Reversal of Cardiac Dysfunction After Long-Term Expression of SERCA2a by Gene Transfer in a Pre-Clinical Model of Heart Failure

Yoshiaki Kawase, MD,* Hung Q. Ly, MD, MSC,*† Fabrice Prunier, MD, PhD,* Djamel Lebeche, PhD,* Yanfen Shi, MD,† Hongwei Jin, PhD,* Lahouaria Hadri, PhD,* Ryuichi Yoneyama, MD, PhD,* Kozo Hoshino, MD,|| Yoshiaki Takewa, MD, PhD,* Susumu Sakata, PhD,¶ Richard Peluso, PhD,# Krisztina Zsebo, PhD,** Judith K. Gwathmey, VMD, PhD,†† Jean-Claude Tardif, MD,† Jean-François Tanguay, MD,† Roger J. Hajjar, MD*

New York, New York; Montreal, Quebec, Canada; Angers, France; Tokyo, Kyoto, and Nara, Japan; Seattle, Washington; La Jolla, California; and Boston, Massachusetts

Objective

The aim of this study was to examine the effects of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA2a) gene transfer in a swine heart failure (HF) model.

Background

Reduced expression and activity of SERCA2a have been documented in HF. Prior studies have reported the beneficial effects of short-term SERCA2a overexpression in rodent models. However, the effects of long-term expression of SERCA2a in pre-clinical large animal models are not known.

Methods

Yorkshire-Landrace pigs were used (n = 16) to create volume overload by percutaneously severing chordae tendinae of the mitral apparatus with a biopreme to induce mitral regurgitation. At 2 months, pigs underwent intracoronary delivery of either recombinant adeno-associated virus type 1 (rAAV1) carrying SERCA2a under a cytomegalovirus promoter (rAAV1.SERCA2a) (n = 10; group 1) or saline (n = 6; group 2).

Results

At 2 months, study animals were found to be in a compensated state of volume-overload HF (increased left ventricular internal diastolic and systolic diameters [LVIDd and LVIDs]). At 4 months, gene transfer resulted in: 1) positive left ventricular (LV) inotropic effects (adjusted peak left ventricular pressure rate of rise (dP/dt)\textsubscript{max}P, 21.2 ± 3.2 s\textsuperscript{-1} group 1 vs. 15.5 ± 3.0 s\textsuperscript{-1} group 2; p < 0.01); 2) improvement in LV remodeling (% change in LVIDs = 3.0 ± 10% vs. +15 ± 11%, respectively; p < 0.01). At follow-up, brain natriuretic peptide levels remained stable in group 1 after gene transfer, in contrast to rising levels in group 2. Further, cardiac SERCA2a expression was significantly decreased in group 2 whereas in group 1 it was restored to normal levels. There was no histopathological evidence of acute myocardial inflammation or necrosis.

Conclusions

Using a large-animal, volume-overload model of HF, we report that long-term overexpression of SERCA2a by in vivo rAAV1-mediated intracoronary gene transfer preserved systolic function, potentially prevented diastolic dysfunction, and improved ventricular remodeling. (J Am Coll Cardiol 2008;51:1112–9) © 2008 by the American College of Cardiology Foundation

Despite recent advances in treatment strategies, heart failure (HF) remains a leading cause of mortality worldwide (1) with annual mortality rates ranging from 25% to 30% in post-infarction HF patients (2). These facts highlight an important gap between current therapeutic approaches and key underlying biological processes relat-
ing to cardiomyocytes in the setting of chronic cardiac dysfunction.

At the cellular level, HF is characterized by disturbances in excitation-contraction coupling (3,4). Alterations in calcium (Ca2+) handling lead to impaired efflux of intracellular Ca2+ with defects in sarcoplasmic reticulum Ca2+ loading and release. The pivotal role of the sarcoplasmic reticulum Ca2+ ATPase (SERCA2a) pump in Ca2+ homeostasis has been described, with reports of consistently decreased levels of SERCA2a protein expression and activity in the failing human heart (5–7). Prior reports have shown that targeting SERCA2a by gene transfer is feasible and represents a novel therapeutic modality to improve diastolic and systolic disturbances in HF (8–10).

Short-term expression, low efficiency, and immune response elicited by current vectors has mitigated the therapeutic applicability of gene transfer (11). Adeno-associated viruses (AAVs) have emerged as promising viral vectors due to more efficient uptake and stable, long-term expression of transferred genes (12). Moreover, the AAV1 serotype may be ideal for targeting the heart due to its tropism for muscle tissues. Using a swine model of volume-overload HF, we investigated the impact of SERCA2a overexpression by AAV1 gene transfer. Herein, we show that the latter can lead to long-term increases in SERCA2a tissue expression with both structural and functional cardiac improvement.

Methods

Animal care and all procedures were approved by local institutional committees and were performed in accordance with the “Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals” (NIH Publication No. 86–23, revised 1985).

Volume-overload HF model creation. Yorkshire-Landrace swine (n = 26, 33.5 ± 1.8 kg) underwent the procedure to induce volume-overload HF. All study animals were sedated with telazol 6.0 mg/kg intramuscularly and mechanically ventilated with isoflurane 0.5% to 2% and oxygen. As previously reported (13), mitral regurgitation (MR) was created by inserting of an 8-F sheath into the internal carotid artery then under fluoroscopic guidance, a bioprobe (BIOPAL 7, Cordis Corp., Miami, Florida) was positioned in the left ventricle (LV) to rupture the chordae tendinae of the mitral valve apparatus. Serial hemodynamic were obtained with a high-fidelity, pressure transducer catheter (Millar Instruments Inc., Houston, Texas). Severe MR at creation was confirmed angiographically (contrast dye reflux into the pulmonary veins) and hemodynamically (increased LV end-diastolic pressure >16 mm Hg). Subsequently, study animals were allowed to recover and treated with daily oral furosemide (20 mg) and nitroglycerin (2 mg).

Recombinant AAV1 carrying SERCA2a (rAAV1.SERCA2a) vector construction. Vector production, harvest, purification, and testing were done as previously described (14). The rAAV1.SERCA2a vector used in this study contains an AAV serotype 1 viral capsid and a single-stranded ~4.5 kb DNA containing the human SERCA2a cDNA driven by a CMV immediate-early promoter/enhancer, a hybrid intron, and a bovine growth hormone polyadenylation signal, all flanked by 145 nt AAV2 inverted terminal repeat sequences necessary for replication and packaging of the vector DNA in the capsid. The vector was manufactured using standard calcium phosphate transfection methods in adherent 293 cells. Three plasmids were used, 1 containing helper functions from adenovirus, 1 containing the AAV rep2 and cap1 genes, and the third containing the vector genome. Final vector preparations were more than 95% pure as judged by SDS-PAGE (Invitrogen, Carlsbad, California).

Vector delivery. At 2 months, swine underwent intra-coronary injection of either rAAV1.SERCA2a or saline. On the day of injection, the virus was kept on ice after thawing. Subsequently, 1012 DNase resistant particles of rAAV1.SERCA2a solution or a similar volume of saline was delivered via a standard injection pump over 10 min.

Cardiac parameters measurements. All parameters were collected at baseline (pre-MR creation), at 2 months (pre-virus/saline injection) and at 4 months (sacrifice). The average of at least 3 consecutive cardiac cycles was used for each measurement.

A 6-F Millar MIKRO-TIP catheter (Millar Instruments Inc., Houston, Texas) positioned within the LV cavity was used to collect the following parameters: systolic pressure, LV end-diastolic pressure, peak LV pressure rate of rise (dP/dt), and decline (dP/dt), tau value (time constant of isovolumic relaxation), (dP/dt)max and (dP/dt)min and Tau value (time constant of isovolumic relaxation).
Transthoracic echocardiograms were performed under similar conditions (i.e., while study animals were mechanically ventilated with isoflurane 0.5% to 2% with breath holds during image acquisition) using the GE Vivid 7 cardiovascular ultrasound system (GE Medical Systems, Milwaukee, Wisconsin) with a 3-MHz transducer. Serial echocardiographic data were collected in a blinded fashion. The Teichholz method was employed to calculate LV volumes and ejection fraction (LVEF) (15). The parasternal long-axis view was used to measure left ventricular diameters at end-diastole (LVIDd) and end-systole (LVIDs). Mitral regurgitation volume was measured using the PISA method (16).

**Tissue and blood samples.** At study completion (4 months), intracoronary cardioplegic solution (Plegisol, Hospira Inc., Lake Forest, Illinois) was used to perform humane euthanasia. Tissue samples from various organs (heart, brain, skeletal muscle, liver, adrenal glands, kidneys, lymph nodes, and ovaries) were placed in 10% formalin solution for histological assessment. Tissues collected for Western blot and enzymatic analysis were snap-frozen with liquid nitrogen. Serial blood samples were collected for brain natriuretic peptide (BNP) measurements.

**Immunoblotting.** The microsomal fraction was prepared from snap-frozen swine heart (5 to 10 g of heart muscle), pulverized in liquid nitrogen, and homogenized in a buffer solution containing 5 mM Tris–HCl pH 7.4, 2 mM EDTA, and 8.5% sucrose with a Potter homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min. The supernatant was then centrifuged for 15 min at 9,000 × g, the resultant supernatant was spun twice for 15 min at 20,000 × g. Sarcoplasmic reticulum vesicles present in this latter 20,000 × g supernatant were pelleted by a 1-h 110,000 × g spin. The pellet was resuspended in 500 μl of the homogenization buffer. All centrifugations were performed at 0 to 4°C. Protein samples were prepared from isolated swine microsomal fractions, matched for protein concentration (using the Bradford method), and separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membrane blots were incubated with antibodies against SERCA2a and phospholamban (ABR-Affinity Bioreagents, 1:400 dilution, Golden, Colorado) overnight at 4°C. Reactive bands were visualized by chemiluminescence (Bioreagents, 1:400 dilution, Golden, Colorado) overnight at 4°C. Protein samples were prepared from isolated swine microsomal fractions, matched for protein concentration (using the Bradford method), and separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membrane blots were incubated with antibodies against SERCA2a and phospholamban (ABR-Affinity Bioreagents, 1:400 dilution, Golden, Colorado) overnight at 4°C. Reactive bands were visualized by chemiluminescence (Bioreagents, 1:400 dilution, Golden, Colorado) overnight at 4°C.

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).** The mRNA levels of SERCA2a were measured using RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and by adding chloroform after tissue disruption. After centrifugation, the RNA in the supernatant solution was precipitated by adding an equal volume of ice-cold isopropanol. RNA pellets were obtained by centrifugation at 12,000 g for 10 min at 4°C and washed by 75% ethanol. Pellets were resuspended in RNase-free water (Invitrogen). The cDNA was synthesized from 1 μg of total RNA using iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, California) in a final volume of 20 μl. GAPDH mRNA levels served as internal controls. The annealing temperature for PCR reaction cycles was adjusted for each specific primer set. Density values of bands of interest from 3 independent experiments were normalized against GAPDH values.

**Statistical analysis.** Continuous variables were reported as mean ± standard deviation. The 2-sided paired Student t test was used to analyze differences within the same group whereas the unpaired t test, for between-group differences. A p value <0.05 was considered statistically significant.

**Results**

A total of 26 female Yorkshire-Landrace swine were subjected to MR creation. Throughout the study, no significant difference in body weight was documented between study groups. At 2 months, the 16 surviving swine were injected either with rAAV1.SERCA2a (n = 10, group 1) or Saline (n = 6, group 2). Three animals died after virus injection: 1 was due to a post-catheterization stroke–like syndrome; another presented a persistent febrile state with deteriorating general status, and, lastly, 1 expired from worsening HF. Thirteen animals (n = 7 from group 1; n = 6 from group 2) survived to 4 months.

**Cardiac function and remodeling.** This severe MR model resulted in a compensated state of HF as shown by the absence of overt systolic dysfunction in both groups (Table 1). No significant difference was documented between the saline and SERCA2a groups for the following parameters: heart rate (2 months: 94 ± 26 beats/min vs. 83 ± 10 beats/min, 4 months: 88 ± 13 beats/min vs. 86 ± 17 beats/min, respectively; p = NS), peak LV pressure (2 months: 94.9 ± 24.0 mm Hg vs. 92.4 ± 8.0 mm Hg, 4 months: 103.4 ± 14.8 mm Hg vs. 100.6 ± 16.8 mm Hg, respectively; p = NS), and end-diastolic pressure (2 months: 10.9 ± 5.5 mm Hg vs. 9.7 ± 3.5 mm Hg, 4 months: 14.0 ± 1.2 mm Hg vs. 13.9 ± 8.4 mm Hg, respectively; p = NS). A significant difference was documented between the saline and SERCA2a groups for

<table>
<thead>
<tr>
<th>Table 1: LVEF Over Time Between Study Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LVEF</strong></td>
</tr>
<tr>
<td>Before MR creation</td>
</tr>
<tr>
<td>At 2 months</td>
</tr>
<tr>
<td>At 4 months*</td>
</tr>
</tbody>
</table>

*p < 0.02; saline group versus sarcoplasmic reticulum Ca2+ ATPase (SERCA2a) group at 4 months.

LVEF = left ventricular ejection fraction; MR = mitral valve regurgitation.
LVEF (61.5 ± 7.4% vs. 70.6 ± 3.5%, respectively; p < 0.05) (Table 1), (dP/dt)max (1,396 ± 383 mm Hg/s vs. 1,822 ± 317 mm Hg/s, respectively; p < 0.05), and adjusted rate of rise, (dP/dt)max/P (15.5 ± 3.0 s⁻¹ vs. 21.2 ± 3.2 s⁻¹, respectively; p < 0.01) (Fig. 1).

Diastolic function was also evaluated after MR creation. Both groups had comparable Tau values from at 2 months. However, after injection, a trend favoring the SERCA2a group was found with isovolumic relaxation abnormalities progressing in the saline group in contrast to the SERCA2a group (Fig. 2).

At 2 months, the volume-overload HF model was successful in inducing significant changes in ventricular parameters LVIDd as well as LVIDs compatible with remodeling in both groups (Table 2). Moreover, when looking at the progression of ventricular dimensions over time (indirect evidence of ventricular remodeling), there was significant difference between study groups favoring the SERCA2a group (Fig. 3).

Of note, no difference in MR regurgitant volume, as measured by PISA method, was noted in both groups at the time of injection (47.9 ± 26.4 ml, saline group vs. 52.3 ± 21.8 ml, SERCA2a group; p = NS). At follow-up, the values for the regurgitant volume in the saline group at 2 months and 4 months, respectively, were 47.9 ± 26.4 ml to 62.4 ± 35.6 ml, and in the SERCA2a group 52.3 ± 21.8 ml to 47.9 ± 25.8 ml.

The difference between 2 months and 4 months in terms of the

![Figure 1 Differences in Adjusted Peak LV Pressure Rate of Rise](image)

Relative to the saline group, study animals that underwent gene transfer with recombinant adeno-associated virus type 1 (rAAV1) sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a) had: 1) comparable peak left ventricular pressure rate (dP/dt)max/P at the 2-month time point (state of compensated volume-overload heart failure); and 2) significant improvement in (dP/dt)max/P. This confirms a positive effect on left ventricular (LV) inotropy of SERCA2a overexpression.

![Figure 2 Differences in Time Constant of Isovolumic Relaxation](image)

Both study groups had no discernable difference in diastolic function at the time of gene transfer. Over the course of the next 2 months after gene transfer, there was a clear trend favoring the SERCA2a group for lack of deterioration of diastolic function. This confirms a potential positive effect on LV lusitropy of SERCA2a overexpression. Tau = time constant of isovolumic relaxation; other abbreviations as in Figure 1.

![Figure 3 LV Internal Systolic Diameters at 4 Months](image)

Despite comparable diameters at baseline and at the time of gene transfer, the saline group showed evidence of progression of their LV internal systolic diameters at the term of the study. Conversely, animals that underwent rAAV1.SERCA2a gene transfer were documented with persistent, stable LV internal systolic diameters. This confirms a positive effect on LV remodeling of SERCA2a overexpression. Abbreviations as in Figure 1.
regurgitant fraction in the saline group was \( \frac{22.1 \pm 30.2}{30.2} \) while in the SERCA2a group it was \( \frac{-4.4 \pm 16.3}{0.1} \). Cardiac tissue expression of SERCA2a and phospholamban. Cardiac tissue expression of SERCA2a was significantly decreased in the saline group relative to the normal heart group. Conversely, SERCA2a expression levels were restored to normal levels in the SERCA2a group (Fig. 4A). Further, despite a lack of difference in SERCA2a mRNA levels between the saline group and normal heart group, mRNA levels were significantly higher in the SERCA2a group when compared with levels seen in either the saline group or the normal heart group (Fig. 4B). Finally, the relative protein expression of phospholamban was comparable among the different groups as shown in Figure 5.

Additional assessments. Adjusting for body weight, the SERCA2a group had significantly smaller total LV muscle mass \( (3.0 \pm 0.4 \text{ mg/kg} \text{ vs. } 3.6 \pm 0.4 \text{ mg/kg}, \text{ respectively; } p < 0.05) \) and smaller total right ventricular muscle mass \( (0.9 \pm 0.1 \text{ vs. } 1.1 \pm 0.1 \text{ mg/kg}, \text{ respectively; } p < 0.01) \) relative to saline group. In collected cardiac tissue samples, no evidence of histological changes compatible with acute myocardial toxicity (inflammation response, myocarditis, or vasculitis) related to rAAV1.SERCA2a gene transfer was documented. In addition, histological analyses did not reveal any direct evidence of toxicity after gene transfer to other end-organs (results not shown). Despite a wide range of total creatine kinase (CK) levels (between 293 to 1,469 U/l), none of the CK-MB fractions reached significant levels in both groups.

The rAAV1.SERCA2a gene transfer did not adversely affect hematologic parameters. No reticulocytosis or white blood cell count increases (including eosinophils) were documented nor were there increases in fibrinogen levels in the SERCA2a group. Further, at 4 months, no altered renal (electrolytes, creatinine, blood-urea-nitrogen) and hepatic
failing hearts (5,7). Prior reports have shown the successful restoration of the SERCA2a pump levels with a catheter-based technique in a small-animal model of HF with homogenous expression patterns obtained after gene transfer by adenoviral vector (22). However, the latter vector system only allows for transient transgene expression (on the order of days to weeks) (23). In an effort to favor clinical applicability and effectively target chronic progressive disease states (such as HF), more efficient vector systems associated with long-term transgene expression are required.

Adeno-associated viruses are replication-defective, non-pathogenic paroviruses with a single-stranded DNA genome (24,25). These viruses are emerging as promising vectors, exhibiting serotype-specific tissue or cell-type tropism, increased in vivo transduction efficiency to both dividing and nondividing target cells, sustained expression (from several months to years), and reduced immunogenicity compared with other viral vector systems (12,26). With documented greater tissue-specific tropism for certain AAV serotypes, transition towards clinical applicability is increasingly feasible; AAV serotype 1 offers attractive biological profiles, such as muscle tropism and ability to bypass pre-existing immunity (i.e., neutralizing antibodies) due to natural infection (27,28). Concerns of adverse immunologic response from prior experience with adenoviral-mediated gene delivery do not seem to apply to rAAV1 since our histological specimens did not reveal any evidence of inflammatory reaction. In this study, we have thus shown that, using the rAAV1 vector, SERCA2a mRNA and protein levels can be successfully and safely overexpressed in swine hearts up to 2 months after delivery.

**Cardiac gene transfer.** An important challenge to clinical translation of pre-clinical findings has been the efficiency of current myocardial gene transfer strategies (11). Direct intramyocardial viral vectors injection has been reported in pre-clinical studies. However, major technical drawbacks of this approach remain: 1) a limited surface area of the myocardium efficiently transfected; and 2) the surgical nature of this route would conceivably not represent the ideal strategy for the majority of HF patients.

Intracoronary delivery remains an attractive alternative due to its limited invasiveness as well as for the possibility to attain widespread myocardial gene delivery. Initial studies in large-animal models using adenovirus have reported conflicting results in terms of consistency of transfection rates of this delivery strategy (29,30). Potential hurdles hampering efficient viral vector transfection are size and type of vector employed, vehicle composition, coronary flow and pressure, and time of exposure of the vector to the coronary endothelium (31). Our group has previously reported on the reproducibility, feasibility, and safety of percutaneous antegrade myocardial adenoviral gene transfer with concomitant coronary venous blockade in swine hearts (32).

In this study, to simplify the process of intracoronary injection and to test the myocardial transfection efficiency of a different, more cardiotropic viral vector, we chose slow,
selective left coronary artery rAAV1.SERCA2a delivery. This approach not only resulted in successful transfection (increased SERCA2a protein and mRNA levels), but, more importantly, in documented improvement in functional and physiological parameters.

Effect of SERCA2a on cardiac function and structure. Results from transgenic animals (33) as well as small-animal studies have convincingly shown enhancement of contractility resulting from overexpression of SERCA2a. With respect to disturbances of Ca$^{2+}$ handling, the need to better define gene transfer in large-animal models is underscored by known discrepancies between species. Whereas in rats 90% of cytosolic Ca$^{2+}$ is handled by the SERCA2a pump, in human hearts, the latter’s contribution falls to 75% with the remainder of cytosolic Ca$^{2+}$ being managed by the Na$^+$/Ca$^{2+}$ exchanger (20). Further, surprising findings of human dilated cardiomyopathy caused by mutation leading to reduced expression of phospholamban, a direct inhibitor of SERCA2a activity, stands in stark contrast to pre-clinical reports (34). Translational research thus represents not just a logical but necessary step before clinical application.

Bearing in mind known limitations of LVEF as a reliable estimate of systolic function in the setting of severe MR, we believe that our HF model is physiologically relevant in that study groups were most likely in a compensated state of dilated cardiomyopathy. This state represents a commonly occurring, clinically pertinent scenario with increasing numbers of compensated HF patients due to advances in both medical and device therapies. In light of this, the present study represents, to our knowledge, the first report documenting that gene transfer (with rAAV1.SERCA2a vector) significantly impacted on parameters of inotropy, showed a favorable trend for lusitropy, and improved remodeling in the setting of a large-animal model of progressive HF.

Study limitations. An important drawback of this study is the small number of study animals. Though associated with high mortality, this physiologically relevant large-animal model of HF allowed us to show discernable differences (for example, in systolic function and ventricular parameters of remodeling) or trends towards significance (such as was the case for diastolic dysfunction) between study groups. In addition, in our model of MR, Tau as one of the indexes of diastolic dysfunction may be hard to interpret. Greater number of surviving animals would have probably provided more robust statistical comparisons. Moreover, a more extended period of follow-up after gene transfer might have been more insightful for the progression of the HF model and to better contrast the positive effects of SERCA2a overexpression relative to the saline group. The potential pro-arrhythmic effect of SERCA2a gene transfer remains controversial (35,36). While we did not monitor for arrhythmias with continuous loop-recordings or Holter monitors, no documented deaths after gene transfer were sudden. In contrast to previous reports in pre-clinical studies, 2 months after SERCA2a transfection, there was no evidence of sudden death or clinically significant ventricular arrhythmias.

Clinical implications. The delivery methodologies we describe in this study can be directly transferred to the clinical setting in patients. Since issues with biodistribution and safety profiles of rAAV1.SERCA2a have been resolved, gene therapy trials with rAAV1.SERCA2a have begun.

Conclusions

In a volume-overload model of HF, long-term overexpression of SERCA2a by in vivo AAV1-mediated gene transfer (via intracoronary delivery) can preserve systolic function, potentially prevent diastolic dysfunction, and improve ventricular remodeling. These encouraging results pave the way for further studies to better define safety and feasibility of this promising therapeutic approach to HF.

Acknowledgment

The authors would like to thank Kim Wagner for her review of this article and Catherine McMahon and James Lough for their special assistance.

Reprint requests and correspondence: Dr. Roger J. Hajjar, Mount Sinai School of Medicine, Cardiovascular Research Center, One Gustave Levy Place, Box 1030, New York, New York 10029. E-mail: roger.hajjar@mssm.edu.

REFERENCES


