Granulocyte Colony-Stimulating Factor Mobilizes Functional Endothelial Progenitor Cells in Patients With Coronary Artery Disease

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Objective—Endothelial progenitor cells (EPCs) that may repair vascular injury are reduced in patients with coronary artery disease (CAD). We reasoned that EPC number and function may be increased by granulocyte colony-stimulating factor (G-CSF) used to mobilize hematopoietic progenitor cells in healthy donors.

Methods and Results—Sixteen CAD patients had reduced CD34 \(^+\)/CD133 \(^+\) (0.0224±0.0063\% versus 0.121±0.038\% mononuclear cells [MNCs], \(P<0.01\)) and CD133 \(^+\)/VEGFR-2 \(^+\) cells, consistent with EPC phenotype (0.0033±0.00015\% versus 0.0017±0.0006\% MNCs, \(P<0.01\)), compared with 7 healthy controls. Patients also had fewer clusters of cells in culture, with out-growth consistent with mature endothelial phenotype (2±1/well) compared with 16 healthy subjects at high risk (13±4/well, \(P<0.05\)) or 14 at low risk (22±3/well, \(P<0.001\)) for CAD. G-CSF 10 \(\mu\)g/kg per day for 5 days increased CD34 \(^+\)/CD133 \(^+\) cells from 0.5±0.2/\(\mu\)L to 59.5±10.6/\(\mu\)L and CD133 \(^+\)/VEGFR-2 \(^+\) cells from 0.007±0.004/\(\mu\)L to 1.9±0.6/\(\mu\)L (both \(P<0.001\)). Also increased were CD133 \(^+\) cells that coexpressed the homing receptor CXCR4 (30.4±8.3/\(\mu\)L, \(P<0.05\)). Endothelial cell-forming clusters in 10 patients increased to 27±9/well after treatment (\(P<0.05\)), with a decline to 9±4/well at 2 weeks (\(P=0.06\)).

Conclusions—Despite reduced EPCs compared with healthy controls, patients with CAD respond to G-CSF with increases in EPC number and homing receptor expression in the circulation and endothelial out-growth in culture. (Arterioscler Thromb Vasc Biol. 2005;25:296-301.)

Key Words: coronary disease ■ atherosclerosis ■ angiogenesis ■ cell adhesion molecules ■ cells

Progenitor cells circulate in the bloodstream, with the potential for cardiovascular repair after injury. In animal models of hind limb, myocardial, or cerebral ischemia, vascular progenitor cells have been shown to incorporate into areas of active vascular growth,\(^1,2\) suggesting that they may have therapeutic potential either by providing endothelial cells for new vessel growth or through secretion of angiogenic growth factors that activate neighboring cells. A lineage of particular interest derives from a subset of CD34 \(^+\) hematopoietic stem cells that expresses one of the receptors for vascular endothelial growth factor (VEGFR-2), with the cell surface marker CD133 further defining early forms of this lineage.\(^3\,\,7\) This population of cells has been referred to as endothelial progenitor or precursor cells (EPCs) because under specific culture conditions, CD34 \(^+\) and CD133 \(^+\) cells differentiate into a mature endothelial phenotype based on microscopic appearance, functional characteristics (uptake of acetylated low-density lipoprotein [LDL], nitric oxide synthesis), and expression of cell surface markers (E-selectin, von Willebrand factor, vascular endothelial cadherin, platelet-endothelial cell adhesion molecule, \(\alpha_\text{v}\beta_\text{3}\) integrin, c-kit) associated with loss of CD133 expression.

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In clinical studies, designation of EPCs has not been consistent, with differences among groups in flow cytometric analysis or cell culture techniques used for processing mononuclear cells (MNCs) from patient samples. Two groups have demonstrated differences in what they defined as EPCs between patients with coronary artery disease (CAD) or its risk factors and healthy subjects. Vasa et al\(^8\) reported that EPCs—defined as dual staining of MNCs, plated on fibronectin-coated dishes for 4 days, for 1,1\'-diocadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated LDL (DiI-LDL) and Ulex europaeus agglutinin-1 (lectin) using fluorescent microscopy—are significantly reduced in...
patients with CAD. The number of CAD risk factors was inversely correlated with number of circulating CD34+/VEGFR-2+ MNCs measured by flow cytometry, with smoking being a significant independent risk factor for lower EPC levels. This group also examined the migratory activity of EPCs from their culture assay in response to VEGF, and determined that this property was significantly diminished in CAD patients compared with healthy controls and inversely related to the number of CAD risk factors. Hill et al extended these findings to healthy subjects with risk factors for CAD, using an assay of endothelial outgrowth from MNCs initially nonadherent to fibronectin after 48 hours in culture dishes and replated in fibronectin-coated wells with growth media. This step was performed to remove circulating mature endothelial cells that may be of vascular rather than bone marrow progenitor cell origin. After 7 days in culture, clusters of rounded cells emanating thin, flat cells at their periphery were designated as EPC colony-forming units. Numbers of these colony-forming units correlated inversely with the number of CAD risk factors and linearly with endothelial function assessed in the brachial artery after increased shear stress with postischemic hyperemia, consistent with the notion that diminished EPC release or survival in the circulation may contribute to endothelial dysfunction and cardiovascular risk.

EPCs of hematopoietic lineage, defined by CD34+/CD133+/VEGFR-2+ cell surface markers, circulate in small numbers, even in healthy individuals (~0.002% of total MNCs), and thus further reduction in number or differentiation potential may compromise endothelial repair or limit cardiac adaptive responses to atherosclerotic cardiovascular disease, including CAD. Accordingly, stimulation of EPC release into the circulation may be an effective strategy for vascular repair in patients with advanced CAD in whom more conventional treatment has failed, an approach supported by experimental studies. However, this may occur only if there are sufficient numbers of cells mobilized from bone marrow with expression of receptors that might promote homing to ischemic myocardium. In this regard, it is possible that CAD patients, who have associated medical conditions and require multiple medications for management, have low numbers of EPCs in the circulation because of impaired production within bone marrow or reduced survival. We hypothesized that EPC release into the circulation may be increased in CAD patients by administration of granulocyte colony-stimulating factor (G-CSF), which is known to mobilize hematopoietic progenitor cells into the peripheral blood in healthy subjects, and that these cells might have potential for differentiation into endothelial cells. We used flow cytometry to study the effects of G-CSF administration on mobilization of cells of hematopoietic stem cell (CD34+) lineage with expression of EPC markers (CD133, VEGFR-2) and cells expressing the chemokine receptor CXCR4, which may promote homing of EPCs to ischemic tissue. We additionally determined the endothelial differentiation capacity of MNCs using an ex vivo colony-forming assay.

### Methods

#### Study Populations

All patients had extensive CAD by recent (within 6 months) coronary arteriography and were Canadian Cardiovascular Society class 3 (chest pain with routine activity) or 4 (chest pain on minimal exertion or at rest), despite previous attempts at surgical or percutaneous coronary revascularization. All patients were either unsuitable for further revascularization procedures or declined further attempts, and had evidence of inducible myocardial ischemia by dobutamine stress cardiac magnetic resonance imaging, with resting left ventricular ejection fractions >30%. No patient had evidence of systemic disease, except for adult-onset diabetes mellitus without retinopathy or renal disease. Medical therapy for all patients included β-blockers, aspirin, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (statin) therapy; these medications were maintained through the study. Sixteen patients (11 men and 5 women; age range, 42 to 71 years) fulfilled eligibility criteria and received G-CSF (filgrastim; Amgen, Thousand Oaks, Calif) 10 μg/kg per day subcutaneously for 5 days after baseline testing. Three blood samples were drawn from each patient: baseline (before the first dose of G-CSF), 6 to 24 hours after the last dose of G-CSF (day 6), and 2 weeks after the first dose of G-CSF (day 14). This protocol was approved by the Scientific Review Committee and the Institutional Review Board of the National Heart, Lung, and Blood Institute, and informed written consent was obtained from all patients. Seven healthy subjects (4 men and 3 women; age range, 19 to 50 years) who had no risk factors for cardiovascular disease received the identical treatment with G-CSF as participants in other protocol and served as controls for flow cytometric measurements. Thirty healthy men served as controls for cell culture measurements: 16 (age 58.9 ± 2.4 years) were considered at high risk for CAD based on Framingham Risk Scores ranging from +6 to +15 (+7.8 ± 0.6) and 14 (age 40.1 ± 2.7 years) were considered at low risk based on scores ranging from −4 to −2 (−0.2 ± 0.4).

#### Flow Cytometry Analysis

Flow cytometry was performed on buffy coat cells isolated from EDTA-anticoagulated peripheral blood to quantitate expression of cell surface markers on MNCs. Blood samples were diluted with phosphate-buffered saline (PBS) and peripheral blood MNCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Recovered cells were washed twice with PBS and resuspended in 2 mL PBS. The number of MNCs was determined using a Coulter Counter (Beckman Coulter, Inc, Fullerton, Calif). One million to 2 million MNCs were aliquoted into 5 mL polystyrene tubes (Falcon; Beckton Dickinson, Franklin Lakes, NJ) and incubated with 50 μL mouse serum (β-Aldrich, St. Louis, Mo) at room temperature to block nonspecific binding of antibodies. Each tube of aliquoted cells was stained with PE or fluorescein isothiocyanate isothiocyanate-conjugated CD34 monoclonal antibody (BD Biosciences, San Jose, Calif) and PerCP/Cy5.5-conjugated CD45 monoclonal antibody (BD Biosciences). Up to 2 additional monoclonal antibodies for EPC and endothelial cell markers were also added to each tube of cells from the following antibodies: biotin-conjugated VEGFR-2 (Sigma-Aldrich), PE-conjugated CD133 or activated protein C (APC)-conjugated CD133 (Miltenyi Biotec, Auburn, Calif), and PE-conjugated CXCR4 (BD Pharmingen). One million to 2 million cells were incubated with appropriate volumes of the antibodies at room temperature and protected from light for ~25 minutes with the combinations of 4 antibodies. Combinations of isotype controls were used as negative controls based on the species and IgG subclass of each antibody (Figure 1). For samples stained with biotin-VEGFR-2 or its biotin control, samples were incubated an additional 45 minutes with 10 μL streptavidin–APC diluted in a 100 μL solution with PBS. After incubation with the antibodies, cells were washed with 2 mL PBS and centrifuged at 2000 rpm for 5 minutes. Stained cells were resuspended in 0.3 μL PBS and 0.3 μL 1% paraformaldehyde solution. The FACSCalibur flow cytometer (BD Biosciences) and CellQuest Software (version 3.3; San Jose, Calif) were...
EPC Isolation and Colony-Forming Assay

Twenty-four mL venous blood was collected at each time point into BD Vacutainer CPT Mononuclear Cell Preparation Tubes (Becton Dickinson). MNCs recovered by density-gradient centrifugation in these tubes were washed twice with PBS and once in EPC growth media consisting of medium199 (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were resuspended in media, plated at a density of $5 \times 10^6$ per well on dishes coated with human fibronectin (BIOCOAT; Becton Dickinson Labware, Bedford, Mass), and incubated at 37°C in humidified 5% CO₂. After 48 hours, nonadherent cells suspended in the growth media were replated onto fibronectin-coated 24-well plates at a density of $10^6$ per well. Media was changed every 3 days, and EPC colony-forming units, defined as a central core of rounded cells surrounded by elongated and spindle-shaped cells, were counted after 7 days. An endothelial progenitor cell colony-forming unit (EPC-CFU) consisted of a central core of rounded cells surrounded by elongated and spindle-shaped cells (A), with confirmation of endothelial phenotype by DiIl-LDL uptake and staining for UEA-1 lectin, CD31, VEGFR-2, and von Willebrand factor in selected samples. Clusters of cells without emerging spindle cells (B) were not counted as an EPC-CFU.

Statistical Analysis

Comparisons of responses to G-CSF from baseline measurements were made by Student t test for paired data. Comparisons between patient and control groups were made by Student t test for unpaired data. Data are reported as mean±SEM.

Results

Flow Cytometry Analysis of Progenitor Cells

Before G-CSF administration to CAD patients, CD34⁺/CD133⁺ cells in blood averaged 0.0224±0.0063% of total MNC (0.53±0.17 cells/µL), which was significantly less than values in healthy subjects (Table). CD133⁺/VEGFR-2⁺ cells averaged 0.00033±0.00015% of total MNCs (0.007±0.004 cells/µL) in patients, which was also significantly lower than values in healthy subjects.

After G-CSF administration for 5 days, CD34⁺/CD133⁺ cells in blood increased to 1.004±0.144% of total MNCs.
(59.5±10.6 cells/μL), returning to baseline by day 14 (Figure 3A). By comparison, in healthy subjects receiving the same regimen of G-CSF, CD34+/CD133+ cells averaged 2.647±0.421% of total MNCs, significantly higher than the response measured in patients (Table). CD133+/VEGFR-2+ cells increased in patients to 0.035±0.0107% of total MNCs (1.9±0.6 cells/μL), similar to the response measured in healthy subjects, and returned to baseline at day 14 (Figure 3B).

Chemokine Receptor Expression
CD133+ cells coexpressing CXCR4, the chemokine receptor for SDF-1, were lower at baseline in patients versus controls (Table) and increased in both groups after G-CSF, albeit to significantly lower at baseline and remained elevated (224±66/μL) after G-CSF (P<0.05, CAD patients vs patients at low risk; *P<0.05, CAD patients vs patients at high risk).

Figure 3. G-CSF increases circulating hematopoietic progenitor cells identified by CD34+/CD133+ surface markers (A), endothelial progenitor cells identified by CD133+/VEGFR-2+ surface markers (B), and CD133+ coexpressing the chemokine receptor CXCR4 (C) within 24 hours of the fifth dose of G-CSF (10 μg/kg per day), with return to baseline by day 14. Data are normalized to patients’ absolute mononuclear cell counts at each time point and expressed as mean±SEM.

Figure 4. Baseline EPC colony-forming units (CFU) per well (average of at least 4 wells) at 7 days of mononuclear cell culture in fibronectin-coated dishes after the pre-plating process described for Figure 2 are higher in healthy volunteers, whether at high risk (n=16) or low risk (n=14) for cardiovascular disease based on their Framingham global risk scores, compared with CAD patients (**P<0.001, CAD patients vs patients at low risk; *P<0.05, CAD patients vs patients at high risk).

Figure 5. G-CSF administered to CAD patients (n=10 for this assay) for 5 days increases EPC colony-forming units (CFU). *P<0.05, day 6 versus day 0; †P=0.06, day 14 vs day 0).

Discussion
Progenitor cells in the circulation, including EPCs of hematopoietic lineage, may serve to repair vascular injury and maintain functional endothelium, as suggested by recent demonstrations of an inverse correlation between EPCs—whether measured in the circulation by flow cytometry or in culture by endothelial cell-forming ability—and risk factors.
in patients with CAD or apparently healthy subjects.\textsuperscript{8,9} Reduced nitric oxide-dependent flow-mediated dilation of brachial arteries of healthy subjects with risk factors for CAD suggests that impaired release of EPCs from bone marrow or survival in the circulation may promote endothelial dysfunction, pathophysiologically linked to atherosclerosis, and risk of future cardiovascular events.\textsuperscript{14–16} We now demonstrate that CAD patients have reduced circulating EPCs of hematopoietic lineage, as defined by CD34\textsuperscript{+}/CD133\textsuperscript{+} and CD133\textsuperscript{+}/VEGFR-2\textsuperscript{+} expression, compared with healthy subjects. Further, CAD patients have reduced endothelial differentiation capacity of MNCs in culture compared with healthy subjects, including those with risk factors for CAD. Although our culture technique—with replating of nonadherent cells after 48 hours in culture and determination of EPC colony-forming units—differs from that of Vasa et al,\textsuperscript{8} our pretreatment findings are similar. This was true of CAD patients in our study, despite universal use of statin therapy, previously shown to increase EPCs in the circulation of CAD patients after 1 month of treatment.\textsuperscript{17} There was no correlation between numbers of EPCs by flow cytometry and endothelial cell-forming colonies in our study, which may indicate that MNCs with endothelial cell differentiation capacity derive from lineages additional to hematopoietic stem cells, consistent with recent reports regarding CD14\textsuperscript{+} monocytes in culture.\textsuperscript{18,19}

We considered whether cytokine stimulation of progenitor cell release into the circulation might be possible in CAD patients, even though reduced numbers of EPCs could indicate irreversible depletion or enhanced apoptosis within bone marrow. Although basal levels of EPCs defined by CD133\textsuperscript{+}/VEGFR-2\textsuperscript{+} cell surface markers were reduced in patients compared with healthy subjects, consistent with the findings of Vasa et al,\textsuperscript{8} we found that G-CSF administration increased EPCs of hematopoietic lineage in the circulation; however, the absolute number of cells in blood was small, \textasciitilde{} 2 cells/\textmu{}L or 10 million cells in the circulation. G-CSF also increased the capacity of MNCs to form colonies capable of endothelial cell maturation and proliferation. We cannot be certain that the endothelial colony-forming units in our culture assay are derived from CD34/CD133/VEGFR-2 population of cells within the circulation, despite the increase in circulating cells after G-CSF administration. However, the clusters of cells emanating cells of endothelial phenotype are similar to findings from assays that used CD34\textsuperscript{+} or CD133\textsuperscript{+}-selected cells instead of unselected MNCs.\textsuperscript{3,5} An additional observation made in our study may be important for the success of a cytokine mobilization strategy to initiate angiogenesis in ischemic myocardium. G-CSF not only increased the numbers of EPCs from baseline values but also activated cells in a manner of potential importance to cell homing,\textsuperscript{20} with increased expression of the chemokine receptor CXCR4. Regarding the mechanism of CXCR4 expression after G-CSF, Kollet et al\textsuperscript{21} reported that CD34\textsuperscript{+} cells contain intracellular CXCR4 that can be induced to functional expression on the cell surface upon stimulation with cytokines. Accordingly, the increase in CXCR4 expression in CD133\textsuperscript{+} cells as measured by flow cytometry in our study is consistent with increased binding of monoclonal antibody to CXCR4 translocated to the cell surface after G-CSF activation of the CD133\textsuperscript{+} subpopulation of hematopoietic stem cells. Increased cell surface expression of CXCR4 may enhance trafficking and homing of progenitor cells to sites of myocardial ischemia in response to its ligand, SDF-1.\textsuperscript{22} In support of the functional significance of CXCR4 receptor expression in our study, levels of SDF-1 in serum declined significantly after G-CSF administration, likely caused by receptor binding on circulating MNCs.

Our findings establish that G-CSF administration to patients with CAD mobilizes hematopoietic progenitor cells into the circulation, including the EPC subset of cells expressing mature endothelial markers and the chemokine receptor CXCR4, and augments EPC colony-forming capacity. This cytokine approach to EPC mobilization was tested in a clinical trial of percutaneous coronary intervention with stenting after myocardial infarction.\textsuperscript{23} This group reported an increased incidence of in-stent restenosis at the 6-month follow-up coronary angiogram, and the clinical trial was stopped. Whether EPCs mobilized into the circulation by G-CSF, with or without collection by leukapheresis and direct administration, will be useful for initiating vascular growth and myocyte repair in patients with chronic ischemic heart disease who have not undergone recent intravascular intervention must be tested in clinical trials.

References


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