Antiapoptotic Effect of Implanted Embryonic Stem Cell-Derived Early-Differentiated Cells in Aging Rats After Myocardial Infarction

Meixiang Xiang,1,2 Jianan Wang,1 Emel Kaplan,2 Peter Oettgen,2 Lewis Lipsitz,3 James P. Morgan,2,4 and Jiang-Yong Min2

1The 2nd Affiliated Hospital, Medical College of Zhejiang University, Hangzhou, China. Divisions of 2Cardiology and 3Gerontology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts. 4Division of Cardiovascular Medicine, Department of Medicine, Caritas St. Elizabeth’s Medical Center and Tufts University School of Medicine, Boston, Massachusetts.

This study tested whether implanted embryonic stem cell-derived early-differentiated cells (EDCs) lead to improvement in cardiac function by preventing cardiac apoptosis in aging rats after myocardial infarction. Cardiac apoptosis after transplantation of EDCs was assessed in situ by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling reaction (TUNEL) staining as well as by measurements of protein levels of cleaved caspases 3, Bax, and Bcl-2. Our results indicate that cell transplantation improved cardiac function at 6-months observation. The frequency of apoptotic cells in the peri-infarcted myocardium 3 days after cell transplantation was significantly decreased in the cell transplantation group. EDC therapy decreased the protein levels of cleaved caspase 3 and Bax, and increased the level of Bcl-2 in comparison to myocardial infarction control. Additionally, the number of apoptotic cells decreased significantly in cardiomyocytes precultured with EDCs. This study demonstrates that functional improvement of EDC transplantation may at least in part be related to a reduction in cardiomyocyte apoptosis.

Advanced age, even in healthy individuals without apparent cardiac disease, is associated with changes in heart structure and compromised cardiac reserve (1,2). Congestive heart failure, caused primarily by myocardial infarction (MI), is the major cause of hospitalization for people older than 65 years (3). Despite treatment strategies developed in the past few decades that were aimed at different pathophysiological mechanisms of myocardial disease, morbidity and mortality due to cardiac dysfunction after MI remains a clinical challenge. Aging decreases the functional reserve of the heart that is associated with the loss of cardiomyocytes due to the progressive process of apoptosis. Previous studies (4,5) demonstrated that there is an increase in the number of apoptotic myocytes after MI. Among the treatment strategies available, the maintenance of contractile mass, determined by the number of functional cardiomyocytes, is a major goal in the therapy of heart disease. Inhibition of myocyte apoptosis could prevent the loss of contractile cells and thus provide a new approach to cardiac dysfunction in the aging heart after MI.

In recent years, cell transplantation has emerged as a potential therapeutic approach for repairing damaged myocardium. Our previous studies (6,7) and those of others (8–11) have demonstrated that engrafted stem cells can survive and differentiate into functional cardiac tissue in an animal model of infarction. However, the mechanisms of cell therapy have not been fully elucidated. The transdifferentiation ability of hematopoietic stem cells has been challenged (12–14), and an intense debate over cell fusion versus transdifferentiation has resulted (15). Our previous studies indicate that newly regenerated cardiomyocytes after early-differentiated cell (EDC) transplantation compose approximately 5.3%–7.3% of the total left ventricle (LV) (6,16). Cell fusion appears to be an infrequent event, occurring perhaps once in 10,000–100,000 cells (17). Thus, the small percentage of stem cell–derived cardiomyocytes and the low frequency of cell fusion cannot explain the significant improvement of cardiac function after stem cell transplantation. One potential mechanism by which stem cells might lead to improvement in cardiac function in the setting of MI is by preventing apoptosis of cardiomyocytes. The present study was designed to test the hypothesis that EDCs prevent apoptosis of cardiomyocytes and lead to functional improvement after local implantation in aging rats in the setting of MI.

Materials and Methods

EDC Preparation, Experimental MI, and EDC Transplantation

The mouse embryonic stem cells (ESC) line, ES-D3, was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured using a method previously described (6,7). Cultured EDCs were finally
trypsinized and resuspended in Joklik’s modified medium (Sigma, St. Louis, MO) with a density of $2 \times 10^7$ cells/mL for cell transplantation.

Experiments were performed in a total of 80 senescent male Fischer 344 rats aged 24 months (obtained from the National Institute on Aging, Bethesda, MD) 1 week after arrival. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the protocol was approved by our Institutional Animal Care Committee. Rats were anesthetized with pentobarbital sodium (60 mg/kg) by intraperitoneal injection, and were ventilated by using a small animal ventilator (Harvard Apparatus, South Natick, MA). MI was induced by ligation of the left anterior descending coronary artery and verified by observing blanching of the myocardium distal to the ligation (6,7). Intramyocardial injection of the EDC suspension (50 μL, $\sim 10^6$ cells) was performed in three different sites in the LV, one within the infarct area and two in the myocardium bordering the infarct area. MI-Control rats received the same volume of the cell-free medium as the rats receiving EDC transplantation. The Sham group underwent an identical surgery with neither ligation of the coronary artery nor cell transplantation. The study comprised two experimental cohorts: a subgroup of aged rats (15 in Sham, 20 in MI-Control, and 20 in MI-EDC) was followed-up for 6 months after MI to determine the long-term functional benefit of EDC transplantation. Another cohort of aged rats (7 rats for each group) was observed for 3 days after MI to determine the antiapoptotic role of implanted EDCs. Furthermore, an in vitro apoptotic assay in cardiomyocytes precocultured with EDCs under conditions of hypoxia was applied to verify the antiapoptotic role of EDCs.

Measurements of Cardiac Function and Infarct Size

Hemodynamic measurements in vivo were performed with a method described previously (6,7) in pentobarbital-anesthetized rats 6 months after EDC transplantation. Briefly, a carotid artery was isolated and cannulated with a 3-Fr high-fidelity Mikro-tip catheter connected to a pressure transducer (Millar Instruments, Houston, TX). The Millar catheter was carefully advanced into the LV. The LV systolic pressure (LVSP), the LV end-diastolic pressure (LVEDP), the maximum rate of LV systolic pressure rise (+dP/dtmax), and the maximum rate of LV systolic pressure fall (–dP/dtmax) were recorded on a computer and analyzed by a PowerLab data-acquisition system (model ML820; ADInstruments, Colorado Springs, CO). The rat heart was harvested after hemodynamic measurement under deep anesthesia with pentobarbital (100 mg/kg). Subsequently, the hearts were transversely sectioned into 4 pieces from the apex to the base, and were prepared for hematoxylin and eosin staining. Infarct size was calculated by dividing the sum of the planimetered endocardial and epicardial circumferences of the infarcted area by the sum of the total epicardial and endocardial circumferences of the LV (6,7).

Assessments of Cardiac Apoptosis In Situ and the Western Blot

In another cohort of aging rats ($n=38$), cardiac apoptosis in situ was assessed by examination of morphological features under light microscopy and by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end-labeling reaction (TUNEL) labeling of the 3’OH ends of DNA in paraffin-embedded heart sections 3 days after MI and EDC transplantation. The hearts were harvested from MI rats after measurement of hemodynamics, fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm. Following incubation with terminal deoxyribonucleotidyl transferase (TdT) enzyme, apoptotic nuclei (brown) were identified by staining with DAB (ApoTag plus peroxidase in situ Apoptosis detection kit; Chemicon, Temecula, CA). Nonapoptotic nuclei (blue) were identified by staining with 0.5% methyl green.

The protein levels of Bax, Bcl-2, and cleaved caspase 3 were measured in aged rat ventricles from Sham, MI-Control, and MI-EDC rats 3 days after acute surgery. Briefly, frozen tissues were homogenized in RIPA buffer containing protein inhibitor on ice and then sonicated. Protein concentrations were determined with a modified Bradford reaction (Bio-Rad, Hercules, CA) and then equal amounts of total protein were separated on a precast sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Bio-Rad). Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane blocked in 5% (wt/vol) nonfat dry milk in 0.1% tris buffered saline with tween (TBST). After incubation with primary antibodies (1:1000 dilution of Bax monoclonal antibody obtained from Cell Signaling Technology, Inc. [Danvers, MA], 1:400 dilution of Bcl-2 monoclonal antibody obtained from Santa Cruz Biotechnology, Inc. [Santa Cruz, CA], or 1:1000 dilution of cleaved Caspase 3 antibody obtained from Cell Signaling Technology, separately) and the second antibodies (Santa Cruz Biotechnology), the membranes were visualized with enhanced chemiluminescence (ECL) plus (Amersham Bioscience, Piscataway, NJ) and exposed to Kodak MR films. The relative amounts of Bax, Bcl-2, and cleaved caspase 3 were determined densitometrically using a NIH image system, and the protein levels of GAPDH were used as an internal control.

Aptoptotic Assay In Vitro With Isolated Cardiac Myocytes Cocultured With EDCs

There is no method available to culture aging cardiomyocytes. Therefore, we performed an in vitro apoptotic assay by using adult cardiomyocytes that were isolated by enzymatic dissociation to further determine the antiapoptotic effect of EDCs in vitro. Briefly, 3-month-old adult rat hearts were harvested under pentobarbital anesthesia and then perfused for 3 minutes with Ca$^{2+}$-free Krebs–Henseleit buffer containing (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 25 NaHCO$_3$, 25 HEPES, pH 7.4 at room temperature. The perfusate was then switched to an enzyme solution containing collagenase-II at 0.3 mg/mL (Worthington type II at 266 U/mg) and hyaluronidase at 0.3 mg/mL (Sigma type II at 667 U/mg) for another 20 minutes.
Ventricular tissue was then finely minced and shaken gently in enzyme solution containing trypsin and DNase I for another 20 minutes. Isolated cardiac myocytes were filtered through a nylon mesh, and resuspended in Medium-199 containing albumin at 2 mg/mL, creatine at 0.4 mg/mL, taurine at 0.6 mg/mL, insulin at 2 μL/mL, and penicillin/streptomycin at 100 U/mL. Thereafter, myocytes isolated from each experimental rat were plated onto laminin-precoated culture plates consisting of three groups for hypoxia–apoptosis assay: normoxic cardiomyocytes, hypoxic cardiomyocytes, and hypoxic cardiomyocytes precultured for 12 hours with EDCs. Each group consisted of five rats. Experimental cardiomyocytes were placed in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) containing 0.5% of oxygen for 12 hours. The oxygen level in the chamber was monitored with an oxygen analyzer (Vascular Technology Inc., Nashua, NH). Annexin-V (10 μL/mL medium; Calbiochem, San Diego, CA) was added to each plate to detect apoptotic cardiomyocytes. Ten randomly selected fields of cultured cardiomyocytes isolated from each experimental rat were counted for green fluorescent cells and total cardiomyocytes under Nikon videomicroscopy (Eclipse TE2000-U). Image capture and analysis were performed by using IPLab 3.6 software (Scanalytics Inc., Fairfax, VA). The number of Annexin-V-labeled cells was normalized to the total number of cells as counted by phase-contrast microscopy of the same field.

Data Analysis
All values are presented as mean ± standard deviation. Data derived from three groups (Sham, MI-Control, and MI-EDCs) were evaluated by using analysis of variance (ANOVA) with repeated measurements. Differences between two groups were compared by using the unpaired Student t test with Bonferroni’s correction. Survival during the 6-month observation period was analyzed by standard Kaplan–Meier analysis, and a statistical comparison among curves was made with the log-rank test. A level of p < .05 was considered significant.

RESULTS

Measurement of Cardiac Function after EDC Transplantation
Approximately 15% of rats (from a total of 80) died within the first day after surgery. In the surviving rats, the 6-month survival rate evaluated by Kaplan–Meier analysis was significantly increased in the group of experimental rats that received stem cell transplantation in comparison to MI-Control rats (Figure 1). Cardiac function assessed by hemodynamics was measured at two different time points (3 days and 6 months) after MI and stem cell transplantation. After 3 days of MI, ventricular function significantly decreased in the groups of MI rats as compared to age-matched Sham rats (Figure 2). The decrease was mainly reflected by a reduction in diastolic function (i.e., LVEDP). Three days of EDC treatment did not provide significant functional benefit to the aging hearts after MI. However, our data indicate that stem cell transplantation significantly improved cardiac function at 6 months after MI and EDC transplantation. This improvement was reflected by an increase of LVSP, +dp/dt, and –dp/dt, as well as a decrease in LVEDP (Figure 3) compared to these measurements in the MI-Control rats that received cell-free medium only. Additionally, infarct size was significantly reduced in the aging MI rats compared to the MI-Control rats 6 months after EDC transplantation (36 ± 3% in MI-EDCs vs 42 ± 4% in MI-Control, p < .05).

Cardiac Apoptosis in Infarcted Myocardium and the Western Blot
Apoptotic cardiomyocytes in the peri-ischemic areas from Sham, MI-Control, and MI-EDCs rats 3 days after surgery (5 hearts for each group) were detected by in situ TUNEL staining. High-power microscopy (×200) demonstrated that TUNEL-positive staining was localized primarily in the nuclei of cardiomyocytes. There were rare TUNEL-positive nuclei in Sham aging hearts. The TUNEL-positive cells increased at 3 days after MI in the peri-ischemic myocardium (Figure 4). Additionally, the TUNEL-positive myocytes in the peri-infarction area were counted and normalized to 100 total nuclei in the same sections (Figure 4). The number of TUNEL-positive myocytes was significantly increased in the infarcted hearts 3 days after MI, whereas EDC transplantation significantly reduced apoptotic cardiomyocytes in comparison to the MI-Control myocardia. To elucidate the molecular mechanisms involved in the antiapoptotic effect of EDC therapy, we measured protein levels of Bcl-2, Bax, and cleaved caspase 3 in additional LVs from Sham, MI-Control, and MI-EDCs rats (5 for each group) at 3 days after MI surgery. Levels of measured proteins were analyzed as a ratio compared to GAPDH. As shown in Figure 5, there was a significant increase of
cleaved caspase 3 in MI rat hearts. The protein level of cleaved caspase 3 was reduced in rat hearts at 3 days after MI and EDC transplantation. The level of Bax increased in the MI hearts compared to the Sham hearts at 3 days after MI operation. EDC treatment at 3 days after cell transplantation significantly decreased the expression of Bax. In contrast, the protein levels of Bcl-2 increased significantly in the rat hearts that received EDC transplantation as compared to the MI-Control hearts.

Assessments of Cardiac Apoptosis on Cultured Cardiomyocytes

We next examined the antiapoptotic effect of EDC therapy in an in vitro culture system. Cellular viability was determined in cultured cardiomyocytes 12 hours after hypoxia. There was a significant increase in Annexin-V-positive cells after 12-hour hypoxia (Figure 6) compared to the control group under normoxic conditions. However, the number of apoptotic cells significantly decreased in cardiomyocytes precocultured for 12 hours with EDCs, as indicated by a reduction the number of Annexin-V-positive cells.

DISCUSSION

It is known that, in the normal heart (18), the rate of cell death increases with age and is not balanced by a concomitant increase in new myocyte formation after middle age. The excess cell death results in a net reduction in cardiomyocyte number (2,19). The remaining viable myocytes become hypertrophic to compensate for the reduced number of functional cardiomyocytes. However, aged hypertrophic myocytes do not respond normally to growth stimuli and are prone to undergo cell death. Apoptosis is much more frequent, particularly in hypertrophic myocytes expressing p16INK4a (19). Aged hearts also have impaired angiogenic response as a result of a decreased ability to release platelet-derived growth factor (20). Our recent study demonstrated a significant decrease in myocardial blood perfusion and a reduction in the number of cardiomyocytes in aged hearts (2). The combination of age-dependent increase in myocyte death, coupled with reduction in vascular perfusion, further impairs functional adaptation in the senescent heart. Apoptosis of cardiomyocytes has also been observed in cardiac tissue from patients with chronic cardiomyopathy later after MI (21). This observation leads
to a concept that myocardocyte apoptosis contributes to the chronic progression of myocardial failure.

In the past few years stem cells have been engrafted into a broad spectrum of tissues, including regenerating bone, neuron, dystrophic skeletal muscle, as well as cardiac tissue. The biological principle is that stem cells contain a unique capacity of tissue-directed differentiation. Our previous studies indicate that EDCs can differentiate into cardiac-like cells and improve cardiac function in postinfarcted adult rat hearts at 6 weeks (6) and 32 weeks (7) after cell transplantation. More recently, we found that EDC transplantation restored the number of cardiomyocytes toward normal values (2). The present study demonstrated that transplantation of EDCs improved cardiac function and enhanced survival rate in the aging rats with MI during 6 months of observation. The underlying mechanism might at least in part be attributed to the antiapoptotic effect of the implanted EDCs.

There is a growing body of evidence supporting cardiac regeneration and enhanced angiogenesis in infarcted hearts as a part of stem cell therapy. However, the percentage of regenerated cardiac tissues is not sufficiently robust to significantly improve cardiac function after MI. A linear regression analysis did not indicate a stronger correlation between a single variable (either myocyte number or blood flow) and ventricular function (2). Myogenesis, angiogenesis, as well as other factors (e.g., the antiapoptotic role) may contribute synergistically to the functional benefit of stem cell transplantation. In the present study we demonstrated that apoptosis of cardiomyocytes significantly increased 3 days after MI in the peri-ischemic myocardium. EDC transplantation significantly reduced the number of apoptotic nuclei in the peri-infarcted area compared to MI-Control group. Furthermore, we found that there was a significant reduction in the number of apoptotic cardiomyocytes after 12 hours of hypoxia when they were cocultured with EDCs. These results suggest that EDCs may rescue ischemia-induced apoptosis of cardiomyocytes and may partially explain an improvement in cardiac function at 6 months in the aging rats after MI and cell transplantation. However, the detection of apoptotic nuclei by TUNEL staining has been challenged (22). Electron microscopy showed that TUNEL-positive cardiomyocytes exhibited irreversible oncosis with ruptured plasma membranes (23). Thus, using electron microscopy in our future study to observe ultrastructural evidence of apoptosis should be important to address antiapoptotic effect of stem cell therapy.

Figure 3. Cardiac function assessed by a Millar catheter in aging rats at 6 months after myocardial infarction (MI) and early-differentiated cell (EDC) transplantation. A, Left ventricular systolic pressure (LVSP); B, left ventricular end-diastolic pressure (LVEDP); C, maximum rate of peak left ventricular systolic pressure rise (+dP/dtmax); D, maximum rate of peak left ventricular systolic pressure fall (−dP/dtmax). Sham (n = 8), sham-operated rats; MI-Control (n = 10), MI rats with cell-free medium injection; MI-EDCs (n = 10), MI rats with EDC injection. *p < .05, **p < .05 vs Sham; #p < .05, ##p < .01 vs MI-Control.
In an in vivo experiment in rats, DNA strand breaks were seen as early as 3 hours after MI (24). Apoptosis can be initiated by the mitochondria, which release cytochrome c into the cytosol in response to extrinsic stimuli, for example, oxidative insults. The mitochondria-mediated pathway is modulated by the Bcl-2 family of proteins (25,26). At least 18 members of the Bcl-2 family have been identified, and these can be either pro-apoptotic (e.g., Bax) or antiapoptotic (e.g., Bcl-2). Apoptosis was accompanied by a decrease in Bcl-2 protein values and an increase in the protein levels of Bax, indicating a role of these proteins in the regulation of ischemia-induced apoptosis. In contrast, myocardial reperfusion injury was reduced in transgenic mice overexpressing Bcl-2 (27). The caspases are a group of cysteine proteases that play a crucial role in initiating and executing apoptosis. A short prodomain-characterized effector, caspase 3, acts downstream in the common pathway to carry out the final biochemical change seen in apoptosis (28). Our present study shows that the protein levels of Bax and cleaved caspase 3 were significantly reduced in the aging rats that received EDC transplantation as compared to the MI-Control rats. However, the protein levels of Bcl-2 increased significantly in infarcted rat hearts that received EDC transplantation at 3 days after treatment as compared to those in the hearts of the MI-Control rats. Our findings are consistent with those of many previous studies, which demonstrated that caspase 3, Bax, and Bcl-2 are involved in the process of apoptosis after cardiac ischemia.

The underlying mechanism of antiapoptosis from engrafted EDCs remains unclear. Cardiac-protecting factors released from implanted stem cells (e.g., insulin-like growth factor [IGF]) appear to be crucial to explain the antiapoptotic effect. In neonatal rat cardiomyocytes, IGF prevents apoptosis after hypoxia, which also could be suppressed with genistein, a tyrosine kinase inhibitor (29). Subsequently, it has been found that IGF exerts its antiapoptotic effect through extracellular signal-regulated kinases (ERK1 and ERK2), resulting in activation of the transcription factor cyclic AMP response element binding protein, which induces Bcl-2 expression (30). A recently
published article indicated that the release of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1α (SDF-1α) from engrafted mesenchymal stem cells leads to efficient vascular regeneration and also attenuates the apoptotic pathway (31). Another study reported that the release of paracrine growth factors from mesenchymal stem cells could promote neoangiogenesis (32). Engrafted stem cells over-expressing Akt intended to increase stem cell viability that may have resulted from induced paracrine effects to perform antiapoptotic effects and salvage on adjacent ischemic myocardium (32). There is growing evidence supporting the hypothesis that paracrine mechanisms mediated by factors released from stem cells play an essential role in the reparative process observed after the donor cells are injected into infarcted hearts, in addition to the direct regenerative potential of the stem cells. The low differentiation rate of transplanted stem cells to functional myocytes could not alone explain the structural and functional improvements reported. Based on the present study we believe that, in addition to cardiac regeneration by engrafted stem cells, myocardial salvage, derived from rescuing apoptotic cardiomyocytes through the paracrine mechanism, plays an important role in cardiac repair after stem cell transplantation.

**Summary**

Our data demonstrated that EDC transplantation improved cardiac function in aging rats at 6 months after MI. The antiapoptotic effect from EDC transplantation with
either in vivo or ex vivo experiments may play an important role on the functional benefit. Further studies should be designed to clarify the specific cardioprotecting factors released from engrafted stem cells and determine their potential for therapeutic application in preventing cardiac apoptosis during myocardial ischemia or infarction. Thereafter, instead of transplanting stem cells, it may be possible to administer specific proteins made by stem cells for cardiac repair in the aged population.

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Address correspondence to James P. Morgan, MD, PhD, Division of Cardiovascular Medicine, Department of Medicine, Caritas St. Elizabeth’s Medical Center, 736 Cambridge Street, Boston, MA 02135. E-mail: james.morgan@caritaschristi.org or Jiang-Yong Min, MD, Cardiovascular Division, Department of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail: jmin@bidmc.harvard.edu

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Figure 6. Cellular viability was determined in cultured cardiomyocytes 12 hours after hypoxia. Ten randomly selected fields of cultured cardiomyocytes isolated from each experimental rat were counted to obtain an average value of cardiomyocytes. Each study group consisted of five rats. Positive staining to Annexin-V indicates apoptotic cardiomyocytes. Data show a significant increase of apoptotic cells after 12-hour hypoxia compared to the control group that was under normoxic conditions. However, the number of apoptotic cells significantly decreased in cardiomyocytes precultured for 12 hours with early-differentiated cells (EDCs). Control = Cardiomyocytes cultured under normoxia; Hypoxia = cardiomyocytes cultured under condition of hypoxia; Hypoxia-EDCs = cardiomyocytes precultured for 12 hours with EDCs under hypoxic conditions. *p < .05, **p < .01 vs Control; **p < .01 vs Hypoxia.


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