Myocardial hibernation is a common clinical condition affecting patients with advanced coronary artery disease,1 for whom cell-based therapies are targeted. In hibernation, repetitive episodes of ischemia and reperfusion lead to metabolic and functional changes in cardiomyocytes, ultimately impairing left ventricular (LV) function. Current therapies such as coronary artery bypass grafting or percutaneous coronary intervention are well established but not suitable for all patients; in previous clinical research from our group, more than one third of patients referred for revascularization are well established but not suitable for all patients; in previous clinical research from our group, more than one third of patients referred for revascularization were not suitable for all patients. Nevertheless, their phenotype is still debated,2 they were shown to uptake acetylated low-density lipoprotein, bind Ulex europaeus agglutinin-1 lectin, and express the pan-leukocyte marker CD45. They can also stain with CD11c and CD14. The imaging protocol was repeated after 7 weeks. Baseline positron emission tomography myocardial blood flow and myocardial flow reserve were reduced in the left circumflex artery territory (both P<0.001), and hibernation (mismatch) was observed. At follow-up, stress myocardial blood flow had increased (P≤0.01) and hibernation decreased (P<0.01) in the cells+matrix group only. Microsphere-measured myocardial blood flow validated the perfusion results. Arteriole density and wall motion abnormalities improved in the cells+matrix group. There was also a strong trend toward an improvement in ejection fraction (P=0.07).

Conclusions—In this preclinical swine model of ischemic and hibernating myocardium, the combined delivery of circulating angiogenic cells and a collagen-based matrix restored perfusion, reduced hibernation, and improved myocardial wall motion. (Circ Cardiovasc Imaging. 2013;6:982-991.)

Key Words: cell transplantation ■ hibernation ■ positron-emission tomography ■ tissue engineering

Clinical Perspective on p 991

Circulating angiogenic cells (CACs) constitute a heterogeneous population of peripheral blood–derived fibroblast–cultured cells. Although what defines their phenotype is still debated,2 they were shown to uptake acetylated low-density lipoprotein, bind Ulex europaeus agglutinin-1 lectin, and express the pan-leukocyte marker CD45. They can also stain with CD11c and CD14. The imaging protocol was repeated after 7 weeks. Baseline positron emission tomography myocardial blood flow and myocardial flow reserve were reduced in the left circumflex artery territory (both P<0.001), and hibernation (mismatch) was observed. At follow-up, stress myocardial blood flow had increased (P≤0.01) and hibernation decreased (P<0.01) in the cells+matrix group only. Microsphere-measured myocardial blood flow validated the perfusion results. Arteriole density and wall motion abnormalities improved in the cells+matrix group. There was also a strong trend toward an improvement in ejection fraction (P=0.07).

Conclusions—In this preclinical swine model of ischemic and hibernating myocardium, the combined delivery of circulating angiogenic cells and a collagen-based matrix restored perfusion, reduced hibernation, and improved myocardial wall motion. (Circ Cardiovasc Imaging. 2013;6:982-991.)

Key Words: cell transplantation ■ hibernation ■ positron-emission tomography ■ tissue engineering

Myocardial hibernation is a common clinical condition affecting patients with advanced coronary artery disease,1 for whom cell-based therapies are targeted. In hibernation, repetitive episodes of ischemia and reperfusion lead to metabolic and functional changes in cardiomyocytes, ultimately impairing left ventricular (LV) function. Current therapies such as coronary artery bypass grafting or percutaneous coronary intervention are well established but not suitable for all patients; in previous clinical research from our group, more than one third of patients referred for revascularization did not undergo intervention because of unsuitable vessel anatomy, comorbidities, or other reasons.1 These patients are at high risk of cardiac events, and novel approaches such as cell-based vasculogenic therapy could provide them with an alternative form of therapy.

Received January 10, 2013; accepted September 13, 2013.


The online-only Data Supplement is available at http://circimaging.ahajournals.org/lookup/suppl/doi:10.1161/CIRCIMAGING.113.000185/-/DC1. Correspondence to Marc Ruel, MD, MPH, University of Ottawa Heart Institute, 40 Ruskin St, Suite 3402, Ottawa, Ontario, Canada K1Y 4W7. E-mail mruel@ottawaheart.ca.
CACs has been confirmed in animal models of ischemia and infarction. Moreover, the benefits of cell transplantation, using CACs or other cell types, have not been investigated in the setting of myocardial hibernation, which is frequently encountered in patients with advanced coronary artery disease. Furthermore, the enhancement of such therapies with biomaterials remains poorly studied in large animal models.

We and others have previously showed that biomaterial scaffolds, such as collagen-based polymer matrices, are attractive for regenerative medicine and may reduce relocation of transplanted cells, improve LV function, and improve vascular density. In the present study, we induced and validated a preclinical model of myocardial ischemia and hibernation and used it to investigate the benefits of biopolymer-supported CAC delivery in this clinically relevant setting.

Methods

General Experimental Sequence and Animal Protocol

All experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The general experimental sequence is shown in Figure 1. Briefly, a total of 32 female Yorkshire pigs (8–10 kg at first surgery) underwent a small left thoracotomy for placement of an ameroid constrictor (1.5–2.0 mm; Research Instruments, Escondido, CA) around the proximal left circumflex artery (LCx; Figure I in the online-only Data Supplement). Two weeks later, pigs underwent (1) blood harvest for CAC isolation, (2) transthoracic echocardiography, (3) rest 13N-ammonia positron emission tomography (PET), (4) dipyridamole-induced stress 13N-NH3 PET, and (5) rest 18F-fluorodeoxyglucose (FDG) PET. The following week, the swine underwent a second thoracotomy and the lateral wall of the heart was exposed to allow direct intramyocardial delivery of treatment; animals were randomized to receive injections of PBS alone (control, n=10), CACs (n=8), or the combination of CACs+collagen-based matrix (n=7). Twelve 0.25 mL injections (total volume of 3 mL) were made in the hibernating portion of the lateral wall after the hibernating area was determined by PET and reviewed before injections. In the 2 groups that received cells, a mean of 32.7±3.9×106 cells was used.

At week 6, animals underwent follow-up PET and echocardiography. Rest and dipyridamole-induced stress myocardial blood flow (MBF; was also determined at 3 and 7 weeks by using isotope-labeled microspheres (ILMs). At week 7, animals were euthanized and hearts were harvested and sliced for microsphere and immunohistochemical analyses, as described in the online-only Data Supplement.

CAC Isolation and Culture

Approximately 60 mL of autologous blood was used to isolate and culture CACs. Cultures were supplemented with endothelial basal media with EGM-2-MV SingleQuots (Clonetics, Guelph, Canada), and adherent cells were used for injection on day 6. Cells were resuspended in 3 mL of PBS or collagen-based matrix. Details of CAC isolation and culture are provided in the online-only Data Supplement.

Collagen Matrix Preparation

Collagen-based matrices were prepared on ice by blending type I rat tail collagen (0.4% wt/vol; BD Bioscience, Oakville, Canada) with chondroitin sulfate C (40% wt/vol; Wako Chemicals, Osaka, Japan), as described previously. Briefly, components were mixed on ice and cross-linked with glutaraldehyde (1.5%). Residual aldehyde groups were inactivated by the addition of glycine (20%). The pH was adjusted to 7.2 to 7.5, and cells were added to the matrix before injection. This preparation was liquid at 4°C and solidified at 37°C.

Echocardiography

Rest transthoracic echocardiography images were acquired with a Phillips Sonos 5500 ultrasound machine and analyzed with Xcelera (Phillips Healthcare, Andover, MA). Simpson's LV ejection fraction (LVEF) was determined on 2-dimensional (2D) acquisitions. Using either a 2- or 4-chamber view, regional wall motion was evaluated by visual analysis of systolic thickening and reported according to the 16-segment model recommended by the American Society of Echocardiography. A normal or hyperkinetic segment was assigned a score of 1; hypokinetic, 2; akinetic, 3; and dyskinetic, 4. Wall motion score index (WMSI) was calculated by dividing the total wall motion score for each animal by the number of segments visualized.

Positron Emission Tomography

Imaging Sequence

After an overnight fast, pigs were anesthetized, intubated, mechanically ventilated, and placed in a right lateral position on the bed in one of the following PET scanners: ECAT ART (Siemens), Discovery RX or 690 (General Electric). A 12-lead ECG was used to monitor the heart. After a low-dose 133Cs transmission scan or x-ray computed tomography scan for attenuation correction, each animal underwent rest and dipyridamole stress (0.56 mg/kg) imaging with 13N-NH3 (20 minutes) and rest imaging with 18F-FDG (60 minutes) to obtain regional estimates of MBF (mL·min−1·g−1) and glucose uptake (MBq/mL), respectively. Each acquisition was dynamic and was separated by a 40-minute period to allow decay of 13N-NH3 activity. For each scan, radiotracers were injected via an ear catheter using a syringe pump (infusion time, 30 s; volume, 10 mL), and all

Thoracotomy (Sx1)
Ameroid constrictor
Microsphere shadow labeling

Thoracotomy (Sx2)
Rest & stress microspheres
Treatment injection:
PBS, CACs, CACs+matrix

Euthanasia (Sx3)
Rest & stress microspheres
Tissue harvest

Imaging (baseline)
Rest & stress 13N-NH3 PET
Rest 18F-FDG PET
Echocardiography
Withdrawal of 60 mL of blood

Imaging (follow-up)
Rest & stress 13N-NH3 PET
Rest 18F-FDG PET
Echocardiography

Figure 1. General experimental sequence. CACs indicates circulating angiogenic cells; FDG, fluorodeoxyglucose; PET, positron emission tomography; and Sx, surgery.
activity residuals were flushed from the intravenous line at the end of infusion using an additional 10 mL of saline. The total activity injected was ≥200 to 550 MBq for ammonia scans and 150 to 300 MBq for FDG scans (dose by weight; ≈15 kg at scan 1, and 40 kg at scan 2). To increase myocardial glucose uptake and image quality on the FDG scans, a euglycemic hyperinsulinemic clamp was used \( \text{II} \) (Figure II in the online-only Data Supplement). The protocol is detailed in the online-only Data Supplement.

**Image Processing**

Images were processed using FlowQuant \( ^{22} \) (Ottawa, Canada). For MBF quantification, time activity curves were derived from sampling regions of interest in the myocardium and LV cavity, base, and atrium blood pools. The rates of \( ^{13} \text{N}-\text{NH}_3 \) uptake at rest and stress were quantified using a 1-compartment model. Absolute MBF (mL·min\(^{-1} \)·g\(^{-1} \)) was quantified and reported according to a standard 17-segment model. Mismatch analyses were performed by comparing relative \( ^{13} \text{N}-\text{NH}_3 \) and FDG uptake at rest, as previously described. Briefly, the LV was divided into 460 sectors, and each sector was expressed as a percent of the maximum tracer uptake, defining perfusion and FDG scores. Mismatch (hibernating myocardium) was defined as a segment with reduced perfusion with FDG score greater than the perfusion. A match defect representing scar tissue was defined in regions where FDG score was less than or equal to perfusion. Mismatch and match scores were also reported following a 17-segment model.

PET analyses did not include explicit coregistration with coronary anatomy, but all images were analyzed in a standard myocardial orientation, with the interventricular septum centered on segment 14. To report regional analyses, we used the standard combination of segments corresponding to each coronary artery territories from the 17-segment model (excluding the apex from the left anterior descending [LAD] territory), therefore assuming similar coronary anatomy between animals.

**MBF Determination With Microspheres**

MBF was assessed at surgeries 2 (week 3) and 3 (week 7) using 15 µm ILMs (BioPAL, Worcester, MA). ILMs of different isotopic mass were used for each measurement. MBF was calculated based on the number of ILMs in myocardial tissue segments. The detailed protocols used to slice the heart (Figure III in the online-only Data Supplement) and for ILM-MBF are provided in the online-only Data Supplement.

**Immunohistochemistry Analyses**

Slides were stained with \( \alpha \)-smooth muscle actin to detect arterioles (see the detailed protocol in the online-only Data Supplement).

**Statistical Analyses**

The study animals were randomly selected to receive 1 of the 2 treatments or control injections. All analyses were performed by blinded observers, and study groups were revealed only after completion of quantitative determinations.

Results are presented as mean±SE. Statistical analyses were performed by using SigmaStat (Ashburn, VA). Paired Student \( t \) tests were used to compare values between the affected and remote regions of the heart, and between baseline and follow-up. Comparisons between groups were done by using a 1-way ANOVA, and 2-tailed Student \( t \) tests, assuming equal variances. Bonferroni correction procedures were not used. Significance is reported for \( \text{P}<0.05 \).

**Results**

Three pigs died during the first surgery, 1 died at induction of the first anesthesia, 2 died during the first PET stress scan, and 1 animal from the cell group died during the second stress scan. No autopsy was performed on these animals considering that the incidence of sudden cardiac deaths attributable to fibrillation or massive myocardial infarction can reach up to 30%, particularly during the period of ameroid closure. All remaining pigs (25 in total) completed the study protocol. Unless otherwise specified, findings are reported for the LCx territory. A summary of all outcome measures is provided in Table 1.

**Ameroid Constrictor Model Induced Myocardial Ischemia, Hibernation, and Wall Motion Abnormalities**

Two and 3 weeks after ameroid placement, both PET and microsphere analyses showed a marked reduction in MBF at rest, MBF at stress, and myocardial flow reserve (MFR) in the LCx region-at-risk, compared with the left anterior

### Table 1. Summary of All Outcome Measures for Each Experimental Group

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells+Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PET MBF (n=25)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Rest 0.84±0.05</td>
<td>0.89±0.04</td>
<td>0.54</td>
</tr>
<tr>
<td>Follow-up</td>
<td>0.83±0.10</td>
<td>0.74±0.04</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td>1.15±0.18</td>
<td>1.12±0.09</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>MFR</strong></td>
<td>1.41±0.21</td>
<td>1.25±0.06</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>PET viability (n=14)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mismatch</strong></td>
<td>10.99±2.39</td>
<td>5.47±1.74</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Match</strong></td>
<td>5.31±2.17</td>
<td>4.04±0.83</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Microspheres (n=17)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rest</strong></td>
<td>0.44±0.08</td>
<td>0.53±0.08</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td>0.85±0.23</td>
<td>1.13±0.12</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>MFR</strong></td>
<td>2.00±0.58</td>
<td>2.39±0.57</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>Echocardiography (n=19)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LVEF</strong></td>
<td>60.53±2.19</td>
<td>58.56±1.26</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>WMISI</strong></td>
<td>1.22±0.08</td>
<td>1.17±0.09</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Values are for the whole left circumflex artery territory and are expressed as mean±SE. LVEF indicates left ventricular ejection fraction; MBF, myocardial blood flow; MFR, myocardial flow reserve; PET, positron emission tomography; and WMISI, wall motion score index.
descending (LAD) remote normal region (Figure 2A–2C and Table 2). Mean mismatch (9.07±1.81 versus 3.24±1.14; P<0.001) and match (7.24±1.27 versus 3.81±1.05; P=0.002) scores were higher in the LCx than in the LAD region (Figure 2E and 2F). Wall motion abnormalities were observed in the lateral and inferoposterior walls, consistent with the LCx region-at-risk, and the mean WMSI was 1.29±0.04.

CACs Delivered in a Collagen-Based Matrix Increased MBF During Stress and Positively Altered MFR on PET
At baseline, there was no difference between groups in rest MBF (P=0.98), stress MBF (P=0.59), and MFR (P=0.41). Three weeks after treatment, rest MBF remained unchanged in all groups, whereas stress MBF increased in the cells+matrix group only (0.95±0.15 to 1.24±0.12 mL·min⁻¹·g⁻¹; P=0.04; Figure 3A). MFR did not change in the control and cells groups (P=0.40 and P=0.66, respectively), whereas there was a strong trend toward an improvement when cells were delivered within the matrix (from 1.08±0.14 to 1.47±0.12; P=0.05).

The highest increase in stress MBF was in the cells+matrix group (+53.89±31.10%), which was significantly higher than PBS and cells alone (−1.13±23.3%; P=0.02). Similarly, the increase in MFR was greatest in the cells+matrix group (+52.42±25.06%), compared with PBS and cells alone (+2.45±24.15%; P=0.04).

CACs Delivered in a Collagen-Based Matrix Increased MBF During Stress Measured by Microspheres
There was no difference in rest MBF (P=0.14), stress MBF (P=0.86), and MFR (P=0.56) between the groups at baseline. Under resting conditions, MBF remained unchanged in all treatment groups between baseline and follow-up. Stress MBF increased from 0.80±0.14 to 1.92±0.39 mL·min⁻¹·g⁻¹ (P=0.02) in the cells+matrix group, showed a trend toward

Table 2. Absolute Myocardial Blood Flow (mL·min⁻¹·g⁻¹) 2 Weeks (PET) and 3 Weeks (Microspheres) After Ameroid Placement on the Proximal LCx

<table>
<thead>
<tr>
<th></th>
<th>PET (n=25)</th>
<th>Micorspheres (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LCx</td>
<td>LAD</td>
</tr>
<tr>
<td>Rest</td>
<td>0.85±0.04</td>
<td>0.95±0.05</td>
</tr>
<tr>
<td>Stress</td>
<td>1.15±0.10</td>
<td>1.51±0.13</td>
</tr>
<tr>
<td>MFR</td>
<td>1.40±0.11</td>
<td>1.62±0.14</td>
</tr>
</tbody>
</table>

LCx indicates left circumflex artery; LAD, left anterior descending; MFR, myocardial flow reserve; and PET, positron emission tomography.
increasing from 0.72±0.15 to 1.14±0.23 (P=0.07) in the cells group, whereas it did not change in a statistically significant manner in the PBS group (P=0.10; Figure 3B).

CACs Delivered in a Collagen-Based Matrix
Increased Myocardial Viability on PET
Mismatch (P=0.51) and match scores (P=0.84) did not differ between groups at baseline. At follow-up, mismatch scores significantly decreased only in the animals that were treated with the combination of cells+matrix (P=0.006; Figure 4). There was no difference in match scores between baseline and follow-up in any of the groups. In the control group, 2 pigs had a global reduction in mismatch paralleled by a global increase in match, suggesting progression from viable to scar tissue. In a subanalysis of segments with the highest mismatch at baseline (see segment selection in the online-only Data Supplement), mismatch scores at follow-up decreased in the cells+matrix (0.19±0.09) and cells (1.06±0.7) groups compared with controls (9.38±2.73; P=0.02 and P=0.04, respectively), and match scores only significantly decreased in the cells+matrix group (P=0.007).

CACs Delivered in a Collagen Matrix
Improved Regional Wall Motion and Positively Impacted LVEF
At baseline, LVEF trended to be higher in the control group compared with the 2 other groups (P=0.06; Table 1), and there was no difference in regional wall motion score (P=0.16) between groups. Regional WMSI improved from 1.41±0.06 to 1.27±0.06 (P=0.049) when cells were delivered within the collagen matrix. When cells were delivered alone, there was a trend toward an improvement in WMSI (baseline WMSI=1.24±0.06; follow-up WMSI=1.17±0.04; P=0.08), and when only PBS was delivered, there were no changes in WMSI (baseline WMSI=1.22±0.08; follow-up WMSI=1.17±0.09; P=0.22).

From baseline LVEF, the cells+matrix group showed a +16.29±6.7% increase in LVEF, which was higher than the variation in the PBS group (−2.76±2.7%; P=0.02) but not different from the changes in the cell group (+11.45±9.14%; P=0.68). There was no difference between the cells and the PBS groups (P=0.14; Figure 5).

CACs Delivered in a Collagen-Based Matrix
Increased Arteriolar Density
Cells+matrix-treated hearts (n=5) demonstrated greater arteriole number (10.9±1.3 arterioles per field of view) in the LCx territory compared with cells- (n=6; 6.3±0.9; P=0.01) and controls (n=8; 4.3±0.6; P=0.001; Figure 6). Similar results were found in the LAD territory where arterioles were more abundant in the cells+matrix-treated animals (10.9±1.55 arterioles per field of view) compared with both the cells- (5.7±0.7; P=0.004) and the PBS-treated animals (3.8±0.8; P<0.001). In the LCx, there was a trend toward a higher mean arteriole number in the cells group compared with PBS group (P=0.08). There was a strong positive correlation between follow-up microsphere MBF at stress and the number of arterioles in the LCx (r=0.7; P=0.008) and LAD (r=0.66; P=0.01) territories.
Discussion

To our knowledge, this study is the first to demonstrate, in a preclinical model of hibernating myocardium, that the vasculogenic potential of transplanted CACs is enhanced when cells are delivered in a biomaterial. In our opinion, this is important because myocardial hibernation is present in up to 50% of patients with coronary artery disease and more than one third of these may not get appropriate treatment. Consequently, the applicability of cell-based therapies is particularly relevant in this population, as early cell and biomaterial delivery may
promote revascularization before irreversible damage to the myocardium occurs, in a context where currently available therapies may commonly not be feasible.

The combination of autologous angiogenic cells and naturally occurring extracellular matrix components successfully improved stress MBF and MFR in our study. Improved MFR suggests an increased response capacity to higher oxygen demands by recruitment or generation of new vasculature. These animals also displayed lower amounts of mismatch and match, suggesting a better recovery of myocardial viability following matrix delivery of CACs. Histological findings were also supportive of the enhanced benefits conferred by the matrix because cells delivered in the matrix doubled the number of arterioles in the myocardium. In the presence of myocardial hibernation, enhanced vasculogenesis may constitute an important mechanism of improved LV function in animals or patients undergoing successful cell therapy. In this study, vasculogenic improvements were paralleled by an improvement in wall motion and a strong trend toward an improvement in LVEF. This occurred despite the relatively small area of hibernation, the early follow-up time points, and the physiological and metabolic rather than functional emphasis of the experiments. A mixture of hibernating and nonvascularized scar tissue partly explains the persistence of low LCx resting flow at follow-up in all groups. In addition, it is likely that in the PBS and cell groups, supply of resting MBF required maximum capillary recruitment, whereas it was not exhausted in the cells+matrix group which allowed additional recruitment with higher MBF at stress.

Because the region of ischemia may not encompass all the LCx territory segments in every animal, we conducted a subanalysis using 2 segments with the lowest stress MBF at baseline. The effects of treatment were of higher magnitude and significance compared with the whole LCx region because the results were less diluted as a consequence (Table 1 in the online-only Data Supplement). For viability, the 2-segment analysis showed a significant decrease in mismatch in every group, with the most significant decrease observed in the cells+matrix group.

Although detailed comparisons of absolute microsphere and PET MBF were beyond the scope of this study, both modalities confirmed the establishment of myocardial ischemia after 2 weeks and supported the enhanced benefits of cell delivery within the matrix. In addition, a strong positive correlation between stress MBF and arteriole density validated those findings, although evaluation of capillary density was not performed. In a sample of 2 animals, we also visually confirmed a good geographic correspondence of ischemic regions between PET and microsphere-measured stress MBF, where microsphere MBF was measured in 41 samples covering the entire LV and represented in a polar map (Figure IV in the online-only Data Supplement). This subanalysis also confirmed that the segments selected for histological analyses were representative of the area at risk as defined on the stress PET MBF images.

Notably, the transplantation of cells alone was not successful in increasing MBF, reducing match scores and significantly increasing the number of arterioles compared with controls. Similar findings were reported by Chen et al., who were unsuccessful at improving the ischemic pig heart with CAC transplantation. In the majority of studies using cultured mononuclear cells from bone marrow or peripheral blood origin and conducted in chronic ischemic models, the number of cells injected range from 2.5 million to 17 million, and the observed functional improvements vary. A dosing study in ischemic pig myocardium showed a dose-dependent effect of bone marrow mononuclear cells on capillary density and suggested that the local number of cells was a better predictor of increased capillary density and reduced fibrosis than the total number of cells injected. Segments with >20 million cells had the highest capillary density and the lowest amount of fibrosis. We can hypothesize that cells delivered within the matrix may have been better protected against the adverse effects of syringe needle flow. The viability of a variety of stem and progenitor cells during syringe needle flow was shown to be decreased when cells were delivered in PBS compared with the use of a cross-linked alginate hydrogel. In addition, the low engraftment and survival of transplanted cells limit their benefits. In this regard, we have previously demonstrated by tracking cells with PET and by histology that our collagen-based matrix can improve cell retention and prevent excessive relocation of transplanted cells to nontarget tissues, in addition to improving capillary density. We also previously showed that CACs exposed to the matrix demonstrated higher levels of phosphorylated Akt (PI3K/Akt pathway) and increased survival under hypoxia. Based on the ability of the collagen matrix to modulate the therapeutic phenotype and function of CACs, it may have induced similar effects in the pig model of this study. The increased retention and viability of cells within the matrix may also have provided more prolonged paracrine effects because transplanted cells secrete proangiogenic factors and upregulate host-derived cytokine secretion.

In our study, cells were delivered intramyocardially during the second thoracotomy. Clinically, intramyocardial cell transplantation can be done surgically, and newer techniques such as 3D electromechanical mapping (3D NOGA EMM) can evaluate ischemia less invasively and allow for catheter-based transcendocardial delivery of cells. This approach has been used in large animals studies and in small clinical trials. In line with the higher cell retention rates when delivered intramyocardially, a recent meta-analysis demonstrated that LVEF was improved by 8.4% when bone marrow progenitor cells were delivered directly to the myocardium.
viability are representative of scar tissue. They were detected adjacent to hibernating segments but to a smaller extent, which is consistent with the findings that normal, stunned, hibernating, and scarred segments cohabit within the hibernating heart. The minimal metabolic defects observed in the LAD territory may be explained by the variability in coronary anatomy and perfusion patterns of pigs, partial-volume averaging effects at the apex attributable to reduced wall-thickness and increased motion compared with the adjacent heart walls, or may be a consequence of severely reduced blood flow as expected in territories most distal to the occlusion. Previous studies with the constrictor model reported hibernation with aging effects at the apex attributable to reduced wall-thickness but none has deliberately studied this model for hibernation. In this regard, we successfully created hibernation in our animals, accompanied by impaired wall motion, similar to what is also observed clinically.

Limitations of this study included the absence of a matrix-only group, motivated by animal economy and costs, because the purpose of this study was to (1) optimize the delivery of cells over the effects of cells alone and (2) use a preclinical setting with economy of treatment groups. Another limitation is that no angiography was performed to evaluate the occlusion status of the vessel at the time of cell injection; however, closure was histologically documented in all animals. Given this limitation and assuming random distribution among animals, the observation that the cells+matrix group was superior to the other treatments could be an understimation of its true potential to restore perfusion and function to the heart. Finally, although the study analyzed multiple measures, we did not correct for repeated testing to avoid reducing statistical power, also affected by small sample sizes; for this reason, it is possible that type I (repeat testing) as well as type II (small sample size) errors may have occurred.

Conclusions
Within the aforementioned limitations, this study demonstrated the successful development of myocardial hibernation using a well-established ameroid constrictor model of chronic ischemia. This study was also the first to demonstrate that delivering CACs within a collagen matrix can improve MBF, reduce the extent of hibernation, and reduce wall motion abnormalities to a greater extent than cells alone. These results, obtained in a preclinical model and in a blinded fashion, constitute an important step toward the application of collagen matrices to support cell therapy and treat myocardial hibernation in the clinical setting.

Acknowledgments
We thank Suzanne Crowe, Wilson Miranda, Mike Blakely, Dan deVette, and the Animal Care staff for their assistance.

Sources of Funding
This work was supported by the Canadian Institutes of Health Research (CIHR; Grant MOP-77536 to Drs Ruel and Suuronen), the Heart and Stroke Foundation of Ontario (HSFO; Grant 6783 to Drs Ruel and Beanlands), and the HSFO Molecular Function and Imaging Program Grant (PRG No. 6242). Dr Beanlands is a career investigator of the HSFO. C. Giordano was supported by a CIHR Frederick Banting and Charles Best Canada Graduate Scholarships, Master’s Awards, and by a Master’s Studentship award from the HSFO. Dr Thorn was supported by an HSFO Doctoral Research award and Dr Zhang was a recipient of the Lawrence Soloway Research Fellowship Award in Cardiac Surgery. Dr Kuraitis was supported by a CIHR Canadian Graduate Scholarship.

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

After a myocardial ischemic event, cardiomyocytes can have damage that ranges from stunning to hibernation and to cell death. Current revascularization strategies aim at restoring perfusion and, consequently, at recovering stunned or hibernating cardiomyocytes; however, approximately one third of patients with ischemic, viable myocardium cannot undergo revascularization because of comorbidities, anatomic reasons, or other incompatibilities. These patients are at higher risk of cardiac events and could benefit from effective cell-based therapies. Despite the above, multiple studies that have evaluated cell therapy in the experimental setting have almost always not determined its effects in myocardial hibernation; furthermore, the recently recognized adjuvant role of injected biomaterial in providing a better adhesion, proliferation, differentiation, and maturation environment to support cell therapy has not been evaluated in the setting of myocardial hibernation. The present experimental study is novel in that it evaluates the effects of a clinically relevant combination of collagen-based matrix injected with autologous transplanted cells in a validated, large animal model of myocardial hibernation. We show that the combination of matrix and cells improves revascularization, decreases hibernation, and improves left ventricular wall motion. Whether the combination of progenitor cell therapy and biocompatible injectable materials aims at regenerating cardiomyocytes, their supportive vasculature, or both, studies such as ours using more clinically relevant models of coronary artery disease are key to the investigation of regenerative strategies which, ultimately, could positively alter the progression of injured cardiomyocytes to heart failure.
Preclinical Evaluation of Biopolymer-Delivered Circulating Angiogenic Cells in a Swine Model of Hibernating Myocardium

Céline Giordano, Stephanie L. Thorn, Jennifer M. Renaud, Talal Al-Atassi, Munir Boodhwani, Ran Klein, Drew Kuraitis, Girish Dwivedi, Pingchuan Zhang, Jean N. DaSilva, Kathryn J. Ascah, Robert A. deKemp, Erik J. Suuronen, Rob S.B. Beanlands and Marc Ruel

*Circ Cardiovasc Imaging*. 2013;6:982-991; originally published online October 2, 2013; doi: 10.1161/CIRCIMAGING.113.000185

*Circulation: Cardiovascular Imaging* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2013 American Heart Association, Inc. All rights reserved.

Print ISSN: 1941-9651. Online ISSN: 1942-0080

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circimaging.ahajournals.org/content/6/6/982

Data Supplement (unedited) at:

http://circimaging.ahajournals.org/content/suppl/2013/10/02/CIRCIMAGING.113.000185.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation: Cardiovascular Imaging* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation: Cardiovascular Imaging* is online at:

http://circimaging.ahajournals.org//subscriptions/
Pre-Clinical Evaluation of Biopolymer-Delivered Circulating Angiogenic Cells in a Swine Model of Hibernating Myocardium

SUPPLEMENTAL MATERIAL

Céline Giordano, MSc; Stephanie L. Thorn, PhD; Jennifer M. Renaud, MSc; Talal Al-Atassi, MD; Munir Boodhwani, MD; Ran Klein, PhD; Drew Kuraitis, PhD; Girish Dwivedi, MD; Pingchuan Zhang, MD; Jean N. DaSilva, PhD; Kathryn J. Ascah, MD; Robert A. deKemp, PhD; Erik J. Suuronen, PhD; Rob S. B. Beanlands, MD; and Marc Ruel, MD, MPH *

* Correspondence to: Marc Ruel, 40 Ruskin Street, Suite 3402, Ottawa, Ontario, Canada K1Y 4W7. Tel: 1-613-761-4893. Fax: 1-613-761-5367. E-mail: mruel@ottawaheart.ca
Supplemental methods

Surgical procedure

Pigs were anesthetised with a mixture of midazolam (0.5 mg/kg) and ketamine (11 mg/kg), maintained with 0.75-3% isoflurane, and intubated and mechanically ventilated at 12-20 breaths per minute. Buprenorphine (0.03 mg/kg) was administered before and after surgery as needed. The above protocol was used for the 3 surgeries and 2 imaging sessions.

At surgery 1 and following adequate surgical preparation, a 4-5 cm left thoracotomy was performed in the 4th intercostal space. The pericardium was opened and the coronary groove exposed by retraction of the atrial appendage. The proximal left circumflex (LCx) artery was carefully isolated and two vessel loops were placed around the artery (Figure 1A). Isotope-labelled microsphere (ILM) shadow labelling was performed during a 2-minute occlusion of the LCx artery. Following the 2 minutes, nitroglycerine was sprayed on the artery for vasodilation, and the ECG was allowed to normalise before proceeding. An ameroid constrictor (1.5-2.0 mm; Research Instruments SW, Escondido, USA) was then inserted around the vessel upstream of the first marginal branch, and it was visually confirmed that the device did not acutely constrict the artery (Figure 1B). The ribs were brought together, the muscle layer closed and the incision was closed with absorbable sutures in a subcuticular fashion. After surgery and for the full length of the study, animals received daily aspirin (81 mg). Three weeks after the first surgery, the animals underwent a second left thoracotomy via the original incision. The adhesions were dissected and the lateral wall of the heart as well as the left atrial
appendage were exposed. The anatomy of the LCx marginal branches was thereafter visually confirmed. Prior to cell injection, baseline PET scans were reviewed by two different operators among which at least one participated in surgery. To facilitate applicability to the live heart, three-dimensional representations of mismatch and MBF in the left ventricle were used when reviewing the scans. Intramyocardial injections were performed in twelve different locations in the area previously identified as the myocardial region at risk. A small incision was also made in the groin area, and a femoral artery catheter was inserted. Simultaneously to each ILM injection in the atrium, an arterial blood sample was collected from the catheter for ILM analyses. The femoral catheter was removed and the artery ligated. The incision was closed as described previously. Seven weeks after the first surgery, animals were anesthetized and a median sternotomy was performed. Adhesions were dissected for direct access to the left atrium. Similarly to surgery 2, an arterial catheter was inserted into the femoral artery for ILM reference blood sample harvest. ILMs were injected in the left atrium under rest and dipyridamole induced stress while the reference blood sample was collected. Pigs were euthanized by exsanguination and the heart was harvested, washed under running water and sliced for ILM and immunohistochemical analyses.

**Circulating angiogenic cell isolation and culture**

Under sterile conditions, approximately 60 mL of autologous arterial blood was harvested from a femoral artery branch according to the Canadian Council on Animal Care guidelines \(^1\) and collected in ethylene diamine tetraacetic acid (EDTA)-containing
Vacutainers (Greiner Bio-One, North Carolina, USA). Blood was layered on Histopaque 1077 (Sigma-Aldrich, Oakville, Canada) and centrifuged for 30 minutes at 800 × g (2000 rpm). The plasma layer was removed, the buffy coat was collected and subsequently washed (1.5 mL foetal bovine serum (FBS); 2.5 mL of EDTA and 150 mL of phosphate buffered solution (PBS; Sigma Aldrich, Oakville, Canada)) and centrifuged at 450 × g (1400 rpm) for 10 minutes. The supernatant was removed and this washing step repeated twice. Following the last centrifugation, cells were resuspended in Endothelial Basal Media (EBM-2; Clonetics, Guelph, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics, Guelph, Canada) and counted with an automated Vicell™ Cell Viability Analyzer/Cell Counter (Beckman Coulter; Miami, USA) using the trypan blue exclusion method. Culture dishes were covered with approximately 2 mL of 0.1% human fibronectin for coating (Sigma Aldrich, Oakville, Canada). After 30 minutes, the fibronectin was removed and plates were washed 2 times with PBS. Approximately 20 million mononuclear cells were plated on each fibronectin-coated dish. EGM (10 mL) was added to the dish, and cells were cultured for 6 days at 37°C with 5 % CO₂ in a humidified atmosphere, according to standard protocol. After 4 days, non-adherent cells were removed by washing with PBS and 10 mL of new media was applied. On the day of injection, culture media was removed and cells were incubated with 1 mL of 1% trypsin 37°C. After 8 minutes, the same amount of EGM was added to stop the effects of trypsin and cells were lifted from the culture plate by repetitive washes with PBS. Cells were brought to a pellet by centrifugation at 450 × g for 10 minutes. The supernatant was removed and CACs prepared for injection. For injection of CACs alone, the pellet was
resuspended in 3mL of PBS. When mixed to the collagen matrix, cells were not resuspended and were directly added to 3mL of collagen-based matrix.

**Dipyridamole-induced stress protocol**

Myocardial blood flow (MBF) measurements by ILMs and positron emission tomography (PET) were performed under rest and stress conditions. Myocardial stress was induced by continuous injection of dipyridamole (0.56 mg/kg) over a 4-minute period. Exactly 4 minutes post total injection, $^{13}$N-NH$_3$ was injected and the stress scan was started. During surgery, blood pressure was stabilised with phenylephrine (10µg/min – 60µg/min; dosed to effect) before injection of ILMs.

**Myocardial blood flow determination with microspheres**

MBF was assessed at surgeries 2 (week 3) and 3 (week 7) by using 15 µm isotope-labeled microspheres of different masses (BioPAL, Worcester, MA). At the time of ameroid placement, 12.5 million ytterbium-labelled microspheres were injected in the left atrium over 30 seconds during temporary occlusion of the LCx to determine the myocardial area supplied by this artery (shadow labelling). For MBF determination, 12.5 million ILM were injected in the left atrium over 30 seconds while a reference blood sample was drawn from the femoral artery at a rate of 8mL/min. Lutetium and europium-labelled microspheres were used during the second procedure (week 3) to determine MBF at rest and stress, respectively. Lanthanum- and samarium-labelled microspheres
were injected during the final surgery (week 7) to evaluate follow-up MBF at rest and stress, respectively. Following euthanasia, the heart was harvested and 12 circumferential transmural left ventricle samples (approximately 1g) were collected, weighed and dried for 24h at 60°C. Each tissue and blood sample was exposed to neutron beams and ILM densities were measured in a gamma counter. MBF was calculated using the following equation:

\[
MBF (\text{ml/min/g}) = \frac{\text{withdrawal rate (ml/min)}}{\text{weight tissue sample (g)}} \times \frac{\text{isotope counts (tissue sample)}}{\text{isotope counts (reference blood sample)}}
\]

**Protocol for heart slicing used for microsphere analyses and immunohistochemistry**

Following harvest, hearts were oriented as shown in supplemental Figure 3A and two 1.5 cm slices were cut at the midventricular level of the LV, between the atroventricular groove and the apex. Each slice was additionally cut in 6 transmural sections starting from the anterior junction of the right and left ventricle (left anterior descending (LAD) territory). Samples were identified clockwise from 1 to 6 starting from the LAD. Only one septal segment was used; the rest of the septum and the right ventricle were discarded (supplemental Figure 3B). Attention was given to preserve the *in-vivo* orientation of the samples, and grossly visible pericardium and adhesions were removed from each section. Approximately 1g of tissue from each of the 12 sections were used for ILM analyses.
**Immunohistochemistry**

For immunohistochemistry analyses, 3 transmural sections from the above-described LAD1 territory and 3 transmural sections from the above-described LCx4 territory were systematically collected and fixed in 4% paraformaldehyde for 24h. One of the three transmural sections from each territory was randomly selected and prepared for analyses. After being fixed, sections were dehydrated, paraffin-embedded and sectioned. Each slice was then stained using a pre-diluted rabbit polyclonal antibody against α-smooth muscle actin (SMA) to identify arterioles (Abcam, Cambridge, USA). Texas Red (Vector, Burlington, Canada) diluted 1:100 was used as a secondary antibody. To determine arteriole density, 6 random pictures per slide were taken at a total magnification of x100 using a fluorescent microscope. Positively stained vessels with a diameter <100µm were counted by two independent blinded examiners. Results are expressed as the number of vessels/field of view.

**Positron emission tomography**

*Euglycemic hyperinsulinemic clamp detailed protocol*

Following a 10 minutes 20% Dextrose front load (Baxter, Mississauga, Canada), insulin (Novolin®ge Toronto, Novo Nordisk, Mississauga, Canada; dose = weight (kg) × 0.25 IU in 50mL of saline) was administered for 4 minutes at a rate of 48mL/min. At the end of the 3rd minute, FDG was injected and the scan started. Insulin injection rate were lowered to 24 mL/min for 3 minutes and stabilised at 12mL/min for the rest of the scan.
Insulin was stopped 10 minutes prior to scan completion. Serum glucose levels were closely monitored throughout the scan, and adjustments were done by altering the rate of glucose administration (Figure 2).

**Segment selection for MBF and viability sub-analyses**

For a sub-analysis of the most affected regions of the ischemic territory, segments were selected as follows: at baseline (two weeks after constrictor placement), the 2 adjacent segments with the lowest stress MBF in the LCx territory were selected as the ischemic region-at-risk. Rest and stress MBF and stress/rest MFR were calculated as the average value within these region-at-risk segments. For metabolic analyses, the 2 adjacent segments within the LCx territory with the highest mismatch score at baseline were selected as the region of hibernating myocardium. The same segments were used for match analyses.
**Supplemental Table**

**Table 1:** A comparison of 2 and 5 segments PET results. Values are presented for the LCx territory and segments selection is described in the supplemental methods section. Values are presented as mean±SE

### Rest MBF in the LCx (n= 25)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells + Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 segments</td>
<td>0.80±0.05</td>
<td>0.84±0.05</td>
<td>0.77±0.10</td>
</tr>
<tr>
<td>5 segments</td>
<td>0.84±0.05</td>
<td>0.89±0.04</td>
<td>0.83±0.10</td>
</tr>
</tbody>
</table>

### Stress MBF in the LCx (n= 25)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells + Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 segments</td>
<td>0.94±0.16</td>
<td>0.97±0.10</td>
<td>0.86±0.09</td>
</tr>
<tr>
<td>5 segments</td>
<td>1.15±0.18</td>
<td>1.12±0.09</td>
<td>1.05±0.11</td>
</tr>
</tbody>
</table>

### MFR in the LCx (n= 25)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells + Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 segments</td>
<td>1.18±0.17</td>
<td>1.16±0.07</td>
<td>1.18±0.11</td>
</tr>
<tr>
<td>5 segments</td>
<td>1.41±0.21</td>
<td>1.25±0.06</td>
<td>1.32±0.11</td>
</tr>
</tbody>
</table>

### PET Mismatch LCx (n=14)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells + Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 segments</td>
<td>23.24±3.68</td>
<td>9.38±2.73</td>
<td>19.2±5.14</td>
</tr>
<tr>
<td>5 segments</td>
<td>10.99±2.39</td>
<td>5.47±1.74</td>
<td>9.81±2.45</td>
</tr>
</tbody>
</table>

### PET Match LCx (n=14)

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Cells</th>
<th>Cells + Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>Baseline</td>
</tr>
<tr>
<td>2 segments</td>
<td>2.44±2.18</td>
<td>3.63±2.68</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>5 segments</td>
<td>5.31±2.17</td>
<td>4.04±0.83</td>
<td>7.80±1.25</td>
</tr>
</tbody>
</table>
**Supplemental Figures**

**Supplemental Figure 1**

**Figure 1: Pig ameroid constrictor model.** During a left mini thoracotomy, the left circumflex coronary artery was isolated between two vessel loops (A) and an ameroid constrictor is inserted around the artery (B).
Supplemental Figure 2

Figure 2: $^{13}$N-NH$_3$ and $^{18}$F-FDG image acquisition protocols
Figure 3: Sectioning protocol of the heart for microsphere and immunohistochemical analyses. Following euthanasia, the heart was harvested and washed under running water. Two circumferential slices were isolated (A) and further sectioned as described in (B). For immunohistochemistry, cross-samples from the LCx4 territory were collected.
Figure 4: Polar map showing stress MBF measured by microspheres. The left ventricle was sectioned in 41 samples used for analyses. A good geographic correspondence is seen between PET and microsphere measurements.
Supplemental References

1. Ccac guidelines on: The care and use of farm animals in research, teaching and testing. 2009;2012