**PRECLINICAL STUDY**

**Percutaneous Cardiac Recirculation-Mediated Gene Transfer of an Inhibitory Phospholamban Peptide Reverses Advanced Heart Failure in Large Animals**

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**Objectives**
The purpose of this study was to develop a clinically applicable high-efficiency percutaneous means of therapeutic gene delivery to the failing heart.

**Background**
Substantial advances in the understanding of the cellular and molecular basis of heart failure (HF) have recently fostered interest in the potential utility of gene and cell therapy as novel therapeutic approaches. However, successful clinical translation is currently limited by the lack of safe, efficient, and selective delivery systems.

**Methods**
We developed a novel percutaneous closed-loop recirculatory system that provides homogeneous myocardial delivery for gene transfer in the failing large animal heart. After 4 weeks' rapid pacing in adult sheep to induce HF, the animals were randomly allocated to receive either adenovirus expressing a pseudophosphorylated mutant (AdS16E) of phospholamban (PLN) or Ad–β-galactosidase (AdLacZ).

**Results**
Two weeks after gene delivery, in the presence of continued pacing, left ventricular (LV) ejection fraction had significantly improved in the AdS16E-treated animals (27 ± 3% to 50 ± 4%; p < 0.001), whereas a further decline occurred in the AdLacZ group (34 ± 4% to 27 ± 3%; p < 0.05). In conjunction, AdS16E delivery resulted in significant reductions in LV filling pressures and end-diastolic diameter (both p < 0.05). In conjunction, AdS16E-treated animals showed significant improvement in the expression of PLN and Ca2+–adenosine triphosphatase activity. In separate animals, recirculating AdLacZ delivery was shown to achieve superior myocardial gene expression in contrast to intracoronary delivery and was associated with lower systemic expression.

**Conclusions**
We report the development of a novel closed-loop system for cardiac gene therapy. Using this approach delivery of AdS16E reversed HF progression in a large animal HF model. (J Am Coll Cardiol 2007;50:253–60) © 2007 by the American College of Cardiology Foundation

The development of new technology for the local organ-restricted delivery of molecular-based therapeutic agents would represent a major advance in the design of biologically targeted therapy by achieving satisfactory local concentrations without the potential for systemic toxicity. Toward this end, the convergence of device technology and biologic therapy has recently become a major focus for research and development in the translational area. For example, the development of drug-eluting stents for the prevention of vascular restenosis represents a prime example of the combination of a drug with a device, in this case to selectively inhibit the proliferation of intimal cells within the device itself.

Although some disease paradigms may require very localized delivery, other disease processes may require more homogeneous high-efficiency delivery to specific organs. Heart failure (HF) is a common clinical cardiovascular disorder, characterized by complex pathophysiology and by substantial morbidity and mortality. While the key underlying mechanism in HF is contractile failure of the myocardium, the only currently available pharmacotherapeutic strategies that prolong life are somewhat indirectly acting agents, in particular directed at the sympathetic nervous system and renin-angiotensin system (1). Thus, although the cellular and molecular causes of myocardial failure are
well understood, current therapies do not directly target these disorders. Accordingly, in HF there is a substantial interest in the potential role of gene therapy or in therapies that control gene expression as a therapeutic tool in the treatment of HF, given that the extensive identification of the molecular deficiencies of the failing heart provides a logical basis for the application of specific gene therapy (2–4).

In particular, in HF an extensive body of data indicate that key defects in the regulation of intracellular Ca\(^{2+}\) play a pivotal role in the development and progression of contractile failure. In both experimental models and clinical heart failure, it has been reported that expression and activity of the sarcoplasmic reticulum Ca\(^{2+}\)-adenosine triphosphatase (ATPase) type 2 (SERCA2) is markedly reduced, typically in conjunction with an increase in the expression and the activity of the regulatory protein phospholamban (PLN) (5–7). Together, these defects provide a clear molecular basis for the observations of reduced sarcoplasmic reticulum (SR) Ca\(^{2+}\) release and diastolic Ca\(^{2+}\) overload in cardiomyocytes from failing myocardium (8,9). In support of these observations, studies performed in transgenic animals or by using gene-transfer strategies in small animals confirm the role of these molecular defects in causing myocardial failure (10,11).

The major current limitation for the clinical translation of gene therapy in HF is the development of suitable delivery tools (12,13). Preferably, an ideal delivery mechanism would provide safe, homogeneous myocardial delivery with limited systemic “spillover” or expression and would require as little delivery vector as possible. In the present study, we report the therapeutic delivery of an adenoviral vector encompassing a pseudophosphorylated mutant of PLN, using a novel closed-loop percutaneous catheter-based recirculating cardiac perfusion system in large animals with HF.

Methods

Recirculating delivery of myocardial perfusate and detection of viral distribution through fluorescent imaging. Recirculating perfusate delivery was achieved with the use of a novel cardiac perfusion circuit. Under fluorescent guidance, coronary venous blood was recaptured from the coronary sinus with the use of a percutaneously positioned occlusive balloon recovery catheter. The draining catheter was placed in such a position to exclude theazygous vein, either by occluding it with the balloon or beyond the entry point of theazygous vein. Further, the recovery catheter incorporated a structural element to prevent dynamic col-lapse during the application of suction (VFocus, VKardia Inc., Minneapolis, Minnesota). Venous return was facilitated by the use of a roller pump, followed by reoxygenation using an oxygenator membrane. Oxygenated perfusate was then directed to the left coronary territory via a nonocclusive catheter placed percutaneously in the left main coronary artery. Right coronary artery cannulation was not performed, given that in the sheep this vessel is generally small, with a limited myocardial distribution. Optimization of pump flow is determined by progressively increasing the roller pump speed to achieve a pump head pressure of \(-80\) to \(-100\) mm Hg on the venous side, which we demonstrated as corresponding to a coronary sinus pressure of 0 to 5 mm Hg. At the conclusion of the recirculation period, blood continued to be removed from the coronary sinus for an additional 2 min and this, together with the remaining circuit blood, was discarded to avoid systemic delivery of residual perfusate.

To determine the distribution of myocardial perfusate delivered in this manner we characterized the pattern of delivery of the fluorophore indocyanine green (ICG). Ten milliliters of ICG (2 mg/ml; Akorn, Decatur, Illinois) was injected into the circuit and recirculated for 10 min. Hearts were then explanted and examined using near-infrared spectroscopy (NIRS). Spectroscopic images were acquired as described in detail previously (14).

Animal procedures and gene delivery. Heart failure was induced in sheep by rapid ventricular pacing (180 beats/min) for a period of 4 weeks, as previously described (15). On the day of gene transfer and on the final study day, echocardiographic (Cypress, Acuson, Malvern, Pennsylvania) and left ventricular hemodynamic assessments (Millar catheter) were performed 1 h after rapid pacing was stopped. For gene delivery, animals were randomized to receive either adenoviral serine-to-glutamate “pseudophosphorylated” PLN mutant (AdS16EPLN; \(1 \times 10^{12}\) vp; \(n = 9\)) or adenoviral \(\beta\)-galactosidase (AdLacZ; \(1 \times 10^{12}\) vp; \(n = 6\)), delivered in the antegrade recirculating mode as described in the preceding section. Both vectors were driven by a cytomegalovirus promoter and the AdLacZ was non-nuclear localizing. Repeat hemodynamic and echocardiographic assessment was performed 2 weeks after gene transfer. At the conclusion of the studies, left ventricular samples were collected, rapidly frozen in liquid nitrogen and stored at \(-80^\circ\)C for subsequent molecular, biologic, and biochemical analysis. In a separate cohort of normal animals, we compared the myocardial, pulmonary, and hepatic expression of \(\beta\)-galactosidase after intracoronary (\(n = 3\)) and recirculating (\(n = 3\)) delivery of AdLacZ (\(1 \times 10^{12}\) vp). All animal studies were performed with the approval of the Institutional Ethics Review Committee.

Histology and immunohistochemistry. For histology, paraffin-embedded sections were stained with hematoxylin and eosin and examined under light microscopy. For \(\beta\)-galactosidase immunohistochemistry, sections were deparaffinized in histolene and rehydrated with ethanol. Sections were then washed in 1% \(\mathrm{H}_2\mathrm{O}_2\) to block endoge-
nous peroxidase activity. After brief rinsing, nonspecific antibody binding was suppressed by 1% normal goat serum diluted in Tris-buffered saline (TBS; 10 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl) with 1% bovine serum albumin (BSA). Sections were then incubated with rabbit anti-β-galactosidase antibody (Abcam, Cambridge, United Kingdom) diluted 1:1,500 in TBS with 1% BSA and 0.1% Tween overnight at 4°C. After rinsing in TBS the sections were incubated with horseradish peroxidase-conjugated goat antirabbit antibody (BioRad) diluted 1:250 in TBS with 0.1% Tween for 1 h at room temperature. After several rinsings, the bound antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride as chromogen. Control sections incubated without the primary antibody, as well as sections from non-treated animals, showed very low background.

**Molecular and biochemical analyses.** Expression of PLN and phospho (S16) PLN was analyzed by Western blot analysis using commercially available antibodies (Affinity Bioreagents, Golden, Colorado; and Abcam). For the determination of myocardial SERCA activity, samples were pulverized in liquid nitrogen, and Ca$^{2+}$-dependent ATPase activity was determined using a pyruvate-nicotinamide adenine dinucleotide 3-step coupled reaction (16). Free Ca$^{2+}$ was determined as previously described (17,18). Assays were run in quadruplicate, and conditions were validated in independent reactions using the mycotoxin cyclopiazonic acid (10 μmol/l) to inhibit SR Ca$^{2+}$-ATPase (19), butanedione monoxime (30 mmol/l) to inhibit potential residual levels of Ca$^{2+}$-dependent actomyosin ATPase, and EGTA (4 mmol/l) to determine basal ATPase activity.

**Statistics.** Data are presented as mean ± SEM. Between-group comparisons were performed using an unpaired t test for normally distributed data or a Mann-Whitney test for data that were not normally distributed (as assessed by the Kolmogorov-Smirnov test). Within-group comparisons were performed using a paired t test as appropriate. A p value <0.05 was considered to be statistically significant.

**Results**

**Establishment of recirculating perfusate delivery to the heart.** We developed a novel recirculating technique for the selective percutaneous delivery of gene therapy to the failing heart. As shown in Figures 1A and 1B, the coronary sinus is selectively cannulated with a balloon drainage catheter system that incorporates a retractable nitinol device which maintains venous patency during coronary sinus drainage. This venous recovery system is connected to an extracorporeal pump-oxygenator circuit, with return to the myocardium via a percutaneously placed left coronary arterial catheter for antegrade perfusate delivery. To demonstrate the homogeneity of perfusion, the pattern of distribution of indocyanine green throughout the myocardium was examined using near-infrared spectroscopy. After 10 min of recirculation, a homogeneous transmural pattern of perfusate delivery was evident in a regional pattern consistent with the perfusion territory of the left coronary arteries (Fig. 2). In conjunction, there was no significant accumulation of lactate within the circuit blood during this period (baseline vs. 10 min: 2.9 ± 0.8 mmol/l vs. 3.1 ± 0.6 mmol/l; p = NS), and circuit blood oxygen saturation was maintained at 100% throughout the procedure. The combined application of a recirculating approach to myocardial perfusion with adenoviral delivery showed no histologic evidence of myocardial inflammation or infarction (Fig. 3).

To evaluate the relative efficacy of this novel approach over intracoronary gene delivery, we examined the intensity of myocardial β-galactosidase expression in the myocardium by immunohistochemistry. In preliminary studies we compared myocardial expression of β-galactosidase after delivery of $10^{10}$ versus $10^{12}$ vp AdLacZ. Delivery of the lower amount resulted in minimal or undetectable levels (data not shown). Accordingly, delivery of adenovirus in the range of $10^{12}$ vp was subsequently used. Subsequently, we compared intracoronary (n = 3) with recirculating delivery (n = 3) of $1 \times 10^{12}$ vp AdLacZ in normal sheep. These studies visually indicated greater LacZ expression in animals treated with recirculating
gene delivery. In conjunction, we attempted to quantify β-galactosidase enzymatic activity in myocardial tissue samples. However, we were not able to distinguish activity distinct from that in control samples, possibly owing to the influence of endogenous β-galactosidase as has been previously reported (20). Next, we also determined the capacity of the recirculation system to limit the systemic leakage of adenovirus by investigating the expression of β-galactosidase in the liver and lungs.
As shown in Figure 4, the use of the recirculating approach yielded a higher intensity of β-galactosidase expression in the myocardium and lower expression in the liver and lungs. Notably, during direct intracoronary infusion of AdLacZ there was some evidence of alveolar infiltration consistent with previous reports (21).

**Echocardiographic, hemodynamic, and molecular effects of recirculating S16EPLN delivery.** To evaluate the therapeutic potential of percutaneous recirculating gene delivery in the failing myocardium, we compared the effects of delivery of AdLacZ to those of AdS16EPLN in sheep with pacing-induced HF. Four weeks after the commencement of rapid ventricular pacing, echocardiographic and hemodynamic evaluation demonstrated the presence of significant ventricular dysfunction. Before gene delivery, the group mean left ventricular ejection fraction (LVEF) was 29 ± 2% and the group mean left ventricular end-diastolic pressure was 24 ± 3 mm Hg (n = 15). Despite a further 2 weeks’ rapid ventricular pacing, animals treated with AdS16EPLN showed significant hemodynamic improvement in conjunction with reverse ventricular remodeling, whereas animals treated with AdLacZ demonstrated progressive ventricular failure, as indicated in Figure 5. Specifically, in the AdLacZ animals there was a continued reduction in LVEF from 35 ± 6% to 27 ± 3% (p < 0.05), whereas in the AdS16EPLN animals the LVEF increased from 27 ± 3% to 50 ± 9% (p < 0.001). Consistent with the induction of a process of reverse remodeling, the left ventricular end-diastolic area fell significantly in AdS16EPLN-treated sheep (37.5 ± 1.7 cm² to 32.4 ± 1.7 cm²; p = 0.01), whereas a continued increase in the left ventricular end-diastolic area was observed in the AdLacZ-treated animals (36.2 ± 2.6 cm² to 41.3 ± 2.0 cm²; p < 0.01). Measures of diastolic function also appeared to be favorably affected by AdS16EPLN delivery. In treated animals, the left ventricular end-diastolic pressure fell from 26.3 ± 2.6 mm Hg to 19.9 ± 1.8 mm Hg (p < 0.05), whereas it remained unchanged in the AdLacZ group (Fig. 5). Directionally similar changes also occurred in the peak −dP/dt (AdLacZ: −1,957 ± 219 mm Hg/s to −1,562 ± 206 mm Hg/s; p = 0.09; AdS16EPLN: −1,572 ± 166 mm Hg/s to 1,671 ± 116 mm Hg/s; p = NS) and in the time constant of relaxation, tau (AdLacZ: 39.2 ± 2.4 ms to 41.8 ± 3.5 ms; p = NS; AdS16EPLN: 47.6 ± 1.4 ms to 41.8 ± 3.3 ms; p = NS). In animals receiving AdS16EPLN, the heart rate did not significantly change with gene delivery (64 ± 2 beats/min to 69 ± 3 beats/min). The AdLacZ-treated animals had a significantly higher baseline heart rate (p < 0.05), driven by 2 animals with a significant resting tachycardia; however, AdLacZ delivery did not significantly alter heart rate (95 ± 13 beats/min to 78 ± 9 beats/min).

**Figure 5 Hemodynamic Effect of S16E Gene Delivery on the Failing Heart**

Echocardiographic and hemodynamic effects of AdS16EPLN (n = 9) gene transfer to the failing ovine heart compared with AdLacZ (lacZ) (n = 6) treated animals. (A) Left ventricular (LV) ejection fraction; (B) LV end-diastolic area; (C) LV end-diastolic pressure (EDP); (D) Peak positive dP/dt. Solid bars = before gene delivery; open bars = 2 weeks after gene delivery. AdLacZ = adenovirus β-galactosidase; AdS16EPLN = adenovirus serine-to-glutamate “pseudo-phosphorylated” phospholamban mutant.
To determine the molecular basis for the functional improvement in animals that underwent Ad-S16EPLN gene transfer, we measured the expression of total and phosphor- ylated (S16) PLN by immunoblotting and SERCA activity. As illustrated in Figure 6, immunoblot analysis demonstrated an increase in the abundance of phospho-PLN in S16E-treated animals, and, consistent with this finding, SERCA activity was significantly increased in S16EPLN HF animals compared with those treated with AdLacZ (Fig. 7).

Discussion

Over the past decade, exponential growth in the molecular characterization of HF has led to the identification of a growing number of targets for disease modification. However, many of these are proteins that play critical roles in signaling in other organ systems and would require restricting their expression to the heart in vivo. In the case of HF, the rapid identification of a myriad of molecular targets also presents the same underlying issues in the quest to translate research into the development of a therapeutic tool. For HF, many of the most appropriate targets are intracellular proteins involved in the regulation of intracellular calcium and/or involvement in signaling (4). Accordingly, manipulation of the expression of these proteins is likely best addressed by a gene therapy-based approach, which for safe and effective translation into clinical practice requires the conjunction of several key elements. These include the use of a high-efficiency viral vector that has the capacity to incorporate an appropriate therapeutic gene.

Defective cellular Ca\(^{2+}\) handling, particularly that mediated by the sarcoplasmic reticulum, is a well recognized feature of HF. This phenomenon has been reported in both clinical and experimental HF and has been variously attributed to a reduction in the expression of SERCA protein or its activity (6,22). Alterations in the amount or activity of SERCA have been shown to significantly influence the rate and amplitude of myocardial contraction as well as the extent of relaxation (23). In further support of this notion, SERCA2a gene transfer has been shown to improve both systolic and diastolic function and survival in experimental HF models (24,25). In conjunction with the central role of SERCA in the pathophysiology of HF, the regulatory protein PLN is also pivotal, given its role as a major regulator of SERCA activity. In the nonphosphorylated state, PLN inhibits SERCA activity, whereas when it undergoes phosphorylation this negative regulation is relieved. As such, many studies have shown that PLN is a major determinant of cardiac contractility and of the myocardial response to β-adrenoceptor agonists (26). Given the important role of PLN as a regulator of SERCA, particular emphasis has also been placed upon the influence of the ratio of PLN to SERCA myocardial contractility (27).

In conjunction with altered SERCA expression, disordered PLN expression per se has also been suggested to possibly play a role in HF, although this remains controversial. In particular, it has been shown that PLN is relatively hypophosphorylated in HF, which contributes to the reduction in SERCA activity.
formed cardiac catheterization techniques combined with the application of this approach uses commonly per-
loop cardiac recirculation. From a practical clinical perspec-
infusion of AdLacZ, which was not evident with closed-
sible evidence of lung inflammation following intracoronary
ventricular function is not clear (29). Consequently, several
precise impact of this heterogeneous pattern of expression on
(28). Evidence also exists to indicate that PLN expression
protein structure, have been accompanied by cardiomyopathy
safely tolerated delivery tool that provides homogeneous
tissue distribution. Preferably, such an approach would also
avoid the potential adverse effects of systemic leakage with its attendant risk of systemic gene expression and an immune
response to the viral capsid (36,37). Although many attempts
have been made to develop techniques for somatic gene
transfer in HF, many are inefficient. Current techniques with
possible relevance to HF in particular range from simple
intracoronary injection, to coronary sinus retroperfusion and
myocardial perfusion during cardiopulmonary bypass. Al-
though direct single bolus intracoronary injection may be the
simplest approach, its limitations include the need for coad-
ministration of potentially toxic adjuvant agents and an inher-
ent inability to prevent systemic leakage of the delivery vector
(13,38,39). Coronary sinus retroperfusion (40) has been shown
to provide some myocardial gene delivery; however, it has not
been evaluated in the context of HF. Of note, we have previously observed that balloon coronary sinus occlusion alone
causes a rapid rise in the coronary venous pressure (unpub-
lished observation), which would likely be poorly tolerated by the failing heart. Gene delivery to the heart during full
cardiopulmonary bypass has also been reported (38), although
this approach would have limited application to a broader HF
population.

With these issues in mind, in the present study we developed a percutaneous closed-loop system for myocardial
gene delivery which could be applied to the failing heart in large animals and ultimately in man. We hypothesized that a
closed-loop recirculation system for myocardial gene delivery might achieve several desirable features. First, by
avoiding peripheral systemic delivery or even single-pass intracoronary infusion the concentration of vector reaching the myocardium would be higher. In the present study we showed that the expression of a reported gene was higher
during recirculation compared with intracoronary delivery. Second, we also aimed to reduce systemic delivery of vector
to avoid potential deleterious effects of transgene expression in other organs. Indeed, in the present study we demon-
strated a reduction in the pulmonary and hepatic expression of β-galactosidase. In conjunction, we observed some possible evidence of lung inflammation following intracoronary
infusion of AdLacZ, which was not evident with closed-loop cardiac recirculation. From a practical clinical perspective,
the application of this approach uses commonly performed cardiac catheterization techniques combined with
standard blood perfusion methods commonly used in the cardiothoracic and intensive care settings. In the present
study the recirculating approach was well tolerated in animals with evidence of moderate to severe HF.

On the basis of data suggesting that the restoration of defective control of intracellular calcium homeostasis may be beneficial in HF (5,12,25), we elected to deliver a pseudophosphorylated mutant of PLN, which has been previously shown to rescue the HF phenotype in small animals with HF (10). In the present study, we showed that the recirculating delivery of S16EPLN was able to significantly improve indices of both systolic and diastolic myocardial function in concert with a reversal of the
process of ventricular remodeling that typifies progressive HF. These observations are consistent with earlier studies (10).

Although in the present study we documented that delivery of S16EPLN resulted in an increase in the ratio of
phospho-PLN to total PLN, we were not able to specifically quantitate the relative abundance of endogenous phospho-
PLN to S16EPLN, because of the lack of an antibody specific to S16EPLN. In the present study we elected to measure SERCA activity rather than SERCA protein expression, given that activity per se rather than protein
levels contributes to myocardial function (27). In earlier studies, SERCA delivery has been shown to reduce the
frequency of ventricular arrhythmias (41); however, in the present study we did not perform detailed electrophysiologic
testing or telemetric monitoring.

Going forward, the likely ultimate utility of adenoviral vectors in the clinical setting is limited by their short duration of expression, inflammatory potential, and low transduction efficiency. Additionally, the immunogenic properties of the encoded protein also play a significant role in the ultimate level of expression. Accordingly, current attention is focused on the utility of recombinant adeno-
associated viruses (rAAV), which demonstrate higher effi-
ciency and greater tissue specificity (36,42–44). Despite the
attraction of rAAV as vectors for human gene therapy, translation into the clinic also potentially remains limited.
Although tissue specificity may be greater for specific rAAV serotypes (36), recent studies indicate that inflammatory responses may significantly limit transgene expression and elicit significant target organ damage (45). Adeno-
associated virus gene expression is also increasingly under-
stood to be limited by the prevailing level of AAV antibod-
ies (46). In addition, rAAV are substantially more complex to generate in quantities likely required for clinical use if
delivered systemically, thereby limiting possible clinical translation. Together, these potential limitations of AAV
application to somatic gene transfer will also require the
development of an interventional approach for targeted therapeutic delivery.

In conclusion, we report the development of a novel enabling technology for the percutaneous delivery of mo-
lemar or cellular therapy to the failing human heart.
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