Transplanted embryonic stem cells following mouse myocardial infarction inhibit apoptosis and cardiac remodeling

Dinender K. Singla,1 Gary E. Lyons,2 and Timothy J. Kamp3,4

1Department of Medicine, College of Medicine, University of Vermont, Colchester, Vermont; 2Department of Anatomy, 3Department of Medicine, and 4WiCell Research Institute, University of Wisconsin, Madison, Wisconsin

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MATERIALS AND METHODS

MYOCARDIAL INFARCTION (MI) leads to cardiac remodeling, a complex, dynamic, and time-dependent phenomenon characterized by 1) death of both cardiomyocytes and nonmyocytes in part due to increased apoptosis; 2) fibrosis marked by increased collagen deposition in response to myocyte loss and stimulation by hormones, cytokines, and growth factors; and 3) hypertrophy of cardiomyocytes (2, 3, 10, 11). A variety of interventions have been developed to blunt post-MI remodeling and improve the prognosis of patients post-MI, including prompt revascularization of the myocardium and standard pharmacological therapies such as angiotensin-converting enzyme inhibitors and β-blockers (11, 25, 31). However, many patients, including those presenting late after an MI or with large MIls, still exhibit significant remodeling that can lead to heart failure and death (11, 25, 31). Thus new therapies to repair the damaged myocardium and prevent post-MI remodeling are under active investigation, including strategies involving cellular transplantation approaches (23, 26).

Over the past decade, cell transplantation post-MI has been studied in animal models testing a variety of cell sources for therapy, including skeletal myoblasts, smooth muscle cells, fetal and embryonic cardiomyocytes, bone marrow stromal and hematopoietic stem cells, and mouse embryonic stem (ES) cells (6, 8, 12, 15, 18, 28). Pluripotent ES cells hold significant appeal for cell therapy, because these cells have the ability to differentiate into any cell type in the body, including all of the cell types found in the heart. ES cells can theoretically be expanded to large numbers of cells that could be available for off-the-shelf uses (18, 28, 29). Results of recent studies suggest that mouse ES cells transplanted to the post-MI heart can differentiate into cardiomyocytes, vascular smooth muscle cells, and endothelial cells (5, 8, 28). Importantly, transplanted ES cells improve left ventricular function, but the extent of functional and structural improvement appears greater than the meager regeneration of new myo-cardium observed in our group’s prior study (28). The present study examines whether ES cell transplantation post-MI impacts adverse remodeling by blunting cardiac apoptosis, hypertrophy, and fibrosis.

Mouse ES cells. Mouse ES cell lines were maintained on mitomycin C (Sigma)-treated STO fibroblasts (ATCC). The cell culture medium contained 15% fetal bovine serum (Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, and 0.1% (vol/vol) leukemia inhibitory factor. R1 ES cells were labeled with β-galactosidase by using a gene trap insertion into the ubiquitously expressed RNF4 gene, as described earlier (4). HM1 ES cells were transfected with enhanced green fluorescent protein (EGFPN1 vector; BD-Clontech) and selected in the presence of neomycin.

Mouse MI and ES cell transplantation. Adult male and female C57BL/6 mice (8–10 wk of age, 18–22 g) were maintained and used for the study as reported previously (28). Animals were divided into two study groups: MI+ cell culture medium and MI+ mouse ES cells. Forty animals were used to produce MI by coronary artery ligation (16, 28). Three to five hours later, left thoracotomy was repeated, and three intramyocardial injections of 10 μl of medium ± 1 × 10^6 ES cells were delivered into the infarction, border, and normal zones via

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Address for reprint requests and other correspondence: D. K. Singla, Biomolecular Sciences Center, Burnett College of Biomedical Science, Univ. of Central Florida, Orlando, FL 32816 (e-mail: dsingla@mail.ucf.edu).

This article is dedicated to Dr. Darrell S. Davis in recognition of his contribution to cardiovascular research and in memory of him.


a 29-gauge floating needle. Two weeks after the surgery, animals were killed by pentobarbital (40 mg/kg ip) injection, and the hearts were removed and fixed with 10% buffered formalin to perform histological, terminal deoxynucleotidyl nick end labeling (TUNEL), and immunofluorescence staining.

Preparation of tissue sections and histopathology. Heart tissue was cut at the mid level, as reported in previous studies (15, 20, 33), into transverse blocks and embedded in paraffin. Serial sections (5 μm) were cut and placed on microscope slides. Tissue sections were deparaffinized by incubation in xylene for 5 min at room temperature, followed by transfer into fresh xylene for an additional 5 min. Sections were rehydrated using sequential incubation in 100, 95, and 70% ethanol for 5 min each at room temperature, followed by washing in distilled water and phosphate-buffered saline (PBS) for 5–10 min. Heart sections were then stained with hematoxylin and eosin, Mason’s trichrome, and Picrosirius red (PSR).

Cardiac fibrosis was quantitated in a blinded fashion by measuring the total blue area (mm^2) with a Carl Zeiss quantitative automatic program in the Masson’s trichrome-stained heart sections. Cardiac hypertrophy in the infarct, noninfarct, and peri-infarct region was assessed using the Excel program’s paired t-test, and all values are means ± SE. Statistical significance was assigned when P < 0.05.

Data analysis. Significance of differences between values was assessed using the Excel program’s paired t-test, and all values are means ± SE. Statistical significance was assigned when P < 0.05.

Detection of apoptosis by TUNEL and caspase-3 staining. Tissue sections were deparaffinized and permeabilized with proteinase K (25 μg/ml in 100 mM Tris·HCl). An in situ apoptotic cell death detection kit (TMR red; Roche Applied Bio Sciences) based on the TUNEL assay was used as per the manufacturer’s instructions to detect apoptotic cells. For colabeling, these sections were incubated with primary antibody, either active anti-caspase-3 rabbit polyclonal (1:50 dilution; Santa Cruz Biotechnology and Cell Signaling) or sarcomeric cardiac α-actin mouse monoclonal antibody (1:20; Sigma). Sections were incubated in a humidified chamber at 37°C for 1 h. Sections were washed with PBS and incubated with anti-rabbit Alexa 635 or anti-mouse Alexa 488 secondary antibodies (Molecular Probes). Negative controls were used in each case by omitting primary or secondary antibody. Sections were mounted with Antifade Vectashield mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to stain nuclei. Sections were examined with a Zeiss Axiosvert 200 microscope and Zeiss LSM 510 META laser scanning confocal microscope.

Quantitative analysis of apoptotic nuclei were performed on two to three heart sections from four to five different hearts as reported by various investigators (15, 20, 33). The percentage of apoptotic nuclei per section was calculated by counting the total number of TUNEL-staining nuclei divided by the total number of DAPI-positive nuclei in 8–10 randomly selected fields at ×20 magnification.

Atrial natriuretic peptide staining. Tissue sections were deparaffinized and washed with PBS. Our standard immunolabeling protocol was used. In brief, sections were incubated with primary antibody against atrial natriuretic peptide (ANP), rabbit polyclonal (1:20 dilution; Santa Cruz Biotechnology). Sections were incubated in a humidified chamber at 37°C for 1 h and then washed with PBS and incubated with anti-rabbit Alexa 568 secondary antibody (Molecular Probes). Negative controls were used in each case by omitting primary or secondary antibody. Sections were mounted with Antifade Vectashield mounting medium containing DAPI (Vector Laboratories) to stain nuclei. Sections were examined with a Zeiss Axiosvert 200 microscope and Zeiss LSM 510 META laser scanning confocal microscope.

Fig. 1. Effects of transplanted embryonic stem (ES) cells on cardiomyocyte apoptosis in C57BL6 mice. Cells were transplanted 3–5 h postmyocardial infarction (post-MI), and hearts were examined 2 wk following cell transplantation. Representative photomicrographs of total nuclei stained with 4’,6-diamidino-2-phenylindole (DAPI) in blue (A and D), apoptotic nuclei stained with terminal deoxynucleotidyl nick end labeling (TUNEL) in red (B and E), and merged images of nuclei in pink (C and F). Magnification, ×40. G: histogram shows quantitative apoptotic nuclei per section from MI + medium and MI + ES cells groups. *P < 0.05. H: scatter plot of the percentage of apoptotic nuclei relative to infarct size [%LV (left ventricular) area] for each heart examined in the MI-alone (○) and MI + ES cells (●) groups.

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RESULTS

MIs were produced in C57BL/6 mice, and ES cells or medium were transplanted 3–5 h post-MI. Hearts were removed and examined at 2 wk after cell transplantation. To determine whether ES cell transplantation exerted an antiapoptotic effect, TUNEL staining and colabeling with anti-caspase-3 were performed. TUNEL staining showed that apoptosis post-MI was significantly reduced in the ES cell-transplanted hearts compared with the medium-transplanted hearts (MI+ES cells: 6.6 ± 1% vs. MI+medium: 12 ± 1.5% apoptotic nuclei/section, respectively, Fig. 1F). ES cell-transplanted hearts also exhibited a reduction in infarct size based on Mason’s trichrome-stained sections cut at the midpapillary muscle level compared with MI-alone hearts (MI+ES cells: 23 ± 3% vs. MI+medium: 43 ± 8%, P < 0.05, Fig. 1H). The reduction in apoptosis observed was not a simple reflection of smaller infarct size in the ES cell-treated hearts based on the lack of correlation of infarct size and TUNEL staining seen in the two data groups in Fig. 1H.

Sections colabeled for sarcomeric cardiac α-actin demonstrated TUNEL-positive nuclei in cardiomyocytes (Fig. 2). The presence of apoptotic nuclei was confirmed by colabeling with caspase-3 antibody (Fig. 3).

To determine the effect of transplanted ES cells on cardiac fibrosis, we quantified the fibrotic area in the myocardial sections. The MI+ES cell group demonstrated 57% less fibrosis compared with the MI+medium group (Fig. 4, A and B). Figure 4A shows a large anterolateral MI with fibrosis and ventricular dilatation in an MI+medium heart compared with a smaller MI with less fibrosis in an MI+ES cell-treated heart. Figure 4C shows an enlarged area of infarct and peri-infarct regions. Increased islands of viable cardiac muscle were observed in the infarct and peri-infarct regions of the MI+ES cell group compared with the MI+medium group (Fig. 4C). Adjacent sections were stained with PSR to determine collagen content. These sections were stained in an area of infarction similar to that stained with Masson’s trichrome. The amount of collagen deposition assessed in the infarct, peri-infarct, and normal regions in PSR-stained sections was reduced following ES cell transplantation (Fig. 5).

To determine the effect of ES cell transplantation on cardiomyocyte hypertrophy, we measured cardiomyocyte cell size. The MI+ES cell group demonstrated a significant decrease in cardiomyocyte area (μm²) in the infarct and noninfarct regions of the heart compared with the MI+ medium group (MI+ES cells: infarct zone = 626 ± 47 μm² and noninfarct zone = 502 ± 50 μm² compared with MI+ medium: infarct zone = 750 ± 72 μm² and noninfarct zone = 601 ± 50 μm²; means ± SE, P ≤ 0.05, Fig. 6, A and C). However, there was no significant difference in the cardiomyocyte cell size in the peri-infarct zone, but there was a trend toward decreased cardiomyocyte cell size in the ES cell-treated group (MI+ES cells: peri-infarct zone = 711 ± 52 μm² and MI+ medium: peri-infarct zone = 769 ± 75 μm²; means ± SE, P = nonsignificant, Fig. 6B). In addition, hearts were also stained with the hypertrophy marker ANP in the hearts with and without ES cell transplantation. Our data suggest that the amount of ANP staining assessed in the infarct, peri-infarct, and normal regions in ANP-stained sections was reduced following ES cell transplantation (Fig. 7).
ES CELLS REDUCE POST-MI APOPTOSIS

Fig. 3. Cardiac sections were stained for apoptosis using TUNEL staining and colabeled with caspase-3 antibody. Representative photomicrographs show total nuclei stained with DAPI in blue (A), and apoptotic nuclei stained with TUNEL in red (B), and caspase-3 antibody staining in green (C). Merged images of triple-labeled sections are shown in D.

Fig. 4. A: photomicrographs from histological sections stained with Masson’s trichrome after 2 wk of left coronary artery ligation from 3 different hearts in each group: MI+medium (top) and MI+ES cells (bottom). Magnification, ×1.25. B: histogram shows less fibrosis (blue stain in A) in the MI+ES cells compared with the MI+medium group (*P < 0.05). C: enlarged photomicrographs of Masson’s trichrome-stained heart sections in the infarct and peri-infarct regions from the MI+ES cells group that show less fibrosis (blue) in the infarct and peri-infarct zone compared with the MI+medium group. Arrows indicate viable area, stained red. Representative histological sections are shown. Magnification, ×20.
Overall, there were significant decreases in myocyte hypertrophy post-MI in the MI+ES cell group compared with the control MI+ medium group in the heart, confirmed by decreased myocyte cell size as well as ANP staining.

Echocardiography was used to determine the impact of ES cell transplantation on cardiac size and function in mice 2 wk post-MI. The functional data from these animals demonstrating improved cardiac function has already been published by our group (28).

DISCUSSION

Apoptosis has been shown to be a key factor in the development and progression of post-MI remodeling that can lead to chronic heart failure (3, 17). Numerous studies have demonstrated that apoptosis in the heart is inhibited by antioxidants, angiotensin II inhibitors, and expression of the antiapoptotic protein Bcl-2 (1, 17, 19, 21). The inhibition of apoptosis has been linked to improved cardiac function (1, 17, 19, 21). In the present study we have demonstrated that apoptosis of the native cardiomyocytes, confirmed by TUNEL staining and caspase-3 colabeling, was significantly reduced in the ES cell transplanted hearts. Recent studies have demonstrated that mobilized endothelial progenitor cells and mesenchymal stem cells transplanted to the heart can likewise reduce apoptosis (14, 32). Therefore, a reduction in post-MI apoptosis of the native myocardium is a common mechanism by which cellular transplantation post-MI exerts its beneficial effects, perhaps through a paracrine mechanism.
Post-MI remodeling involves enhanced ECM degradation, collagen deposition, and myocyte hypertrophy (9–11). In this study we have shown that collagen deposition and cardiomyocyte cell size were significantly reduced following ES cell transplantation in the infarcted heart, demonstrating an inhibition of cardiac fibrosis and cardiomyocyte hypertrophy. A previous study showed that bone marrow-derived stem cells transplanted into infarcted rat heart significantly attenuated mRNA expression of collagen types I and III and of transforming growth factor-β1 at 7 and 28 days. Thus bone marrow stem cells may act in a similar paracrine fashion to inhibit cardiac fibrosis (34). Kudo et al. (15) have demonstrated that marrow stem cells transplanted in the mouse heart decrease MI size and fibrosis. The increased levels of ANP in the myocardial tissue as well as in the plasma have been shown to be associated with cardiac hypertrophy, remodeling, and heart failure (7, 24, 27). Accordingly, in the present study we have demonstrated that the level of expression of the hypertrophic marker ANP was reduced following ES cell transplantation, suggesting a decrease in hypertrophy and cardiac remodeling. Therefore, reduction in post-MI fibrosis and myocyte hypertrophy may be a shared mechanism of benefit by cellular transplantation post-MI employing ES cells or a certain population of adult stem cells.

In the present study we suggested that inhibition of apoptosis and fibrosis contributes to the improved cardiac function reported previously (28) by using the same animal groups following transplantation of 3 × 10⁴ ES cells. However, future studies are warranted to optimize these observed effects, including defining a complete cell-dose response relationship.

Next, it is important to determine whether the beneficial effects of inhibition of apoptosis, fibrosis, and hypertrophy are due to the released factors from ES cells. Our unpublished cell culture data (Singla DK and McDonald D) suggest that conditioned medium prepared from ES cells contains the antiapoptotic proteins cystatin C, osteopontin, clusterin (13, 22, 35), and the antifibrotic tissue inhibitor metalloproteinase-1 (TIMP-1) (30). We hypothesize that these released factors have provided beneficial effects to the inhibited apoptosis and fibrosis observed in the present study. However, further in vivo studies are required to test the roles of these factors directly.

In conclusion, this is the first report demonstrating that ES cells transplanted post-MI inhibit apoptosis, fibrosis, and hypertrophy. These beneficial effects contribute to the reduction in adverse post-MI remodeling in addition to the benefit from myocardial regeneration. Critical questions for future investi-

**Fig. 7. Effects of transplanted ES cells on hypertrophy marker atrial natriuretic peptide (ANP) following MI.** Representative photomicrographs of ANP staining in the infarcted hearts with and without transplantation of ES cells. ANP staining is identified by anti-ANP antibody labeling in red (B, H, and N, E, K, Q), and nuclei are stained with DAPI (A, G, and M; D, J, and P) in blue. Merged images of x-labeled sections are shown in C, I, and O and in F, L, and R. Images in A–C (infarct zone), G–I (peri-infarct region), and M–O (noninfarct region) are from the medium-transplanted hearts, and images in D–F (infarct zone), J–L (peri-infarct region), and P–R (noninfarct region) are from the ES cell-transplanted group, demonstrating a decrease in the expression of ANP staining in all 3 heart regions. Data are representative of 1–2 sections studied from 4–6 different hearts in each group. Magnification, ×40.
gation remain, including defining the signaling pathways and potential paracrine factors responsible for the antiapoptotic and antifibrotic effects. Ongoing efforts using various cellular sources for transplantation need to carefully compare and optimize these multiple impacts of cellular therapies on the post-MI myocardium. The potential of human ES cells for cardiac therapy merits further consideration given the demonstration that, in mouse models, multiple potential mechanisms of benefit from ES cell transplantation post-MI have been demonstrated, including regeneration of new myocardium and inhibition of adverse remodeling.

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