Targeted Cell Delivery Into Infarcted Rat Hearts by Retrograde Intracoronary Infusion: Distribution, Dynamics, and Influence on Cardiac Function

Ken Suzuki, MD, PhD; Bari Murtuza, MD, PhD; Satsuki Fukushima, MD; Ryszard T. Smolenski, MD, PhD; Anabel Varela-Carver, PhD; Steven R. Coppen, PhD; Magdi H. Yacoub, FRS

Background—Intracoronary infusion for cell transplantation has potential advantages in disseminating cells globally into the myocardium with less injury over direct intramuscular injection. Arterial route, however, has a risk of coronary embolism and a limitation in cell delivery into ischemic or infarcted areas. We assessed the efficiency of retrograde intracoronary cell implantation into infarcted hearts using a novel rat model.

Methods and Results—After left coronary artery ligation in rat, a catheter was inserted into the left cardiac vein, which drains the left ventricular free wall. Through this, $1\times10^6$ skeletal muscle precursor cells expressing nuclear β-galactosidase were infused retrogradely into the vein. In situ staining demonstrated that β-galactosidase–expressing donor cells had disseminated throughout the left ventricular free wall, including both infarcted and surrounding border areas, at 10 minutes after infusion. At 28 days, in contrast, positively stained multinuclear myotubes were found in border zones, whereas no positive cells were seen in infarcted areas. Measurement of β-galactosidase enzyme activity estimated that 29.8±6.9% of total infused cells were retained within the myocardium at 10 minutes and that this number decreased to 23.7±8.1% at 3 days but rapidly increased thereafter, reaching a plateau at 90.2±17.1% by 14 days. Echocardiography and Langendorff perfusion demonstrated that cell implantation improved cardiac function and dimensions by 28 days, compared with both sham-treated and phosphate-buffered saline-infused infarcted hearts, and this was associated with decreased collagen deposition.

Conclusion—Retrograde intracoronary cell transplantation could provide an effective cell delivery into infarcted hearts and could be a useful strategy for treating myocardial infarction. (Circulation. 2004;110[suppl II]:II-225–II-230.)

Key Words: cell transplantation ■ coronary vein ■ intracoronary infusion ■ myocardial infarction ■ skeletal myoblast

Experimental research has shown that cell transplantation is promising for treating heart failure, and this has encouraged clinicians to inject autologous skeletal muscle precursor cells (MPCs) or bone marrow stem cells into patients.1,2 The most popular method for cell transplantation into the heart is direct intramuscular injection, which enables selective cell delivery into either normal or infarcted myocardium.1,3 However, it has been shown that this injection method causes mechanical injury and subsequent acute inflammation, resulting in poor survival of grafted cells and myocardial damage.4–6 Furthermore, MPCs infused by this method usually produce localized islet-like formations, which are reportedly isolated from the native myocardium.7

Cell delivery via the intracoronary route has theoretical advantages over direct intramuscular injection in global cell dissemination within the myocardium.8,9 This method is also likely to result in less myocardial and graft injury. We have shown that MPCs grafted by antegrade intracoronary infusion disseminate throughout cardiac layers, proliferate, differentiate, and integrate into host myocardium in rat.8,9 We have further reported that antegrade intracoronary MPC transplantation results in improvement of cardiac function of doxorubicin-induced failing hearts in rat.10 This route has recently been applied for infusion of bone marrow stem cells into infarcted human hearts.2 However, antegrade intracoronary infusion is considered to have a limitation in delivering cells to ischemic or infarcted areas in addition to a risk of coronary embolism.8–10

Retrograde intracoronary delivery has been reported to be useful in administrating cardioplegic solution, peptide, and vectors for gene therapy into the myocardium using large animal models.11–14 Emigration of inflammatory cells into the myocardial interstitium is known to take place at postcapillary venules rather than at arterioles or capillaries.15 One
could therefore hypothesize that retrograde intracoronary infusion might offer a safer and more efficient cell dissemination within the myocardium even in ischemic or infarcted areas where antegrade arterial delivery may have limited efficacy. We have recently developed a retrograde intracoronary infusion model in rat, by which MPCs were disseminated selectively within the left ventricular (LV) free wall of intact hearts with little myocardial damage.16 In the present study, by applying this method to the rat heart with acute myocardial infarction (MI), we have investigated the distribution, dynamics, and influence on cardiac function of MPCs that have been implanted into MI hearts by retrograde intracoronary infusion.

Methods
Retrograde Intracoronary Infusion of Dye Into MI Hearts
All studies were performed with the approval of the institutional ethics committee and the Home Office, UK. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication 85-23, 1996). Male Lewis rats (250 to 300 grams, Charles River Laboratories, UK) underwent left thoracotomy under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal) and mechanical ventilation. After left coronary artery (LCA) ligation,17,18 a 24-gauge catheter (Vialon, UK) was inserted into the left cardiac vein, which drains the LV free wall, via left superior vena cava with a purse-string suture to control bleeding from the insertion site. Through this catheter, dye, PBS, or cell suspension was infused with the snare tied. LA indicates left atrium. After LCA ligation,17,18 one million donor MPCs resuspended in 1 mL phosphate-buffered saline (PBS) were retrogradely infused into the left cardiac vein with 5-minute temporal occlusion using the method described (cell group). The PBS group received PBS infusion after LCA ligation, whereas the sham group underwent LCA ligation only. The catheter was then removed and the purse suture was tied. Surgical wounds were repaired and the rats were extubated and returned to cages to recover. FK506 (2.5 mg/kg, subcutaneous, donated by Fujisawa GmbH, Germany) was injected daily for immunosuppression as described previously.16

Assessment of Cardiac Function
At 28 days after treatment, rats were again anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal) for echocardiography (n = 8 in each group) using a Sequoia 512 system and 15-MHz probe (Acuson). All images of 2-dimensional and M-mode were taken at the mid papillary level and analyzed by 2 experienced researchers (K.S. and B.M.) in a totally blinded, duplicated manner. Ejection fraction was calculated as described.20 After this, the hearts were quickly excised after systemic heparinization and perfused using a Langendorff apparatus.5,10,13 Parameters were measured using a thin-walled balloon with LV ventricular end-diastolic pressure stabilized at 10 mm Hg. Data were recorded and calculated using a computer-associated recorder (Linton).

β-gal Enzyme Activity Measurement
To evaluate the number of MPCs existing in the myocardium, hearts were excised at selected times (n = 4 at each time point of 10 minutes, day 3, 7, 14, or 28 after MPC implantation) and β-gal activity of the samples was measured using o-nitrophenyl-β-D-galactopyranoside (Sigma, UK).10,15 A standard curve of β-gal activity plotted against known numbers (1 × 10^6, 5 × 10^5, 1 × 10^5, 5 × 10^4, 1 × 10^4) of donor MPCs (mixed with intact hearts) was generated as described before.16

Histological Study
To investigate the distribution and differentiation of MPCs implanted, hearts were collected at 10 minutes (n = 4 in the cell group) and at day 28 (n = 4 in each group) after implantation. These were cut and frozen in OCT compound. The embedded samples were cut into 10-μm cryosections and stained for β-gal as described previously.9,16 This was followed by counter-staining with 1% neutral red. For investigating collagen deposition as a result of ventricular remodeling after MI, 10-μm cryosections were fixed in 10% neutral-buffered formal saline and stained with 0.1% picrosirius red F3B.21 Fibrosis in the border zone surrounding infarcts was semiquantitatively

Figure 1. Method of retrograde intracoronary infusion into LCA-ligated hearts. Intra-operative picture (A) and the schematic drawing (B) of retrograde intracoronary infusion method into LCA-ligated hearts in rats. After LCA ligation (ligation site shown with a red cross), a catheter was inserted into the left cardiac vein via left superior vena cava (LSVC) with a purse-string suture. A temporal snare suture was placed at the distal left cardiac vein to prevent flush-back of injected solution. Through this catheter, dye, PBS, or cell suspension was infused with the snare tied. LA indicates left atrium.
assessed by morphometric determination of collagen volume fraction using National Institutes of Health image analysis software.

**Statistical Analysis**
All values are expressed as means±SEM. Statistical comparison of the data was performed using 1-way ANOVA followed by Bonferroni test for individual significant difference. A value of $P<0.05$ was considered statistically significant.

**Results**

**Operative Mortality**
LCA ligation, followed by retrograde intracoronary infusion of MPCs or PBS, was well-tolerated, with no persistent arrhythmias, hemorrhage, or cardiac swelling during surgery. During the period up to 28 days, 3 of 11 (27%), 4 of 12 (33%), and 11 of 39 (28%) rats were lost in the sham, PBS, and cell groups, respectively. The majority of these losses occurred within 24 hours of the procedure, presumably caused by acute heart failure.

**Perfusion Area by Retrograde Intracoronary Infusion in LCA-Ligated Hearts**
Evans blue dye was intravenously injected into rats, in which the LCA had been ligated. The dye stained right ventricular free wall (RVFW) and interventricular septum (IVS) in blue, whereas a wide area of LV free wall (LVFW) was not stained (shown in red) and thus not perfused. Retrograde intracoronary infusion of Evans blue dye into LCA-ligated hearts. The dye positively stained (blue color) a wider area of LVFW. RVFW or IVS was not stained (shown in red). Comparison between (A) and (B) indicates that the perfusion area by retrograde intracoronary infusion encompasses the infarct/ischemic and the border zones induced by LCA ligation. Arrowheads show the LCA ligation site.

**Histological Study**
In situ β-gal staining showed that positively stained MPCs were widely disseminated throughout LV free wall, both in infarcted or surrounding border zones, at 10 minutes after implantation (Figure 4A through 4C). By day 28, surviving MPCs had differentiated into multinuclear myotubes that had aligned with the cardiac fiber axis in the border zone (Figure 4E and 4F), whereas no positive cells were found in infarcted areas (Figure 4D). Picrosirius red staining demonstrated that collagen had markedly accumulated within extracellular spaces in the sham and PBS groups, whereas this was clearly reduced in the cell group (Figure 5A through 5C). Collagen

**Figure 2.** Perfusion area by retrograde intracoronary infusion in LCA-ligated hearts. A, Intravenous injection of Evans blue dye into rats, in which the LCA had been ligated. The dye stained right ventricular free wall (RVFW) and interventricular septum (IVS) in blue, whereas a wide area of LV free wall (LVFW) was not stained (shown in red) and thus not perfused. B, Retrograde intracoronary infusion of Evans blue dye into LCA-ligated hearts. The dye positively stained (blue color) a wider area of LVFW. RVFW or IVS was not stained (shown in red). Comparison between (A) and (B) indicates that the perfusion area by retrograde intracoronary infusion encompasses the infarct/ischemic and the border zones induced by LCA ligation. Arrowheads show the LCA ligation site.

**Figure 3.** Dynamics of MPCs implanted by retrograde intracoronary infusion. After implantation of β-gal–expressing MPCs into LCA-ligated hearts by retrograde intracoronary infusion, β-gal activity was measured to evaluate the number of MPCs existing in the heart. By using the standard curve of β-gal activity plotted against known numbers of MPCs (mixed with intact hearts), it was estimated that ~30% of total grafted MPCs existed within the heart 10 minutes after implantation. The number decreased at day 3 but rapidly increased thereafter, and reached a plateau by day 14. Data are shown as percent of total grafted number (one million); n=4 at each point.

**Dynamics of MPCs Grafted by Retrograde Intracoronary Infusion Into LCA-Ligated Hearts**
By using a standard curve of β-gal activity plotted against known numbers of β-gal–expressing MPCs (mixed with intact hearts), the number of MPCs existing within the myocardium after retrograde intracoronary infusion was estimated (Figure 3). The result indicated that 29.8±6.9% of total grafted MPCs were initially retained within the myocardium and that the number decreased to 23.7±8.1% by day 3, rapidly increased to 81.2±15.2% by day 7, and reached a plateau of ~90% by day 14 (88.8±14.9%) and day 28 (90.2±17.1%).
volume fraction in the border zones surrounding infarcts was significantly smaller in the cell group compared with the other 2 groups (Figure 5D). No tumor-like formation was observed in any samples.

**Improved Cardiac Function by Retrograde Intracoronary MPC Implantation**

Cardiac function and dimensions of LCA-ligated hearts were measured by echocardiography and Langendorff perfusion system at 28 days after retrograde intracoronary MPC delivery. Echocardiography showed that LV ejection fraction was significantly improved, with reduced LV end-diastolic dimension, in the cell group, as compared with both the sham and PBS groups (Table). These data corresponded to the results obtained by Langendorff perfusion: LV developed pressure, and maximum and minimum dp/dt were significantly improved in the cell group compared with the PBS and sham groups. Coronary flow was also significantly higher in the cell group than in either the PBS or the sham group.

**Discussion**

Using a novel experimental model in rat, we have demonstrated that infusion of MPCs via the retrograde intracoronary route can provide selective cell dissemination within the heart with acute MI induced by LCA ligation. The perfusion area of this infusion method was the LV free wall, which overlaps the injured area by LCA ligation (Figure 2). Thus, this method delivered MPCs into injured (both infarcted and surrounding border) areas as shown in Figure 4. In contrast, at 28 days after implantation, MPCs were observed only in the border area surrounding infarcts, suggesting that MPCs delivered into the infarcts had died because of the severe pathological conditions or had migrated into the surrounding areas. Furthermore, these observations were associated with improved cardiac function and decreased collagen deposition. There are several possible mechanisms by which MPC transplantation affects failing hearts.22 Grafted MPCs may be able to contribute to cardiac contraction in an active manner and also support ventricular walls by a passive mechanical effect. In addition, a paracrine effect by secreted growth factors or cytokines is also believed to be an important mechanism.

**Figure 4.** Histological findings after retrograde intracoronary MPC delivery. In situ staining for β-gal with neutral red counterstaining showed that positive (dark blue) MPCs were widely disseminated throughout LV free wall of LCA-ligated hearts, including both infarcted (A) and surrounding border zones (B, C), at 10 minutes after implantation via retrograde intracoronary route. By day 28, surviving MPCs had differentiated into multinuclear myotubes (dark blue nuclei) that had aligned with the cardiac fiber axis in the border zone (E, F), whereas no positive cells were found in infarcted areas (D). Scale bar: 100 μm for A, B, D, E; 25 μm for C and F.

**Figure 5.** Reduced collagen deposition by retrograde intracoronary MPC delivery. At 28 days after LCA ligation and subsequent treatment, the hearts of the sham (A) and PBS (B) groups showed marked deposition of extracellular collagen (red color) as shown by picrosirius red staining. Cardiomyocytes were stained yellow. In contrast, collagen deposition was attenuated in the cell group (C). Scale bar: 100 μm. Collagen volume fraction, semi-quantitatively measured by computer-associated morphometry, was significantly lower in the cell group (D). *P<0.05 versus the PBS group; †P<0.05 versus the sham group; n=4 in each group.
These effects are assumed to directly or indirectly modulate extracellular collagen deposition.

The number of initially retained MPCs within the LCA-ligated heart at 10 minutes after retrograde intracoronary infusion was estimated to be $29.8\pm6.9\%$ of total grafted MPCs. This initial entrapment efficiency was similar to that of grafted cells into intact hearts by the same procedure of retrograde intracoronary infusion ($31.4\pm4.8\%$ as shown in our previous report).$^{16}$ This suggests that the efficiency of initial MPC retention within the myocardium after retrograde intracoronary infusion is not affected by occlusion of the supplying coronary arteries. The remaining 70% of infused MPCs were considered to be lost by leakage via the venous supply or by flush-out into the coronary sinus after retrograde intracoronary infusion (31.4\%--4.8\% as shown in our previous report).$^{16}$ This suggests that the efficiency of initial MPC retention within the myocardium after retrograde intracoronary infusion is not affected by occlusion of the supplying coronary arteries. The remaining 70% of infused MPCs were considered to be lost by leakage via the venous supply or by flush-out into the coronary sinus after retroperfusion. In contrast, we have shown that 90\% of MPCs infused by antegrade intracoronary infusion were entrapped in the heart immediately after implantation.$^{9}$ However, this does not necessarily indicate that antegrade infusion achieves 3-fold higher efficiency of cell delivery into the myocardium than retrograde infusion. The entrapment rate after antegrade infusion includes not only MPCs that successfully emigrated into extravascular spaces (myocardial interstitium) but also cells that could not emigrate across the endothelial barrier and were retained in intravascular spaces.$^{8}$ However, after retrograde infusion, the majority of cells retained in the intravascular space were likely to be flushed out into the coronary sinus by reperfusion. It is presumed that cells remaining within intravascular spaces cannot survive long and that only cells that have successfully migrated into the extravascular space would survive and contribute to the therapeutic effect induced by cell transplantation. Further study needs to be performed to establish the “true” efficiency of cell delivery into the extravascular myocardial interstitial spaces after antegrade infusion. Although the mechanism of emigration of infused cells from the intravascular to myocardial interstitial spaces via the endothelial barrier is not fully understood, one could speculate that this migration may work more favorably for retrograde infusion, because emigration of inflammatory cells into the myocardial interstitium takes place at postcapillary venules, rather than at capillaries or arterioles.$^{15}$ Acute inflammation encourages adherence of leukocytes to myocardial endothelial cells lining the postcapillary venules and stimulates subsequent migration of these cells to the extravascular tissue by passing through the interendothelial junction and the basement membrane.$^{15}$ Higher infusion pressure, longer incubation time, or use of drugs to increase vascular permeability may be useful in achieving higher efficiency of cell delivery into the myocardial interstitium via the retrograde intracoronary route.$^{11–13}$

Changes in the number of surviving MPCs in MI hearts between 10 minutes and day 3 after retrograde intracoronary infusion were distinct from one in intact hearts. The number decreased from 29.8\% to 23.7\% in MI hearts, whereas it increased from 31.4\% to 33.0\% in intact hearts during this period.$^{16}$ This may be caused by the different behavior of grafted MPCs during this 72-hour period, including death and proliferation in the infarcted/ischemic versus healthy myocardium. In contrast, from day 3 onward, the pattern of change in the MPC number after retrograde infusion into MI hearts was similar to that of intact hearts.$^{16}$ The numbers quickly increased from 23.7\% to 81.2\% by day 7 and reached a plateau of $\approx90\%$ by day 14 in MI hearts, as compared with 33.0\%, 117.3\%, 132\% in intact hearts at day 3, 7, and 14, respectively.$^{16}$ This pattern suggests that surviving MPCs proliferate in the early period and, perhaps on reaching a particular cellular density, stop proliferation and begin to differentiate. It was postulated that MPCs that had survived the initial hazardous period in MI hearts, mostly within infarct border areas, could proliferate and differentiate in a similar way as those grafted into intact hearts.$^{16}$ Therefore, the lower MPC survival in MI hearts at day 3 might be directly responsible to the smaller final graft size at 28 days: $\approx90\%$ in MI hearts compared with 130\% in intact hearts. In this study, we have examined the grafted cells up to 28 days after implantation and speculate that most of the cells surviving for 28 days will continue to survive for a longer period as reported previously.$^{24}$ However, further investigations are needed to clarify the long-term survival, integration, and cardiomyogenic differentiation of the grafted cells.

In conclusion, we have shown that retrograde intracoronary cell transplantation can provide safe and effective targeted cell delivery into MI hearts and may thus be a useful strategy for treating MI. This rat model of retrograde intracoronary infusion after LCA ligation would be useful to investigate the efficiency of the retrograde intracoronary route in delivering not only other cell types including bone marrow stem cells but also vehicles for gene transfection or cardioprotective reagent into the heart with acute MI.

### Cardiac Function and Dimension After Retrograde Intracoronary Cell Delivery

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVEF (%)</th>
<th>LVEDD (mm)</th>
<th>LVDP (mm Hg)</th>
<th>Max dp/dt (mm Hg/s)</th>
<th>Min dp/dt (mm Hg/s)</th>
<th>CF (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>40.4±2.7*†</td>
<td>7.4±0.3*†</td>
<td>137.8±4.3*†</td>
<td>2767±104*†</td>
<td>−2119±76*†</td>
<td>22.5±1.1*†</td>
</tr>
<tr>
<td>PBS</td>
<td>29.7±2.9</td>
<td>9.0±0.4</td>
<td>117.0±5.0</td>
<td>2309±106</td>
<td>−1787±87</td>
<td>17.8±0.6</td>
</tr>
<tr>
<td>Sham</td>
<td>29.3±2.6</td>
<td>8.9±0.4</td>
<td>118.4±5.9</td>
<td>2256±113</td>
<td>−1810±80</td>
<td>18.2±1.1</td>
</tr>
</tbody>
</table>

At 28 days after LCA ligation followed by retrograde intracoronary infusion of MPCs (cell group), PBS (PBS group), or sham (sham group), LVEF (LV ejection fraction) and LVEDD (LV end-diastolic dimension) were measured by echocardiography. LVDP (LV developed pressure), dp/dt, and CF (coronary flow) were measured using Langendorff perfusion.

*P<0.05 vs. the PBS group.
†P<0.05 vs. the sham group.

n=8 in each group.
Acknowledgments
We thank the Magdi Yacoub Institute (former Harefield Research Foundation) for financial support. K.S. is a Senior Clinical Fellow and B.M. is a Research Training Fellow of the Medical Research Council, UK.

References
Targeted Cell Delivery Into Infarcted Rat Hearts by Retrograde Intracoronary Infusion: Distribution, Dynamics, and Influence on Cardiac Function
Ken Suzuki, Bari Murtuza, Satsuki Fukushima, Ryszard T. Smolenski, Anabel Varela-Carver, Steven R. Coppen and Magdi H. Yacoub

doi: 10.1161/01.CIR.0000138191.11580.e3

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/11_suppl_1/II-225