Cytokine-Induced Mobilization of Circulating Endothelial Progenitor Cells Enhances Repair of Injured Arteries

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Background—The existence of circulating endothelial progenitor cells (CEPCs) has previously been documented. These cells can be mobilized by cytokines and are recruited to sites of injury, where they may participate in tissue repair. In the present study, we examined the hypothesis that mobilization of CEPCs by exogenous granulocyte-colony stimulating factor (G-CSF) enhances repair of injured arteries by facilitating reendothelialization and inhibiting neointima development.

Methods and Results—Male rats were injected daily with 50 μg/kg recombinant human G-CSF or 0.9% NaCl SC for 8 days. On the fifth day of treatment, 1 mL of blood was collected for fluorescence-activated cell sorting analysis of mononuclear cells, and the animals underwent balloon angioplasty of the common carotid artery. The animals were killed at 2 or 4 weeks after injury, and the carotid arteries were harvested and processed for immunohistochemistry, scanning electron microscopy (SEM), and morphometric analysis of endothelialization and neointimal formation. G-CSF increased the number of circulating mononuclear cells that express endothelial cell lineage markers several-fold. SEM and immunohistochemical staining with the endothelial marker, platelet and endothelial cell adhesion molecule-1, showed rapid and nearly complete (>90%) reendothelialization of the denuded vessels in the G-CSF–treated animals compared with <20% in the control animals. Reendothelialization was paralleled by a decrease in inflammation in the vessel wall. Neointima thickness was reduced by ~60% in the G-CSF–treated animals compared with control animals at 2 and 4 weeks after injury.

Conclusion—We postulate that cytokine-induced mobilization of CEPCs may be a suitable therapeutic strategy for prevention of restenosis after revascularization procedures. (Circulation. 2004;110:2039-2046.)

Key Words: growth substances ■ restenosis ■ endothelium ■ immunohistochemistry

Vascular injury leads to pathological repair and remodeling that involve vascular smooth muscle cell migration and proliferation, resulting in neointimal hyperplasia.1 Endothelial cell (EC) loss is a major contributing factor to the pathological repair of the injured blood vessel.2 The disruption of endothelial integrity leads to a concomitant reduction in the production of vasculoprotective mediators, such as nitric oxide and prostacyclin, and increased vasoconstrictor and growth-promoting substances,3,4 resulting in elevated vascular tone, platelet adhesion, enhanced inflammation, and medial smooth muscle cell proliferation.3,4 The resultant neointimal hyperplasia is the pathological basis for restenosis after revascularization procedures such as angioplasty, stenting, and bypass grafting.1,2

Because EC loss plays a pivotal role in the pathogenesis of intimal hyperplasia after vascular injury, we postulated that a therapeutic strategy that promotes early reendothelialization of the injured vessels would inhibit intimal lesion development, facilitate vascular repair, and improve long-term vessel patency. Progenitor cells originating from the bone marrow have previously been isolated from the mononuclear cell fraction of peripheral blood.5,6 These cells have high proliferative potential3 and under specific growth conditions, differentiate into ECs,7 suggesting that they may be suitable as a substrate for the reendothelialization of damaged vessels. We showed recently that transplantation of autologous circulating endothelial progenitor cells (CEPCs) onto balloon-denuded arteries led to rapid reendothelialization of the injured artery.8 Others have reported that transplantation of CEPCs onto the decellularized porcine iliac artery led to formation of a bioactive endothelial monolayer and prolonged graft patency when these preparations were implanted as carotid interposition grafts.9

We hypothesized that mobilization of CEPCs by hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) may provide a potentially effective noninva-
sive strategy to enhance reendothelialization of injured vessels. Several studies have shown that exogenous administration of cytokines increases the number of CEPCs. For example, pretreatment with vascular endothelial growth factor was reported to double the number of circulating CEPCs in humans, and administration of G-CSF and stem cell factor was found to mobilize EPCs from the bone marrow. Recently, Bhattacharya et al and Shi et al reported that G-CSF enhances endothelialization of small-caliber prosthetic implanted grafts, whereas Walter et al showed a comparable effect in injured carotid arteries of mice after statin therapy for several weeks before injury.

In the present study, we evaluated the efficacy of short-term G-CSF pretreatment as a strategy for promoting reendothelialization and inhibition of neointimal hyperplasia in injured arteries. We chose to use G-CSF because of its ability to efficiently mobilize hematopoietic precursor cells from the bone marrow and to stimulate EC migration and proliferation. Our results show that short-term treatment with G-CSF before balloon angioplasty leads to accelerated reendothelialization and marked inhibition of neointimal formation in the injured vessels.

Methods

Animals
Male Sprague-Dawley rats (200 to 250 g body weight) were purchased from Harlan Laboratories (Indianapolis, Ind). The animals were maintained on a 12-hour:12-hour light/dark cycle under ambient conditions of 24°C and 60% humidity. Food and water were provided ad libitum. The Harvard Medical Area Standing Committee on Animal Care approved all animal procedures.

Cytokine-Induced Mononuclear Cell Mobilization
The animals were anesthetized by intraperitoneal injection with ketamine (70 mg/kg) and xylazine (4 mg/kg) and splenectomized to eliminate the spleen as a source of CEPCs. Two weeks after splenectomy, the animals were injected daily with 50 μg/kg SC recombinant human G-CSF (a generous gift from Dr Pamela Baker, University of Massachusetts, Amherst) for 8 days. Control animals were treated with an equivalent volume of saline.

Balloon-Injury Model
Balloon injury of the left common carotid artery was performed on the fifth day after initiation of G-CSF treatment, as previously described. In brief, a 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences) was inserted through the external carotid artery, inflated with 200 μL air, and passed 3 times along the length of the segment.

Figure 1. FACS analysis of cytokine-induced mononuclear cell mobilization in splenectomized rats. A, Mononuclear cell mobilization by G-CSF. B, Percentage of mononuclear cells coexpressing hematopoietic and endothelial lineage markers CD45 and CD31. C, Percentage of mononuclear cells coexpressing endothelial lineage markers CD34, endothelial nitric oxide synthase (eNOS), Flk-1, stem cell factor receptor c-kit, and E-selectin. D, FACS histograms of hematopoietic and EC markers. *P<0.05 saline vs G-CSF. PE indicates phycoerythrin; FITC, fluorescein isothiocyanate; eGFP, enhanced green fluorescence protein; and APC, allophycocyanin. All other abbreviations are as defined in text.
Characterization of Cytokine-Mobilized Mononuclear Cells

On the fifth day of G-CSF or saline treatment, 1 mL blood was harvested from the inferior vena cava of each rat. The mononuclear cells were isolated by density-gradient centrifugation and counted with a Z1 Coulter particle counter. Approximately 10^6 cells from each animal were suspended in 50 μL phosphate-buffered saline containing 5 mmol/L EDTA and 0.5% bovine serum albumin and incubated for 30 minutes on ice with 20 μg/mL phycoerythrin-conjugated anti-mouse Flk-1, allophycocyanin-conjugated anti-mouse c-kit, and fluorescein isothiocyanate-conjugated anti-mouse CD45 or anti-mouse vascular endothelial (VE)-cadherin. All primary antibodies were purchased from BD Pharmingen with the exception of VE-cadherin, which was purchased from R&D Systems. Unlabeled cells served as negative controls. The cells were analyzed by 3-color fluorescence activated cell sorting (FACS) with a FACSCalibur flow cytometer (Beckman Dickinson) equipped with a 488-nm and a 635-nm red diode laser. Propidium iodide staining was used to gate out dead cells.

Immunohistochemistry

Carotid arteries were harvested 7 and 14 days after injury and frozen in OCT compound (Miles). Sections (5 μm) were incubated in a 1:50 dilution of anti-rat monoclonal CD-51 (platelet and endothelial cell adhesion molecule-1, BD Biosciences). Adjacent sections were incubated in 1:50 monoclonal anti-rat vascular cell adhesion molecule-1 (VCAM-1), 1:100 polyclonal anti-rat CD45 (BD Biosciences), or monoclonal rat endothelial cell antibody (RECA-1, Abcam). All sections were then incubated in a 1:400 dilution of biotinylated anti-mouse rat IgG (Vector Laboratories), treated with alkaline phosphatase, and developed in Sigma Fast Red (Sigma). The sections were counterstained with hematoxylin.

Morphometric Analysis of Endothelialization and Neointimal Hyperplasia

Morphometric assessment of endothelialization and neointimal hyperplasia was performed in frozen sections 2 and 4 weeks after injury, as previously described. The integrity of the reconstituted endothelium was also verified by scanning electron microscopy (SEM). For morphometric analysis of neointimal hyperplasia, 6 to 8 individual, elastin-stained, paraffin sections sampled from 4 different regions of the injured segment were used to calculate neointimal and medial thicknesses, cross-sectional areas, and luminal diameter with the use of ImagePro software.

Statistical Analysis

All results are presented as mean±SEM. An unpaired t test was used for comparisons between control and treated groups. One-way ANOVA followed by Bonferroni multiple-comparison test was used for comparisons between groups at different time points. A probability value ≤0.05 was considered to indicate statistical significance.

Results

Hematopoietic Cell Mobilization and FACS Analysis

The effect of G-CSF treatment on hematopoietic cell mobilization is shown in Figure 1A. After 5 days of G-CSF treatment, the number of circulating mononuclear cells increased by 2-fold (control, 5.96±0.35×10^6 cells; G-CSF, 13.07±1.376×10^6 cells; P=0.001, n=5 for each group). FACS analysis of the whole mononuclear fraction at this time showed a 3.3-fold increase in the percentage of mononuclear cells expressing the common leukocyte antigen CD45 (control, 2.84×10^6 cells; G-CSF, 9.41×10^6 cells/mL blood; Figure 1B and 1D). Comparable numbers were seen for cells expressing CD34, a surface molecule in ECs (Figure 1B and 1D). Approximately 6% of the cells expressed the hematopoietic lineage marker CD34 in the G-CSF–treated group compared with 2% in the saline control, corresponding to a 7-fold increase in the number of positive cells (control, 1.2×10^5; G-CSF, 8.4×10^5 cells; Figure 1C and 1D). The endothelial markers endothelial nitric oxide synthase, Flk1, and E-selectin were expressed in 8.7%, 6.1%, and 2.7% of the mononuclear cells from G-CSF–treated animals compared with 1.3%, 3.0%, and 2.5%, respectively, of the controls, corresponding to an =15-, 4.5-, and 2.3-fold increase in the number of cells expressing these markers (Figure 1C and 1D). Approximately 2.4×10^5 cells expressed the stem cell marker c-kit in the G-CSF–mobilized group compared with 8.7×10^4 in the control group (Figure 1C and 1D). These results suggest that G-CSF treatment significantly increases the number of circulating cells expressing an endothelial lineage phenotype.

Balloon Injury and Inflammation

The morphology of the intact and balloon-injured carotid vessel wall is shown in Figure 2. Histological analysis of hematoxylin-and-eosin–stained cross sections and topographic SEM of the luminal surface of uninjured vessels...
showed a continuous endothelial monolayer lining the vessel lumen (Figure 2A and 2B). The integrity of the endothelium was further confirmed by immunohistochemical detection of RECA-1 (data not shown). The angioplasty procedure led to nearly complete disruption of the endothelium, as evidenced by the presence of an interrupted intima exposing the subendothelial fibers (Figure 2C and 2D). Intense VCAM-1 immunoreactivity was detected in the medial and intimal layers of carotid vessels from control animals on day 4 after injury (Figure 3A) but was markedly reduced in G-CSF–treated animals (Figure 3B). After 2 weeks, VCAM-1 activity was still detectable in the control sections, albeit at a lower intensity (Figure 3C). In contrast, no VCAM-positive immunoreactivity was present in the sections from G-CSF–treated animals (Figure 3D). A small number of CD45-positive cells was found in the adventitia in saline-treated (Figure 3E and 3G) and G-CSF–treated (Figure 3F and 3H) animals, but no leukocyte infiltration was seen in the intima at either time point.

Reendothelialization of Denuded Carotid Artery

We assessed the effect of G-CSF treatment on reendothelialization of denuded vessels in sections stained with CD31 and RECA-1 14 days after injury, as well as by SEM. Sections from control animals showed patchy and interrupted CD31– (Figure 4A) and RECA-1– (Figure 4B) positive staining. A topographic SEM view of the untreated vessels showed incomplete and sparse endothelium (Figure 4C). In contrast, a nearly complete and continuous monolayer of CD31– (Figure 4D) and RECA-1– (Figure 4E) positive cells was found lining the lumen of G-CSF–treated animals. SEM of the luminal surface of these vessels revealed the presence of densely packed, continuous endothelium-like cells covering the luminal area (Figure 4F). Morphometric analysis of the CD31-positive area showed >90% EC coverage of the lumen in the G-CSF–treated animals compared with <20% in the control animals (Figure 4G).

Inhibition of Neointimal Hyperplasia

We determined the ability of G-CSF pretreatment to inhibit neointimal hyperplasia of the balloon-injured carotid vessels at 2 and 4 weeks after injury. Two weeks after injury, a prominent, concentric neointima had developed in the untreated vessels (Figure 5A). By 4 weeks the neointima had slightly expanded, but this did not reach statistical signifi-
cance relative to 2 weeks (Figure 5B). Neointimal formation was markedly reduced in the G-CSF–treated animals at both time points (Figure 5C and 5D). Morphometric analysis of serial sections showed a decrease of ≈58% in neointimal thickness in the G-CSF group at 2 (0.0148±0.0025, n=6) and 4 (0.0171±0.0039, n=6) weeks after injury relative to saline controls (2 weeks, 0.0356±0.0034, n=6; 4 weeks, 0.0412±0.0057, n=5, P<0.05; Figure 5E). Neointimal cross-sectional area decreased by 160% in the G-CSF group at 2 (0.0182±0.0032, n=4) and 4 (0.0128±0.0037, n=5) weeks compared with the control animals (2 weeks, 0.0475±0.0102, n=4; 4 weeks, 0.0341±0.005, n=5, P<0.05). Comparable changes were seen in neointima-media ratios in the G-CSF–treated group (2 weeks, 0.195±0.035; 4 weeks, 0.311±0.84) compared with the control group (2 weeks, 0.506±0.069; 4 weeks, 0.656±0.074, P<0.05; Figure 5F). Concomitant with the changes in neointimal thickness, there was a 9% to 10% increase in luminal diameter in the G-CSF–treated animals at both time points (2 weeks, 0.765±0.023; 4 weeks, 0.749±0.021) compared with the saline group (2 weeks, 0.691±0.011; 4 weeks, 0.684±0.0049, P<0.05; Figure 5G). Luminar area was correspondingly increased in the G-CSF group by 23% at 2 (0.461±0.028, n=4) and by 21% at 4 (0.334±0.0185, n=5) weeks relative to the saline controls (2 weeks, 0.376±0.012; 4 weeks, 0.274±0.004, n=4, P<0.05), suggesting eccentric remodeling in the G-CSF–treated group. Medial thickness did not differ between the G-CSF–treated and control animals at either time point (Figure 5H).

**Discussion**

Disruption of the endothelium triggers a number of signaling cascades that converge on medial smooth muscle cells to stimulate their proliferation and migration, leading to pathological repair and the development of neointimal hyperplasia. Although the injury itself may stimulate reendothelialization of the denuded vessel, the time required for this native repair process to restore endothelial function appears too long to prevent the early critical events leading to activation of vascular smooth muscle cells and neointimal hyperplasia. Accordingly, we postulated that a therapeutic approach that could promote early reendothelialization of the injured vessels would potentiate the endogenous repair process. In the present study, we used G-CSF pretreatment as a strategy to stimulate the mobilization of circulating putative EPCs to enhance rapid reendothelialization of balloon-injured vessels. Our results show that stimulation with G-CSF increases the abundance of circulating mononuclear cells expressing the endothelial lineage phenotype. More significantly, we demonstrate that G-CSF pretreatment accelerates the rate of reendothelialization and inhibits neointimal thickening in balloon-injured carotid arteries.

We and several other groups have recently reported that autologous CEPCs can be harvested from peripheral blood and transplanted into denuded vessels. However, cell transplantation protocols for vessel repair are time consuming and technically challenging. Moreover, the ability to obtain a sufficient number of cells may be limited by the onset of cell senescence. Here, we show that a noninvasive approach based on a well-established protocol of cytokine-induced mobilization of hematopoietic precursor cells, when administered in advance of vascular injury, confers significant protection against the subsequent development of neointimal hyperplasia. This appears to be, at least in part, due to the increased availability of circulating progenitor cells capable of inducing rapid reendothelialization of the injured vessel.
Figure 5. Neointimal hyperplasia in balloon-denuded carotid arteries. Van Gieson elastic staining of vessel profiles from untreated animals at A, 2 weeks and B, 4 weeks after injury. Elastic staining of vessel profiles from G-CSF–treated animals at C, 2 weeks and D, 4 weeks after injury. Sections were viewed at ×100. Planar morphometric analysis of E, neointima thickness; F, neointima-media ratio; G, lumen diameter; and H, medial thickness in untreated and G-CSF–treated animals at 2 and 4 weeks after balloon injury. Significant differences were seen in neointimal thickness, neointima-media ratios, and lumen diameter between G-CSF–treated and control animals at 2 and 4 weeks after injury. *P<0.05, saline vs G-CSF. All abbreviations are as defined in text.
and preventing the activation of medial smooth muscle. This is suggested by the accelerated rate of reendothelialization and the accompanying reduction in inflammation in the injured vessels of the G-CSF–treated animals. We hypothesize that the rapid reconstitution of the denuded vessel by the mobilized CEPCs leads to timely restoration of endothelial function and vascular homeostasis, resulting in inhibition of neointimal formation.

We note that several groups have reported mobilization of EPCs by cytokine treatment.9–13 Kocher et al11 reported that stimulation with C-CSF increases the number of CD34-positive cells expressing endothelial markers. Also, using G-CSF treatment, Bhattacharya et al12 and Shi et al13 showed enhanced endothelialization of small-caliber prosthetic grafts in association with an elevation in CEPCs. Our present findings provide further evidence of the therapeutic potential of cytokine-induced mobilization of progenitor cells in vessel repair. Specifically, we demonstrate for the first time the injury site.16 From a therapeutic standpoint, our results suggest that several groups have reported mobilization of CEPCs by cytokine treatment.9–13 Kocher et al11 reported that stimulation with C-CSF increases the number of CD34-positive cells expressing endothelial markers. Also, using G-CSF treatment, Bhattacharya et al12 and Shi et al13 showed enhanced endothelialization of small-caliber prosthetic grafts in association with an elevation in CEPCs. Our present findings provide further evidence of the therapeutic potential of cytokine-induced mobilization of progenitor cells in vessel repair. Specifically, we demonstrate for the first time the therapeutic benefit of G-CSF pretreatment as a strategy for prevention of restenosis after angioplasty-induced vascular injury. Others have reported that statin therapy enhances reendothelialization and reduces neointimal formation in injured vessels,14 apparently due to sustained mobilization of CEPCs from the bone marrow. However, the mechanism by which statins mobilize CEPCs may differ from that of G-CSF and other cytokines. The effect of G-CSF on CEPC mobilization could be seen in the short term, whereas the effect of statin therapy may require several weeks of treatment. This difference in time course may have therapeutic implications, in that the timing of administration and response could differ, especially in association with angioplasty and revascularization procedures.

Our study did not specifically examine the sources and the mechanisms governing the mobilization, homing, and differentiation of CEPCs and their role in endothelial repair at the site of injury. The bone marrow has been reported to be the primary site of origin of progenitor cells5,6,9,12; however, contributions from other hematopoietic and nonhematopoietic tissues to the circulating progenitor cell pool cannot be completely excluded. In addition, resident EPCs and native ECs may also contribute to vascular repair. In the present study, we eliminated the spleen as a source of CEPCs20 by splenectomizing the animals before cytokine administration. Regarding the potential mechanisms involved in mobilization and homing of the CEPCs, several groups have reported that the cells are recruited predominantly to sites of injury,7,11,14 suggesting that signals emanating from the injury site may play a role in the mobilization, homing, and differentiation processes. In addition, G-CSF may stimulate the migration and proliferation of resident EPCs and ECs in the vicinity of the injury site.18 From a therapeutic standpoint, our results may have clinical implications for the treatment of vascular-proliferative disease. Mobilization of progenitor cells in advance of angioplasty may provide a strategy for inhibition of restenosis in injured vessels. This may represent a novel antirestenosis paradigm that focuses on disease prevention by harnessing the therapeutic potential of the native repair and regeneration processes. The simplicity and cost-effectiveness of this approach would be major advantages compared with the stent and drug-based therapies currently in use. However, our observations are based on a splenectomized animal model. Clearly, the clinical feasibility of this approach will need to be evaluated in an intact animal model of vasculo-proliferative disease. Despite this limitation, our results clearly show a significant therapeutic effect of G-CSF treatment in reendothelialization of injured blood vessels. On the other hand, the potential stimulation of inflammatory cells by G-CSF needs to be carefully evaluated. Recent evidence suggests that G-CSF exerts counterbalancing effects on inflammation by promoting neutrophilia while inhibiting proinflammatory cytokine production.21 In addition, there is the possibility that mobilized bone marrow progenitors may differentiate into vascular smooth muscle cells,22 which could potentially aggravate the severity of restenosis of treated vessels. Clearly, the long-term outcome of this strategy and its safety and feasibility for use in patients will have to be established before the complete therapeutic potential of this approach can be assessed.

In conclusion, the present study shows that bone marrow stimulation with G-CSF before balloon-induced vascular injury significantly increases the availability of CEPCs and accelerates the rate of reendothelialization of injured vessels, leading to marked inhibition of neointimal formation and favorable vascular remodeling. These findings suggest that pretreatment with G-CSF may be a feasible and efficient therapeutic strategy for prevention of restenosis after revascularization procedures, such as such as percutaneous transluminal angioplasty, stenting, and atherectomy.

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