VEGF is critical for stem cell-mediated cardioprotection and a crucial paracrine factor for defining the age threshold in adult and neonatal stem cell function

**METHODS**

**CORONARY ARTERY DISEASE is a source of tremendous morbidity and mortality (28).** The medical management of the more severe cases of cardiac ischemia is often inadequate, thereby warranting revascularization via percutaneous stenting or arterial bypass. The subsequent reperfusion injury associated with the ischemic episode is also detrimental and results in significant aberrations to the inflammatory cascade (3, 8, 22). In this regard, bone marrow mesenchymal stem cells (MSCs) represent a novel treatment modality with increasing therapeutic potential (1, 12, 17, 23, 27).

Many studies have previously demonstrated that MSC differentiation is not absolutely required for stem cell-mediated tissue protection (18). The infusion of MSCs into myocardium subjected to acute ischemia and reperfusion injury was noted to improve functional recovery, decrease proinflammatory cytokine production, and decrease the activation of proapoptotic caspases in the absence of differentiation and incorporation into the myocardium (34, 38). This led to the important appreciation that stem cells release a variety of paracrine factors which may confer protection to injured cardiac tissue (5, 34, 41).

Although MSCs from hosts of varying ages are able to show multipotent potential, the increasing age of stem cells and their hosts has been associated with a decreased functional capacity under conditions of stress (2, 29). Specifically, older age has been associated with telomere shortening and dysfunction, mesenchymal progenitor cell dysfunction, a reduced capacity of bone marrow stromal cells to maintain functional hematopoietic stem cells, and noted changes in cytokine production (15). Despite the proposed benefits of younger stem cells, we have observed that neonatal stem cells produce lower levels of vascular endothelial growth factor (VEGF) in cell culture and have observed that neonatal stem cells produce lower levels of VEGF in adult MSCs (αMSCs) would equalize the differences observed between αMSC- and neonatal stem cell (nMSC)-mediated cardioprotection. Female adult Sprague-Dawley rat hearts were subjected to ischemia-reperfusion injury via Langendorff-isolated heart preparation (15 min equilibration, 25 min ischemia, and 60 min reperfusion). MSCs were harvested from adult and 2.5 wk-old neonatal mice and cultured under normal conditions. VEGF was knocked down in both cell lines by VEGF siRNA. Immediately before ischemia, one million αMSCs or nMSCs with or without VEGF knockdown were infused into the coronary circulation. The cardiac functional parameters were recorded. VEGF in cell supernatants was measured via ELISA. αMSCs produced significantly more VEGF than nMSCs and were noted to increase postischemic myocardial recovery compared with nMSCs. The knockdown of VEGF significantly decreased VEGF production in both cell lines, and the pretreatment of these cells impaired stem cell-mediated myocardial function. The knockdown of VEGF in adult stem cells equalized the myocardial functional differences observed between adult and neonatal stem cells. Therefore, VEGF is a critical paracrine mediator in facilitating postischemic myocardial recovery and likely plays a role in mediating the observed age threshold during stem cell therapy.

**ischemia-reperfusion**

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quarantine room for 1 wk before the experiments. Female neonatal C57/B6J mice (2.5 wk old, Jackson) were allowed to birth naturally and were kept with their mothers until the time of death. The animal protocol was reviewed and approved by the Indiana Animal Care and Use Committee of the Indiana University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Preparation of mouse bone marrow stem cells. A single-step stem cell purification method using adhesion to cell culture plastic was employed as previously described (26). Briefly, nMSCs and aMSCs were collected from bilateral femurs and tibias after death 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 26-gauge needle. Cells were disaggregated by vigorous pipetting several times and were passed through a 30-µm nylon mesh to remove the remaining clumps of tissue. Cells were washed by adding complete media and centrifuged for 5 min at 300 g at 24°C, and the supernatant was removed. The cell pellet was then resuspended and cultured in 75-cm² culture flasks with complete media at 37°C in 5% CO₂-95% room air. MSCs preferentially attached to the polystyrene surface; after 48 h, nonadherent cells in suspension were discarded. Fresh complete media was added and replaced every 3 or 4 days thereafter. When the cultures reached 90% of confluence, the stem cell culture was passaged; cells were recovered by the addition of a solution 0.25% trypsin-EDTA (GIBCO Invitrogen) and replated in flasks. Adult and neonatal stem cells maintained similar MSC markers. Both cell lines were negative for the hematopoietic markers CD34, CD45, and CD117 and were positive for the MSC marker CD105 (21). Cells were used for experimentation between passages 3–7.

VEGF knockdown via siRNA. siRNA was designed to target the common sequence of the three mouse VEGF-A isoforms. The sense sequence of the 19 nucleotide (plus 2 amino acids overhanging at 5’)-VEGF siRNA was 5’-CCG ACG AGA UAG AGU ACAU-3’. The uniqueness of the designed siRNA was examined by using NCBI BLAST. VEGF siRNA as well as scrambled-control siRNA were obtained from Dharmacon (Lafayette, CO).

MSCs were prepared for transfection by seeding 10⁵ cells into each well of a six-well culture plate with complete media. After we allowed the cells to adhere for 24 h, the media was removed, the cells were washed with PBS, and the anti-biotic free/low serum Optimem media (GIBCO Invitrogen) was added. siRNA was then complexed with Lipofectamine 2000 (Invitrogen) in Optimem media and allowed to incubate at room temperature for 20 min. Complexes were then added to culture wells to yield a final siRNA concentration of 100 nM/well. After 24 h of incubation, the media and siRNA complexes were removed, normal complete media was added, and the cells were allowed to incubate for an additional 72 h. Transfection did not appear to affect cellular viability since a similar number of cells were recovered from the wells for each experiment.

Isolated heart preparation: Langendorff. Rats were anesthetized (pentobarbital sodium, 60 mg/kg ip) and heparinized (500 U ip), and the hearts were rapidly excised via median sternotomy and placed in 4°C Krebs-Henseleit solution containing (in mM) 119 NaCl, 20.8 NaHCO₃, 11 Dextrose, 2-H₂O, 47 KCl, 11.7 MgSO₄·7H₂O, and 11.8 K₂HPO₄. The aorta was cannulated and the heart was perfused under constant pressure (mean, 75 mmHg) with oxygenated Krebs-Henseleit solution (37°C). A left atrial resection was performed before the insertion of a water-filled latex balloon through the atrium into the ventricle. The balloon was initially adjusted to a desired mean end-diastolic pressure (EDP) of 5 mmHg, and the hearts were allowed to equilibrate for 15 min. Pacing wires were fixed to the atrium, and the hearts were paced at ~6 Hz, 3 V, and 2 ms (350 beats/min) during equilibration and reperfusion to ensure a standard heart rate between groups. A three-way stopcock above the aortic root was used to create warm global ischemia, during which time the heart was placed in a 37°C degassed organ bath. After 25 min of ischemia, the hearts were reperfused for 60 min. The cardiac contractility (+dP/dt), the rate of myocardial relaxation (−dP/dt), EDPs were continuously recorded using a PowerLab 8 preamplified/digitizer (AD Instruments, Milford, MA) and an Apple G4 PowerPC computer (Apple Computer, Cupertino, CA).

Experimental isolated heart groups. Rat hearts were divided into the following groups (n = 5 per group): 1) vehicle control, 2) adult stem cell infusion, 3) neonatal stem cell infusion, 4) adult stem cell VEGF siRNA infusion, 5) adult stem cell Scramble siRNA infusion, 6) neonatal stem cell VEGF siRNA infusion, and 7) neonatal stem cell Scramble siRNA infusion. Stem cells were recovered from 10 culture wells by the addition of 0.25% trypsin-EDTA (GIBCO Invitrogen). The cells were counted with the aid of a hemocytometer and trypan blue exclusion, and one million viable cells were isolated. The cells were centrifuged at 300 g, the media was removed, and the cells were resuspended in 1 ml of Krebs-Henseleit solution (37°C). Over the course of 1 min immediately before ischemia, the MSC solution was infused into the coronary circulation.

Enzyme-linked immunosorbent assay. VEGF in the MSC supernatant was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA set (R&D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. All samples and standards were measured in duplicate (n = 6–26/group).

Real-time polymerase chain reaction. Total RNA was extracted from both MSCs or nMSCs using RNA STAT-60 (TEL-TEST, Friendswood, TX). Total RNA (0.5 µg) was subjected to cDNA synthesis using cloned AMV first-strand cDNA synthesis kit (Invitrogen Life Technologies). cDNA from each sample was analyzed for 18S (assay identification number: Hs99999901_s1) and VEGF-A (assay identification number: Mm00437304_m1) by using TaqMan gene expression assay (RT-PCR) (Applied Biosystems, Foster City, CA). The experiments were repeated to confirm the results.

Presentation of data and statistical analysis. All reported values are means ± SE, and P < 0.05 was considered statistically significant. Myocardial functional indexes are displayed at end reperfusion, and +dP/dt and −dP/dt are presented as a percentage of baseline during equilibration. Data were compared using two-way ANOVA with post hoc Bonferroni or Student’s t-test when appropriate.

RESULTS

MSC VEGF production. nMSCs produced lower levels of VEGF compared with aMSCs following 72 h of normal culture (Fig. 1A). The transfection of MSCs with VEGF siRNA also significantly decreased cellular VEGF RNA levels in both cell lines compared with Scramble siRNA (Fig. 1B). No significant differences were noted in VEGF production from nontransfected and Scramble siRNA-transfected groups.

Adult stem cells improve myocardial functional recovery following ischemic injury. The pretreatment of the myocardium with aMSCs before ischemia resulted in significantly improved postischemic functional recovery compared with vehicle controls. At end reperfusion, +dP/dt and −dP/dt remained significantly higher in the aMSC groups. Although EDP was significantly higher throughout reperfusion in the aMSC group compared with controls (data not shown), there was no significant difference in EDP at end reperfusion (Fig. 2). The infusion of neonatal stem cells before ischemia did not improve the postischemic protection of the injured myocardium. This was denoted by similar myocardial functional indexes between vehicle control hearts and those infused with nMSCs.
VEGF is a critical component of stem cell-mediated cardioprotection. Intracoronary infusion of aMSCs transfected with VEGF siRNA significantly impaired postischemic myocardial function compared with aMSCs transfected with Scramble siRNA. This was noted by a significant decrease in dP/dt and dP/dt at end reperfusion in VEGF siRNA-treated groups. There were no differences in cardiac function at end reperfusion between nontransfected aMSCs and Scramble siRNA-transfected groups (Fig. 3).

Knockdown of VEGF in adults equalizes the cardioprotective differences observed between aMSCs and nMSCs. Not only did the application of VEGF siRNA to aMSCs impair their ability to promote postischemic myocardial recovery, but it also equalized the myocardial functional differences observed between adult and neonatal stem cell therapy. Specifically, the average EDP and −dP/dt were equivalent between hearts exposed to VEGF siRNA-transfected aMSCs and Scramble siRNA-transfected nMSCs. Moreover, the average contractility from hearts infused with VEGF siRNA-transfected aMSCs was actually lower at end reperfusion compared with Scramble siRNA-transfected nMSCs (Fig. 5).

DISCUSSION

Stem cells have previously been shown to improve myocardial functional recovery following ischemic injury. Herein we confirmed that aMSC infusion increased postischemic myocardial functional recovery, whereas nMSC infusion had no benefit above vehicle controls. In addition, we showed that 1) VEGF RNA and protein production were decreased in MSCs after VEGF knockdown with siRNA, 2) VEGF was a critical component of stem cell-mediated myocardial protec-
tion following ischemia, and 3) VEGF may be partially responsible for the age threshold seen in stem cell-mediated tissue protection.

Stem cells are thought to aid injured tissue via several different mechanisms. Some feel that stem cells differentiate into specific end-organ cells, which then become incorporated into the tissue to increase postinjury functional recovery (19). However, others have shown in cardiac, pulmonary, and renal tissue that protection from injury can be achieved in the absence of stem cell differentiation, thereby suggesting that stem cells aid native tissues via cell-cell interactions or via the release of protective paracrine substances during their transit through injured tissue (25, 33, 36, 39). Many studies have applied stem cell therapy in the postischemic setting and have seen mixed results. Our studies, however, used the preischemic application of stem cells to prevent impending myocardial infarction and heart failure. This model may be more applicable to planned ischemic episodes, such as may occur during cardiac surgery or during other operative interventions that may apply major stress to the myocardium. Preischemic stem cell therapy may therefore have the ability to prevent major cardiac morbidity in those patients who are at high risk for surgical intervention.

Stem cell paracrine properties aid the recovery of injured tissue via a variety of mechanisms. These include the production of antioxidants such as catalase, glutathione peroxidase, and manganese superoxide dismutase, which work to decrease the number of damaging oxygen-free radicals present in ischemic tissue (7). In addition, stem cells are an abundant source of growth factors, such as VEGF, hepatocyte growth factor, and insulin-like growth factor-1, which are believed to protect ischemic tissues via the promotion of angiogenesis, the inhibition of apoptosis, and the stimulation of cellular proliferation (4, 37).

VEGF has been shown to chronically inhibit leukocyte/epithelial cell adherence and the effects of chronic inflammation (30). In addition, VEGF promotes angiogenesis during acute inflammation and ischemia and may protect transplanted stem cells during therapy (10, 35, 40). Therefore, elevated local concentrations of VEGF may be beneficial to the injured tissue. However, VEGF levels may be increased in the presence of ischemia, which may indicate that VEGF is critical in the recovery of injured tissue.
myocardium. In fact, our group has recently observed that the infusion of recombinant VEGF before planned ischemia improved cardiac function following injury (11). In this regard, the elevated levels of VEGF produced by MSCs may be an important mediator in their ability to provide protection to ischemic tissues (5).

In the present study, we saw that the knockdown of VEGF by siRNA significantly impaired both MSC VEGF production as well as stem cell-mediated myocardial recovery following ischemia. Impaired function was seen by significantly decreased $+dP/dt$ and $-dP/dt$ after VEGF siRNA-transfected stem cell infusion. Although EDP was elevated in the VEGF siRNA groups compared with the Scramble siRNA groups, thereby indicating a higher degree of injury, there were no statistically significant differences in values. The lack of significance in measured EDPs was likely due to the decreased sensitivity of this parameter in the Langendorff model.

Interestingly, the knockdown of VEGF in the aMSCs ablated their cardioprotective properties and functionally made the aMSCs appear like the nMSCs in terms of their ability to facilitate postischemic myocardial recovery. We have shown in this study that VEGF is a critical paracrine factor in stem cell-mediated myocardial protection. Furthermore, aMSCs have been shown to produce, on average, 200 pg/ml more VEGF in culture compared with neonates (21). The noted differences in VEGF production between aMSCs and nMSCs have been associated with differential p38, ERK, and NF-κB activation among these cell lines (21, 24). Despite the differences in VEGF protein levels, VEGF mRNA levels were similar between adults and neonates, suggesting a mechanism for posttranslational modification of VEGF. Given the results of studies demonstrating that the recombinant VEGF application increased myocardial recovery following injury, we suspect that manipulating neonatal stem cells to overexpress VEGF would also facilitate a better myocardial recovery following injury. It therefore stands to reason that neonatal MSCs, due to lower VEGF levels and an activation of different intracellular signaling cascades, might not protect the injured myocardium as well as adult stem cells. This would suggest that VEGF plays an integral role in postischemic cardioprotection and may be involved, at least in part, in facilitating the age threshold seen in stem cell-mediated tissue protection.

Understanding the mechanisms of VEGF-induced tissue protection in the adult stem cell population is of particular interest, since methods to further increase VEGF in the heart after injury would likely be beneficial (11). In this regard, sex hormones have been shown to play a role in stem cell function, tissue protection, and intracellular signaling cascades (6, 14, 16, 32). This protection is thought to be due to the downregulation of proinflammatory cytokines and the upregulation of VEGF through the activation of estrogen receptor-α by endogenous estrogen (9, 13, 31). Because the female adult stem cell hosts used in this study had achieved sexual maturity, their levels of endogenous estrogen were higher than the neonatal hosts before stem cell harvest. Therefore, chronic in vivo exposure to estrogen in the adult stem cell population may have enhanced VEGF production within these cells, thereby providing them with a greater potential for therapeutic protection. It is therefore highly plausible that the activation of intracellular signaling cascades associated with adolescence has a positive effect on stem cells and may work to increase the protective properties of stem cells during therapeutic intervention. Further studies examining sex differences in neonatal stem cell cytokine production are therefore warranted to determine the role that sex hormones play in stem cell activation.

In conclusion, MSCs confer postischemic cardioprotection by a VEGF-dependent mechanism. VEGF also appears to play a role in facilitating the age threshold seen in stem cell-mediated tissue protection. It is likely, though, that VEGF is not the only paracrine factor responsible for the differences observed between adult and neonatal stem cell function. Further studies designed to explore the effects that these other mediators induce is therefore warranted. Defining the genes that initiate protective signaling mechanisms within younger stem cells may allow for the genetic amplification of these vital genes in autologous stem cells, thereby allowing for the generation of “super stem cells” that provide maximum protection to ischemic tissues.

GRANTS

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