Engraftment, Differentiation, and Functional Benefits of Autologous Cardiosphere-Derived Cells in Porcine Ischemic Cardiomyopathy

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Background—Cardiosphere-derived cells (CDCs) isolated from human endomyocardial biopsies reduce infarct size and improve cardiac function in mice. Safety and efficacy testing in large animals is necessary for clinical translation.

Methods and Results—Mesenchymal stem cells, which resemble CDCs in size and thrombogenicity, have been associated with infarction after intracoronary infusion. To maximize CDC engraftment while avoiding infarction, we optimized the infusion protocol in 19 healthy pigs. A modified cocktail of CDCs in calcium-free PBS, 100 U/mL of heparin, and 250 μg/mL of nitroglycerin eliminated infusion-related infarction. Subsequent infusion experiments in 17 pigs with postinfarct left ventricular dysfunction showed CDC doses ≥10⁷ but <2.5×10⁸ result in new myocardial tissue formation without infarction. In a pivotal randomized study, 7 infarcted pigs received 300 000 CDCs/kg (~10⁷ total) and 7 received placebo (vehicle alone). Cardiac magnetic resonance imaging 8 weeks later showed CDC treatment decreased relative infarct size (19.2% to 14.2% of left ventricle infarcted, \(P=0.01\)), whereas placebo did not (17.7% to 15.3%, \(P=0.22\)). End-diastolic volume increased in placebo, but not in CDC-treated animals. Hemodynamically, the rate of pressure change (dP/dt) maximum and dP/dt minimum were significantly better with CDC infusion. There was no difference between groups in the ability to induce ventricular tachycardia, nor was there any tumor or ectopic tissue formation.

Conclusions—Intracoronary delivery of CDCs in a preclinical model of postinfarct left ventricular dysfunction results in formation of new cardiac tissue, reduces relative infarct size, attenuates adverse remodeling, and improves hemodynamics. The evidence of efficacy without obvious safety concerns at 8 weeks of follow-up motivates human studies in patients after myocardial infarction and in chronic ischemic cardiomyopathy. (Circulation. 2009;120:1075-1083.)

Key Words: cells, stem □ myocardial infarction □ heart failure

Despite remarkable advances in the treatment of coronary disease, nearly one third of patients who survive a myocardial infarction (MI) develop heart failure within 5 years.¹ This burden of disease has driven investigation of bone marrow–derived cells.²–⁷ More recently, the discovery of resident cardiac stem cells, which possess a predetermined cardiac fate and can generate new heart tissue, has spurred interest in their clinical application.⁸–¹² Although present, cardiac stem cells exist in small numbers in vivo, and their isolation and expansion ex vivo present a challenge to their clinical use. Prior work in our laboratory developed a practical method to isolate and expand resident cardiac stem cells from endomyocardial biopsy specimens.¹³ These “cardiosphere-derived cells” (CDCs) express surface markers typical of stem cells (c-kit, CD105) and, delivered to the heart after coronary ligation, can generate new myocardium and improve cardiac function in mice. Successful clinical application could provide a novel therapy for postinfarct left ventricular (LV) dysfunction and cardiomyopathy in general.

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Given the results in mice, we hypothesized that CDC isolation and delivery after infarction in a large animal model...
could be performed safely and would result in formation of new cardiac tissue, which in turn would limit negative remodeling and improve cardiac function. We sought to test this hypothesis in a relevant clinical model, making every effort to determine whether the safety concerns that have plagued certain modalities of stem cell therapy would apply to CDC therapy.\(^{14,15}\)

**Methods**

**Isolation of Porcine Progenitor Cells**

Studies were performed according to the “Position of the American Heart Association on Research Animal Use,” with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Under anesthesia, 3 types of progenitor cells were isolated from pigs. Mesenchymal stem cells (MSCs) and bone marrow mononuclear cells (BMMCs) were isolated and collected as reported previously.\(^{16,17}\) CDCs were isolated and cultured from endomyocardial biopsies. Briefly, 5F endomyocardial biopsy forceps (Argon Medical, Athens, Tex) were advanced to the right ventricle (RV) through a jugular venous sheath under fluoroscopic guidance. Biopsies from the septum were stored in ice-cold cardioplegia and processed within 3 hours to yield CDCs as previously described\(^{18}\) (also see online-only Data Supplement). CDCs used for intracoronary infusion were frozen in Pentaspan (B. Braun Medical, Bethlehem, Pa) containing 5% dimethyl sulfoxide with 2% albumin and stored in liquid nitrogen until the day of infusion. Freezing did not affect CDC viability (data not shown).

**CDC Reporter Gene Transduction**

CDCs used for engraftment studies were transduced with lentiviral luciferase or nuclear-localized LacZ. Cells at 50% confluence were exposed to lentivirus at a multiplicity of infection of 20 in media containing Polybrene. After exposure, fresh media was added to the culture flask, and when confluent, CDCs were passaged per routine. Luciferase activity was measured (Promega, Madison, Wis), to establish a standard luciferase activity curve for each animal. LacZ expression was confirmed by X-gal stain (Applied Biosystems, Norwalk, Conn).

**Ex Vivo Thrombus Assay and Tissue Factor ELISA**

Progenitor cells (10^4 or 10^5 cells in 1 mL of Ca\(^{2+}\)-free PBS [PBS−]) were mixed with 20 mL of fresh blood to measure thrombus formation. The mixture was placed in a 50 mL conical tube and kept at room temperature, with gentle inversion every 5 minutes. One and 8 hours after mixing, thrombus was collected for analysis by centrifugation. Tissue factor ELISA of separate progenitor cell lysate samples was performed per manufacturer instructions (R&D Systems, Minneapolis, Minn).

**Intracoronary CDC Delivery**

After induction of anesthesia, intracoronary infusion of CDCs was performed in 19 healthy farm pigs and 33 pigs with infarcted hearts (9 farm and 24 miniature pigs). Infarcted animals had anterior MI created as previously described.\(^{18}\) followed immediately by endomyocardial biopsy. Four weeks later, intracoronary infusion was performed via a 3.0×8 mm over-the-wire coronary balloon (Boston Scientific, Natick, Mass) placed in the mid left anterior descending artery. CDCs or placebo (carrier solution alone) were infused in 3 cycles of intermittent balloon inflation. Heart rate, blood pressure, end-tidal carbon dioxide, pulse oximetry, and 12-lead ECG were monitored throughout (online-only Data Supplement).

**Measurement of Short-Term CDC Engraftment by Luciferase**

Animals receiving luciferase+ CDCs were euthanized 24 hours after infusion (see online-only Data Supplement Figure I for schematic of this experiment). One-gram tissue samples were taken from sites in the LV and RV and from the lungs, liver, spleen, and kidneys and were then flash-frozen and stored at −80°C. Within 24 hours, tissue was homogenized with 10% fetal bovine serum, then centrifuged. Luciferase activity of the supernatant was measured and converted to CDCs/g tissue using the standard curve created for each cell line.

**Assessment of Long-Term CDC Engraftment by LacZ**

Animals receiving lacZ+ CDCs were euthanized 8 weeks after infusion (see online-only Data Supplement Figure II for schematic of this experiment). Tissue from the peri-infarct zone and remote areas of the heart was fixed in 4% paraformaldehyde. Frozen sections were used for X-gal and immunohistochemical staining, with the latter using antibodies for β-galactosidase conjugated to fluorescein isothiocyanate (Abcam, Cambridge, Mass) and α-sarcomeric actin (Abcam) with Alexa 568 secondary antibody (Invitrogen, Carlsbad, Calif).

**Placebo-Controlled Experiment**

To determine whether engrafted CDCs influence postinfarct remodeling, 16 miniature pigs were randomized to CDC (300 000 CDCs/kg) or placebo infusion. Four weeks after infarction, 3T cardiac magnetic resonance imaging (MRI; Siemens, Munich, Germany) was performed to determine LV systolic function and chamber size using 8-mm contiguous short axis slices; infarct size was measured using 6-mm contiguous slices with delayed gadolinium enhancement.\(^{19}\) Analysis was performed using Cine Tool 3.4 software (GE Healthcare, Milwaukee, Wis). Immediately before infusion, LV hemodynamic measurements were performed (Millar Instruments, Houston, Tex). Blood samples were taken at infusion and 24 hours later to measure troponin I (Tnl). Eight weeks later, animals had repeat MRI, hemodynamic measurement, coronary angiography, and provocative electrophysiologic testing (EPS) followed by comprehensive autopsy including examination of the heart, lungs, liver, spleen, kidneys, and brain. During EPS, up to 3 extra stimuli were performed at the RV apex and outflow tract to attempt to induce ventricular tachycardia (see online-only Data Supplement Figure III for schematic of this experiment).

**Statistical Analysis**

All data are presented as mean±SEM. Statistical analyses were performed with the use of statistical software SAS (version 9.2, Cary, NC), where \(P<0.05\) was deemed significant. Due to the limited sample size, the nonparametric Wilcoxon signed rank test was used to perform paired and unpaired comparisons. For MRI and hemodynamic analyses, where measurements were repeatedly taken from the same pig over time, a stratified linear mixed model was used.\(^{20}\) The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Isolation and Expansion of CDCs**

CDC isolation from endomyocardial biopsy specimens was highly successful: 33 of 35 animals (94%) were successfully biopsied, with a mean total biopsy weight of 52.6±6.7 mg. From these, CDCs were isolated in 30 of 33 cases (91%), for a mean final yield of 24.5±2.9 million cells grown in 2.7±0.1 passages and 26.9±1.3 days.
Comparison of CDCs With MSCs and BMMCs
MSCs have been shown to produce microvascular occlusion and infarction after intracoronary infusion, unless certain precautions are taken. This is not surprising, as their average cell diameter measured by light microscopy is 21.0 ± 3.3 μm, exceeding the typical capillary luminal diameter of 7 to 10 μm. At 20.6 ± 3.9 μm, CDCs are similar in size to MSCs (P = 0.52) and larger than BMMCs, which measured 8.6 ± 1.8 μm (P < 0.0001; Figure 1A). Like MSCs, a pilot study of intracoronary infusion of 5 × 10^7 allogeneic CDCs resuspended in PBS containing Ca^2+ resulted in a large infarction (online-only Data Supplement).

Assessment of CDC Clumping and Thrombogenicity
Two major factors that contribute to microvascular obstruction from cell infusion include clumping and thrombus formation. To determine whether CDCs would clump before infusion, we suspended CDCs in PBS with Ca^2+ for 1 hour at room temperature. Microscopic examination showed no clumping, regardless of whether the solution contained Ca^2+ (Figure 2A).

To assess CDC thrombogenicity, we mixed cells suspended in PBS− or PBS+ with allogeneic arterial blood and measured thrombus formation at room temperature. Visible thrombus began to form after 90 minutes in PBS−, but after only 40 minutes in PBS+. Total thrombus in either solution reached its peak in about 8 hours (Figure 2B). To rule out an immunologic reaction, we performed the same experiment using autologous blood instead of allogenic blood. We found no difference in the rate or amount of thrombus formed compared with allogeneic samples (data not shown).

To compare the relative thrombogenicity of different progenitor cells, we measured thrombus formation using 1 × 10^7 BMMCs, MSCs, or CDCs at 1 hour (Figure 2C) or 8 hours (Figure 2D) after mixture with allogeneic arterial blood. In this condition, MSCs and CDCs are more thrombogenic than BMMCs. The presence of Ca^2+ in the cell infusion solution significantly increased the amount of thrombus formed at 1 hour in all cell types, although the difference was no longer significant at 8 hours. To begin to determine why CDCs and MSCs are more thrombogenic than BMMCs, we measured tissue factor. CDC and MSC lysates had similar tissue factor concentrations; both were significantly higher than BMMCs (P < 0.001; Figure 1B).

To limit ex vivo thrombus formation, we added heparin (100 U/mL or 200 U/mL) to the PBS− cell suspension solution; 100 U/mL sufficed to eliminate cell-related thrombus formation in all 3 cell lines at both 1 and 8 hours (Figure 2C and 2D).

Early Engraftability and Myocardial Damage in Intact Heart
To achieve measurable engraftment, but no infarction after intracoronary CDC infusion, we delivered 10^7, 10^8, or 10^9 luciferase+ CDCs using multiple infusion solutions in healthy farm pigs (n = 2 for each condition); luciferase activity was measured 24 hours later. We defined the target delivery area of the heart as the LV septum and anterior wall (Figure 3A, areas 1 to 4), nontarget areas as LV posterolateral and RV free walls (areas 5, 6), and nontarget organs as lung, liver, spleen, and kidney. Luciferase activity was reliably detected in the target area only when we infused 10^7 CDCs. At lower doses, minimal activity was detected, even in the target area (Figure 3B). No luciferase activity was detected in nontarget areas or organs at any dose. Distribution of lucif-
erase activity within the target area was consistent in every animal: maximum engraftment in the septum with lower levels in the anterior wall (Figure 3B).

To assess MI after CDC delivery, we measured serum TnI 24 hours after infusion (Figure 3C and 3D). Delivery of $10^7$ CDCs resuspended with PBS− caused a sizable infarction with TnI of 21.0 ± 14.6 ng/mL. The addition of 100 U/mL of heparin and 250 μg/mL of nitroglycerin (NTG; table below y axis shows composition of suspension solution). Maximal engraftment (cells/g) was detected in the septum (areas 1 and 2). Total luciferase activity is sum of target areas and was increased by the addition of heparin. TnI concentration was significantly reduced with the addition of heparin and NTG (n=2). (D) Luciferase activity and serum TnI 24 hours after cell infusion in the infarcted heart. Maximum and total luciferase activity in the target area, as well as serum TnI, were all dependent on the number of CDCs infused (n=2).

Figure 3. Short-term CDC engraftment. (A) Gross example of porcine heart; arrowhead marks infusion site; boxes indicate sampling areas: 1 to 4 are target areas; areas 5 and 6 are nontarget areas. (B) Luciferase activity in heart and nontarget organs after infusion of $1 \times 10^5$, $1 \times 10^6$, or $1 \times 10^7$ CDCs (n=2) shows virtually no off-target engraftment (numbers on y axis above “heart” label correspond to areas from panel A). (C) Luciferase activity and troponin I (TnI) concentration after infusion of CDCs in PBS− with or without 100 U/mL of heparin and 250 μg/mL of nitroglycerin (NTG; table below y axis shows composition of suspension solution). Maximal engraftment (cells/g) was detected in the septum (areas 1 and 2). Total luciferase activity is sum of target areas and was increased by the addition of heparin, TnI concentration was significantly reduced with the addition of heparin and NTG (n=2). (D) Luciferase activity and serum TnI 24 hours after cell infusion in the infarcted heart. Maximum and total luciferase activity in the target area, as well as serum TnI, were all dependent on the number of CDCs infused (n=2).

Early Engraftment and Myocardial Damage in the Infarcted Heart Using Optimized Infusate

Using the optimized delivery protocol in the infarcted heart, we infused $10^6$, $10^7$, $2.5 \times 10^7$, or $5 \times 10^7$ luciferase+ autologous CDCs in farm pigs 4 weeks post-MI, measuring short-term engraftment by luciferase activity, and TnI 24 hours later to exclude infarction. CDC engraftment was detectable at all CDC doses, with highest engraftment in the septum; engraftment was minimal in nontarget areas and organs. Maximum and total luciferase activity increased with progressively increasing CDC dosage (Figure 3D). TnI remained within normal limits (<0.5 ng/mL), with <2.5 × 10^7 CDCs. Above this threshold, TnI increased in a dose-dependent fashion (Figure 3D), indicating that infusion of 10^7 CDCs in the infarcted heart achieves measurable early engraftment of CDCs without thromboembolic complication; doses ≥2.5 × 10^7 achieve greater engraftment, but risk infarction.

Long-Term CDC Engraftment

To assess long-term CDC engraftment and differentiation, intracoronary infusion was performed in 8 adult miniature pigs 4 weeks after infarction, 2 animals each receiving placebo (infusate alone), $10^5$, $10^6$, or $10^7$ autologous lacZ+ CDCs. At euthanasia 8 weeks later, mature lacZ+ cardiomyocytes and vascular cells were identifiable in the peri-infarct zone of both animals receiving $10^7$ CDCs. Figure 4A shows examples of cardiomyocytes with lacZ+ nuclei separated by connective tissue in the infarct border zone; Figure 4B illustrates cells with lacZ+ nuclei lining an arteriole.
virtue of their location, size, and appearance, the latter are likely endothelial cells.13 To confirm the cardiomyocyte identity of the large nonvascular cells, coexpression of β-galactosidase with α-sarcomeric actin was confirmed by immunohistochemistry (Figure 4C). No lacZ+ cells were identified in animals receiving placebo or lower doses of CDCs.

**CDC Infusion Reduces Infarct Size and Improves Hemodynamic Function Compared With Placebo**

Having shown that CDCs can engraft and survive after infusion at doses that do not cause infarction, we set out to determine whether CDC delivery affects post-MI remodeling. Animals in the short-term engraftment experiment had an average weight at infusion of 38.1 kg, thus those animals receiving 107 CDCs received ≈260 000 CDCs/kg. For extrapolation to clinical trials, we used a dose of 300 000 CDCs/kg, approximating the dose that resulted in engraftment and well below the 650 000 CDCs/kg (≈2.5×107) dose that caused significant infarction. To this end, 16 adult miniature swine were randomized to receive 300 000 autologous CDCs/kg or placebo 4 weeks after infarction. Fourteen animals (7 CDC-treated, 7 placebo-treated) completed the protocol and were used for final analysis. Of the 2 animals that did not complete the protocol, 1 animal randomized to CDCs died unexpectedly 10 days after infusion; an autopsy showed significant heart failure. The second animal was randomized to placebo, but had a noncardiac injury forcing removal from the protocol before infusion.

At MRI before infusion, animals on average had large infarcts (18.5±1.8% of LV mass) and significant LV systolic dysfunction (left ventricular ejection fraction [LVEF] 38.6±2.3%); there was no difference in infarct size, LVEF, or other MRI parameters between animals assigned to either group (Table). At the time of infusion, all animals had a patent left anterior descending artery, and hemodynamic function did not differ between the 2 groups. Twenty-four hours after infusion, TnI was mildly elevated in both groups (0.74±0.37 ng/mL in CDC versus 0.95±0.48 ng/mL in placebo), but with no significant difference between them (P=0.73).

Eight weeks later, analysis of delayed gadolinium-enhancement MRI data from 7 CDC-treated animals and 6 placebo-treated animals (baseline gadolinium-enhanced images were not obtained in 1 placebo-treated animal, precluding comparison) showed that relative infarct size (% of LV infarcted as a fraction of LV mass) decreased significantly in the group that received CDCs (19.2±2.5% to 14.2±3.1%, P=0.01), but not placebo (17.7±2.9% to 15.3±3.0%, P=0.22; Figure 5). Total LV mass increased with CDC infusion (58.8±3.8 g to 76.7±6.1 g, P=0.02), but was of only borderline significance with placebo (60.0±2.8 g to 72.1±5.0 g, P=0.05). Absolute infarct mass decreased modestly, albeit insignificantly, with CDCs (11.0±1.3 g to 10.6±2.6 g, P=0.89), but remained unchanged in placebo (10.5±1.6 g to 10.6±1.7 g, P=0.92). Thus the decrease in relative infarct size in the CDC-treated animals reflects an underlying increase in LV mass coupled with a modest decrease in infarct mass. There was, however, no significant difference in final infarct size or LV mass between CDC-treated animals and placebo in unpaired analysis (Table).

Analysis of LV chamber size and LVEF by MRI at the time of infusion and 8 weeks later (n=7 in both groups) suggests that CDC infusion slows adverse remodeling. Although CDC-treated animals had nonsignificant increases in both end-diastolic volume (40.7±3.4 mL to 49.6±4.3 mL; P=0.10) and end-systolic volume (25.8±3.3 mL to 31.8±4.4 mL; P=0.26), in placebo animals, end-diastolic volume was significantly increased (45.3±1.8 mL to 58.6±4.3 mL; P<0.01), and end-systolic volume was nearly so (27.7±2.5 mL to 37.5±4.3 mL; P=0.06). LVEF was preserved with CDC infusion (37.8±3.4% to 37.6±3.4%, P=0.98), but tended to decrease modestly in placebo (39.5±3.5% to 37.0±3.5%, P=0.59).

Hemodynamic measurement 8 weeks after infarction revealed animals treated with CDCs (n=7) had a greater rate of
We found that, after the systematic optimization of intracoronary infusion, autologous CDCs may be safely delivered in a minimally invasive manner, and has been proven to be feasible in a primate model of ischemic cardiomyopathy. Furthermore, delivered CDCs have the ability to engraft, form mature cardiac cells, and exert positive effects on infarct size, LV remodeling, and hemodynamic function. The study was conducted using standard clinical equipment and techniques in a model designed to replicate a clinical scenario in which CDCs might be used, namely the treatment of postinfarct LV remodeling and dysfunction.

**Optimization of Intracoronary Cell Infusion**

Intracoronary infusion is an attractive method for cell delivery to the heart because it is widely available clinically, can be performed in a minimally invasive manner, and has been demonstrated to have clinical benefit in a primate model of ischemic cardiomyopathy. Furthermore, delivered CDCs have the ability to engraft, form mature cardiac cells, and exert positive effects on infarct size, LV remodeling, and hemodynamic function. The study was conducted using standard clinical equipment and techniques in a model designed to replicate a clinical scenario in which CDCs might be used, namely the treatment of postinfarct LV remodeling and dysfunction.

**Table. MRI and Hemodynamic Measurements in Placebo-Controlled Study Before Intracoronary Infusion and 8 Weeks Later**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Preinfusion</th>
<th>8 Weeks After Infusion</th>
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<tbody>
<tr>
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<td>Placebo</td>
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<td>Percent LV infarcted by mass (%)</td>
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<td>17.7±2.9</td>
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<tr>
<td>End-diastolic pressure (mm Hg)</td>
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CDC indicates cardiosphere-derived cells; LV, left ventricular; and MRI, magnetic resonance imaging.

**Discussion**

We found that, after the systematic optimization of intracoronary infusion, autologous CDCs may be safely delivered in a minimally invasive manner, and has been demonstrated to have clinical benefit in a primate model of ischemic cardiomyopathy. Furthermore, delivered CDCs have the ability to engraft, form mature cardiac cells, and exert positive effects on infarct size, LV remodeling, and hemodynamic function. The study was conducted using standard clinical equipment and techniques in a model designed to replicate a clinical scenario in which CDCs might be used, namely the treatment of postinfarct LV remodeling and dysfunction.

**Optimization of Intracoronary Cell Infusion**

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**Figure 5.** Measurement of relative infarct size before infusion and 8 weeks later showed a significant decrease in animals treated with CDCs (P=0.01, n=7), but not in the placebo group (P=0.22, n=6).

**Figure 6.** Eight weeks after infusion, animals treated with CDCs (n=7) had significantly greater dP/dt maximum (P=0.04, A) and significantly lower dP/dt minimum (P=0.03, B) than placebo animals (n=6).
used in a number of clinical trials. The larger diameter and greater ex vivo thrombogenicity of MSCs and CDCs compared with BMMCs described here may explain why high doses of BMMCs have been delivered in clinical studies (as many as $3 \times 10^8$ cells) without report of thrombotic complications, whereas MSCs have been reported to cause infarction after intracoronary infusion unless special precautions are taken. Our results show that with optimization, CDCs, like MSCs, can be safely delivered via intracoronary infusion, and furthermore, that at this dose, CDCs can engraft and positively affect LV remodeling after infarction. Also, although BMMCs have been delivered at much higher doses in clinical trials, long-term follow-up has suggested that the benefits of BMMC transplantation may be transient. Indeed, the smaller size and nonthrombogenic characteristics of BMMCs may paradoxically result in lower relative retention in the heart after infarction. Ultimately, clinical trials are necessary to compare the relative effectiveness and durability of different cell types. This study, however, clearly shows that optimization of intracoronary CDC infusion is critical to achieve a positive effect without complications.

**Endomyocardial Biopsy and CDC Isolation After Infarction Is Safe and Effective**

The paradigm proposed for clinical CDC isolation tested in this study shows that endomyocardial biopsy after infarction can be safely performed and results in growth of large numbers of CDCs. Clinically, it is unlikely that patients would undergo endomyocardial biopsy immediately after infarction, and one would expect that biopsy performed days to weeks after infarction would only be safer than in the model described here. The data presented here also indicate that, despite an acute infarction, in the majority of cases (91% of animals in this study), large numbers of CDCs can be isolated and grown. In humans, CDC isolation has also been successful in the vast majority of cases, even with biopsies from patients with chronic heart failure. Furthermore, our results suggest that CDCs delivered to the heart after such isolation are effective, having a positive impact on infarct size, remodeling, and hemodynamic function.

**Effects of CDC Treatment Are Not Limited to the Targeted Delivery Zone**

Although relative infarct size was reduced in CDC-treated animals, it should be noted that there was not a significant change in absolute infarct size in either group, or between groups. Instead, the change in relative infarct size in the CDC-treated animals was due primarily to a significant increase in LV mass, which was not seen with placebo. If cardiac regeneration were to occur after cell therapy, one would predict either an increase in viable myocardium, a decrease in infarcted myocardium, or both. The present data suggest the first possibility, and the improved hemodynamics indicate that the increase in LV mass had positive functional benefits.

The global effects on LV mass and adverse remodeling seen in this study also suggest that CDCs may exert effects on sites outside the targeted delivery zone. Although our work here showed that CDCs have the ability to form both mature cardiac myocytes and vascular cells in the peri-infarct zone, no evidence of significant engraftment was found in remote, “off-target” myocardium. This suggests that any remote effects of intracoronary CDC delivery results from translation of mechanical effects in the infarct zone to remote areas or from paracrine factors. There is evidence that CDCs generate growth factors such as fibroblast and hepatocyte growth factors both in vitro and in vivo. Further study is necessary to determine how important either of these effects might be and whether the resulting increase in LV mass is primarily due to hypertrophy of existing cardiomyocytes or to tissue regeneration.

**Comparison With Other Methods of Cardiac Stem Cell Isolation and Expansion**

The ability of several different types of stem cells (both cardiac and noncardiac) to improve cardiac function is currently being evaluated. Although the limitations of CDCs include the need to obtain autologous myocardial tissue and the length of time necessary to grow the cells, the method described here is minimally invasive and highly successful (91% of biopsies resulted in CDCs). In addition, the time necessary for CDC growth is significantly shorter than that required for other methods to produce cardiac-derived stem cells. Although large numbers of bone marrow–derived stem cells can be obtained in a few days, the clinical effects of these treatments remain modest and the ability of such cells to truly regenerate myocardium remains uncertain. Although it can be argued that the effects of CDCs reported here were modest as well, these effects were achieved with what appears to be low-level engraftment. Recent work suggests that the use of basic fibroblast growth factor can enhance CDC engraftment and synergistically increase the positive effect of CDCs after infarction. Synergistic improvement of the results reported here could provide an important new tool for the treatment of cardiomyopathy.

**Limitations of this Study**

Although we made every effort to replicate the clinical conditions in which CDCs might be employed, this study was nevertheless performed in an iatrogenic model of MI in a surrogate species, and admittedly, in a small number of animals. In this model, we have demonstrated that tissue for CDC growth can be safely obtained after MI, that the resulting cells can be safely delivered via intracoronary infusion, and that there is a potential benefit from doing so. Taken together, the results show the potential promise of CDCs in the treatment of post-MI ventricular dysfunction; however, only clinical trials will determine whether this promise can be translated to patients. To this end, the CArdiosphere-Derived aUtologous stem CElls to reverse ventricular dysfunCTion study, based on the technology presented here, has recently been initiated (see http://www.clinicaltrials.gov for details).

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Disclosures

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References


CLINICAL PERSPECTIVE

Every year, more than 1 million Americans suffer a myocardial infarction; nearly one third will go on to develop heart failure. This burden of ischemic cardiomyopathy has driven interest in stem cell therapy, in the hope that damaged myocardium might be repaired. Cardiosphere-derived cells (CDCs) are cardiac-derived stem cells and supporting cells that can be isolated from minimally invasive percutaneous endomyocardial biopsies and grown to large numbers (millions) in a few weeks. CDCs have the advantage of being preprogrammed to differentiate into cardiac and vascular lineages, but not into cell types that are alien to the heart. In mice, human CDCs delivered postinfarction engraft, reduce infarct size, and preserve left ventricular function compared with placebo. To further translate these findings, we studied CDCs in a porcine model of anterior myocardial infarction and resultant ischemic cardiomyopathy. We found that endomyocardial biopsy after myocardial infarction is safe, and CDC isolation from these biopsies is highly successful. After optimization of the delivery solution, autologous CDCs may be safely delivered via intracoronary infusion at doses that result in engraftment and formation of new cardiac myocytes and vascular cells. As compared with placebo infusion, animals receiving CDCs had decreased infarct size, less postinfarct ventricular remodeling, and improved hemodynamic function. No neoplastic or proarrhythmic side effects were observed. Together, these results indicate that CDCs are a novel, safe, and potentially effective therapy for ischemic cardiomyopathy. Recently, the National Institutes of Health–sponsored phase I CARDiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction trial was initiated to study intracoronary delivery of autologous CDCs in patients with mild-to-moderate ischemic cardiomyopathy.
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Supplemental Materials

Supplemental Methods:

**Infarct model and endomyocardial biopsy**

Anesthesia was induced in pigs using intramuscular delivery of solution containing 50 mg/mL ketamine HCl, 50 mg/mL xylazine and 100mg/mL Telazol to allow for endotracheal intubation and subsequent administration of 1.5% isoflurane. Using sterile surgical technique a cutdown was performed to access the right jugular vein and carotid artery. Following anticoagulation with heparin, a 7 French (F) coronary catheter (Cordis, Miami Lakes, FL) was advanced to the left main coronary under fluoroscopic guidance; iodinated contrast dye (Omnipaque, GE Healthcare, Milwaukee, WI) was injected to confirm placement and patency of the left anterior descending artery. A 3.0 x 8 mm angioplasty balloon (Boston Scientific, Natick, MA) was advanced to the mid-LAD over the wire. The balloon was inflated to obstruct flow, confirmed by administration of contrast and ST elevation on ECG. Within 15 minutes of balloon inflation, all animals received 50 mg of intravenous (IV) amiodarone and 25 mg of lidocaine to suppress arrhythmias. Additional boluses of 25 mg of lidocaine were administered in the event of arrhythmia. In the case of sustained ventricular tachycardia or fibrillation, external cardioversion was performed immediately. After 2½ hours, the balloon was deflated to allow for reperfusion.

As soon after reperfusion as animals were judged to be hemodynamically stable (usually within 5 to 10 minutes), percutaneous biopsy was performed of the right ventricular septum. Endomyocardial biopsy forceps (Argon Medical, Athens, TX) were advanced from the internal jugular vein to the right ventricle under fluoroscopic guidance. Up to 6 biopsy specimens were taken from the septum and transferred to ice-cold cardioplegia containing 5% dextrose, mannitol 68.6 mmol/L, potassium chloride 1.6 mmol/L, sodium bicarbonate 3.1 mmol/L and heparin 1000 USP units/L in sterile Ca++ and Mg++-free phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA); biopsies were kept on ice until processed.

**Tissue Processing and CDC Isolation**

All tissue was processed within 3 hours of biopsy using sterile technique. Each biopsy was weighed then partially minced and placed in type IV collagenase (Sigma, St. Louis, MO) diluted 1 mg/mL in Dulbecco’s Modified Eagle Media 1:1 with Ham’s F-12 supplement (Invitrogen, Carlsbad, CA) at 37° C for 1 hour. The tissue pieces (“explants”) were then placed on tissue culture plates coated with fibronectin (diluted 25 µg/mL fibronectin in sterile water; Becton Dickinson, Franklin Lakes, NJ), covered with media containing 20% fetal bovine serum, gentamicin 50 µg/mL, L-glutamine 2 mmol/L, and 2-mercaptoethanol 0.1mmol/L in Iscove’s modified Dulbecco medium (called 20% cardiac explant medium or “20% CEM”) and maintained at 37° C. After 8-10 days, outgrowth from the explants formed a confluent layer of cells; these cells were then gently trypsinized and collected. Following pelleting and resuspension, the pooled cells were counted and plated in 24 well plates coated with poly-d-lysine (Sigma) diluted 20µg/mL in Dulbecco’s Modified Eagle Media 1:1 with Ham’s F-12 supplement (Invitrogen) at 50,000 cells/well in 0.5 mL of serum containing 10% FBS, gentamicin 50 µg/mL, L-glutamine 2 mmol/L, and 0.1mmol/L 2-mercaptoethanol in Iscove’s modified Dulbecco medium (called 10% cardiac explant medium or “10% CEM”). After 2-3 days a portion of the cells formed free-floating spherical clusters called “cardiospheres” (CSps).
Any CSps were collected and placed in fibronectin coated tissue culture flasks (as above) in fresh 10% CEM. After 3-5 days the CSps attached to the culture surface and began to multiply, forming a confluent monolayer, at which point they were trypsinized, counted and split into new fibronectin coated plates in fresh 10% CEM. Following the 2nd or 3rd passage, these “cadiosphere derived cells” (CDCs) were trypsinized, suspended, and counted, after which they were frozen in in Pentaspan (B. Braun Medical, Bethlehem, PA) with containing 5% DMSO with 2% albumin. CDCs were kept frozen in liquid nitrogen until the day of infusion.

**Intracoronary CDC Delivery**

For intracoronary delivery pigs were anesthesized as described above. An 8F arterial sheath was placed in the right femoral artery using ultrasound guidance, after which animals were anticoagulated with heparin. A 7F coronary catheter was used to engage the left main coronary and a 3.0 x 8 mm over-the-wire coronary balloon (Boston Scientific, Natick, MA) was advanced to the mid-LAD as described above. Autologous CDCs or placebo (carrier solution alone) were infused through the balloon lumen during intermittent inflation: 1/3 of the solution was infused during each of three 3-minute long inflations. Between each inflation the balloon was deflated for 3 minutes to allow for reperfusion. Following infusion, the balloon, catheter and femoral sheath were removed and hemostasis was achieved via manual compression.

Infusate was prepared immediately prior to delivery. CDCs were thawed and diluted in sterile PBS without calcium (PBS-) then pelleted and resuspended in fresh sterile PBS- for counting. The desired number of CDCs was taken, pelleted and resuspended in infusate containing 100 U/mL of heparin and 250 \( \mu \)g/mL of nitroglycerin in PBS-. Placebo animals received infusate alone.

The timing of infarct creation, biopsy, CDC infusion and subsequent animal follow-up are described in detail and diagrammed in supplemental figures 2 to 4.

**Supplemental Results:**

**Pilot study of CDC infusion**

In a pilot study of CDC infusion we delivered \( 5 \times 10^7 \) CDC resuspended with PBS containing \( \text{Ca}^{2+} \) through a balloon catheter placed in the mid-LAD. After CDC infusion, the surface ECG showed persistent ST elevation in leads V1 to V3. Coronary angiography performed 24 hours after infusion showed disruption of contrast media in the mid-LAD, where the cells had been infused (supplemental figure 1a). The animal was sacrificed immediately after angiography. The excised heart showed a large infarction accompanied with hemorrhage (supplemental figures 1b and c). Another attempt was made using coronary venous infusion of CDCs through a coronary venous balloon catheter, but likewise resulted in massive infarction (supplemental figure 1d). This revealed that unlike prior work with BMMCs simple infusion of CDCs via the coronary artery or vein was complicated by massive infarction. These early mishaps motivated a systematic optimization of CDC delivery via the intracoronary route, focusing on avoidance of microembolization and intravascular thrombosis.
Supplemental Figure Legends

Supplemental Figure 1. Examples of infarction following intracoronary and retrograde venous CDC infusion without optimization. A. Angiography 24 hours after intracoronary CDC infusion showing contrast dye disruption (arrow) and occlusion of mid-LAD. B. Superficial myocardial hemorrhage (arrows) 24 hours after intracoronary CDC infusion. C. Intramuscular hemorrhage (arrows) 24 hours after intracoronary infusion. D. Large area of myocardial infarction (arrows) following retrograde venous CDC infusion.

Supplemental Figure 2. Schematic of protocol examining short term CDC engraftment in the infarcted heart. Myocardial infarction and biopsy occurred at time zero. Four weeks later autologous, luciferase+ CDCs were delivered via intracoronary infusion. Animals were sacrificed 24 hours later and tissues were taken for luciferase measurement to determine engraftment. Serum troponin I was measured at the time of infusion and 24 hours later to determine whether myocardial infarction had occurred.

Supplemental Figure 3. Schematic of protocol examining long term CDC engraftment in the infarcted heart. Myocardial infarction and biopsy occurred at time zero. Four weeks later autologous LacZ+ CDCs or control (carrier solution alone) were delivered via intracoronary infusion. Eight weeks later animals were sacrificed and tissues taken for histologic analysis.

Supplemental Figure 4. Schematic of protocol comparing the effects of intraocoronary CDC infusion to placebo on post-infarct remodeling. Myocardial infarction and biopsy occurred at time zero. Four weeks later animals had cardiac MRI performed to evaluate LV systolic function, chamber and infarct size (by gadolinium enhancement). Within 3 days animals received either autologous CDC infusion (300,000 CDCs/kg) or placebo (carrier solution alone). Hemodynamic measurements were performed at the time of infusion. Eight weeks later animals had repeat cardiac MRI then underwent cardiac catheterization with repeat hemodynamics and provocative electrophysiologic study. A complete autopsy was then performed with gross and histologic examination of vital organs.
Supplemental Figure 2.

MI, Biopsy | CDC Infusion | Sacrifice
---|---|---
4 weeks | 24 hours | Tissue for Luciferase Assay
Troponin I | Troponin I

Supplemental Figure 3.

MI, Biopsy | CDC Infusion | Sacrifice (Histology)
---|---|---
4 weeks | 8 weeks | Catheterization
Supplemental Figure 4.

<table>
<thead>
<tr>
<th>MI, Biopsy</th>
<th>CDC Infusion</th>
<th>Sacrifice (Autopsy)</th>
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<tbody>
<tr>
<td></td>
<td>4 weeks</td>
<td>8 weeks</td>
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- **Imaging:**
  - MRI

- **Invasive Studies:**
  - Catheterization
  - Catheterization Hemodynamics
  - Catheterization Hemodynamics Electrophysiologic Study
Supplemental Table 1. Standard clinical laboratory values (mean +/- standard deviation) for CDC and Placebo groups at each protocol timepoint.

<table>
<thead>
<tr>
<th>Lab Value</th>
<th>Group</th>
<th>Baseline</th>
<th>Pre-Infusion</th>
<th>Sacrifice</th>
<th>p-value</th>
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<tr>
<td>Creatinine</td>
<td>CDCs</td>
<td>0.783±0.075</td>
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<tr>
<td>AST*</td>
<td>CDCs</td>
<td>36.17±6.62</td>
<td>46.83±13.06</td>
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<td>Placebo</td>
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<td>ALT†</td>
<td>CDCs</td>
<td>40.17±6.77</td>
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<td>White Blood Cells</td>
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<td>p-value</td>
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<td>Hemoglobin</td>
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<td>Platelets</td>
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* Aspartate aminotransferase; † Alanine aminotransferase