Intramyocardial Transplantation of Autologous Endothelial Progenitor Cells for Therapeutic Neovascularization of Myocardial Ischemia

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Background—We investigated whether catheter-based, intramyocardial transplantation of autologous endothelial progenitor cells can enhance neovascularization in myocardial ischemia.

Methods and Results—Myocardial ischemia was induced by placement of an ameroid constrictor around swine left circumflex artery. Four weeks after constrictor placement, CD31+/H11001 mononuclear cells (MNCs) were freshly isolated from the peripheral blood of each animal. After overnight incubation of CD31+/H11001 MNCs in noncoated plates, nonadhesive cells (NA/CD31+/H11001 MNCs) were harvested as the endothelial progenitor cell–enriched fraction. Nonadhesive CD31− cells (NA/CD31− MNCs) were also prepared. Autologous transplantation of 10⁷ NA/CD31+/H11001 MNCs, 10⁷ NA/CD31− MNCs, or PBS was performed with a NOGA mapping injection catheter to target ischemic myocardium. In a parallel study, 10⁵ human CD34+/H11001 MNCs, 10⁵ human CD34+/H11002 MNCs, or PBS was transplanted into ischemic myocardium of nude rats 10 minutes after ligation of the left anterior descending coronary artery. In the swine study, ischemic area by NOGA mapping, Rentrop grade angiographic collateral development, and echocardiographic left ventricular ejection fraction improved significantly 4 weeks after transplantation of NA/CD31+/H11001 MNCs but not after injection of NA/CD31− MNCs or PBS. Capillary density in ischemic myocardium 4 weeks after transplantation was significantly greater in the NA/CD31+/H11001 MNC group than the control groups. In the rat study, echocardiographic left ventricular systolic function and capillary density were significantly better preserved in the CD34+/H11001 MNC group than in the control groups 4 weeks after myocardial ischemia.

Conclusions—These favorable outcomes encourage future clinical trials of catheter-based, intramyocardial transplantation of autologous CD34+/H11001 MNCs in the setting of chronic myocardial ischemia. (Circulation. 2003;107:461-468.)

Key Words: transplantation ■ cells ■ catheters ■ ischemia ■ vasculogenesis

Endothelial progenitor cells (EPCs) were first isolated as CD34+ mononuclear cells (MNCs) from adult peripheral blood.1,2 Tissue ischemia mobilizes EPCs from bone marrow to peripheral blood, and mobilized EPCs home specifically to sites of nascent neovascularization and differentiate into mature endothelial cells (ECs).3 The demonstrated role of EPCs in the physiological response to ischemia has led to the development of strategies of cell therapy for neovascularization in ischemic diseases. Intravenous transplantation of cultured human EPCs enhances neovascularization and improves limb salvage in nude mice with hindlimb ischemia.4 A similar strategy applied in a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs incorporated into rat myocardial neovascularization, differentiated into mature ECs in ischemic myocardium, enhanced neovascularization, preserved left ventricular (LV) function, and inhibited myocardial fibrosis.5 Recently, Kocher et al6 attempted intravenous infusion of freshly isolated (not cultured) human CD34+ MNCs (EPC-enriched fraction) into nude rats with myocardial ischemia. This strategy resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings in immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

Although these previous reports indicate a potential therapeutic role for EPCs in ischemic diseases, 2 major obstacles exist that must be overcome before considering actual clinical
applications: dosage and immunologic rejection. In the previous study by our laboratory,\textsuperscript{3} $1 \times 10^5$ cultured EPCs were used for each $\approx 200$-g rat. Kocher et al\textsuperscript{4} transplanted $1 \times 10^4$ freshly isolated EPCs/100-g rat. On a weight-adjusted basis, this would translate into $3 \times 10^4$ to $6 \times 10^4$ cells for an average-size human, requiring 8.5 to 120 L of peripheral blood. Although it may be possible to obtain enough EPCs from bone marrow in the clinical situation, it is far from a realistic strategy to isolate EPCs from peripheral blood by the previous methods. Moreover, these previous studies used an immunodeficient rat model to circumvent issues of cell rejection.

Accordingly, we designed a series of in vivo investigations to address the limitations of these previous approaches. First, we tested the hypothesis that local transplantation of EPCs, rather than systemic infusion, would permit a significant reduction in the number of EPCs required. Second, we developed a strategy that relies on freshly isolated, autologous EPCs that would allow us to evaluate the therapeutic potential of autologous EPC transplantation. We therefore performed catheter-based transplantation of a freshly isolated, autologous EPC-enriched fraction in a swine chronic myocardial ischemia model. To verify the therapeutic usefulness of the freshly isolated, human EPC-enriched fraction, we also performed intramyocardial transplantation in immunodeficient rats with myocardial ischemia using freshly isolated human CD34+ MNCs.

**Methods**

**Animal Models of Myocardial Ischemia**

Acute myocardial ischemia was induced by ligating the left anterior descending coronary artery (LAD) of male athymic nude rats (Hsd: RH-rnu rats, Harlan Sprague Dawley, Indianapolis, Ind) 6 to 8 weeks old.\textsuperscript{5} Male Yorkshire swine (Pine Acre Rabbitry Farm, Norton, Mass) were 20 to 25 kg were used as in vivo models of myocardial ischemia. After left thoracotomy, an amniotic constrictor (Resonant Instruments SW) was placed around the proximal portion of the left circumflex (LCx) coronary artery.\textsuperscript{7}

**Isolation and Autologous, Percutaneous, Intramyocardial Transplantation of Swine EPCs**

Four weeks after constrictor placement, 150 mL of peripheral blood was obtained from the ear vein of each pig. Total peripheral blood MNCs were isolated by density-gradient centrifugation. The MACS bead selection method for CD31 (Miltenyi Biotech) was used to isolate the EPC-enriched fraction from total MNCs (anti-swine CD34 antibody is not available). CD31+ MNCs resuspended in EC basal medium-2 (EBM-2, Clonetics) were cultured overnight in noncoated plastic plates at a density of $5 \times 10^4$ cells/10-cm plate. To remove macrophages, only nonadhesive CD31+ (NA/CD31+) MNCs were collected as the EPC-enriched fraction. CD31− MNCs were treated similarly, and nonadhesive CD31− MNCs (NA/CD31− MNCs) were obtained as a negative control.

To elucidate in vivo differentiation to endothelial lineage, $10^7$ NA/CD31+ or the same number of NA/CD31− autologous MNCs were labeled with fluorescent carbocyanine 1,1'-dioctadecyl-3,3',3'-tetramethylindocarboxyamine perchlorate (DiI) dye (Molecular Probes) and were injected via a 27-gauge needle to the LV lateral wall 4 weeks after constrictor placement. Four weeks after cell transplantation, 5 mg of Bandeiraea simplicifolia lectin I (BS-1 lectin) (Vector Laboratories), which is a murine- and porcine-specific (not human) EC marker, was infused into the left coronary artery, and the pigs were killed by an overdose of pentobarbital.

Fluorescence microscopy was performed to examine incorporation of transplanted cells into foci of myocardial neovascularization. After these preliminary studies, we examined the therapeutic potential of autologous, percutaneous, intramyocardial transplantation of an EPC-enriched MNC fraction in the swine chronic myocardial ischemia model. Four weeks after constrictor placement, NOGA nonfluoroscopic LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia. The NOGA system (Biosense-Webster) of catheter-based mapping and navigation has been described in detail previously.\textsuperscript{8,9} Ischemic myocardium was defined as a zone with unipolar voltage greater than the automatically determined cutoff, signified by red color on the unipolar voltage map and linear local shortening $<3\%$ on the linear local shortening map. This definition was consistent in all examinations throughout this study. Immediately after the ischemic territory was identified by NOGA mapping, $10^7$ NA/CD31+ MNCs in 1 mL of PBS (n=7), $10^6$ NA/CD31−MNCs in 1 mL of PBS (n=8), or 1 mL of PBS without cells (n=9) were injected into 5 sites within the ischemic myocardium (200 μL to each site) with the NOGA injection catheter (Biosense-Webster).

**Fresh Isolation and Intramyocardial Transplantation of Human EPCs**

Human total peripheral blood MNCs were isolated from healthy volunteers by density-gradient centrifugation, and CD34+ MNCs were isolated from total MNCs by the MACS bead selection method (Miltenyi Biotech) as the EPC-enriched fraction.\textsuperscript{1} After the isolation, CD34− MNCs were also collected. CD34+ MNCs and CD34− MNCs were labeled with DiI. Ten minutes after the LAD of nude rats (n=2) had been ligated, $10^7$ DiI-labeled CD34+ MNCs in 100 μL of PBS or $10^5$ DiI-labeled CD34− MNCs in 100 μL of PBS were injected into 2 sites in the ischemic LAD territory with a 27-gauge needle (50 μL to each site). The ischemic zone was macroscopically identified by the pale color of the anterior and lateral walls after LAD ligation. This subgroup of rats was killed 10 days after myocardial ischemia. Thirty minutes before euthanization by overdose of pentobarbital, 500 μg of BS-1 lectin was administered intravenously. The hearts were fixed with 4% paraformaldehyde. The fixed tissues were embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen for fluorescence microscopy. After this preliminary study to evaluate the incorporation of the cells into myocardial neovascularization, the therapeutic potential of CD34+ MNCs in myocardial ischemia was examined. Ten minutes after the LAD had been ligated, $10^7$ human CD34+ MNCs in 100 μL of PBS (n=6), $10^6$ human CD34− MNCs in 100 μL of PBS (n=6), or 100 μL of PBS (n=7) were injected into the myocardium as described above.

**Physiological Assessment of LV Function and Ischemia**

In the rat study, transthoracic echocardiography (SONOS 5500, Agilent Technologies) was performed to evaluate LV function 2 days before (baseline) and 4 weeks after myocardial ischemia. LV dimensions in end diastole (LVDd) and end systole (LVDs), fractional shortening (FS), and LV regional wall motion score\textsuperscript{10} were examined.

In the swine study, transthoracic echocardiography (SONOS 5500), selective coronary angiography, and NOGA LV electromechanical mapping were performed 4 weeks after constrictor placement (just before injection of cells or PBS) and 4 weeks after the injections. LV ejection fraction was quantified by a computerized analysis system using a proprietary software package in the echo unit\textsuperscript{11,12} in the LV short-axis view at the mid–papillary muscle level. Collateral flow to the LCx territory was graded angiographically in a blinded manner by use of the Rentrop scoring system.\textsuperscript{13,14} The area of ischemia was quantified by NOGA mapping as previously described.\textsuperscript{15} All data were evaluated by blinded observers (echocardiography by Y.-S.Y., coronary angiography by J.-I.Y., and postprocessing analysis of the NOGA mapping by C.M.).
Histological Assessment of Animals Receiving Transplants
Both the rats and swine were killed 4 weeks after treatment. At necropsy, rat hearts were sliced in a bread-loaf manner into 8 transverse sections from apex to base and fixed with 100% methanol. To elucidate the severity of myocardial fibrosis, elastic tissue–trichrome staining was performed on paraffin-embedded sections from each tissue block, and the percentage area of fibrosis was calculated. Immunohistochemical staining with antibody prepared against the EC marker isolectin B4 (Vector Laboratories) was performed, and capillary density was evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4. Immunohistochemical staining for the human-specific EC marker *Ulex europaeus* lectin type 1 (UEA-1 lectin) (Vector Laboratories) was also performed to identify transplanted human MNCs that had differentiated into mature ECs in the ischemic myocardium.

At necropsy, swine hearts were also sliced in a bread-loaf manner into 4 transverse sections from apex to base, and each section was separated into anterior, lateral, and posterior LV free wall; interventricular septum; and right ventricular free wall. All tissues obtained from each portion were fixed with 100% methanol. Immunohistochemistry for isolectin B4 was also performed to evaluate capillary density in the ischemic myocardium identified by NOGA mapping.

All morphometric studies were performed by 2 examiners (H.M. and A.H.) who were blinded to treatment.

Statistical Analysis
All values were expressed as mean±SEM. Student’s paired t test was performed for comparison of data before and after treatment. ANOVA was performed to compare data among 3 groups. A probability value of *P*<0.05 was considered to denote statistical significance.

Results
Transplanted Autologous Swine EPCs Attenuate Chronic Myocardial Ischemia
Ischemic area determined by NOGA mapping before transplantation was not significantly different between the NA/CD31+, NA/CD31−, and PBS groups. A decrease in the size of the ischemic area was observed only after NA/CD31+ transplantation (before, 27.3±8.5%; after, 12.3±6.3%; *P*=0.0034), whereas the zone of ischemia increased in size after NA/CD31− or PBS injection. Similarly, the change in percentage ischemic area after transplantation was significantly improved only in the CD31+ group (*P*=0.0017 versus NA/CD31+ group and *P*=0.038 versus PBS group) (Figure 1).

Transplanted Autologous Swine EPCs Enhance Neovascularization
Selective left coronary angiography was performed to evaluate collateral development before and after transplantation.
in the swine study. The mean value of the Rentrop score of collateral development to the LCx territory at baseline was 0.6±0.4 in the NA/CD31+ group, 1.1±0.4 in the NA/CD31− group, and 1.1±0.3 in the PBS group (P=NS). Rentrop scoring was improved significantly only after NA/CD31+ transplantation (0.6±0.4 versus 2.0±0.4, P=0.02) and not after NA/CD31− or PBS injection. Similarly, the change in the Rentrop score was significantly greater in the NA/CD31+ group than in either the NA/CD31− or PBS groups (P=0.002 versus NA/CD31− MNCs and P=0.006 versus PBS) (Figure 2).

Histochemical staining of isolectin B4 was performed to identify capillaries in ischemic myocardium 4 weeks after cell transplantation. Capillary density was significantly greater in the NA/CD31+ group than in the NA/CD31− and PBS groups (P=0.003 versus NA/CD31− MNCs and P=0.004 versus PBS). Capillary density in the NA/CD31− group was similar to that in the PBS group (Figure 3, a and b).

Transplanted Autologous Swine EPCs Improve LV Function
LV ejection fraction measured by echocardiography in the NA/CD31+ group was similar to that in the NA/CD31− and PBS groups 4 weeks after constrictor placement (Figure 3c). However, LV ejection fraction improved significantly only after NA/CD31+ transplantation (P=0.0037) and not after NA/CD31− or PBS injection. LV ejection fraction 4 weeks after transplantation was significantly greater in the NA/CD31+ group than in the NA/CD31− and PBS groups (P=0.0018 versus NA/CD31− and P=0.0017 versus PBS) (Figure 3c).

Swine EPCs Differentiate Into Endothelial Lineage After Catheter-Based Injection in Vivo
To examine in vivo differentiation of swine autologous EPCs after transplantation into ischemic myocardium, Dil-labeled NA/CD31+ or NA/CD31− MNCs were injected into the lateral LV wall 4 weeks after constrictor placement. Four weeks after transplantation, the majority of NA/CD31+ MNCs were positive for BS-1 lectin in the ischemic myocardium. In contrast, transplanted NA/CD31− MNCs positive for BS-1 lectin were rarely observed in the ischemic myocardium (Figure 3d).

Transplanted Human EPCs Enhance Neovascularization and Inhibit Myocardial Fibrosis
In the rat study, capillary density was significantly greater in the CD34+ group than in the CD34− and PBS groups (P=0.003 versus CD34− MNCs and P=0.003 versus PBS). Capillary density in the CD34− group was not significantly different from that in the PBS group (Figure 4, a and b). Elastic tissue–trichrome staining was performed to identify LV fibrosis after myocardial ischemia. The fibrotic area was significantly smaller in the CD34+ group than in either the CD34− or PBS group (P=0.001 versus CD34− and P=0.01 versus PBS) (Figure 5, a through d).
Transplanted Human EPCs Preserve LV Function

In the rat study, baseline LVDd, LVDs, FS, and regional wall motion score were similar between rats receiving human CD34+ MNCs, rats receiving CD34- MNCs, and rats receiving PBS. In all groups, all echocardiographic parameters worsened significantly 4 weeks after induction of myocardial ischemia ($P<0.01$ in all groups). Echocardiography performed 4 weeks after treatment revealed that LVDd was similar among the 3 treatment groups (Figure 5e). However, LVDs 4 weeks after ischemia was significantly smaller.

Figure 3. a, Representative immunohistochemical findings for isolectin B4 in swine ischemic myocardium 4 weeks after cell transplantation. b, Capillary density in swine ischemic myocardium 4 weeks after transplantation. **$P<0.01$; ***$P<0.001$. c, Echocardiographic LV ejection fraction (EF) before and 4 weeks after intramyocardial cell transplantation in swine with chronic myocardial ischemia. Circle, pigs receiving NA/CD31+ MNCs; square, pigs receiving NA/CD31- MNCs; triangle, pigs receiving PBS. d, Representative fluorescence microscopic findings of swine ischemic myocardium 28 days after cell transplantation. Red (DiI) fluorescence marks all autologously transplanted cells; green fluorescence indicates BS-1 lectin binding, identifying ECs. Therefore, yellow fluorescence marks double-positive cells, ie, cells harvested from systemic circulation, that were autologously transplanted into myocardium and now express a marker of endothelial phenotype. A majority of transplanted NA/CD31+ MNCs differentiated into EC lineage in vivo, indicated by high percentage of double-positive (yellow) cells (arrows) in 2 left panels. In contrast, most of transplanted NA/CD31- MNCs are positive only for ex vivo Dil label and negative for endothelial phenotype (arrowheads). Double-positive cells were rarely observed in CD31- transplanted animals.

Figure 4. a, Representative immunohistochemical findings for isolectin B4 in ischemic myocardium of nude rats 4 weeks after cell transplantation. b, Capillary density in rat ischemic myocardium 4 weeks after cell transplantation. **$P<0.01$. 

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(\(P=0.013\) versus CD34\(^{-}\) MNCs and \(P=0.005\) versus PBS) (Figure 5f), FS was significantly greater (\(P=0.007\) versus CD34\(^{-}\) MNCs and \(P=0.001\) versus PBS) (Figure 5g), and regional wall motion score was significantly better (\(P=0.005\) versus CD34\(^{-}\) MNCs and \(P=0.0002\) versus PBS) (Figure 5h) in rats receiving CD34\(^{+}\) MNCs compared with those treated with CD34\(^{-}\) MNCs or PBS. LVDs, FS, and regional wall motion score 4 weeks after transplantation in the CD34\(^{-}\) MNCs group were not significantly different from those in the PBS group (Figure 5, f through h).

**Transplanted Human EPCs Incorporate Into Foci of Myocardial Neovascularization and Differentiate Into Mature ECs**

Both Di-1 labeled human CD34\(^{+}\) MNCs (EPC-enriched fraction) and CD34\(^{-}\) MNCs (EPC-poor fraction) were distributed principally in the ischemic area of the rat myocardium. However, the number of cells incorporated into tubular structures consistent with neovascularization was much greater in rats receiving CD34\(^{+}\) MNCs than in those in which CD34\(^{-}\) MNCs were transplanted (Figure 6a).

Differentiated human ECs derived from transplanted MNCs were frequently identified by UEA-1 lectin staining in the vasculature of the ischemic myocardium in rats receiving CD34\(^{+}\) MNCs. In contrast, mature human ECs were rarely identified in the ischemic myocardium of rats receiving CD34\(^{-}\) MNCs (Figure 6b). Thus, locally transplanted human EPCs were incorporated into foci of neovascularization and differentiated into mature ECs in ischemic myocardium.

**Discussion**

In the present study, we demonstrate the therapeutic potential and technical feasibility of percutaneous, intramyocardial transplantation of autologous EPCs in the setting of chronic myocardial ischemia. This strategy was designed to overcome the 2 inherent limitations of previous approaches that would prevent application in humans. First, the requirement for a large number of EPCs was avoided by delivering the cells directly to the ischemic myocardium with the use of a novel, real-time ischemia mapping system. Second, the issue of immunologic compatibility was resolved by the use of autologous cells. Although transplantation of autologous cells, such as bone marrow MNCs\(^{16}\) or skeletal myoblasts,\(^{17}\) has been reported, the present study is the first to elucidate the therapeutic potential of autologous EPC transplantation.

Catheter-based, percutaneous intramyocardial transplantation of swine EPCs resulted in histological, angiographic, and functional evidence of enhanced neovascularization of ischemic myocardium. The incorporation of transplanted EPCs into the neovascularization was documented in pilot studies using labeled NA/CD31\(^{+}\) cells. Increased vascularity of the myocardium was observed only in animals in which EPCs were
delivered. The notion that inflammation is induced either by needle injury or trauma resulting from injection of cells is completely dispelled by these data.

The porcine model of chronic myocardial ischemia was chosen for these preclinical studies to evaluate the strategy of local delivery via the NOGA injection catheter. Although CD34/H11001 MNCs would be used in future clinical situations, anti-swine CD34 antibody is not available. Therefore, we performed cell selection with anti-swine CD31 antibody instead. To complement these studies and verify that selected CD34 cells could also yield similar clinical benefit, we transplanted freshly isolated human CD34/H11001 cells into the myocardium in a nude rat model of myocardial ischemia. The locally transplanted CD34+ cells incorporated into foci of myocardial neovascularization, differentiated into mature ECs, enhanced vascularity in the ischemic myocardium, preserved LV systolic function, and inhibited LV fibrosis. Once again, these benefits were absent after injection of negatively selected cells or PBS, providing further evidence against the “injury hypothesis” of neovascularization. These positive outcomes are similar to those in previous studies involving intravenous EPC transplantation. However, the number of transplanted human CD34+ MNCs in this study was 20 times less than that in these previous studies of intravenous transplantation, providing a practical solution to the requirement for large numbers of cells in these previous investigations.

These data suggest that percutaneous delivery of autologous, freshly isolated EPCs targeted to sites of ischemia may represent a practical strategy for revascularization of patients with chronic myocardial ischemia.

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