Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction

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Endothelial progenitor cell (EPC) mobilization has been reported following tissue damage, whereas no data are available regarding the mobilization of hematopoietic progenitor cells (HPCs). We performed the phenotypic and functional analysis of circulating CD34$^+$ progenitor cells in patients with acute myocardial infarction (AMI), assessed from admission up to 60 days, in patients with stable angina pectoris (SA), and in healthy controls (CTRLs). In patients with AMI at admission (T0), the number of circulating CD34$^+$ cells was higher ($P < .001$) than in CTRLs and became comparable with CTRLs within 60 days. Both the number of CD34$^+$ cells coexpressing CD33, CD38, or CD117 and the number of HPCs was higher ($P < .02$ for all) in patients with AMI at T0 than in CTRLs, as was the number of hematopoietic colonies ($P \leq .03$). Patients with AMI (T0) had a significantly increased number of CD34$^+$ vascular endothelial growth factor receptor 2–positive (VEGFR-2$^+$) cells ($P < .002$) with respect to CTRLs, including CD34$^+$ CD133$^+$VEGFR-2$^+$ and CD34$^+$ CD117$^+$ VEGFR-2$^+$ EPCs. The number of endothelial colonies was higher in patients with AMI (T0) than in CTRLs ($P < .05$). No significant difference was documented between patients with SA and CTRLs. Spontaneous mobilization of both HPCs and EPCs occurs within a few hours from the onset of AMI and is detectable until 2 months. (Blood. 2005;105:199-206)

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Introduction

Acute myocardial infarction (AMI) has always been closely associated with the idea of irreversible tissue damage followed by scar replacement. This dogma has been translated into therapeutic terms by mainly concentrating on early reperfusion in order to limit myocardial necrosis and subsequent remodeling. However, recent experimental data documenting myocardial tissue regeneration suggest the possibility of adopting a stem cell–based therapeutic approach.[1-7] In animal models, bone marrow cells can act as a source of progenitors capable of homing, differentiating, and replacing cardiac necrotic cells when given by intramyocardial, intracoronary, or intravenous injection,[1-3] or by pharmacologic mobilization.[4,5] In postnatal human life, the expression of the CD34 surface antigen characterizes a heterogeneous population of cells including hematopoietic progenitor cells (HPCs), endothelial progenitor cells (EPCs), and mature endothelial cells[8] that in healthy subjects are localized in the bone marrow, with very small amounts circulating in the peripheral blood (PB).[9,10] The possibility of using extracardiac progenitor cells for myocardial regeneration in humans has been suggested by studies performed in transplanted hearts in which, albeit at an extremely low rate, host progenitors can migrate and possibly differentiate into cardiomyocytes.[6,7]

It has been reported that the coexpression of surface CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR-2) antigens identifies early EPCs[11]; the lack of the CD133 antigen characterizes a mature endothelial cell, whereas the lack of the VEGFR-2 defines a subset of mainly immature/noncommitted HPCs.[12] In addition, recent papers suggested that bone marrow–derived cells coexpressing CD34, CD117, and VEGFR-2 antigens may also represent a population containing EPCs that can induce both vasculogenesis and angiogenesis, at least in experimental animal models of myocardial infarction.[3-5,13-15]

EPCs and their relationships with a number of cytokines have been studied in burns, diabetes, coronary artery disease (CAD), aging, and smoking,[16] but little is known about spontaneous EPC mobilization in response to AMI. It has been reported that both CD34$^+$ cells and VEGF increase and reach a peak 7 days after the onset of symptoms,[17] whereas, to the best of our knowledge, no data are available on the mobilization of cell subsets that include hematopoietic progenitors. A better understanding of the cell subsets involved in mobilization processes after AMI could be of help when related therapeutic options are considered, including their potential manipulations.

The aim of this study was to evaluate the possible physiologic mobilization of both hematopoietic and endothelial CD34$^+$ cells after AMI.[16] We investigated the number of circulating HPCs and EPCs by both cytofluorimetric analysis and cell culture from the onset of AMI up to 60 days; in addition, we measured plasma levels of cytokines potentially related to bone marrow cell mobilization.[18]

Patients, materials, and methods

Study population

Patients with AMI. From September 2002 to March 2003, we studied 26 patients ($<$ 75 years old) admitted to our Institution, because of a first AMI within 6 hours from the onset of symptoms, who were eligible for primary percutaneous transluminal coronary angioplasty (PTCA). AMI was defined according to the presence of prolonged ($>$ 20 minutes) cardiac chest pain associated with persistent (nitroglycerine refractory) ST-segment elevation more than 1 mm in 2 or more contiguous peripheral, or more than 2 mm in 2 or more contiguous precordial, electrocardiographic leads. Exclusion criteria were previous MI; history of renal, hepatic, hematologic-coagulative disorders, acute-chronic inflammatory diseases, malignancies, recent trauma, or surgical-percutaneous interventions ($<$ 6 months); cardiogenic shock; or major bleeding requiring blood transfusion.

Control groups. We studied 10 patients with stable angina pectoris (SA) according to the exclusion parameters described for AMI and meeting the following criteria: (a) history of transient episodes of typical chest pain on effort, lasting unchanged for more than 3 months and not associated with rest angina; (b) documented exercise-induced myocardial ischemia; and (c) angiographically proven CAD.

Seventeen age- and sex-matched healthy volunteers represented the healthy control group (CTRL).

Consent. The study protocol was approved by our Institutional Ethical Committee and all the enrolled subjects gave their written informed consent according to the Declaration of Helsinki.

Study design

PB samples for flow cytometric analysis and determination of plasma cytokine levels were obtained from patients with AMI at admission (T0); immediately and 24 hours after primary PTCA; and 7, 15, and 60 days later. The clinical follow-up was performed in all the patients; on the other hand, after the discharge from the hospital, for logistical reasons, the phenotypic analysis of progenitor cells could not be evaluated at all the established time points in some of the patients enrolled. As heparin can hamper the detection of plasma VEGF levels,[19,20] patients who had received heparin before T0 (n = 10) were excluded from VEGF analysis; in the remaining 16 patients plasma VEGF levels were evaluated at T0 and then after heparin discontinuation (ie, on days 7, 15, and 60). At admission, 7 patients with AMI also underwent a heparinized PB sampling for in vitro cell cultures.

A PB sample for cytofluorimetric analysis, cytokines, and high-sensitivity C-reactive protein (hsCRP) evaluation was collected from patients with SA (asymptomatic subjects at least 48 hours after any documented episodes of myocardial ischemia) and from CTRLs. In 7 cases of each group, a further heparinized PB sample for in vitro cell cultures was obtained.

Blood samples for cytofluorimetric analysis and in vitro cultures were processed within 2 hours after drawing, whereas plasma samples were stored at $-70°$C until used for cytokine determinations.

Coronary angiography, primary PTCA, and medical treatment

All patients with AMI underwent emergency coronary angiography from the right femoral approach, followed by primary PTCA and stent deployment according to standard techniques. The procedural pharmacologic protocol included intravenous aspirin (250 mg), unfractionated heparin (70 IU/kg; maximum, 7000 IU), and abciximab (0.25 mg/kg, followed by 0.125 μg/kg/min for 12 hours). Aspirin (100 mg orally daily) plus ticlopidine (500 mg orally daily) were then administered for 1 month, followed by aspirin indefinitely. Short-term subcutaneous heparin (mean treatment 4 $\pm$ 1 days) and nitroglycerine, beta-blockers, and calcium antagonists were administered according to the indications of the referring physician. Ten patients were chronically treated with statins and 14 with angiotensin-converting enzyme (ACE) inhibitors.

In the SA group, 5 patients had statins and 4 patients had ACE inhibitors on board when studied. All patients were receiving 100 mg aspirin orally daily.

None of the CTRLs was treated by any drug in the 3 months before the study, except for 2 who were on therapy with statins and 4 who were on therapy with ACE inhibitors.

Enzymatic and echocardiographic quantification of myocardial damage

In patients with AMI, the levels of creatine kinase (CK) and CK isoenzyme myocardial and brain (MB) (CKMB) were measured at admission, every 6 hours during the first 24 hours, and then every day until normalization. Peak CK (CKp) and CKMB (CKMBp) levels, as well as CK and CKMB areas under the curve (AUCs), were considered reliable indexes of myocardial tissue necrosis. The echocardiographic left ventricular ejection fraction (LVEF) as well as the LV wall motion score index (LVWMSI, 16-segment model) were calculated and used to quantify the extent of myocardial damage, both in the acute phase (within 24 hours from admission) and in the follow-up (after 1 year).

High-sensitivity CRP concentrations

The assay for hsCRP was conducted according to the manufacturer's instructions (Dade Behring, Düdingen, Switzerland) in all patients with SA and CTRLs.

Flow cytometric analysis

Fifty microliters of PB collected in EDTA (ethylenediaminetetraacetic acid)–containing tubes was incubated for 30 minutes at 4°C with 10 μL of 7-amino-actinomycin D (7-AAD), 20 μL of fluorescein (FITC)–conjugated anti-CD34 and phycoerythrin (PE)–conjugated anti-CD38, or PE–anti-CD117 (Becton Dickinson, Pharmingen, San Jose, CA). The staining with FITC–anti-CD34, PE–anti-CD33, and biotin-conjugated anti–VEGFR-2 (Sigma Chemical, St Louis, MO) revealed using peridinin-chlorophyll-protein (PerCp)–streptavidin (Becton Dickinson, Pharmingen) was also evaluated. Appropriate isotype controls were used for each staining procedure. After red cell lysis, the samples were centrifuged and the pellets resuspended in 300 μL of phosphate buffer with 0.5% of fetal calf serum (FCS). Cells (2×10^5) were acquired by flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA) and analyzed by CellQuest software (BD Bioscience, San Jose, CA). The analysis was performed excluding cellular debris in a side scatter/forward scatter (SSC/FSC) dot plot, whereas dead (7-AAD bright) and apoptotic (7-AAD dim) cells were excluded by a SSC/7-AAD dot plot. The percentage of positive cells was calculated subtracting the value of the appropriate isotype controls. This specific percentage of positive cells was converted into the absolute number of positive cells/μL using the following formula: specific percentage of positive cells $\times$ white blood cell (WBC) count/100.[21]

Flow cytometric analysis of CD34+ hematopoietic progenitor cells

The flow cytometric analysis of PB cells from 15 CTRLs and from 15 patients with AMI at time of admission, 24 hours, and 7, 15, and 60 days later was performed adopting the gating strategy defined by the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines[22] using the CD34 and CD45 expression patterns as well as their morphologic qualities for detection. For this purpose we stained 100 μL of PB collected in EDTA-containing tubes with an appropriate amount of FITC–anti-CD45 and PE–anti-CD34 antibodies for 20 minutes at 4°C. In addition, we added a PE mouse immunoglobulin G1 (IgG1) antibody to a second anti-CD45–stained blood sample as isotype control. Following lysis of red blood cells, we acquired at least 200 000 CD45+ cells, and the absolute number of HPCs/μL was obtained using the following formula: percentage CD34+ cells $\times$ WBC/100. Two investigators in blinded experiments independently assessed the number of HPCs.

CD34+ cell selection and staining

Briefly, 15 mL of heparinized PB from patients with AMI at admission and 50 mL of buffy coats from healthy CTRLs underwent density gradient

centrifugation (1077 g/mL; Biocoll, Seromed Biochrom, Berlin, Germany) and CD34$^+$ cell purification was performed using a MiniMacs CD34 separation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. The selected cells were stained with FITC–anti-CD34, biotin-conjugated anti–VEGFR-2 monoclonal antibody (moAb) revealed with PerCp-streptavidin, and PE–anti-CD133 (10 μL; Miltenyi Biotech) or PE–anti-CD117. The isotype control was performed staining the immunomagnetically selected cells with FITC–anti-CD34, biotin-conjugated IgG1 revealed with PerCp-streptavidin, and PE-IgG1. The samples were analyzed by flow cytometry and a minimum of 100 000 events were acquired; selected CD34$^+$ cells (80%-90% pure) were further electronically gated and their positivity to anti-CD133, anti-CD117, and anti–VEGFR-2 moAbs was expressed as percentage of gated CD34$^+$ cells.

Cytokine measurements

Platelet-poor plasma samples from EDTA PB were stored at −70°C. VEGF or stromal-derived growth factor-1α (SDF-1α) levels were measured using commercially available kits (VEGF-*duoset* and Quantikine SDF-1α; R&D Systems, Minneapolis, MN). Stem cell factor (SCF) was measured using an enzyme-linked immunosorbent assay with a SCF moAb for coating, a biotin-conjugated SCF antibody for detection, and recombinant human SCF as standard (all from R&D Systems). The detection limits of the assays were 7.5 pg/mL for VEGF, 156 pg/mL for SDF-1α, and 5.8 pg/mL for SCF.

In vitro cultures of HPCs

PB mononuclear cells (PBMCs; 5×10^5) were plated in 35-mm Petri dishes in methylcellulose with 30% FCS, 10 ng/mL interleukin 3 (IL-3), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), and 50 ng/mL SCF. After 14 days at 37°C, 5% CO_2, the numbers of erythroid burst-forming units (BFU-Es) and granulocyte macrophage colony-forming units (CFU-GMs) were counted using standard criteria.[23]

In vitro cultures of EPCs

PBMCs (1×10^6) were plated in liquid culture in a 24-well plate coated with fibronectin in Endomed medium (StemCell Technologies, Vancouver, BC, Canada) containing 20% FCS and VEGF. After 7-day incubation at 37°C, 5% CO_2, the endothelial colonies, defined as central clusters of cells surrounded by spindle-shaped cells,[24] were counted using an inverted microscope (Leitz GmbH, Oberkochen, Germany). The endothelial nature of the colonies was confirmed by in situ staining with antibodies anti-CD34 (CS37; Exalpha Biologicals, Watertown, MA), anti-CD31 (1F11; Immuno-tech, Marseille, France), anti–VE-cadherin (Bender Med System, San Bruno, CA), anti-CD45 (2D1; BD Bioscience), and anti–von Willebrand factor (anti-VWF; Dako, Glostrup, Denmark). Double-labeling experiments were performed by using anti-CD31 and anti–VE-cadherin antibodies or anti-CD45 and anti-VWF antibodies. Antibody binding was revealed through an Alexa Fluor-488– or Alexa Fluor-594–conjugated goat anti-mouse or antirabbit antibody (Molecular Probes, Eugene, OR). Negative controls were performed by replacing the primary antibody with an isotype-matched mouse moAb or an irrelevant rabbit polyclonal antibody.

Statistical analysis

The data are presented as median (range). Data were compared between diagnostic groups by means of Kruskal-Wallis analysis of variance (ANOVA). Bonferroni correction for multiple tests was applied for posthoc pair-wise comparisons. The changes over time of the (log-transformed) biologic parameters were evaluated by fitting a regression model for repeated measures (with the calculation of Huber-White robust standard errors). The AUC was calculated for CK over 48 hours and for circulating cell subsets from T0 to 7 days, following the trapezoidal rule.[25] Single missing values during the observation time were interpolated. The correlations between the variables were assessed using the Spearman correlation coefficient. A P value of less than .05 was considered statistically significant. All computations were performed with STATISTICA software (Statsoft, Tulsa, OK).

Results

Patient characteristics and clinical, angiographic, and procedural data

As shown in Table 1, there were no significant differences among patients with AMI, patients with SA, or CTRLs regarding age, sex, and body mass index (BMI). Patients with AMI and SA had comparable frequency of cardiovascular risk factors (other than more smokers in patients with AMI). Two patients with AMI (8%) suffered from preinfarction angina pectoris and, accordingly, they showed a less diffuse CAD than patients with SA (multivessel disease 31% vs 70%); collateral flow toward the culprit artery was rare (4%). Mechanical recanalization was quickly performed (median, 195 minutes from the onset of symptoms) with a high success rate (thrombolysis in myocardial infarction grade 3 [TIMI 3] flow rate 92%). Left ventricular function was only marginally compromised in the acute phase and slightly improved after 1 year. The rate of major adverse cardiovascular events was low (n = 5; 3 re-infarctions and 2 target lesion revascularization), all but 1 cardiovascular event occurred after the end of the study (60 days). White blood cell (WBC) count was significantly higher ($P < .01$) in patients with AMI at admission (median, 13.6×10^9/L [13.6×10^3/μL]; range, 7.3-20.2 $\times$ 10^9/L [7.3-20.2 $\times$ 10^3/μL])

Table 1. Clinical, angiographic, and procedural data of patients with AMI, patients with SA, and healthy CTRLs

	AMI, n = 26	SA, n = 10	CTRLs, n = 17
Age, y, mean (range)	60 (41-74)	60 (46-75)	51 (40-70)
Males (%)	21 (80)	8 (80)	14 (80)
Body mass index	26.7	29.7	25.4
Cardiovascular risk factors (%)			
Family history	6 (25)	4 (40)	3 (18)
Hypertension	8 (30)	4 (40)	4 (23)
Dislipidemia	5 (20)	5 (50)	3 (18)
Smoking	18 (70)*	2 (20)	2 (12)
Diabetes	2 (7)	3 (30)	1 (6)
Previous angina pectoris (%)	2 (8)	0 (0)	0 (0)
Statins (%)	10 (38)	5 (50)	2 (12)
ACE inhibitor (%)	14 (53)	4 (40)	4 (23)
Anterior AMI (%)	12 (45)	—	—
Multivessel CAD (%)	8 (31)†	7 (70)	—
Before PTCA (%)			
TIMI 0-2	23 (88)	—	—
TIMI 3	3 (12)	—	—
Collateral flow	1 (4)	—	—
Time to balloon, min (range)	195 (180-300)	—	—
After PTCA (%)			
TIMI 0-2	2 (8)	—	—
TIMI 3	24 (92)	—	—
pCK, mU/mL (range)	2055 (161-3907)	—	—
pCK-MB, mU/mL (range)	221 (77-372)	—	—
LVEF (%)			
24 h	47 ± 9	—	—
1 y	53 ± 8	—	—
LVWMSI (%)			
24 h	1.8 ± 0.31	—	—
1 y	1.5 ± 0.3	—	—

— indicates not applicable; ACE, angiotensin-converting enzyme; CAD, coronary artery disease; PTCA, primary percutaneous transluminal coronary angioplasty; TIMI, thrombolysis in myocardial infarction; LVEF, left ventricular ejection fraction; and LVWMSI, left ventricular wall motion score index.

*$P \leq .008$ versus SA and CTRLs.

†$P = .03$ versus SA.

and 24 hours after the onset of AMI (median, 10.0×10^9/L [10.0×10^3/μL]; range, 7.0-15.8 × 10^9/L [7.0-15.8 × 10^3/μL]) with respect to either patients with SA (median, 7.7 × 10^9/L [7.7 × 10^3/μL]; range, 5.8-8.2 × 10^9/L [5.8-8.2 × 10^3/μL]) or CTRLs (median, 6.2 × 10^9/L [6.2 × 10^3/μL]; range, 4.0-8.9 × 10^9/L [4.0-8.9 × 10^3/μL]); WBC count of patients with AMI tested 7, 15, or 60 days after AMI was comparable to that of patients with SA or CTRLs. In patients with SA, hsCRP serum levels (median, 0.16 mg/dL; range, 0.05-0.45 mg/dL) were comparable to those found in CTRLs (median, 0.12 mg/dL; range, 0.05-0.39 mg/dL), further confirming the biologic and clinical stability of the disease.

Flow cytometric analysis of circulating CD34+ cells

The percentage of total circulating CD34+ cells was significantly higher in patients with AMI at admission ($P < .01$) than CTRLs (Figure 1A). In order to evaluate the absolute number per microliter of circulating CD34+ cells we used the following formula: specific percentage of CD34+ cells × WBC/100. We found that in patients with AMI, the median value of the number of circulating CD34+ cells/μL was 5.8-fold increased with respect to that of CTRLs ($P < .001$; Figure 1B). In patients with SA, both the percentage and the absolute number per microliter of CD34+ cells was not significantly different from that of CTRLs (Figure 1A-B). Five of 10 patients with SA were tested twice within 7 days and the values obtained were comparable (not shown). The longitudinal study of patients with AMI showed a decreasing trend of the number per microliter of circulating CD34+ cells, which at day 7 was statistically lower than at T0 ($P < .001$) although higher than that of CTRLs ($P < .02$) and became comparable to that of CTRLs within 60 days (Figure 1C).

Flow cytometric analysis of the circulating CD34+ cell subsets

The number per microliter of circulating CD34+ cells coexpressing CD33, CD38, or CD117, and that of endothelial cells evaluated as CD34+VEGFR-2+ (including mature and immature endothelial cells) was significantly higher in patients with AMI at T0 than in CTRLs (Figure 2A-D; Figure 2E-H for staining dot plots of one representative patient with AMI). In patients with SA, the number per microliter of these cell subsets was not statistically different from CTRLs (Figure 2A-D).

The longitudinal study performed in patients with AMI showed that the number per microliter of circulating CD34+CD33+ cells gradually decreased and at 60 days became statistically lower than at T0 ($P < .01$; Figure 2A). On the other hand, within 60-day follow-up, the number per microliter of circulating CD34+CD38+, CD34+CD117+, and CD34+VEGFR-2+ cells was never significantly lower than at T0 (Figure 2B-D).
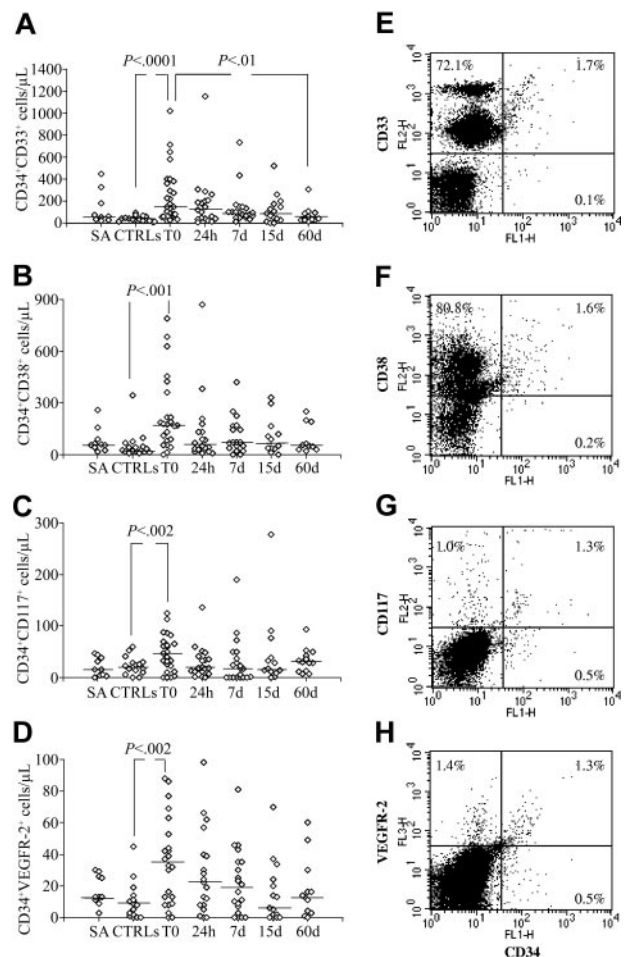
Figure 2. Longitudinal study of CD34+ cell subsets in patients with AMI. Longitudinal study of the number per microliter of CD34+CD33+ cells (A), CD34+CD38+ (B), CD34+CD117+ (C), and CD34+VEGFR-2+ cells (D) in patients with AMI at admission (T0); after 24 hours; and after 7, 15, and 60 days. Patients with stable angina pectoris (SA) and healthy controls (CTRLs) are shown for comparison but not included in the statistical analysis performed by the regression model for repeated measures. All the cell subsets are significantly increased in patients with AMI at T0 with respect to CTRLs. The number per microliter of CD34+CD33+ cells at T0 significantly decreases within 60 days ($P < .01$; A) becoming comparable to that found in CTRLs. CD34+CD38+ (B), CD34+CD117+ (C), and CD34+VEGFR-2+ cell subsets (D) of patients with AMI, although showing a decreasing trend, on day 60 are not significantly lower than at T0. Solid lines represent the median values. The panels at the right of the figure show the dot plots of peripheral blood cells from one representative patient with AMI stained with FITC–anti-CD34 combined with PE–anti-CD33 (E), PE–anti-CD38 (F), PE–anti-CD117 (G), or PerCp-streptavidin–biotinylated anti–VEGFR-2 (H). Markers were set according to the appropriate isotype controls.
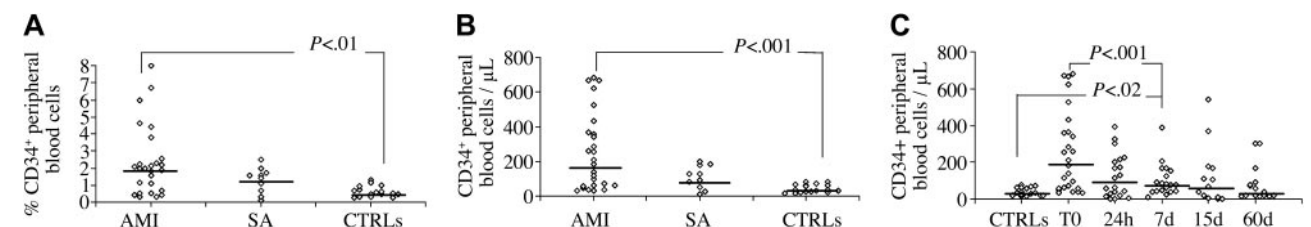
Figure 1. Evaluation of circulating total CD34+ cells in patients with AMI, SA, and CTRLs. Percentage (A) and absolute number per microliter (B) of circulating CD34+ cells in patients with acute myocardial infarction (AMI) at admission, in patients with stable angina pectoris (SA), and in healthy controls (CTRLs). The longitudinal study of patients with AMI (C) shows a decreasing trend of the number per microliter of circulating CD34+ cells, which at day 7 is statistically lower than at T0 ($P < .001$) but higher than that of CTRLs ($P < .02$) and becomes comparable to that of CTRLs within 60 days. Solid lines represent the median values. In panel C, the CTRLs are shown but not included in the statistical analysis performed by the regression model for repeated measures.

Table 2. Number per microliter of CD34$^+$ HPCs evaluated by ISHAGE guidelines in peripheral blood of patients with AMI at T0; 24 hours after onset; and 7, 15, and 60 days after onset and in healthy CTRLs

	AMI					CTRLs
	T0	24 h	7 d	15 d	60 d	
n	15	15	15	10	10	15
HPCs, number per microliter	2.8 (0-7.5)*	0.9 (0-4.7)	1.8 (0-6.7)	0.77 (0-1.54)	0.7 (0.5-0.9)	0.6 (0-1.2)

Results are shown as median (range).
*$P < .02$ versus CTRLs.

We also evaluated the number per microliter of CD34$^+$CD38$^-$ cells in patients with AMI from T0 to 60 days, in patients with SA, and in CTRLs. No difference was found between AMI at admission, patients with SA, and CTRLs; in addition, there was no variation of this cell subset in patients with AMI tested at the different time points (not shown). No difference in the proportion of circulating CD34$^+$ or CD34$^+$ cell subsets before and after PTCA was found (not shown).

Characterization and functional evaluation of circulating HPCs

We assessed by cytofluorimetric analysis (ISHAGE guidelines) and by in vitro culture both the number of CD34$^+$ HPCs and the frequency of BFU-E and CFU-GM colonies. The number per microliter of CD34$^+$ HPCs was significantly higher in patients with AMI at T0 than in CTRLs ($P < .02$) and normalizes within 60 days after the onset of AMI (Table 2; Figure 3 for one representative patient evaluated at T0). The increased number per microliter of CD34$^+$ HPCs at T0 was in keeping with the finding that the number of BFU-Es and CFU-GMs/5×10^5 PBMCs was also higher ($P \leq .03$) in patients with AMI at T0 than in CTRLs (Table 3). The number of colonies derived from PBMCs of patients with SA was comparable to that of CTRLs (Table 3).

Characterization and functional evaluation of circulating EPCs

To test whether the increased number per microliter of CD34$^+$VEGFR-2$^+$ cells found in patients with AMI was due to an increased number of EPCs, we purified by immunomagnetic selection circulating CD34$^+$ cells from 16 patients with AMI evaluated at T0 and 8 buffy coats from healthy CTRLs. By flow cytometric analysis, we found that the percentage of CD34$^+$ cells coexpressing CD133 and VEGFR-2 antigens (early EPCs) and CD34$^+$CD133$^-$VEGFR-2$^+$ cells was higher ($P < .001$ and $P < .02$,

respectively) in patients with AMI than in healthy CTRLs. The percentage of CD34$^+$CD133$^+$VEGFR-2$^-$ cells was not significantly different in healthy CTRLs and in patients with AMI at T0 (Table 4; Figure 4A-B for one representative patient and Figure 4D-E for one healthy CTRL). Similarly, we found a significantly higher percentage of CD34$^+$ cells coexpressing CD117 and VEGFR-2 antigens ($P < .03$) than in CTRLs (Table 5; Figure 4A-C for 1 representative patient and Figure 4D-F for 1 healthy CTRL). The percentage of CD34$^+$CD117$^-$VEGFR-2$^+$ cells was higher ($P < .03$) in patients with AMI than in CTRLs, whereas the percentage of CD34$^+$CD117$^+$ VEGFR-2$^-$ was not significantly different in patients and CTRLs (Table 5).

The number of endothelial colonies/10^6 PBMCs was higher ($P < .05$) in AMI at admission than in CTRLs (Table 3); the longitudinal study of the number of colonies showed a significant decrease at 24 hours and 7 days with respect to T0 ($P < .04$), with values comparable to those of CTRLs (Figure 5A). PBMCs from patients with SA formed a number of colonies comparable to that of CTRLs (Table 3).

The colonies had a typical in vitro morphology,[24] costained for VE-cadherin and CD31 (Figure 5B-C) and also stained for VWF and CD34 endothelial antigens, whereas they did not express CD45. No morphologic difference was found between colonies from patients with AMI, patients with SA, and CTRLs.

Plasma cytokine levels

Plasma VEGF levels were higher in patients with AMI at admission than in CTRLs ($P < .04$) and significantly decreased during the 60-day follow-up ($P < .02$, regression model for repeated measures), with values on day 7 being significantly lower than those found at T0 ($P = .036$) and comparable to those of CTRLs (Table 6). In patients with SA, plasma VEGF levels showed intermediate values between AMI and CTRLs not significantly different from either of them. Plasma SCF or SDF-1α levels were comparable in patients with AMI, patients with SA, and CTRLs; no significant variation was observed during the 60-day follow-up of patients with AMI.
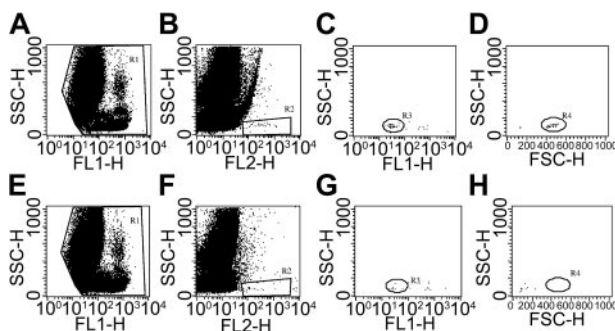
Table 3. Evaluation of the total number of colonies derived from hematopoietic (CFU-hem's) and endothelial progenitor cells (CFU-end's) obtained by in vitro cultured PBMCs from patients with AMI at T0, patients with SA, and healthy CTRLs

	AMI (T0), n = 7	SA, n = 7	CTRLs, n = 7
CFU-hem's	202 (94-250)*	44 (0-189)	86 (13-100)
CFU-GMs	90 (36-108)*	18 (0-100)	34 (5-42)
BFU-Es	94 (59-150)*	26 (0-88)	52 (8-60)
CFU-end's	39 (20-57)†	21 (12-35)	21 (8-26)

The number of committed erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells is also shown. The results, expressed as number of CFU-hem/5×10^5 PBMCs or number of CFU-end/10^6 PBMCs, are shown as median (range).
*$P \leq .03$ versus CTRLs.
†$P < .05$ versus CTRLs.

Figure 3. Gating strategy for detection of circulating hematopoietic progenitor cells (HPCs) on the basis of ISHAGE guidelines. The top panels (A-D) represent a patient with AMI at T0 stained with anti-CD45–FITC (FL1-H) and anti-CD34–PE (FL2-H). The bottom panels (E-H) show the same sample using an isotype control for anti-CD34. We first counted 200 000 CD45$^+$ cells (A,E). From this primary gate, HPCs were identified by means of additional expression of CD34 (B,F). The CD45 antigen expression (C,G) and the characteristic light-scatter properties (D,H) are shown.

Table 4. Immunomagnetically purified CD34⁺ cells from PBMCs of patients with AMI at admission and healthy CTRLs: staining with anti-CD133 and anti–VEGFR-2 antibodies

	AMI, n = 16	CTRLs, n = 8	P
CD34⁺CD133⁺VEGFR-2⁺	6.7 (3.2-18.2)	0.9 (0.4-1.1)	< .001
CD34⁺CD133⁻VEGFR-2⁺	8.2 (0-38.1)	0.6 (0.1-1.1)	< .02
CD34⁺CD133⁺VEGFR-2⁻	49.5 (11.9-84.6)	58.5 (47.9-84.8)	NS

Results, expressed as percentage of CD34⁺ cells, are shown as median (range). NS indicates not significant.

Table 5. Immunophenotypically selected CD34⁺ cells from PMBCs of patients with AMI at admission and healthy CTRLs: staining with anti-CD117 and anti–VEGFR-2 antibodies

	AMI, n = 13	CTRLs, n = 5	P
CD34⁺CD117⁺VEGFR-2⁺	23.0 (14.8-66.4)	2.6 (2.1-2.8)	< .03
CD34⁺CD117⁻VEGFR-2⁺	11.3 (1.3-18.5)	0.2 (0.1-0.4)	< .03
CD34⁺CD117⁺VEGFR-2⁻	50.7 (20.2-67.0)	62.2 (53.3-65.5)	NS

Results, expressed as percentage of CD34⁺ cells, are shown as median (range). NS indicates not significant.

Correlations among plasma cytokine levels, number of circulating CD34⁺ cells, and clinical data

In patients with AMI at admission, the increased plasma VEGF levels correlated with the number per microliter of circulating CD34⁺VEGFR-2⁺ cells (n = 16, R = 0.57, P = .04) as well as of CD34⁺CD33⁺ cells (n = 16, R = 0.56, P = .023); in addition, CKp values were correlated with the number per microliter of circulating CD34⁺VEGFR-2⁺ cells (n = 16, R = 0.58, P = .008).

No correlation was found between indexes of myocardial damage, such as CKp, CKp-MB, myocardial enzyme AUCs, or echocardiographic parameters (LVEF, LVWMSI), and plasma VEGF levels nor between VEGF levels and any other CD34⁺ cell subset. In patients with AMI, no chance existed to test possible correlations between mobilized cell subsets and prognosis, owing to the extremely low rate of major adverse cardiovascular events in the follow-up. Moreover, no relation was observed between VEGF plasma levels and any of the following parameters: age, sex, BMI, cardiovascular risk factors, infarct location, or extension of CAD (not shown). The number per microliter of circulating CD34⁺ cells or CD34⁺ cell subsets was not significantly different in patients that at the onset of AMI were or were not receiving statins or ACE inhibitors (see Table 7 for the analysis of CD34⁺, CD34⁺VEGFR-2⁺, and CD34⁺CD117⁺ cells). No significant correlation was found between the number of hematopoietic or endothelial colonies and CD34⁺ cells, CD34⁺ cell subsets, or the clinical parameters of

Figure 4. Cytofluorimetric analysis of immunomagnetically purified CD34⁺ cells. Immunomagnetically purified CD34⁺ cells from PBMCs of one representative patient with acute myocardial infarction (AMI) at admission were stained with FITC–anti-CD34, PE-IgG1, and PerCp-streptavidin–biotinylated IgG1 (A), with FITC-CD34, PE-CD133, and PerCp-streptavidin–biotinylated anti–VEGFR-2 (B), or with FITC-CD34, PE-CD117, and PerCp-streptavidin–biotinylated anti–VEGFR-2 (C). The same staining procedure was performed on purified CD34⁺ cells from one representative healthy control (CTRL; D-F). Results are expressed as percentage of CD34⁺ cells.

age, sex, BMI, cardiovascular risk factors, infarct location, or CAD extension (not shown).

Discussion

This study shows that patients with AMI have a 5.8-fold increase in the median number per microliter of total circulating CD34⁺ cells, which is appreciable within a few hours (median, 195 minutes) after symptom onset, along with higher plasma VEGF levels. Both HPCs and EPCs, assessed by cytofluorimetric analysis and by in vitro cultures, contribute to this spontaneous mobilization, which peaks early after the onset, decreases after 7 days, and normalizes within 2 months.

If compared with CTRLs and patients with SA, patients with AMI at admission have a significant increase in the number per microliter of circulating CD34⁺CD33⁺, CD34⁺CD38⁺, CD34⁺CD117⁺ cell subsets, as well as of CD34⁺CD45⁺ cells, representing HPCs according to ISHAGE analysis. The increment in circulating CD34⁺VEGFR-2⁺ cells observed in patients with AMI reflected a higher percentage of CD34⁺CD133⁺VEGFR-2⁺ and CD34⁺CD117⁺VEGFR-2⁺ EPCs; these cell subsets have been shown both in vitro[11,26] and in animal models[5,13,15] to play a role in neoangiogenic processes and cardiac repair. The result of the phenotypic analysis was confirmed by the larger number of hematopoietic and endothelial colonies obtained from PBMCs of patients with AMI at admission than from PBMCs of patients with SA or CTRLs. Although at T0 the number of progenitor cells observed in patients with AMI could be in part magnified by the number of WBCs, the increase of circulating CD34⁺ cells was also significant when we expressed our data as percentage of peripheral blood cells; in addition, the associated increased plasma VEGF levels and the higher frequency of cell colonies grown in vitro at that time point indicate that the phenomenon is real. The persistent elevation of the number per microliter of the different cell subsets in the longitudinal study of patients with AMI, unrelated to WBC count (which was comparable to that of CTRLs beginning from day 7 after AMI), further supports this interpretation. Finally, the increased number of circulating progenitor cells in patients with AMI was observed despite a higher incidence of smoking and hypertension, 2 conditions known to be associated with a reduction of EPCs.[27,28]

To the best of our knowledge, this is the first study that describes a mobilization of both hematopoietic and endothelial cells a few hours from the onset of AMI. There is only one other report that investigated a spontaneous mobilization of CD34⁺ cells after AMI,[17] but in that study, the increase of CD34⁺ cells was attributed exclusively to an increase of circulating EPCs and it was observed only 7 days after the onset of AMI. We also found a quantitatively comparable increased number of circulating CD34⁺ cells at day 7 with respect to CTRLs, but, in our hands, the highest spontaneous mobilization of CD34⁺ cells was found at T0. The
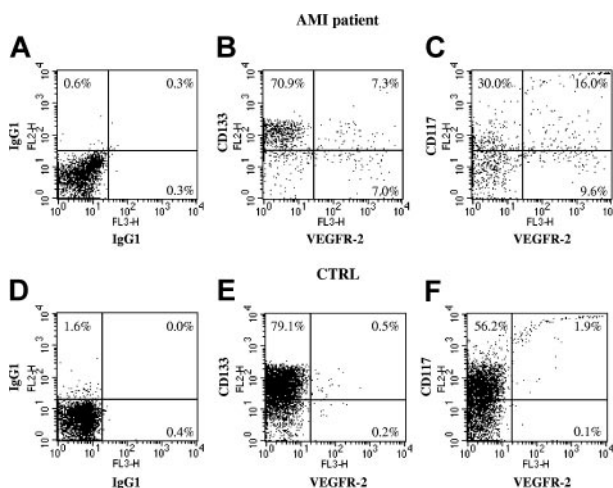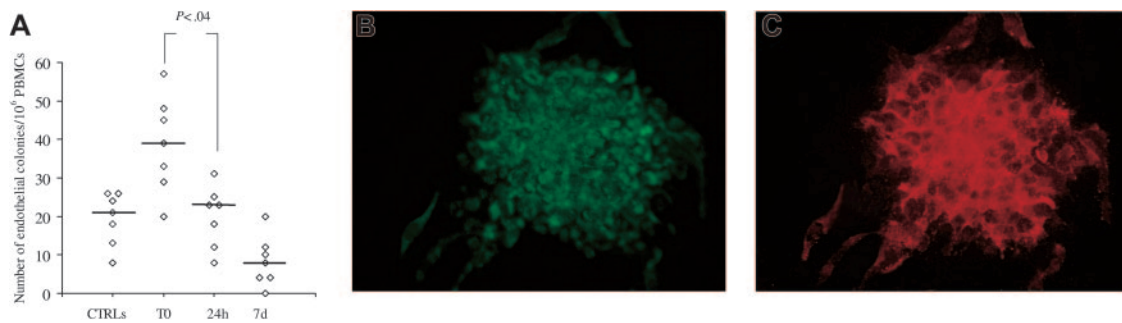
Figure 5. Follow-up study and phenotype of endothelial colonies from patients with AMI. Number of endothelial colonies obtained from PBMCs of healthy controls (CTRLs) or patients with AMI tested at T0, 24 hours, (24h), and 7 days (7d) after onset of AMI (A); the lines represent the median values. Panels B and C show an endothelial colony coexpressing both VE-cadherin (B; green) and CD31 (C; red) antigens. Specimens were stained by indirect immunolabeling using secondary antibodies conjugated with AlexaFluor-988 (green) or AlexaFluor-594 (red) fluorochromes (Molecular Probes, Eugene, OR). Mowiol4-88 (Calbiochem, La Jolla, CA) was used for mounting medium. Micrographs were captured using an Olympus IX71 inverted microscope (Olympus Europa GmbH, Hamburg, Germany) equipped with a CPlan F1 10×/0.30 objective, an Olympia Camedia C-3030 zoom with a 3× optical plus 2.5× digital zoom (Olympus), and Camedia Master 2.0 software (Olympus). Original magnification, × 250.

reasons for this discrepancy may be due to different timing in blood sampling (from day 1 to day 28 rather than from admission to day 60) and evaluation of CD34$^+$ cells as frequency (MNC^{CD34+}/10^6 WBCs) rather than absolute number per microliter. Interestingly, our data are in agreement with a previous report[26] describing an early and transient increase in circulating CD34$^+$VEGFR-2$^+$ cells following burns or cardiac surgery, peaking after 6 to 12 hours and normalizing 24 hours after the injury.

Another novel finding of our study is that levels of SCF and SDF-1α factors, affecting the number of circulating CD34$^+$ cells,[18,29,30] are comparable to CTRLs. This does not rule out the possibility that an increased production of these cytokines may be present at the site of the damage. To this regard it has been recently described that SDF-1α may constitute a homing signal in the recruitment of circulating progenitor cells to enhance endogenous repair mechanisms after ischemia.[31] In addition, a rapid kinetic of these cytokines cannot be excluded. On the other hand, we observed that plasma VEGF levels were increased in patients with AMI at admission. We did not find any relation between the extent of myocardial infarction, evaluated either by enzyme release or echocardiography, and plasma VEGF levels. However, both CKp and plasma VEGF levels at T0 correlated with circulating CD34$^+$VEGFR-2$^+$ cells. This observation is in keeping with the idea that VEGF really acts as a mobilizer of CD34$^+$ cells.[32]

The analysis of the relation between progenitor cell mobilization and prognosis of patients with AMI was beyond the aim of the study; the limited population and the low rate of adverse events in the follow-up preclude any attempt of speculation. Nevertheless, no relation was found between HPC or EPC mobilization and some prognostic surrogated end points, such as LV function (in terms of LVEF and LVWMSI) and extension of CAD (single- versus double- or triple-vessel disease). In addition, no relation was found between a number of anamnestic (age, sex, number of cardiovascu-lar risk factors, BMI) or procedural (time to PTCA, TIMI flow) characteristics and progenitor mobilization.

Although a reduction in progenitor cells has been described in patients with CAD,[27] we did not observe any significant difference in the number per microliter of circulating CD34$^+$ cells or cell subsets in patients with SA if compared with CTRLs. Accordingly, plasma VEGF, SCF, and SDF-1α levels as well as the frequency of hematopoietic and endothelial colonies of patients with SA and CTRLs were comparable. These findings suggest that (1) if any reduction in circulating progenitors does exist in chronic CAD, it must be subtle under basal conditions; and (2) transient, possibly repeated but not actual, episodes of myocardial ischemia are unable to induce a sustained stem/progenitor cell mobilization, whereas severe tissue damage is required.[33]

Our findings can be a matter of some speculation. First, it could be surprising that immature HPCs like CD34$^+$CD38$^-$ cells did not increase after AMI; however, it is not exactly known yet which subset of HPCs takes part in tissue repair after AMI, and available clinical trial in humans all rely, so far, on injection or mobilization of either CD34$^+$ cells[34,35] or CD133$^+$cells,[36] with no further selection of any cell subsets. Second, it is intriguing to think that HPCs and EPCs can play different roles, if any, in tissue repair, with EPCs devoted to neoangiogenesis and HPCs, due to their "plasticity," to new myocardium generation. Although in rodents such a scenario has been reported,[37] a recent observation, based on a nonhuman primate model,[38] showed that mobilized cells can contribute to neoangiogenesis but not to muscle regeneration in the infarcted region. The possible different roles of the 2 classes of progenitor cells also bring up the question of whether, in future clinical trials, it could be better to use G-CSF or erythropoietin

Table 6. Plasma cytokine levels in patients with AMI at admission and 7 days after AMI, in patients with SA, and in healthy CTRLs

	AMI, n = 16	SA, n = 10	CTRLs, n = 17
VEGF, T0	45.9 (15.6-983.0)*	21.6 (15.6-63.8)	< 7.5 (< 7.5-130.6)
VEGF, day 7	< 7.5 (< 7.5-433)†	—	—
SCF	< 5.8 (< 5.8-273.0)	< 5.8 (< 5.8-24)	< 5.8 (< 5.8-346.3)
SDF-1α	2252 (< 156-5636)	2694 (< 156-4902)	2046 (< 156-3178)

Results, expressed as pg/mL, are shown as median (range).

— indicates not done.

*P < .04 versus CTRLs.

†P = .036 versus values obtained in patients with AMI at T0.

Table 7. Correlation of CD34$^+$ cells, or CD34$^+$ cell subsets, in patients with AMI at T0 divided according with statins or ACE inhibitor treatment

	n	CD34$^+$	CD34$^+$VEGFR-2$^+$	CD34$^+$CD117$^+$
Statins				
Yes	10	246 (46-682)	39 (8-44)	49 (3-82)
No	16	160 (33-668)	32 (0-88)	60 (0-124)
ACE inhibitors				
Yes	14	190 (38-682)	38 (0-44)	41 (22-124)
No	12	232 (33-668)	32 (0-88)	61 (0-113)

Results, expressed as number per microliter of cells, are shown as median (range).

P values were not significant.

(EPO)[39] with the aim to mobilize mostly HPCs or EPCs, respectively.

In conclusion, this study documents the spontaneous significant increase of circulating hematopoietic and endothelial progenitor cells, peaking already a few hours after the onset of symptoms of AMI, and therefore independently from any kind of mechanical or pharmacologic intervention. These data may contribute to a better understanding of the repairing process spontaneously occurring after AMI and, in turn, may help in identifying the best strategy for the use of a cell-based therapy in the regeneration of human myocardium.

References

1. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature. 2001;410:701-705.

2. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest. 2001;107:1395-1402.

3. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001;7:430-436.

4. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med. 1999;5:434-438.

5. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A. 2001;98:10344-10349.

6. Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. N Engl J Med. 2002;346:5-15.

7. Laflamme MA, Myerson D, Saffitz JE, et al. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res. 2002;90:634-640.

8. Krause DS, Fackler MJ, Civin CI, May S. CD34: structure, biology, and clinical utility. Blood. 1996; 87:1-13.

9. Mutin M, Canavy I, Blann A, et al. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. Blood. 1999;93:2951-2958.

10. Reyes M, Dudek A, Jahagirdar B, et al. Origin of human endothelial progenitors in human post natal bone marrow. J Clin Invest. 2002;109:337-346.

11. Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. Blood. 2000;95: 952-958.

12. Yiu AH, Miraglia S, Zanjani ED, et al. AC133 a novel marker for human hematopoietic stem and progenitor cells. Blood.1997;90:5002-5012.

13. Li TS, Hamano K, Nisihida M, et al. CD117+ stem cells play aa key role in therapeutic angiogenesis induced by bone marrow cell implantation. Am J Physiol Heart Circ Physiol. 2003;285: H931-H937.

14. Bailey AS, Jiang S, Afentoulis M, et al. Tras-

planted adult hematopoietic stem cells differentiate into functional endothelial cells. Blood. 2004; 103:13-19.

15. Li TS, Hayashi M, Liu ZL, et al. Low angiogenic potency induced by the implantation of ex vivo expanded CD117+ stem cells. Am J Physiol Heart Circ Physiol. 2004;286:H1236-H1241.

16. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: isolation and characterization. Trends Cardiovasc Med. 2003;13:201-206.

17. Shintani S, Murohara T, Ikeda H, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation. 2001; 103:2776-2779.

18. Petit I, Lapidot T. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol. 2002;30:973-981.

19. Jelkmann W. Pitfalls in the measurement of circulating vascular endothelial growth factor. Clin Chem. 2001;47:617-623.

20. Kawamoto A, Kawata H, Akai Y, et al. Serum levels of VEGF and basic FGF in the subacute phase of myocardial infarction. Int J Cardiol. 1998;67:47-54.

21. Barosi G, Viarengo G, Pecci A, et al. Diagnostic and clinical relevance of the number of circulating CD34+ cells in myelofibrosis with myeloid metaplasia. Blood. 2001;98:3249-3255.

22. Sutherland DR, Anderson L, Keeney M, et al. The ISHAGE guidelines for CD34+ cell determination by flow cytometry: International Society of Hematotherapy and Graft Engineering. J Hematother. 1996;5:213-226.

23. Sutherland HJ, Eaves CJ, Eaves AC, et al. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 1989;74:1563-1570.

24. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275:964-967.

25. Altman D. Practical Statistics for Medical Research. Oxford, United Kingdom: Blackwell Scientific Publications; 1990;433.

26. Gill M, Dias S, Hattori K, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2+AC133+ endothelial precursor cells. Circ Res. 2001;88:167-174.

27. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk fac-

tors for coronary artery disease. Circ Res. 2001; 89:E1-E7.

28. Hill JM, Zalos G, Holcox JP, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med. 2003;348:593-600.

29. Hitchon C, Wong K, Ma G, et al. Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. Arthritis Rheum. 2002;46:2587-2597.

30. Papayannopoulou T. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood. 2004;103:1580-1585.

31. Britten MB, Abolmaali ND, Assmus B, et al. Infarct remodeling after intracoronary progenitor cell tretment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. Circulation. 2003;108:2212-2218.

32. Hattori K, Dias S, Heissig B, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate post-natal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med. 2001;193:1005-1014.

33. Perera D, Kanaganayagam G, Marber M. Stem cells in unstable angina: the dynamic duo. Eur Heart J. 2004;25:999-1000.

34. Kang H, Kim H, Zhang S, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet. 2004;363:751-756.

35. Wollert KC, Meyer GP, Lotz J, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet. 2004;364:141-148.

36. Stamm C, Westphal B, Kleine H-D, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 2003;361:45-46.

37. Rafii S, Lyden D. Therapeutic stem and progenitor cells transplantation for organ vascularization and regeneration. Nat Med. 2003;9:702-712.

38. Norol F, Merlet P, Isnard R, et al. Influence of mobilized stem cells on myocardial infarct repair in a non human primate model. Blood. 2003;102: 4361-4368.

39. Heeschen C, Aicher A, Lehmann R, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood. 2003; 102:1340-1346.