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

Margherita Massa, Vittorio Rosti, Maurizio Ferrario, Rita Campanelli, Isabella Ramajoli, Roberta Rosso, Gaetano M. De Ferrari, Marco Ferlini, Lucio Goffredo, Alessandra Bertoletti, Catherine Klersy, Alessandro Pecci, Remigio Moratti, and Luigi Tavazzi

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 ≤ .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) © 2005 by The American Society of Hematology

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. 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, or by pharmacologic mobilization. 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 that in healthy subjects are localized in the bone marrow, with very small amounts circulating in the peripheral blood (PB). 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. It has been reported that the coexpression of surface CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR-2) antigens identifies early EPCs; 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. In addition, recent papers suggested that bone marrow–derived cells coexpressing CD34, CD117, and VEGF-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. 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) © 2005 by The American Society of Hematology

From the Biotechnology Laboratory, Organ Transplantation Laboratory, Department of Cardiology, Internal Medicine and Medical Oncology, Biometry and Clinical Epidemiology, Clinica Medica III, and Department of Clinical Chemistry, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy.

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Reprints: Margherita Massa, IRCCS Policlinico San Matteo, Laboratorio Biotecnologie, 19, Pie Golgi, 27100 Pavia, Italy; e-mail: m.massa@smarteo.pv.it.

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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); cardiovascular 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 hPMP can hamper the detection of plasma VEGF levels, 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 ±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 everyday 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 (ethylene-diaminetetraacetic acid)–containing tubes was incubated for 30 minutes at 4°C with 10 µL of 7-aminactinomycin 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 (2×10⁵) 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 × white blood cell (WBC) count/100.

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 Hemotherapy and Graft Engineering (ISHAGE) guidelines 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-CD34 and PE–anti–CD38, or PE–anti–CD117 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 × 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-duseum 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 × 10⁸) 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% CO₂, the numbers of erythroid burst-forming units (BFU-Es) and granulocyte macrophage colony-forming units (CFU-GMs) were counted using standard criteria.

**In vitro cultures of EPCs**

PBMCs (1 × 10⁹) 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% CO₂, the endothelial colonies, defined as central clusters of cells surrounded by spindle-shaped cells, 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; Immunotech, Marseille, France), anti–VE-cadherin (Bender Med System, San Bruno, CA), anti-CD45 (CS37; Exalpha Biologicals, Watertown, MA), and anti–VEGFR-2 monoclonal antibody (anti-VWF; Dako, Glostrup, Denmark). Double-labeling experiments were performed by using anti-CD31 and anti–VE-cadherin antibodies or anti-CD45 and anti-VEGFR-2 antibodies. Antibody binding was revealed through an Alexa Fluor-488– or Alexa Fluor-594–conjugated goat anti-mouse or anti-rabbit 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. 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 0.05 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 × 10⁹/L [13.6 × 10⁹/µL]; range, 7.3-20.2 × 10⁹/L [7.3-20.2 × 10⁹/µL])

| Table 1. Clinical, angiographic, and procedural data of patients with AMI, patients with SA, and healthy CTRLs |
|---------------------------------|----------------|----------------|----------------|
| Age, y, mean (range)            | AMI, n = 26    | SA, n = 10     | CTRLs, n = 17  |
| 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)         |
| Dyslipidemia                    | 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, µ/L (range)                | 2055 (161-3907)| —              | —              |
| pCK-MB, µ/L (range)             | 221 (77-372)   | —              | —              |
| LVF (%)                         |                |                |                |
| 24 h                            | 47 ± 9         | —              | —              |
| 1 y                             | 53 ± 8         | —              | 1 y            |
| LVWMSI (%)                      | 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; LVF, left ventricular ejection fraction; and LVWMSI, left ventricular wall motion score index.

*P = .008 versus SA and CTRLs.
†P = .03 versus SA.
and 24 hours after the onset of AMI (median, 10.0 × 10⁹/L [10.0 × 10⁹/µL]; range, 7.0-15.8 × 10⁹/L [7.0-15.8 × 10⁹/µL]) with respect to either patients with SA (median, 7.7 × 10⁹/L [7.7 × 10⁹/µL]; range, 5.8-8.2 × 10⁹/L [5.8-8.2 × 10⁹/µL]) or CTRLs (median, 6.2 × 10⁹/L [6.2 × 10⁹/µL]; range, 4.0-8.9 × 10⁹/L [4.0-8.9 × 10⁹/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 higher in patients with AMI at admission (Figure 1A). In order to evaluate the absolute number per microliter of CD34⁺CD33⁺, CD34⁺CD38⁺, and CD34⁺CD117⁺, and CD34⁺VEGFR-2⁺ cells (A), CD34⁺CD33⁺, CD34⁺CD38⁺, and CD34⁺CD117⁺ cells (B), CD34⁺VEGFR-2⁺ cells (C), and CD34⁺VEGFR-2⁺ cells (D) of 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) were found in CTRLs. CD34⁺ 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.

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.
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 × 10⁶ PBMCs was also higher (P ≤ .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-F 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/10⁶ 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.

![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.](image)
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 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 cytfluorimetric 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\textsuperscript{11,26} and in animal models\textsuperscript{5,13,15} to play a role in neangiogenic 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.\textsuperscript{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,\textsuperscript{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
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 describing 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 recruitment of circulating progenitor cells to enhance endogenous and 7 days after AMI, in patients with SA, and in healthy CTRLs.

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

<table>
<thead>
<tr>
<th></th>
<th>AMI, n = 16</th>
<th>SA, n = 10</th>
<th>CTRLs, n = 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, T0</td>
<td>45.9 (15.6-983.0)*</td>
<td>21.6 (15.6-63.8)</td>
<td>&lt; 7.5 (&lt; 7.5-130.6)</td>
</tr>
<tr>
<td>VEGF, day 7</td>
<td>&lt; 7.5 (&lt; 7.5-433)†</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SCF</td>
<td>&lt; 5.8 (&lt; 5.8-273.0)</td>
<td>&lt; 5.8 (&lt; 5.8-24)</td>
<td>&lt; 5.8 (&lt; 5.8-346.3)</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>2252 (&lt; 156-5636)</td>
<td>2694 (&lt; 156-4902)</td>
<td>2046 (&lt; 156-3178)</td>
</tr>
</tbody>
</table>

Results, expressed as pg/mL, are shown as median (range). * indicates not done.

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

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>CD34+</th>
<th>CD34+VEGFR-2+</th>
<th>CD34+CD117+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>246 (46-682)</td>
<td>39 (8-44)</td>
<td>49 (3-82)</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>160 (33-668)</td>
<td>32 (0-88)</td>
<td>60 (0-124)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>190 (38-682)</td>
<td>38 (0-44)</td>
<td>41 (22-124)</td>
</tr>
<tr>
<td>No</td>
<td>12</td>
<td>232 (33-668)</td>
<td>32 (0-88)</td>
<td>61 (0-113)</td>
</tr>
</tbody>
</table>

Results, expressed as number per microliter of cells, are shown as median (range). P values were not significant.
(EPO) 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

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

Margherita Massa, Vittorio Rosti, Maurizio Ferrario, Rita Campanelli, Isabella Ramajoli, Roberta Rosso, Gaetano M. De Ferrari, Marco Ferlini, Lucio Goffredo, Alessandra Bertoletti, Catherine Klersy, Alessandro Pecci, Remigio Moratti and Luigi Tavazzi

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