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Mobilization of Endothelial Progenitor Cells in Patients With Acute Myocardial Infarction

Satoshi Shintani, MD; Toyoaki Murohara, MD; Hisao Ikeda, MD; Takafumi Ueno, MD; Tomoki Honma, MD; Atsushi Katoh, MD; Ken-ichiro Sasaki, MD; Toshifumi Shimada, MD; Yuichi Oike, MD; Tsutomu Imaizumi, MD

Background—Endothelial progenitor cells (EPCs) circulate in adult peripheral blood (PB) and contribute to neovascularization. However, little is known regarding whether EPCs and their putative precursor, CD34-positive mononuclear cells (MNC^{CD34+}), are mobilized into PB in acute ischemic events in humans.

Methods and Results—Flow cytometry revealed that circulating MNC^{CD34+} counts significantly increased in patients with acute myocardial infarction (n=16), peaking on day 7 after onset, whereas they were unchanged in control subjects (n=8) who had no evidence of cardiac ischemia. During culture, PB-MNCs formed multiple cell clusters, and EPC-like attaching cells with endothelial cell lineage markers (CD31, vascular endothelial cadherin, and kinase insert domain receptor) sprouted from clusters. In patients with acute myocardial infarction, more cell clusters and EPCs developed from cultured PB-MNCs obtained on day 7 than those on day 1. Plasma levels of vascular endothelial growth factor significantly increased, peaking on day 7, and they positively correlated with circulating MNC^{CD34+} counts ($r=0.35$, $P=0.01$).

Conclusions—This is the first clinical demonstration showing that lineage-committed EPCs and MNC^{CD34+}, their putative precursors, are mobilized during an acute ischemic event in humans. (*Circulation*. 2001;103:2776-2779.)

Key Words: angiogenesis ■ endothelium ■ stem cells ■ ischemia

Postnatal neovascularization has been thought to result exclusively from the proliferation and migration of pre-existing vascular endothelial cells (ECs),¹ a process referred to as angiogenesis. However, in a previous study, we identified circulating endothelial progenitor cells (EPCs)² that contributed to neovascularization in a manner consistent with postnatal vasculogenesis.³ Although tissue ischemia could mobilize EPCs from bone marrow into peripheral blood (PB) in animals,³ this issue was not investigated in humans. Accordingly, we examined whether EPCs and their putative precursor, CD34-positive mononuclear cells (MNC^{CD34+}), were mobilized in patients with acute myocardial infarction (AMI).

Methods

Patients

Patients with AMI (mean age, 64 years; 11 men and 5 women) and control subjects (mean age, 65 years; 5 men and 3 women) who had atypical chest pain but no evidence of cardiac ischemia were enrolled. AMI patients met the following criteria: chest pain lasting for >30 minutes that was not relieved by sublingual nitroglycerin, abnormal Q waves on the ECG, and elevated serum creatine kinase levels. Angiography revealed the total occlusion of one of the 3 major coronary artery branches. AMI patients underwent angioplasty

and were treated with heparin, isosorbide dinitrate, and aspirin. Control subjects underwent coronary angiography to rule out ischemic heart disease on the day of admission. There were no significant differences regarding the use of β -adrenoceptor blockers, calcium antagonists, and ACE inhibitors between the control and AMI patients. The protocol was approved by the Institutional Ethics Committee, and informed consent was obtained from all subjects.

Quantification of MNC^{CD34+}

The circulating MNC^{CD34+} count was quantified on days 1, 3, 7, 14, and 28. In brief, peripheral white blood cells were stained with a fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibody (Becton-Dickinson). Samples were subjected to a 2D side scatter-fluorescence dot plot analysis (FACScan, Becton-Dickinson).⁴ After appropriate gating, the number of MNC^{CD34+} with low cytoplasmic granularity (low sideward scatter) was quantified and expressed as number of cells per 10⁶ white blood cells. In control subjects, circulating PB-MNC^{CD34+} were quantified on days 1 and 7.

Cell Culture Assay for Circulating EPCs

PB (20 mL) was obtained on days 1 and 7, and MNCs were isolated by a density-gradient centrifugation method.⁵ MNCs were cultured on gelatin-coated 6-well plates in medium-199 containing 20% FBS, EC growth supplement, antibiotics (Gibco), and heparin (10 U/mL). EPCs were defined by the expression of EC lineage-markers (kinase insert domain receptor [KDR], vascular endothelial [VE]-cadherin, CD31, and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine

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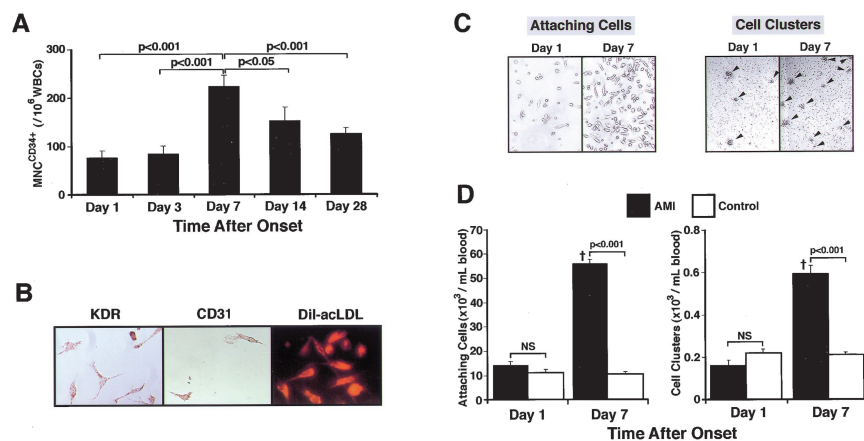


Figure 1. A, Time course of circulating MNC^{CD34+} counts after AMI. B, Spindle-shaped and attaching cells expressed EC lineage-markers and took up DiI-acLDL. C, Typical morphological structures of EPCs and cell clusters are shown. D, More EPCs and cell clusters developed from PB-MNCs obtained on day 7 than those obtained on day 1 in AMI patients, whereas they did not increase in controls.

perchlorate-labeled acetylated LDL [DiI-acLDL] incorporation) and by negative CD45 antigen.^{2,5} Thirty microscopic fields from 6 randomly selected wells were examined in each sample at day 7 of culture, and numbers of EPCs and cell clusters were expressed as number of cells or clusters per original PB (1 mL).

Biochemical Measurements

PB (5 mL) was collected from patients with AMI on days 1, 3, 7, 14, and 28. Complete cell counts and serum creatine kinase levels were examined as routine tests, and plasma levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-8 levels were measured using commercially available ELISA kits. In control subjects, PB (5 mL) was obtained on days 1 and 7 to measure plasma VEGF levels.

Statistics

Values are expressed as mean±SE. Data were subjected to 1-way ANOVA followed by Fisher’s test for comparison between any 2 means. Differences of *P*<0.05 were considered significant.

Results

Circulating MNC^{CD34+}

Figure 1A shows the time course of circulating MNC^{CD34+} counts. MNC^{CD34+} increased after the onset of AMI and peaked on day 7. MNC^{CD34+} then gradually decreased, but the number on day 28 was still greater than that on day 1. In controls, circulating MNC^{CD34+} counts on days 1 and 7 were similar (113±8 and 117±13/10⁶ WBCs). The MNC^{CD34+}

counts did not differ between the AMI and control groups on day 1, but they were significantly greater in the AMI group than in the control group on day 7 (*P*<0.001).

EPC Culture Assay

From cultured PB-MNCs, cell clusters developed and spindle-shaped attaching cells sprouted from the clusters. The morphological appearance of attaching cells resembled that of EPCs differentiated from human PB, which we reported previously.^{2,5} More than 80% of attaching cells expressed EC lineage-markers (KDR, VE-cadherin, and CD31) and took up DiI-acLDL. Representative photomicrographs of KDR- and CD31-immunostaining and DiI-acLDL incorporation are shown in Figure 1B. Attaching cells, however, did not express CD45, a common leukocyte antigen (data not shown). Attaching cells thus expressed multiple EC antigens, and we defined attaching cells as a major population of EPCs.

Cell culture assays revealed that more cell clusters and EPCs developed from MNCs obtained on day 7 than those obtained on day 1 in AMI patients (Figures 1C and 1D). In contrast, the numbers of EPCs and cell clusters did not change between day 1 and day 7 in controls (Figure 1D).

Plasma Levels of Cytokines

The Table shows the time course of circulating white blood cell counts, serum creatine kinase, and plasma VEGF, bFGF, G-CSF, GM-CSF, IL-3, and IL-8 levels after the onset of AMI. Only plasma VEGF levels were significantly elevated,

Time Courses of White Blood Cell Counts and Levels of Creatine Kinase and Plasma Cytokines

	Day 1	Day 3	Day 7	Day 14	Day 28	<i>P</i>
WBC, ×10 ³ /mm ³	11.8±1.0	7.7±1.1	7.5±0.8	7.0±0.6	4.8±0.6	<0.01
CK, U/L	1650±300	701±304	164±69	51±6.0	35±10	<0.01
VEGF, pg/mL	85±11	150±46	171±31	143±17	142±33	<0.01
bFGF, pg/mL	45±5.0	40±3.1	43±2.8	43±4.4	37±2.5	NS
G-CSF, pg/mL	98±1.0	95±10	82±7.5	74±4.5	77±3.2	NS
GM-CSF, pg/mL	9.8±0.5	8.8±0.3	9.1±0.2	9.1±0.1	9.2±0.4	NS
IL-8, pg/mL	16±3.7	13±0.8	13±1.0	13±1.0	13±0.7	NS
IL-3, pg/mL	64±4.0	72±2.6	70±4.6	74±3.2	70±4.8	NS

Values are mean±SE. WBC indicates white blood cell; CK, creatine kinase.

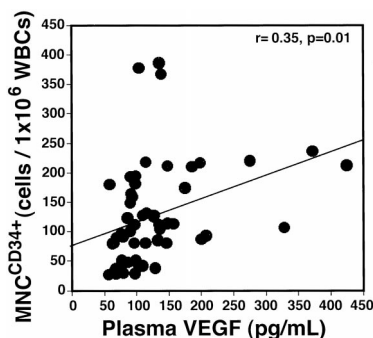


Figure 2. Circulating MNC^{CD34+} counts correlated with plasma VEGF levels in patients with AMI.

peaking on day 7; the levels of the other 5 cytokines were unchanged. Because it was only VEGF that was elevated in the plasma of AMI patients, we measured only plasma VEGF levels in the control group. VEGF levels slightly but significantly increased on day 7 compared with day 1 (100 ± 6 versus 72 ± 2 pg/mL) in the control group. However, on day 7, plasma VEGF levels were significantly greater in the AMI group than in the control group (171 ± 31 versus 100 ± 6 pg/mL, $P < 0.01$).

Circulating VEGF and MNC^{CD34+}

We examined the potential relationship between MNC^{CD34+} counts and plasma cytokine levels in AMI. Simple regression analysis revealed that the number of circulating MNC^{CD34+} positively correlated with the plasma levels of VEGF ($r=0.35$, $P=0.01$; Figure 2). There were no significant relationships between MNC^{CD34+} counts and bFGF ($P=0.06$), G-CSF ($P=0.07$), GM-CSF ($P=0.24$), IL-3 ($P=0.56$), or IL-8 ($P=0.09$) (plots not shown). The MNC^{CD34+} counts did not correlate with maximum serum creatine kinase levels ($P=0.52$). In control subjects, plasma levels of VEGF did not correlate with MNC^{CD34+} counts (plots not shown).

Discussion

Because EPCs differentiate from MNC^{CD34+} and mobilize from bone marrow,^{2,3} we first quantified circulating MNC^{CD34+}. Circulating MNC^{CD34+} counts significantly increased in patients with AMI, peaking on day 7, but they did not change in controls. Our results suggest that AMI likely stimulated bone marrow, and MNC^{CD34+} were subsequently mobilized.

Currently, little is known about how MNC^{CD34+} are mobilized after AMI. Inflammatory cytokines are released from ischemic tissues and may stimulate bone marrow to release EPCs and MNC^{CD34+}.³ Indeed, Asahara et al^{3,6} reported that hematopoietic/angiogenic cytokines (eg, VEGF and GM-CSF) mobilized EPCs in animals. Thus, we analyzed the time course of plasma levels of major hematopoietic/angiogenic cytokines in AMI patients. Among the 6 cytokines we measured, only plasma VEGF levels were significantly elevated (Table). The precise origin(s) of circulating VEGF is unknown, but ischemic cardiac tissues likely secrete VEGF because the promoter sequence of the *VEGF* gene contains

hypoxia-responsive elements. In fact, a recent study showed that myocardial VEGF expression was enhanced in patients with AMI.⁷ Interestingly, plasma VEGF levels positively correlated with the numbers of MNC^{CD34+} in AMI (Figure 2) in the present study, which is consistent with a recent report showing that VEGF functions as a mobilizer for EPCs in patients with coronary artery disease receiving therapeutic *VEGF* gene transfer.⁸

Because MNC^{CD34+} give rise to EPCs and to hematopoietic progenitors, we analyzed the number of circulating EPCs by PB-MNC culture assay. In our previous studies, a subset of PB-MNCs differentiated into EPCs during culture.^{2,5} In the present study, PB-MNCs formed multiple cell clusters, and spindle-shaped attaching cells sprouted from the clusters. The morphological appearance of attaching cells resembled that of EPCs originating from human PB, which we reported recently.^{2,5} In addition, >80% of attaching cells expressed EC-lineage markers and function (KDR, VE-cadherin, CD31, and DiI-acLDL uptake). Thus, we defined attaching cells as a major population of EPCs. A greater number of EPCs and cell clusters developed from a culture of PB-MNCs obtained on day 7 than those obtained on day 1 in AMI patients. Given the fact that EPCs derive from MNC^{CD34+},^{2,5} the results of the culture assay for circulating EPCs are consistent with the time course of the circulating MNC^{CD34+} counts (Figure 1A).

The present study has several limitations. First, we do not know whether EPCs participate in neovascularization after AMI. Because one cannot obtain cardiac tissues from AMI patients and because bone marrow-derived ECs cannot be distinguished from native ECs due to the lack of exclusive markers, this issue may be difficult to prove. Nevertheless, a recent study showed that bone marrow-derived EPCs had participated in neovascularization in patients with fatal AMI with preceding allogeneic bone marrow transplantation.^{9,10} Also, Asahara et al³ showed that EPCs were mobilized from bone marrow and accumulated within the ischemic border zone after AMI in animals. Thus, the elevated circulating EPCs likely contribute to neovascularization (postnatal vasculogenesis), although it is unknown whether EPCs can vascularize scar tissues as well. Second, mature ECs may also circulate in the PB. However, Mutin et al¹¹ reported that the number of circulating mature ECs was low, even in patients with AMI (10 to 100 cells/mL blood), whereas circulating EPCs ranged between 0.3 to 1×10^4 cells/mL.^{5,8} Moreover, mature ECs have a low proliferative activity and do not participate in neovascularization.^{9,12,13} Third, doses of medications, such as isosorbide dinitrate and aspirin, were different between the AMI and control groups. However, no study has reported that such drugs alter either hematopoiesis or MNC^{CD34+} mobilization. Thus, it is less likely that EPCs were mobilized in response to these drugs in AMI patients. Finally, all subjects underwent cardiac catheterization using heparin; thus, the catheterization procedure itself did not likely account for the augmented EPC mobilization.

In summary, the present study is the first to demonstrate that EPCs and their putative precursor, MNC^{CD34+}, are mobilized into PB during an acute ischemic event in humans. The functional roles of mobilized EPCs should be determined further.

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