Restoration of Deficient Membrane Proteins in the Cardiomyopathic Hamster by In Vivo Cardiac Gene Transfer

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Background—One of the most important problems in developing in vivo cardiac gene transfer has been low transfection efficiency. A novel in vivo technique was developed, tested in normal hamsters, and the feasibility of restoring a deficient structural protein (δ-sarcoglycan) in the cardiomyopathic (CM) hamster evaluated.

Methods and Results—Adenoviral (AdV) vectors encoding either the lacZ gene or δ-sarcoglycan gene were constructed. Hypothermia was achieved in hamsters by external body cooling to a rectal temperature of 18 to 25°C. Through a small thoracotomy, the ascending aorta and the main pulmonary artery were occluded with snares, and cardioplegic solution containing histamine was injected into the aortic root; viral constructs were delivered 3 to 5 minutes later followed by release of the occluders and rewarming. Four days later, homogeneous β-galactosidase expression was detected throughout the ventricles of the normal hearts (average 77.3±9.0% [SEM] of left ventriclar myocytes). At 1 and 3 weeks after transfection, immunostaining showed extensive restoration of δ-sarcoglycan as well as α- and β-sarcoglycan proteins to the myocyte membranes, despite loss of β-galactosidase expression at 3 weeks. Also, at 3 weeks after gene transfer, there was significantly less progression of left ventricular dysfunction assessed as percent change in fractional shortening compared with controls.

Conclusions—This study demonstrates the feasibility of high efficiency in vivo myocardial gene transfer and shows application in improving the level of a deficient cardiac structural protein and cardiac function in CM hamsters. The approach should be useful for assessing effects of expressing other genes that influence the structure or function of the normal and failing heart. (***Circulation***. 2002;105:502-508.)

Key Words: gene therapy ■ cardiomyopathy ■ adenovirus

Myocardial gene therapy using viral vectors offers a potential approach for treating a number of cardiac disorders, including acquired and inherited forms of cardiomyopathy and congestive heart failure.[1] One of the most important problems has been low transfection efficiency of in vivo cardiac gene transfer, even though the cardiac myocyte is highly competent to receive viral vectors.[2] Recent in vivo studies using aortic root injection of adenoviral (AdV) constructs in normal[3] and aortic-banded rats,[4] aortic root,[5] or selective intracoronary injections[6] of AdV constructs in normal rabbits have reported significant changes in global left ventricular (LV) function. However, the extent of gene transfer throughout the LV either was not quantified[3,4] or reported to be primarily regional in distribution.[5,6] Therefore, it would be desirable to have delivery methods capable of achieving consistent, homogeneous, and efficient expression of transgenes, particularly for the delivery of structural gene products to cardiac myocytes. To our knowledge, in vivo cardiac transfer of a structural membrane protein has not yet been reported in a model of primary dilated cardiomyopathy (DCM).

The hamster is of particular interest in this regard because of the natural occurrence of autosomal recessive cardiomyopathy caused by a mutation in the δ-sarcoglycan (SG) gene,[7,8] with loss of this component and other sarcoglycans of the dystrophin-dystroglycan complex from cardiac myocyte and vascular smooth muscle cell membranes.[9,10] The cardiomyopathic (CM) hamster heart has many phenotypic features of the failing human heart.[10] This report describes a novel in vivo gene transfer approach for achieving high-efficiency, short-term cardiac gene transfer using an AdV vector in normal and CM hamster hearts, and we demonstrate the
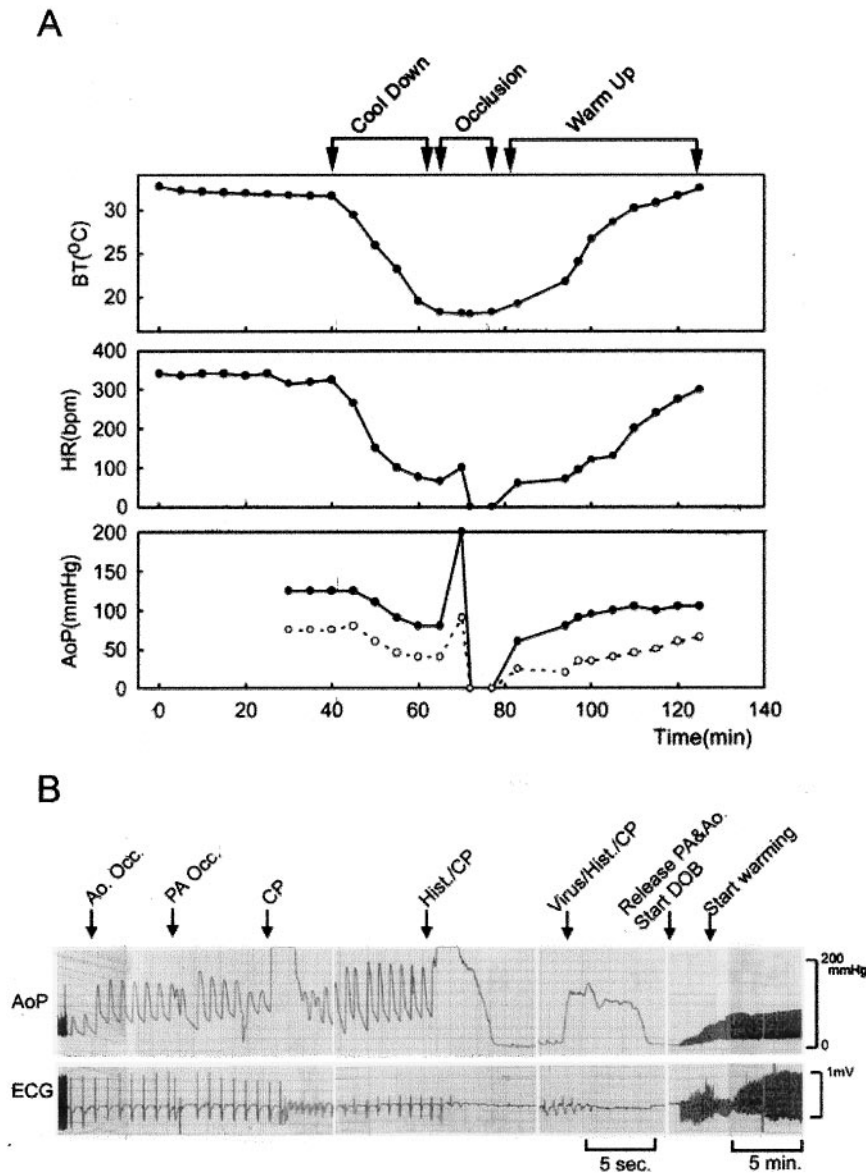
A

B

Figure 1. A, Representative time course of the procedure using deep hypothermia in a normal hamster. BT indicates body temperature; HR, heart rate; AoP, aortic pressure. Occlusion of the aorta and the pulmonary artery is indicated. Cooling time was 20 to 30 minutes. B, Representative tracings acquired during the procedure. Ao indicates aorta; PA, pulmonary artery; Occ, occlusion; CP, cardioplegic solution; Hist, histamine; and DOB, dobutamine. After cooling, the Ao and PA were occluded, and aortic pressure increased to 150 to 200 mm Hg with rapid near-cardiac arrest after Hist/CP injection.

feasibility of using this approach for replacing the deficient δ-SG protein in BIO14.6 CM hamster hearts.

Methods

Animals

Golden male hamsters (n=28, 8 to 12 weeks old) were obtained from Charles River Laboratory (Wilmington, Mass). BIO14.6 male cardiomyopathic (CM) hamsters (n=29, 7 to 9 weeks old) were obtained from BIO Breeders (Fitchburg, Mass). Protocols were approved by the University of California San Diego Animal Subjects Committee.

AdV Vector Construct

Hamster and human δ-SG DNA was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR). β-Galactosidase (β-gal) DNA with nuclear encoding signal sequences or δ-SG DNA were inserted into an AdV shuttle vector PacCMV.pApL.[11] Replication-deficient recombinant AdV-5 vectors were generated as previously described[11] (AdV.CMV.LacZ and AdV.CMV.δ-SG, respectively). The range of the Ad.CMV.LacZ titer was 1.9 to 9.0×10^{10} PFU/mL with 0.61 to 1.16×10^{12} particles/mL; in each

animal, 1.0×10^{11} particles (100 to 250 μL of stock solution) were delivered. The range of the Ad.CMV.δ-SG titer was 2.0 to 3.0×10^{10} PFU/mL with 0.40 to 1.40×10^{12} particles/mL; in each animal, 0.7 to 1.0×10^{11} particles (200 to 250 μL of stock solution) were given. The dilution factor of the viral stock solution with cardioplegic solution was approximately 2 to 2.5.

In Vivo Transfection Method

Hamsters were anesthetized with sodium pentobarbital (75 mg/kg, IP), intubated, ventilated, ECG electrodes placed on the limbs, and a thermistor inserted into the rectum. A small (<1 CM) anterior thoracotomy was performed in the left 2nd intercostal space; ligatures were looped around the ascending aorta and main pulmonary artery and threaded through occluder tubes. The right carotid artery was cannulated with flame-stretched polyethylene tubing for measurement of arterial pressure and performing injections; its tip was placed just above the aortic valve and below the aortic snare.

In initial experiments, bags filled with ice water were placed around the supine animal, and the heart rate and temperature were monitored every 5 minutes until the core temperature reached 18°C (Figure 1A). The aorta and pulmonary artery were then occluded, and solutions bolus-injected into the aorta as follows (Figure 1B): (1)

cardioplegic solution[12] (NaCl 110, KCl 20, CaCl$_2$ 1.2, MgCl$_2$ 16, and NaHCO$_3$ 10 mmol/L, 2 μL/g body weight [BW]); 10 seconds later, (2) cardioplegic solution plus histamine 20 mmol/L, volume 2.5 μL/g BW; 3 to 5 minutes later, (3) adenovirus construct diluted with cardioplegic-histamine solution (histamine 10 mmol/L, total volume 5 μL/g BW). Both snares were released 30 seconds to 1 minute after the final injection (total occlusion time about 7 to 10 minutes), and intraaortic infusion of dobutamine (10 μg/kg/min) was started. When arterial pressure reached about 50 mm Hg, the animals were placed on a heating pad (42°C). The chest was then closed and intrathoracic air evacuated by suction.

In later experiments, it was found that the level of hypothermia could be reduced to 25 to 26°C and the duration of aortic occlusion limited to 4 to 5 minutes, with only modest reduction of transfection efficiency.

Gene transfer experiments using conventional intraaortic root injection[3,4] during 10 to 20 seconds of aortic and pulmonary artery occlusion also were performed with or without use of histamine.

Protocol for In Vivo δ-SG Gene Transfer

Preoperative echocardiography was performed in all animals. Normal hamsters were studied at 4 days after in vivo LaZ gene delivery. Twenty nine CM hamsters (7 to 8 weeks old at transfection) were randomly divided into 3 groups (lacZ, n=11; δ-SG, n=12; vehicle, n=6) and underwent in vivo cardiac gene transfer. The experiment was terminated in 8 transfected and 6 vehicle-treated hamsters at 1 week and in the remaining animals at 3 weeks after gene transfer. Prior to euthanasia, LV function was assessed as below in a blinded manner in all animals, and heart tissue was then acquired for analysis by immunohistochemistry and assay of protein expression.

β-Gal Staining

The heart was transversely sliced into 3 pieces and the apical piece was quick frozen for β-gal enzyme assay. Sections (10 μm) from basal and mid-LV slices were used for β-gal staining.[13] For calculating β-gal efficiency in normal hamsters, the percentage of positive myocyte nuclei was estimated using a stereological point-counting method.

Immunohistochemistry

Monoclonal anti-α, -β, and –δ-SG antibodies were obtained from Novocastra Laboratory (Newcastle, UK). The polyclonal anti–δ-SG antibody has been described.[14] Immunohistochemistry was performed as previously described.[15]

Echocardiography

Transthoracic echocardiography was performed as previously described[10] before, 4 days (some of these hamsters were used for the analysis of protein expression) or 1 and/or 3 weeks after gene transfer.

Hemodynamic Measurements

Pressure in the LV was measured at 1 and 3 weeks after transfection using a fidelity pressure manometer (Millar Inc) introduced through the LV apex by a small midline thoracotomy. Maximum and minimum LV dP/dt and the time constant of relaxation, τ (using an exponential function), were calculated from LV pressure as described.[16]

Statistics

Comparison between 2 groups was performed by Student unpaired or paired t tests. Comparisons between repeated values were done by repeated measure ANOVA, followed by post hoc test (Student-Newman-Keuls test). A nonparametric test (Mann Whitney U test) was used to compare the 2 groups when appropriate. A value of $P<0.05$ was considered significant. Data are mean±SEM, unless otherwise noted.

Results

Transfection Procedures

Conventional Method

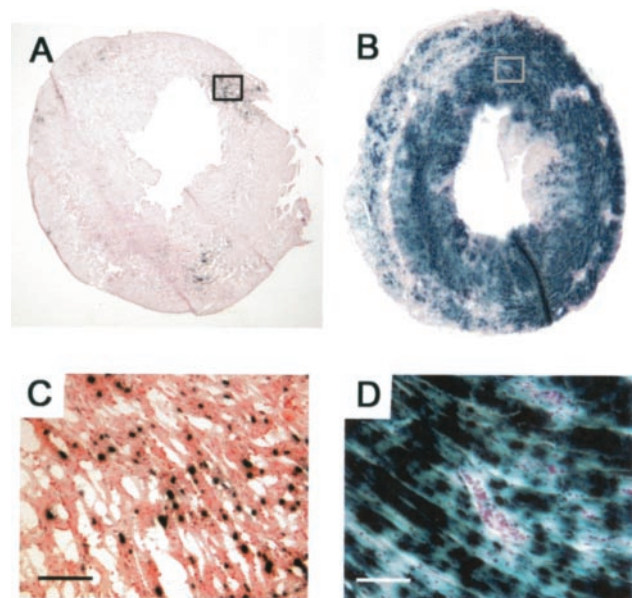In normal hamsters transfected using brief normothermic aortic and pulmonary artery occlusions with AdV injection,[3]

Figure 2. A, β-Gal staining from the heart transfected with the conventional method. B, β-Gal staining from the heart transfected with the hypothermic-cardioplegia method. C, High magnification of a stained area in A (rectangular area). D, High magnification of B (rectangular area). Bar in C and D=100 μm.

there was no acute mortality and all animals survived until day 4 after operation.

Hypothermic-Cardioplegia Method

In normal hamsters, operative mortality in initial deep hypothermia operation was approximately 10%. All animals recovered from hypothermic operation and did not show any evidence of neurological or other abnormalities. In the later experiments using AdV.CMV.LacZ with milder hypothermia (25 to 26°C) and shorter aortic occlusion, total mortality was <5% in normal hamsters and 10% in CM hamsters, and no animals appeared abnormal during follow-up.

The initial injection of cardioplegic solution alone had little effect on heart rate (Figure 1B); however, subsequent bolus injection of the cardioplegic-histamine solution markedly slowed the heart rate during hypothermia in all hamsters (Figure 1B). During the 5-minute interval between the cardioplegic-histamine injection (Hist/CP, Figure 1B) and injection of virus construct (Virus/Hist/CP, Figure 1B), low voltage, low frequency ECG activity with transient increases of the aortic pressure were sometimes observed, indicating that the cardioplegia usually was not complete.

Expression of β-Galactosidase in Normal Hamsters

Conventional Method

In the normal hamster hearts transfected using the normothermic aortic occlusion method,[3] β-gal expression was sparse and uneven in the LV, as shown in Figures 2A and 2C, and the average transfection efficiency was highly variable (range 0% to 18.9%) averaging 4.5±3.6% (n=5). The use of histamine with this technique (n=4) did not improve transfection efficiency.
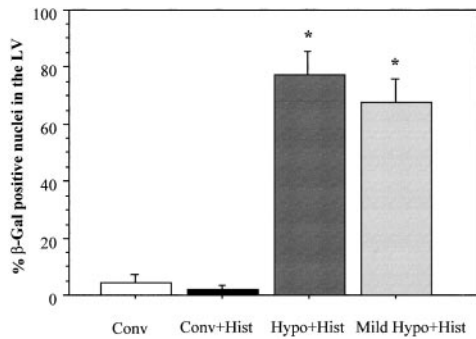
Figure 3. Percent β-gal positive nuclei in LV of normal hearts. Conv indicates conventional injection method; Conv+Hist, conventional plus histamine; Hypo+Hist, hypothermic-cardioplegia method with histamine; and Mild Hypo+Hist, mild hypothermic-cardioplegia method with histamine. *$P<0.05$ vs the conventional method.

Hypothermic-Cardioplegia Method

This approach produced a marked increase in transfection efficiency (Figure 2B), with heavy and homogeneous β-gal staining of the normal heart, both LV and right ventricle. Most of cardiac myocyte nuclei were deeply stained with β-gal (Figure 2D). The average estimated percent of positive myocardial nuclei was 77.3±9.0% (range 35.6% to 97.2%) ($P<0.05$ versus the conventional aortic root injection groups). The mild hypothermic-cardioplegic method also produced high efficiency (67.5±8.8% [range 45.9% to 97.3%], not significantly different from the original procedure) (Figure 3).

No significant inflammatory reaction was observed histologically in hearts obtained 4 days after transfection, but at 6 to 7 days, there was inflammatory cell infiltration, as anticipated. LacZ expression was not detected in kidney spleen, skeletal muscle, or aorta in any of the hamsters, and there was only an occasional positive cell in the lung. β-Gal staining was frequently observed in hepatic cells surrounding the central hepatic venules, probably due to venous stasis during pulmonary artery occlusion (Figure 4).

Immunohistochemistry After δ-SG Gene Transfer

At 1 and 3 weeks after δ-SG transfer in CM hamster hearts, δ-SG protein was diffusely stained at the sarcolemma and t-tubules of cardiac myocytes throughout the LV as shown in Figures 5A (1 week) and 5B (3 weeks), indicating clear restoration of δ-SG protein compared with controls (Figure 5C). Overall gene transfer efficiency of δ-SG was approximately 50% to 60% at both 1 and 3 weeks after gene transfer (not determined quantitatively), whereas that of β-gal was markedly reduced at 3 weeks, compared with 1 week after gene transfer (approximately 10% of the LV cross-section; data not shown). Interestingly, the intensity of sarcoglycan staining at the sarcolemma and t-tubules at 3 weeks was even stronger than at 1 week (Figures 5A and 5B). In addition, δ-SG protein appeared to be evenly distributed in the membranes of individual myocytes at 3 weeks (Figure 5B), whereas there was uneven δ-SG distribution at 1 week (arrows in Figure 5A).

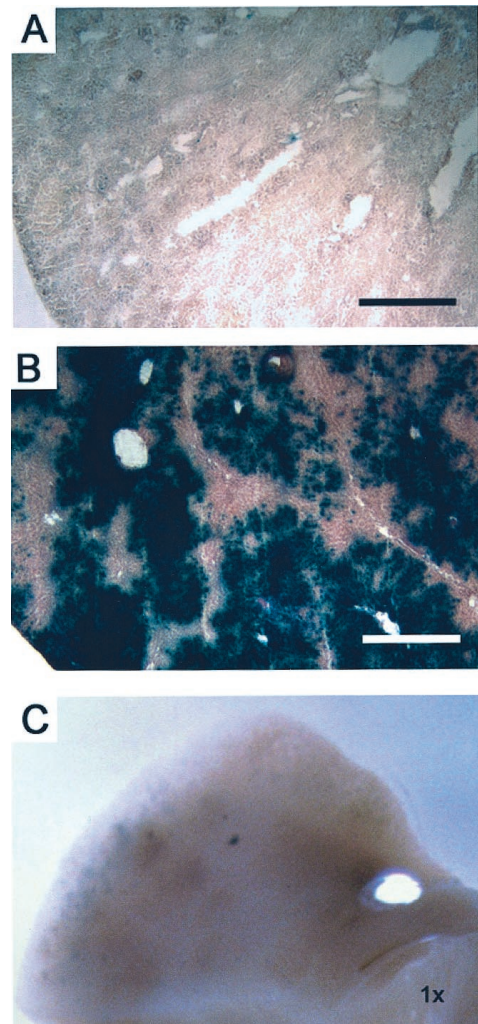Other sarcoglycan (β-SG and α-SG) also were restored to the membranes (Figures 5D through 5I).

Figure 4. Representative lacZ staining of the other organs. A, Kidney; B, Liver; C, Lung. Bars in A and B=100 μm.

Cardiac Function Before and After Gene Transfer

Echocardiographic data are summarized in Table 1. In normal hamsters studied before and at 4 days after LacZ gene transfer, the percent LV fractional shortening (%FS), VCF, and end-diastolic dimension (LVDd) did not change significantly after transfection (Table 1A). In vehicle-treated CM hamsters, echocardiographic variables at 1 week also did not change significantly (Table 1A).

LacZ- and δ-SG–treated CM hamsters were also studied before and after gene transfer. At 1 week, changes between the pre- and postoperative studies were not significant, further suggesting that no functional deterioration was caused by the gene transfer procedure (Table 1B). At 3 weeks after transfection (Table 1B), LacZ hamsters showed significantly decreased %FS compared with pretransfection data, together with significant enlargement of LVDd, whereas in δ-SG–transfected hamsters there was no significant change in %FS (Figure 6) and the LVDd was increased but was not significantly different from the LacZ group. The increases in LVDd may, in part, reflect normal growth (see Discussion). The mild increase in the percent change of %FS from the pretransfection level after δ-SG gene transfer was signifi-
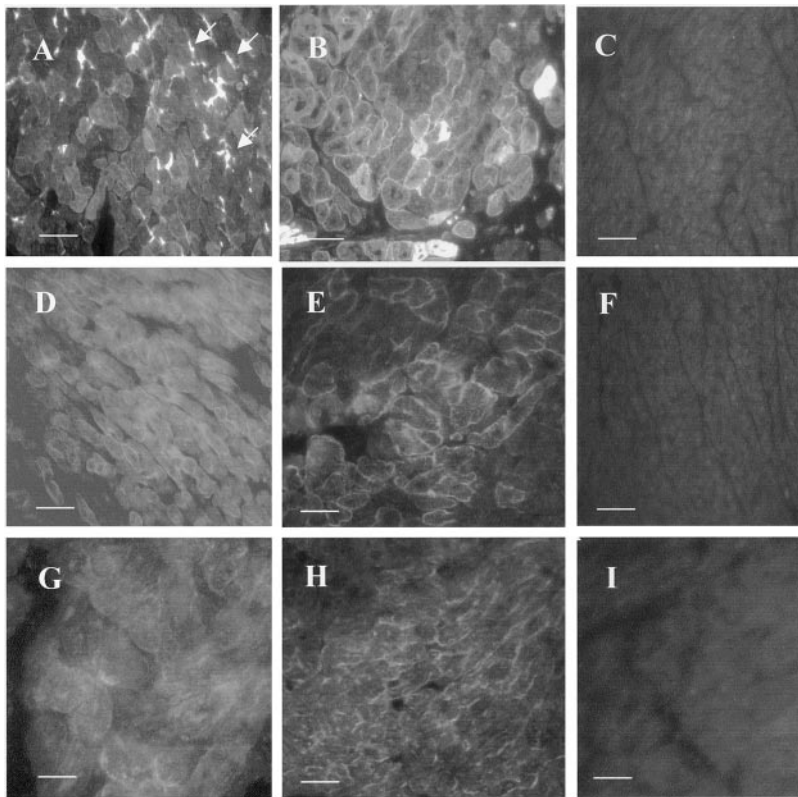
Figure 5. Immunohistochemistry of sarcoglycans 1 week and 3 weeks after gene transfer in CM hamster hearts. δ-SG staining: A, 1 week; B, 3 weeks; C, lacZ control at 3 weeks. β-SG staining: D, 1 week; E, 3 weeks; F, lacZ control. α-SG staining: G, 1 week; H, 3 weeks; I, lacZ control. Bars=25 μm.

cantly different compared with the decrease observed in lacZ-transfected hamsters (Figure 6).

Hemodynamic data at the terminal study are summarized in Table 2. There were no significant differences between the lacZ- and δ-SG–transfected groups; cardiac catheterizations were not performed before transfection due to use of only 1 carotid artery for the surgical procedure, so that paired analysis was not feasible.

Discussion

Cardiac gene transfer efficiency in the isolated retrograde-perfused rabbit heart model was affected by multiple factors,

TABLE 1. Echocardiographic Data Before and After Transfection

	HR, bpm	LVDd, mm	PWT, mm	%FS	VCF
A					
Normal, LacZ pre (n=8)	414±47	4.12±0.30	1.07±0.12	46.8±2.4	7.77±0.57
Normal, LacZ post 4 days (n=8)	405±30	4.38±0.22	1.12±0.08	43.9±5.4	7.66±0.57
CM, pre (n=6)	409±25	4.66±0.32	0.96±0.06	30.5±3.1	4.42±0.65
CM, post 1 week (n=6)	451±16	4.78±0.39	0.93±0.13	27.6±4.5	4.38±0.80
B					
CM, LacZ pre (n=9)	439±35	4.25±0.25	1.04±0.12	40.2±2.8	5.47±0.55
LacZ post 1 week (n=3)	451±34	4.40±0.29	1.00±0.05	34.4±7.94	4.87±1.11
LacZ post 3 weeks (n=6)	407±55	4.99±0.40*	1.06±0.10	33.97±2.1*	5.06±0.6
CM, DSG pre (n=11)	441±20	4.41±0.37	1.03±0.13	36.9±6.0	5.0±1.0
DSG post 1 week (n=5)	436±53	4.54±0.29	0.98±0.12	35.0±3.5	5.0±0.4
DSG post 3 weeks (n=6)	400±30	5.14±0.13*	1.13±0.08	37.7±5.5	5.4±1.3

A, Data before (pre) and after (post) transfection in normal LacZ-treated hamsters and vehicle-treated cardiomyopathic (CM) hamsters. B, Data with Ad.δ-SG gene transfer between before, after 1 week, and after 3 weeks in CM hamsters.

HR indicates heart rate; LVDd, left ventricular (LV) end-diastolic dimension; %FS, percent fractional shortening of the LV; VCF, LV circumferential fiber shortening rate; PWT, LV posterior wall thickness at end-diastole. Values are mean±SD. *$P<0.05$ vs Pre.
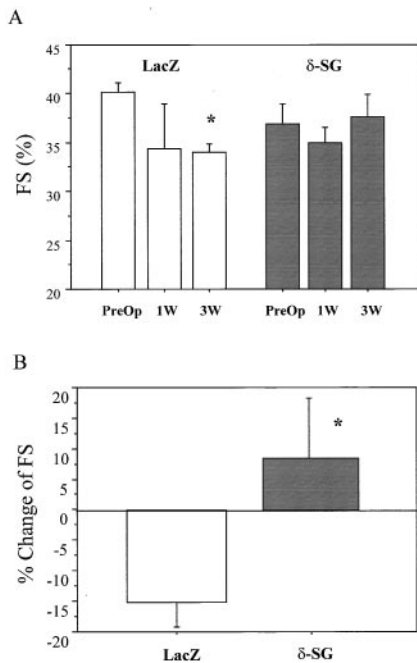
A

B

Figure 6. Echocardiographic data before and at 1 week and 3 weeks after gene transfer. A, Percent fractional shortening (FS) of the LV in lacZ- and δ-SG–transduced CM hamster hearts before and at 1 and 3 weeks after gene transfer. *$P<0.05$ vs preop lacZ-treated hearts. B, Percentage change of %FS between before and after gene transfer at 3 weeks. *$P<0.05$ vs lacZ. Values are mean±SD.

including coronary flow, virus exposure time, virus concentration, temperature, and the presence of red blood cells[17]; it was also reported to be augmented by low calcium concentration and serotonin.[18] Histamine has been shown to augment transfection efficiency in skeletal muscle when viral vectors were delivered by intraarterial injection.[19] To optimize several of these factors under in vivo conditions in a rodent model (in which direct intracoronary delivery is not feasible), we applied hypothermia to protect the brain, heart, and other organs during aortic occlusion, with intraaortic delivery proximal to the occlusion of cardioplegic treatment and histamine, followed 3 to 5 minutes later by injection of the AdV constructs. This procedure consistently resulted in high expression of the transgene in normal and BIO14.6 cardiomyopathic hamsters.

Our hypothermic technique is based on that used for human cardiovascular surgery in infants performed in the 1960s, which allowed cardioplegia for up to 1 hour.[20] The present method using hypothermia with cardioplegic treatment markedly reduced the metabolic requirement for coronary blood flow, allowing prolongation of virus exposure time and was coupled with use of an agent to enhance endothelial permeability. During the development of this transfection technique, AdV injections with cardioplegia during hypothermia without the use of histamine resulted in very limited transgene expression (data not shown). Because histamine requires several minutes to activate endothelial fenestration in capillary vessels in vitro,[18] a period of 2 to 3 minutes was provided between histamine injection and the viral injection. Histamine also appeared to contribute to the achievement of cardioplegia or marked slowing of heart rate, perhaps by improving microvascular perfusion, although its main effect undoubtedly was to increase microvascular permeability by endothelial gap fenestration,[21] allowing adenovirus to enter the extravascular space.

In CM hamsters, gene transfer of δ-SG in the CM hearts strikingly increased δ-SG protein expression together with restoration of other SG proteins at the sarcolemmal membrane and t-tubules, resembling findings with gene transfer of δ-SG by direct injection into myopathic skeletal muscle of hamsters.[19,22] The adenovirus vector is generally recognized to express marker protein for about 2 weeks, and β-gal staining was markedly reduced at 3 weeks; however, sustained expression of all SG proteins was observed in the myocardial sarcolemma at 3 weeks. The intensity of δ-SG at 3 weeks appeared consistently greater than that at 1 week and was more evenly distributed in the sarcolemmal membrane and t-tubules (Figure 5D and 5E); this phenomenon perhaps reflects slow protein turnover rate and the time required for membrane integration and stabilization of the sarcoglycan complex. These findings may explain the improved LV function expressed as %FS, compared with that in the LacZ group at 3 weeks. Thus, it seems possible that the lack of effect on LV function at 1 week after transfection is related to the longer period required for widespread expression of δ-SG in the sarcolemmal membranes. A progressive decline occurs in %FS after 4 weeks of age in CM hamsters,[10] so that a decrease in %FS during a 3-week interval could be expected in the controls.

It will be of interest to determine if δ-SG gene transfer can further retard progression of LV dysfunction over a longer period, compared with the known rate of deterioration in the untreated CM hamster,[10] and improve survival. In this regard, use of an adeno-associated virus (AAV) vector would likely be optimal for this purpose. Longer term and cell type

TABLE 2. Hemodynamic Data After Transfection in Cardiomyopathic Hamsters

	HR, bpm	LVP, mm Hg	LVEDP, mm Hg	Max dP/dt, mm Hg/s	Min dP/dt, mm Hg/s	τ, ms
LacZ post 1 week (n=3)	369±28	95±17	4±2	7185±2516	−5888±1258	13.3±1.6
LacZ post 3 weeks (n=6)	334±17	102±9	10±3	7413±770	−4951±512	17.9±3.4
DSG post 1 week (n=5)	371±28	88±16	4±1	6484±2567	−4700±1179	13.5±3.0
DSG post 3 weeks (n=6)	349±32	101±11	10±3	6769±1881	−4685±1182	20.8±6.6

HR indicates heart rate; LVP, left ventricular (LV) peak systolic pressure; LVEDP, LV end diastolic pressure; max dP/dt, maximum LV dP/dt; min dP/dt, minimum LV dP/dt; τ, time constant estimated from isovolumic LV pressure decay.

Values are mean±SD. None of the values show significant difference between LacZ vs DSG group.

specific δ-SG viral vectors also will be required to determine whether vascular[23] or myocyte sarcolemmal[10] mechanisms predominate in causing myocardial damage in the CM hamster.

The method for gene transfer described herein should be useful for investigating effects of expressing genes coding for nonstructural as well as other structural proteins in rodents. The hamster is midway in size between rat and mouse, and studies currently are underway to adapt the current protocol for gene transfer in those species. This method may hold potential as a new approach for cardiac gene delivery in clinical settings, such as during cardiovascular surgery using extracorporeal cardiopulmonary bypass.

Mutations in the δ-SG gene recently have been described in human dilated CM,[24] and several other mutant sarcoglycan genes also have been reported in hereditary dilated CM in humans.[25] As in the CM hamster,[16] in some hereditary muscular dystrophies in humans such as Duchenne's, death most commonly results from heart failure due to cardiomyopathy rather than effects of the skeletal muscle myopathy. Therefore, this initial demonstration of the feasibility of high efficiency cardiac transfer of a structural protein gene may carry implications for the future treatment of some forms of genetic human CM.

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