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The Impact of the Capability of Circulating Progenitor Cell to Differentiate on Myocardial Salvage in Patients With Primary Acute Myocardial Infarction

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Background—Circulating endothelial progenitor cells (EPCs) are known to be involved in vasculogenesis and mobilized after acute myocardial infarction (AMI). To test the hypothesis that the angiogenic function of EPCs affects post-myocardial infarction (MI) myocardial salvage, we evaluated the number and potential differentiation of EPCs and compared these data with clinical parameters 6 months after MI.

Methods and Results—Consecutive 51 patients (age, 61 ± 8 years, mean ± SD) with primary AMI who were successfully treated with stenting were enrolled. EPC identified as CD45low, CD34+, CD133+, and VEGFR2+ was quantified by a flow cytometry. The potential of EPCs to differentiate into endothelial cells (EPC differentiation) was also confirmed by the upregulation of CD31 and VEGFR2 after 7 days of culture. According to the proportion of EPC fraction, patients were divided into 2 groups (cut-off value = median). Although no difference was seen in myocardial damage shown by mean peak CK leakage and mean area at risk between the differentiated group (n = 26) and nondifferentiated group (n = 25), the number of attached cell was greater in differentiated group than in the nondifferentiated group (P = 0.023). Left ventricular function and ischemic damaged area were assessed by scintigraphic images of 123I-BMIPP in the acute phase and 99mTc-tetrofosmin in the chronic phase. We found that a greater increase in myocardial salvage (P = 0.0091), decrease in end-systolic volume (P = 0.012), and recovery of ejection fraction (P = 0.011) occurred in the group with differentiated EPCs than in the nondifferentiated group.

Conclusions—In patients with primary AMI, the capability of EPCs to differentiate influences the functional improvement and infarct size reduction, indicating that manipulation of EPCs could be a novel therapeutic target to salvage ischemic damage. (Circulation. 2006;114[suppl I]:I-114–I-119.)

Key Words: ischemia □ myocardial infarction □ myocardial salvage □ progenitor cell □ reperfusion □ scintigraphy

T he progress in percutaneous coronary interventions (PCI) such as the development of drug-eluting stents and antithrombus therapy has dramatically decreased the mortality and morbidity in the acute and subacute phases in patients with myocardial infarction (MI).1–3 However, heart failure followed by MI, which is often accompanied with myocardial remodeling, is established in the chronic phase and often remains refractory to conventional drug therapy. Therefore, salvaging the jeopardized myocardium to protect remodeling is one of the most important clinical goals from revascularization in the acute phase to management as an outpatient in the chronic phase.4–6 Recent studies have provided evidence that the formation of capillary vessels in postnatal life does not only result from the sprouting of preexisting vessels (angiogenesis)7 but also involves the recruitment of bone marrow-derived progenitors for endothelial cells (vasculogenesis).8 Furthermore, we and other groups have reported a significant mobilization of circulating endothelial progenitor cells (EPCs) and hematopoietic stem cells in the subset of acute MI (AMI).9–11 Regarding myocardial regeneration, bone marrow-derived and peripheral tissue-derived progenitor cells appear to be able to regenerate a myocardium by enhancing the neovascularization. However, little is known about the degree to which these...
mobilized EPCs in the acute phase could salvage the damaged myocardium after AMI in a clinical setting. A better understanding of the cell subsets and function involved in mobilization processes after AMI could help in determining therapeutic options, including their potential manipulations.

We hypothesized that increased numbers and function of EPCs would improve such pathophysiologically and reparative processes, potentially contributing to recovery from myocardial damage, and these characteristics of EPCs might be reflected in the healing potential after ischemic myocardial damage. In the present study, we assessed the association between the number and function of EPCs and myocardial salvage in the subset of AMI. To quantify the degree of myocardial salvage, we collected single photon emission computed tomography (SPECT) images of $^{123}$I-β methyl-p-iodophenyl-pentadecanoic acid (BMIPP) for measuring the areas at risk at days 5 to 7 after MI and of $^{99m}$Tc-tetrofosmin (TF) for the infarct area at 6 months, and subsequently compared these with the functional characteristics of mobilized EPCs in the peripheral circulation.12–16

Methods

Statement of Responsibility

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Patient Population

Patients between 45 and 75 years of age were enrolled in this study if they had a primary ST segment-elevated MI of the left anterior descending coronary artery alone that was treated successfully by coronary stenting. Exclusion criteria were the occurrence of major adverse cardiovascular events during the observation period, major bleeding requiring blood transfusion during the PCI procedure, a history of hepatic or renal dysfunction, evidence of malignant neoplasms, and an unwillingness to participate. Age-matched healthy subjects were recruited as controls for the analysis of EPC mobilization (age = 60 ± 5 years, mean ± SD, n = 20). The protocol was reviewed and approved by the ethics committee of the Nagoya University School of Medicine, Japan, and the study was conducted in accordance with the Declaration of Helsinki. All patients enrolled in this study provided written informed consent.

PCI Procedure

The PCI procedure was performed by experienced interventionists according to the guidelines for coronary angiography and PCI of the American College of Cardiology and the American Heart Association.12 All digital images of cineangiograms were analyzed by an independent cardiologist (T.S.) without knowledge of the results of PCI Procedure.17 All digital images of cineangiograms were analyzed by an independent cardiologist (T.S.) without knowledge of the results of PCI Procedure.17 All digital images of cineangiograms were analyzed by an independent cardiologist (T.S.) without knowledge of the results of PCI Procedure.17

Gated SPECT Imaging for BMIPP and Tetrofosmin

SPECT imaging was performed with 3-head gamma cameras equipped with low-energy high-resolution collimators. A 10% to 20% window was centered on the 141- and 159-keV photopeak. At 5 to 7 days after MI (6.5 ± 0.5 days), patients in a fasting condition were injected with 111 MBq of $^{123}$I-BMIPP, with SPECT image acquisition for the early BMIPP image beginning 20 minutes later. The total acquisition time was 16 minutes. At 180 minutes after injection, a second BMIPP SPECT acquisition was started, with a total acquisition time of 32 minutes. Images were acquired over 360° (20 projections per head in a 64×64 matrix) with a 3-head camera in the step-and-shoot mode. The early images of BMIPP were used for SPECT analysis of myocardial salvage. Three to 6 months after MI (5.1 ± 0.5 months), patients were injected with 147 MBq of $^{99m}$Tc-tetrofosmin and SPECT images were acquired within 2 hours. Reconstruction was performed with a conventional filtered back-projection algorithm at an in-plane resolution of 10-mm full width half maximum.

SPECT Image Analysis

All SPECT images were interpreted by 3 expert nuclear cardiologists in a blinded manner after images were transferred to the Radiosotope Imaging Analysis Center, Ogaki Municipal Hospital. The independent cardiologists interpreted the studies by consensus using a 20-segment model of the myocardium and a quantitative gated SPECT analysis program for end systolic and end diastolic volumes and ejection fraction, and a quantitative perfusion SPECT analysis program for perfusion defects following the recommended guidelines.

EPC Preparation

Peripheral blood samples were collected in a fasting condition between 7:00 AM and 9:00 AM. Mononuclear cells (MNCs) were isolated by a density-gradient centrifugation method.18 The cell surface markers CD34, CD45, and CD133 and vascular endothelial growth factor receptor type 2 (VEGFR2) were analyzed by flow cytometry (FACS; FACScan, Becton-Dickinson) as described previously.19 The numbers of CD45$^{+}$CD34$^{+}$, CD45$^{+}$CD34$^{+}$CD133$^{+}$, and CD45$^{+}$CD34$^{+}$ CD133$^{+}$VEGFR2$^{+}$ cells were counted.

Cell Culture and Characterization of Progenitor Cells

MNCs were isolated from 20 mL of peripheral blood by a density-gradient centrifugation method.18 MNCs were cultured on 2% gelatin/fibronectin-coated dishes at 10$^{5}$ cells/cm$^{2}$ as described previously.18,19 Cells were cultured in M199 supplemented with 20% fetal bovine serum (Invitrogen/GIBCO, Carlsbad, Calif), 2 mmol/L glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and bovine pituitary extract (Invitrogen/GIBCO). At 7 days of culture, to visualize the upregulation of endothelial markers, cells were fixed, incubated with anti-human antibodies, and stained with biotinylated anti-mouse or rabbit Ig antibodies, and with FITC and Texas red-avidin with DAPI counterstaining, and observed using an epifluorescence microscope (Olympus). The marker proteins were vascular endothelial growth factor receptor (VEGFR2/KDR) (LabVision), and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Invitrogen) (see supplementary material online at).

Definitions and Follow-Up Protocol

A scatter plot of the EPC fraction as analyzed by flow cytometry and immunocytochemistry showed non-parametric distribution (Figures 1 and 2). The median number of EPCs at differentiation for the whole study population was 15 cells with CD45$^{+}$CD34$^{+}$CD133$^{+}$ VEGFR2$^{+}$10$^{3}$ leukocytes. Patients were divided into 2 groups based on the number of differentiated EPCs: <15 cells with CD45$^{+}$CD34$^{+}$CD133$^{+}$ VEGFR2$^{+}$10$^{3}$ leukocytes and >15 cells with CD45$^{+}$CD34$^{+}$CD133$^{+}$ VEGFR2$^{+}$10$^{3}$ leukocytes.

The Salvage index was defined as [(1 – percentage infarct area)/(percentage area at risk)]; the initial defect size was obtained by BMIPP SPECT image and the final infarct size by TF SPECT image in accordance with the methods of Ndrepepa et al with modifications.15

Up to the time of re-investigation by coronary angiography at 6 months after MI, all patients were followed-up clinically every month at an outpatient clinic, and compliance with medication of 200 mg of ticlopidine, 160 to 200 mg of aspirin, angiotensin type 1 receptor antagonist (ARB), and statins was confirmed.
56 patients with acute STEMI treated successfully with stent revascularization

Figure 1. Flow chart of the study protocol. Of 56 patients enrolled, 26 and 25 patients were assigned to the differentiated EPC and nondifferentiated groups, respectively (the other 5 patients were excluded from this analysis). The chart depicts each reason for and the timing of exclusion. BMIPP indicates 123I-BMIPP scintigraphy; CAG, coronary angiogram; EPC, endothelial progenitor cell; FACS, fluorescence-activated cell sorting; STEMI, ST segment-elevated myocardial infarction; TF, technetium-tetrofosmin scintigraphy; VT, ventricular tachycardia.

Statistical Analysis
Continuous variables are presented as mean and SD values. Categorical variables were compared by the χ² test or Fisher exact test. Statistical comparisons between 2 groups were performed nonparametrically using the Mann-Whitney U test. Spearman’s rank correlation test was used to assess the correlation between circulating EPC counts and left ventricular (LV) function. Statistical significance was assumed if P<0.05. All statistical analyses were performed using StatView-J (version 5.0; SAS Institute Inc).

Results

Patients
A flow chart of the study protocol is shown in Figure 1. Of 56 consecutive patients with primary acute STEMI who were successfully treated with stent implantation (62 bare metal stents for 50 lesions, and 6 drug-eluting stents, Cypher, Cordis, for 6 lesions), 5 patients were excluded. Therefore, a total of 51 patients (age, 61±8 years) were analyzed. The average of the peak of creatine kinase leakage was (3180±1732 IU/L), and the size of infarct area assessed by BMIPP image (55±8%), indicating a moderate level of ischemic damage after MI.

Endothelial Progenitor Cell Counts and Differentiation
First, the EPC fraction was counted by FACS (supplemental Figure, A and B, available online at http://circ.ahajournals.org). To evaluate the mobilization of EPCs after AMI, we compared the abundances of the EPC fractions between MI patients and healthy volunteers who had no evidence of coronary heart disease. The mobilization of CD34⁺ cells and CD34⁺CD133⁺ KDR⁺ cells were 3.3-fold (P=0.002) and 1.7-fold (P=0.01) greater in MI patients than in healthy controls, respectively (supplemental Table I and Figure, B, available online at http://circ.ahajournals.org).

In peripheral blood of MI patients, scattered dots corresponding to the EPC fraction showed a nonparametric distribution (Figure 2A). To determine the functional capacity of circulating EPCs, we cultured MNCs for 7 days to quantify CD31, VEGFR2, (supplemental Figure, C to H). The distribution pattern of VEGFR2⁺ cells was also parametric, or even biphasic (Figure 2B).

In cell culture, attached cell number was greater in differentiated group if compared with those of nondifferentiated group (5.1±0.3 versus 2.1±0.1, ×10² cells/field, respectively, P=0.023). EPCs in differentiated group were more differentiated into an endothelial lineage with upregulation of VEGFR2/KDR than those of nondifferentiated group (482±36 versus 212±12, per 10³ attached cells, respectively, P<0.001).

Table 1.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Differentiated (n=26)</th>
<th>Nondifferentiated (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60±8</td>
<td>61±9</td>
<td>NS</td>
</tr>
<tr>
<td>Female, n</td>
<td>4</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker (current and ex.), n</td>
<td>8</td>
<td>8</td>
<td>NS</td>
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<tr>
<td>Hypertension, n</td>
<td>14</td>
<td>13</td>
<td>NS</td>
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<tr>
<td>Diabetes mellitus, n</td>
<td>8</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Hyperlipidemia, n</td>
<td>11</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>Killip class (I/II/III), n</td>
<td>14/8/3</td>
<td>12/9/2</td>
<td>NS</td>
</tr>
<tr>
<td>Onset to needle, min</td>
<td>221±32</td>
<td>239±35</td>
<td>NS</td>
</tr>
<tr>
<td>Diameter stenosis, %</td>
<td>91±15</td>
<td>90±13</td>
<td>NS</td>
</tr>
<tr>
<td>Peak CK, IU/L</td>
<td>3035±1754</td>
<td>3126±1711</td>
<td>NS</td>
</tr>
<tr>
<td>Area at risk, %</td>
<td>56±8</td>
<td>55±8</td>
<td>NS</td>
</tr>
<tr>
<td>Progenitor cell fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34⁺/10⁶ leukocyte</td>
<td>106±28</td>
<td>94±18</td>
<td>0.012</td>
</tr>
<tr>
<td>CD34⁺CD133⁺/10⁷ leukocyte</td>
<td>89±21</td>
<td>78±15</td>
<td>0.015</td>
</tr>
<tr>
<td>CD34⁺CD133⁺KDR⁺/10⁷ leukocyte</td>
<td>20±4</td>
<td>9.1±2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Differentiated indicates differentiated EPC group; Nondifferentiated, nondifferentiated EPC group; Data, mean±SD; Peak CK, peak creatine kinase leakage; area at risk, percent area at risk assessed by BMIPP imaging; CD34⁺, CD34⁺CD133⁺, and CD34⁺CD133⁺KDR⁺, each fraction counted by flow cytometry.
Baseline Clinical Characteristics

The baseline clinical characteristics of the 2 groups categorized on the basis of EPC differentiation are listed in Table 1, which indicates that none of the parameters differed significantly between the 2 groups including the peak value of creatine kinase leakage and the size of area at risk (Table 2).

EPC Differentiation and Clinical Outcome 6 Months After MI

The clinical outcomes at the 6-month follow-up after MI are listed in supplemental Table II, available online at http://circ.ahajournals.org. We generally found a moderate correlation between the EPC fraction and myocardial salvage ($P=0.012$, $r=0.43$), and in the difference between the ejection fractions in the acute and chronic phases ($P=0.023$, $r=0.41$). Although the LV end-diastolic volume did not differ between the 2 groups, significant differences were observed in infarct size as assessed in TF images ($P<0.0001$), salvage index ($P=0.0091$, Figure 3A), LV end-systolic volume ($P=0.012$), and ejection fraction ($P=0.022$). Figure 3B shows representative cases of the differentiated and nondifferentiated groups.

Discussion

There is accumulating evidence from both experimental animal studies and clinical human trials that cell therapy improves myocardial perfusion in MI. Recent studies have found that the magnitude of circulating EPCs appearing as CD34+/KDR$^+$ after MI may predict the clinical outcome and prognosis. However, there is little information from clinical studies on whether circulating EPCs are originally involved in the healing process of MI and, if they are, the degree to which these cells are involved and by what kind of functions (other than the number of EPCs). Our investigations revealed diversity in the capability of differentiation to an endothelial lineage, in both the circulating fraction of EPCs and cultured EPCs. A greater area of myocardial salvage accompanied with a reduction of the ischemic area and greater recovery of LV function were observed in patients with differentiated EPCs compared with those with nondifferentiated EPCs.

In the process of myocardial regeneration, bone marrow-derived and peripheral tissue-derived progenitor cells appear to be able to regenerate a myocardium by enhancing the neovascularization. These proangiogenic mechanisms may allow EPCs to positively contribute to the regeneration of an ischemic myocardium after reperfusion therapy. Regarding EPC differentiation, we first measured the abundance of EPC fraction defined as CD45$^+$CD34$^+$CD133$^+$KDR$^+$ in the sample of patients with AMI and healthy volunteers and compared this with parameters of clinical outcome. The EPC fraction was also present in the samples of healthy subjects, although the abundance was less than that in MI patients. Regarding the mobilization of EPCs, the increase of CD34$^+$ cells and CD34$^+$CD133$^+$KDR$^+$ cells at 7 days were 3.3-fold ($P=0.002$) and 1.7-fold ($P=0.01$) greater in MI patients than in healthy controls, respectively. Massa et al recently observed that the EPC mobilization was 5.8-fold higher on the day of AMI onset and 2-fold higher for up to 15 days after AMI onset in 26 AMI patients compared with healthy control subjects. Our findings are consistent with previous reports, and together these findings support the notion that even in a healthy condition, EPCs circulate to maintain the vascular function and that after the onset of AMI, these EPCs are mobilized to repair the damaged myocardium and capillary network.

MI is a complex syndrome with many factors involved in its prognosis, and hence we carefully considered patient criteria and the follow-up protocol. During the follow-up period, all patients received statins and ARB (both of which may affect the prognosis of MI) in addition to the antiagulants aspirin and ticlopidine. Four cases (11%) of the differentiated group showed impaired myocardial salvage that was even less than the average in the nondifferentiated group. Although each of the risk area sizes did not differ significantly from those of other patients of the same group, 3 of 4 patients resumed smoking after hospital discharge, and all of these 4 patients had at least 4 cardiovascular risk factors. Previous studies and those of others have shown that smoking deteriorates EPC attachment and differentiation, and that other risk factors also prominently affect EPC mobilization and differentiation, which may directly lead to EPC engraftment to the myocardium after ischemic injury. In these cases, such unfavorable behavior during the recovery period may reverse the good symptoms of EPC differentiation in the

TABLE 2.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Differentiated (n=26)</th>
<th>Nondifferentiated (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter stenosis, %</td>
<td>12±9</td>
<td>11±10</td>
<td>NS</td>
</tr>
<tr>
<td>Final infarct area, %</td>
<td>19±8</td>
<td>27±9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regional wall motion, mm</td>
<td>5.2±2</td>
<td>4.7±2</td>
<td>0.017</td>
</tr>
<tr>
<td>Salvage index</td>
<td>0.66±0.1</td>
<td>0.51±0.1</td>
<td>0.0091</td>
</tr>
<tr>
<td>LVESEV, mL</td>
<td>52±7</td>
<td>57±8</td>
<td>0.012</td>
</tr>
<tr>
<td>LVEDV, mL</td>
<td>124±25</td>
<td>126±28</td>
<td>NS</td>
</tr>
<tr>
<td>Ejection fraction (EF), %</td>
<td>59±8</td>
<td>56±12</td>
<td>0.022</td>
</tr>
<tr>
<td>$\Delta$EF</td>
<td>12±2</td>
<td>8±1</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Clinical outcome at 6 months. LVESEV indicates left ventricular end-systolic volume; LVEDV, left ventricular end-diastolic volume; $\Delta$EF, difference between EF in the acute phase and EF in the chronic phase.
acute phase. A future study should consider other effectors that are relevant to the recovery process of a damaged myocardium in the chronic phase.

Positron emission tomography is currently the gold standard for assessing myocardial perfusion imaging. However, the required equipment is not widespread, and the preparation of tracers involves a complex procedure. In contrast, SPECT has been widely used for more than 2 decades in clinical cardiology. Regarding isotope tracers, myocardial perfusion imaging with $^{99m}$Tc-sestamibi or TF is efficacious for evaluating myocardial ischemia in an emergency room because it provides real-time perfusion images of the jeopardized myocardium, those agents are available for emergency use, and radioisotope signals are stronger than the signals from thallium agents. However, the perfusion area gradually changes before and after PCI, and sometimes the administration of tracer on admission and the collection of images are inappropriate in patients with AMI in a serious condition. The uptake of the iodinated fatty acid analogue, BMIPP, reflects activation of fatty acids by coenzyme A and indirectly reflects cellular ATP production resulting from fatty acid metabolism. Therefore, in contrast to myocardial perfusion imaging, the high myocardial uptake and long retention time of BMIPP associated with BMIPP SPECT images allow the identification of alterations to fatty acid metabolism long after successful reperfusion (so-called ischemic memory), allowing us to estimate areas at risk at a remote period such as 7 days after AMI. Kawai et al examined 65 patients with AMI who underwent successful reperfusion therapy. They found that TF on the same day of MI onset and BMIPP at 1 week after MI were similarly effective at identifying areas at risk. Recently, Dilsizian et al confirmed that BMIPP metabolic defects at rest were similar to those of exercise-induced thallium perfusion defect after demand ischemia. Taken together, these results indicate that BMIPP imaging is useful in evaluating the size and degree of impaired myocardial metabolism and is safe in clinical settings such as AMI.

In conclusion, in patients with primary AMI, the capability of EPCs to differentiate is associated with functional improvement and infarct size reduction, indicating that manipulation of EPCs could become a novel therapeutic target to salvage ischemic damage.

Acknowledgments

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Disclosures

None.

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