Granulocyte colony-stimulating factor promotes tumor angiogenesis via increasing circulating endothelial progenitor cells and Gr1+CD11b+ cells in cancer animal models

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Abstract

Recombinant granulocyte colony-stimulating factor (G-CSF) is used for cancer patients with myelosuppression induced by chemotherapy. G-CSF has been reported to progress tumor growth and angiogenesis, but the precise mechanism of tumor angiogenesis activated by G-CSF has not been fully clarified. N-terminal-mutated recombinant human G-CSF administration increased WBCs and neutrophils in peripheral blood and reduced bone marrow stromal cell-derived factor-1 in mice, indicating its biological relevance. Mice were inoculated with Lewis lung carcinoma cells (LLCs) or KLN205 cells and treated with G-CSF. G-CSF accelerated tumor growth and intratumoral vessel density, while it did not accelerate proliferation of LLCs, KLN205 cells or human umbilical vein endothelial cells in vitro. In the absence of tumors, G-CSF did not increase circulating cells that displayed phenotypic characteristics of endothelial progenitor cells (EPCs). In the presence of tumors, G-CSF increased circulating EPCs. In addition, G-CSF treatment increased immune suppressor and endothelial cell-differentiating Gr1+CD11b+ cells in tumor-bearing mice. We conclude that G-CSF promotes tumor growth by activating tumor angiogenesis via increasing circulating EPCs and Gr1+CD11b+ cells in cancer animal models.

Introduction

Neutropenia and the resultant infection are life-threatening side effects of cancer chemotherapy. The use of dose-intensive chemotherapeutic regimens has made the control of myelosuppression increasingly important. Granulocyte colony-stimulating factor (G-CSF) is commonly used to treat these patients to ameliorate neutropenia and prevent severe infections.

G-CSF can shorten the duration of chemotherapy-induced neutropenia, but recent reports show that it has no benefit in reducing the rate of hospitalization of febrile neutropenia (1, 2) or in prolonging survival (3), and question the preventive effects of G-CSF on neutropenia.

Although a few earlier reports show the inhibitory effect of G-CSF on solid tumor growth (4), many recent reports show their accelerating effects on tumor growth (5–7). Since blood supply is essential for solid tumors, growth is highly dependent on angiogenesis and the formation of new capillaries from pre-existing blood vessels. In the conventional view, angiogenesis is mediated by the local proliferation and migration of vessel wall-associated endothelial cells (ECs) that emerge from their resting state in response to angiogenic growth factor (8). However, recent works suggest that circulating cells with the potential to differentiate into mature ECs, the so-called circulating endothelial progenitor cells (EPCs), may also contribute to tumor angiogenesis (9, 10).

Granulocyte macrophage colony-stimulating factor and vascular endothelial growth factor (VEGF) have been reported to mobilize EPCs into peripheral circulation from the bone marrow and promote angiogenesis (11–14). Although it was reported that G-CSF administration promotes tumor growth...
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and angiogenesis (7), the effect of G-CSF on EPCs in cancer animal models has not been investigated. On the other hand, a recent study demonstrated that Gr1+CD11b+ cells in spleens contribute to tumor angiogenesis by directly differentiating into ECs (15). But the relationship between Gr1+CD11b+ cells and G-CSF has not been investigated. In this study, we investigated the effect of G-CSF on tumor growth and angiogenesis. To clarify how G-CSF activates tumor angiogenesis, we investigated the effect of G-CSF on circulating EPCs and Gr1+CD11b+ cells. Our findings suggest that G-CSF promotes tumor angiogenesis by increasing circulating EPCs and Gr1+CD11b+ cells in tumor-bearing mice.

Methods

Cell culture

Lewis lung carcinoma cells (LLCs) and squamous carcinoma KLN205 cells were purchased from American Type Culture Collection (Manassas, VA, USA). LLCs were cultured in high-glucose DMEM containing 10% FCS and 100 μg ml⁻¹ kanamycin. KLN205 cells were cultured in MEM containing 10% FCS, 1% non-essential amino acids and 100 μg ml⁻¹ kanamycin. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan), and were cultured in HuMedia-MvG (Kurabo). KLN205 cells were purchased from American Type Culture Collection (Manassas, VA, USA), and were cultured for 48 h. KLN205 cells and HUVECs were cultured for 72 h. Then the cell number was determined by water-soluble terazolium (WST) assay using a Cell Counting kit (Dojindo, Tokyo, Japan).

In vivo tumor models

LLCs were injected (3 × 10⁵ cells per animal) subcutaneously into the flank of male 6- to 9-week old C57BL/6 mice on day 0. KLN205 cells were injected (5 × 10⁵ cells per animal) subcutaneously into the flank of male 6- to 9-week old BDF1 mice on day 0. Tumor size was quantified daily as width² × length × 0.52 (16). For tumor growth rate models, N-terminal-mutated human recombinant G-CSF (nartograstim, Kyowa Hakko Kogyo, Tokyo, Japan; 8 μg kg⁻¹ body weight for G-CSF high and 50 μg kg⁻¹ body weight for G-CSF low) was injected into mice subcutaneously for 3 days daily from day 10, and every following week, G-CSF was injected daily for 3 days. Mice inoculated with LLCs were sacrificed on day 28 (total of nine G-CSF injections per mouse). Mice inoculated with KLN205 cells were sacrificed on day 36 (total of 12 G-CSF injections per mouse). For culture assay of EPCs, G-CSF was injected subcutaneously for 3 days daily from day 25, and the mice were sacrificed on day 28. For tumor growth inhibition models by SU1498 (Calbiochem, San Diego, CA, USA), mice were inoculated with LLCs on day 0. From day 9 and every following week, mice were injected intramuscularly with SU1498 three times a week. G-CSF (8 μg kg⁻¹) was injected into mice subcutaneously daily for 3 days from day 10, and every following week, G-CSF was injected daily for 3 days (total of nine G-CSF injections per mouse). Mice were treated with 400 μg of SU1498 dissolved in 100 μl dimethyl sulfoxide (DMSO) for each injection. Control mice were treated with DMSO. Mice were sacrificed on day 28. For an analysis of Gr1+CD11b+ cells in spleens, mice were inoculated with LLCs on day 0. From day 19, G-CSF (8 μg kg⁻¹) was injected into mice subcutaneously daily for 3 days and spleens were isolated on day 22. G-CSF dissolved in 100 μl PBS was used for each injection, and control mice were injected subcutaneously with PBS.

Cell growth assays

Cell proliferation assay was performed as previously described (17). Briefly, LLCs (5 × 10⁵ cells), KLN205 cells (5 × 10⁵ cells) or HUVECs (3 × 10⁵ cells) were plated onto 96-well plates and incubated with 0, 0.1, 1 or 10 ng ml⁻¹ of G-CSF. LLCs were cultured for 48 h. KLN205 cells and HUVECs were cultured for 72 h. Then the cell number was determined by WST assay using a Cell Counting kit (Dojindo, Tokyo, Japan).

Culture assay of circulating EPCs

Mononuclear cells were isolated, cultured and characterized as previously described with some modifications (18). First, mononuclear cells were isolated from 700 μl of peripheral blood from each mouse using density gradient centrifugation with lymphosepar II (IBL, Fujioka, Japan). Following isolation, 4 × 10⁶ cells were plated on dishes coated with human fibronectin (Becton Dickinson, Bedford, MA, USA) and maintained in HuMedia-MvG (Kurabo), supplemented with 20% FCS and 100 ng ml⁻¹ recombinant mouse VEGF (R&D, Minneapolis, MN, USA). The medium was changed on day 4. On day 7, medium was changed and cells were washed with PBS. Adherent cells were incubated with 2.4 μg ml⁻¹ acetylated low-density lipoprotein–DiI complex (DiI–acLDL, Molecular Probes, Eugene, OR, USA) for 1 h. Cells were fixed in 2% PFA for 10 min and stained with 10 μg ml⁻¹ FITC-labeled lectin from Ulex europaeus (Sigma) for 1 h. Fluorescent microscopy identified double-positive cells as EPCs. Two independent investigators evaluated the number of double-positive cells in each well by counting three randomly selected high-power fields.

Flow cytometry

FITC-labeled anti-CD34, purified rat anti-CD144 (VE-cadherin) and APC-labeled anti-Gr1 antibodies were purchased from BD PharMingen (San Diego, CA, USA), and control rat IgG2a and FITC-labeled anti-CD11b were purchased from eBioscience (San Diego, CA, USA). For staining of EPCs, mononuclear cells in peripheral blood were isolated and cultured for 7 days as shown in Culture Assay of Circulating EPCs. Then the cells were washed with PBS and adherent cells were scraped off with a cell scraper (Iwaki, Tokyo, Japan) and suspended in PBS. For staining of splenocytes, single-cell suspensions were made from spleens. The cells were first incubated with unlabeled anti-CD16/32 mAb (eBioscience) to block non-specific binding to the FcyR. After washing, the cells were incubated on ice with FITC-, APC- or non-labeled mAbs. The cells incubated with anti-CD144 were then incubated with FITC-labeled anti-rat IgG (H + A) (eBioscience). After washing again, the cells were subjected to flow cytometry on a FACSscan (BD Bioscience, San Jose, CA, USA) and the data were analyzed with CellQuest software (BD Bioscience). For all samples, dead cells were excluded from the analysis by propidium iodide staining.

Immunohistochemistry

When the diameter of the tumor became ~1 cm, tumor tissues were fixed in 10% formalin, embedded in paraffin and sectioned. Control mice were sacrificed on day 21, and the
G-CSF (8 μg kg⁻¹)-treated mice were sacrificed on day 19 (total of six G-CSF injections). They were blocked with 10% normal goat serum and incubated with polyclonal anti-human factor VIII-related antigen antibody (DAKO, Carpinteria, CA, USA). Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA), and then with ABC kit (Vector), and were detected by 3-amino-9-ethylcarbazole (Vector) and counterstained with hematoxylin.

**Determination of microvessel density**

The intratumoral microvessel density (MVD) was determined as previously described (19, 20). In brief, the intratumoral vessels were stained immunohistochemically with anti-human factor VIII-related antigen antibody. The image that contained the highest number of microvessels was chosen for each section by an initial scan at 100× magnification. Then the vessels were counted in the selected image at 200× magnification.

**Peripheral blood analysis**

G-CSF (8 μg kg⁻¹) was injected into mice subcutaneously daily for 3 days. Eight hours after the last injection, 700 μl of peripheral blood was collected from each mouse. Total WBC numbers and differential leukocyte counts were obtained using hematology analyzer LH750 (Beckman Coulter, Fullerton, CA, USA).

**Cytokine ELISA**

The concentrations of stromal cell-derived factor-1 (SDF-1) in serum and bone marrow were determined using a murine SDF-1 ELISA kit (R&D) according to the manufacturer’s recommendation. G-CSF (8 μg kg⁻¹) was injected into mice subcutaneously for 1 or 3 days daily. PBS was injected into control mice. Then the serum was isolated and subjected for ELISA. For tumor models, LLCs were injected into mice on day 0. From day 25, G-CSF (8 μg kg⁻¹) was injected into mice for 3 days daily. PBS was injected into control mice. On day 28, serum was isolated and subjected for ELISA.

**Data analysis**

Statistical analysis of the results was performed using analysis of variance with Fisher’s least significant difference test for multiple comparisons. Values of \( P < 0.05 \) were considered to be significant.

**Results**

**G-CSF treatment increased WBCs and neutrophils in peripheral blood and reduced the bone marrow SDF-1 level**

We treated mice with N-terminal-mutated recombinant human G-CSF, nartograstim (8 μg kg⁻¹), and examined the number of total WBCs and neutrophils in peripheral blood to confirm its biological effects. Three days of G-CSF treatment significantly increased both WBCs and neutrophils (Fig. 1A). G-CSF has been reported to reduce SDF-1 level in bone marrow without changing the serum SDF-1 level, and mobilizes leucocytes from bone marrow to peripheral circulation (21). We examined the SDF-1 level in serum and bone marrow after 1 or 3 days of G-CSF treatment. G-CSF treatment did not change the serum SDF-1 level (Fig. 1B), but...
indicates 50 μg kg⁻¹ G-CSF treatment. Similar results were obtained from three independent experiments.

**G-CSF treatment accelerated the tumor growth rate in vivo, but not in vitro**

LLCs were inoculated into the flank of C57BL/6J mice subcutaneously on day 0. From day 10, we injected G-CSF or PBS into the mice daily for 3 days, and every following week, we injected G-CSF or PBS daily for 3 days. As shown in Fig. 1, 8 μg kg⁻¹ G-CSF (indicated as G-CSF low) had a biological effect, but this dose was relatively low. Therefore, we treated mice with 50 μg kg⁻¹ G-CSF as a positive control (indicated as G-CSF high). Compared with PBS treatment, G-CSF treatment accelerated the tumor growth statistically significantly (Fig. 2A). There was no significant difference in tumor growth between high- and low-dose G-CSF treatment (Fig. 2A).

Similar results were obtained from mice inoculated with KLN205 cells (Fig. 2B). Thus, we performed the following experiments using the low-dose G-CSF (8 μg kg⁻¹). We next examined the direct effect of G-CSF on the proliferation of LLCs and KLN205 cells in vitro. We cultured LLCs and KLN205 cells in various concentrations of G-CSF. G-CSF had no effect on cell proliferation in vitro (Fig. 2C). We also cultured LLCs and KLN205 cells with 0, 0.1, 1 or 10 ng ml⁻¹ of G-CSF and counted the cells on days 3, 4, 5 and 6. We could not find an effect of G-CSF at any concentration on the cell proliferation rate either (data not shown).

**G-CSF treatment induced the increase of vessel density in tumor tissues, but did not affect the proliferation of HUVECs**

Hematoxylin and eosin staining of the tumor tissues revealed hyper-neovascularization in tumors from G-CSF-treated mice (Fig. 3A and C; total of six G-CSF injections). To confirm the vessels, we performed immunohistochemistry staining using an antibody against factor VIII-related antigen (Fig. 3B and D). Factor VIII-related antigen is a well-established cell-surface marker of vascular ECs (20). Compared with control mice, we found an increase of tumor vessel density in G-CSF-treated mice. The difference in MVD between control and G-CSF-treated mice was statistically significant (Fig. 3E). To investigate whether G-CSF induced the proliferation, differentiation and development of sprouts from pre-existing ECs, we examined the direct growth effect of G-CSF on HUVECs. We cultured HUVECs with various concentrations of G-CSF. G-CSF had no effect on the proliferation of HUVECs in vitro (Fig. 3F). We also cultured HUVECs with 1 or 10 ng ml⁻¹ of G-CSF and counted the cells on days 2, 3, 4 and 5. We could not find an effect of G-CSF on the proliferation rate or morphological changes (data not shown).

**G-CSF treatment to cancer animal models increased the circulating EPCs**

As G-CSF did not have a direct growth effect on ECs, we hypothesized the enhanced neovascularization as the result of an increase of circulating EPCs. We cultured mononuclear cells and characterized EPCs as adherent cells double positive for Dil–acLDL uptake and lectin binding as previously described (Fig. 4A) (13, 14, 22). We further confirmed the expression of well-established murine endothelial-specific markers CD34 and VE-cadherin on adherent cells by FACS analysis (Fig. 4B) (22, 23). To investigate the effects of G-CSF on the increase of Dil–acLDL uptake and lectin-binding cells in peripheral blood, mice were injected with G-CSF for 3 days, and mononuclear cells in peripheral blood which displayed phenotypic characteristics of EPCs were counted. G-CSF treatment itself did not significantly increase the circulating EPCs (Fig. 4C). To further investigate the effect of G-CSF on the increase of circulating EPCs in cancer animal models, we inoculated LLCs into mice at day 0. At 25 days after inoculation, we treated them with G-CSF for 3 days. This treatment significantly reduced the bone marrow SDF-1 level (Fig. 1C) as previously shown (21). These results indicated that G-CSF at this dose would be sufficient to exert its biological effects.

![Fig. 2. G-CSF treatment accelerated the tumor growth in vivo, but not in vitro. G-CSF low indicates 8 μg kg⁻¹ G-CSF treatment and G-CSF high indicates 50 μg kg⁻¹ G-CSF treatment. (A) Mice were injected subcutaneously with LLCs on day 0 and treated with PBS or G-CSF. Results are indicated as mean ± SD of eight mice in each group, and the difference in tumor volume on day 28 between control and G-CSF-treated mice was statistically significant (**P < 0.01). (B) Mice were injected subcutaneously with KLN205 cells on day 0 and treated with PBS or G-CSF. Results are indicated as mean ± SD of eight mice in each group, and the difference in tumor volume on days 32 and 36 between control and G-CSF-treated mice was statistically significant (**P < 0.01). (C) LLCs and KLN205 cells (5 × 10⁵ cells) were cultured with the indicated amounts of G-CSF. LLCs were cultured for 48 h, and KLN205 cells were cultured for 72 h. Cell number was determined by WST assay. Data are shown as mean ± SD of triplicate samples. Similar results were obtained from three independent experiments.](image-url)
significantly increased the number of circulating EPCs in G-CSF-treated mice (Fig. 4D). Similar results were obtained from mice inoculated with KLN205 cells (data not shown).

**LLCs inoculation elevated the serum VEGF level, and VEGFR-2 kinase inhibitor SU1498 inhibited the tumor growth**

VEGF has been shown to mobilize bone marrow-derived EPCs (12, 14). We examined the serum level of VEGF in mice after G-CSF treatment for 3 days. VEGF was not detectable in the serum from the control or G-CSF-treated mice (Fig. 5A). We next inoculated LLCs into mice and examined the serum VEGF level. Inoculation of the LLCs induced the elevation of the serum VEGF level in control and G-CSF-treated mice (Fig. 5A). VEGF has been reported to contribute to angiogenesis through activation of VEGFR-2 (VEGFR-2/KDR/Fk-1) and SU1498 is a potent and selective inhibitor of the VEGFR-2 tyrosine kinase (24). To investigate the possible role of VEGF on LLC tumor growth, we treated mice with SU1498 and G-CSF. SU1498 inhibited the tumor growth in mice both with and without G-CSF injection. However, tumor growth in mice treated with G-CSF had the SU1498-insensitive part (Fig. 5B). These results suggested that VEGF might not be involved in the differential tumor growth between G-CSF-treated and non-treated mice.
G-CSF treatment increased Gr1+CD11b+ cells both in tumor-free and tumor-bearing mice

In tumor-bearing hosts, increase of Gr1+CD11b+ cells and immunosuppressive effect of these cells have been reported (25). Moreover, Gr1+CD11b+ cells in spleens of tumor-bearing mice were shown to directly differentiate into ECs in tumors and contribute to tumor angiogenesis and growth (15). They also indicated that Gr1+CD11b+ cells were different from EPCs. To investigate whether G-CSF increases Gr1+CD11b+ cells in spleens, mice were treated with G-CSF for 3 days. The spleens were isolated and the splenocytes were subjected to FACS analysis. The cell population of Gr1+CD11b+ cells in spleens of control mice was 3.61 ± 0.17%, and G-CSF treatment significantly increased the cell population of Gr1+CD11b+ cells up to 7.52 ± 1.04% (Fig. 6A; mean ± SD). As the G-CSF treatment induced splenomegaly, the difference in the absolute number of Gr1+CD11b+ cells in spleens became much more evident between control and G-CSF-treated mice (Fig. 6C). Gr1+CD11b+ cells in spleens were indicated to increase 21–28 days after LLC inoculation (15). We treated mice with G-CSF daily for 3 days from 19 days after LLC inoculation, and isolated spleens 22 days after LLC inoculation. On day 22, there was no significant difference in tumor size between control and G-CSF-treated mice. The cell population of Gr1+CD11b+ cells in spleens of tumor-bearing mice was 21.6 ± 2.2% (Fig. 6B). G-CSF treatment in tumor-bearing mice significantly increased the cell population of Gr1+CD11b+ cells up to 41.1 ± 0.5% (Fig. 6B; mean ± SD). G-CSF treatment also significantly increased the absolute number of Gr1+CD11b+ cells in spleens of tumor-bearing mice (Fig. 6D).

Discussion

G-CSF has been reported to activate angiogenesis in malignancy (7), but its precise mechanism has not been fully clarified. Here, we demonstrated that accelerated tumor growth by G-CSF was accompanied with angiogenesis activation via increase of circulating EPCs and Gr1+CD11b+ cells (Figs 2–4 and 6). G-CSF had no accelerating effects on cell proliferation in both cancer cells and ECs in vitro (Figs 2 and 3), suggesting that the effect on tumor progression and angiogenesis cannot be explained by their direct action on cells in situ. Recently, it was reported that G-CSF enhanced tumor neovascularization in which bone marrow cells participated (7). However, in the study, the involvement of EPCs was not shown. In our study, G-CSF significantly increased the ratio of circulating EPCs in the tumor-bearing mice (Fig. 4). Since EPCs are known to become a part of tumor vessels (9, 10), these studies might
suggest that the circulating EPCs mobilized after G-CSF treatment contributed to the vessel formation in the tumors. In contrast to the tumor-bearing mice, G-CSF did not increase the ratio of the EPCs in the mice without tumors (Fig. 4), and these might indicate that G-CSF requires some other factors to increase EPCs. VEGF has been reported to mobilize EPCs from bone marrow (12, 14). Previously, we have shown that LLCs continuously release VEGF (16), and the baseline serum VEGF level was elevated in mice inoculated with LLCs (Fig. 5A). VEGFR-2 tyrosine kinase inhibitor SU1498 partially inhibited...
the tumor growth effect of G-CSF (Fig. 5B), and this might indicate a crucial role of a certain level of VEGF to increase EPCs in the periphery.

In addition to suggesting the involvement of circulating EPCs in tumor angiogenesis after G-CSF treatment, here we showed the possibility that the increase of Gr1+CD11b+ cells might contribute to G-CSF-induced activation of tumor angiogenesis (Fig. 6B and D). Previously, Gr1+CD11b+ cells were reported to increase in tumor-bearing hosts, and these cells were also reported to impair immune responses (25). Moreover, very recently, these cells were reported to directly differentiate into ECs and to contribute to tumor angiogenesis (15). EPCs in peripheral blood (or in circulation) are also called circulating endothelial precursor cells, and these cells are described as CD11b negative (9, 26). Therefore, Gr1+CD11b+ cells might represent another population of cells that participate in tumor angiogenesis in addition to EPCs. These results suggest that Gr1+CD11b+ cells contribute G-CSF-induced acceleration of tumor growth not only by immunosuppressive action but also by inducing angiogenesis.

A recent study showed that the majority of cultured EPCs expressed monocyte/macrophage markers and only a minority expressed specific endothelial markers and questioned the nomenclature EPC (27). Our results indicated that cultured EPC expressing CD34 and VE-cadherin is not a minority (Fig. 4). This discrepancy might be due to the difference in culture conditions (concentration of VEGF or FCS or cell density). Moreover, transplanted EPCs cultured in the essentially same conditions as in Fig. 4 were shown to functionally improve neovascularization after hind-limb ischemia in mice, and transplanted EPCs were shown to differentiate into ECs (28, 29). They also showed that macrophages or dendritic cells were significantly less effective in improving neovascularization than EPCs (28). These results indicate the importance of culture conditions for EPCs and difficulties of EPC characterization.

In a previous study, 20 µg kg⁻¹ human recombinant G-CSF was reported to promote tumor angiogenesis in mice (7). Here, we showed that relatively low-dose (similar to clinical dose) G-CSF has the capability to promote tumor angiogenesis. We used N-terminal-mutated human recombinant G-CSF, nartograstim. Nartograstim is known to have a three to five times higher potency than human recombinant G-CSF (filgrastim) (30). In order to confirm the biological effects of nartograstim at this dose, we showed that nartograstim increased the number of WBCs and neutrophils in peripheral blood (Fig. 1A), and reduced the SDF-1 level in bone marrow (Fig. 1C) as previously described (21). Moreover, for tumor growth models, we treated mice with 50 µg kg⁻¹ G-CSF as a positive control.

As previously shown, a 1-day G-CSF treatment did not change the serum SDF-1 level (Fig. 1) (21). They also showed that a 5-day G-CSF treatment reduced the serum SDF-1 level in mice but not in humans (21). Here, we showed that a 3-day G-CSF treatment to mice at our dose did not change the serum SDF-1 level (Fig. 1). The bone marrow SDF-1 level we showed here (Fig. 1) was lower than that of Petit et al. (21), but comparable to that of Hattori et al. (31). Petit et al. obtained bone marrow by flushing femurs, tibias, humeri and pelvis with 500 µl of PBS. In contrast, we obtained bone marrow by single flush of a right femur with 500 µl of PBS. Moreover, a large difference of SDF-1 levels in bone marrows among different strains of mice was indicated (32). The SDF-1 level in bone marrow has also been described as SDF-1 per femur, and various SDF-1 levels have been reported (ranging from 100 pg per femur to 2.7 ng per femur) (32–34). The bone marrow SDF-1 levels in Fig. 1 were shown as 110–220 pg per femur. Taken together, the different method in obtaining bone marrow and the difference in strains [BALB/c mice by Petit et al. (21) and C57BL/6 mice in this paper] might be the reason for the discrepancy of the SDF-1 level in the bone marrow.

In summary, we demonstrated that G-CSF increased circulating EPCs and Gr1+CD11b+ cells, which activated tumor angiogenesis that led to the acceleration of tumor growth in cancer animal models. Moreover, we demonstrated that relatively low-dose G-CSF (nartograstim) showed such effects. These results may suggest that clinicians should be careful with an excessive use of G-CSF in cancer patients with residual tumors.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>Dil-acLDL</td>
<td>acetylated low-density lipoprotein–Dil complex</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cell</td>
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<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>KDR</td>
<td>kinase insert domain containing receptor</td>
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<tr>
<td>LLC</td>
<td>Lewis lung carcinoma cell</td>
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<td>MVD</td>
<td>microvessel density</td>
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References

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