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

Tiffany M. Powell, Jonathan D. Paul, Jonathan M. Hill, Michael Thompson, Moshe Benjamin, Maria Rodrigo, J. Philip McCoy, Elizabeth J. Read, Hanh M. Khuu, Susan F. Leitman, Toren Finkel, Richard O. Cannon III

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 (0.00033±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 μg/kg per day for 5 days increased CD34+/CD133+ cells from 0.5±0.2/μL to 59.5±10.6/μL and CD133+/VEGFR-2+ cells from 0.007±0.004/μL to 1.9±0.6/μL (both P<0.001). Also increased were CD133+ cells that coexpressed the homing receptor CXCR4 (30.4±8.3/μL, 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;25:1-6.)

Key Words: coronary disease ■ atherosclerosis ■ angiogenesis ■ cell adhesion molecules ■ cells
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.4–7,10,11 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),6 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.2,12 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,13 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 throughout 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 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).9

**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, 6t o 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 used for data acquisition. Data were gated on the mononuclear population during data acquisition and 250 000 events were collected in the gated region for each sample of cells. Summit software (version 3.1; Dako Cytomation, Ft Collins, Colo) was used for data analysis. The percent of each of the following subpopulations was expressed as a percent of the total MNCs: CD34/CD133, CD133/VEGFR-2, CD34/CD144,
CD34/CD51–61, CD34/CD31, and CD133/CXCR4. Cell populations in patients were also expressed as the number of circulating cells per volume of peripheral blood, based on the nucleated white blood cell count from the automated counter.

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 5x10⁶ per well on dishes coated with human fibronectin (BIOCOAT; Becton Dickinson Laboratory, 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 5x10⁶ per well. Media was changed every 3 days, and EPC colony-forming units 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 DiI-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 were not counted as an EPC-CFU.

Figure 2. Assay for endothelial cell formation by progenitor cells. PBMCs were initially suspended in media and plated on fibronectin-coated wells (5x10⁶ cells per well). After 48 hours, nonadherent cells were replated onto fibronectin-coated wells, and colonies 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, with confirmation of endothelial phenotype by DiI-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 were not counted as an EPC-CFU.

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 MNCs (0.53±0.17 cells/μL), which was significantly less than values in healthy subjects (Table). CD34+/VEGFR-2+ cells averaged 0.0063±0.0015% 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 0.121±0.038% of total MNCs (5.03±0.129 cells/μL) at baseline, which increased to 0.344±0.017% of total MNCs (25.34±1.024 cells/μL) after 5 days, returning to baseline by day 14 (Figure 3A).

Expression of Hematopoietic Progenitor (CD34+/CD133+), Endothelial Progenitor (CD34+/VEGFR-2+), and Chemokine Receptor (CD133+/CXCR4+) Cell Surface Markers Measured by Flow Cytometry in Peripheral Blood Mononuclear Cells From 16 Coronary Artery Disease Patients and From 7 Healthy Controls Before and After Administration of Granulocyte Colony-Stimulating Factor

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After G-CSF</th>
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</thead>
<tbody>
<tr>
<td>CD34+/CD133+ (% MNC)</td>
<td>0.0224±0.0063*</td>
<td>1.004±0.144†</td>
</tr>
<tr>
<td>Controls</td>
<td>0.121±0.038</td>
<td>2.647±0.421†</td>
</tr>
<tr>
<td>CD34+/VEGFR-2+ (% MNC)</td>
<td>0.0003±0.00015*</td>
<td>0.035±0.00107†</td>
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<tr>
<td>Controls</td>
<td>0.0017±0.0006</td>
<td>0.009±0.002†</td>
</tr>
<tr>
<td>CD34+/CXCR4+ (% MNC)</td>
<td>0.031±0.012*</td>
<td>0.471±0.129†</td>
</tr>
<tr>
<td>Controls</td>
<td>0.165±0.037</td>
<td>2.346±0.344†</td>
</tr>
</tbody>
</table>

Data: mean±SEM. MNC indicates mononuclear cell; CAD, coronary artery disease; G-CSF, granulocyte colony-stimulating factor. *P<0.01 vs controls. †P<0.001 vs baseline.

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.
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 higher levels in controls compared with patients. CD133+/CXCR4+ cells returned toward baseline values by day 14 (Figure 3C). Levels of SDF-1, the ligand for CXCR4, measured in plasma by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn), declined from 1715±1715 pg/mL before treatment to 1357±1715 pg/mL after G-CSF (P<0.05 versus baseline), and remained significant above baseline at day 14 (67±25/μL; P<0.05 versus baseline).

Differentiation of Mononuclear Cells Into Endothelial Cells
The effect of G-CSF on differentiation of MNCs into endothelial cells was assessed by flow cytometry and by cell culture assay. CD34+ cells coexpressing markers of endothelial phenotype were measured in blood before and after G-CSF administration: CD31+ (platelet–endothelial cell adhesion molecule [PECAM]) cells increased in blood from 54±28 to 183±66/μL after G-CSF (P<0.05 versus baseline) and remained elevated (224±60/μL) at day 14 (P<0.05 versus baseline); CD144+ (vascular endothelial cadherin [VE-cadherin]) cells increased from 16±12 to 107±90/μL after G-CSF (P<0.05 versus baseline), and remained significantly above baseline at day 14 (67±25/μL; P<0.05 versus baseline); and CD51/61+ (αvβ3 integrin) cells increased from 14±14 to 81±64/μL after G-CSF (P<0.05 versus baseline), and tended to remain above baseline at day 14 (49±24/μL blood), although this difference did not achieve statistical significance.

To determine whether G-CSF changed the capacity of MNCs to differentiate into endothelial cells in culture conditions, MNCs from 10 CAD patients were plated on fibronectin with EPC growth media for 1 week after replating of nonadherent cells at 48 hours of initial culture and assayed for clusters of rounded cells with out-growth of mature endothelial cells (confirmed by fluorescent Dil-acetylated LDL and lectin staining; Figure 2). Patients with CAD had lower EPC colony-forming units at baseline in comparison with 30 healthy subjects, regardless of whether the Framingham Risk Score was low or high (Figure 4).

After G-CSF, colonies with out-growth of endothelial cells increased to 10-fold over baseline measurements, with persistent increase at day 14 (Figure 5). There was no correlation between numbers of CD133+/VEGFR-2+ cells measured by flow cytometry and EPC colony-forming units in culture, at baseline or after G-CSF administration.

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. Reduced nitric oxide-dependent flow-mediated dilation of brachial arteries of healthy subjects with risk factors for CAD demonstrates 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 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.)
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\(^+\)/CD133\(^−\) and CD133\(^+\)/VEGFR-2\(^−\) 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\(^+\)CD133\(^−\) 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\(^+\)/VEGFR-2\(^−\) 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, \(\approx 2\) cells/\(\mu\)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\(^+\) or CD133\(^+\)—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\(^+\) cells contain intracellular CXCR4 that can be induced to functional expression on the cell surface on stimulation with cytokines. Accordingly, the increase in CXCR4 expression in CD133\(^+\) 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\(^+\) 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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*Arterioscler Thromb Vasc Biol.* published online November 29, 2004;
*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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