Improvement of Collateral Perfusion and Regional Function by Implantation of Peripheral Blood Mononuclear Cells Into Ischemic Hibernating Myocardium

Hiroshi Kamihata,* Hiroaki Matsubara, Takashi Nishiue,* Soichiro Fujiyama,* Katsuya Amano,* Osamu Iba, Takanobu Imada, Toshiji Iwasaka

Objective—This study was performed to evaluate the angiogenic effect of implantation of peripheral blood mononuclear cells (PB-MNCs) compared with bone marrow mononuclear cells (BM-MNCs) into ischemic hibernating myocardium.

Methods and Results—A NOGA electromechanical system was used to map the hibernating region and to inject cells. PB-MNCs and BM-MNCs contained similar levels of vascular endothelial growth factor and basic fibroblast growth factor, whereas contents of angiogenic cytokines (interleukin-1β and tumor necrosis factor-α) were larger in PB-MNCs. Numbers of endothelial progenitors were ≈500-fold higher in BM-MNCs. In BM-MNC–implanted myocardia of pigs, an increase in systolic function (ejection fraction from 33% to 52%) and regional blood flow (2.1-fold) and a reduction of the ischemic area (from 29% to 8%) were observed. PB-MNC implantation reduced the ischemic area (from 31% to 17%), the extent of which was less than that seen with BM-MNCs. In saline-implanted myocardium, the ischemic area expanded (from 28% to 38%), and systolic function deteriorated. Angiography revealed an increase in collateral vessel formation by PB-MNC or BM-MNC implantation. Capillary numbers were increased 2.6- and 1.7-fold by BM-MNC and PB-MNC implantation, respectively. BM-MNCs but not PB-MNCs were incorporated into neocapillaries.

Conclusions—Catheter-based implantation of PB-MNCs can effectively improve collateral perfusion and regional function in hibernating ischemic myocardium by its ability to mainly supply angiogenic factors and cytokines. (Arterioscler Thromb Vasc Biol. 2002;22:1804-1810.)

Key Words: angiogenesis ■ myogenesis ■ bone marrow ■ stem cells ■ ischemic myocardium

Therapeutic angiogenesis is a new and promising strategy to revascularize ischemic myocardium by stimulation of the growth of new blood vessels or maturation of preexisting collaterals. Angiogenesis is induced by surgical or catheter-based delivery of angiogenic molecules, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Double-blind placebo-controlled clinical studies have indicated that intracoronary infusion of VEGF or FGF2 does not improve myocardial perfusion, whereas catheter-based gene transfer of VEGF produces an increase in regional perfusion in patients with ischemic myocardium.

Myogenic cell grafting into the damaged myocardium is a promising approach for the treatment of heart failure. We and others have shown previously that intramyocardial transplantation of bone marrow (BM) mononuclear cells (MNCs) improves regional perfusion and systolic function in animal models of ischemic heart failure. Marked increase in cardiac function after BM-MNC implantation may be due not only to neovascularization but also to cardiomyogenesis derived from marrow hematopoietic cells (see review) and/or marrow mesenchymal cells. Hamano et al reported the clinical efficacy and safety of BM-MNC implantation in 5 patients with ischemic heart disease in combination with bypass surgery. Thus, BM-MNC implantation may be feasible to salvage myocardial ischemia, although this procedure requires a minithoracotomy to expose the myocardium for intramuscular injection and has some risk associated with the administration of general anesthetics, limiting the feasibility of repeat administration.

An earlier study in our laboratory indicated that BM-MNC implantation into swine myocardium after acute occlusion of coronary artery improves regional perfusion and systolic function via a local supply of endothelial progenitor cells (EPCs) as well as angiogenic factors. However, it remains to be determined which of EPC inclusion and the supply of angiogenic factors contributes more to BM-MNC–induced neovascularization. Peripheral blood (PB)-MNCs synthesize and release high levels of VEGF, which are known to be prerequisites for the investment of stable vessels with peri-}

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From the Department of Medicine II and Cardiovascular Center, Kansai Medical University, Moriguchi, Osaka, Japan.
*These authors contributed equally to the present study.
Correspondence to Hiroaki Matsubara, MD, Department of Medicine II, Kansai Medical University, Moriguchi, Osaka 570-8507, Japan. E-mail matsubah@takii.kmu.ac.jp
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plantation have been reported in ischemic limbs in an animal model. Although implantation of PB-MNCs into ischemic myocardium may enhance collateral vessel formation by supplying angiogenic factors rather than EPCs, their efficacy and availability for therapeutic angiogenesis remain undefined. To investigate the potential efficacy of PM-MNC implantation as well as the precise role of EPCs in BM-MNC implantation, we examined angiogenic effects by catheter-based implantation of PB-MNCs compared with BM-MNCs into the hibernating myocardium.

Methods

Chronic Ischemic Model and Treatment Groups

Twenty specific pathogen-free domestic pigs weighing 35 to 40 kg underwent ameroid constrictor implantation around the proximal left circumflex artery (LCX) because the procedure to place an ameroid constrictor around the left anterior descending coronary artery can easily cause total occlusion for anatomic reasons, resulting in acute myocardial infarction. The right coronary artery is very short in pigs, and the contribution of the LCX in supplying myocardial blood perfusion is relatively large. Four weeks after implantation, all pigs underwent (1) selective coronary angiography, (2) echocardiography, (3) regional myocardial blood flow assessment using microspheres, and (4) NOGA LV electromechanical mapping (EMM, Biosense-Webster) and percutaneous catheter-based injection of cells (BM-MNC implantation, n=7; PB-MNC implantation, n=7; and saline injection, n=6). The injected cell number was 5×10⁶ cells, according to our previous observations. The animals showing total occlusion of LCX 4 weeks after ameroid implantation were selected for the experiment. This animal experiment was approved by the Animal Care Committee of Kansai Medical University.

NOGA LV EMM

Pigs underwent nonfluoroscopic EMM immediately before cell implantation to guide the injections of cells to foci of hibernating ischemic myocardium. The NOGA system of catheter-based mapping and navigation has been described in detail previously. The raw data were edited by using the NOGA system computer. Local functional analysis was based on linear local shortening, a parameter that calculates the fractional shortening of regional endocardial surfaces at end systole and is correlated with wall motion. Unipolar (UpV) and bipolar endocardial potentials recorded from the tip electrode, based on local intracardial signal amplitudes, were used to map myocardial viability. Myocardial area with UpV ≥5 mV (suggestive of viable myocardium) and normal local shortening ≥12% (suggestive of normal contraction) would imply normal myocardium. UpV <5 mV and abnormal local shortening <4% (severe regional hypokinesis or akinesis) would denote LV infarction. In contrast, UpV ≥5 mV and abnormal local shortening of 4% to 12% (mild to moderate impairment of contractility) would suggest an area of ischemic hibernating myocardium. To quantify the area of ischemia, a 2D algorithm based on a standard reference frame was used to calculate the area of ischemia and the total surface area in the 2D view depicting maximal ischemia.

Six weeks after ameroid implantation, measurement of regional blood flow with colored dye microspheres and coronary angiography were performed. A fluid-filled catheter was introduced into the femoral artery, and dP/dt and LV end-diastolic pressure were analyzed.

Percutaneous Catheter-Based Cell Transplantation

After the completion of EMM, the mapping catheter was replaced by the injection catheter (Myostar, Biosense-Webster), which was a modified 8F mapping catheter, the distal tip of which incorporated a modified 27-gauge needle that could be advanced or retracted by 4 to 6 mm. The catheter was advanced via femoral arteriotomy across the aortic valve into the LV, and it was manipulated to acquire stable points on the basis of the parameters described above within the target region that had been superimposed on the 3D map acquired previously. Once a stable point was attained, the needle was advanced 5 mm into the myocardium; the intracardiac electrode tip was transected, and myocardial injury and/or premature ventricular contractions as evidence of needle penetration into the myocardium. A total of 20 injections (total 5×10⁶ cells) were made into areas of hibernating ischemia (suggested by the combination of preserved voltage and abnormal wall motion). Each injection consisted of 0.1 mL of solution delivered from a 1-mL syringe. By dye-injection experiments (n=4), cells deep in the injected site were found to advance by 5 mm, and intramyocardial retention percentage of the injected volume was almost 100%.

Preparation of BM-MNCs and PB-MNCs

The procedure for BM-MNC isolation has been described previously. Briefly, pigs were anesthetized with ketamine hydrochloride, followed by halothane. BM cells (~60 mL) were aspirated from the ilium. MNCs were isolated by Percoll gradient centrifugation (Lymphoprep, NYCOMED). Cell sorting of PB-MNCs was performed by using a CS3000-Plus separator (Baxter), in which the number of CD34⁺ cells in human PB-MNCs was shown to be ~500-fold less than that in BM-MNCs. On the basis of May-Grünwald staining (n=20), the sorted BM-MNCs contained lymphocytes (69±3%), erythroblasts (9±2%), monocytes (15±2%), and granulocytes (7±1%). Sorted PB-MNCs contained lymphocytes (79±3%), monocytes (19±2%), and granulocytes (2±0.3%).

Immunohistochemistry and Analyses of Vessel Numbers

Paraffin-embedded sections were immunostained with rabbit von Willebrand factor (vWF, DAKO). To analyze the vessels, 5 fields (5 mm²) were chosen from the random field from the ischemic area. Researchers, who were unaware of the group identity of the slides, evaluated the density of arteries and capillaries in each field by counting vessels in 5 unit areas chosen at random (500 μm²) by using an ocular micrometer as described previously. The total number of vessels in 25 unit areas (5 fields with 5 unit areas in each field) were counted. Interobserver variation was <5%. Endothelial-lineage cells were analyzed by using endothelial markers, DII-acetylated LDL (DII-acLDL) incorporation, and ulex lectin binding, as described previously.

To detect transdifferentiation of implanted cells, BM-MNCs (n=4) or PB-MNCs (n=4) were prelabeled with green fluorescence cell linker (PKH2-GL, Sigma Chemical Co) as previously described and injected into the hibernating myocardium via the NOGA system. Four weeks after implantation, cardiac samples were snap-frozen and cut with cryostat. These were incubated with anti-desmin (clone DE-R-11, DAKO) or anti-vWF antibody to detect cardiomyocytes or vascular endothelial cells, respectively, followed by incubation with FITC- or TRITC-conjugated secondary antisera.

Quantitative Angiographic Analysis and Echocardiography

Numbers of visible vessels (>100 μm in diameter) branching from the left anterior descending coronary artery in the direction of the ischemic area were counted with the use of myocardial grids by at least 2 experienced cardiologists who were unaware of the group identity of the angiographic film as previously described. Interobserver variation was <5%. Echocardiographic studies were performed immediately before and 6 weeks after implantation with the use of Agilent Technology Sonos 5500 with an ultrahard S4 sector transducer as described.

Quantification of mRNA Levels

The mRNA levels for VEGF (3.3 kb), bFGF (2.8 kb), interleukin (IL)-1β (3.5 kb), or tumor necrosis factor (TNF)-α (2.5 kb) were evaluated by Northern blotting analysis with the use of cRNA...
riboprobes. As an internal RNA control, U3 rRNA was examined with the use of a cDNA probe.

Statistical Analysis
Statistical analyses were performed by paired t test. Data (mean±SE) were considered statistically significant at a value of P<0.05.

Results
Endothelial-Lineage Cells and Angiogenic Factors in BM-MNCs and PB-MNCs
Fluorescence-activated cell sorter (FACS) analysis indicated that 26±1.8% and 28±1.5% of BM-MNCs incorporated Dil-acLDL and bound ulex lectin (n=5), respectively, and 23±1.5% of cells were positive for both markers (see online Figure I, available at www.ahajournals.org). Endothelial-lineage cells have been reported to be included in this double-positive fraction. PB-MNCs exhibited much lower ratios of Dil-acLDL incorporation (4%) and ulex lectin binding (6%), and the ratio of endothelial-lineage cells was 2.8±0.4% (n=5). Northern blotting showed that levels of VEGF and bFGF mRNA were similar between BM-MNCs and PB-MNCs, whereas IL-1β and TNF-α levels were lower in BM-MNCs than in PB-MNCs (see online Figure I).

LV EMM
Areas of electrically viable myocardium (UpV >5 mV) associated with abnormal/impaired wall motion (linear local shortening <12%), ie, electromechanical uncoupling diagnostic of hibernating ischemia by the NOGA system, were detected in all pigs that underwent ameroid constrictor implantation. Transient unifocal ventricular ectopic activity was observed at the time the needle was extended into the myocardium. Sporadic premature ventricular contractions occurred during injection, but no episodes of sustained ventricular arrhythmia were observed. Continuous ECG monitoring for 24 hours after cell injection indicated no sustained ventricular arrhythmia. Compared with basal levels before injection, creatine kinase-MB levels were not elevated after cell injection.

NOGA Electromechanical Assessment of Cell Implantation
Mean UpV and bipolar voltage recordings, defining myocardial viability in the ischemic segments, did not change appreciably after BM-MNC or PB-MNC implantation (Figures 1 and 2, left panels). As indicated in the color change from the red zone (reduced wall motion) to the purple zone (normal motion), wall motion assessed by local linear shortening in segments of hibernating ischemia was greatly improved in all BM-MNC–implanted pigs (n=7) 6 weeks after BM-MNC implantation (Figure 1). The area of ischemic myocardium was consequently reduced from 29% before implantation to 8% at 6 weeks after implantation (P<0.001,

Figure 1. NOGA electromechanical analysis of BM-MNC–implanted myocardium. NOGA images in left lateral projection before BM-MNC implantation (BMI) show myocardial viability and wall motion; the red zone (which depicts abnormal wall motion) on the linear local shortening map (top right) and the observed viability (purple/blue) on the unipolar voltage map (top left) constitute the focus of electromechanical uncoupling that suggests ischemic hibernating myocardium. The same projection 6 weeks after BMI showed almost complete resolution of wall motion in the hibernating region (shift from red to purple in wall motion view). Circular black icons in the top panels represent injection sites of BM-MNCs. The horizontal axis is indicated by a white line. The red line represents the long axis through the apex. The data are representative of BM-MNC–implanted myocardium (n=7).
The ejection fraction (EF) was improved from 33±1.9% to 52±2.6% by BM-MNC implantation ($P<0.001$, Figure 3), consistent with improvement in EF values simultaneously determined by echocardiography (from 34±2.1% to 50±2.8%). There was a significant correlation between EF evaluated by the NOGA system and echocardiography ($r=0.82$, $P<0.001$), suggesting the accuracy of NOGA analysis. As shown in Figure 3, there were no significant differences in ischemic areas (percentage) among 3 experimental groups, suggesting that the degree of the ameroid-induced myocardial ischemia was comparable among the 3 experimental groups.

In the PB-MNC–implanted myocardium (n=7), wall motion in the ischemic myocardium improved to the normal...
motion in 3 pigs, as observed in BM-MNC-implanted myocardium, whereas in 4 pigs, regional blood flow was below the normal level (change from red to blue/green color zone) 6 weeks after BM-MNC implantation (Figure 2). Quantification of the ischemic area and EF showed significant changes after PB-MNC implantation (Figure 3), whereas the percent changes were smaller than those in the BM-MNC-implanted myocardium ($P<0.001$). In contrast, wall motion in the myocardium, endocardial blood flow and epicardial blood flows were decreased (from 1.8 to 1.4 mL/min per gram [endocardial] and from 1.7 to 1.4 mL/min per gram [epicardial]; Figure 3).

Assessment of Regional Blood Flow
Regional blood flow in the ischemic region was measured by using colored dye microspheres. In the BM-MNC-implanted myocardium, endocardial blood flow and epicardial blood flow in the ischemic myocardium were increased ($P<0.001$) from 1.7 and 1.5 mL/min per gram, respectively, before implantation to 3.6 and 3.3 mL/min per gram, respectively, at week 6 (Figure 3). PB-MNC implantation improved endocardial blood flow (from 1.6 to 2.2 mL/min per gram, $P<0.001$) but did not significantly increase epicardial blood flow, whereas in saline-injected myocardium, endocardial and epicardial blood flows were decreased (from 1.8 to 1.4 mL/min per gram [endocardial] and from 1.7 to 1.4 mL/min per gram [epicardial]; Figure 3).

Immunohistological Analysis and Differentiation Into Endothelial Cells
Vascular endothelial cells in ischemic regions were immunohistologically stained with anti-vWF antibody, and capillary vessel numbers were quantified. Numbers of visible collateral vessels (>50 μm and <50 μm in diameter) were markedly increased in BM-MNC-implanted myocardium (2.4- and 3.4-fold, $P<0.001$) or PB-MNC-implanted myocardium (1.7- and 2.2-fold, $P<0.001$) compared with saline-injected myocardium (Figure 4A). Most of green-labeled BM-MNCs corresponded to vWF-positive capillaries (12±1.5% of total vWF-positive cells), whereas apparent transdifferentiation into desmin-positive cardiomyocytes was not observed (Figure 4B). Green-labeled PB-MNCs were not observed in the tissue samples (data not shown).

Coronary Angiography
Coronary angiography showed that distal portions of constricted arteries were apparently visible by increased collateral vessel formation in BM-MNC- or PB-MNC-implanted myocardium but not in saline-injected myocardium and that the numbers of collateral vessels branching in the direction of the ischemic area were increased in BM-MNC-implanted myocardium (3.4±0.8-fold, $P<0.001$) and PB-MNC-implanted myocardium (2.1±0.4-fold, $P<0.001$) compared with saline-injected myocardium (please see online Figure III, available at www.ahajournals.org).

Discussion
Catheter-based delivery of autologous BM cells to amiodarone-induced chronic myocardial ischemia was reported in pigs by Fuchs et al. They implanted whole bone marrow cells, including neutrophils and erythrocytes, and demonstrated improvement of collateral blood flow in the implanted myocardium. They concluded that VEGF and monocyte chemotactic protein-1 released from implanted BM cells augmented collateral perfusion by inducing endothelial cell proliferation. The
present study extended the study by Fuchs et al by comparing the angiogenic effects between PB-MNC and BM-MNC implantation and thus attempted to investigate the angiogenic effects of PB-MNC implantation on ischemic hibernating myocardium and also to determine which of the endothelial progenitors and angiogenic factors supplied by implanted BM-MNCs contributes more to neovascularization.

The major findings of the present study included the following: (1) PB-MNC implantation that supplies angiogenic factors (VEGF and bFGF) and angiogenic cytokines (IL-1β and TNF-α) but not endothelial progenitors augmented neocapillary formation, leading to inhibition of the progression of myocardial ischemia and improvement in cardiac function, although its angiogenic effect was significantly weaker than that of BM-MNC; (2) implanted BM-MNCs differentiated into vascular endothelial cells, whereas implanted PB-MNCs were not incorporated into neocapillaries; and (3) catheter-based cell delivery associated with NOGA EMM can be safely and successfully achieved to ameliorate regional perfusion in hibernating myocardium in a relatively site-specific fashion. Thus, our results demonstrate the potential efficacy of therapeutic angiogenesis with the use of PB-MNC implantation in ischemic myocardium and also confirm the role of EPCs in BM-MNC–mediated angiogenic therapy.

Myogenic cell grafting in damaged myocardium is a promising approach in the treatment of heart failure. We and others have demonstrated the efficacy of intramyocardial transplantation of BM-MNCs in animal models of ischemic heart failure.7–10 Side effects, such as increases in cardiac enzymes, malignant arrhythmia, or differentiation into cells of other lineages, were not observed in the BM-MNC–implanted myocardium.7 Because BM-MNCs contain cells of various lineages, such as hematopoietic cells, fibroblasts, osteoblasts, and myogenic cells, as well as cells of endothelial lineage, such mixed populations of BM-MNCs can work beneficially and harmfully in ischemic myocardium. In our previous study, we showed that in the ischemic myocardium, some surviving factors to stabilize BM fibroblasts or BM osteoblasts are lacking, and we also showed that BM-derived endothelial-linkage cells can effectively and selectively differentiate into mature endothelial cells. Recently, it has been reported that cardiomyocytes can be regenerated from marrow hematopoietic cells (see review11) or marrow mesenchymal cells,12 whereas our present study did not clearly prove transdifferentiation of implanted BM-MNCs into cardiomyocytes. Collectively, these findings suggest that the increase in cardiac function as observed in the present study mainly results from an increase in blood supply into the hibernating myocardium.

Marrow cells secrete a broad spectrum of inflammatory angiogenic cytokines.20,22 In the present study, we found that PB-MNCs and BM-MNCs contain VEGF and bFGF to a similar extent, whereas IL-1β and TNF-α levels are higher in PB-MNCs than in BM-MNCs. IL-1β upregulates the expression of VEGF and VEGF receptor-2 in coronary endothelial cells, and IL-1β and TNF-α have been shown to have angiogenic activity.20,21 Kobayashi et al20 also reported the involvement of IL-1β in BM cell–induced angiogenesis in ischemic rat hearts. Thus, it is likely that such angiogenic cytokines and angiogenic factors (VEGF and bFGF) are involved in the increase in endocardial blood flow observed in PB-MNC–implanted myocardium.

Losordo et al6 reported that percutaneous catheter-based injection of the myocardial VEGF gene by the NOGA electromechanical system was safely performed in human subjects. There were no adverse procedural outcomes, including ventricular arrhythmia, myocardial infarction, systemic embolization, or ventricular perforation. In the present preclinical study using catheter-based cell delivery, it was most important to selectively inject cells into the hibernating myocardium, including sites that are less accessible from minithoracotomy. This catheter-based transcatheter injection method has been used successfully to deliver solutions such as methylene blue.23 Because there is no need for general anesthesia or operative dissection through adhesions related to the placement of previous bypass conduits, the catheter-based approach facilitates placebo-controlled double-blind clinical trials, and the intervention can be performed as an outpatient procedure and is also repeatable. Targeted catheter-based implantation of PB-MNCs into the ischemic myocardium was shown to improve cardiac function by increasing regional blood perfusion in the hibernating zone. Although the angiogenic action of BM-MNCs, including endothelial progenitors, was more effective than that of PB-MNCs, this procedure was needed to aspirate bone marrow cells. Catheter-based implantation of PB-MNCs and function analysis using the electromechanical mapping system will provide a novel and safe therapeutic strategy for the treatment of ischemic heart disease.

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References


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In the November 2003 issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, in the article by Kamihata et al (Myocardial Angiogenesis by Blood Cell Implantation; pp 1804–1810), there were errors in Figure 4A and online Figure III. The corrected Figure 4A, and the correct Figure III corresponding to the new Figure 4A, are shown.