Embryonic stem cells attenuate myocardial dysfunction and inflammation after surgical global ischemia via paracrine actions

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Stem cell therapy has been explored as a potential solution to attenuate tissue injury and subsequently improve functional outcomes after myocardial ischemia. Local and systemic proinflammatory responses associated with the onset of myocardial ischemia often precede irreversible injury. Numerous studies have explored the use of a variety of cell types, including adult bone marrow-derived stem cells and embryonic stem cells (ESCs), to improve myocardial contractile function. The cardiac protective effects of ESCs have been shown in multiple in vivo studies, yet the mechanisms of protection remain to be defined. Here, we examined the paracrine protective mechanisms of ESCs in the setting of surgical global ischemia.

Adult Sprague-Dawley rat hearts were subjected to 25 min of warm ischemia and 40 min of reperfusion and were randomly assigned into one of four groups: 1) vehicle treated; 2) BMSC or ESC preischemic treatment; 3) BMSC or ESC posts ischemic treatment; and 4) BMSC- or ESC-conditioned media treatment. Myocardial function was recorded, and hearts were analyzed for expression of tissue cytokines and growth factors (ELISA). Additionally, ESCs and BMSCs in culture were assessed for growth factor production (ELISA). ESC-treated hearts demonstrated significantly greater postischemic recovery of function (left ventricular developed pressure, end-diastolic pressure, and maximal positive and negative values of end-diastolic pressure) than BMSC-treated hearts or controls at end reperfusion. ESC-conditioned media (without cells) also conferred cardioprotection at end reperfusion. ESC-conditioned hearts demonstrated increased VEGF and IL-10 production compared with BMSC hearts. ESC hearts also exhibited decreased proinflammatory cytokine expression compared with MSC hearts. Moreover, ESCs in cell culture demonstrated greater pluriotropy than BMSCs. ESC paracrine protective mechanisms in surgical ischemia are superior to those of adult stem cells.

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Fig. 1. Comparison of myocardial function [left ventricular developed pressure (LVDP), end-diastolic pressure (EDP), and maximal positive (+dP/dt) and negative (−dP/dt) values of the first derivative of pressure] at end reperfusion in hearts with and without intervention. 

A–D: LVDP, EDP, +dP/dt, and −dP/dt in hearts infused with stem cells before ischemia. Results are means ± SE; *P < 0.05 vs. controls, †P < 0.05 vs. corresponding mesenchymal stem cell (MSC) group. ESC, embryonic stem cell group. 

E–H: LVDP, EDP, +dP/dt, and −dP/dt in hearts infused with stem cells after ischemia. Results are means ± SE; *P < 0.05 vs. controls, †P < 0.05 vs. MSC. 

I–L: LVDP, EDP, +dP/dt, and −dP/dt in hearts infused with stem cells or conditioned media derived from stem cells. Results are means ± SE; *P < 0.05 vs. controls, †P < 0.05 vs. corresponding stem cell media group. Statistics: ordinary ANOVA with post hoc Bonferroni test.
We hypothesized that ESCs would be acutely cardioprotective against myocardial surgically induced I/R injury and that embryonic stem cells would confer greater protection than adult MSCs due to increased paracrine effects. The purposes of the present study were to investigate the effect of ESCs or adult MSCs on myocardial I/R as measured by 1) functional indices; 2) myocardial inflammatory cytokine production; 3) myocardial growth factor production; and 4) in vitro growth factor production.

MATERIALS AND METHODS

Animals. Normal C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) and normal (250–350 g, 9–10 wk old) Sprague-Dawley male rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 1 wk before the experiments. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the Guide for the...
Preparation of mouse bone marrow stromal cells. A single-step purification method using adhesion to cell culture plastic was used as previously described (27), with the following modifications: 9- to 10-wk-old male mice were euthanized, and bone marrow stromal cells were collected from bilateral femurs and tibias by removing the epiphyses and flushing the shaft with complete media (Iscove’s modified Dulbecco’s medium; GIBCO Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (GIBCO Invitrogen) using a syringe with a 23-gauge needle. Cells were disaggregated by vigorous pipetting several times. Cells were passed through 30-μm nylon mesh to remove remaining clumps of tissue. Cells were washed by adding complete media, centrifuging for 5 min at 300 rpm at 24°C and removing supernatant. The cell pellet was then resuspended and cultured in 75-cm² culture flasks with complete media at 37°C. MSCs were preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete medium was added and replaced every 3 or 4 days thereafter. MSC cultures were maintained at 37°C in 5% CO₂ in air. When the cultures reached 90% of confluence, the MSC culture was passaged; cells were recovered by the addition of a solution of 0.25% trypsin-EDTA (GIBCO Invitrogen) and were replated in 75-cm² culture flasks.

Preparation of embryonic mouse stem cells. Male embryonic W4/129S6 stem cells were purchased from Taconic (Hudson, NY). ESCs were harvested and cultured from normal 129S6/SvEvTac mice. Thawing of cells and initiation of culture process were performed according to the manufacturer’s instructions. ESCs were plated in 100-mm tissue culture petri dishes (BD Falcon, Fisher, NY) and cultured with Dulbecco’s minimal essential medium with high glucose (GIBCO) supplemented with 15% fetal bovine serum (GIBCO), 100 nM nonessential amino acids, 2 mM L-glutamine, 0.5% penicillin-streptomycin (GIBCO), 100 nM mercaptoethanol (Sigma) and 1,000 U/ml leukemia inhibitory factor (Chemicon International) at 37°C, 5% CO₂, and 90% humidity. Medium was changed every 3 days. Light microscopy and yellow-green fluorescent detection of alkaline phosphatase (marker of pluripotency) were performed to verify that ESCs remained pluripotent.

Isolated heart experimental groups. All isolated rat hearts were subjected to the same I/R protocol: 15-min equilibration period, 25-min global index ischemia (37°C), and 40-min total reperfusion. Rats were divided into six experimental groups: 1) control hearts without intervention (n = 9); 2) isolated hearts infused with two million MSCs before I/R (n = 7); 3) isolated hearts infused with three million ESCs before I/R (n = 9); 4) isolated hearts infused with two million MSCs 1.5 min after I/R (n = 3); 5) isolated hearts infused with three million ESCs 1.5 min after I/R (n = 9); and 6) isolated hearts infused with 1 ml ESC-conditioned media prior to I/R (n = 3). MSCs or ESCs were washed with PBS, resuspended in warm (37°C) oxygenated Krebs-Henseleit (KH) solution, and infused (1 ml of 2 or 3 million cells) via the Langendorff apparatus an antegrade intracoronary route 1 min immediately before global index ischemia. The dose of MSCs or ESCs was determined using a dose-response curve (data not shown). At higher concentrations of MSCs or ESCs, significant coronary cell trapping occurred, causing a dose-dependent decline in myocardial function. Two million MSCs and three million ESCs conferred maximal cardioprotection without a decline in myocardial function.

Stem cell therapy most often entails a postinjury treatment protocol. We have previously demonstrated that a pretreatment protocol, which is particularly useful in surgically induced ischemia, may not only allow the ultimate recovery of viable tissue following injury, but may also act as a stabilizing/protective “helper cell” during I/R. Thus, both a preischemic and a postischemic treatment were used in the present study.
(50 ng/ml); 24-h LPS (200 ng/ml); or 24-h hypoxia (1.0% O2; IN VIVO 300 hypoxia chamber without substrate deprivation). After 24-h incubation, supernatants were harvested for VEGF. With the use of a Nikon TE2000U microscope at ×200 magnification, cell morphology was also assessed.

Conditioned media for isolated heart experiments were generated using identical conditions for the hypoxic stimulus in cell culture experiments. After 24 h of hypoxic exposure, supernatants from ESCs or MSCs were harvested for isolated heart infusion.

Isolated heart preparation (Langendorff). Hearts were isolated as previously described (35). Briefly, rats were anesthetized (60 mg/kg ip pentobarbital sodium) and heparinized (500 units ip), and hearts were rapidly excised via median sternotomy and placed in 4°C KH solution. The aorta was cannulated, and the heart was perfused in the isolated, isovolumetric Langendorff mode (70 mmHg) with KH solution (in mM: 11 dextrose, 110 NaCl, 1.2 CaCl2, 4.7 KCl, 20.8 NaHCO3, 1.18 KHPO4, and 1.17 MgSO4) at 37°C. The KH solution was bubbled with 95% O2-5% CO2 (Medipure) to achieve a PO2 of 450 to 460 mmHg, PCO2 of 39 to 41 mmHg, and pH 7.39 to 7.41. Total ischemic time was less than 45 s. The perfusion buffer was continuously filtered through a 0.45-μm filter to remove particulates. A pulmonary arteriotomy and left atrial resection were performed before insertion of a water-filled latex balloon through the left atrium into the left ventricle. The preload volume (balloon volume) was held constant during the entire experiment to allow continuous recording of the left ventricular developed pressure (LVDP). The balloon was adjusted to

Fig. 4. Changes in myocardial function following I/R in hearts without intervention (controls), hearts infused with ESCs before ischemia, hearts infused with ESC-conditioned media before ischemia (n = 3), hearts infused with MSCs before ischemia, and hearts infused with MSC media before ischemia (n = 3). A: ESC LVDP (% of equilibration). B: EDP (mmHg). C: ESC −dP/dt maximum (% of equilibration). D: ESC −dP/dt maximum (% of equilibration). E: MSC LVDP (% of equilibration). F: MSC EDP (mmHg). G: MSC −dP/dt maximum (% of equilibration). H: MSC −dP/dt maximum (% of equilibration). Results are means ± SE; *P < 0.05 vs. ESC media. Statistics: repeated-measures ANOVA with post hoc Bonferroni test.
Myocardial function. I/R resulted in markedly decreased LVDP in all groups. Postischemic recovery of LVDP (expressed as percentage of preischemic function) was significantly higher \((P < 0.05, \text{ANOVA and Bonferroni})\) in hearts with preischemic MSC or ESC infusion than control hearts \((35.7 \pm 3.5\%)\) at end reperfusion (Fig. 1A). However, preischemic infusion of ESCs conferred significantly greater protection \((P < 0.05)\) of LVDP \((75.2 \pm 8.4\%)\) compared with MSC infusion \((56.6 \pm 5.9\%)\) at \(3 \times 10^6\) cells/ml (Fig. 2E) but not at \(2 \times 10^6\) cells/ml (Fig. 2A).

Left ventricular EDP was elevated in response to I/R (Fig. 2, B and F). Hearts infused with MSCs or ESCs before ischemia demonstrated significantly lower EDP at end reperfusion than hearts with no intervention (Fig. 1B). Furthermore, hearts with ESCs exhibited significantly improved EDP recovery compared with hearts with MSC infused at \(3 \times 10^6\) cells/ml (Fig. 2F) but not at \(2 \times 10^6\) cells/ml (Fig. 2B).

Maximum positive and negative dP/dt were impaired at the start of reperfusion (Fig. 2, C and G). Control hearts demonstrated more depression of \(+\text{dP/dt}\) and elevation of \(\text{dP/dt}\) compared with hearts infused with MSCs or ESCs at end reperfusion (Fig. 1, C and D). However, hearts with ESCs exhibited improved contractility compared with hearts with MSCs at \(3 \times 10^6\) cells/ml (Fig. 2, G and H) but not at \(2 \times 10^6\) cells/ml (Fig. 2, C and D).

MSC infusion postischemia did not improve postischemic recovery of function, whereas ESC infusion 1.5 min into reperfusion did confer protection at end reperfusion as measured by EDP, \(+\text{dP/dt}\), and \(\text{dP/dt}\) (Fig. 1, E–H). Using repeated-measures ANOVA, neither MSC nor ESC postischemic infusion improved myocardial function significantly (Fig. 3).

ESC-conditioned media alone (without cells) also conferred protection of myocardial recovery but not to the same degree as ESC infusion (with cells) as measured by LVDP \((59.7 \pm 11.9\%)\) at end reperfusion (Fig. 1, I–L). MSC-conditioned media at end reperfusion did not demonstrate significant improvement of myocardial function. Furthermore, ESC- and MSC-conditioned media alone (without cells) demonstrated a trend of increased myocardial function, which did not prove significant (Fig. 4).

Measure of pluripotency. To objectively confirm whether ESCs remained undifferentiated, ESC and MSC alkaline phosphatase activity was measured. ESCs demonstrate elevated levels of alkaline phosphatase, an enzyme associated with undifferentiated pluripotent stem cells (data not shown). Alkaline phosphatase was not detectable in MSCs, however, confirming their more limited potency.
ESC and adult MSC morphology. MSCs exhibit greater cell diameter and differing morphology than ESCs viewed under a Nikon TE2000U microscope at ×200 magnification (Fig. 5).

ESC and MSC activation in cell culture. Stem cell production of paracrine factors may be important components of stem cell-mediated acute repair. To determine whether differences in paracrine factor production exist between ESCs and MSCs, basal and stimulated ESC or MSC growth factor production was measured. Hypoxia, LPS, or TNF resulted in significant activation of ESCs and MSCs (Fig. 6). ESC VEGF and IL-10 as well as MSC VEGF production was increased compared with control groups in response to hypoxia, LPS, or TNF. However, basal levels of VEGF production were greater in MSCs in culture compared with ESCs in culture.

Myocardial response to stem cell infusion and I/R. Myocardial production of TNF, IL-1β, IL-6, IL-10, VEGF, and IGF-I was measured via ELISA. ESC-infused hearts exhibited decreased TNF, IL-1β, and IL-6 expression ($P < 0.05$) compared with MSC-infused hearts as shown in Fig. 7, A–C. Conversely, ESC-infused hearts demonstrated increased IL-10, VEGF, and IGF-I production ($P < 0.05$) compared with MSC-infused hearts as shown in Fig. 7, D–F.

Interestingly, hearts treated with ESC-conditioned media exhibited greater inflammatory cytokine and VEGF production and similar IL-10 production compared with ESC-treated hearts (Fig. 7).

DISCUSSION

These results constitute the initial demonstration that pretreatment with murine embryonic stem cells protects native myocardium from injury. Furthermore, we have shown that embryonic stem cell infusion provides greater postischemic functional recovery and decreased myocardial tissue proinflammatory cytokine production compared with adult MSCs. These effects may be mediated at least in part by paracrine growth factor production of VEGF and IL-10 and modulation of local inflammation.

Stem cell therapy is a promising treatment modality for injured cardiac tissue (28). However, we now appreciate that this stem cell-mediated protection may not result from differentiation into cardiac myocytes (3, 23). We previously demonstrated that human and murine MSC administration immediately before cardiac ischemia improved functional recovery, decreased proinflammatory cytokine production, and decreased proapoptotic signaling (35). In the present study, we found that acute delivery of ESCs into injured cardiac tissue before ischemia provided greater attenuation of I/R injury and postischemic decrease of LVDP, $+dP/dt$, and $-dP/dt$ compared with adult MSCs. The preischemic stem cell delivery protocol used previously and in the present study allowed the MSCs and ESCs to increase recovery of myocardial function after ischemia. Interestingly, ESC-treated hearts exhibited a different rate of recovery in comparison with MSC-treated hearts, which may suggest different mechanisms of action. In addition to our established preischemic stem cell delivery protocol in the isolated heart, a posts ischemic MSC delivery protocol was also used; the acute use of stem cells and measure of function in both protocols precluded immediate stem cell differentiation as a cause of myocardial protection. Hearts treated with posts ischemic ESCs demonstrated improved recovery of myocardial function compared with MSC hearts at end reperfusion. However, we found that the postischemic protocol conferred less cardioprotection than the preischemic protocol; indeed, both ESC- and MSC-treated hearts demonstrated no difference

Fig. 6. Stem cell activation after 24-h TNF-α (TNF; $n = 4$), endotoxin (LPS; $n = 4$), or hypoxic (1.0% O$_2$) $(n = 4)$ exposure. A: expression of VEGF in ESCs. B: expression of VEGF in adult MSCs. C: expression of IL-10 in ESCs. D: expression of IL-10 in adult MSCs. Results are expressed as pg/ml, means ± SE; *$P < 0.05$ vs. control (Ctrl).
throughout ischemia using repeated-measures ANOVA compared with vehicle-treated hearts. This difference in cardioprotection between preischemic delivery of stem cells and postischemic delivery may have occurred for two reasons: 1) the exposure to beneficial stem cell effects is shorter (reperfusion only vs. ischemia + reperfusion) and 2) less powerful stimulus for stem cell activation (I/R only vs. ischemia + I/R injury). These data suggest that embryonic stem cell treatment may provide greater preservation of myocardial function compared with adult MSCs. Furthermore, both embryonic as well as adult stem cells may acutely improve cardiac contractile performance and limit myocardial infarct size by mechanisms other than differentiation. Also, preischemic delivery of stem cells, applicable in the clinical scenario of surgically induced ischemia, may be more effective than postinjury stem cell treatment. However, it remains unclear through which acute mechanisms ESCs and MSCs exert their myocardial protection following ischemia and reperfusion.

Stem cells may mediate their acute protective effects via complex paracrine actions. In response to stress like TNF, stem cells release greater levels of protective factors VEGF and IGF-I (9, 19, 34, 36). In congruence with these findings, we found that both ESC and adult MSC stimulation with hypoxia, TNF, or LPS resulted in significantly increased release of VEGF in cell culture. Moreover, ESCs and MSCs with or without stimulation produced antiinflammatory IL-10 (10). This growth factor production in the face of stress may represent the stem cell acting to release substances that limit local inflammation to enhance its own survival (11). Concurrently, these growth factors may play an important paracrine role in the MSC-induced myocardial protection from ischemia (15). Indeed, embryonic stem cells and adult mesenchymal stem cells both significantly elevated myocardial production of VEGF compared with control hearts. Furthermore, ESC-treated hearts demonstrated greater VEGF production than MSC-treated hearts, which may be associated with the greater ESC protection of myocardial function. Determining whether cell release of VEGF confers protection to surrounding tissue via reduced apoptosis (1, 2, 30, 37), decreased proinflammatory cytokines (29, 38, 39), or other mechanistic pathways (20, 32) requires further investigation.
In vitro studies of cardiomyocytes injured in response to monocyte chemoattractant protein-1 (26) or hypoxia (12) demonstrated protection with MSC-derived conditioned medium that did not contain stem cells. In the current study, ESC-conditioned media also elicited increased myocardial functional recovery and myocardial production of VEGF compared with controls. However, in the present study, embryonic stem cells produced lower basal levels of growth factor production than adult mesenchymal stem cells. In addition, hearts treated with ESCs demonstrated less paracrine factor production than hearts treated with ESC-conditioned media. These interesting findings suggest that further study is needed to determine the role of cell-cell interactions and paracrine effects in myocardial stem cell-mediated protection.

While this cardioprotection may be due in part to paracrine growth factor release by stem cells, other stem cell protective mechanisms include salvage of tenuous or malfunctioning cardiomyocytes at the infarct border zone; alteration of the extracellular matrix, resulting in more favorable postischemic remodeling; and activation of resident cardiac stem cells. Indeed, in the present study, stem cells may have employed cardioprotection via modulation of local inflammation; embryonic and adult mesenchymal stem cells increased myocardial antiinflammatory IL-10 and attenuated myocardial proinflammatory production of TNF, IL-1β, and IL-6. However, hearts treated with ESCs further exhibited decreased myocardial inflammatory cytokines compared with hearts treated with MSCs. Thus, increased myocardial protection in hearts treated with ESCs may be associated with increased attenuation of myocardial inflammation during I/R.

These results demonstrate that murine embryonic stem cells are superior to adult mesenchymal stem cells in attenuation of surgically induced warm global myocardial I/R injury. Planned ischemic events such as those that occur during cardiac surgery, angioplasty, or transplantation may allow an additional opportunity to observe the potential paracrine clinical benefit of adult stem cell pretreatment. Stem cells are a novel potential agent for myocardial protection against I/R, but further investigations of the limitations are necessary to maximize this protection.

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