Heat Shock Treatment Enhances Graft Cell Survival in Skeletal Myoblast Transplantation to the Heart

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Background—Graft survival after skeletal myoblast transplantation is affected by various pathological processes caused by environmental stress. Heat shock is known to afford protection of several aspects of cell metabolism and function. We hypothesized that prior heat shock treatment of graft cells would improve their survival after cell transplantation.

Methods and Results—L6 rat skeletal myoblasts expressing β-galactosidase (β-gal) were subjected to heat shock (42°C, 1 hour). Increased expression of heat shock protein 72 was detected 24 hours later in the heat-shocked cells. After hypoxia-reoxygenation in vitro, lactate dehydrogenase leakage was significantly attenuated in the heat-shocked cells; in addition, the percentage of early apoptosis was lower in this group measured by flow cytometry with annexin V staining. For the in vivo study, 1×10⁶ heat-shocked (hsCTx) or normal-cultured (CTx) myoblasts were infused into the explanted rat hearts through the coronary artery followed by heterotopic heart transplantation. β-gal activity was significantly higher in the hsCTx group after cell transplantation, with an estimated 8×10⁶ surviving cells per heart in the hsCTx group and 5×10⁶ cells in the CTx group on day 28. Discrete loci of grafted cells were globally observed in the myocardium of the hsCTx and CTx groups, with a higher frequency in the hsCTx group. Surviving myoblasts occasionally differentiated into myotubes and had integrated with the native cardiomyocytes.

Conclusions—Heat-shocked skeletal myoblasts demonstrated improved tolerance to hypoxia-reoxygenation insult in vitro and enhanced survival when grafted into the heart. Heat shock treatment could be useful in improving graft cell survival in cell transplantation. (Circulation. 2000;102[Suppl III]:III-216-III-221.)

Key Words: cells ■ transplantation ■ apoptosis ■ survival

The irreversibility of end-stage heart failure is to a large extent caused by limited regenerative capacity of adult myocardium.1 Cell transplantation is thus a promising strategy to treat patients with end-stage heart failure who have lost cardiomyocytes. The ability to augment the number of cardiomyocytes could be of therapeutic value if sufficient numbers of new myocytes will functionally integrate with the preexisting myocardium. Several types of cells, including neonatal cardiomyocytes2 and smooth muscle cells,3 have been examined as a graft in cellular cardiomyoplasty. We and others4–6 consider skeletal myoblasts to be among the most promising cells for grafting. Skeletal myoblasts retain the capacity to fuse with surrounding myoblasts or with damaged muscle fibers to regenerate functional skeletal muscle. Moreover, this differentiation ability has been observed even with engraftment into myocardial tissue.4–6 Recently, it has been reported that skeletal myoblast transplantation improved cardiac function of cryoinjured rabbit heart.4 In the clinical setting, skeletal myoblasts could be isolated from patients themselves for use as autografts,7 avoiding the necessity of immunosuppressive agents.

Improvement of graft cell survival after skeletal myoblast transplantation is a major concern. Graft cell survival is limited by various pathological processes such as the inflammatory response, rejection, and ischemia-reperfusion, which induce cell death not only through necrosis but also by apoptosis.4,8 It is expected that improvement of graft survival will greatly contribute to refinement of the efficiency of cell transplantation. Heat shock treatment is known to induce several kinds of self-protective proteins, including heat shock protein 70, and protects cells in vitro9–12 and organs in vivo13,14 from various environmental insults. We accordingly hypothesized that prior heat shock treatment of graft cells would improve their survival in cell transplantation to the heart.

Methods

Generation of β-Galactosidase–Expressing L6 Myoblasts and Heat Shock Treatment

Generation of L6 Myoblasts Expressing β-Galactosidase
The L6 rat skeletal muscle cell line (American Type Culture Collection) was maintained with Dulbecco’s modified Eagle’s me-
dium (DMEM, Sigma) supplemented with 10% FCS, 2 mmol/L glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified chamber equilibrated with 5% CO₂ in air. LacZ reporter gene was transfected into L6 cells with MFGNlacerZ retrovirus-mediated gene transfection as previously described. Clonal cells expressing β-galactosidase (β-gal) were selected by following 2 rounds of limiting dilution at a density of 0.2 cell/well. The culture was split before reaching subconfluence to maintain the undifferentiated state and used during the 6th to 11th passages.

To confirm β-gal expression, cells were incubated on 8-well chamber slides (Nunc). After fixation in 2% formaldehyde and 0.2% glutaraldehyde, cells were washed and stained for β-gal activity by 24-hour incubation at 37°C with 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L MgCl₂.

Heat Shock Procedure and Heat Shock Protein 72 Expression
Subconfluent cultured cells in 35-mm dishes (for in vitro experiments) or 25-cm² flasks (for in vivo experiments) were subjected to hyperthermia of 42°C for 1 hour with a water bath. As a control, treatment.

Tolerance to hypoxia-reoxygenation of β-gal–expressing L6 myoblasts was evaluated in vitro with or without prior heat shock treatment. First, the level of heat shock protein 72 (HSP72) was evaluated in heat-shocked or control cells with Western blotting (n = 5 in each group). The cells were harvested by scraping confluent 60-mm plates in 500 μL of 1% SDS containing 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin and homogenated. Protein (25 μg) was loaded onto an SDS 10% polyacrylamide gel. After 1-dimensional electrophoresis, the proteins were transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with a 1:1000 dilution of anti-HSP72 mouse monoclonal antibody (StressGen Biotechnologies Corp) for 8 hours at 4°C. The blots were incubated with a 1:1000 dilution of horseradish peroxidase–conjugated rabbit anti-mouse IgG antibody (Dako) for 1 hour. Blots were visualized with the use of an enhanced chemiluminescence detection system (Amersham) and scanned with a Molecular Dynamics 300A laser densitometer to determine protein levels with Quantity One software (PDI).

In Vitro Experiments
Tolerance to hypoxia-reoxygenation of β-gal–expressing L6 myoblasts was evaluated in vitro with or without prior heat shock treatment.

Hypoxia-Reoxygenation Protocol
Confluent monolayers of heat-shocked and normal cultured L6 myoblasts grown on 35-mm dishes (n = 7 in each group) were subjected to 18 hours of hypoxia followed by 3 hours of reoxygenation. The remaining 7 dishes of cultured cells in each group were incubated for 21 hours without hypoxia-reoxygenation. The hypoxic conditions were generated by using a GasPak system (Becton Dickinson Microbiology System) in a plastic jar.

Measurement of LDH Leakage
At the end of reoxygenation, the conditioned medium was collected to evaluate the degree of plasma alteration by measuring LDH leakage. LDH concentration in the samples was assayed with a spectrophotometer in the presence of 0.5 mmol/L pyruvate and 0.15 mmol/L NADH.

Detection of Apoptosis Caused by Hypoxia-Reoxygenation Insult
Apoptosis caused by hypoxia-reoxygenation insult was evaluated with annexin V staining with flow cytometry, as described before. After sampling of supernatant for LDH measurement, adherent cells were detached gently by trypsinization. After washing with DMEM containing 10% FCS followed by rinsing with binding buffer, 5 × 10⁶ cells were incubated with 0.5 μg/mL FITC-conjugated annexin V and 2.5 μg/mL propidium iodide for 15 minutes at 25°C in the dark (AnnexinV Apoptosis Kit, Clontech). Analysis of cell fluorescence was performed with flow cytometry with a single laser-emitting excitation light at 488 nm (EPICS XL-MCL, Beckman Coulter). More than 10⁶ cells were examined from each sample.

In Vivo Experiments
β-gal–expressing L6 myoblasts subjected to the heat shock treatment were infused into rat heart through the coronary artery in vivo.

Animal Care
All studies were performed with the approval of the institutional ethics committee for animal research. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Intracoronary Infusion of Skeletal Myoblasts
The myoblasts were harvested with the use of trypsin, resuspended in serum-free DMEM at a concentration of 2 × 10⁷ cell/mL, and stored at 4°C until infusion to the heart. The hearts of Sprague-Dawley rats (250 g) were arrested with cardioplegic solution and removed under anesthesia with sodium pentobarbital (50 mg/kg IP) and anticoagulation with heparin (200 USP units IV). Just before infusion, the cells were filtered through a 20-μm membrane filter (Millipore) and adjusted to a concentration of 1 × 10⁶ cell/mL in serum-free DMEM. The hearts were infused with 1 × 10⁶ cells through the coronary artery, with the venae cavae, pulmonary arteries, and veins ligated. Heat-shocked myoblasts were infused for the heat-shocked (hsCTx) group and normal-cultured myoblasts for the normal-cultured (CTx) group. For the control group, the same volume of cell-free DMEM was infused. After 10 minutes of incubation, the pulmonary artery was incised and the coronary circulation was flushed with cold PBS. The hearts were then heterotopically transplanted into the abdomens of 350-g recipient rats of the same strain. The hearts were collected on days 3, 7, 14, and 28 after cell transplantation for analysis.

Assay for β-Gal Activity
Assay for β-gal activity was done to evaluate the number of β-gal–expressing L6 myoblasts existing in the heart. Under anesthesia, the transplanted hearts were quickly excised from the abdomen (n = 5 in each point) and washed out of blood. The hearts were frozen in liquid nitrogen and homogenized in 0.25 mol/L Tris-HCl (pH 7.8). The homogenates were centrifuged at 3500g and then 12 000g. Thirty microliters of this supernatant was mixed with 66 μL of 4 mg/mL ONPG (O-nitrophenyl-β-D-galactopyranoside; Sigma) dissolved in 0.1 mol/L sodium phosphate (pH 7.5), 3 μL of 4.5 mol/L β-mercaptoethanol dissolved in 0.1 mol/L MgCl₂, and 200 μL of 0.1 mol/L sodium phosphate. The mixture was incubated at 37°C for 30 minutes, and the reaction was stopped by adding 500 μL of 1 mol/L Na₂CO₃. Values of OD₄₂₀ were read on a spectrophotometer and divided by protein concentration measured with the Bradford assay.

A standard scale was produced to evaluate graft cell survival from the data of β-gal activity. Nontreated rat hearts were collected under terminal anesthesia, washed out of blood, and mixed with known numbers (1 × 10⁶, 1 × 10⁷, 5 × 10⁶ or 1 × 10⁷) of β-gal–expressing L6 cells. The mixtures (n = 5 in each) were homogenated to measure the β-gal activity as described above.

In Situ β-Gal Staining
The remaining hearts (n = 5 at each point) were frozen in an embedding medium with liquid nitrogen for histochemical staining for β-gal. The samples were cut into 10-μm sections, fixed, and stained with X-gal as mentioned above. The samples were counterstained with 1% neutral red for 10 minutes.
**Statistical Analysis**

All values are expressed as mean±SEM. Comparison of the data for β-gal activity was performed with repeated-measures ANOVA. If a significant F ratio was obtained, further comparisons were determined by Bonferroni’s post hoc test. Differences in LDH leakage and apoptosis were determined by Bonferroni’s post hoc test. A low level of LDH activity in the culture medium was detected in normal culture incubation without hypoxia-reoxygenation insult. However, this percentage was significantly increased in both groups after the insult (0.70±0.06 U/well for control cells and 0.46±0.06 U/well for heat-shocked cells, n=7). There was a significant (P=0.02) reduction in the LDH leakage in heat-shocked cells compared with control cells, suggesting less plasma membrane damage (Figure 2B).

**Results**

**Generation of β-Gal–Expressing L6 Skeletal Myoblasts and Heat Shock Treatment**

To identify grafted skeletal myoblasts from native heart cells, L6 cells were genetically labeled to express β-gal in the nucleus. β-gal staining in vitro demonstrated that all cells express nuclear β-gal (A, magnification ×100). These cells could be induced to undergo myodifferentiation in vitro to form multinucleated myotubes in differentiation medium (B, magnification ×100, hematoxylin and eosin staining).

**Induction of HSP72 by Heat Shock Treatment**

β-gal–expressing cells were subjected to heat shock (42±0.3°C, 1 hour) or normal incubation (control) and examined 24 hours later in vitro or in vivo. No dead (rounded) cells were found in either group under microscopic observation. Western blotting confirmed a higher level expression of HSP72 in heat-shocked cells as compared with control-treated cells (A). LDH leakage increased in both groups after hypoxia-reoxygenation insult, whereas there was significant reduction in LDH leakage in heat-shocked cells compared with control cells (B). H-R (+) indicates myoblasts subjected to hypoxia-reoxygenation; H-R (−), myoblasts cultured without hypoxia. *P<0.01 vs H-R (−) in each group. †P=0.02 vs control L6 cells, n=7 in each point. Values are expressed as mean±SEM.

**Apoptotic Cell Death**

Apoptotic cell death caused by hypoxia-reoxygenation was detected with the use of flow cytometry with annexin V staining. Cells showing positive annexin V staining and negative propidium iodide (PI) staining (area 4 in Figure 3A) were counted as early apoptotic cells. Representative samples in each point are demonstrated in Figure 3A. Early apoptosis was detected in both control and heat-shocked cells even without hypoxia-reoxygenation insult, with no significant difference between the two groups (Figure 3B). The percentage of early apoptosis increased in both groups after hypoxia-reoxygenation. However, this percentage was significantly (P=0.01, n=7) reduced in heat-shocked cells (23.6±2.4%) as compared with the control cells (57.6±5.2%).

**In Vivo Experiments**

**Graft Cell Survival**

One million heat-shocked (hsCTx group) or normal-cultured (CTx group) β-gal–expressing L6 myoblasts were infused into the isolated heart through the coronary arteries, followed by 10-minute incubation, followed by heterotopic heart transplantation. The operative mortality rate was ≈6%, mainly related to ileus and intra-abdominal infection. Assay for β-gal activity with ONPG was performed to evaluate graft cell survival after cell transplantation. β-gal activity of the standard samples, which were mixtures of hearts and a known number of β-gal–expressing cells, were 17.2±3.5, 57.8±6.2, 120.6±15.7, and 173.1±20.5 OD550/μg protein for the 1×10^6, 1×10^5, 5×10^4, and 1×10^3 cell samples, respectively (n=5 in each group). The time course of β-gal activity after cell transplantation is shown in Figure 4. β-gal activity was significantly highest in the hsCTx group among the three groups. Using the standard scale, we estimated that ≈1×10^6 surviving cells on day 3 quickly increased to ≈5×10^6 on day...
7, with a slow rise until day 28 (≈8×10⁶ cells) in the hsCTx group. For the CTx group, the pattern of change in cell number was similar: 5×10⁵ cells on day 3 increased quickly to ≈2.5×10⁶ on day 7, with a slow rise to ≈5×10⁶ cells. The values for the control hearts were <10 OD₄₂₀/g protein throughout the postoperative period.

**Histological Examination**

β-gal–positive cells were detected at discrete loci widely distributed throughout the cardiac layers of both left and right coronary territories. The frequency of the loci tended to be greater in the hsCTx hearts as compared with the CTx hearts (Figure 5, A, B, and C). At some of these loci, surviving cells formed a colony composed of the β-gal–positive myoblasts that appeared to be undifferentiated (Figure 5D). In contrast, it was observed that in other loci, surviving myoblasts had completely differentiated into β-gal–positive multinucleated myotubes that aligned with the cardiac fiber axis and had integrated with the native myocardium (Figure 5E). The rate of differentiation did not appear to be affected by heat shock treatment. Histological evidence of myocardial thrombosis or infarction was not detected. Further, any findings suggestive of significant immunorejection were not found over the 1-month period after transplantation, even though no immunosuppressive reagents were used.

**Discussion**

We have reported here that prior heat shock treatment of skeletal myoblasts enhances their survival after cell transplantation to the heart in vivo. Parallel in vitro experiments demonstrated enhanced self-protective capacity in heat-
shocked myoblasts to attenuate cell death, including apoptosis caused by hypoxia-reoxygenation insult. Heat shock treatment could thus be useful in improving the efficiency of cell transplantation.

Skeletal myoblast transplantation is a promising alternative to treat end-stage heart failure. Survival and integration of skeletal myoblast grafts in experimental models have, however, been limited.4-8 Inflammation and free radicals generated by environmental stress such as engraftment itself, ischemia-reperfusion injury, and mechanical stress in the failing recipient myocardium may lead to not only necrosis but also apoptosis of the grafted myoblasts. This is likely to be a major factor hindering graft function. The in vivo experiments in this work have demonstrated that heat shock treatment before skeletal myoblast grafting doubled the cell survival at all time points examined after cell transplantation. It is reported that overexpression of HSP70 lasts at most for 48 to 72 hours.9,10 Accordingly, it can be speculated that this improvement in survival might be due to an enhanced tolerance of grafted myoblasts to various pathological processes, especially in the early period after grafting. This procedure of heat shock treatment for graft cell before cell transplantation is a simple method to accomplish improved graft cell survival with little risk. We would suggest that this system could be applied to not only cellular cardiomyoplasty but also cell transplantation for other organs such as skeletal muscle, liver, and pancreas.

In our data, ~90% of the myoblasts infused were entrapped in the lumina of small capillaries or had migrated into the myocardial interstitium immediately after cell transplantation according to the results of β-gal activity staining (data not shown). We therefore speculate that most of the infused cells are entrapped in the coronary bed rather than flushed out into the circulation and then permeate into the myocardial interstitium through the coronary endothelial barrier. Even though the detailed mechanism of translocation of grafted myoblasts into the myocardial interstitium remains unclear, the 10-minute incubation under increased intracoronary pressure in our method might help this process of cell migration into myocardium.

As a semiquantitative marker of cell number surviving in the heart, we applied β-gal activity, which has already been established in the field of cell transplantation to skeletal muscle.8,15 Stably transfected clonal cells can be expected to express β-gal constantly and uniformly as a whole, although the level of expression of an individual cell may be affected by cell cycle and circumstance. Therefore, one can expect the β-gal activity to be directly proportional to the number of cells (proliferation). β-gal activity as a semiquantitative marker of cell number would be considered reliable enough to be used alone; other kinds of cell labeling such as BrdU might be useful in supporting our data on proliferation. Using this β-gal activity measurement, we estimated that ~1×10⁶ heat-shocked myoblasts survived compared with 5×10⁵ nontreated cells on day 3 after engraftment of 1×10⁶ cells. The existing cell number in the myocardium quickly increased 5-fold by day 7, with a slow rise until day 28 in both groups. This observation may suggest that the surviving myoblasts proliferated mainly in the early period and then began differentiation after exit from the cell cycle. The mechanism of switching the proliferation into the differentiation state would involve cyclin-dependent kinases and their inhibitors,21 which could potentially be modulated by various local growth factors.22 Further experiments are required to clarify these issues. Another possible concern in our study might be immunoreaction, which might have been caused by not only infused L6 rat skeletal myoblasts but also transplanted hearts and expression of the foreign protein, β-gal. Any findings suggestive of significant immunorejection, however, were not found over the 1-month period after transplantation without immunosuppressive reagents. In addition, it is certainly demonstrated that a certain number of grafted cells survived, proliferated, and differentiated. We therefore speculate that immunorejection was not significantly serious in our system.

Figure 4. Graft cell survival after cell transplantation. One million heat-shocked (hsCTx) or normal cultured (CTx) β-gal-expressing L6 myoblasts were infused into heart through coronary artery. Control hearts were infused with cell-free medium. By standard scale on right of graph, 1×10⁶ myoblasts survived in hsCTx group on day 3 and increased quickly to ~5×10⁶ on day 7, with slow rise until day 28 to final number of 8×10⁶. *P<0.05 vs control group; †P<0.05 vs control and CTx groups, n=5 in each point. Values are expressed as mean±SEM.

Figure 5. Histological findings. A: After skeletal myoblast transplantation through coronary artery, β-gal-positive myoblasts were detected at discrete loci throughout myocardium. Frequency of loci appeared to be greater in hsCTx heart (A, magnification ×100) compared with CTx hearts (B, magnification ×100). No staining was observed in control hearts (C, magnification ×100). Some surviving myoblasts in hsCTx group differentiated into β-gal-positive multinucleated myotubes that aligned with cardiac fiber axis (E, magnification ×400); others formed colony composed of β-gal-positive undifferentiated myoblasts (D, magnification ×400).
It was also found in the present experiments that some surviving myoblasts differentiated into multinucleated myotubes and integrated with the native myocardium, whereas others formed undifferentiated colonies. The rate of differentiation did not appear to be affected by heat stress. The reason that some remained in an undifferentiated state and others differentiated is unknown. Although the differentiation ability of the β-gal-expressing myoblasts was confirmed in vitro, it may not be possible to conclude this for the in vivo experiments. For the purpose of enforcing the differentiation ability to allow more myoblasts to differentiate in myocardium in vivo, genetic engineering of skeletal myoblasts to overexpress appropriate beneficial proteins might be advantageous.

Both necrosis and apoptosis are potentially involved in the mechanism of grafted cell death after transplantation to the heart, though the precise mechanism remains unclear. It has been reported that heat shock treatment attenuates both necrotic and apoptotic cell death induced by several kinds of environmental stress in a variety of cells and that this is associated with induction of high levels of expression of HSP72.9–12 This protective effect against apoptosis is suggested to be associated with downregulation of p5310,12 and induction of Bax/Bcl-2.10,11 In the present studies, we have also demonstrated that heat shock treatment induced a high level expression of HSP72 in L6 skeletal myoblasts and protected them against apoptosis caused by hypoxia-reoxygenation injury in vitro. We have not provided direct evidence of a relation between HSP72 induction and attenuation of apoptosis because heat shock treatment is reported to attenuate hypoxic injury in rat.13 The present experiments, however, have shown that HSP72 itself protects cells against apoptosis through a mechanism of inhibiting activation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and CED-3–related protease caspase-3.21,23,24 Accordingly, we consider that heat shock treatment protects cells from apoptosis, resulting at least in part from induction of HSP72.

In conclusion, we have demonstrated that heat shock treatment improves the tolerance of skeletal myoblasts to a hypoxia-reoxygenation insult associated with high level expression of HSP72 in vitro. In addition, we have shown a significant enhancement in the survival of heat-shocked skeletal myoblasts in vivo when infused into the heart via coronary artery. Heat shock treatment could therefore be a useful strategy for improving graft cell survival in cellular cardiomyoplasty.

References
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