Effects of Granulocyte Colony Stimulating Factor on Functional Activities of Endothelial Progenitor Cells in Patients With Chronic Ischemic Heart Disease

Joerg Honold, Ralf Lehmann, Christopher Heeschen, Dirk H. Walter, Birgit Assmus, Ken-Ichiro Sasaki, Hans Martin, Judith Haendeler, Andreas M. Zeiher, Stefanie Dimmeler

Objective—Bone marrow–derived circulating endothelial progenitor cells (EPCs) may contribute to regeneration of infarcted myocardium and enhance neovascularization. Granulocyte colony-stimulating factor (G-CSF) is well-established to mobilize hematopoietic stem cells (HSCs) and might, thereby, also increase the pool of endogenously circulating EPC. Therefore, we investigated the effects of G-CSF administration on mobilization and functional activities of blood-derived EPC in patients with chronic ischemic heart disease (CIHD).

Methods and Results—Sixteen patients with CIHD received 10 μg/kg per day subcutaneous G-CSF injection for 5 days. Leukocyte counts, the number of HSCs and EPCs, and the migratory response to VEGF and SDF-1 were analyzed before and after G-CSF-therapy. At day 5 of G-CSF treatment, the number of circulating leukocytes, CD34<sup>+</sup>/CD45<sup>−</sup> and CD34<sup>+</sup>/CD133<sup>+</sup> cells was significantly increased. Likewise, G-CSF treatment augmented the numbers of colony forming units with endothelial cell morphology (EC-CFU). However, the functional activity of the EPC as assessed by the migratory response to VEGF and SDF-1 was significantly reduced after G-CSF treatment (P<0.01). Because G-CSF was previously shown to cleave the CXCR4 receptor, we determined the surface expression of the 6H8 epitope of the CXCR4 receptor by fluorescence-activated cell sorter (FACS) analysis. Consistent with the reduced migratory capacity, the surface expression of the functionally active CXCR4 receptor was significantly reduced. To test the functional activity of the cultivated EPCs in vivo, cells were intravenously infused in nude mice after hind limb ischemia. EPCs, which were cultivated before G-CSF administration, increased blood flow recovery and prevented limb necrosis. However, infusion of EPCs, which were isolated 5 days after G-CSF treatment from the same patient, showed a reduced capacity to augment blood flow recovery and to prevent necrosis by 27%.

Conclusion—G-CSF treatment effectively mobilizes HSCs and EPCs. However, the migratory response to SDF-1 and in vivo capacity of G-CSF-mobilized EPCs was significantly reduced. (Arterioscler Thromb Vasc Biol. 2006;26:2238-2243.)

Key Words: chronic ischemic heart disease ■ endothelial progenitor cells ■ granulocyte colony stimulating factor

Bone marrow–derived circulating endothelial progenitor cells (EPCs) contribute to regeneration of infarcted myocardium by enhancement of neovascularization and putative paracrine effects such as secretion of pro-angiogenic factors.1-3 Several studies described the safety, feasibility and beneficial effects of autologous progenitor cell transplantation on left ventricular dysfunction and cardiac remodeling in patients with acute myocardial infarction (AMI)4-6 and patients with chronic ischemic heart disease (CIHD).7-10

Patients with coronary artery disease (CAD) are known to have reduced numbers of ex vivo cultivated EPCs, defined as adherent cells with uptake of diacetylated low-density lipoprotein (LDL) cholesterol and expression of surface markers such as vascular endothelial growth factor (VEGF) receptor 2, von Willebrand factor (vWF) and lectin.11 Furthermore, the functional activities of progenitor cells in CAD patients are compromised12 and might thereby limit the potential for neovascularization processes within the myocardium after AMI. Such a numeric and functional impairment of the endogenously circulating EPC pool might also limit the therapeutic effects of further stem cell therapy trials.

The granulocyte colony-stimulating factor (G-CSF) is well-established to mobilize hematopoietic stem cells (HSCs) from the bone marrow in patients with hematologic disorders undergoing radiochemotherapy and stem cell transplantation. Thereby, G-CSF activates progenitor cell releasing factors such as neutrophile elastases and matrix-metalloproteinase to release the stem cells from the bone marrow.13 Within the
bone marrow, activated elastases were shown to cleave receptors and integrins. Bone marrow stromal cells express vascular cell adhesion molecule (VCAM)-1/CD 106 that adheres to the integrin α4β1 or very late antigen-4 (VLA-4) expressed by HSCs and EPCs. Similarly, the CXCR-4 receptor is cleaved by elastases at its N-terminal extracellular domain 6H8 leading to an inactivation of the receptor and interruption of the coupling of HSCs and EPCs to the bone marrow.

However, the interaction between the chemokine stromal-derived factor 1 (SDF-1) and its unique cellular receptor CXCR-4 is crucial not only for retention in the bone marrow but also for functional properties of EPCs in terms of their neovascularization capacity. Moreover, it is shown that VEGF as a mediator of postnatal neovascularization transactivates the CXCR4-signaling pathway.

Recently, it has been demonstrated that G-CSF can mobilize EPCs not only in healthy subjects, but also in patients at high risk for CAD and patients with documented CAD. In the clinical setting, G-CSF was also used for stem cell mobilization in AMI and CAD patients. However, the effects of G-CSF mobilization on functional properties of EPCs needed for neovascularization have not been described so far. Therefore, we investigated the effects of G-CSF-administration on mobilization and functional activities of blood-derived EPCs in patients with CIHD.

Materials and Methods

Patients and Study Design

Sixteen patients with CIHD and significant left ventricular dysfunction under optimized pharmacological therapy were treated with subcutaneous injections of 10 μg/kg per day G-CSF (Filgrastim) for 5 subsequent days. Every day, a complete blood count was performed. The patient characteristics are summarized in the Table. The ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Isolation, Cultivation, and Characterization of EPCs

On day 0 and day 5 of therapy, mononuclear cells (MNCs) were isolated from venous blood via Ficoll density gradient centrifugation and 4×10^6 MNCs were plated on fibronectin-coated 24-well dishes in EBM medium containing 20% patient serum with 0.1 mmol/L atorvastatin.

After 3 days of culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and adherent cells underwent cytochemical analysis for DiLDL uptake as described previously.

Colony Assay

After 3 days of culture, adherent cells were washed twice with PBS and detached with EDTA; 5×10^4 isolated EPCs were seeded in methylcellulose plates (Methocult GF H4434; CellSystems) with 100 ng/mL human recombinant VEGF. Plates were studied under phase-contrast microscopy, and colonies were counted after 14 days of incubation. Colonies that contained a minimum of 50 cells were defined as endothelial cell colony-forming unit (EC-CFU).

Flow Cytometry Analysis

On day 0 and day 5, a volume of 100 μL peripheral blood was incubated for 15 minutes in the dark with monoclonal antibodies against human CD34 (Becton Dickinson), followed by phycoerythrin (PE)-conjugated secondary antibody, with the fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against human CD45 (Becton Dickinson), with the PE-conjugated monoclonal antibody against human CD133 (Milteny) and an antibody against the N-terminal extracellular domain 6H8 of the CXCR4-receptor (kindly provided by Dr. Arenzana-Seisdesdos, Pasteur Institute, Paris). After incubation, cells were lysed, washed with PBS, and fixed in 2% paraformaldehyde before analysis.

Migration Assay

Isolated EPCs were detached using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μL endothelial basal medium (EBM) and counted; 2×10^4 EPCs were then placed in the upper chamber of a modified Boyden chamber assay. The chamber was placed in a 24-well culture dish containing EBM and human recombinant VEGF (50 ng/mL) or SDF-1 (20 ng/mL). After 24 hours incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with DAPI. Cells migrating into the lower chamber and attached to the lower side of the filter were counted manually in 3 random microscopic fields.

Hindlimb Ischemia Model

The neovascularization capacity of EPCs was investigated in a murine model of hindlimb ischemia by use of 8- to 10-week-old
Efficiency of G-CSF Mobilization in CAD Patients

To evaluate the efficiency of G-CSF for mobilization of HSCs in patients with CHD, 16 patients with documented CAD, reduced left ventricular function and optimized pharmacological treatment (for patient characteristics see the Table) received Filgrastim doses of 10 μg/kg for 5 subsequent days. The described side effects of this treatment like headache and skeletal pain relieved after analgetic therapy. Before the first G-CSF-dose and on day 5 of treatment, venous blood was drawn and examined in a routine white blood count for the increase of leukocytes. Figure 1A illustrates the increase of patient leukocytes before and after G-CSF-treatment from 7435±1858/μL blood versus 31 490±7872/μL blood (P<0.01).

To evaluate the mobilization of hematopoietic stem cells, flow cytometry analysis of the patient peripheral blood was performed for the hematopoietic stem cell markers CD34, CD45, and the early marker CD133. After G-CSF treatment, the numbers of circulating CD34+CD133+ cells (Figure 1B) and circulating CD34+CD45+ cells (Figure 1C) increased significantly by 435% and 185%, respectively.

To determine the specific mobilization of endothelial progenitor cells by G-CSF, EPCs were cultivated from mononuclear cells isolated on day 0 and on day 5 of G-CSF administration. Adherent EPCs were characterized by DilLDL uptake and were counted. As illustrated in Figure 2A, the numbers of cultivated and harvested EPC per μL blood significantly increased after G-CSF treatment.

The influence of G-CSF on the colony forming capacity was investigated before and after G-CSF-mobilization of the same patients. The colony forming capacity of mobilized EPCs, determined as cell clusters with endothelial morphology after 14 days in a methylcellulose assay, increased significantly by 196% (Figure 2B).

Results

Efficiency of G-CSF Mobilization in CAD Patients

The functional capacity of G-CSF mobilized EPCs was determined in vitro in a modified Boyden chamber assay, using SDF-1 and VEGF as physiological chemoattractants for EPCs to migrate to sites of ischemia. The migratory response to both SDF-1 and VEGF gradients of G-CSF mobilized EPCs was profoundly impaired (see Figure 3A and 3B), compared with the same patient’s cells cultivated before therapy.
The CXCR4 receptor plays a crucial role for migration of HSCs and EPCs. Therefore, we investigated whether a cleavage and subsequent reduction of the CXCR4 receptor may contribute to the migration impairment observed in patients after G-CSF administration. Indeed, the number of EPC expressing the CXCR4 epitope 6H8 was significantly reduced in EPCs isolated from patients after G-CSF administration (Figure 3C), suggesting that the G-CSF treatment may impair the functional expression of the CXCR4 receptor on EPC. Additionally, FACS analysis of circulating noncultivated cells showed a decrease in the epitope 6H8 in CXCR4 cells to a similar extent as analysis of cultured EPC (Figure 3D). These data indicate that G-CSF treatment stimulates the cleavage of the CXCR4-receptor epitope 6H8, which is important for the functional activity of the CXCR4 receptor. To exclude that G-CSF directly influences EPC migration, we directly added G-CSF in equivalent doses to ex vivo cultivated EPC. However, the direct addition of G-CSF to EPC ex vivo did not affect the migratory capacity of EPC (1.3±0.7 migrated EPC/high power field cultivated without G-CSF versus 2.1±0.3 migrated EPC/high power field cultivated with G-CSF–enriched medium, mean±SEM, n=12, P=NS) supporting the assumption that in vivo CXCR4 receptor cleavage by G-CSF-induced activation of bone marrow residing proteases contributes to EPC impairment.

Effects of G-CSF on the Neovascularization Capacity In Vivo
A reduced migratory capacity of bone marrow–derived mononuclear cells is associated with an impaired capacity of the cells to improve neovascularization in animal models. Moreover, ex vivo migration of bone marrow–derived cells or EPC correlates with functional recovery of patients after cell therapy. Therefore, we investigated the neovascularization improvement of EPCs isolated before and after G-CSF administration by using a hind limb ischemia model. Infusion of EPCs from patients before G-CSF administration reduced the number of necrotic limbs 2 weeks after ischemia, compared with mice without cell therapy. However, the incidence of toe or limb necrosis was significantly higher when similar numbers of EPC from patients after G-CSF treatment were infused into the mice (Figure 4A). Likewise, the Laser Doppler-derived measurement of recovery of blood flow showed a slight, but significant decrease when G-CSF–mobilized EPCs were infused, compared with blood flow recovery after infusion of nonmobilized EPC from the same patients (Figure 4B).

Discussion
The present study describes the effects of G-CSF on the mobilization and function of circulating progenitor cells. The investigated patients with CAD and impaired left ventricular

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**Figure 3.** A and B, Migratory activity of nonmobilized and G-CSF-mobilized EPC derived from the same patients in a modified Boyden chamber assay using VEGF and SDF-1 as chemoattractant shows significant impairment of migration toward both the VEGF and SDF-1 gradient after mobilization. n=15, *P*<0.01. Data are given as mean±SEM. C, Flow cytometry analysis of the functional N-terminal epitope 6 H 8 of the CXCR4-receptor on cultivated EPC before and after G-CSF treatment: the number of cells expressing the epitope 6 H 8 decreases significantly after G-CSF-mobilization. n=9, *P*<0.05. Data are given as mean±SEM. D, Flow cytometry analysis of peripheral blood before and after G-CSF-treatment, normalized to the number of gated events for CXCR4 6H8 cells: in peripheral blood, a similar loss of the epitope 6H8 in CXCR4 cells than in cultivated EPC was observed (n=7; data are given as mean±SEM, *P*<0.02).

**Figure 4.** A, Number of gangrenic amputations in animals that received mobilized EPC compared with animals treated with nonmobilized EPC, *P*<0.05, n=10). B, Laser Doppler-derived blood flow 14 days after intravenous infusion of 5×10⁶ cultivated nonmobilized and mobilized EPCs from the same patients (n=10) before and after G-CSF-administration, *P*<0.05, data are given as mean±SEM.
function were treated with a state-of-the-art pharmacotherapy including statins, ACE-inhibitors and beta-blockers, excluding an intrinsic mobilization effect initiated by statins as described.\textsuperscript{27,28} Consistent with findings from other groups, we could show that G-CSF leads to a mobilization of CD34\textsuperscript+ or CD133\textsuperscript+ progenitor cells from the bone marrow in CAD patients\textsuperscript{21} with left ventricular dysfunction. We demonstrated that the number of cultivated endothelial progenitor cells and their clonal capacity significantly increased after G-CSF administration, indicating a potential use of G-CSF in cell-based studies for cardiac repair. Our next goal was to investigate whether G-CSF leads to an alteration of the functional properties of EPCs. Therefore, we tested the migratory capacity toward the chemoattractant factors SDF-1 and VEGF, which are believed to play an important role for in vivo homing of EPCs to ischemic tissues.\textsuperscript{29–33} However, VEGF-induced and SDF-1–induced migration of EPCs isolated from G-CSF mobilized patients was significantly reduced. Moreover, we demonstrated a significant reduction of the 6H8 epitope of the CXCR4 receptors on EPCs cultivated from G-CSF–treated patients. Because the cleavage of the 6H8 epitope by G-CSF–activated proteases in vivo reduces the functional activity of CXCR4, one may envision that the impairment of the CXCR4 receptor signaling underlines the inhibition of EPC migration after G-CSF treatment.

The reduced response toward VEGF might be rationalized by the finding, that the CXCR4 receptor is transactivated by other growth factor receptors. Indeed, the addition of CXCR4 neutralizing antibodies also impaired the response of EPC toward VEGF,\textsuperscript{32} suggesting that the G-CSF-mediated activation of proteases leading to cleavage of the CXCR4 receptor in vivo might be the reason for the functionally impaired response of ex vivo cultivated EPC toward SDF-1 and VEGF. However, we cannot rule out that EPCs from G-CSF–mobilized patients exhibit additional signaling defects. Because G-CSF did not affect the migratory response of EPC in vitro, we at least can exclude a direct negative effect of G-CSF on EPC function.

Consistent with the migration impairment, EPCs from patients after G-CSF treatment showed a significant lower capacity to augment blood flow after ischemia despite a higher clonal expansion capacity. Because the migration capacity correlates with in vivo homing and incorporation of progenitor cells to sites of ischemia and might represent a predictor for the clinical outcome of cell therapy-treated patients,\textsuperscript{27} one may argue that G-CSF mobilized EPCs may exhibit a reduced recruitment to the ischemic tissue. However, the results of recent clinical trials using G-CSF for treatment of patients with acute myocardial infarction demonstrating no effect of G-CSF on functional improvement may support this assumption.\textsuperscript{33} Whether this G-CSF mobilization-induced impairment of functional capacities of EPC indeed leads to an attenuation of improvement in cardiac function in CAD patients after cell therapy remains to be elucidated. The use of other mobilizing substances, such as AMD3100, which lack a negative influence on the migratory capacity of circulating progenitor cells\textsuperscript{34} may be a preferred option for future treatment of ischemic disease.

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Disclosures

None

References


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