Mechanisms of Bone Marrow–Derived Cell Therapy in Ischemic Cardiomyopathy With Left Ventricular Assist Device Bridge to Transplant

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ABSTRACT

BACKGROUND Clinical trials report improvements in function and perfusion with direct injection of bone marrow cells into the hearts of patients with ischemic cardiomyopathy. Preclinical data suggest these cells improve vascular density, which would be expected to decrease fibrosis and inflammation.

OBJECTIVES The goal of this study was to test the hypothesis that bone marrow stem cells (CD34+ ) will improve histological measurements of vascularity, fibrosis, and inflammation in human subjects undergoing left ventricular assist device (LVAD) placement as a bridge to cardiac transplantation.

METHODS Subjects with ischemic cardiomyopathy who were scheduled for placement of an LVAD as a bridge to transplantation underwent bone marrow aspiration the day before surgery; the bone marrow was processed into cell fractions (bone marrow mononuclear cells, CD34+, and CD34− ). At LVAD implantation, all fractions and a saline control were injected epicardially into predetermined areas and each injection site marked. At the time of transplantation, injected areas were collected. Data were analyzed by paired Student t test comparing the effect of cell fractions injected within each subject.

RESULTS Six subjects completed the study. There were no statistically significant differences in complications with the procedure versus control subjects. Histological analysis indicated that myocardium injected with CD34+ cells had decreased density of endothelial cells compared to saline-injected myocardium. There were no significant differences in fibrosis or inflammation between groups; however, density of activated fibroblasts was decreased in both CD34+ and CD34− injected areas.

CONCLUSIONS Tissue analysis does not support the hypothesis that bone marrow–derived CD34+ cells promote increased vascular tissue in humans with ischemic cardiomyopathy via direct injection. (J Am Coll Cardiol 2015;65:1424–34) © 2015 by the American College of Cardiology Foundation.

Ischemic heart disease is characterized by a loss of myocardial cells and vasculature leading to end-stage heart failure. Cell therapy offers an appealing solution to replace these components and restore normal structure and function. Bone marrow cells are an easily accessible source of cells that have been shown to differentiate into cardiac components that potentially provide paracrine factors to improve healing of injured myocardium.
More than a decade ago, studies in small animal models showed that injection of bone marrow–derived stem cells improved cardiac remodeling after myocardial infarction (MI) (1). Specifically, injection of either bone marrow stem cells (CD34+) or bone marrow mononuclear cells (BMMC) improved vascularity, decreased scar burden, and resulted in improved cardiac function compared to noninjected animals (2,3). Follow-up studies using large animal models of bone marrow cell injection after MI showed similar results (4,5).

**METHODS**

Twenty-six subjects with ischemic cardiomyopathy scheduled for placement of a left ventricular assist device (LVAD) as a bridge to transplantation (BTT) were screened for the trial (Figure 1). Inclusion criteria included age >18 years, ischemic cardiomyopathy, and need for LVAD implantations as a BTT. Exclusion criteria included presence of an intra-aortic balloon pump, emergent LVAD, and inability of the subject to consent. Eight subjects consented to the study. One subject withdrew and a second was too unstable for injection. Of the 18 subjects who refused consent or met exclusion criteria, 13 consented to safety data collection as controls.

The day before LVAD implantation, 15 to 20 ml of marrow was collected from the iliac crest and transported to the Fred Hutchinson Cell Therapeutics facility. Marrow was processed into 3 fractions for injection: 0.5 x 10⁶ CD34+ cells, 1.0 x 10⁶ CD34− cells, and 1.0 x 10⁶ BMMC cells, and placed in numbered vials. An additional vial was loaded with 1 ml of Plasmalyte (Baxter International Inc., Deerfield, Illinois) as a control. The contents of all vials were blinded to the investigators (Online Figure 1). The doses of cells correspond to the average cell number per injection site reported in earlier clinical trials (Online Table 1).

Preoperatively, technetium single-photon emission computed tomography rest scans were reviewed and areas of 50% to 75% perfusion identified for injection. In the operating room, the contents of each vial were drawn up into a sterile syringe fitted with a sterile 25-gauge needle. Pilot sheering studies indicated minimal cell death (viability >98% by trypan blue stain) with this diameter. Areas to be injected were identified on the heart’s surface and a 1-cm diameter marker sewn on. The cells for each fraction and for saline were injected separately into the area’s 4 quadrants. Thus, each area received 4 250-μl injections of a single cell fraction or saline. After injection, the LVAD implantation proceeded per routine.

Case report forms were completed 24 h and 7 days after the LVAD implantation. Usage of blood products (packed red blood cells [PRBCs], fresh frozen plasma [FFP], and platelets [PLT]) was recorded at each time point. Chart notes, 12-lead electrocardiograms, and telemetry were reviewed to define episodes of ventricular tachycardia in the postoperative period. Safety data were reviewed by the principal investigator and the safety monitor after each subject enrollment to assess for safety endpoints that would preclude enrollment of additional subjects.
**TISSUE HARVEST AND ANALYSIS.** At the time of transplantation, the explanted heart was obtained and the markers identified. The area below each marker was excised from the epicardial to endocardial surface. After excision, this cube was cut into 4 sections to correspond to each injected region. Two sections were processed for histology and 2 were frozen in liquid nitrogen. One histological sample was placed in sucrose formaldehyde and embedded in paraffin. The second histological sample was flash frozen in OCT. For each endpoint, analysis was performed on 5 50-micron step sections. Antibodies or staining was used as follows for histological endpoints: Picrosirius Red for fibrillar collagen, CD31 (1A10, Leica Microsystems Inc., Buffalo Grove, Illinois) for endothelial cells, CD68 (M0876, Dako North America, Inc., Carpinteria, California) for macrophages, alpha-smooth muscle actin (alpha-SMA) (1A4, Dako) for activated fibroblasts, and Ki-67 (M7240, Dako) for proliferating cells. Quantification of Picosirius Red, CD31, and alpha-SMA was performed by an automated image analysis system. To quantify activated fibroblast content, alpha-SMA+ cells in blood vessel walls (smooth muscle cells) were manually excluded. Quantification of macrophage density was performed by manual count of CD68+ cells. Quantification of blood vessels was performed by manual count of circular CD31+ structures. Vessels with 2 or fewer nuclei and no smooth muscle media were counted as microvessels. Vessels with more than 2 nuclei or containing a smooth muscle rich media were counted as macrovessels. All analyses were performed by blinded observers.

**STATISTICAL ANALYSIS.** To account for the effect of injection on tissue structure, a saline-injected segment was used as the control. Because each subject had their own control measurement within 1 of the 4 quadrants, we were able to examine the difference of each treatment from control within each subject. Outcome measurements were averaged across samples for each subject, so each outcome measure has 1 value per treatment for each subject. The comparison of each treatment to the control is a distinct biological question of interest. For each outcome measure, we computed a 2-tailed paired...
Student t test to compare each treatment to control. No adjustments for multiple comparisons were applied. Analysis of safety endpoints was performed using independent sample Student t tests and Fisher exact test to compare patients in the study group and the control group. Exploratory endpoints were examined using Pearson correlations to compare results from CD34+ and CD34-depleted treatments.

RESULTS

Six patients completed both bone marrow aspiration and cell injection at the time of LVAD placement; their clinical characteristics are shown in Table 1. Age and incidence of diabetes was similar to previous populations of patients with ischemic heart disease eligible for cardiac transplantation. Eleven subjects were on hydroxymethyl glutaryl coenzyme A reductase inhibitors. Content of CD34+ cells varied between subjects; however, all subjects had robust representation of CD133+ cells in the CD34+ cell fraction. One subject had insufficient CD34+ cells for injection, but received injection of BMMC fraction and saline. Harvest of injected tissue occurred at time of cardiac transplantation, and time between LVAD placement and transplantation ranged between 47 and 374 days.

Comparison of safety endpoints between injected patients and controls who underwent LVAD implantation without injection showed no statistically significant differences in bleeding as quantified by blood product use at 1 and 7 days postoperatively (PRBC 4 ± 2.2 U vs. 4.6 ± 3.8 U; p = 0.66; FFP 6 ± 7.8 U vs. 6 ± 3.2 U; p > 0.9; PLT 1.3 ± 1.2 vs. 2.3 ± 2.4 6-pack units; p = 0.25 by independent samples Student t test) (Online Figure 2). Furthermore, there were no statistically significant differences in the incidence of ventricular arrhythmias in the first week post-LVAD between injected subjects and un-injected controls (4 of 6 vs. 8 of 13; p > 0.9 by Fisher exact test). There were no deaths or reoperations in either group.

PRIMARY ENDPOINTS. To test the hypothesis that injection of bone marrow–derived CD34+ stem cells would improve vascularity, we quantified the density of endothelial cells as measured by CD31 staining using automated image analysis. Surprisingly, the areas injected with CD34+ cells had significantly fewer endothelial cells in comparison to saline injected areas (0.0058 ± 0.0032 vs. 0.013 ± 0.0065 % CD31+ area; n = 5; p = 0.02 by paired Student t test) (Figure 2). Although not statistically significant, there was a trend toward a reduction in endothelial density in regions injected with both the BMCC and CD34-depleted cell fractions (BMCC 0.0068 ± 0.0043 vs. saline 0.011 ± 0.0079, n = 6; p = 0.2; CD34-depleted 0.0085 ± 0.0071 vs. saline 0.013 ± 0.0065 % CD31-positive area, n = 5; p = 0.2). Measurement of microvascular density showed no statistically significant difference between saline and CD34+ cell–injected areas (55.7 ± 4.7 vs. 59.8 ± 16.7 vessels/mm²; p = 0.6).

Because stem cells may modulate cardiomyocyte survival, we measured fibrosis as a common endpoint for a beneficial effect of bone marrow cells on cardiac structure. Overall, there was no statistically significant improvement in fibrosis between tissue injected with CD34+, CD34-depleted, or BMMC compared to saline-injected tissue (32.4 ± 11.4, n = 5; 31.7 ± 15.8, n = 5; 33.3 ± 16.1, n = 6; 28.0 ± 10.6, n = 6, % Picrosirius Red–positive area, respectively; p > 0.5 for each treatment compared to saline; Figure 3A). Analysis of individual results showed a marked variability in response of fibrosis to cell therapy (Figure 3B), although histology showed that significant fibrosis remained in treated subjects independent of response (Figures 3C to 3F).

SECONDARY ENDPOINTS. To determine if injection of specific bone marrow cell fractions modulated inflammation, activation of myofibroblasts, or cell proliferation, we quantified the density of CD68 (a marker of macrophages), alpha-SMA (a marker of activated fibroblasts and vascular smooth muscle cells), and Ki-67 (present in the nuclei of dividing cells). There were no statistically significant differences in macrophage density between saline-injected tissues and tissues injected with any cell type (208.6 ± 36.3, n = 5; 206.2 ± 30.7, n = 5; 201.3 ± 50.2, n = 6; 197.9 ± 95.5, n = 6; cells/mm² for CD34+, CD34-depleted, BMMC, and saline, respectively; p > 0.5 for each treatment comparison to saline) (Figure 4A).

<table>
<thead>
<tr>
<th>TABLE 1 Patient Characteristics</th>
<th>Patient</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Injection to Transplant (Days)</th>
<th>Pre-LVAD Diabetes (Y/N)</th>
<th>Pre-LVAD Insulin (Y/N)</th>
<th>% CD34+ in Bone Marrow</th>
<th>% CD34+ in CD34+ Fraction</th>
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*Insufficient CD34+ cells (<0.5 × 10^6) could be isolated for injection. Value included in calculation of mean and SD. T values are mean ± SD or %.

LVAD = left ventricular assist device.
On the other hand, there were significantly fewer activated myofibroblasts (defined as blood vessel–independent alpha-SMA–positive) in tissues injected with either CD34+ or CD34-depleted cells in comparison to saline-injected areas (CD34+ 4.6 ± 1.2 vs. saline 5.6 ± 0.5, n = 5; p = 0.04; CD34-depleted 4.8 ± 0.9 vs. 5.6 ± 0.5, n = 5; p = 0.014, % alpha-SMA positive area) (Figure 4B).

Proliferating cells were observed in the interstitial space in all segments as measured by Ki-67 positivity. However, there were no differences in proliferating cells detected in tissue injected with CD34+ stem cells compared to saline-injected tissue (24.4 ± 6.4 vs. 14.7 ± 6.7 Ki-67 positive cells/mm², n = 5; p = 0.08) (Figures 4C and 4D). Ki-67 positivity was not observed in nuclei contained within myofibrils.

To determine if bone marrow cells can transdifferentiate into cardiac cell components, our protocol included incubation of a fraction of the BMMC with Feridex, a solution of iron nanoparticles. Our labeling protocol resulted in 82.5% iron positivity in BMMC indicating that multiple cell populations were labeled. The first 2 subjects in the protocol underwent injection of Feridex-labeled BMMC cell fractions. However, soon after their enrollment, Feridex was taken out of clinical production and further subjects were unable to undergo cell labeling. Analysis of tissues for iron-labeled cells using Prussian blue stain and matching with adjacent sections with various immunohistochemistry cell phenotypes showed that most iron-labeled cells were also positive for the macrophage marker CD68 (Figures 5A and 5B). No Prussian blue positive, CD31+ cells were detected.

**EXPLORATORY ENDPOINTS.** Because our results were contrary to the published literature and human subjects have great genetic variability, we performed several exploratory studies to determine if analysis of individual patient results would be illuminating. Analysis of the relationship of the time between cell injection and tissue harvest revealed no differences in our primary endpoints of endothelial density or fibrosis between subjects harvested early (<100 days) versus late (>170 days) after injection of cells. To test whether cell effects were specific to CD34+ stem cells, we correlated results from areas injected with CD34+ and CD34-depleted areas. Intriguingly, there was a tight correlation in the density of macrophages, activated myofibroblasts, and fibrosis between tissue injected with the stem cell versus the non-stem cell fraction (Pearson correlation of 0.98, 0.90, 0.93, and p = 0.004, 0.035, 0.023, respectively) (Figures 5C to 5E).

**DISCUSSION**

In this unique trial we show the safety and feasibility of direct injection of cell therapeutic products into the hearts of human subjects with ischemic cardiomyopathy undergoing LVAD implantation (Central Illustration). Furthermore, we show that this strategy can be used in a BTT population to determine the mechanisms by which these cells alter cardiac structure. Our data support the importance of these mechanistic studies, as the effects of the cell fractions in humans with advanced heart disease after LVAD implantation were not as predicted from other models. Specifically, injection of CD34+ stem cells did not result in a statistically significant increase in vascularity in ischemic myocardium as defined by
2 separate measures: density of CD31+ endothelial cells and number of manually counted blood vessels. Furthermore, there were no statistically significant differences in fibrosis or macrophage density in cell-versus saline-injected segments. Although the study lacked the power to detect small changes, the lack of dramatic improvements in histological structure makes it unlikely that these cell therapeutics would result in meaningful functional improvements in this patient population.

On the other hand, several of our results support the hypothesis that cell therapy may modulate some elements of cardiac biology in subjects after LVAD implantation and provide targets for future studies. Areas injected with either CD34+ or CD34-depleted cells had a significantly decreased density of myofibroblasts. Although this finding may represent a Type 2 error, it was intriguing that the measurements of myofibroblasts, fibrosis, and macrophage density were highly correlated between areas injected with CD34+ and CD34-depleted fractions, raising the hypothesis that changes in myofibroblast number are insufficient to alter fibrosis and inflammation in this population. Furthermore, our data showing the
persistence of iron particles from labeled cells imply that most injected cells eventually died, consistent with animal studies (21). However, as only 2 subjects received labeled cells it does not rule out the possibility of persistence or transdifferentiation of small numbers of injected cells.

A substantial literature in animal models and ex vivo human cell culture has shown the ability of bone marrow-derived CD34+ stem cells to both differentiate into vascular cells and promote vessel formation via paracrine factors (2,22–24). Thus, use of these cells to therapeutically increase perfusion in patients with ischemic heart disease was rapidly translated to clinical trials. These trials have targeted different patient populations (post-MI, refractory angina, and ischemic cardiomyopathy); used a variety of cell fractions (BMMC, CD34+, CD133+, and mesenchymal stem cells); and delivered cells via intracoronary, direct epicardial injections, and catheter-delivered endocardial injections. A meta-analysis and Cochrane Review of published trials to date has shown modest improvements in ventricular function with these cells at both early and late time points after injection (12,25). Moreover, many of these trials showed improvements in noninvasive measurements of myocardial perfusion supporting the hypothesis that bone marrow cell therapy directly increases vascularity (26).

Patients with ischemic cardiomyopathy represent a growing population and increasingly many of these patients are undergoing LVAD placement (27). There are reports of myocardial recovery after LVAD implantation; however, these cases have been predominantly in subjects with idiopathic cardiomyopathy (28,29). It is presumed that in patients with ischemic disease, both the presence of fibrosis and decreased myocardial perfusion contribute to a process that is irreversible. We sought to determine if recovery could be augmented in this population via injection of cells to improve vascularity with
subsequent decrease in fibrosis. Unfortunately, our study failed to show dramatic improvements in vascularity or decreases in fibrosis on direct histologic assessment.

There are several possibilities why our data may be discordant with other preclinical and clinical studies. First, this is the first study to test the angiogenic properties of bone marrow cells in unloaded myocardium. Myocardial unloading by the LVAD results in decreased oxygen use by the heart (30) and other changes that may independently alter the angiogenic properties of the cardiac tissue (31). Furthermore, the aortic valve is closed during the majority of cardiac cycles and flow is nonpulsatile in LVAD patients, which may modulate flow-mediated signals for angiogenesis (32,33). On the other hand, compared to other clinical populations, the consistent unloading of the LVAD provided a relatively homogenous environment for the cells that may obviate the need to control for changes in filling pressures or new ischemic events in our population.

Second, the quality of the cell product could have contributed to the lack of effect. Several studies have noted a correlation between patient characteristics and angiogenic activity of stem cells. Stem cells from older subjects or those with comorbid risk factors, such as hypertension and diabetes, may be less robust at promoting angiogenesis by both transdifferentiation and paracrine mechanisms (34). All of our subjects were older, and 5 of our 6 subjects had diabetes potentially impacting the angiogenic capacity of the bone marrow cell fractions (35–37). Because of the small amount of bone marrow collected, we had insufficient cells to test angiogenic properties of the cell fractions. However, we did confirm significant enrichment of CD133+ cells in the CD34+ fractions in all subjects. Furthermore, when analyzed as cells injected per volume of tissue, our doses were close to other studies in patients with similar clinical characteristics (Online Table 1), if extrapolated to cells injected per cubic centimeter (13,16). On the other hand, these doses are less than...
doses in more recent studies focused on the anti-anginal effect of cells in patients with similar risk factors (38,39).

A final explanation for the discrepancy between our data and other models is the significant difference in the pathophysiology of advanced human cardiomyopathy versus acute MI in both human subjects and animal models. Unlike models in which a discrete infarction is generated and cell therapeutics given soon afterward, our human subjects had longstanding neurohormonal activation with chronic remodeling of even normally perfused areas of myocardium. This fibro-inflammatory milieu may alter tissue response to cell therapy. Furthermore, as opposed to other studies, all of our subjects had been exposed to many years of medical therapy, which may alter the response of their myocardium to cell therapies.

**STUDY LIMITATIONS.** Limitations of the study include: 1) left ventricle is unloaded during LVAD and remodeling and effect of cell injection may not be the same as in a non-unloaded heart; 2) small numbers increase risk of both Type I and Type II statistical errors; and 3) older age of donor cells may have affected capacity for regeneration.

**CONCLUSIONS**

Injection of bone marrow–derived cells at the time of LVAD implantation is safe and feasible and may provide mechanistic insights into cell therapy. CD34+ stem cells do not increase vascularity in the unloaded, ischemic ventricle. Injection of cells may induce cellular changes that are independent of the origin of the cell therapeutic delivered. However, these data suggest that preclinical studies of cardiovascular cell therapies are insufficient to predict results in human subjects with chronic cardiomyopathy and LVADs. Similar mechanistic studies should be performed before large-scale clinical trials of other therapeutic cell products.

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**REPRINT REQUESTS AND CORRESPONDENCE:** Dr. April Stempien-Otero, Department of Medicine, University of Washington, 850 Republican Street, Box 35850, Seattle, Washington 98109. E-mail: april@uw.edu.
COMPETENCY IN MEDICAL KNOWLEDGE: Fibrosis and inflammation are common features of the myocardium of patients with end-stage heart failure. Although bone marrow–derived stem cell therapy promotes vascularity in preclinical models of acute myocardial injury, this approach does not translate into histological benefit in patients with chronic ischemic heart disease supported with left ventricular assist devices who are awaiting cardiac transplantation.

TRANSLATIONAL OUTLOOK: Better mechanistic models that more closely mimic chronic human heart disease are needed to inform the design of clinical trials of stem cell therapy.

REFERENCES

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**KEY WORDS** angiogenesis, cell therapy, heart failure, ischemia

**APPENDIX** For a supplemental table and figures, please see the online version of this article.