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).

Conclusions—rhG-CSF promoted endothelial migration and tube formation in vitro. Local injection of low doses rhG-CSF effectively increased ischemia-induced angiogenesis in vivo. This treatment regimen of low-dose rhG-CSF may become a new and safe modality for therapeutic angiogenesis.

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 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-phenolchloroform 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-transcribe (Superscript II; Invitrogen) with 1 µg total RNA per sample. The primer set for detecting human G-CSF receptor mRNA was: for (sense) 5'-TCTTCCAC-CCTGATGACTTGGA-3' and for (antisense) 5'-GAAGTTGAGC-AGTGCCCACAAG-3'. RT-PCR of G-CSF receptor was expected to yield 3 PCR products sized 620 bp (transmembrane form, GenBank MS9818), 701 bp (insert in the cytoplasmic domain, GenBank MS9820), and 548 bp (soluble form, GenBank MS9819). The primers for human GAPDH were 5'-CTTCAACCACAT-GGAGGGG-3' and 5'-TGAAGTCAGAGGAGACCAC-3', yielding a 575-bp PCR product.

**Cell Migration Assay**

We examined the effects of rhG-CSF (lenograstim; Chugai Pharmaceuticals) on HUVECs migration. Migration activity assay was performed using a modified Boyden chamber apparatus (Neuroprobe, Gaithersburg, Md) as described previously. Polyvinylpyrrolidone (PVP)-free polycarbonate filters with multiple pores (8 µm in diameter) were coated with 0.1% gelatin and 50 µg/mL fibrinogen and were dried under air for 1 hour. Culture medium (250 µL) was placed in the upper chamber. The whole apparatus was then incubated for 3 hours at 37°C. Then, absorbance was measured at 490 nm/655 nm for (antisense) 5'-H11032/GA-AGGAGG-3' and for (sense) 5'-H11032/GA-AGGAGG-3'.

**Formation on Matrix Gel Culture**

In Vitro Angiogenesis Model: Endothelial Network Formation on Matrix Gel Culture

We examined the effects of rhG-CSF on endothelial network formation in Matrigel. 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 vivo 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. 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. 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.

**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. 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.
using anti-CD45 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) in ischemic tissues as described previously.\textsuperscript{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.

\textbf{Analysis of EPC Mobilization After rhG-CSF Treatment}

At day 3 after starting rhG-CSF injection (2, 10, or 20 \(\mu\)g/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 Dil-labeled acetylated low-density lipoprotein and Ulex lectin binding.\textsuperscript{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.

\textbf{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 \(P<0.05\).

\textbf{Results}

\textbf{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).

\textbf{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).

\textbf{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).
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 1B). 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 1B). 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 (P<0.05) (Figure 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.
HSCs and EPCs (2% to 3% of total BM-MNCs) significantly facilitate EPC-mediated postnatal vasculogenesis (ie, de novo synthesis of vascular tissues).

rhG-CSF could mobilize not only HSCs but also EPCs and other lineage cells such as EPCs to facilitate postnatal tissue regeneration in the cardiovascular system.21,22 We and other investigators recently found that circulating rhG-CSF has been used widely to mobilize CD34

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.4 Moreover, tissue implantation of CD34

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, would induce 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.12,13 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 discrepancies between our data and those by Bussolino et al regarding endothelial proliferation. It is known that there are differences in the formal dose of rhG-CSF.

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Regarding the potency for inducing leukocytosis by the rhG-CSF in rodents, previous studies showed that rhG-CSF (100 to 300 µg/kg per d) in murine species was almost equivalent to rhG-CSF (10 to 20 µg/kg per day) administered in humans to elicit stem cell mobilization and peripheral leukocytosis. In the present study, therefore, the regimen of rhG-CSF (ie, 2, 10, and 20 µg/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 µg/kg per day in humans in terms of the potency of rhG-CSF. Interestingly, all of the three rhG-CSF treated groups (2, 10, and 20 µg/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 µg/kg per day of the rhG-CSF groups, respectively. The lowest dose of rhG-CSF (ie, 2 µg/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.

Acknowledgments

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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
- LDBF
- WBC Count

(B) Control vs. rhG-CSF treatment in rats with limb ischemia and without limb ischemia.