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Enhanced cell transplantation: preventing apoptosis increases cell survival and ventricular function

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Nakamura, Yoshinobu, Tamotsu Yasuda, Richard D. Weisel, and Ren-Ke Li. Enhanced cell transplantation: preventing apoptosis increases cell survival and ventricular function. *Am J Physiol Heart Circ Physiol* 291: H939–H947, 2006. First published March 31, 2006; doi:10.1152/ajpheart.00155.2006.—Cell transplantation prevents cardiac dysfunction after myocardial infarction. However, because most implanted cells are lost to ischemia and apoptosis, the benefits of cell transplantation on heart function could be improved by increasing cell survival. To examine this possibility, male Lewis rat aortic smooth muscle cells (SMCs; 4×10^6) were pretreated with antiapoptotic Bcl-2 gene transfection or heat shock and then implanted into the infarcted myocardium of anesthetized, syngenic female rats ($n = 23$ per group). On the first day after transplantation, apoptotic SMCs were quantified by using transferase-mediated dUTP nick-end labeling staining. On *days 7* and *28*, grafted cell survival was quantified by using real-time PCR, and heart function was assessed with the use of echocardiography and the Langendorff apparatus. SMCs given antiapoptotic pretreatments exhibited improvements in each measure relative to controls. Apoptosis was reduced in Bcl-2-treated cells relative to all other groups ($P < 0.05$), whereas survival ($P < 0.01$) was increased. Heat shock also significantly decreased apoptosis and increased survival relative to control groups ($P < 0.05$ for group effect), although these effects were less pronounced than in the Bcl-2-treated group. Further, scar areas were reduced in both Bcl-2- and heat shock-treated groups relative to controls ($P < 0.05$), and fractional area change and cardiac function were greater ($P < 0.05$ for both measures). These results indicate that antiapoptosis pretreatments reduced grafted SMC loss after transplantation and enhanced grafted cell survival and ventricular function, which was directly related ($r = 0.72$; $P = 0.002$) to the number of surviving engrafted cells.

cell therapy; Bcl-2; heat shock; ventricular modulation; angiogenesis

SKELETAL MYOBLAST TRANSPLANTATION prevents scar thinning and ventricular dilatation after a myocardial infarction and has been associated with improved regional and global function in both animal experimentation (5, 17, 26) and the initial clinical trials of this intervention (6, 15). The mechanism responsible for this beneficial effect has not been elucidated but may include angiogenesis, altering the elasticity of the ventricular wall, and/or modifying matrix remodeling. These mechanisms were suggested because none of the myriad of implanted cells have been demonstrated to differentiate into functioning cardiomyocytes, nor have they been demonstrated to beat synchronously with the remaining recipient cardiomyocytes. Therefore, the goal of cell transplantation is to establish a graft of viable cells within the infarct region to modify ventricular remodeling and prevent congestive heart failure. If the grafted cells could contribute to the contractility of the infarct scar, the beneficial effect would be enhanced. In the present study, our first aim was to evaluate the efficacy of smooth muscle cell (SMC) transplantation to augment cardiac function, because these cells routinely induce angiogenesis and matrix remodeling and might be ideally suited to efficiently prevent cardiac dilatation and congestive heart failure (10, 30). In addition, SMCs are easily harvested and rapidly proliferate in culture to provide a large number of cells for implantation soon after the infarct.

The major limitation of muscle cell transplantation has been the high attrition rate of the injected cells. Both the regional and global functional improvement has been demonstrated to be proportional to the number of cells surviving implantation (18). However, several studies (16, 13) demonstrated that the survival rate of implanted SMCs or cardiomyocytes (28) was limited to 20–30%, possibly due to ischemia, apoptosis, inflammation, or immunological rejection. In addition to ischemic injury, potential mechanisms for grafted cell death after transplantation include the activation of apoptotic pathways, the loss of matrix attachments (anoikis), or the effects of inflammatory cytokines (19). To improve grafted cell survival, several preconditioning methods have been evaluated, including antiapoptotic gene transfection and physiological pretreatment. We found that transfection with the insulin growth factor gene induced increased grafted SMC survival (13), and the combination of VEGF gene and cardiac cell therapy also improved both cell survival and postinfarction ventricular function (29). Suzuki et al. (25) reported that heat shock pretreatment improved the survival of implanted skeletal myoblasts. However, none of these previous reports clearly demonstrated a relationship between grafted cell survival and cardiac function, and none compared the efficacy of these pretreatments. The second aim of the current study was to identify a strategy to improve cell survival rate and correlate the implanted cell number with the resultant improvement in cardiac function after coronary occlusion.

Several investigators (19, 11, 22) estimated grafted cell survival using histological methods. Most of these methods are semi-quantitative, and the survival rates varied depending on the quantification techniques employed. Using a quantitative real-time PCR technique, we have quantified the survival of transfected cells (13, 28). The real-time PCR method provided a very good correlation between DNA copy number of the transplanted cells and cycle numbers detected by fluorescence exceeding threshold (28).

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In the present study, we compared the effects of Bcl-2 transfection and heat shock pretreatment on the survival of transplanted SMCs in the infarcted myocardium using the real-time PCR method, and evaluated the functional improvement associated with both treatments.

MATERIALS AND METHODS

Experimental Animals

The experimental animals used in this study were male adult Lewis rats weighing 150–200 g as the donors and female adult Lewis rats weighing 150–200 g as the recipients (Charles River). All procedures were approved by the Animal Care Committee of the Toronto General Research Institute and carried out in compliance with *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care and *Guide for the Care and Use of Laboratory Animals,* published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985).

Preparation of Donor Cells

SMCs were isolated as previously described (10). Third passage cells were employed for transplantation. The SMCs were identified immunohistochemically, using a monoclonal antibody against smooth muscle myosin heavy chain (SM1, 1:2,000; Yamasa, Tokyo, Japan). The cell pellet was resuspended at a concentration of 4×10^6/50 μl for cell transplantation.

Heat Shock Pretreatment

Subconfluent cultured cells in 100-mm dishes (for in vitro experiments) or 175-cm^2 flasks (for in vivo experiments) were subjected to hyperthermia of 42°C for 1 h with a water bath (25). Heat shock did not produce irreversible injury to the cells, and no rounded cells were found after exposure to heat shock. Histological examination did not show any differences between heat shock and nontreated cells (exposed to media without heat shock).

In Vitro Heat Shock Protein 72 Levels

The level of heat shock protein 72 (HSP72) was evaluated in the heat shock-treated and control cells by Western blotting on 1, 3, and 7 days after heat shock to determine the optimal time to inject heat shock-treated cells. The cells were disrupted in a lysis buffer [50 nM Tris·HCl (pH 8.0), 10 nM EDTA, 4 M urea, and 1% Triton X-100], and 10 μg protein samples were loaded onto 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was rinsed and blocked. The membrane was incubated with a 1:1,000 dilution of anti-HSP72 monoclonal antibody (Stressgen Biotechnologies, Victoria, BC, Canada). The membrane was then incubated with the second antibody (goat anti-mouse horseradish peroxidase-conjugated antibody, Bio-Rad, Mississauga, ON, Canada). An enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ) was used to visualize the protein bands.

Human Bcl-2 Gene Transfection

The plasmid PUC19-hBcl-2, containing green fluorescence protein, was kindly provided by Dr. Tsujimoto, Osaka University (Osaka, Japan). The SMCs, seeded and cultured for 24 h, were transfected with either 10 μg of the plasmid PUC19-hBcl-2 or the vector plasmid using 10 μg of lipofectamine (Lipofectamine 2000; Invitrogen, Burlington, ONT, Canada).

In Vitro Human Bcl-2 Protein Levels

On *days 3* and *7* after gene transfection, protein levels of Bcl-2 in the gene or plasmid vector-transfected cells were evaluated using Western blotting. In brief, intracellular proteins were isolated using a lysis buffer, and 15 μg of protein from each sample were loaded onto 10% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane. After it was rinsed and blocked, the membrane was incubated with a monoclonal antibody against human Bcl-2 oncoprotein (code no. sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was next incubated with the secondary antibody (goat anti-mouse horseradish peroxidase-conjugated antibody, Bio-Rad). An ECL kit (Amersham) was used to visualize the protein bands.

Apoptosis Induced by Oxidative Stress

Cells in each group were exposed to oxidative stress by hydrogen peroxide. Cultured SMCs (1×10^6 cells) transfected with the human Bcl-2 gene (BCL), or plasmid vector (VEC), heat shock treated SMCs (HS), or nontreated SMCs (CEL) ($n = 7$ per each group) were exposed to 100 μmol/l hydrogen peroxide for 8 h as described previously (27). Next, caspase-3 activities were measured by using a Caspase-3 colorimetric assay kit (Chemicon).

Creation of Myocardial Infarction

Recipient female rats were anesthetized with an intramuscular injection of ketamine (20 mg/kg), followed by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). The animals were then intubated and ventilated with oxygen-supplemented room air with a Harvard ventilator at a rate of 60 breaths/min and a tidal volume of 2.0 ml. The heart was exposed through a 2-cm left thoracotomy, and an anterior myocardial infarction was created by ligation of the proximal coronary artery [left anterior descending artery (LAD)] as previously described (14). After LAD ligation, the animals recovered from the operation and were given Penlong XL (150,000 U/ml benzathine penicillin G and 150,000 U/ml procaine penicillin G; 0.3 ml im) every 3 days and buprenorphine (0.01–0.05 mg/kg subcutaneously) every 12 h for the first 48 h after the operation.

Cell Transplantation

Six days after LAD ligation was performed, the Bcl-2 gene-transfected (BCL group), heat shock-treated (HS group), plasmid transfected (VEC group) or nontreated control (CEL group) SMCs (4×10^6/50 μl/rat) were injected into the infarcted myocardium ($n = 23$ rats per group). To confirm the efficacy of SMC transplantation, culture media (50 μl) (MED group) were also injected ($n = 23$). Under general anesthesia, the heart was exposed through a median sternotomy. Before cell injection, a purse-string suture was placed in the anterior wall of the left ventricle to prevent leakage of cells from the injection site. One minute after transplantation, the purse-string suture was ligated. After cell transplantation, the chest was closed and the animals were treated in the same manner as described after LAD ligation.

Apoptosis of Grafted Cells in Infarcted Area

Twenty-four hours after cell transplantation, the number of grafted SMCs containing fragment DNA in the BCL, HS, CEL, and VEC groups ($n = 5$ rats per group) was detected by in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an in situ cell death detection kit according to the manufacturer's specifications (Boehringer Ingelheim, Burlington, ON, Canada). The sample sections were counterstained with hematoxylin. Ten microscopic fields (×400 by Eclipse-TE200; Nikon, Tokyo, Japan) of each slide were randomly selected and digitally photographed. To identify the grafted SMCs, the sections adjacent to the section used for TUNEL staining were also stained with the smooth muscle myosin heavy chain (SM1) antibody (described in *Preparation of Donor Cells*). The number of grafted and apoptotic SMCs was counted separately with the NIH Image program (NIH, Springfield, VA) and Adobe Photoshop (Adobe Systems, San Jose,

CA). Apoptosis was expressed as the number of apoptotic grafted nuclei per 1,000 grafted cell nuclei.

Quantitative Analysis of Cell Survival

The survival rate of grafted male cells in the hearts of the female recipients was analyzed by real-time PCR to quantify the Y chromosome on *days 7* and *28* after cell transplantation, as described in previous publications (13, 28). The infarcted myocardium from rats in the BCL, HS, CEL, and VEC groups ($n = 6$ rats per group per time point; assessed after echocardiographic measurements at corresponding time points) was dissected from the left ventricle and ground into fine powder with a precooled mortar and pestle. A piece of normal myocardium from a remote area was also collected for analysis. The DNA was extracted using a Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen, Mississauga, ON, Canada), and the amount of DNA was measured by spectrophotometry. Real-time PCR was employed using SYBR-Green (Applied Biosystems, Foster City, CA). The SYBR-Green I dye binds to the double-stranded product, resulting in an increase in fluorescence detected by the ABI 7900HT Sequence Detection System (Applied Biosystems). A specific sequence of rat "Sry3" gene in the Y chromosome was targeted by using the primer pairs of 30 nM RTA (GCA TTT ATG GTG TGG TCC CGC GG) and 30 nM RTE (GGC ACT TTA ACC CTT CGA TGA GGC). The cycling condition was 5 min at 50°C, 10 min at 95°C for activation of polymerase, 30 s at 95°C for denaturation, 60 s at 62°C, to induce annealing, and 30 s at 72°C for extension. Forty-five cycles were employed. The genomic DNA taken from four known numbers of male SMCs was used to obtain a standard curve, which was then used to evaluate the number of grafted male SMCs. Cell survival rate was calculated as number of surviving cells/total number of cells injected $\times$ 100.

Cardiac Function

Cardiac function of the rats in the BCL, HS, CEL, and VEC groups was evaluated with echocardiography on *days 7* and *28* after cell transplantation and with isolated Langendorff heart perfusion on *day 28* after cell transplantation.

Echocardiography. At 7 and 28 days after cell transplantation, the rats ($n = 6$ rats per group per time point; these same rats were subsequently used for PCR analysis at corresponding time points) were sedated with isoflurane, and left parasternal images were taken in the right lateral decubitus position with a 13-MHz transducer (Sequoia C256 and 15L8, respectively; Acuson, Mountain View, CA). Short-axis two-dimensional images at the midpapillary level of the left ventricle were stored as digital loops, and the end-systolic (ESA) and end-diastolic (EDA) cavity areas were determined by tracing the endocardial borders. The fractional area change (FAC) was calculated as (EDA $-$ ESA)/EDA $\times$ 100. For each measurement, three consecutive cardiac cycles were traced and averaged by an experienced examiner in a blinded fashion, according to the American Society for Echocardiology Leading Edge Method.

Isolated Langendorff heart perfusion. At 28 days after cell transplantation, global heart function ($n = 6$ rats per group; these same rats were subsequently used for morphological and histological studies) was also evaluated by using Langendorff preparation as previously described (10). Left ventricular peak systolic, end-diastolic, and developed pressures were measured as the ventricular volumes were increased in 0.01-ml increments until the end-diastolic pressure reached 30 mmHg. Developed pressure was calculated as the difference between the peak systolic and end-diastolic pressures at each ventricular volume by an automated real-time computer software program (Ponemah Physiology Platform; Gould Instrument Systems, Valley View, OH).

Hearts were then arrested with 10 ml of KCl solution (20 mmol/l). Passive diastolic pressures were recorded at each balloon volume in 0.04-ml increments until the diastolic pressure was over 60 mmHg. The heart was then fixed at a ventricular pressure of 30 mmHg with 10% phosphate-buffered formalin solution for 48 h.

Morphological and Histological Studies

At 28 days after cell transplantation, formalin-fixed hearts ($n = 6$ rats per group; assessed after Langendorff heart perfusion measurements) were cut into 2-mm-thick sections, and both apical and basal sections were digitally photographed (Coolpix, Nikon, Tokyo, Japan) and quantified using the public domain NIH Image program. The thickness of the left ventricular free wall and the scar area were measured as previously described (10). The left ventricular chamber volume was also calculated from planimetric measures. At the end of the study, the hearts used for functional analysis using the Langendorff aparatus were fixed at a ventricular pressure of 30 mmHg with 10% phosphate-buffered formalin solution for 48 h. The formalin-fixed hearts were cut into 2-mm-thick sections, and both apical and basal aspects of each section were digitally photographed. The area of the left ventricular chamber was measured on both sides of each section and averaged. The area was multiplied by the 2- mm thickness of each section, and the sum of all sections was calculated as the left ventricular chamber volume. Finally, the sections were stained with hematoxylin and eosin as described in the manufacturer's specifications (Sigma, St. Louis, MO). Immunohistochemical staining with a monoclonal antibody against SM1 was used to identify SMCs in the scar.

Statistical Analysis

The mean and SE are provided in the figures. StatView and the Statistical Analysis System software (SAS Institute, Cary, NC) was used for analysis. Comparisons of continuous variables among all groups were performed by a one-way analysis of variance. Scheffé's test was used to specify differences among groups. Echocardiography function data were evaluated by an analysis of covariance (ANCOVA), and group and time interactions were analyzed. Langendorff function data were also analyzed by ANCOVA: the main effects were group, left ventricular end-diastolic pressure, and the interaction between group and left ventricular end-diastolic pressure. Probability values <0.05 were considered statistically significant.

RESULTS

In Vitro Studies

Most of the cultured SMCs before cell transplantation stained positively for SM1 in 90.0 $\pm$ 3.0% ($n = 6$).

Bcl-2 protein elevated in transfected SMCs. The Bcl-2 gene was introduced into cultured SMCs with a transfection efficiency of 14 $\pm$ 3% on the third day after transfection (Fig. 1*A*). Figure 1*B* illustrates the expression of Bcl-2 protein in the BCL and CEL groups. On *days 3* and *7* after transfection, the SMCs in the BCL group expressed more Bcl-2 protein compared with the CEL group. However, the Bcl-2 protein level in the BCL group decreased to normal at 2 wk after gene transfection (data not shown). Therefore, we implanted Bcl-2-transfected SMCs on the third day after transfection.

Heat shock increased HSP72 in cultured SMCs. Figure 1*C* illustrates the expression of HSP72 in the HS and CEL groups. The peak overexpression of HSP72 protein was detected on the first day after exposure to heat shock. On *day 7*, the level of HSP72 protein was almost the same as that of the control cells. Therefore, we implanted the heat shock-treated cells at 24 h after the treatment.

Caspase-3 activity after oxidative stress. Caspase-3 activity in the BCL and HS groups was significantly lower than in the

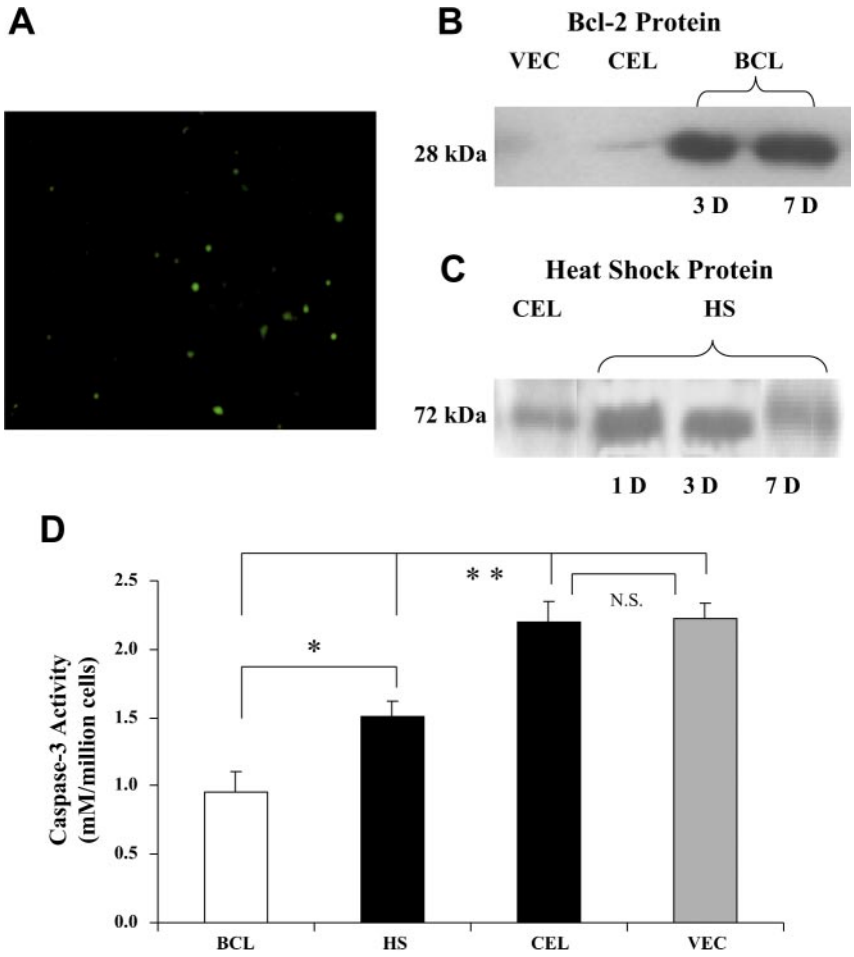

Fig. 1. *A*: Smooth muscle cells (SMCs) were transfected with plasmid vector containing Bcl-2 gene; transfected efficacy was evaluated by expression of green fluorescent protein (×100). *B*: intracellular Bcl-2 protein levels in the transfected groups were evaluated by Western blotting at 3 and 7 days after gene transfection. Less Bcl-2 protein was found in cultured SMCs (CEL) and vector-transfected cells (VEC) than in Bcl-2-transfected cells (BCL). *C*: Western blot showing heat shock protein 72 (HSP72) protein expression in cells from CEL group and in cells from heat shock-treated (HS) group at 1, 3, and 7 days after induction of heat shock. *D*: caspase-3 activity was measured with a colorimetric assay in cultured cells after they were exposed to 100 μmol/l hydrogen peroxide for 8 h. Caspase-3 activity in the BCL group was lower than in the other groups. Caspase-3 activity in the HS group was less than in the VEC and CEL groups. $n = 7$ tests per group. *$P < 0.05$; **$P < 0.01$.

VEC and CEL groups after oxidative stress ($P < 0.01$; Fig. 1*D*). Further caspase-3 activity in the BCL group was much lower than that in the HS group ($P < 0.05$).

In Vivo Studies

Apoptosis of grafted SMCs. The number of SMC nuclei with DNA fragmentation was determined by TUNEL staining. A typical apoptotic nucleus appeared red-purple (Fig. 2). The apoptotic nuclei were quantified in relation to normal nuclei in 10 fields of adjacent tissue sections at ×400 magnification. The number of apoptotic SMCs in the BCL group (76.9 ± 8.9/1,000 nuclei) was significantly lower ($P < 0.05$) than in the other groups (HS: 133.4 ± 21.3, $P < 0.05$; VE: 232.8 ± 27.8, $P < 0.01$; and CEL: 244.2 ± 22.7/1,000 nuclei, $P < 0.01$) at 24 h after transplantation. The number of apoptotic cells in the HS group was significantly less ($P < 0.01$) than in the VEC and CEL groups.

Cell survival. Figure 3 shows that more extensive engraftment was achieved in the pretreatment groups. Cell survival was significantly greater in the BCL group than in the other groups ($P < 0.01$, for all groups), with 30.8 ± 5.3% survival on *day 7* and 22.7 ± 2.9% on *day 28* (HS: 20.5 ± 3.5 and 17 ± 4.5; VEC: 14.5 ± 3.0 and 9.0 ± 3.5; and CEL: 15.5 ± 3.5 and 9.5 ± 4.0% on *days 7* and *28*, respectively; Fig. 3*A*). Additionally, cell survival in the HS group was significantly higher ($P < 0.05$) on *days 7* and *28* than in the VEC and CEL groups. Twenty-eight days after implantation, SMCs were identified within the infarct region in all groups with the use of SM1 staining (Fig. 3, *B–E*). In the VEC and SMC groups, many fewer SMCs were detected compared with the BCL and HS groups at 28 days after grafting. Grafted cells were not detected in the remote myocardium.

Cardiac Function

Echocardiography. There were no differences before cell transplantation among the five groups. In the analyses of FAC, group and time effects as well as the group-by-time interaction were strongly significant (Fig. 4). The BCL group had better FAC ($P < 0.05$) than the other groups 7 days after cell transplantation. At 28 days, the BCL group had better FAC than the VEC and CEL groups ($P < 0.05$), although there were no significant differences between the BCL and HS groups at this time point. FAC in the HS group was significantly greater than in the VEC group ($P < 0.05$) at 7 days after cell transplantation. At 28 days, FAC in the HS group was greater than in the CEL and VEC groups ($P < 0.05$). Figure 5 indicates that FAC after cell transplantation strongly correlated with cell survival ($r = 0.74$, $P = 0.002$, $n = 24$).

Langendorff study. Heart rate and coronary flow were similar among the five groups during the Langendorff study, but the peak systolic pressure was significantly greater in the BCL group compared with the other groups ($P < 0.01$ for all groups; Fig. 6*A*). The peak systolic pressure was significantly greater in the HS group than in the VEC and CEL groups ($P < 0.01$ for

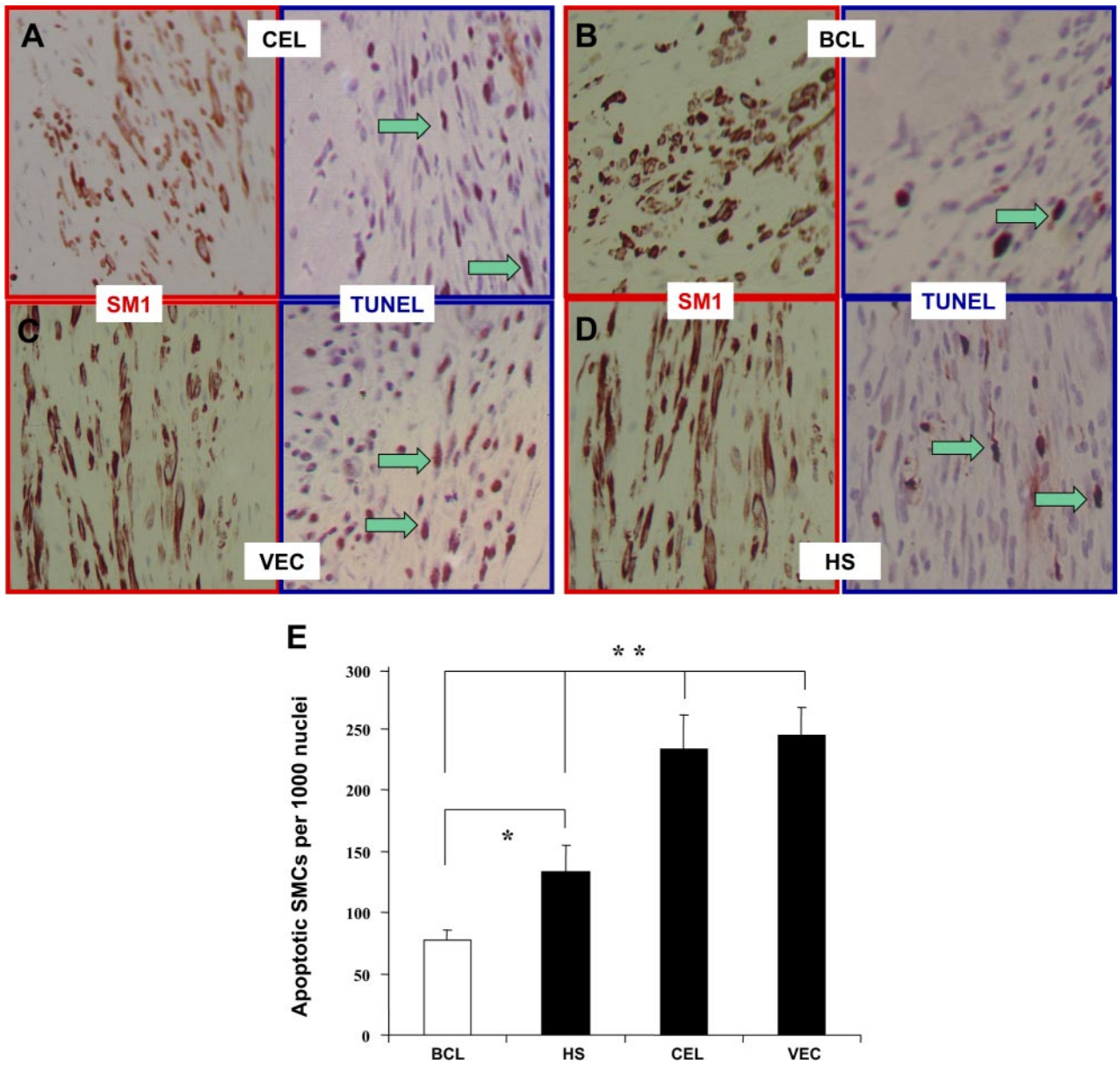

Fig. 2. *A–D*: microphotographs of myocardial scar tissue from animals transplanted with SMCs (*A*), Bcl-2 transfected cells (*B*), plasmid vector-transfected cells (*C*), or heat shock-treated cells (*D*). Tissue has been stained with smooth muscle myosin heavy chain (SM1) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (×400 in all panels). TUNEL-positive SMCs (red-purple-stained nuclei; arrows) were observed at 24 h after cell transplantation in all groups. Numbers of TUNEL-positive grafted SMCs were significantly reduced in BCL and HS groups relative to other groups (*E*). n = 5 rats per group. *$P < 0.05$; **$P < 0.01$.

all groups). The CEL group had significantly better systolic function than the medium injection group ($P < 0.01$), in agreement with our previous reports (10, 30). Maximum rate of increase in left ventricular pressure (dP/d*t*) in the BCL group was significantly greater than in the other groups (Fig. 6*B*). Minimum dP/d*t* was significantly lower in the BCL group than in the other groups (Fig. 6*C*).

Ventricular Morphology

At 28 days after implantation, left ventricular volumes were significantly smaller ($P < 0.05$) in the BCL group compared with the HS group and all control groups (VEC, CEL, MED). Similarly, scar areas were significantly smaller ($P < 0.01$) in the BCL group than in the other groups, whereas scar thicknesses were significantly greater ($P < 0.01$). Furthermore, whereas the HS group had significantly smaller ($P < 0.05$) scar areas than those of the VEC and MED groups, the VEC group showed greater ($P < 0.05$) scar thicknesses than those of either the HS or MED group. Mean group values for left ventricular morphological data collected at 28 days after cell transplantation are summarized in Table 1.

DISCUSSION

Recent studies suggest that skeletal myoblasts improve regional and global cardiac function without the conversion of the myoblasts to a cardiogenic phenotype and without their

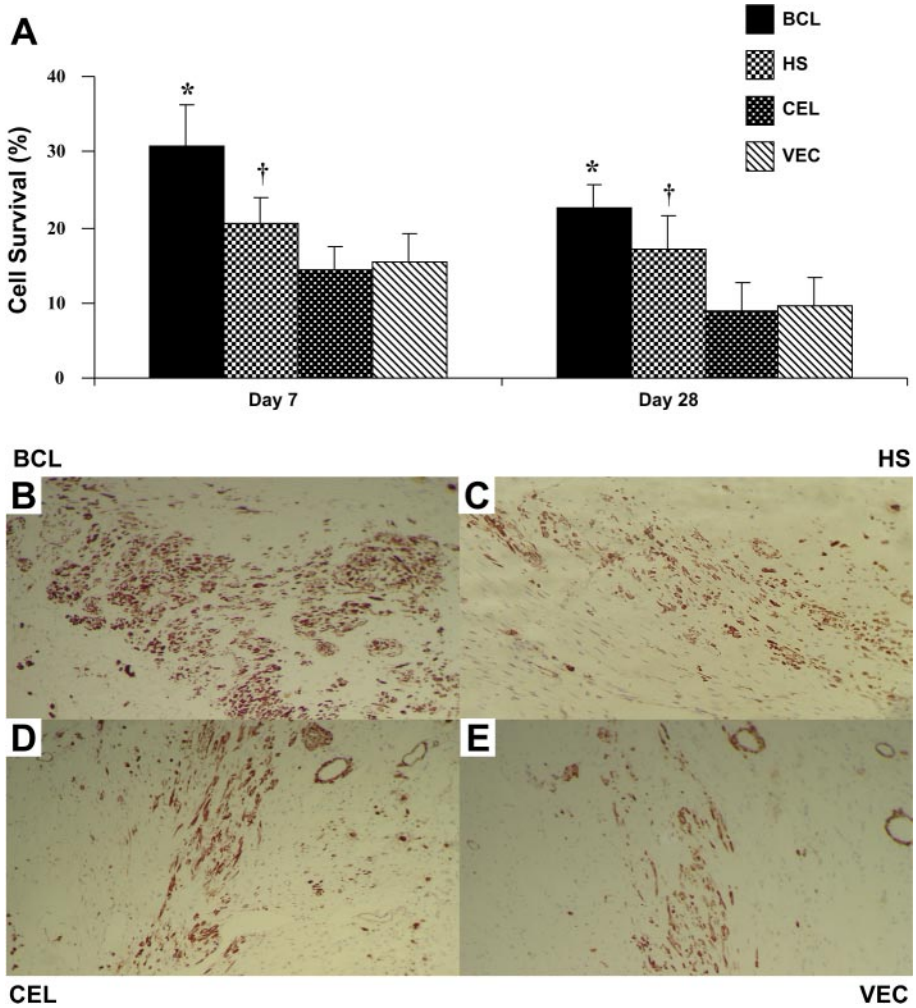

Fig. 3. *A*: percent survival of engrafted SMCs at 7 and 28 days after cell implantation was determined by real-time PCR targeting Y chromosome of male cells implanted into female hearts. Percent survival of cells in BCL group was greater than that of cells in the other groups at both 7 and 28 days (*$P <$ 0.01). Heat shock-treated cells experienced greater survival than either the cell transplanted (CEL) or vector-transfected cells at 7 and 28 days after implantation (†$P < 0.05$). $n = 6$ rats per group. *B–E*: immunohistochemical staining for SM1 28 days after cell transplantation. SM1 staining appears brown in color in SMC cytoplasm of engrafted cells within the infarct region (×100 in all panes).

synchronous contraction with the remaining recipient cardiomyocytes (5, 10, 16, 18, 26, 30). These data demonstrate that noncontractile skeletal muscle cell transplantation improves cardiac function after implantation. The present investigation indicates that cardiac function is also augmented by the implantation of SMCs, which is a significant finding because these cells may have therapeutic advantages over other types of muscle cells. For example, SMCs have a greater chance for postimplantation survival because of their rapid adherence to the matrix in the infarct region; such anchorage is necessary for

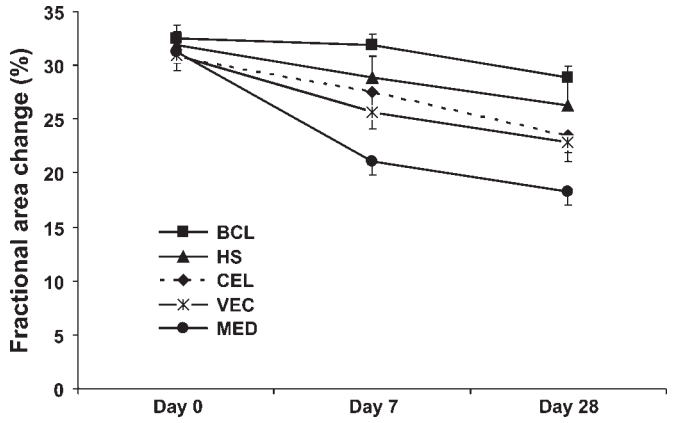

Fig. 4. Echocardiographic assessment demonstrated that fractional area changes were significantly higher in BCL and HS groups than in CEL and VEC groups ($P < 0.05$ for all groups) over the study period. Medium (MED) control group showed significantly smaller fractional area change than all other groups on *days 7* and *28* after transplantation ($P < 0.01$ for all groups). $n =$ 6 rats per group.

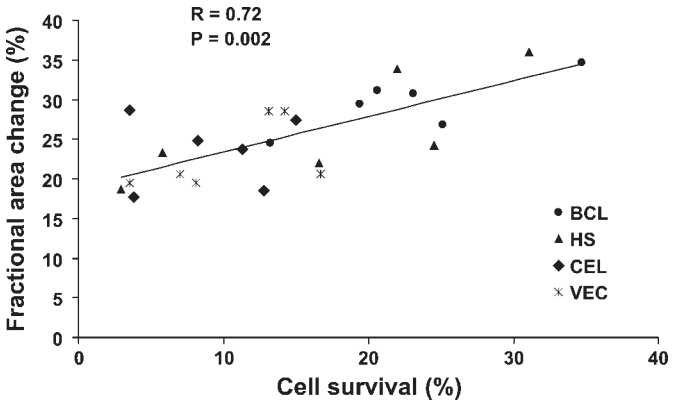

Fig. 5. Cardiac function at 28 days after cell transplantation. A positive correlation was found between fractional area change measured by echocardiography and survival of grafted cells in the BCL, HS, VEC, and CEL groups. *R*, correlation coefficient.

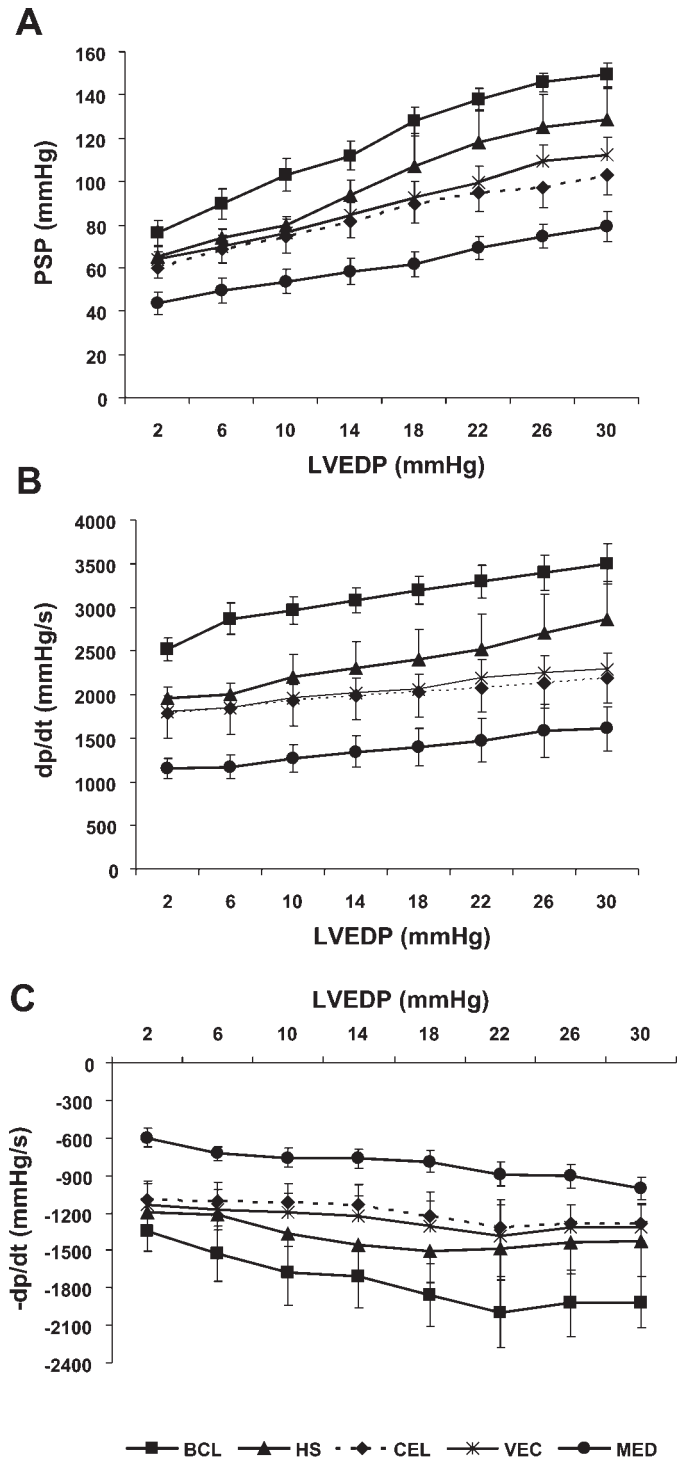

Fig. 6. Cardiac function assessed during Langendorff perfusion 28 days after implantation. Peak systolic pressure (PSP; *A*) and maximum rate of increase in left ventricular (LV) pressure (dP/d*t*; *B*) in BCL group were significantly greater ($P < 0.05$) than those in HS group, which were higher ($P < 0.05$ for all groups) than those in CEL and VEC groups. MED group showed significantly lower measures ($P < 0.01$ for all groups) than all other groups. −dP/d*t* (*C*) in BCL group was significantly lower than in all other groups (LVEDP, LV end-diastolic pressure); $n = 6$ rats per group.

Table 1. *Summary of LV morphological data collected for groups receiving implantation of BCL, HS, CEL, or VEC cells or medium at 28 days after implantation*

Group	Scar Thickness, cm	Scar Area, cm^2	LV Volume, cm^3
BCL	0.16±0.004*	0.71±0.032*	0.16±0.006†
HS	0.12±0.005	0.90±0.047‡	0.20±0.009
CEL	0.13±0.007	1.02±0.074	0.21±0.018
VEC	0.13±0.005	1.08±0.056	0.20±0.018
MED	0.12±0.003	1.30±0.031	0.22±0.010

Values are means ± SE. Scar thickness was significantly increased ($P < 0.01$) in Bcl-2 transfected (BCL) group relative to all other groups, whereas left ventricular (LV) volume was decreased ($P < 0.05$). Scar area was decreased ($P < 0.01$) in BCL group relative to heat shock-treated (HS) group and was decreased ($P < 0.05$) in both groups relative to vector-transfected (VEC) and medium (MED) control groups. CEL, nontreated SMC control group. *$P < 0.01$ compared with all other groups; †$P < 0.05$ compared with all other groups; ‡$P < 0.05$ compared with VEC and MED groups.

survival. SMCs also produce extracellular matrix and elastin (23, 12), which may increase their survival in the infarct region if the matrix has been damaged by the ischemic injury. Finally, SMCs can be easily harvested from the saphenous vein or radial artery, where they readily proliferate in vitro and respond to hemodynamic stresses by hypertrophy and hyperplasia. However, determination of the best cell to improve human cardiac function after a myocardial infarction will require well-controlled clinical trials.

The most important benefits of cardiac cell transplantation are the prevention of ventricular dilatation, dysfunction, and congestive heart failure. Because most implanted cells remain noncontractile in the implanted area, we hypothesize that they enhance contractility in a recipient heart after infarction by the following multiple mechanisms: *1*) induction of angiogenesis, which may preserve hibernating or apoptotic cardiomyocytes and reduce scar tissue formation (7); *2*) strengthening of the damaged tissue, which prevents scar expansion (10); *3*) prevention of matrix degredation, which may help to preserve extracellular structure (4); and *4*) secretion of cytokines that increase stem cell homing (3). In the present study, the multiple pathways by which implanted SMCs exerted their positive effects might explain the exaggerated effect of cell survival on improved ventricular function.

The functional improvement associated with cell transplantation has been demonstrated to correlate with the number of surviving engrafted cells (18). However, accumulated evidence suggests that the survival rate of graft cells in the normal or infracted myocardium is low (16, 19, 28), so strategies to improve grafted cell survival are likely to enhance the beneficial effects of implanted cells on cardiac function. In this study, we showed that antiapoptotic pretreatment of SMCs increased the survival of grafted cells, which was inversely correlated with scar size and directly correlated with cardiac function. After a myocardial infarction, injured cardiomyocytes are replaced by fibroblasts and collagen fibers. With time, the number of cellular elements decreases, and nonelastic fibrotic tissue expands. Cell implantation increases the cellularity within the myocardial scar tissue—preserving scar thickness, increasing the elastic properties of the scar tissue, and, in turn, producing functional benefits for the infarcted heart. This study demonstrates that antiapoptotic strategies can enhance the positive

effects of transplanted cells on ventricular structure and function after an infarction.

Both the cell type and the environment into which the cells are injected influence the number of grafted cells that survive. For example, implanted cell survival is limited by ischemia, apoptosis, inflammation, and rejection (19). The major impediment to the survival of the grafted cells is sustained ischemic injury before and after transplantation. Prolonged ischemia of the cells during transportation and before implantation may contribute to early cell loss. After implantation into scar tissue, the ischemic environment will also increase cell loss. In the present study, we found that ~40% of the grafted SMCs survived at 24 h after cell transplantation in the CEL and VEC groups, whereas ~20% were TUNEL positive in our preliminary study. Zhang et al. (31) also reported a similar rate of cell survival early after cell transplantation and a similar rate of apoptosis 4 days after grafting but no evidence of apoptosis 7 days after cell transplantation Therefore, a technique to prevent apoptosis early after cell transplantation should improve the long-term cell survival and prolonged efficacy of cell therapy in augmenting ventricular function.

Although alteration of the environment in the recipient infarct region into which the cells are grafted might improve the survival of implanted cells (20), increased cellular tolerance to ischemia may be more beneficial. The induction of angiogenesis in the infarct region before implantation of cells was associated with only a modest improvement in ventricular function (21). The present study demonstrated that antiapoptotic strategies to increase cellular tolerance to ischemia, specifically, Bcl-2 gene transfection and heat shock pretreatment, produced major improvements in ventricular function. The in vitro studies demonstrated that both Bcl-2 and HSP72 proteins were overexpressed for at least 7 days, and both increased cellular resistance to the ischemic injury.

Activation of caspases, a family of proteases that participates in the cellular disassembly, plays an important role during cellular apoptosis, inducing a cascade of proteolytic enzymes. Normally, this process is tightly regulated. The Bcl family reduces caspase activation and regulates apoptosis (1, 2). Overexpressed for at least 7 days after gene transfection, in the present in vitro studies, Bcl-2 proteins can limit capase-3 activation and prevent irreversible cellular damage. The in vivo data showed that Bcl-2 overexpression within implanted cells had a profound inhibitory effect on apoptosis and effectively improved both cell survival and cardiac function.

Other possible mechanisms for the beneficial effect of Bcl-2 overexpression include decreases in inflammation, which may be influenced by the injection process itself, as well as rejection, both of which may contribute to the early cell loss after transplantation. Cellular exposure to animal proteins during ex vivo expansion in culture may induce the rejection of the autologous or syngenic cells. Both inflammation and rejection might therefore impede the survival of the grafted cells. Kobayashi et al. (8) reported that Bcl-2 overexpression prevented immunological rejection in a hamster-to-rat cardiac xenograft model.

Heat shock treatment induces several self-protective proteins, including HSP72, which protect cells from environmental insults (25). Heat shock proteins act as molecular chaperones to maintain cellular homeostasis despite environmental stresses. HSP72 can prevent oligomerized Apsf-1 from recruiting pro-caspase-9 by its chaperone function, resulting in the attenuation of apoptosis (9). HSP72 can also induce the overexpression of antiapoptotic proteins such as Bcl-2 (24). Heat shock not only renders cells resistant to ischemic injury, but the self-protective proteins may also improve grafted cell survival within an ischemic scar. The present in vitro studies demonstrated that HSP72 protein was overexpressed for at least 7 days, increasing cellular resistance to the ischemic injury. In vivo data demonstrated that heat shock treatment improved cell survival after implantation into the damaged myocardium. Still, the Bcl-2-pretreated group demonstrated greater cell survival and better ventricular function than the heat shock-pretreated group.

The efficiency of Bcl-2 transfection with lipofectamine was low (14 ± 3%) in this study, and the duration of protein production was limited, suggesting that only a limited expression was required to achieve a significant improvement in cell survival and ventricular function. Acting via antiapoptotic pathways, Bcl-2 increased grafted cell survival and produced beneficial effects on heart function after a myocardial infarction, effects which may have become exaggerated by the multiple pathways responsible for the improved ventricular function after cell engraftment. Transfection efficiency could be increased by using an alternate vector, such as an adenovirus, which may produce prolonged overexpression of the Bcl-2 protein. However, viruses may induce excessive inflammation and can induce a cardiomyopathy. Future studies should therefore examine the effects of prolonged Bcl-2 overexpression. In addition, dose-response relationships should be determined for Bcl-2 expression and cell survival rates at various time points after engraftment by using differing numbers of injected cells and differing doses of Bcl-2.

In summary, we found that the antiapoptosis pretreatment of SMCs reduced cell loss after transplantation and improved cardiac function after a myocardial infarction. In this study, both Bcl-2 transfection and heat shock were effective treatments. Antiapoptosis pretreatment may further enhance the benefits of cell transplantation on ventricular function after a myocardial infarction.

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