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Granulocyte-Colony Stimulating Factor Augments Neovascularization Induced by Bone Marrow Transplantation in Rat Hindlimb Ischemia

Yasuhiro Takagi[1], Takashi Omura[1,*], Minoru Yoshiyama[1], Ryo Matsumoto[1], Soichiro Enomoto[1], Takanori Kusuyama[1], Daisuke Nishiya[1], Kaname Akioka[1], Hiroshi Iwao[2], Kazuhide Takeuchi[1], and Junichi Yoshikawa[1]

[1]*Department of Internal Medicine and Cardiology,* [2]*Department of Pharmacology, Osaka City University Graduate School of Medical Sciences, Osaka 545-8585, Japan*

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Abstract. Because granulocyte-colony stimulating factor (G-CSF) mobilizes bone marrow cells including endothelial progenitor cells, we examined whether G-CSF augments angiogenesis and collateral vessel formation induced by bone marrow-mononuclear cells transplantation (BMT). Unilateral hindlimb ischemia was surgically induced in Lewis rats. One week after surgery, administration of 100 mg/kg per day G-CSF significantly increased the laser Doppler blood perfusion index (LDBPI), number of angiographically detectable collateral vessels (angiographic score), and capillary density determined by alkaline phosphatase staining. In the BMT group (1×10^7 cells/rat) and the group with combined G-CSF treatment and BMT, LDBPI was significantly increased compared with that in the vehicle-treated group. In the BMT group, neovascularization was significantly increased as evidenced by the angiographic score and capillary density compared with the vehicle-treated group. Furthermore, the combination of G-CSF treatment and BMT augmented neovascularization compared with BMT alone, as evidenced by the angiographic score and capillary density. Moreover, G-CSF significantly increased vascular endothelial growth factor mRNA and fibroblast growth factor-2 mRNA in hindlimb muscle. In conclusion, G-CSF was found to augment neovascularization in rat hindlimb ischemia. Combined use of G-CSF treatment and BMT may be a useful strategy for therapeutic neovascularization in ischemic tissues.

Keywords: granulocyte-colony stimulating factor, bone marrow-mononuclear cell transplantation, angiogenesis, hindlimb ischemia, endothelial progenitor cell

Introduction

The formation of new blood vessels from endothelial progenitor cells (EPCs) during embryogenesis begins by the formation of blood islands comprised of EPCs and hematopoietic stem cells (1, 2). EPCs and hematopoietic stem cells may originate from common mesodermal ancestral cells, given the presence of common cell surface antigens such as KDR, Tie-2, and CD34 (3 – 5). Moreover, circulating EPCs have been discovered in peripheral blood (6) and have been shown to participate in postnatal neovascularization after mobilization from bone marrow (BM). It has been shown that BM mononuclear cell (BM-MNC) implantation increases collateral vessel formation in both ischemic limb models and patients with limb ischemia (7, 8). However, additional technical approaches to enhancing neovascularization by BM-MNC transplantation have not been well discussed.

Granulocyte colony-stimulating factor (G-CSF) is widely used clinically for chemotherapy-induced febrile neutropenia, high-dose chemotherapy with progenitor-cell transplantation, and mobilization of peripheral blood stem cells. Recently, transplantation of G-CSF-induced peripheral blood stem cells to induce angiogenesis has been extensively studied not only in animals but humans as well (9, 10). In addition, recent studies

*Corresponding author. FAX: +81-6-6646-6808
E-mail: omura@med.osaka-cu.ac.jp

have also shown that G-CSF is associated with a significant angiogenic response that appears to be mediated by the release of angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (11, 12). However, the effects of G-CSF on angiogenesis in hindlimb ischemia are still unclear. In addition, the effects of G-CSF and BM-MNC transplantation on angiogenesis in hindlimb ischemia remain to be fully elucidated. Accordingly, we tested the hypotheses that 1) G-CSF can induce angiogenesis in hindlimb ischemia and 2) augment neovascularization by BM-MNC transplantation in a rat model of unilateral hindlimb ischemia.

Materials and Methods

Isolation of rat BM-MNCs

Bone marrow was harvested by flushing the tibiae and femurs of Lewis rats (SLC, Shizuoka) with DMEM supplemented with 10% fetal bovine medium. The plug of whole marrow cells was dispersed by passing it through pipettes of decreasing sizes. After a homogeneous cell suspension was obtained, mononuclear cells were isolated by density gradient centrifugation (Nyco Prep 1.077 Animal; Axis-Shield PoC AS, Oslo, Norway).

Unilateral hindlimb ischemia

The rat ischemic hindlimb model is a modification of a 2-stage procedure previously described (13). Ischemia was created in the right hindlimb of inbred 8-week-old male Lewis rats under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally). All right-side branches of the aorta distal to the renal arteries and of the iliac artery were resected. On the same day as the second operation, the femoral artery was resected. In the first series of experiments, 16 rats were subjected to unilateral hindlimb ischemia. At postoperative day 7, 100 μg/kg recombinant human G-CSF (Kyowa Hakko Kogyo Co., Ltd., Tokyo) or saline (n = 6) was injected subcutaneously to the abdomen. G-CSF was administered once a day for 5 days. For the second sets of experiments, we examined the effect of G-CSF combined with BM-MNC transplantation. Additional rats (n = 32) were subjected to unilateral hindlimb ischemia and randomly divided into 4 groups. At day 7, the control group (n = 8) received 2.5 mL saline. The G-CSF group (n = 8) received 100 μg/kg G-CSF subcutaneously once a day for 5 days. The third group (n = 8) received BM-MNCs (1×10^7 cells per animal, BMT group) transplanted into the ischemic thigh skeletal muscle with a 26-gauge needle at 6 different points. The fourth group (n = 8) received G-CSF treatment followed by BM-MNC transplantation (1×10^6 cells per animal, G-CSF + BMT group).

Laser Doppler analysis and angiography

We measured the ratio of ischemic (right) / normal (left) limb blood flow using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments Ltd., Axminster, UK). After blood flow was scanned twice, stored images were subjected to computer-assisted quantification, and the average flows of ischemic and nonischemic limbs were calculated. To minimize variation in data due to ambient light and temperature, the laser Doppler blood perfusion index (LDBPI) was expressed as the ratio of ischemic to nonischemic limb blood flow. Collateral formation was evaluated using a Microfocus X-Ray Television Device (Hitex Co., Ltd., Osaka) on day 28. A catheter was inserted through the right femoral artery and advanced to the lower abdominal aorta. Angiographs were taken at 2 s after the injection of contrast medium (Iopamiron; Nihon Schering, Osaka). To quantitatively assess the extent of collateral vessel formation, we calculated the angiographic score as described previously (14).

Histological analysis

At 4 weeks, rats were euthanized with an overdose of pentobarbital. Four pieces of ischemic tissue from the adductor and semimembranous muscles were obtained. Frozen sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells as described previously (14). Five fields from 2 muscle samples of each animal were randomly selected for capillary counts. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined.

mRNA quantification

Changes in the mRNA levels of VEGF and FGF-2 in ischemic muscle were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from ischemic tissues and then reverse-transcribed using Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Tokyo). The synthesized cDNA was quantified using TaqMan quantitative PCR analysis of each gene with the ABI PRISM 7700 Detection System (Applied Biosystems Japan Ltd., Tokyo) according to the manufacturer's protocol.

Statistics

Results are expressed as means ± S.D. Statistical

significance of differences was determined using ANOVA and the Student-Newman-Keuls test. Differences were considered significant when $P<0.05$.

Results

Blood flow assessed by LDPI analysis

To examine subcutaneous blood perfusion, LDPI analysis was performed once/week after hindlimb ischemia. Figure 1 shows the results of LDBP analysis at 28 days after surgery. The LDBPI was significantly higher in the G-CSF group (0.92 ± 0.05) and BMT group (0.87 ± 0.15) than in the control group (0.68 ± 0.10) at day 28 ($P<0.05$ in each group). Combined treatment (G-CSF + BMT) led to a significant increase in the LDBPI compared with that in the control group. However, there was no difference in LDBPI between the G-CSF + BMT group and the G-CSF group or between the G-CSF + BMT group and the BMT group.

Collateral formation evaluated by angiography

At postoperative day 28, all animals were subjected to iliac angiography (Fig. 2). Collateral vessels had developed in G-CSF-treated rats but not in those of the control group. Quantitative analyses of ischemic tissues using angiographic score revealed a significantly greater number of collateral vessels in the G-CSF-treated group than in the control group (1.6 ± 0.5-fold, $P<0.01$). Monotherapy with BMT increased angiographic scores significantly compared with the control (3.0 ± 0.5-fold compared with the control, $P<0.01$), and G-CSF + BMT improved angiographic scores further (3.7 ± 0.1-fold compared with the control, $P<0.01$).

Capillary endothelial staining with alkaline phosphatase

The medial thigh muscles of the ischemic limbs were histologically examined at day 30. As shown in Fig. 3, alkaline phosphatase staining revealed the presence of numerous capillary endothelial cells in the G-CSF-treated group, but a low number of capillary endothelial cells in the control group. The capillary/muscle fiber ratio was greater (1.4 ± 0.2-fold, $P<0.01$) in the G-CSF-treated group than in the control group. BMT significantly increased the capillary/muscle fiber ratio compared with the control (1.8 ± 0.3-fold compared with the control, $P<0.01$). Moreover, G-CSF + BMT led to a further increase in capillary/muscle fiber ratio compared with BMT (2.0 ± 0.3-fold compared with the control, $P<0.01$).

Time course of VEGF and FGF-2 expression

To elucidate the effects of G-CSF and BMT on the expression of angiogenic growth factors, we investigated VEGF mRNA expression in ischemic hindlimb by

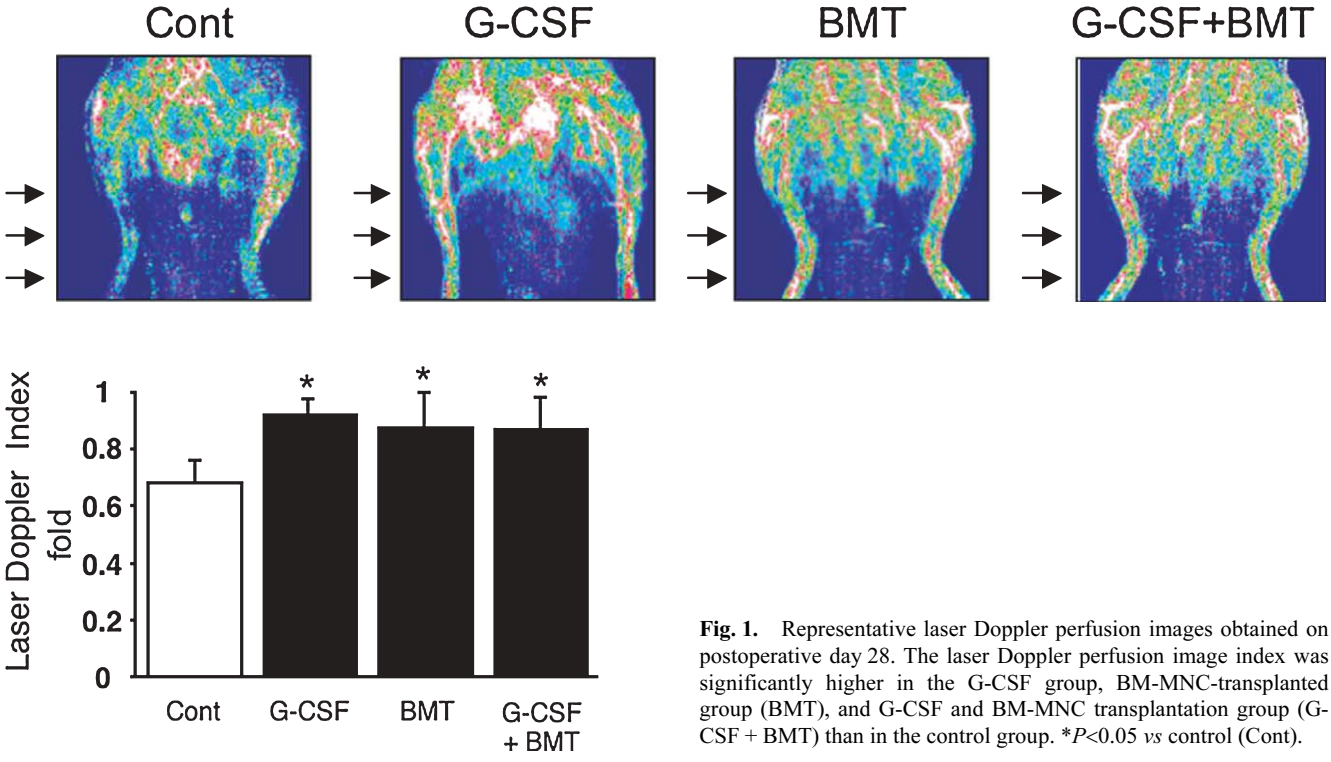

Fig. 1. Representative laser Doppler perfusion images obtained on postoperative day 28. The laser Doppler perfusion image index was significantly higher in the G-CSF group, BM-MNC-transplanted group (BMT), and G-CSF and BM-MNC transplantation group (G-CSF + BMT) than in the control group. *$P<0.05$ *vs* control (Cont).

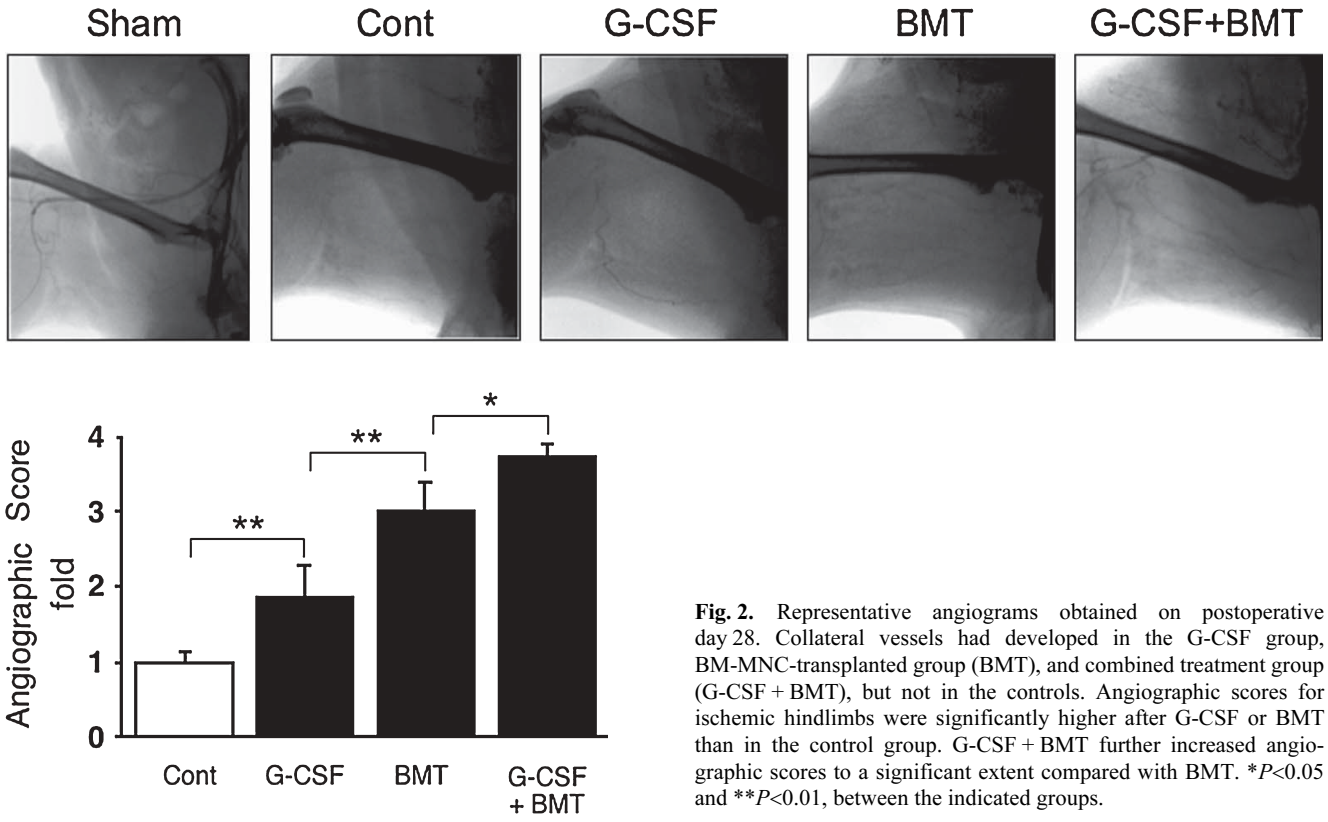

Fig. 2. Representative angiograms obtained on postoperative day 28. Collateral vessels had developed in the G-CSF group, BM-MNC-transplanted group (BMT), and combined treatment group (G-CSF + BMT), but not in the controls. Angiographic scores for ischemic hindlimbs were significantly higher after G-CSF or BMT than in the control group. G-CSF + BMT further increased angiographic scores to a significant extent compared with BMT. *P<0.05 and **P<0.01, between the indicated groups.

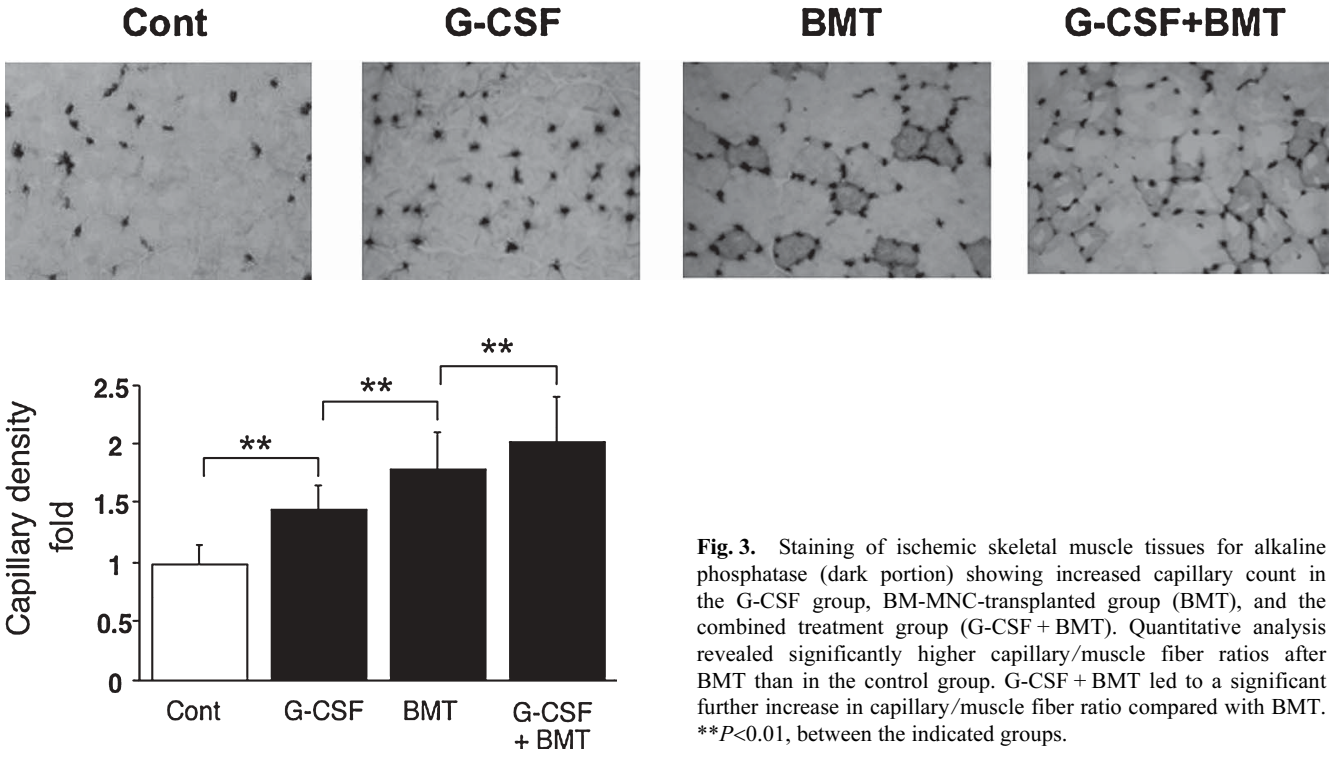

Fig. 3. Staining of ischemic skeletal muscle tissues for alkaline phosphatase (dark portion) showing increased capillary count in the G-CSF group, BM-MNC-transplanted group (BMT), and the combined treatment group (G-CSF + BMT). Quantitative analysis revealed significantly higher capillary/muscle fiber ratios after BMT than in the control group. G-CSF + BMT led to a significant further increase in capillary/muscle fiber ratio compared with BMT. **P<0.01, between the indicated groups.

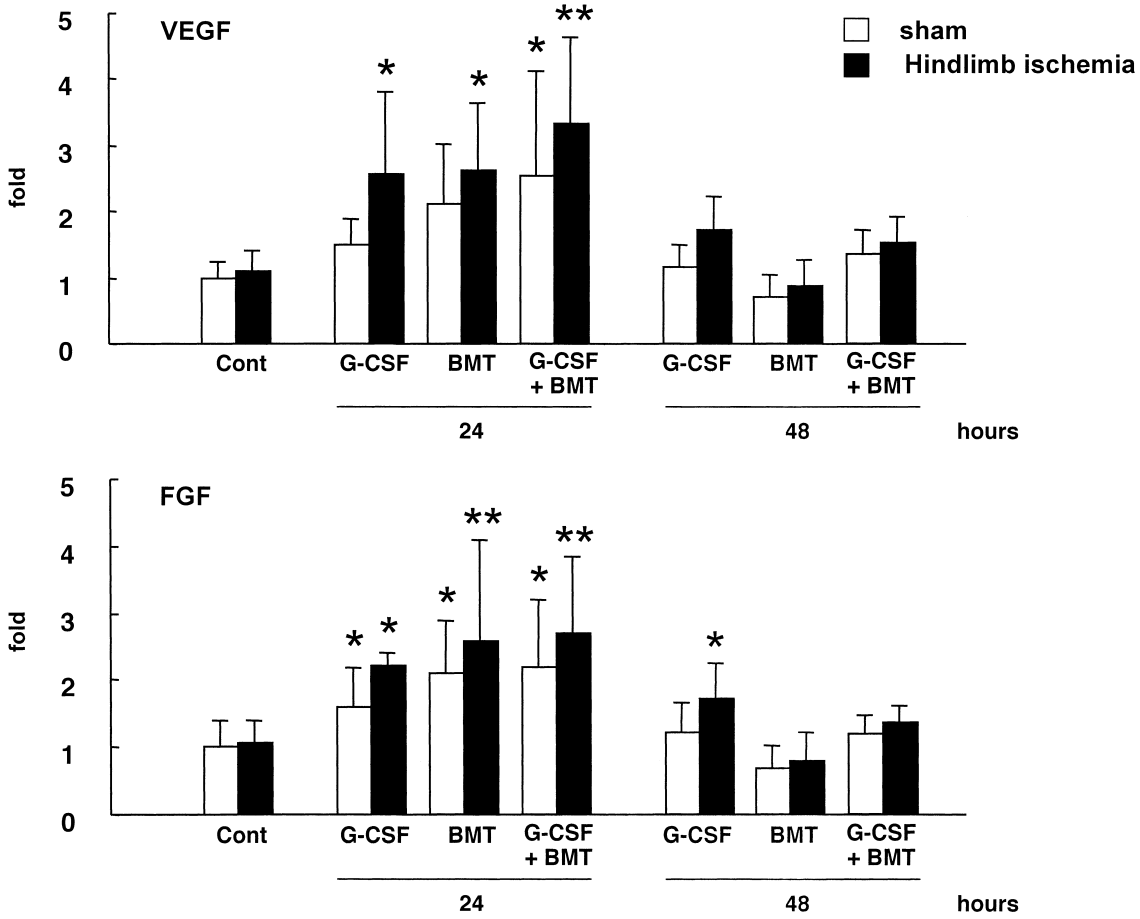

Fig. 4. Quantitative real-time RT-PCR analysis of VEGF and FGF. Data were obtained from 6 independent ischemic muscles for each time point and are expressed as the mean ± S.D. of mRNA level normalized to GAPDH. **P*<0.05 and ***P*<0.01 *vs* control (Cont) of hindlimb ischemia.

real-time RT-PCR (Fig. 4). VEGF mRNA expression was significantly increased at 24 h after G-CSF treatment and/or BMT in ischemic hindlimb (2.6 ± 1.2-fold in G-CSF, 2.6 ± 1.0-fold in BMT, and 3.3 ± 1.9-fold in G-CSF + BMT), compared with the expression of untreated sham-operated rats. Similarly, FGF-2 mRNA expression was significantly increased at 24 h after G-CSF treatment and/or BMT in normal hindlimb (1.6 ± 0.6-fold in G-CSF, 2.1 ± 0.8-fold in BMT, and 2.2 ± 1.0-fold in G-CSF + BMT) in ischemic hindlimb (2.2 ± 0.2-fold in G-CSF, 2.6 ± 1.5-fold in BMT, and 2.7 ± 1.2-fold in G-CSF+BMT), compared with the expression of untreated sham operated rats.

Discussion

The major findings of the present study are as follows: 1) G-CSF induced neovascularization in ischemic hindlimbs, and 2) G-CSF augmented neovascularization by transplanted BM-MNCs in ischemic hindlimb.

Although a previous study suggested that EPCs mobilized by G-CSF from bone marrow contribute to the angiogenic response, the effects of G-CSF on ischemic hindlimb have remained unclear. In the present study, we obtained evidence indicating that G-CSF could induce neovasculization at the levels of collateral arteries and capillaries in rat hindlimb ischemia. Given the abundance of evidence that BM-MNC transplantation induces neovascularization via proliferation and differentiation of EPC or cytokines secreted from BM-MNC, it has been suggested that G-CSF may elicit neovascularization through a similar mechanism by mobilization of BM-MNC (9, 15). On the other hand, G-CSF has been reported to mediate the release of angiogenic growth factors such as VEGF, FGF, and hepatocyte growth factor in several conditions (11, 12). In the present study, we observed that levels of VEGF and FGF-2 mRNA were increased after G-CSF treatment, suggesting that induction of VEGF and FGF-2 partially contributed to neovascularization by G-CSF.

Neovascularization by VEGF in vivo has been attributed to its mitogenic and promigratory effects on endothelial cells, consistent with the concept of angiogenesis as occurring by way of the development of sprouts from pre-existing, fully differentiated endothelial cells (14, 16 – 18). More recent studies have demonstrated that VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived EPCs (19, 20). It appears that the facilitative effect of VEGF on tissue neovascularization, previously attributed exclusively to angiogenesis, may in fact involve the contribution of mobilized EPCs to vasculogenesis. Furthermore, it is likely that direct effects of VEGF on transplanted BM-MNCs contributed to improved limb neovascularization. VEGF is known to be essential for the in vitro differentiation of purified EPCs into mature endothelial cells (21). This observation is consistent with the demonstration by in vivo studies of the importance of VEGF for vasculogenesis (22, 23). Since EPCs express VEGF receptors, VEGF may also enhance EPC proliferation, adhesion, and incorporation into endothelial cell monolayers and differentiation to endothelial cells as demonstrated previously (22 – 25). These studies have demonstrated the pivotal roles played by VEGF and its receptors in blood vessel development. FGF-2 is a powerful stimulator of angiogenesis in vivo and a pleiotropic regulator of the proliferation, migration, differentiation, and survival of many types of cells in vitro, including endothelial cells, smooth muscle cells, and pericytes (26 – 31). FGF-2 is widely expressed during embryonic development and normal wound healing as well as in ischemic diseases (32 – 34). Although what kinds of cells produced VEGF and FGF in skeletal muscle was unclear in the present study, G-CSF could increase the mRNA expression of VEGF and FGF in ischemic muscles rather than the non-ischemic control. These results suggest that ischemia may enhance the effects of G-CSF on the expression of angiogenic factors. Further investigations were needed to examine the response of each cell to G-CSF in skeletal muscle tissue.

In the present study, transplantation of BM-MNCs augmented angiogenesis and collateral vessel formation in ischemic tissue. G-CSF enhanced neovascularization by transplanted BM-MNCs. Because marrow stromal cells, belonging to BM-MNC, augment collateral remodeling through release of several cytokines such as VEGF and FGF, BMT may induce VEGF and FGF not only by response to injury but also by release from BM-MNC (35). In the present study, there was no significant difference in VEGF and FGF mRNA expression between BMT and G-CSF + BMT, thereby suggesting that BMT alone could induce maximum growth factor expression. In addition to the expression of VEGF and FGF-2, angiogenic augmentation by G-CSF may be due in part to the effects of G-CSF on mobilization of BM-MNC from bone marrow. Altogether, the above findings suggest that local overexpression of VEGF, FGF-2, and EPCs may promote neovascularization in target ischemic tissues.

In summary, our findings show that G-CSF can augment neovascularization by transplanted BM-MNCs in the ischemic hindlimb. It is likely that G-CSF induces collateral vessel formation in part by supplying VEGF and FGF-2. G-CSF could be useful clinically for enhancing neovascularization following BM-MNC transplantation.

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