In vivo gene delivery of HSP70i by adenovirus and adeno-associated virus preserves contractile function in mouse heart following ischemia-reperfusion

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Although HSP70i expression shows promise in protecting the heart from damage done by ischemia-reperfusion, little work has been done to demonstrate its therapeutically protective effect in preserving cardiac function when administered in the form of a viral vector to the heart (gene therapy). Previous studies using liposome-mediated gene delivery in rat hearts relied on ex vivo coronary perfusion of the liposomes followed by ectopic abdominal transplantation of the heart in a host animal (7, 8, 20). Although these studies subsequently demonstrated a protection against normothermic and hypothermic ischemia-reperfusion injury, ectopically transplanted hearts are mechanically unloaded and have been shown to undergo remodeling (10, 11). Another study involving localized administration of HSP70i adenovirus to an area subjected to regional ischemia in rabbit heart demonstrated a decrease in infarct size (17); however, the area studied was not of sufficient size to grossly affect cardiac function. In this study, we infected the left ventricular (LV) free wall of a mouse with HSP70i adenovirus in vivo to examine its potential in preserving cardiac function in Langendorff perfused hearts subjected to global ischemia-reperfusion. The mouse was chosen for this study because the thinness of the LV wall allows for transmural infection, and the spread of the virus allows us to cover most of the LV free wall with just a few injections. In addition to our use of HSP70i adenovirus, we have also examined long-term (8 mo) expression of HSP70i delivered by adeno-associated virus (AAV) in protecting the heart following ischemia-reperfusion.

METHODS

Viral Vector Production

Adenovirus expressing HSP70i. The cloning of HSP70i in replication-deficient human adenovirus (type 5) was accomplished as described previously (15). DNA expressing rat HSP70i was cloned in a shuttle vector pACCMV.pLpA (a kind gift from Dr. Robert D. Girard) between the CMV promoter/enhancer and the SV40 polyadenylation signal. This shuttle plasmid was subsequently cotransfected with an adenovirus-derived plasmid pJM17 (a gift from Dr. Frank L. Graham) in 293 cells. Homologous recombination between the two plasmids in cell culture via adenoviral- or plasmid-based vector delivery of the HSP70i cDNA (15). Overexpression in isolated cells prevents cell death and lactate dehydrogenase release when exposed to simulated ischemia, whereas overexpression in the whole heart reduces infarct size and preserves cardiac function following ischemia-reperfusion (14, 15).

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293 cells led to the generation of an adenovirus expressing HSP70i. A large-scale preparation of the adenovirus grown in 293 cells was purified by CsCl gradient centrifugation, and the subsequent adenoviral bands were dialyzed against a viral storage medium (10 mM Tris-HCl, pH 8.0, 0.2 mM MgCl2, 0.9% NaCl, and 4% sucrose) and plaque titered as previously described (1). An adenoviral preparation expressing nuclear localized LacZ was produced in a similar manner. An empty (control) adenoviral vector, termed “SR–”, was created by cotransfection of the shuttle plasmid without any insert and plasmid pJM17 in 293 cells.

**AAV expressing HSP70i.** The AAV shuttle vector (AAV shuttle used in viral construction (AAV type 2) was derived by taking an Xba I fragment of the pShuttle1 plasmid (19) and ligating it to a human CMV enhancer/promoter fragment followed by a multiple cloning site and a polyadenylation signal. The rat cDNA encoding HSP70i was cloned into the AAV shuttle vector. Recombinant clones were verified by DNA sequencing, and large amounts of plasmid along with the plasmid pDG as described by Grimm et al. (6) were purified using a CsCl protocol. For production of infectious virus particles, both the pShuttle1 and plasmid pDG plasmid (70 g/plate) and the pDG plasmid (50 g/plate) were transfected into 293T cells (subconfluent 15-cm plate) using a calcium phosphate coprecipitation method. The following day the medium was changed to fresh DMEM containing 10% FBS, and the cells were incubated for two more days at 37°C and 5% CO2. Harvesting of virus particles was done by scraping 293T cells from each plate in 2.5 ml of DMEM. The cells were then spun down, resuspended in 25 ml DMEM, and subjected to three freeze-thaw cycles to lyse the cells. After this, 100 µg of DNase I and RNase A were added to the suspension and incubated at 37°C for 30 min. Following another centrifugation at 3,000 g, 0.5% deoxycholate was added to the supernatant and incubated for 30 min at 37°C. The supernatant was then filtered sequentially through a 5- and 0.8-µm syringe filter and mixed gently at room temperature for 1 h with 3 ml of a suspension of heparin-agarose. The suspension was then loaded on a glass column, and the heparin-agarose resin was washed with 25 ml PBS containing 0.254 M NaCl. Virus particles were eluted with PBS containing 0.554 M NaCl and kept in small fractions for titer determination. 10 mice each were injected with AAV expressing HSP70i or LacZ were allowed to recover for 8 mo following surgery before measuring cardiac function. In this experiment, 10 mice each were injected with AAV expressing HSP70i or LacZ. One LacZ mouse and 2 HSP70i mice died within 48 h of gene therapy, and although the rest survived the 8 mo postsurgery, hearts from 3 LacZ mice and 2 HSP70i mice were damaged during the set up for the ischemia-reperfusion protocol and had to be discarded.

For those mice injected with adenovirus or AAV expressing LacZ for X-gal staining, gene therapy was performed as described above, except the injection site was limited to between one and three locations. The mice were killed for X-gal staining in the heart at various times as indicated.

**Ischemia-Reperfusion Protocol**

Perfusion experiments were conducted in a blinded manner as to viral treatment. Mice were killed by an overdose of pentobarbital sodium, and the heart was excised from the chest. The aorta was cannulated, and perfusion was initiated with Krebs-Henseleit (KH) buffer consisting of (in mM) 118 NaCl, 4.7 KCl, 2.25 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.5 Na2EDTA, and 5.5 glucose, which was bubbled continuously (at 37°C) with 95% O2-5% CO2. A small balloon was made from polyethylene and inserted in the LV through the pulmonary vein (9). The balloon was fluid filled and inflated to an end-diastolic pressure of 10 mmHg. Pressure development was recorded digitally (1 kHz) by connecting the intraventricular balloon to a 2-Fr Millar pressure transducer (Millar Instruments, Houston, TX), and the resultant pressure waves were analyzed for pressure derivatives (+dp/dt, −dp/dt) and developed pressure. The hearts were paced at 400 beats/min by means of platinum wires placed on the right atrium. This heart rate was chosen to allow a rate fast enough to allow capture for pacing but slow enough to allow time for perfusion of the heart during diastole. Hearts underwent an initial 15-min aerobic perfusion to establish baseline parameters after which they underwent 20 min of global no-flow ischemia. During the ischemic period, pacing was ceased, and the chamber was filled with KH buffer to submerge the heart to maintain uniform temperature (37°C). Following ischemia, coronary perfusion and pacing were reintroduced and continued for 2 h. Aliquots of KH buffer from the coronary perfusion were collected at various times for analysis of creatine kinase (CK) content.

**CK Assay**

CK release was used as a determinant of cellular disruption and damage. CK activity from the coronary effluent was measured spec-

**Adenovirus Treated**

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**Fig. 1.** Western blot showing inducible heat shock protein 70 (HSP70) expression in adenovirus (Adv)-treated hearts 4 days after gene therapy. Note that HSP70i is only observed in hearts treated with HSP70i-expressing adenovirus, whereas those treated with empty (control) adenoviral vector (SR–) adenovirus show no expression despite surgery and viral infection.
trophotometrically at 340 nm using a kit (Sigma, St. Louis, MO) based on NADH production. CK activity was measured during the preischemic perfusion period and at 1, 2, 3, 4, 5, 10, and 120 min during the reperfusion phase and expressed as the increase in NADH absorbance per minute times the volume of coronary perfusate.

Western Blot Analysis

For Western blot analysis, ventricular tissue was homogenized by using a Polytron homogenizer. After protein content of the homogenate (Bradford Protein Assay, Bio-Rad, Hercules, CA) was measured, protein separation was achieved by resolving 50 μg of protein (unless otherwise specified) on a 4–20% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA) and transferring to a nitrocellulose membrane. The nitrocellulose membrane was blocked overnight at 4°C using 5% nonfat milk powder in Tris-buffered saline (+0.05% Tween 20) and exposed for 2 h to primary antibodies directed against HSP70i (mouse monoclonal; Stressgen Biotechnologies) and α-actin (mouse monoclonal; Sigma). The blots were then washed and exposed to secondary antibodies conjugated to horseradish peroxidase to drive a chemiluminescence reaction (Amersham, Little Chalfont, UK) and exposed to film. Band densities were quantified with the NIH image software ImageJ.

Statistical Analysis

Values shown represent means ± SE. All comparisons were performed using Student’s t-test with significance being set at \( P < 0.05 \).

RESULTS

HSP70i Expression in Adenovirus-Treated Mice

Following adenoviral injection in the LV free wall of mice, the expression of HSP70i was confirmed by Western blot analysis (Fig. 1). The protein was observed in mice injected with HSP70i expressing adenovirus but not the SR- (control) adenovirus.

Functional Assessment of Adenovirally Treated Hearts

Contractile function following in vivo gene therapy was measured in isolated Langendorff perfused hearts using an intraventricular balloon inserted in the LV. In preischemic aerobically perfused hearts, developed pressure as well as the rate of contraction (dP/dt) and relaxation (dP/dt) did not differ between mice injected with adenovirus expressing HSP70i or those expressing an empty vector (control; Fig. 2A). Following 20 min of ischemia and 120 min of reperfusion, analysis of pressure tracings generated by the heart indicated a higher developed pressure in the HSP70i group (46% increase). Analysis of the rates of contraction and relaxation also showed a significant (\( P < 0.05 \)) increase in the HSP70i-treated group (Fig. 2, B and C). Analysis of CK release during reperfusion indicated a greater release of CK in the control group than in the HSP70i group early during reperfusion (3–5 min; Fig. 3), suggesting greater cellular damage in the SR-
(control) adenovirus-treated group early during reperfusion. By 120 min of reperfusion, however, no difference could be found between the groups.

Comparison of Adenovirus and AAV Expression of LacZ

To assess the long-term potential for expression of viral constructs, LacZ expression was examined in hearts injected with adenovirus or AAV (Fig. 4). Adenoviral-mediated expression of LacZ can be seen in hearts 4 days to 2 wk after infection, with only a minor scar resulting from the injection site (Fig. 4, A, C, and E). By 8 wk, however, little LacZ expression remains with myocytes being replaced by massive fibrosis with remaining muscle showing signs of compensatory hypertrophy (especially the papillary muscle; Fig. 4E). In contrast, injection of AAV expressing LacZ developed slowly over the first 2 wk and did not show a similar development of
fibrosis at 8 wk (Fig. 4, B, D, and F). In fact, LacZ expression was still observed when sampled at 8 mo (Fig. 4H). These results illustrate the feasibility of using AAV vectors with direct LV injection to obtain long-term expression of proteins of interest such as HSP70i.

**HSP70i Expression in Adeno-Associated-Treated Mice**

To examine the effect of long-term expression of HSP70i on cardiac protection, mice underwent gene therapy with AAV expressing HSP70i and were allowed to recover for 8 mo. An AAV expressing LacZ was used as a control. At the end of 8 mo, the hearts underwent the same ischemia-reperfusion protocol as the adenovirally treated mice. Again, HSP70i expression could only be observed in mice treated with AAV expressing HSP70i but not in LacZ-treated mice (Fig. 5).

**Functional Assessment of AAV-Treated Hearts**

Values for preischemic cardiac function did not differ between the two groups (Fig. 6A). In contrast, developed pressure after 120 min of reperfusion was 78% higher in the HSP70i group than the LacZ-treated group, although a similar increase in the rates of contraction and relaxation were observed in the HSP70i-treated group (Fig. 6, B and C).

**DISCUSSION**

This study examined the application of viral-mediated gene therapy delivering HSP70i to the protection of contractile function in the heart following ischemia-reperfusion. The effect of HSP70i in mediating protection from ischemia has been well documented in heat-shocked animals (2), transgenic mice (14), and in isolated myocytes transfected with HSP70i via viral or plasmid-based vector delivery (15). Despite this wealth of evidence, studies involving a therapeutic application of HSP70i in the protection against ischemia- and reperfusion-mediated damage have been limited. HSP70i expressing adenovirus was employed by Okubo et al. (17) in rabbit hearts to study infarct size in regional ischemia; however, global cardiac function was not affected in that study. A protection of global function against ischemia was observed in rat hearts where liposomes were employed to deliver HSP70i-expressing vectors (7, 8, 20). However, in those studies, hearts had to be excised for ex vivo vector delivery via coronary perfusion followed by abdominal ectopic transplantation to allow for HSP70i expression. In this study, we use viral vector-mediated...
delivery to the LV in vivo to increase HSP70i expression, and we examined the potential of both short- and long-term expression in mediating protection against ischemia-reperfusion injury.

It is interesting to note that HSP70i was only observed in hearts infected with HSP70i expressing adenovirus. Neither the invasive surgery involved in delivery of the viral vectors nor the expression of foreign proteins (such as LacZ) led to a detectable increase in HSP70i expression. The improved recovery of function observed in adenovirally mediated HSP70i-expressing hearts following ischemia-reperfusion confirms previous observations examining short-term HSP70i expression (7, 15). Although this beneficial effect of increased HSP70i could be mediated over short time periods using adenoviral vectors, to be practical therapeutically, HSP70i expression would have to be extended for longer periods of time. Unfortunately, long-term expression via adenoviral infection in the heart is impractical, since it leads to an immune response and myocyte loss (4, 5). We observed this effect in a series of hearts injected with adenovirus vectors expressing LacZ (Fig. 4). Although robust expression of LacZ could be observed at 4 days and 2 wk after viral delivery, most of this expression was lost by 8 wk with extensive scar formation and remodeling of the ventricle. In contrast, AAV-mediated LacZ expression, while slower to develop, was still apparent at 8 wk, confirming previous observations that AAV represents a suitable vector for infecting cardiac myocytes and mediating long-term expression of proteins of interest (3). In using AAV expressing HSP70i, we were able to demonstrate protein expression and improved protection from ischemia-reperfusion 8 mo after viral infection. These results show the feasibility of using AAV expressing HSP70i for long-term preservation of cardiac function following ischemia-reperfusion. The approach would allow for HSP70i expression and the resulting beneficial effects under chronic ischemic conditions. In addition, a “prophylactic” approach could be envisioned for conditions such as unstable angina, where a high probability of future ischemic events exists. The challenge in using virally expressed HSP70i would be in correctly determining when such therapy should be applied in light of predicting when ischemic episodes are likely to take place. It should also be noted that we did not examine the time course of AAV-mediated HSP70i expression with respect to changes in expression levels over time and how this correlates to its capacity to protect against ischemic injury.

This study provides evidence that HSP70i expression via viral vectors preserves cardiac function following ischemia-reperfusion. By the direct injection of viral vectors expressing HSP70i in the LV free wall of a mouse heart, we have been able to moderate functional loss and cellular damage (measured via CK release) following ischemia-reperfusion. Furthermore, this protective effect of HSP70i can be observed up to 8 mo after gene therapy using AAV vectors, demonstrating a potential therapeutic effect for chronic conditions where ischemic damage is likely.

REFERENCES


