Endostatin Inhibits the Vascular Endothelial Growth Factor-Induced Mobilization of Endothelial Progenitor Cells

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ABSTRACT

Circulating endothelial cells (CECs) are present in peripheral blood and have been shown to contribute to normal and pathologic neovascularization. Antiangiogenic molecules can inhibit neovascularization in tumors and other sites, but their effect on CECs has not yet been determined. We hypothesized that angiogenic factors will increase the number of CECs, and conversely, antiangiogenic treatment will reduce these numbers. Mice treated with high levels of vascular endothelial growth factor (VEGF) showed increased numbers of Flk-1-positive cells in peripheral blood and endothelial cell colonies compared with vehicle-treated controls. These changes were accompanied by increased bone marrow neovascularization. In contrast, mice that received VEGF and endostatin had significantly lower numbers of CECs and reduced bone marrow vasculogenesis. Endostatin-induced apoptosis was probably responsible for the decreased number of CECs. Systemic delivery of a VEGF antagonist, soluble Flt-1, also inhibited the VEGF-induced increase in CECs. These results were further confirmed in a Tie2/LacZ mouse model, in which endostatin reduced the number of β-galactosidase-expressing peripheral blood mononuclear cells. We propose that endothelial progenitor cells are a novel target for endostatin and suggest that the relative numbers of CECs can serve as a surrogate marker for the biological activity of antiangiogenic treatment.

INTRODUCTION

Neovascularization is a key process in the growth of solid tumors, and they will not grow beyond a few cubic millimeters unless a vascular network is established (1, 2). To stimulate neovascularization, the tumor cells produce a variety of angiogenic factors such as fibroblast growth factors and VEGF.5 CECs are found in adult blood, but their exact origin is still elusive (3–5). CECs may be mobilized from the bone marrow as EPCs or angioblasts (3, 6, 7), or they could be dislodged from mature blood vessels (8, 9). Identification of CECs within the PBMCs is based on differential expression of hematopoietic and EC markers. In humans, CD133 (AC133) was recently used to distinguish EPCs from mature ECs because CD133 is not expressed by mature ECs (7, 10). Hebbel and colleagues used P1H12 antibodies that recognize CD146 (MUC18), which is expressed on CECs in peripheral blood but not on monocytes, granulocytes, platelets, megakaryocytes, or T or B lymphocytes (8, 11). Other markers common to progenitor and mature ECs are the cell surface receptors VEGF receptor-2 (human KDR and mouse Flk-1) and Tie2 (7, 12). Several models including wound healing, cornea, and tumor angiogenesis have documented incorporation of EPCs into newly formed vessels and their contribution to neovascularization in a process described as postnatal vasculogenesis (7, 13). Further evidence for the contribution of EPCs to tumor angiogenesis came from a study by Lyden et al. (14, 15) in which a mouse genetic model with impaired EPC differentiation was used to demonstrate a dependence of tumor growth on bone marrow-derived EPCs. In addition, the mobilization of EPCs from the bone marrow requires angiogenic growth factor activation, such as VEGF (16, 17).

The development of new therapies for cancer represents a major challenge in medicine. Recently, a number of angiogenesis inhibitors have undergone clinical testing as a single therapy or in combination with other agents (1, 2). Endostatin is a M, 20,000 COOH-terminal fragment of collagen XVIII that dramatically suppressed the growth of primary tumors and metastases in several xenograft mouse models (18). Endostatin treatment induced EC apoptosis in vivo in several mouse xenograft models and in a clinical trial of cancer patients receiving endostatin treatment (19–22). However, the exact mechanism by which endostatin exerts its antiangiogenic activity is not yet clear.

Although antiangiogenic drugs specifically target ECs, their effects on CECs are largely unknown. In the present study, we have tested the effects of VEGF and endostatin on CECs in peripheral blood of mice. We observed that mice treated with VEGF had elevated numbers of CECs, whereas coadministration of VEGF and endostatin or sFLT significantly reduced these numbers. To validate these results, we have analyzed CECs in a Tie2/LacZ transgenic mouse model (12), in which ECs can be stained blue, and found similar effects. These results suggest that CECs are a target for endostatin and that their relative numbers may serve as a surrogate marker for endostatin’s bioactivity.

MATERIALS AND METHODS

Cell Lines and Antibodies

Cells expressing recombinant human VEGF and rEndostatin were cultured and encapsulated as described previously (23, 24). For immunostaining, the following antibodies were used: anti-Flk-1 and antimouse CD31 (BD Biosciences Pharmingen, San Diego, CA). For flow cytometry analysis, the following antibodies were used: antimouse CD45 and Flk-1 (BD Biosciences Pharmingen) in combination with 7AAD (Sigma, St. Louis, MO). Recombinant adenoviruses expressing VEGF (Ad-VEGF), sFLT (Ad-sFLT), GFP (Ad-GFP), and β-galactosidase (Ad-LacZ) were obtained from Harvard Gene Therapy Initiative core (Harvard Institute of Medicine, Boston, MA).

Mouse Models of EPCs and CECs

Implantation of Encapsulated Cells. Under anesthesia, SCID mice (Massachusetts General Hospital, Boston, MA) received s.c. injection of encapsulated cells expressing VEGF, endostatin, or nontransfected cells and combinations (5 × 10^6 cells from each type, per mouse). Five days later, mice were sacrificed, blood samples were collected from the retroorbital venous plexus (approximately 1 ml/mouse), and mononuclear cells were purified over Ficoll-

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Note: John V. Heymach and Masashi Nomi contributed equally to this work.

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Hypaque (Sigma) gradient. Some cells were cultured in vitro, as described below, and some cells were loaded onto Cytoslides using Cytospin centrifuge (Thermo Shandon, Pittsburgh, PA). Femoral bones were retrieved from two mice of each group. The bones were fixed in 37% formaldehyde, decalcified in 0.5 M EDTA (pH 8.0), and embedded in paraffin. Bone sections were immunostained with antineous CD31 antibodies.

Implantation of Myoblasts Infected with Ad-VEGF. Several muscle fibers were isolated from the soleus muscle of FVB/NJ mice (The Jackson Laboratory, Bar Harbor, ME) and cultured in DMEM containing 10% fetal bovine serum. Two weeks later, myoblasts migrating from the muscle fibers were harvested and infected with Ad-VEGF or Ad-LacZ (multiplicity of infection = 50). After 2 days, the myoblasts were harvested by trypsin mixed with type I collagen and injected s.c. to FVB/NJ mice (5 × 10⁶ cells/mouse). Seven days later, half of the VEGF-treated mice received s.c. injection with rEndostatin (EntreMed, Rockville, MD) for 5 days (1 daily injection of 100 mg/kg). Mice were sacrificed, and blood samples were retrieved and prepared for flow cytometry as described below. The levels of serum VEGF were measured using a mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN).

Injections of Recombinant Adenovirus. FVB/NJ mice received i.v. injection with combinations of Ad-VEGF + Ad-GFP [approximately 1 × 10⁸ and 1 × 10⁷ pfu/mouse, respectively (VEGF)] and Ad-VEGF + Ad-sFLT [approximately 1 × 10⁹ and 1 × 10¹⁰ pfu/mouse, respectively (VEGF + sFLT)]. Mice given injection with Ad-GFP (approximately 1 × 10⁸ pfu/mouse) served as control. After 6 days, mice were sacrificed, and blood samples were retrieved and prepared for flow cytometry as described below.

Tie2/LacZ mice (The Jackson Laboratory) received i.v. injection with Ad-VEGF (approximately 5 × 10⁹ pfu/mouse). After 1 day, half of the mice (5 mice/group) received daily i.p. injection of recombinant murine endostatin (20 mg/kg) or saline. Nontreated Tie2/LacZ mice served as normal controls. Five days later, mice were sacrificed, and PBMCs were purified and cultured as described below. Cells were grown in culture for 2 days and then fixed with 4% paraformaldehyde and incubated with a β-galactosidase substrate (5-bromo-4-chloro-3-indolyl β-d-galactoside) that stains cells blue.

Flow Cytometry

CECs in peripheral blood were evaluated using three- or four-color flow cytometry. Red cell lysis was performed using FACSLyse Solution (BD Biosciences, San Jose, CA), as per the manufacturer’s directions, or ammonium chloride lysis buffer for experiments in which apoptosis was evaluated. The following antibodies were used: rat antimon CD45 conjugated to FITC or APC and anti-Flk-1 conjugated to Phycoerythrin (PE) and 7AAD was used to assess apoptosis. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences) with analysis gates designed to remove platelets and cellular debris. Between 50,000 and 100,000 events were typically counted for each mouse. Mouse blood spiked with Flk-1-expressing Hemangiendothelioma (EOMA) cells was used as a positive control.

EPC Culture in Vitro

PBMCs corresponding to 0.5–1 ml of blood were seeded onto a fibronectin-coated 48-well dish. The cells were cultured in EGM-2 medium (Bio-Whittaker, Walkersville, MD), and VEGF and bFGF (5 and 2 ng/ml, respectively) were added every other day. EPC colonies were allowed to grow for 14 days, and the medium was changed every 7 days. At the end of 2 weeks, EPCs from wells with colonies were harvested by trypsin and seeded onto fibronectin-coated dishes.

RESULTS

VEGF Increases the Number of Endothelial Progenitor Colonies. Several pathological conditions associated with high levels of circulating VEGF have been reported to induce differentiation and mobili-
Fig. 2. Opposite effects of VEGF and endostatin on EPC mobilization. SCID mice (10 mice/group) received injection with microcapsules containing cells expressing recombinant VEGF (III and IV) or control cells (I and II), as described under “Materials and Methods.” Half of the mice from each group received coinjection with microcapsules containing cells expressing rEndostatin (II and IV), as indicated. Five days later, mice were sacrificed, and PBMCs were purified and counted, and samples corresponding to 1 ml of blood were loaded onto glass slides and stained with fluorescence-labeled anti-Flk-1 antibodies. The slides were further stained with 4',6-diamidino-2-phenylindole (DAPI), which labels all nuclei, to determine the total number of PBMCs in each slide. Approximately 1.6% Flk-1-expressing PBMCs were detected in the blood of encapsulated control cell-implanted mice, and lower numbers were detected in mice given implants of encapsulated cells expressing rVEGF and cells expressing rEndostatin only (Fig. 2A, IV). In parallel, PBMCs corresponding to 1 ml of peripheral blood were cultured in EC medium containing bFGF and VEGF. PBMCs from encapsulated control cell-injected mice gave rise to a small number of colonies after 2 weeks in culture (Fig. 1A). In contrast, PBMCs from mice treated with rVEGF yielded a large number of colonies (Fig. 1B). At a higher magnification, many cells were elongated and were able to incorporate Dil-labeled acetylated low-density lipoprotein into their membrane (Fig. 1, D and C, respectively). In a parallel experiment, colonies from PBMCs of mice treated with rVEGF were stained with anti-Flk-1 antibodies at the end of 2 weeks in culture (Fig. 1E). Most elongated cells were positively stained, indicating that the majority of the cells in these colonies differentiated into ECs. EPCs from rVEGF-treated mice were successfully grown in culture and further shown to be stained positively with anti-Flk-1, CD31, and von Willebrand factor antibodies (data not shown). These results indicate that VEGF induces mobilization of EPCs to peripheral blood and that these cells can form EC colonies in vitro.

EPC Mobilization Is Increased by High Levels of VEGF and Inhibited by Endostatin. Recombinant endostatin inhibits EC proliferation and migration in vitro, but its effects on EPCs are not known. At present, EPCs cannot be maintained as adherent cells in culture because they differentiate within a few passages in tissue culture dishes (25). Thus, to test the effects of endostatin on EPCs, we used an in vitro model that generates EPCs. To achieve this goal, encapsulated cells expressing rVEGF and cells expressing rEndostatin were implanted in mice (23, 24). Blood samples were retrieved 5 days later, and PBMCs were purified and counted, and samples corresponding to 1 ml of blood were loaded onto glass slides and stained with fluorescence-labeled anti-Flk-1 antibodies. The slides were further stained with 4',6-diamidino-2-phenylindole (DAPI), which labels all nuclei, to determine the total number of PBMCs in each slide. Approximately 1.6% Flk-1-expressing PBMCs were detected in the blood of encapsulated control cell-implanted mice, and lower numbers were detected in mice given implants of encapsulated cells expressing rEndostatin (Fig. 2A, I and II). In contrast, mice that received injection with encapsulated cells expressing VEGF had approximately 3.8% Flk-1-expressing PBMCs (Fig. 2A, III). However, when encapsulated cells expressing rVEGF and cells expressing rEndostatin were coimplanted in mice, the number of Flk-1-positive PBMCs was reduced to the level observed in mice given implants with encapsulated cells expressing rEndostatin only (Fig. 2A, IV). In parallel, PBMCs corresponding to 1 ml of peripheral blood were cultured in EC medium containing bFGF and VEGF. After 2 weeks in culture, a large number of colonies was observed in the wells seeded with PBMCs from VEGF-treated mice (Fig. 2B, III), but not in PBMCs derived from mice given implants with encapsulated control cells and encapsulated
cells expressing rEndostatin only (Fig. 2B, I and II). In contrast, coimplantation of encapsulated cells expressing rEndostatin with cells expressing rVEGF significantly reduced the number of EPC colonies to the levels seen in mice given implants with encapsulated control cells (compare Fig. 2B, IV with Fig. 2B, I). These results indicate that endostatin inhibited VEGF-induced mobilization of EPCs in mice.

We further examined the bone marrow of mice given implants with encapsulated cells expressing rVEGF and cells expressing rEndostatin. Bone sections were immunostained with anti-CD31 antibodies to evaluate bone marrow vascularization. Only a few bone marrow blood vessels were observed in bone sections from mice given implants with encapsulated control cells and encapsulated cells expressing rEndostatin only (Fig. 2C, I and II). The bone marrow of mice given implants with encapsulated cells expressing rVEGF was filled with many capillaries and larger-diameter blood vessels (Fig. 2B, III). Coimplantation of encapsulated cells expressing rEndostatin with cells expressing rVEGF resulted in a dramatic decrease in the number of bone marrow capillaries and a decrease in the size of the bone marrow blood vessels (Fig. 2B, IV). These results indicate that VEGF and endostatin have opposite effects on bone marrow neovascularization, suggesting a systemic effect of the implanted encapsulated cells secreting rVEGF and rEndostatin.

Endostatin Inhibits VEGF-Induced CECs. In the previous experiment, we used encapsulated cells expressing about 10 ng/ml VEGF during a 24-h culture in vitro, resulting in high levels of circulating VEGF in mice (1–5 ng/ml). To validate the previous results, we used a system during a 24-h culture experiment, we used encapsulated cells expressing about 10 ng/ml VEGF and a decrease in the size of the bone marrow blood vessels (Fig. 2B, III). Coimplantation of encapsulated cells expressing rEndostatin with cells expressing rVEGF resulted in a dramatic decrease in the number of bone marrow capillaries and a decrease in the size of the bone marrow blood vessels (Fig. 2B, IV). These results indicate that VEGF and endostatin have opposite effects on bone marrow neovascularization, suggesting a systemic effect of the implanted encapsulated cells secreting rVEGF and rEndostatin.

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Endostatin Inhibits VEGF-Induced CECs in a Tie2/LacZ Transgenic Mouse Model. To confirm the previous results, we used a transgenic mouse model in which the expression of β-galactosidase (LacZ) is driven by an EC-specific gene, the Tie2 promoter (12). PBMCs were purified from peripheral blood of control Tie2/LacZ mice and grown in EC medium for 3 days. LacZ-stained cultured PBMCs showed approximately 200 round and blue cells per 1 ml of blood (Fig. 4). To test the effect of VEGF on CECs, we injected a low-titer Ad-VEGF (5 × 107 pfu/mouse) and collected blood samples 6 days later. VEGF induced a significant 2-fold-increase in the number of blue PBMCs to 400 blue PBMCs/ml. In contrast, daily i.p. injections of recombinant murine endostatin (20 mg/kg) reduced the number of blue-stained PBMCs to 120 blue PBMCs/ml. These results provide further evidence that endostatin can inhibit the VEGF-induced mobilization of EPCs in mice.

DISCUSSION

Vascularization is a crucial factor for tumors to grow and metastasize. The notion that ECs are present in the circulation and can contribute to neovascularization has implications for development of therapeutic agents for cancer and implementation of these agents into clinical trials. In the present study, we have tested the effects of angiogenic and antiangiogenic factors on CECs in mice. We observed that mice treated with VEGF had elevated numbers of CECs, as
shown previously (16, 17), whereas endostatin administration significantly reduced these numbers. These results identify CECs as a target for the antiangiogenic activity of endostatin.

In recent years, EPCs were isolated from peripheral blood and bone marrow and characterized in vitro (3, 28). However, due to rapid differentiation of EPCs to mature ECs when cultured on matrix-coated tissue culture dishes, it is currently not technically feasible to perform extensive in vitro tests on EPCs (25). To achieve detectable levels of EPCs to test the effects of endostatin, we used two different in vivo VEGF delivery models. We transplanted encapsulated cells that continuously release VEGF and injected mice with a recombinant adenovirus expressing VEGF. We observed that high levels of VEGF significantly induced EPC mobilization in mice, as judged by the number of Flk-1$^+/H11001^+/CD45^+/H11002$ PBMCs and LacZ-expressing PBMCs from Tie2/LacZ mice. These numbers were significantly reduced when VEGF was coadministered with the VEGF antagonist sFLT, indicating the specific effects of VEGF in these experiments. Continuous release of VEGF resulted in the formation of a large number of EPC colonies, which, when further cultured in vitro in the presence of VEGF, expressed specific EC markers such as Flk-1. Furthermore, we were able to demonstrate a massive bone marrow vascularization 5 days after implantation of encapsulated cells secreting VEGF. This result indicates the systemic activity of VEGF in our mouse model and suggests that the newly formed bone marrow vessels may facilitate mobilization of EPCs to the peripheral blood. Taken together, these results demonstrate that VEGF induces differentiation and mobilization of EPCs.

The present study shows that endostatin inhibits the VEGF-induced mobilization of CECs in mice. In the first model, we used encapsulated cells that express high levels of rVEGF to induce CECs (24) and coimplanted them with encapsulated cells that express high levels of rEndostatin (23). Endostatin significantly reduced the number of VEGF-induced Flk-1$^+$ PBMCs. These results were confirmed by the absence of EPC colonies from the blood of mice that were given coimplants with encapsulated cells expressing VEGF and endostatin. In the second model, we delivered lower levels of VEGF using myoblasts infected with recombinant adenovirus expressing murine VEGF. The average of circulating VEGF levels in these mice at the end of the experiment was slightly higher than that in age-matched mice but was not significantly different (76.8 ± 13.4 and 64.4 ± 8.9 pg/ml, respectively). In this model, mice received daily injection with clinical-grade rEndostatin, and CECs were evaluated by FACS analysis. Although the levels of circulating VEGF were not dramatically higher than levels normally found in mice, the mice that were given implants with myoblasts secreting VEGF had approximately a 3-fold increase in the number of CECs and approximately a 50% reduction in CEC apoptosis. Endostatin, on the other hand, induced CEC apoptosis, which probably accounted for the reduction in CEC numbers to levels similar to those seen in control mice. Recently, Capillo et al. (29) reported that endostatin reduced CEC numbers in healthy and...
endothelial progenitor cells.

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