

G-CSF and/or M-CSF accelerate differentiation of bone marrow cells into endothelial progenitor cells *in vitro*

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Abstract. It has been reported that granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) can mobilize endothelial progenitor cells (EPCs) in bone marrow cells (BMCs) into peripheral blood (PB) *in vivo*. Previously, we also reported that macrophage-colony stimulating factor (M-CSF) can mobilize EPCs into PB, which results in the rapid recovery of blood flow in induced-ischemia limbs by augmenting the number of intramuscular capillaries *in vivo*. In the present study, we demonstrate that M-CSF and/or G-CSF can increase EPCs from lineage (CD3, B220, Gr-1, Mac-1, CD11c, Ter119, NK1.1 or CD31)-negative BMCs *in vitro*. Lineage-negative BMCs were cultured with or without M-CSF and/or G-CSF. Three days after culture with M-CSF and/or G-CSF, the number of Flk-1⁺/CD45⁻, Sca-1⁺/CD45⁻, CD31⁺/CD45⁻ or CD146⁺/CD45⁻ cells increased in comparison with no cytokines. When the cultured BMCs with or without G-CSF and/or M-CSF were intravenously injected into ischemia-induced hindlimbs of mice, the number of intramuscular capillaries in the ischemia-induced legs increased; BMCs cultured with G-CSF and/or M-CSF were more effective than those of cytokine non-treated BMCs. These results suggest that M-CSF and/or G-CSF can induce the differentiation of BMCs into EPCs, even *in vitro*.

Introduction

It has been reported that BMCs can differentiate into endothelial cells, and EPCs are present in the bone marrow (BM) and PB of animals and humans (1-5). Kalka *et al*

reported that human peripheral blood mononuclear cell (PBMNC)-derived EPCs rescued induced-ischemia legs of nude mice from necrosis (4). Takeishi-Yuyama *et al* reported that an injection of autologous BM mononuclear cells (BM-MNCs) into the ischemic muscles of patients with arteriosclerosis induced the recovery of blood flow in their ischemic legs (5). Thus, BMCs and EPCs in PB are clinically used for the treatment of ischemic diseases of the limbs.

We previously reported that G-CSF and/or M-CSF can mobilize EPCs from BM, and the mobilized EPCs migrate to induced-ischemia muscles and differentiate into endothelial cells in blood vessels, resulting in the augmentation of blood flow in muscle (6).

G-CSF is known to accelerate the differentiation of bone marrow hematopoietic stem cells into granulocytes, and is clinically used for patients after chemotherapy and bone marrow transplantation (BMT) and also for patients with aplastic anemia (7,8). In contrast, M-CSF is known to stimulate the differentiation of hematopoietic stem cells into monocyte-macrophage lineage cells and augment the functions of monocyte-macrophage lineage cells (9,10). However, several side effects of these cytokines have been reported: in G-CSF, thrombocytopenia and the exacerbation of inflammation; and in M-CSF, shock, fever and general fatigue (7). Therefore, if we can utilize EPCs for the treatment of ischemic diseases after their expansion *in vitro*, the side effects of the cytokines could be reduced.

In this study, we show that G-CSF and/or M-CSF can augment the number of EPCs from lineage (Lin)-negative BMCs *in vitro*, and these EPCs can differentiate into endothelial cells *in vivo*, followed by the augmentation of blood flow in the ischemia-induced leg.

Materials and methods

Animals. C57BL/6 (B6, H-2b) mice were purchased from Japan SLC (Hamamatsu, Japan). B6 mice carrying the eGFP transgene (eGFP B6 mice) were kindly donated by Dr Okabe (Osaka University, Osaka, Japan). All mice were maintained under specific pathogen-free conditions in our animal facility until use. All mice were used at 8-12 weeks of age for this experiment.

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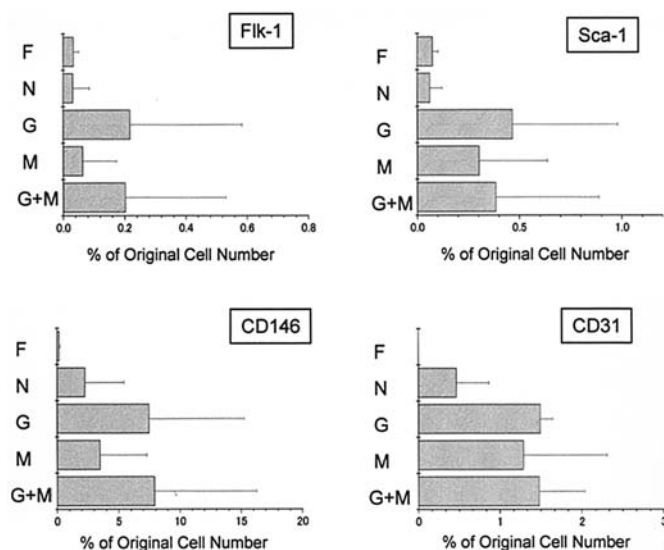


Figure 1. G-CSF and M-CSF augment the number of EPCs. Purified Lin-bone marrow cells (BMCs) were cultured with or without G-CSF (30 ng/ml) and/or M-CSF (30 ng/ml) for 3 days. The cells were harvested, counted, and stained with Cy5.5-labeled anti-CD45 and PE-labeled anti-CD146, anti-Sca-1, anti-CD31 or anti-Flk-1 Abs. The number of CD45/Sac-1⁺ cells, CD45/Flk-1⁺ cells and CD45/CD31⁺ cells was calculated with harvested cell number and percentages of indicated cells. The percentages of indicated cells to the original Lin- cells were calculated with cell number of the indicated cells and original cell numbers, using the following equation: (cell number of the indicated cells) $\times 100 / (\text{original cell number})$. F, freshly isolated Lin-BMCs; G, Lin-BMCs cultured with G-CSF; M, Lin-BMCs cultured with M-CSF; and G+M, Lin-BMCs cultured with G-CSF plus M-CSF.

Antibodies. Biotin-conjugated rat anti-mouse monoclonal antibodies (Abs) against CD3, B220, Gr-1, CD11b, Ter119, CD31, NK1.1 and CD11c were purchased from PharMingen (San Diego, CA, USA). Streptavidin-conjugated magnetic beads (Dynabeads M-280 streptavidin) were from Dynal (Oslo, Norway). Phycoerythrin (PE)-coupled anti-CD31, anti-Flk1 and Sca-1 Abs were also from PharMingen, and PE coupled anti-endothelial cell marker (CD146) Ab and Tricolor (TC)-labeled anti-CD45 Ab were from Chemicon International (Temecula, CA, USA).

Cell preparation. BMCs were collected from the tibias and femurs of B6 or eGFP B6 mice and then rinsed and suspended in phosphate-buffered saline (PBS) containing 2% heat-inactivated fetal calf serum (FCS) (PBS-FCS). After centrifugation, the cells were incubated with a mixture of biotin-coupled mAbs against CD3, B220, TER119, CD11c, CD11b, CD31, Gr-1 and NK1.1 for 30 min on ice, then washed twice with PBS-FCS, followed by incubation with streptavidin-conjugated magnetic-beads (Dynabeads) to negatively enrich the Lin-BMCs. These cells were stained with PE-Cy5-labeled avidin (Dako, Glostrup, Denmark) to detect the residual lineage cells, and sorted using an Epics Altra™ cell sorter (Beckman-Coulter, Fullerton, CA, USA), to purify Lin-BMCs. These cells were cultured with or without M-CSF and/or G-CSF.

Culture of Lin-cells. The sorted cells were equally divided into 4 groups and cultured in a Biocoat endothelial cell growth environment (BD Biosciences, San Diego, CA, USA)

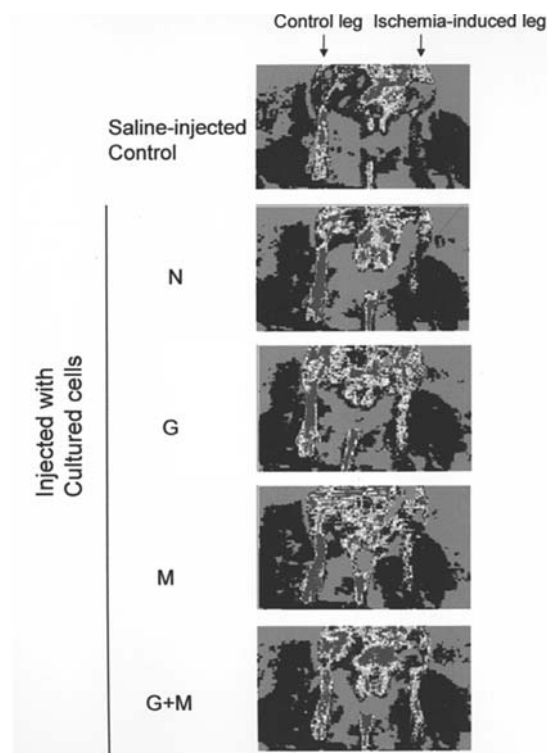


Figure 2. Injection of Lin-BMCs cultured with G-CSF and/or M-CSF is effective in improving the blood flow in ischemia-induced limbs. Purified Lin-bone marrow cells (BMCs) were cultured with or without G-CSF (30 ng/ml) and/or M-CSF (30 ng/ml) for 2 days, and injected into mice in which hindlimb-ischemia had been induced. Three days after the injection, the blood flow in the legs was examined by a laser Doppler perfusion image analyzer.

on a 30-mm collagen type I-coated dish (BD Biosciences) supplemented with or without M-CSF (30 ng/ml) and/or G-CSF (30 ng/ml). The four groups were as follows: i) culture without cytokine; ii) culture with G-CSF; iii) culture with M-CSF; and iv) culture with G-CSF and M-CSF. The cultured cells were harvested after 3-day culture, then stained with the indicated antibodies, and analyzed with a BD LSR (BD Biosciences).

Preparation of hindlimb ischemia. On day 0, unilateral hindlimb ischemia was induced by resecting the right femoral arteries and veins of B6 mice, and the mice were then injected with cultured Lin-BMCs from eGFP B6 mice. Three days after surgery, the mice were sacrificed to study neovascularization. The skeletal muscles (bilateral gastrocnemius muscle) were isolated, embedded in OCT compound (Miles Scientific, Elkhart, IN), and snap-frozen in liquid nitrogen.

Histological analyses. Histological analyses were performed as previously described (11,12). Briefly, 2- μ m sections of frozen muscle were stained with PE-labeled anti-CD31 Ab (Caltag, Burlingame, CA) to detect blood vessels. They were then observed using a confocal laser microscope (Olympus, Tokyo, Japan).

Laser Doppler perfusion image. The hindlimbs of mice were shaved using a razor. The mice were anesthetized with 160 mg/kg pentobarbital, then fixed supine on a cork plate.

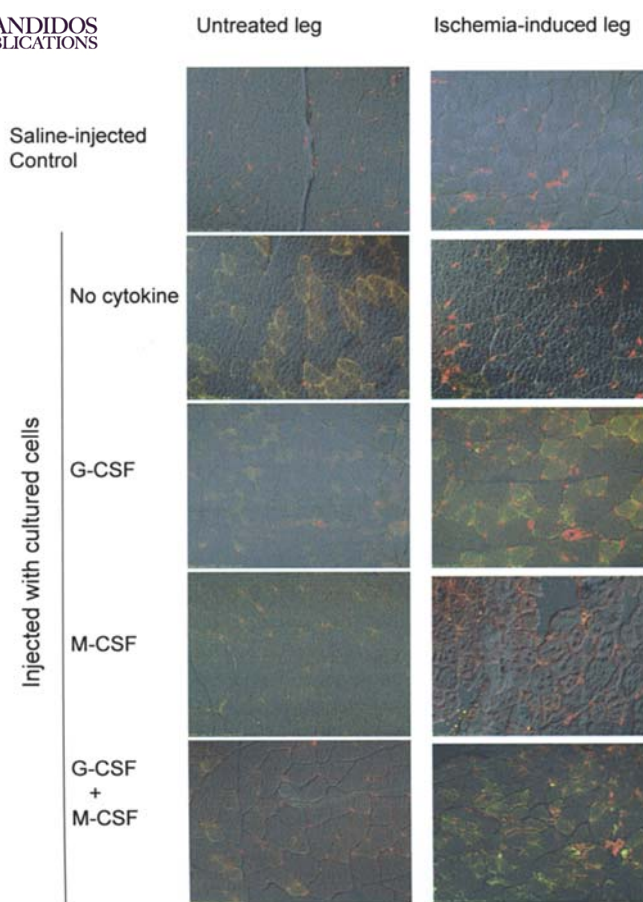


Figure 3. Injection of Lin-BMCs cultured with G-CSF and/or M-CSF augments the number of blood vessels in ischemia-induced limbs. Purified Lin-bone marrow cells (BMCs) were cultured with or without G-CSF (30 ng/ml) and/or M-CSF (30 ng/ml) for 2 days and injected into the mice in which hindlimb ischemia had been induced. Three days after injection, the mice were sacrificed, and muscles from induced-ischemia legs and contralateral control legs were obtained. Sections from the muscles were stained with PE-labeled anti-CD31 Ab and observed using a confocal microscopy.

We next measured the blood flow in the limbs using a laser Doppler perfusion image analyzer (Moor Instrument, Millway, Devon, UK) as described previously (13).

Results

G-CSF and/or M-CSF augment the number of EPCs in vitro. We previously demonstrated that G-CSF and/or M-CSF can mobilize EPC into the PB from the BM, followed by the augmentation of neovascularization in ischemia-induced limbs *in vivo* (6). This encouraged us to analyze the effects of G-CSF and/or M-CSF on the augmentation of the number of EPCs *in vitro*. We cultured lineage-negative BMCs in a Biocoat endothelial cell growth environment with or without G-CSF and/or M-CSF. After 3-day culture, the cultured cells were analyzed for the markers of EPCs or endothelial cells. As shown in Fig. 1, either G-CSF or M-CSF augments the number of CD45/CD146⁺ cells, CD45/CD31⁺ cells, CD45/Sca-1⁺ cells and CD45/Flk-1⁺ cells. Moreover, G-CSF plus M-CSF have synergistic effects on the increase in number of these cells.

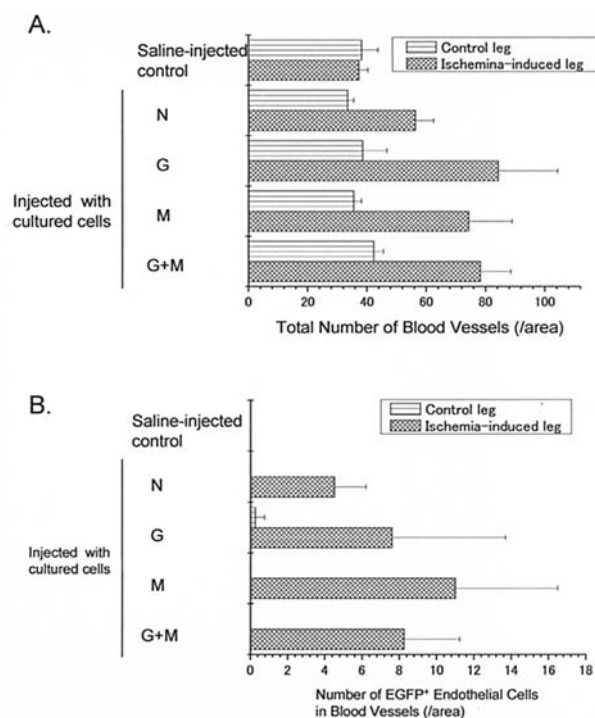


Figure 4. Injection of Lin-BMCs cultured with G-CSF and/or M-CSF can differentiate into endothelial cells in the blood vessels. Purified Lin-BMCs were cultured with or without G-CSF (30 ng/ml) and/or M-CSF (30 ng/ml) for 2 days and injected into the mice in which hindlimb-ischemia had been induced. Three days after injection, the mice were sacrificed, and muscles from induced-ischemia legs and contralateral control legs were obtained. Sections from the muscles were stained with PE-labeled anti-CD31 Ab and observed using a confocal microscopy. The number of blood vessels and eGFP⁺ blood vessels was then counted. The means and standard deviations of vessel numbers in one field are shown.

EPCs after culture with G-CSF and/or M-CSF can induce rapid recovery of blood flow in ischemia-induced legs. We examined whether the EPCs induced from Lin-BMCs can really differentiate into endothelial cells in blood vessels and work functionally. Two-day cultured Lin-BMCs were injected into the vein of ischemia-induced hindlimbs of mice. Three days after injection, the blood flow of the ischemia-induced legs was examined using a laser Doppler perfusion image analyzer. As shown in Fig. 2, the mice that had been injected with the BMCs cultured with G-CSF and/or M-CSF showed a better recovery of blood flow than the mice injected with BMCs cultured without cytokines. We also examined the number of blood vessels in bilateral gastrocnemius muscles, as shown in Figs. 3 and 4. In the ischemia-induced muscle of mice injected with BMCs cultured with G-CSF and/or M-CSF, the number of blood vessels increased in comparison with that of mice injected with BMCs cultured without cytokines. Moreover, the cultured BMC-derived endothelial cells were detected in the ischemia-induced muscle in mice injected with cultured BMCs. The number of blood vessels induced from injected BMCs in the ischemia-induced muscles increased more in mice injected with BMCs cultured with G-CSF and/or M-CSF than in mice injected with BMCs cultured without G-CSF and M-CSF. However, there was no significant difference in the number of blood vessels between the mice injected with BMCs cultured with G-CSF, M-CSF and G-CSF plus M-CSF.

Discussion

In the present study, we demonstrated that G-CSF and/or M-CSF can accelerate the differentiation of BMCs into EPCs *in vitro*. G-CSF has been reported to be able to mobilize not only hematopoietic stem cells (HSCs), but also EPCs from the BM, and that EPCs contribute to the angiogenesis of tumors (14). M-CSF has been reported to be crucial for the differentiation of hematopoietic stem cells into monocytes and macrophages (15,16). At present, these cytokines are clinically used to enhance hematopoiesis. G-CSF is used for: i) the mobilization of HSCs from donor BM into PB for the purpose of peripheral blood stem cell transplantation (PBSCT) (17-19); ii) the augmentation of WBC counts of patients suffering from severe infection and aplastic anemia (20-23); and iii) the acceleration of hematopoietic recovery after BMT and chemotherapy (24,25). In contrast, M-CSF is clinically used not only for the acceleration of hematopoietic recovery after BMT but also for the augmentation of anti-fungal response after BMT (26,27). Therefore, these cytokines can be expected to be safely used even after *in vitro* expansion.

We previously reported that M-CSF can mobilize EPCs and accelerate the neovascularization of ischemic limbs *in vivo* (6). Even in humans, it has been reported that G-CSF has some effects on neovascularization (28). Therefore, it is expected that the injection of these cytokines would clinically induce neovascularization of the ischemic tissues and organs. There are several reports indicating that EPCs are induced from embryonal stem (ES) cells and BMCs using several cytokines *in vitro*. Nishikawa *et al* have shown that ES cells can differentiate into both hematopoietic cells and endothelial cells (29). Kalka *et al* have also shown that BMCs can differentiate into EPCs, which can rescue the ischemia-induced legs from necrosis (4). Therefore, if we can expand EPCs from BMCs and transplant the EPCs into the ischemic organs, we can reduce the various side effects of these cytokines.

In this study, we have shown that G-CSF and/or M-CSF can augment the number of EPCs from BMCs *in vitro*. These results suggest the possibility of using *in vitro*-expanded EPCs by G-CSF and/or M-CSF in a clinical setting.

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