Mesenchymal Stem Cells Differentiate into an Endothelial Phenotype, Enhance Vascular Density, and Improve Heart Function in a Canine Chronic Ischemia Model

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Background—Bone marrow–derived stem cells are under investigation as a treatment for ischemic heart disease. Mesenchymal stem cells (MSCs) have been used preferentially in the acute ischemia model; data in the chronic ischemia model are lacking.

Methods and Results—Twelve dogs underwent ameroid constrictor placement. Thirty days later, they received intramyocardial injections of either MSCs (100 × 10^6 MSCs/10 mL saline) (n=6) or saline only (10 mL) (controls) (n=6). All were euthanized at 60 days. Resting and stress 2D echocardiography was performed at 30 and 60 days after ameroid placement. White blood cell count (WBC), C-reactive protein (CRP), creatine kinase MB (CK-MB), and troponin I levels were measured. Histopathological and immunohistochemical analyses were performed. Mean left ventricular ejection fraction was similar in both groups at baseline but significantly higher in treated dogs at 60 days. WBC and CRP levels were similar over time in both groups. CK-MB and troponin I increased from baseline to 48 hours, eventually returning to baseline. There was a trend toward reduced fibrosis and greater vascular density in the treated group. MSCs colocalized with endothelial and smooth muscle cells but not with myocytes.

Conclusions—In a canine chronic ischemia model, MSCs differentiated into smooth muscle cells and endothelial cells, resulting in increased vascularity and improved cardiac function. (Circulation. 2005;111:150-156.)

Key Words: cells ■ heart failure ■ ischemia ■ revascularization

The prevalence of ischemic heart failure remains markedly high despite several recent therapeutic advances in the treatment of acute myocardial infarction.1,2 Alternative pharmacological treatments are being developed for the failing heart, but the mortality and quality of life in advanced stages of congestive heart failure are still significant issues.3 Recently, stem cell–based therapy has emerged as a promising treatment of severe postinfarction systolic left ventricular dysfunction.4,5 Because stem cells are capable of differentiating into cardiomyocytes, cell grafting within the damaged myocardium may theoretically limit the consequences of the loss of contractile function.6 Another proposed effect of transplanting stem cells involves augmentation of angiogenesis and consequent improvement of myocardial ischemia.7

Bone marrow–derived stem cells have been used in recent attempts at myocardial regeneration and neovascularization.8 However, bone marrow–derived stem cells are composed of a heterogeneous group of cells, and many controversies remain regarding the ideal subtype for cell therapy.5 Among the multipotent cells found in the bone marrow are mesenchymal stem cells (MSCs), which can differentiate into nerve cells, skeletal muscle cells, and vascular endothelial cells.9 MSCs have been used for cardiomyoplasty and to induce neovascularization when they are injected into infarcted myocardium.5,10 Preclinical studies investigating bone marrow–derived cells as treatment for ischemic myocardium have been performed preferentially in the acute ischemia model; data in the chronic ischemia model are lacking. Moreover, chronic myocardial ischemia leading to heart failure is a leading cause of morbidity and mortality in the United States. Accordingly, the aim of the present study was to determine whether bone marrow–derived MSC transplantation would improve the morphology and function of the heart in a chronic canine model of myocardial ischemia.

Methods

This study was reviewed and approved by the University of Texas Health Science Center Houston (UTHSCH) Animal Welfare Committee and conducted at the UTHSCH Center for Laboratory Animal Medicine and Care, located at the Department of Veterinary Medicine and Surgery of the University of Texas M.D. Anderson Cancer Center.
Cell Isolation, Culture, and Labeling

Allogeneic canine MSC isolation was performed at Osiris Therapeutics, Inc (Baltimore, Md) as described previously. Briefly, bone marrow aspirates were passed through a density gradient to eliminate unwanted cell types. When plated, a small number of cells developed in visible symmetric colonies by days 5 to 7. Hematopoietic cells, fibroblasts, and other nonadherent cells were washed away during medium changes. The remaining purified MSC population was further expanded in culture. Cells were then harvested, labeled with the cross-linkable membrane dye CM-Dil (Molecular Probes, Inc) and the nuclear stain DAPI, and frozen in cryocyte bags. Frozen cells were stored in the vapor phase of liquid nitrogen until the time of implantation. Before injection, the cells were thoroughly washed and resuspended in a 10-ML volume of saline (1 × 10^6 MSC/ML). At the time of delivery, the viability of the cell suspension was confirmed to be greater than 70%.

Chronic Canine Ischemia Model, Coronary Angiography, and Cell and Saline Injections

In brief, a left thoracotomy was performed under anesthesia induced with pentothal (17 mg/kg, IV) and maintained with isoflurane (1.5% to 2.0%). Chronic myocardial ischemia was produced by implantation of an ameroid constrictor in the proximal left anterior descending coronary artery (LAD) and diagonal branch ligation. All collateral to the LAD distal bed were permanently ligated, including branches from the right and left circumflex coronary arteries, including the posterior descending coronary artery.

Left coronary angiography was performed immediately before intramyocardial injections (30 days after ameroid placement) and 30 days after intramyocardial injections to assess coronary anatomy and correct placement of the ameroid constrictor. In the treatment group (n = 6), after the LAD occlusion had been confirmed, allogeneic MSCs (1 × 10^6 diluted in 10 ML PBS) were delivered via direct surgical intramyocardial injection (20 sites) within the ischemic area (anterior and lateral walls). The control group (n = 6) received saline injections (20 sites; total of 10 ML) in the same manner. The dogs did not receive any immunosuppression therapy.

Functional Assessment by Echocardiography

Transthoracic rest echocardiography was performed before ameroid placement, 30 days after ameroid placement (immediately before cell or saline therapy), and at 60 days after ameroid placement (30 days after cell or saline treatment). Stress echocardiography was performed immediately before and 30 days after the cell injection procedure. Stress echocardiography was performed after a pacemaker wire was introduced into the right femoral vein and positioned in the right atrium. Stress echocardiography was performed during right atrial pacing (after 2 minutes when a plateau heart rate of 180 bpm was reached).

Echocardiography was performed with a commercially available echocardiographic system (Hewlett-Packard Sonos 1000) equipped with a 10-MHz linear-array transducer. The echocardiography was performed as described elsewhere. The heart was imaged in the 2D mode in the short-axis view of the left ventricle at the level of the papillary muscle. The left ventricular ejection fraction (LVEF) at rest and at 180 bpm was obtained according to the American Society of Echocardiography leading-edge method from at least 3 consecutive cardiac cycles. A blinded, experienced observer (S.C.) performed all measurements.

Laboratory Blood Sample Analysis

Serial blood sample analysis of white blood cell count (WBC), C-reactive protein (CRP), creatine kinase MB fraction (CK-MB), and troponin I was performed before cell or saline injection and then 24 hours, 48 hours, and weekly thereafter up to 30 days of follow-up to assess inflammatory responses (WBC and CRP) and myocardial damage (CK-MB and troponin I).

Histopathological Analysis

Dogs were euthanized 30 days after cell or saline injection. Their hearts were exposed by median sternotomy and quickly removed. The heart weight was recorded. The hearts were sliced in a bread-loaf manner into 4 transverse sections from apex to base. Each section was separated into anterior, anterolateral, lateral, posterolateral, and posterior LV free wall; anterior, mid, and posterior interventricular septum; and right ventricular free wall. Each section thickness was sliced in half; one half was frozen in liquid nitrogen, and the remainder was fixed in 10% formaldehyde.

Measurement of Vascular Density

The effect of stem cell transplantation on angiogenesis was evaluated in paraffin-embedded sections by counting the number of vessels in anterolateral wall sections (10 sections per heart) immunostained for the endothelial cell marker factor VIII (Dako). The number of vessels was counted under a light microscope in 5 random fields (each field measuring 0.58 mm^2); vascular density was expressed as the area of blood vessels in μm^2 per mm^2 of each ventricular section.

Fibrosis Quantification

Trichrome staining was used to evaluate collagen deposition. Ten anterolateral sections from each heart were evaluated in their entirety and quantified. The results were expressed as μm^2 of fibrosis per mm^2 of each ventricular section.

MSC Differentiation

The survival of engrafted cells was identified by Dil- and DAPI-positive cells in frozen sections made from the hearts. Potential transformation to cardiac-like cells from engrafted MSCs was verified by antibody immunostaining for cardiac troponin I Briefly, frozen tissue sections were fixed in acetone at 4°C for 10 minutes and incubated separately with a goat polyclonal immunoglobulin G anti-troponin I antibody (Santa Cruz Biotechnology, Inc) for 60 minutes at room temperature. After a washing with PBS solution, sections were incubated with a rabbit anti-goat conjugated rhodamine immunoglobulin G for troponin I. Neovascular transformation of engrafted MSCs was verified by antibody immunostaining for α-smooth muscle actin (Dako) and factor VIII (Dako) according to the manufacturer’s recommendations. All morphometric studies were performed by 2 examiners (D.V. and S.L.).

Statistical Analysis

All values are expressed as mean ± SEM. All analyses were performed with appropriate software (Statview; SAS Institute, Inc). Comparisons of vascular density and the amount of fibrosis between groups at day 30 were performed by use of an unpaired Student’s t test. Comparison of stress LVEF was performed by use of an unpaired Student’s t test. Differences in cardiac function laboratory data were studied by use of a 2-way repeated-measures ANOVA. A value of P < 0.05 was considered statistically significant.

Results

Procedural Safety

All dogs underwent intramyocardial cell injection without complications. Each injection of 5 × 10^6 cells was delivered in a volume of 0.5 mL. Coronary angiography revealed total LAD occlusion in all dogs at 30 and 60 days after ameroid implantation. There were no arrhythmias, ST-T changes, or Q waves associated with the procedure. No pericardial effusions were observed on 2D echocardiograms.
CK-MB and troponin I were measured at baseline and then 24 hours, 48 hours, and weekly thereafter up to 30 days later (Table 1). CK-MB and troponin measured at baseline and then 24 hours, 48 hours, and weekly thereafter up to 30 days later (Table 1). CK-MB and troponin were increased absolutely from baseline to 48 hours, then returned to baseline over time. Only one dog (a control) had a CK-MB elevation of more than twice the baseline value at 48 hours.

To assess myocardial damage, CK-MB and troponin I were measured at baseline and then 24 hours, 48 hours, and weekly thereafter up to 30 days later (Table 1). CK-MB and troponin I levels increased absolutely from baseline to 48 hours, then returned to baseline over time. Only one dog (a control) had a CK-MB elevation of more than twice the baseline value at 48 hours.

To assess the inflammatory response to cell injections, CRP and WBC were measured at baseline and at 24 hours, 48 hours, and up to 30 days after the procedure. CRP and WBC levels were not significantly different between the 2 groups over time (Table 2).

**Histopathology**

Histological examination of an average of 30 sections from each heart revealed no myocardial infarction in 5 of 6 treated hearts and in 5 of 6 control hearts. The infarcts (both healed) were located subendocardially in the anterolateral wall.

Quantitative morphometry of treated and control hearts revealed a trend toward reduced fibrosis in the anterolateral wall of the treated hearts that did not reach statistical significance (Figure 2A). Areas of interstitial fibrosis next to needle tracks were rare.

| Table 1. CK-MB and Troponin I in Treated vs Control Dogs Over Time* |
|----------------|----------------|----------------|----------------|
|                | CK-MB† Treated | CK-MB† Control | Troponin ‡ Treated | Troponin ‡ Control |
| Baseline       | 0.61±0.3       | 0.81±0.46      | 0.05±0.07        | 0.04±0.03          |
| 24 h           | 2.8±1.3        | 5.9±3.5        | 5.2±1.85         | 3±1.5             |
| 48 h           | 2.45±1.3       | 2±1.3          | 1.12±0.42        | 1.2±0.45          |
| 1 wk           | 0.63±0.23      | 1.1±0.6        | 0.02±0.01        | 0.05±0.01         |
| 2 wk           | 1.21±0.95      | 1.6±1.1        | 0.02±0.01        | 0.03±0.02         |
| 3 wk           | 1.33±1         | 2.2±1.7        | 0.02±0.01        | 0.03±0.02         |
| 4 wk           | 0.81±0.36      | 1±1            | 0.02±0.01        | 0.03±0.01         |

* CK-MB and troponin I levels were determined at baseline (before cell or saline intramyocardial implantation) and at several subsequent time points. No significant differences between the groups were discerned.
† P=0.34, treated vs control dogs.
‡ P=0.8, treated vs control dogs.

**Table 2. CRP and WBC Counts in Treated vs Control Dogs Over Time**

<table>
<thead>
<tr>
<th></th>
<th>CRP† Treated</th>
<th>CRP† Control</th>
<th>WBC‡ Treated</th>
<th>WBC‡ Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.23±0.25</td>
<td>0.2±0.2</td>
<td>7.08±1.37</td>
<td>8.23±2.0</td>
</tr>
<tr>
<td>24 h</td>
<td>0.99±0.71</td>
<td>0.8±0.5</td>
<td>11.7±3.8</td>
<td>14.8±4.0</td>
</tr>
<tr>
<td>48 h</td>
<td>0.72±0.29</td>
<td>0.5±0.4</td>
<td>11.4±4.2</td>
<td>14.7±3.7</td>
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<tr>
<td>1 wk</td>
<td>0.2±0.22</td>
<td>0.3±0.2</td>
<td>12.7±3.6</td>
<td>12.38±4.8</td>
</tr>
<tr>
<td>2 wk</td>
<td>0.2±0.21</td>
<td>0.2±0.2</td>
<td>11.08±1.0</td>
<td>12.4±3.0</td>
</tr>
<tr>
<td>3 wk</td>
<td>0.2±0.21</td>
<td>0.1±0.2</td>
<td>11.4±1.4</td>
<td>12.99±4.9</td>
</tr>
<tr>
<td>4 wk</td>
<td>0.2±0.20</td>
<td>0.24±0.2</td>
<td>8.8±3.4</td>
<td>11.6±5.4</td>
</tr>
</tbody>
</table>

* C-reactive protein levels and complete white blood cell counts were determined at baseline (before cell or saline intramyocardial implantation) and at several subsequent time points. No significant differences between the treatment groups were discerned.
† P=0.61, treated vs control dogs.
‡ P=0.25, treated vs control dogs.
Enhancement of Neovascularization by Transplanted MSCs

Vascular density was markedly higher in the anterolateral wall of treated dogs (mean, 6378 ± 2400 versus 3445 ± 667 μm²/mm²) (Figure 2B). The needle tracks were approximately 1 to 2 mm apart, and no increase in vascular density was present adjacent to them compared with areas away (0.5 to 1 mm) from the tracks.

Stem Cell Differentiation in Ischemic Myocardium

Some epicardial and intramyocardial arteries distal to the flow-limiting epicardial ameroid showed remodeling with intimal infiltration of α-smooth muscle actin–positive cells, leading to a focally significant increase in wall thickness and luminal narrowing (Figure 3) with mural fibrosis (Figure 3A, trichrome stain) and internal elastic lamina reduplication (Figure 3B, elastin stain).

Immunofluorescence studies showed that DAPI- and Dil-positive cells (Figure 4A) localized primarily in the anterolateral wall. However, in every treated dog, some lateral and posterior sections (Figure 4B) showed labeled cells, although these cells were always outnumbered by those in the anterolateral wall. Dil-positive cells colocalized with endothelial (Figure 5) and smooth muscle (Figure 6) cells but did not colocalize with myocytes.

Discussion

The present study describes MSC transplantation in a canine model of chronic ischemia. The results suggest that injecting MSCs into ischemic myocardium results in improved myocardial function and increased vascularity. In experimental studies, bone marrow–derived cells have been shown to regenerate areas of infarcted myocardium and coronary capillaries,14 thus limiting functional impairment after myocardial infarction. Transendocardial injection of autologous bone marrow mononuclear cells has been shown to increase myocardial contractility and perfusion in swine.7 Various cell lineages have been used to generate evidence that bone marrow stem cells differentiate into cardiomyocytes, endothelium, and smooth muscle cells.15,16 However, there is much controversy regarding which stem cell subtype might be responsible for the therapeutic benefit of bone marrow mononuclear cell transplantation into ischemic myocardium.5 The bone marrow mononuclear cell subset, which is quite heterogeneous, is composed of MSCs, hematopoietic progenitor cells, endothelial progenitor cells, and more committed cell lineages such as natural killer lymphocytes, T lymphocytes, and B lymphocytes. In theory, the ideal cell type for cellular therapy is likely to be a less committed one that can undergo full cardiomyocyte differentiation, augment angiogenesis, and trigger vasculogenesis. In that regard, MSCs may have the necessary combination of plasticity17 and viability.
In vitro, MSCs are capable of transdifferentiating into functional cardiomyocytes under differentiation-inducing culture conditions (eg, when treated with 5-azacytidine). In vivo, it has been shown that, in the acute ischemia model or in a myocardial environment, MSCs differentiate into cardiomyocyte-like cells that express desmin, troponin T, and sarcomeric MHC and produce a concomitant functional benefit. However, in vivo transdifferentiation of hematopoietic stem cells into cardiomyocytes after cell transplantation remains an object of controversy. In the present study, colocalization of MSCs with cardiac muscle–specific proteins was not observed. This might be because of a lack of specific cell signaling in the chronically ischemic myocardium. In the setting of chronic ischemia, the tissue injury cascade and compensatory response are different from that present in acute myocardial infarction. Rapid and massive activation of the inflammatory cascade, which is characteristic of acute myocardial infarction and responsible for inflammatory cell infiltration, may be required for MSC myogenesis. It is particularly intriguing, however, that quantitative morphometry revealed a trend toward reduced fibrosis in the anterolateral wall of stem cell–treated hearts compared with controls ($P=0.08$).

In the present study, engrafted MSCs present in vessel walls were positive for $\alpha$-smooth muscle actin, suggesting their transdifferentiation into smooth muscle cells. Yet, transdifferentiation into other cell types, such as endothelial cells, also appears to occur.

Figure 4. A, Dil staining of anterior wall showing labeled cells. Cells had been injected into anterolateral wall 30 days earlier ($\times 20$). B, Dil staining of posterior wall of same heart showing fewer labeled cells, suggesting migration of injected cells.

Figure 5. A, Factor VIII staining with FITC (green) showing a thin vessel wall. B, Dil-positive MSCs (red) in a vessel of anterolateral wall. C, Colocalization (yellow) of MSCs and endothelial cells, indicating transformation of MSCs into endothelial cells. D, DAPI staining showing labeled endothelial nuclei.
MSCs were present predominantly in the luminal face of the endothelium of several vessels and expressed factor VIII, suggesting their transdifferentiation into endothelial cells. This transdifferentiation might have contributed to the significantly higher capillary density in the anterolateral wall of stem cell–treated animals \((P<0.03)\). Therefore, MSCs may have participated in or triggered an angiogenic process, an idea supported by the literature. In previous studies, transplantation of MSCs into ischemic myocardium enhanced angiogenesis. Bone marrow cells can induce angiogenesis by providing angiogenic factors such as vascular endothelial growth factor and basic fibroblast growth factor. More specifically, as recently reported by Kinnaird et al, MSCs produce a wide array of arteriogenic cytokines and improve perfusion and remodeling in a mouse model of hindlimb ischemia, and these effects appear to be mediated through paracrine mechanisms associated with local release of the arteriogenic cytokines. This suggests that chronic ischemia and tissue hypoxia in the absence of acute infarction, as in the present study, may drive MSCs to differentiate into vascular cells. A better understanding of the interstitial milieu produced by each of these models may result in the determination of critical signaling molecules for lineage-specific differentiation. Thus, our findings add to the evidence in the literature that MSCs might induce angiogenesis in the setting of chronic myocardial ischemia.

In our study, we evaluated the functional improvement seen after MSC implantation by 2D echocardiography. After ameroid placement, one would expect a progressive decline in LVEF values at rest, with an additional drop in LVEF during stress once significant myocardial ischemia impairs myocardial contractility. At 30 days after ameroid placement, treatment and control groups showed the expected decline in LVEF. MSCs might have contributed to the preservation of resting LVEF 30 days after intramyocardial injections (60 days after ameroid placement), thus restoring it to near preameroid values in the treated group. In contrast, and as expected, the control group experienced a progressive decline in LVEF (Figure 1A). Also, measurements of LVEF during stress were significantly higher in the treated group. Angiogenesis may contribute to the maintenance of cardiac function by preserving residual, viable cardiomyocytes, and neovascularization might also restore contractility in hibernating areas of myocardium. Furthermore, the grafting of MSCs may augment or preserve the myocardial elasticity after ischemia. Overall, the increase in capillary density and the transdifferentiation into smooth muscle and endothelial cells seen in the treated group might have contributed to the preservation of LVEF at rest and during stress, thus indicating an improvement in total cardiac ischemic burden in both states.

The above-discussed results might have even greater significance because of the allogeneic nature of MSCs and the fact that treated dogs in our study received no immunosuppression therapy. MSCs from humans and other species have a cell surface phenotype that is poorly immunogenic. Recent data from several research groups have demonstrated long-term allogeneic MSC engraftment in a variety of noncardiac tissues in the absence of immunosuppression. Thus, in theory, allogeneic MSCs could have the apparent advantage of clinical availability over autologous hematopoietic stem cells in different clinical settings, such as during cardiac surgery for possible intraoperative cell therapy or in the setting of acute myocardial infarction at the time of coronary reperfusion.

The homing process, which results in cell engraftment, is thought to play a key role in the success of cell therapy. After acute events, serum vascular endothelial growth factor levels rise significantly, and it is to be expected that homing signals may be more intense in acute ischemic syndromes. Accordingly, in the present study, direct intramyocardial
injection was chosen as the delivery mode, under the rationale that homing signals in the chronic ischemia setting may not be as intense as in the acute ischemia setting. Surprisingly, DAPI- and Dil-positive MSCs were found in the noninjected posterior wall of treated hearts associated with small amounts of fibrosis. In this model, collaterals of the posterior descending artery were ligated during ameroid constrictor placement in the LAD. This could account for the injury seen in the posterior wall and might be the homing signal for MSCs to mobilize and engraft in the posterior wall after anterolateral wall implantation.

The major limitation of the present study is the small number of animals in each group, which limits conclusions about efficacy. However, statistically significant differences between the treatment and control groups were shown in regard to echocardiographic parameters and capillary density. In addition, one limitation of our chosen model that could, in theory, limit interpretation of cardiac function data is the fact that dogs might develop substantial collateral circulation. However, coronary angiography was performed both before and 30 days after cell and saline injection to ensure the correct placement of the ameroid constrictor and to assess for the development of new collateral vessels. No significant differences in major collateral development were seen between treated and control hearts. However, nonvisible collaterals may have been present.

In conclusion, the present study suggests that implantation of MSCs into chronically ischemic myocardium is safe and effective. MSCs differentiated into smooth muscle cells and endothelial cells, resulting in increased vascularity and improved cardiac function. Pending future studies with larger sample sizes, the present findings suggest that MSC transplantation might one day become an alternative therapy for ischemic heart failure.

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