 by blinded observers, as previously described.12 There were no statistically significant differences in any histological parameters between treatment groups and control animals that underwent only open-chest manipulation of the heart. There was no association between fibrosis or mononuclear cell infiltration and the presence or type of virus, the presence of poloxamer, or the presence of trypsin, suggesting that the open-chest procedure rather than the gene transfer process caused the fibrosis.

The results of echocardiographic examinations showed no change in left atrial diameter for any of the animals (Figure
2A). Left atrial ejection fraction decreased on day 3 in a dose-dependent manner (Figure 2B). For 0.5% trypsin exposure, serial measurements in the 21-day animals revealed that the decrease in left atrial ejection fraction was transient. There were no significant differences between preoperative measurements and those taken on days 7, 14, or 21 (Figure 2B).

To evaluate the structural integrity of the atrial tissue, strips were cut to a uniform size and tensile strength was measured. Right atrial tensile strength was not changed significantly for any trypsin concentration. Left atrial tensile strength was unaffected for trypsin concentrations up to 0.5%, but the tensile strength of the 1% trypsin exposure group decreased significantly (Figure 3A). For the 0.5% trypsin exposure groups, there were no significant differences in tensile strength when controls were compared with 3- and 21-day post–gene transfer animals (Figure 3B).

Modification of cardiac phenotype was demonstrated with epicardial painting of solutions containing AdHERG-G628S, encoding a dominant-negative mutation for IKr.14 MAPD\(_{90}\) and ERP of atria and ventricles were measured before and 3 or 21 days after virus administration. Electrical homogeneity within individual atria was evaluated by measuring MAPD\(_{90}\) in 10 predetermined regions spread in a gridlike pattern across both atria in all animals (Figure 4A). Baseline measurements on day 0 showed no significant differences within each chamber, but there was an overall increase in MAPD\(_{90}\) in left atrial sites compared with right atrial sites. In the AdHERG-G628S group on days 3 and 21, MAPD\(_{90}\) at all sites was prolonged compared with measurements made on day 0. The homogeneity within left and right atria and the relative differences between the left and the right atrium persisted at these time points, indicating that the electrophysiological effects of HERG-G628S gene transfer were uniformly applied across both atria.

The magnitude of gene transfer effect was evaluated by comparing day-3 and day-21 measurements between AdHERG-G628S and Ad\(\beta\)gal groups. Given the homogeneity of measurements across the 5 areas in each atrium, MAPD\(_{90}\) measurements were taken from area 4 in the right atrium and area 9 in the left atrium (Figure 4B). The Ad\(\beta\)gal group had no significant change in MAPD\(_{90}\) at any time point, indicating that the painting procedure itself was not responsible for any observed repolarization changes. Compared with the Ad\(\beta\)gal group, AdHERG-G628S animals had an MAPD\(_{90}\) prolongation of 15% in the right atrium and 10% in the left atrium on day 3, and on day 21 MAPD\(_{90}\) had been prolonged by 34% in the right atrium and by 46% in the left atrium. Ventricular MAPD\(_{90}\) was measured from the basal region of right and left ventricles adjacent to their respective atria. Consistent with the \(\beta\)-galactosidase results showing no gene transfer to the ventricular myocardium, MAPD\(_{90}\) was unchanged in all animals regardless of treatment.

The ERPs of the 4 chambers were measured by pacing from the center of sites 4 and 9 in the right and left atrium, respectively, and from the basal ventricular region adjacent to both atria. The AdHERG-G628S animals were compared with the Ad\(\beta\)gal animals at days 0, 3, and 21 (Figure 4C). There were no significant differences at day 0. Compared with the Ad\(\beta\)gal group, AdHERG-G628S animals had an ERP prolongation of 5% in the right atrium and of 14% in the left atrium on day 3 and of 31% in the right atrium and of 28% in the left atrium on day 21. Ventricular ERP was unchanged in all animals regardless of treatment.
After baseline measurements on days 3 and 21, the animals received 10 μg/kg dofetilide to pharmacologically block the HERG channel. Measurements of MAPD₉₀ and ERP were repeated for all chambers to compare the level of HERG current in the β-galactosidase and HERG-G628S groups (Figure 4B and 4C). On both days 3 and 21, the Adβgal animals had a prolongation of MAPD₉₀ and of ERP to the same level as pre-dofetilide measurements in the AdHERG-G628S group. The HERG animals had no significant change in MAPD₉₀ or ERP with dofetilide treatment, suggesting that the HERG channel was already blocked by gene transfer and no further blockade was possible. Ventricular MAPD₉₀ and ERP were similarly prolonged in both treatment groups, showing that ventricular HERG function remained intact after gene transfer and therefore could be blocked pharmacologically.

**Discussion**

Treatment of cardiac arrhythmias has evolved significantly since publication of the CAST trial in 1990.¹⁵ That landmark study documented the risk of ventricular arrhythmias from antiarrhythmic drug therapy, leading essentially to abandonment of antiarrhythmic drugs as first-line therapy for ventricular arrhythmias. The question of how best to treat atrial arrhythmias remains open. The recently published AFFIRM and RACE trials documented equivalence of rhythm control (ie, maintenance of sinus rhythm with drugs or electrical cardioversion) and ventricular rate control treatment strategies.¹⁶,¹⁷ Subsequent studies of the AFFIRM patients and of patients undergoing radiofrequency ablation for AF have suggested a survival advantage in maintaining sinus rhythm,¹⁸ implicating the negative effects from antiarrhythmic drugs in the initial AFFIRM and RACE results. With these data in mind, we hypothesized that the specificity afforded by targeted gene transfer would allow modification of atrial electrophysiology without risk of adverse effects on the ventricles. In this report, we have taken the first steps in the direction of AF-targeted gene therapy. Using a novel delivery system, we have documented safety and 100%
efficacy for reporter gene transfer in the targeted region. In addition, we have modified the atrial electrical substrate without affecting ventricular electrophysiology.

The painting delivery model was inspired by 3 observations: (1) previous reports of pericardial vector administration documented patchy epicardial gene transfer and minimal penetration into the myocardium; (2) studies on adenovirus/poloxamer complexes showed increased contact time between virus and target; and (3) early studies on adenovirus behavior reported that exposure of HeLa cells to certain proteases increased the infection efficiency. In addition, a recent article described a similar process for gene transfer ex vivo to canine carotid artery rings and in vivo to rabbit carotid arteries. Those authors found effective gene transfer to the superficial layers without penetration into the muscular layer with the use of trypsin-free solutions and that poloxamer-free solutions required 10 minutes of painting for effective gene transfer.

Poloxamers (or Pluronics) are synthetic polymers that are liquid at colder temperatures and gelatinous at body temperature (for a review, see Kabanov et al. This unique property allows solutions containing poloxamers and other compounds to be mixed at colder temperatures and then solidified into a gel to prolong contact time with a target at body temperature. March et al. were the first to report an increase in gene transfer efficiency after complexing adenoviruses with poloxamers. In cultures of vascular smooth muscle cells, they showed a 10-fold increase in gene transfer after complexing the virus with poloxamer P407.

The beneficial effects of trypsin or chymotrypsin exposure were reported in the early days of adenovirus research. Lonberg-Holm and Philipson demonstrated faster virus attachment to HeLa cell cultures after exposure to trypsin or chymotrypsin. The effect was not universal, however, because exposure to pronase or subtilisin eliminated adenovirus attachment. A conclusion from those studies was that pronase and subtilisin cleaved the receptor and prevented virus attachment, whereas trypsin and chymotrypsin cleaved other surface proteins, thus improving access to the receptor. Fromes et al. extended those findings by including collagenase and hyaluronidase in gene transfer solutions injected into the pericardial space of rats. They observed patchy gene transfer to the ventricular epicardium and to some myocytes penetrating to a depth of roughly half the thickness of the ventricular myocardium. As such, the gene transfer was not transmural; it did not affect 100% of myocytes in any layer, and it was not specific for any portion of the rat heart. There was no comment on any atrial gene transfer with that delivery technique.

Our data demonstrate 100% transmural gene transfer with the epicardial painting method. Complexing the virus to the gelatinous poloxamer matrix and applying it directly to the atria allowed specificity of delivery. Unlike all other reported delivery methods, no evidence of reporter gene expression was found in the cardiac ventricles, lungs, liver, spleen, kidneys, gonads, or skeletal muscle. Dilute concentrations of trypsin allowed transmural penetration without inactivating the virus or cleaving the viral receptor. A potential limitation of this method is the ability to penetrate the epicardial fat often found in human patients. Because our pigs had no epicardial fat, we were unable to evaluate this problem. Another potential limitation is the requirement to open the chest for delivery, but it is easy to conceive of modifications that would allow similar delivery through a thoracoscope.

The overall safety of the method was evaluated by clinical, histological, and functional means. The animals had no spontaneous arrhythmias during limited ECG monitoring and no change in provokable arrhythmias at follow-up. Unfortunately, telemetry was unavailable to completely rule out the possibility of nonsustained arrhythmias during periods between observations. We did not assess animal temperature or blood parameters after adenovirus exposure, but other investigators have found that delivery of similar quantities of adenovirus to pigs causes only a low-grade fever over the first 7 days, minimal inflammatory infiltrates of the target organ, and no significant changes in cardiac function, blood chemistry, or hematology.

A particular concern of ours was the effect that trypsin exposure might have on atrial integrity or function. These safety concerns were allayed by documentation of preserved atrial size and tensile strength for the 0.5% trypsin concentration, a concentration sufficient to achieve transmural gene transfer. A transient decrease in left atrial function was observed on day 3, but this recovered by day 7. Epicardial adhesions and inflammation were found to the same extent in all animals, regardless of which open-chest procedure each experienced. The finding of adhesions, then, is concerning to the same extent that it would be for any other open-chest surgery. Importantly, despite the invasive nature of the delivery method, no adverse events were noted in any animals of this pilot study.

The true test of any new method is its ability to achieve a desired functional effect, in our case, modification of atrial electrophysiology with avoidance of any effects on ventricular function. Our reporter gene studies show complete transmural penetration of gene transfer in the atria and the absence of gene transfer to the ventricles. In addition, HERG-G628S gene transfer caused a homogeneous prolongation of atrial APD and ERP, a finding with significant therapeutic implications (eg, treatment of atrial arrhythmias). Dofetilide treatment of the animals confirmed preexisting block of the HERG channel in the HERG-G628S group. Unlike antiarrhythmic drug therapy, no effect on ventricular polarization was noted after atrially targeted gene transfer with the painting method. Remaining questions include the importance of reaching parts of the atria inaccessible to the epicardial surface (eg, atrial septum, posterior wall), the potential effects of epicardial fat found in most humans but not in the study animals, and the selection of genes appropriate for particular applications.

In summary, we report a novel method for targeted transmural myocardial gene transfer that is safe and effective. AdHERG-G628S data illustrate the use of this method to alter atrial function. Similar application procedures could be used for other thin-walled structures. Ultimately, modification of this technique for use with long-term expression vectors (helper-dependent adenoviruses or adeno-associated virus-es) or less invasive delivery methods (eg, thoracoscopy)
could allow translation into human applications for common diseases such as AF or sinus node dysfunction.

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References

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