Therapeutic Angiogenesis With Intramuscular Injection of Low-Dose Recombinant Granulocyte-Colony Stimulating Factor

Mejeong Lee, Mika Aoki, Takahisa Kondo, Koichi Kobayashi, Kenji Okumura, Kimihiro Komori, Toyoaki Murohara

Objective—In vivo administration of granulocyte colony-stimulating factor (G-CSF) has been shown to facilitate regeneration of cardiovascular tissues. However, G-CSF causes marked leukocytosis that potentially induces adverse cardiovascular events. Earlier studies showed that G-CSF had direct stimulatory actions on mature endothelial cells, resulting in promotion of angiogenesis. We thus examined whether low doses of recombinant human G-CSF (rhG-CSF) locally injected into ischemic tissues would stimulate angiogenesis without inducing severe leukocytosis.

Methods and Results—Reverse-transcription polymerase chain reaction (PCR) revealed expression of G-CSF receptor in human umbilical vein endothelial cells (HUVECs). rhG-CSF (100 ng/mL) enhanced migration and tube formation but not proliferation of HUVECs in vitro. We then examined the effects of rhG-CSF on angiogenesis in a rat model of hindlimb ischemia. Nude rats received in their ischemic skeletal muscles either rhG-CSF (2, 10, 20 μg/kg per day) or saline (control) for 6 days. Laser Doppler blood flowmetry (LDBF) revealed an augmented ischemic/normal limb LDBF ratio and an increased capillary density in the rhG-CSF–treated groups compared with the control at days 14, 21, and 28 (P<0.05). These doses of rhG-CSF induced only mild leukocytosis (~1.4-fold increases versus baseline).


Key Words: angiogenesis ■ endothelial cell ■ ischemia ■ rhG-CSF

Therapeutic angiogenesis is an effective means to treat no-option patients with severe peripheral artery disease and end-stage ischemic heart disease. Single gene or protein therapy of several angiogenic cytokines has been introduced into clinical trials, but enthusiasm was hampered by a series of negative clinical outcomes of randomized trials. However, endothelial progenitor cells (EPCs) have been identified in adult human peripheral blood, bone marrow and cord blood.1–3 Circulating EPCs participate in postnatal neovascularization after mobilization from bone marrow (BM), consistent with postnatal vasculogenesis.1 Subsequently, transplantation of culture-expanded EPCs or autologous BM mononuclear cells (BM-MNCs) has been shown to augment ischemia-induced neovascularization in vivo.4–5 However, BM cell therapy usually requires aspiration of a large amount of BM.

Granulocyte colony-stimulating factor (G-CSF) is the most widely used hematopoietic cytokine that mobilizes CD34+ HSCs.6–9 However, it is known that G-CSF enhances the number of circulating granulocytes that potentially induces adverse cardiovascular events including acute coronary syndrome.10,11 In earlier studies, Bussolino et al demonstrated that G-CSF stimulated angiogenic functions of mature endothelial cells (ECs), resulting in enhanced migration, proliferation and tube formation in vitro.12,13 From the latter findings, we reasoned that low dose of G-CSF injected into local ischemic tissues would enhance neovascularization via direct stimulatory actions on endothelial cells. Moreover, such low-dose local therapy with G-CSF would be able to avoid severe leukocytosis and potential adverse cardiovascular events.

Accordingly, we tested our hypothesis that recombinant human G-CSF (rhG-CSF) would enhance migration, proliferation, and tube formation of mature endothelial cells in vitro, and that local intramuscular injection of low-dose rhG-CSF, which does not induce severe peripheral leukocytosis, would be effective for therapeutic angiogenesis in an animal model of hind limb ischemia in vivo.

Methods

Reverse-Transcription Polymerase Chain Reaction

Using semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis, we examined whether G-CSF receptor
mRNA was expressed in several classes of human mature endothelial cell lines including human umbilical vein ECs (HUVECs) (passages 3 to 11), human dermal microvascular ECs (HMVECs) (passage 6), and human coronary artery ECs (HCAECs) (passage 6). These cells were purchased from Sanko Junyaku (Tokyo, Japan). Total RNA was extracted from ECs using guanidium isothiocyanate-phenol chloroform solution (TRIZol reagent, Invitrogen) quantified by measuring absorption at 260/280 nm, and subjected to RT-PCR analysis. Total RNA was reverse transcribed using oligo dT primers and RNase H reverse-transcriptase (Superscript II; Invitrogen) with 1 μg total RNA per sample.14 The primer set for detecting human G-CSF receptor mRNA was: for (sense) 5'- TCCCTCACCTGATGACCTTTGA-3' and for (antisense) 5'- GGAAGTTGAGCTTATACTTC-3'. RT-PCR of G-CSF receptor was expected to yield 3 PCR products sized 620 bp (transmembrane form, GenBank M59818), 701 bp (insert in the cytoplasmic domain, GenBank M59820), and 548 bp (soluble form, GenBank M59819). The primers for human GAPDH were 5'-CTTACCAACCATGGAGG-3' and 5'-TGAAGTCAGAGGAGACC-3', yielding a 557-bp PCR product.

Cell Migration Assay
We examined the effects of rhG-CSF (filgrastim; Chugai Pharmaceuticals) on HUVECs migration. Migration activity assay was performed using a modified Boyden chamber apparatus (Neuroprobe, Gaithersburg, Md) as described previously.15 Polynvinylpyrrolidone (PVP)-free polycarbonate filters with multiple pores (8 μm in diameter) were coated with 0.1% gelatin and 50 μg/mL fibronectin and were dried under air for 1 hour. Culture medium (25 μL) supplemented with 1% fetal bovine serum and rhG-CSF (1, 10, 100, and 1000 ng/mL) was placed in the lower chamber of the apparatus. rhG-CSF was kindly provided by Chugai Pharmaceuticals (Tokyo, Japan). As a positive control, rhVEGF (10 ng/mL) (R&D Systems, Minneapolis, Minn) was placed in the lower chamber. Subconfluent HUVECs in culture were washed and trypsinized for a minimum time required to achieve cell detachment. After a coated PVP-free polycarbonate filter was placed between the lower and upper chambers, HUVECs (1 × 104 cells) suspended in 100 μL of Medium-199 containing 1% fetal bovine serum without rhG-CSF were seeded in the upper chamber. The whole apparatus was then incubated for 3 hours at 37°C in a humidified incubator supplemented with 95% air and 5% CO2 to render HUVECs to migrate through the membrane. After the incubation, the filter was removed and nonmigrated HUVECs on the upper side of the filter were scraped-off with a rubber. The filters were then fixed with methanol for 10 minutes and stained with May-Gruenwald's solution (Sigma). Migrated HUVECs attached to the lower side of the filter were counted in randomly selected microscopic fields (×200) in each chamber. All experiments were performed 6 times and data were expressed as the number of migrated cells/field.

Cell Proliferation Activity Assay
Proliferative activity of HUVECs was analyzed using a previously validated colorimetric MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay with the electron coupling reagent phenazine methosulfate (Cell Titer 96 AQ; Promega, Madison, Wis). HUVECs were seeded in a 96-well plate (1000 cells/well) in 0.2 mL of Medium-199 containing 2% fetal bovine serum with or without rhG-CSF (0.1, 1, 10, 100, and 1000 ng/mL). MTS/phenazine methosulfate mixture (20 μL) was added per well on day 3 of culture and allowed to incubate for 4 hours at 37°C. Then, absorbance was measured at 490 nm/635 nm using an enzyme-linked immunosorbent assay plate reader (Model 680 Microplate Reader; Bio-Rad).16

In Vitro Angiogenesis Model: Endothelial Network Formation on Matrix Gel Culture
We examined the effects of rhG-CSF on endothelial network formation in Matrigel.17 HUVECs (4×104 cells) suspended in 1 mL of Medium-199 containing 2% fetal bovine serum with control saline or with different concentrations of rhG-CSF (1, 10, and 100 ng/mL) were cultured on growth factor-reduced Matrigel (Becton Dickinson, San Jose, Calif). After a 12-hour incubation period at 37°C under 5% CO2 within humidified atmosphere, endothelial network formation was examined and photographed at randomly chosen fields in each well (×40). To analyze quantitatively, the length of endothelial network formation in each image was calculated by a computerized WIN ROOF program (Mitani, Fukui, Japan). All experiments were performed in quadruplicate and data were expressed as a length of the network (mean length/field).

Animal Model: Nude Rat Ischemic Hind Limb Model
Peripheral Leukocyte Count
In our experimental protocol is shown in Figure IA (available online at http://atvb.ahajournals.org). Male nude rats (F344/N nu/nu) were subjected to unilateral hind limb ischemia by complete resection of the entire left femoral artery and vein as described previously.3 In the 3 rhG-CSF treatment groups, rats received rhG-CSF (2, 10, or 20 μg/kg per day) within ischemic adductor muscles (n=8 in each group) for 6 days starting on the day of surgery for ischemia, whereas control rats (n=8) received equivalent volume of saline for 6 days by a similar manner. In rats, it has been shown that 200 to 300 μg/kg per day for 5 days injection of rhG-CSF induce equipotent hematopoietic efficacy observed in humans who receive 10 to 20 μg/kg per day for similar period.18 Therefore, in the present study, the doses of rhG-CSF (2, 10, or 20 μg/kg per day) were decided because these doses are within low enough range and do not induce severe leukocytosis in rats.

For leukocyte count in peripheral blood, blood samples (0.2 mL from each animal) were taken from the tail vein before operation and at postoperative days 3 and 7. Blood cell count was performed using an automated cell analyzer Sysmex KX-21 (Sysmex, Kobe, Japan). As a control experiment, rhG-CSF (2, 10, or 20 μg/kg per day) was injected into nonischemic hindlimb, and circulating leukocytes were counted as well. All rats were subjected to have regular diet and tap water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine.

Laser Doppler Blood Flow Analysis
We measured the ratio of the ischemic/normal hind limb blood flow using a laser Doppler blood flow (LDBF) analyzer (moorLDI, Moor Instrument) as described previously.3 At 7 predetermined time points (before and on postoperative days 1, 3, 7, 14, 21, and 28), we performed 2 consecutive laser scannings over the region of interest (legs and feet). The average perfusion signals of the ischemic and nonischemic feet were computed on the basis of histograms of the colored pixels relating the extent of blood flow. To minimize variations caused by ambient light, blood flow was expressed as the ischemic/normal hind limb LDBF ratio.

Capillary Density Analysis and Tissue Inflammatory Responses
The effect of rhG-CSF administration (or saline) on microvascular neovascularization was assessed under light microscopy by measurements of the number of capillary endothelium in ischemic tissues.3 The adductor skeletal muscles were embedded in OCT compound (Miles) and snap frozen in liquid N2 at postoperative day 28. The sections were histochemically stained for alkaline phosphatase to detect capillary endothelial cells as described previously. The capillary endothelial cells were counted under light microscopy (×200) to determine the histological capillary density. Five high-power microscopical fields from each muscle sample were randomly selected and counted for alkaline phosphatase-positive endothelial cells.

Because rhG-CSF is a granulopoietic cytokine, we also examined the effects of local injection of rhG-CSF on inflammatory responses in the ischemic tissue. We performed immunohistochemical analysis
using anti-CD45 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) in ischemic tissues as described previously.19 The number of CD45-positive cells in the ischemic tissues at postoperative days 3 and 21 were counted under light microscopy and expressed as a mean number of cells per high-power field.

Analysis of EPC Mobilization After rhG-CSF Treatment
At day 3 after starting rhG-CSF injection (2, 10, or 20 μg/kg per day), peripheral blood mononuclear cells (PB-MNCs) were isolated by a density gradient centrifuge method. During culture of PB-MNCs, cell clusters and spindle-shaped attaching cells appeared. EPC-like attaching cells were characterized by the incorporation of DiI-labeled acetylated low-density lipoprotein and Ulex lectin binding.15 EPC-like AT cells were counted under fluorescence microscopy on day 7 of culture. Five randomly selected microscopic fields in each well were examined, and the mean number of EPC-like attaching cells (per 5 high-power fields) was calculated and compared between the groups.

Statistical Analysis
All data are presented as means ± SE. Comparison between the experimental groups was performed using ANOVA followed by Fischer’s probability test. Significant difference was defined \( P < 0.05 \).

Results

G-CSF Receptor mRNA Expression in ECs in Culture
We examined the expression of G-CSF receptor on mature human endothelial cells by RT-PCR analysis. We confirmed the expression of G-CSF receptor on 3 human mature endothelial cell lines HUVECs, HMVECs, and HCAECs (Figure 1). As a positive control, G-CSF receptor was expressed on human BM-MNCs (Figure 1).

The Effects of rhG-CSF on Migration and Proliferation of HUVECs In Vitro
Both migration and proliferation of endothelial cells are important early processes of angiogenesis. We therefore examined whether rhG-CSF would directly promote migration of HUVECs using a modified Boyden chamber apparatus. We found that rhG-CSF stimulated migration of HUVECs in a dose-dependent manner, and that at 100 ng/mL rhG-CSF significantly enhanced HUVECs migration \( (P < 0.001) \) (Figure 2A and 2B). Although the highest dose of rhG-CSF (1000 ng/mL) slightly increased the migratory response, it was not statistically significant. Thus, rhG-CSF (1 to 1000 ng/mL) induced a bell-shaped dose-response curve regarding HUVEC migration (Figure 2B).

We next examined whether rhG-CSF would influence proliferation of endothelial cells. We found that rhG-CSF (0.1, 1, 10, 100, and 1000 ng/mL) did not induce the proliferation of HUVECs. In contrast, VEGF (10 ng/mL), as a positive control, significantly stimulated the proliferation of HUVECs \( (P < 0.01) \) versus control (Figure 2C).

The Effects of rhG-CSF on Endothelial Network Formation In Vitro
To examine whether rhG-CSF directly stimulates endothelial tube formation in vitro, rhG-CSF was administered to HUVECs cultured on basement membrane matrix gel (Matrigel). After a 12-hour incubation period, rhG-CSF (100 ng/mL) significantly stimulated endothelial network formation \( (P < 0.01) \) versus control (Figure 3A and 3B). Lower doses of rhG-CSF (1 and 10 ng/mL) had a tendency to stimulate endothelial tube formation but the effects were not statistically significant compared with the nontreated control group (Figure 3B).

![Figure 1](http://atvb.ahajournals.org/) RT-PCR analysis for G-CSF receptor mRNA expression in mature human endothelial cell lines. The expression of G-CSF receptor was confirmed in cultured HUVECs, HMVECs, and HCAECs. BM-MNCs as positive control cells expressed G-CSF receptor.

![Figure 2](http://atvb.ahajournals.org/) Effects of rhG-CSF on migration and proliferation of HUVECs. rhG-CSF induced endothelial cell migration, but did not affect the proliferation. A, HUVEC migration assay using modified Boyden chamber apparatus. Stained cells are migrated cells. B, rhG-CSF induced HUVEC migration in a dose-dependent manner. Significantly greater number of HUVECs migrated in response to 100 ng/mL of rhG-CSF. rhG-CSF induced a bell-shaped dose-response curve in HUVEC migration assay. VEGF (10 ng/mL) also significantly stimulated HUVEC migration as a positive control. C, rhG-CSF (0.1, 1, 10, 100, 1000 ng/mL) did not stimulate HUVEC proliferation. VEGF (10 ng/mL) significantly stimulated HUVEC proliferation as a positive control. G = control group. *** \( P < 0.001 \) vs control.
In Vivo Angiogenesis Model

The Effects of rhG-CSF on Circulating Leukocyte Counts

We examined total leukocyte counts as an indication for the mobilizing action of rhG-CSF on circulating white blood cells (WBCs) in rats with hindlimb ischemia. At day 3 after operation and starting administration of rhG-CSF, the leukocyte count was modestly increased by 1.4-fold and by 1.3-fold in the groups with the 2 different doses of rhG-CSF (10 and 20 μg/kg per day) (P < 0.05 versus baseline values, respectively). The WBC count rapidly returned to the levels similar to baseline at day 7 after surgery (not significant versus baseline) (Figure IB). In control rats without hind limb ischemia, rhG-CSF was injected into nonischemic hindlimb, and peripheral leukocyte counts were analyzed. The leukocyte counts responded in a similar manner as those with rhG-CSF injected into the ischemic hindlimb at day 3. Only in the group of rats receiving 20 μg/kg per day, WBC count increased by 1.4-fold at day 7 (P < 0.05 versus baseline) (Figure IB). Overall, the WBC count increased as much as 1.4-fold compared with the baseline value in both ischemic and nonischemic animals in each rhG-CSF group, suggesting the doses used in the present study induced only modest leukocyte mobilizing action.

The Effects of Low-Dose rhG-CSF on Ischemia-Induced Neovascularization in Nude Rats In Vivo

Serial LDBF analyses revealed significantly augmented the ratio of ischemic/normal hind limb LDBF in all 3 rhG-CSF treatment groups compared with the control group at postoperative days 14, 21, and 28 (Figure 4A and 4B). The ischemic/normal LDBF ratios were significantly greater in all the 3 rhG-CSF treatment groups (2, 10, or 20 μg/kg per day) than in the saline-treated control group. However, there was no significant statistical difference in the LDBF ratios among the 3 rhG-CSF treatment groups (Figure 4B).

Tissue Inflammatory Responses

At postoperative days 3 and 21, we analyzed inflammatory cell infiltration in ischemic hindlimb tissues. There were no significant differences in the number of infiltrated CD45-positive leukocytes in tissues among the 4 experimental groups on both postoperative days 3 and 21.

Tissue Capillary Density

At postoperative day 28, histochemical analysis of the ischemic hindlimb skeletal muscle tissues with alkaline phosphatase staining revealed a significant increase in the capillary...
density in all 3 rhG-CSF-treated groups compared with the control group (P<0.01 versus saline control). However, there was no significant statistical difference in the LDBF ratios among the 3 rhG-CSF treatment groups (Figure 5A and 5B).

The Effects of Low-Dose rhG-CSF on Mobilization of EPCs
We examined the effects of low-dose rhG-CSF on EPC mobilization. The number of culture expanded EPC-like attaching cells revealed 2.5-fold to 3-fold increase compared with the control group after injection of rhG-CSF (2, 10, and 20 μg/kg per day) (Figure IIA and IIB, available online at http://atvb.ahajournals.org).

Discussion
rhG-CSF has been used widely to mobilize CD34+ HSCs and to increase circulating granulocytes in patients receiving BM transplantation or chemotherapy. Recently, much interest has focused on the use of rhG-CSF to mobilize not only HSCs but also other lineage cells such as EPCs to facilitate postnatal tissue regeneration in the cardiovascular system. We and other investigators recently found that circulating EPCs are possibly derived from CD34+ cells, otherwise identified as HSCs. These studies raised novel concept that rhG-CSF could mobilize not only HSCs but also EPCs and facilitate EPC-mediated postnatal vasculogenesis (ie, de novo synthesis of vascular tissues).

We previously demonstrated that tissue implantation of autologous BM-MNCs that contain a large number of both HSCs and EPCs (2% to 3% of total BM-MNCs) significantly augmented microvascular angiogenesis and collateral vessel formation (ie, arteriogenesis) in patients with critical limb ischemia. Moreover, tissue implantation of CD34+ cells isolated by cell apheresis from rhG-CSF-mobilized peripheral blood MNCs augmented ischemia-induced neovascularization. Thus, G-CSF administration have been considered as an alternative method for therapeutic angiogenesis. However, Kang et al recently demonstrated a high rate of in-stent restenosis after administration of rhG-CSF (10 μg/kg per day) for 4 days in patients with severe coronary artery disease presumably by increased peripheral leukocytes and platelets, leaving a significant caution for the use of rhG-CSF for patients with coronary artery diseases.

Thus, one should be very careful with G-CSF–mediated therapeutic angiogenesis because of enhanced number of circulating leukocytes and platelets. However, Bussolino et al previously demonstrated that G-CSF and GM-CSF had direct stimulatory actions on mature vascular endothelial cells independent of mobilizing actions on CD34+ HSCs. G-CSF directly stimulated endothelial cell migration, proliferation in culture and angiogenesis-like network formation on Matrix gel culture in vitro. These findings raised a novel concept that local injection of lower doses of rhG-CSF would directly enhance angiogenesis via a direct stimulatory action on mature endothelial cells in situ without inducing severe leukocytosis. However, the effects of low-dose rhG-CSF on ischemia-induced angiogenesis were little known. We investigated whether local injection of low dose rhG-CSF, which does not elicit severe peripheral leukocytosis, would induce angiogenesis in the setting of tissue ischemia in vivo.

The major findings of the present study are that: (1) G-CSF receptor presents on mature human endothelial cell lines; (2) rhG-CSF promoted migration but not proliferation of HUVECs in vitro; (3) rhG-CSF promoted angiogenesis-like tube formation of HUVECs on matrix gel; and (4) direct local injection of low dose rhG-CSF, which did not induce severe leukocytosis in rats, augmented ischemia-induced angiogenesis in a model of unilateral hind limb ischemia in vivo. Our current findings for the first time demonstrate that rhG-CSF induces migration of mature human endothelial cells, and low-dose rhG-CSF augments ischemia-induced angiogenesis in vivo. This method would become a possible new modality for therapeutic angiogenesis in patients with severe peripheral artery occlusive disease.

Bussolino et al previously showed that rhG-CSF induced migration, proliferation, and tube formation of HUVECs.
Our present study further extends their findings that low doses of rhG-CSF have a direct angiogenic action when injected directly into ischemic tissues in vivo. However, there are discrepancies between our data and those by Bussolino et al regarding endothelial proliferation. It is known that there are \( \sim 100 \) derivatives of rhG-CSF created by various gene mutation techniques. These can be classified into glycosylated and nonglycosylated protein.\(^{24}\) Okabe et al also reported that 2 different derivative of rhG-CSF showed different activity in cell proliferation assay.\(^{25}\) Although we do not know exactly about rhG-CSF type that Bussolino et al used, it would be possible to consider the different results caused by the formal difference of rhG-CSF.

Regarding the potency for inducing leukocytosis by the rhG-CSF in rodents, previous studies showed that rhG-CSF (100 to 300 \( \mu \)g/kg/d) in murine species was almost equivalent to rhG-CSF (10 to 20 \( \mu \)g/kg per day) administered in humans to elicit stem cell mobilization and peripheral leukocytosis.\(^{18}\) In the present study, therefore, the regimen of rhG-CSF (ie, 2, 10, and 20 \( \mu \)g/kg per day) was in the range of low doses rhG-CSF for rats. Such doses of rhG-CSF would be equivalent for a range of 0.2 to 2 \( \mu \)g/kg per day in humans in terms of the potency of rhG-CSF.\(^{18}\) Interestingly, all of the three rhG-CSF treated groups (2, 10, and 20 \( \mu \)g/kg per day) in the present study revealed increases in the ischemic/normal hindlimb LDBF ratio and capillary density in ischemic tissues. The number of peripheral leukocytes was increased modestly by 1.3-fold to 1.4-fold in 10 and 20 \( \mu \)g/kg per day of the rhG-CSF groups, respectively. The lowest dose of rhG-CSF (ie, 2 \( \mu \)g/kg per day) did not increase circulating leukocytes significantly. Therefore, the present treatment regimen of low dose rhG-CSF could enhance ischemia-induced angiogenesis without inducing severe leukocytosis. Because our in vitro data clearly showed that rhG-CSF would directly stimulate migration and tube formation of endothelial cells, neovascularization induced by local injection of rhG-CSF is likely mediated via direct stimulatory action on mature endothelial cells in the ischemic tissues rather than mobilizing action on stem/progenitor cells.

### Study Limitations

First, rhG-CSF increases circulating leukocytes, and therefore possibly affects inflammation-induced angiogenesis in ischemic tissues. However, histological analysis revealed that there were no significant differences in CD45-positive leukocyte infiltrated in tissues among the 4 groups, indicating that inflammatory response is not likely involved in the difference of angiogenesis. Second, mobilization of EPC-like attaching cells was stimulated by low doses of rhG-CSF. This result suggests that EPC-mediated vasculogenesis may be also enhanced after local injection of rhG-CSF, and this possibility should be also analyzed in future studies.

In summary, we showed that rhG-CSF directly induced endothelial cell migration and tube formation in culture. Also, local injection of low doses of rhG-CSF, which did not induce severe leukocytosis in the peripheral circulation, effectively augmented neovascularization in the setting of tissue ischemia in vivo. This method would become a novel new therapeutic modality to induce angiogenesis in patients with severe peripheral artery disease without increasing circulating leukocyte counts, avoiding potential adverse cardiovascular effects evoked by leukocytosis.

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### References


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Figure Legends

Figure I. A. In vivo experimental protocol is shown. Either saline or rhG-CSF (2, 10 or 20 µg/kg/day) was directly injected into the ischemic skeletal muscles on indicated time points after the operation for hindlimb ischemia. B. Peripheral blood WBC counts. Administration of rhG-CSF (2, 10 and 20 µg/kg/day) for 6 days modestly increased circulating WBC counts. The number returned to baseline levels at 7 days after the induction of tissue ischemia. * p<0.05 vs. baseline values

Figure II. Low dose rhG-CSF stimulates mobilization of EPC-like attaching cells. A,B. At day 3, culture analysis of PB-MNCs revealed an increase in EPC-like attaching cells (DiI-acLDL = red, Ulex lectin = green, double positive = yellow). * p<0.05 vs. control.
A
rhG-CSF, 6 days

Operation  X
LDBF  X X X X X X X
WBC Count  X X X

B
rhG-CSF

Control
2 µg/kg/d
10 µg/kg/d
20 µg/kg/d

WBC Counts (µL)
6000
5000
4000
3000
2000

day
0 3 7

Rats with limb ischemia

WBC Counts (µL)
6000
5000
4000
3000
2000

day
0 3 7

Rats without limb ischemia