VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells

Takayuki Asahara, Tomono Takahashi, Haruchika Masuda, Christoph Kalka, Donghui Chen, Hideki Iwaguro, Yoko Inai, Marcy Silver and Jeffrey M. Isner

Departments of Medicine (Cardiology) and Biomedical Research, St Elizabeth’s Medical Center, Tufts University School of Medicine, 736 Cambridge Street, Boston, MA 02135, USA

Vascular endothelial growth factor (VEGF) has been shown to promote neovascularization in animal models and, more recently, in human subjects. This feature has been assumed to result exclusively from its direct effects on fully differentiated endothelial cells, i.e. angiogenesis. Given its regulatory role in both angiogenesis and vasculogenesis during fetal development, we investigated the hypothesis that VEGF may modulate endothelial progenitor cell (EPC) kinetics for postnatal neovascularization. Indeed, we observed an increase in circulating EPCs following VEGF administration in vivo. VEGF-induced mobilization of bone marrow-derived EPCs resulted in increased differentiated EPCs in vitro and augmented corneal neovascularization in vivo. These findings thus establish a novel role for VEGF in postnatal neovascularization which complements its known impact on angiogenesis.

Keywords: blood/bone marrow/endothelial progenitor cell/vascular endothelial growth factor/vasculogenesis

Introduction

Identified initially as vascular permeability factor (VPF) (Senger et al., 1983; Keck et al., 1989) due to its potent permeability-enhancing activity, vascular endothelial growth factor (VEGF) subsequently was shown to have a modest mitogenic effect specifically for endothelial cells (ECs) (Ferrara and Henzel, 1989; Leung et al., 1989; Plouet et al., 1989). The finding that VEGF could promote neovascularization in vivo (Banai et al., 1994; Takeshita et al., 1994; Pearlman et al., 1995; Isner et al., 1996; Li et al., 1996) was attributed to its mitogenic and pro-migratory (Ferrara and Henzel, 1989; Keck et al., 1989; Leung et al., 1989; Plouet et al., 1989) effects on ECs, consistent with the concept of angiogenesis, i.e. development of sprouts from pre-existing, fully differentiated ECs (Folkman and Klagsbrun, 1987).

During embryogenesis, however, the role of VEGF in vascular development is not limited to angiogenesis. Gene targeting studies (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996) have demonstrated that VEGF is not only essential for vascular sprouting, but is also required for vasculogenesis. VEGF deficiency in utero delays EC differentiation (Carmeliet et al., 1996; Ferrara et al., 1996), and mice with targeted disruption of the VEGF receptor Flk-1 lack ECs and hematopoietic cells (Shalaby et al., 1995). VEGF is thus an initial determinant of hemangioblast differentiation into endothelial progenitor cells (EPCs), or angioblasts, and hematopoietic stem cells (HSCs) (Pardanaud et al., 1989; Flamme and Risau, 1992; Risau and Flamme, 1995; Wilting et al., 1995; Hatzopoulos et al., 1998).

Recent studies performed in our laboratory and by others have demonstrated that vasculogenesis is not restricted to embryogenesis, but instead contributes to postnatal neovascularization (Asahara et al., 1997; Shi et al., 1998). Given its regulatory role in both angiogenesis and vasculogenesis during fetal development, we considered that VEGF may have a similarly complementary function in adults, namely to promote vasculogenesis. Accordingly, we investigated the hypothesis that VEGF may mobilize EPCs from bone marrow (BM), modulate EPC kinetics and promote EPC differentiation in adult mice.

Results

Effect of VEGF on EPC kinetics in circulation

To evaluate the effect of VEGF on EPC kinetics, recombinant human VEGF165 (rhVEGF) or control buffer was administered to C57BL/6J mice by intraperitoneal (i.p.) injection daily for a period of 1 week. Peripheral mononuclear blood cells were counted during and following rhVEGF or control buffer administration (Figure 1A). The total number of mononuclear cells increased beginning on treatment day 1, peaked on treatment day 4 (174.8% of control) and remained elevated through 7 days post-treatment.

Fluorescence-activated cell sorting (FACS) was performed to identify the cellular population mobilized by rhVEGF administration. Light scatter analysis indicated that the increased mononuclear cell population in peripheral blood was more prominent among monocyte-size (MS) cells (910 ± 67 versus 249 ± 13×10^3/ml; 265.5% increase, p < 0.01) than in the lymphocyte-size (LS) population (2164 ± 115 versus 1541 ± 125×10^3/ml; 40.4% increase, p < 0.05) at treatment day 4 (Figure 1B). We observed a marked increase in the number of cells positive for EC-specific antigens, CD34 (478 ± 66 versus 105 ± 11×10^3/ml; 355% increase, p < 0.01), Flk-1 (124 ± 29 versus 31 ± 4×10^3/ml; 296% increase, p < 0.05) and VE cadherin (535 ± 47 versus 210 ± 52×10^3/ml; 155% increase, p < 0.01), while macrophage antigen positivity for Moma2 was not significantly increased (123 ± 12 versus 88 ± 5×10^3/ml, p = not significant).

Blood mononuclear cells were divided into two groups based on acetylated low density lipoprotein (acLDL)
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Fig. 1. (A) Change in number of PBMCs during and following administration of rhVEGF or control buffer. The total number of mononuclear cells increased beginning on treatment day 1 through day 7 post-treatment with a peak on treatment day 4 (174.8% of control). (B) Light scatter dot plots of monocyte-size (MS) and lymphocyte-size (LS) fractions. In contrast to controls, VEGF treatment was characterized by an increase in the MS fraction. (C) FACS analysis of PBMCs gated into LS or MS fractions using acLDL-DiI and control rat IgG or rat IgG antibody against mouse CD34. Uptake of acLDL is classified as positive (acLDL+) or negative (acLDL−). CD34 staining demonstrates that both CD34-positive populations in acLDL+ of the LS and MS fractions increased in rhVEGF-pre-treated mice versus controls. The illustrated scattergrams were made from the equivalent total PBMC number, followed by gating for LS and MS fractions. (D) FACS analysis for each acLDL uptake group of LS and MS fractions. Endothelial markers, Flk-1, VE-cadherin and CD34, are increased primarily in the acLDL+ group of the LS and MS fraction (VEGF group), while the increase in Moma-2 is not significant.

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uptake, using acLDL-Dil fluorescence. Circulating cells mobilized in response to VEGF administration consisted principally of cells taking up acLDL (acLDL⁺) (83.0%; Figure 1C and D). These mobilized acLDL⁺ cells included significant numbers of putative EPCs expressing CD34 (31.0%), Flk-1 (7.5%) and VE cadherin (26.0%). In contrast to putative EPC populations, no significant increase in macrophage-specific antigen (Moma-2)-positive cells was observed. Mobilized putative EPCs were detected in both the LS (lower side-angle light scatter) and MS cell fraction (higher side-angle light scatter). The size differences indicated for these two EPC populations may reflect differential EPC development.

As a functional index of increased circulating EPCs, a culture assay was employed. The number of cultured EPCs, confirmed by acLDL uptake and BS-1 lectin reactivity (Figure 2A and B), again indicated more frequent EPCs in the peripheral blood of VEGF-treated mice than in controls. The effect of VEGF could be detected by EPC culture assay from treatment day 1 (254% of control, \(p < 0.05\)) through day 14 post-treatment (214%, \(p < 0.05\)) with a peak at treatment day 4 (375%, \(p < 0.01\)).

A neutralizing antibody prepared against rhVEGF protein was employed to confirm the role of VEGF on EPC mobilization. Administration of neutralizing antibody along with rhVEGF protein completely abrogated the impact of rhVEGF administration on the results of the day 1 EPC culture assay (Figure 2C).

**VEGF effect on mitogenic and migratory activity of EPCs**

The effects of VEGF on proliferation and migration of cultured EPCs were also assayed. While the culture assay disclosed a modest effect of VEGF on EPC proliferative activity (Buttke et al., 1993) (28.7% increase at 10 ng/ml, \(p < 0.05\)) (Figure 2D), a more potent dose-dependent effect was documented for EPC migration (Falk et al., 1980) (0 versus 10 ng/ml VEGF = 7.9 ± 0.8 versus 284.9 ± 8.1 cell/well, \(p < 0.05\)) (Figure 2E).

**VEGF effect on BM mononuclear cell chemotaxis**

The effect of VEGF on the chemotactic activity of BM mononuclear cells was investigated to gauge the potency of VEGF on EPC mobilization from BM (Zigmond and Hirsh, 1973). The results demonstrated a dose-dependent chemotactic effect of VEGF on BM mononuclear cells (0 versus 100 ng/ml VEGF = 3041 ± 238 versus 4850 ± 76 cells/well, \(p < 0.05\)) (Figure 2F). These effects were abrogated by coincidental application of a neutralizing antibody against rhVEGF. Peak mobilization in response to the maximum dose of rhVEGF was ~60% of that observed in response to recombinant murine granulocyte–macrophage colony-stimulating factor (rmGM-CSF). Checkerboard assay confirmed the chemotactic but not the chemokinetic activity (data not shown).

**Effect of mobilized EPCs on neovascularization**

To establish the effect of VEGF-induced modulation of circulating EPC kinetics on pathological neovascularization, mice were pre-treated with rhVEGF or control buffer for 7 days prior to cornea micropocket injury (Muthukkaruppan and Auerbach, 1979; Asahara et al., 1998). On day 7 post-injury (i.e. 7 days following the last dose of rhVEGF), the resulting neovasculature was assessed morphometrically followed by in situ BS-1 lectin staining. Augmented corneal neovascularization was found in the rhVEGF group (vascular length = 0.62 ± 0.04 versus 0.50 ± 0.09 mm; circumference = 114 ± 11 versus 82 ± 10) compared with controls (Figure 3A–F).

Finally, a mouse BM transplantation (BMT) model was employed to establish direct evidence of enhanced BM-derived EPC incorporation into foci of cornea neovascularization following rhVEGF administration. FVB/N mice underwent BMT from transgenic mice expressing β-galactosidase encoded by lacZ under the transcriptional regulation of an EC-specific promoter, Tie-2 (Schlaeger et al., 1995). Reconstitution of the transplanted BM yielded Tie-2/LacZ/BMT mice in which expression of lacZ is restricted to BM-derived cells expressing Tie-2/lacZ and is not observed in other somatic cells. Detection of tie-2–lacZ fusion transcripts by RT-PCR in peripheral blood mononuclear cells (PBMCs), BM and spleen of the BMT recipients confirmed reconstitution of the BM and the origin of the incorporated cells in Tie-2/LacZ/BMT mice (Figure 4A). The Tie-2/LacZ/BMT mice underwent corneal assay microsurgery (Muthukkaruppan and Auerbach, 1979; Asahara et al., 1998) 1 day following pre-treatment with rhVEGF or vehicle control. Corneas excised 6 days after micropocket implantation demonstrated BM-derived EPC incorporation into neovascular foci of both groups; β-galactosidase activity, however, was significantly higher in VEGF-pre-treated versus control-pre-treated animals (VEGF pre-treatment = 19.4 ± 3.6 × 10³, control = 7.9 ± 2.4 × 10³, background activity in tissue: 0.2 ± 0.1 × 10³, \(p < 0.05\)) (Figure 4B and C). Histological examination, including X-gal staining, demonstrated incorporation of BM-derived EPCs into capillaries and stromal tissue of cornea.

**Discussion**

These results indicate that VEGF modulates postnatal EPC kinetics. The documented chemotactic effect on BM EPCs supports the notion that VEGF mobilizes EPCs from BM. Mobilization of EPCs into the peripheral circulation was confirmed by an increased number of circulating cells bearing EC-specific antigens after rhVEGF administration in vivo. VEGF-induced EPC mobilization resulted in an increase in the number of differentiated EPCs in vitro, and augmented corneal neovascularization in vivo.

HSCs, believed to derive from an ancestral lineage common to EPCs, migrate to BM from fetal liver during fetal development, and home to the extravascular compartment of BM following transplantation. Conversely, HSCs may be mobilized in the opposite direction, from BM to peripheral blood, in response to cytokines such as GM-CSF, granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and stromal cell-derived factor-1 (SDF-1) (Duhren et al., 1988; Siena et al., 1989; Donahue et al., 1996; Kim and Broxmeyer, 1998).

The current findings thus suggest a novel role for VEGF that is analogous to that described for HSC-modulating cytokines in the regulation of postnatal neovascularization. Just as VEGF promotes angiogenesis in both the embryo and adult, the role of VEGF in modulating embryonic
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Fig. 2. (A) Representative fluorescence photomicrographs of EPCs cultured 4 days following isolation from peripheral blood. EPCs are identified as double-positive cells due to acLDL-DiI uptake (red) and BS-1 lectin reactivity (green). EPCs are more frequent and differentiated in VEGF (right) than in the control group (left). (B) EPC culture assay during and following rhVEGF versus control buffer administration. EPCs in the culture assay are more frequent in the VEGF group beginning on day 1 during treatment (DT) through day 14 post-treatment (PT), with a peak on treatment day 4. (C) Abrogation of VEGF-induced EPC mobilization in vivo. EPC culture assay on day 1 demonstrated that the enhancement of EPC kinetics induced by VEGF administration was completely abrogated using neutralizing antibody against human VEGF protein. (D) MTS assay of mouse EPCs. A moderate dose-dependent mitogenic response of EPCs to rhVEGF was observed following 7 days of culture. (E) Migration assay of mouse EPCs in response to VEGF. Cultured EPCs demonstrated potent dose-dependent migratory activity. (F) The chemotaxis assay disclosed a dose-dependent chemotactic effect of rhVEGF on BM mononuclear cells; the potency of this effect was 60% that of recombinant murine GM-CSF. The checkerboard assay confirmed the chemotactic but not the chemokinetic activity (data not shown).

vasculogenesis is recapitulated in adults as well. Previous reports of endogenous VEGF expression in the BM environment (Katoh et al., 1995) or BM stroma cell lines (data not shown) are consistent with the concept of a physiological regulatory function for VEGF in EPC mobilization, similar to the role established for other BM cytokines expressed locally in the BM microenvironment on HSC mobilization. The fact that VEGF is naturally secreted by intact cells (Tischer et al., 1991) constitutes a critical feature that potentially allows such local paracrine effects as well as hormone-like effects when VEGF is up-regulated in remote tissues in response to hypoxia (Shweiki et al., 1992; Couffinhal et al., 1998). Recently, Springer et al. (1998) demonstrated that local sustained overexpression of VEGF by transferred myoblast implantation resulted in vasculogenesis near the site of injection in normal muscle. Our data suggest that such vasculogenesis may be contributed to not only by local EPC recruitment
Fig. 3. Cornea neovascularization assay 6 days after micropocket implantation. Macroscopic photographs (A and B) and fluorescence photomicrographs following BS-1 lectin in vivo staining (C and D) demonstrate enhanced neovascularization in animals pre-treated with rhVEGF. 

(E and F) Quantitative analysis of neovascularization in the cornea assay.

and proliferation, but also by enhanced EPC kinetics in the circulation as a result of expansion and mobilization of EPCs from BM.

Mechanisms contributing to cytokine-induced HSC mobilization include the chemoattractive effect of the cytokine gradient, cellular proliferation, modulation of adhesion molecules and modulation of the blood–BM barrier (Turner and Sweetenham, 1996; Kim and Broxmeyer, 1998). Like the aforementioned cytokines, VEGF has a chemoattractive effect on a fraction of BM mononuclear cells and cultured EPCs, demonstrated here, as well as on adhesive mononuclear cells (Clauss et al., 1990, 1996; Shen et al., 1993) and monocytes in peripheral blood (Barleon et al., 1996) shown previously. Enhanced EPC differentiation in culture as early as 4 h following rhVEGF injection (treatment day 1) reflects the immediate
effect of VEGF on EPC mobilization, probably related to prompt development of a chemotactic gradient, similar to that associated with hematopoietic growth factors which mobilize HSCs (Duhrsen et al., 1988; Siena et al., 1989; Donahue et al., 1996; Kim and Broxmeyer, 1998).

The proliferative impact of VEGF probably also contributes to EPC mobilization. The documented mitogenic effect of VEGF on cultured EPCs, together with sustained EPC frequency in culture assay up to 2 weeks after rhVEGF pre-treatment, suggests VEGF-induced expansion of BM EPCs, with a subsequent leak into the circulation.

In addition to these direct effects, indirect effects of VEGF may help in regulating EPC mobilization. VEGF has been shown to enhance vascular permeability (50 000 times more than histamine) (Senger et al., 1983; Keck et al., 1989) and modulate adhesion molecule expression on endothelium (Griffioen et al., 1996; Melder et al., 1996; Senger et al., 1996). Permeabilization of the BM vasculature and modification of adhesive molecule expression of BM endothelium by VEGF may contribute to recruitment of EPCs into the circulation.

The expression of lacZ regulated by the Tie-2 promoter potentially could identify BM-derived differentiated ECs as well as EPCs. There are at least two lines of evidence, however, which suggest that the preponderance of such circulating cells are EPCs. First, previous work (Asahara et al., 1997; Hatzopoulos et al., 1998; Shi et al., 1998) indicates that the population of circulating EPCs far exceeds the number of differentiated ECs in peripheral blood (Peretz et al., 1992; Solovey et al., 1997). Secondly, recent work from our own laboratory has shown that in contrast to EPCs, heterologous transplanted differentiated ECs rarely incorporate into foci of neovascularization (Kalka et al., 1998). Accordingly, the majority of the cellular population mobilized into the circulation and then incorporated into neovascular foci following VEGF administration is likely to consist of EPCs derived from BM.

The demonstration that HSC transplants derived from peripheral blood can provide sustained hematopoietic recovery constitutes inferential evidence for circulating stem cells (Kessinger and Armitage, 1991; Sheridan et al., 1992; Shpall et al., 1994; Brugger et al., 1995). This observation is now being exploited clinically as an alternative to BMT (Brugger et al., 1995). Similarly, EPCs mobilized by VEGF from BM into peripheral blood could constitute an available source of EPCs that might facilitate neovascularization. Indeed, we have demonstrated recently that excess cultured EPCs isolated from human peripheral blood can enhance neovascularization of the ischemic hindlimb of immuno-deficient mice (unpublished data); the magnitude of improvement in limb neovascularity is similar to that achieved by VEGF gene transfer in the same animal model (Couffinhal et al., 1998). These dual outcomes suggest that the facilitative impact of VEGF on tissue neovascularization (Banai et al., 1994; Pearlman et al., 1995; Baumgartner et al., 1998; Mack et al., 1998), previously attributed exclusively to angiogenesis, may in part represent the contribution of mobilized EPCs to vasculogenesis.

Materials and methods

Study design

All protocols were approved by St Elizabeth’s Institutional Animal Care and Use Committee. rhVEGF, 10 μg in 100 μl of 0.5% bovine serum albumin (BSA, Sigma), was administered to C57BL/6J mice (n = 3–6 each) by i.p. injection daily for 1 week (day 1–day 7). Control mice received 100 μl of BSA according to the same schedule. Both groups of mice were sacrificed to harvest peripheral blood and BM on days 1, 4 and 7 during VEGF or BSA administration, and on days 1, 7, 14 and 28 post-VEGF administration. To confirm the specificity of rhVEGF-induced EPC mobilization, 100 μg of neutralizing antibody against human VEGF (R&D) in 100 μl of 0.5% BSA was administered i.p immediately prior to 10 μg of rhVEGF (n = 4). Animals sacrificed on day 1 of VEGF, control vehicle or VEGF + antibody administration were euthanized 4 h post-treatment.

All results are expressed as mean ± SE. Statistical significance was evaluated using unpaired Student’s t-test for comparisons between two means, and using ANOVA for comparisons between more than three
means. A value of \( p < 0.05 \) was interpreted to denote statistical significance.

**Mononuclear cell isolation and FACS analysis**

At each time point, blood was obtained from the heart immediately before sacrifice and separated by Histopaque-1083 (Sigma) density gradient centrifugation. Light density mononuclear cells were harvested, washed twice with Dulbecco’s phosphate-buffered saline (PBS; no calcium or magnesium) (Fisher) supplemented with 2 mM EDTA (DPBS-E). Contaminating plasmacytoid cells were eliminated using ammonium chloride solution (Stem Cell Technologies).

Immediately following isolation, PBMCs from each animal were incubated in DPBS-E containing 10 μg/ml Dil-labeled acLDL (Biomedical Technologies) for 1 h at 37°C. The cells were then incubated with fluorescent-labeled antibodies and analyzed by FACS. Accumulated cells were segregated into LS and MS cells by gating using in light scatter analyses. Gating of the MS fraction was programmed arbitrarily so that PBMCs would be accumulated until a total of 5000 MS cells had been counted; at the point when accumulation was complete, the number of cells in the LS fraction was noted as well. The number of MS cells together with the number of LS cells comprised the total number of PBMCs that served as the denominator for calculation of the percentage antigen-positive cells. The absolute number of antigen-positive cells per milliliter of whole blood was calculated by multiplying the percentage of antigen-positive cells by the total number of PBMCs per ml of blood.

Antibodies against Flk-1 (89B3A5, rat IgG1) (generous gift from Dr W.Risau), VE cadherin (19E6, rat IgG2a) (generous gift from Dr E.Dejana) and murine CD34 (RAM34, biotinylated rat IgG2a) (Pharmingen) were used as endothelial lineage markers; the specificity of the latter was confirmed by showing lack of detection in CD34 –/– mice (Buttke et al., 1993). Mouse EPCs were harvested 7 days after culture (Falk et al., 1990). rhVEGF was diluted to specific amounts of chemoattractants to the lower Transwell chamber.

A positive chemoattractant concentration gradient (0/+ 0) was made by adding chemoattractant to the lower chamber, a negative (+ 0) gradient was made by adding chemoattractant to the upper chamber, and a zero gradient was made by either adding chemoattractant to both chambers (+ + 0) or by not adding chemoattractant to either chamber (0/0). Murine GM-CSF (50 ng/ml) (R&D Systems) was used for the positive chemotactic response in this assay. Chambers were incubated at 37°C, 5% CO₂, for 2 h. Cells migrating into the lower chamber were collected in 50 μl of buffer and counted manually using a hemocytometer.

**Cornea neovascularization assay**

Age-matched (8 weeks old) C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME) or Tie2/LZ/BMT mice, described below, were used for murine corneal neovascularization assays. All animals were anesthetized by i.p. pentobarbital injection (160 mg/kg) for subsequent surgical procedures. Corneal pockets were created with a modified von Graefe cataract knife. Into each pocket, a 0.34×0.34 mm sucrose aluminum sulfate (Bukh Meditec, Denmark) pellet coated with hydron polymer type NCC (IFN Science) containing 180–200 ng of VEGF was implanted. The pellets were positioned 1.0 mm from the corneal limbus, and erythromycin ophthalmic ointment was applied to each operated eye. The corneas of all mice were examined routinely by slit-lamp biomicroscopy on post-operative day 6 after pellet implantation. Vessel length and circumference of the limbus showing neovascularization were measured by using the sixth post-operative day when all corneas were photographed. After completing the measurements, mice received 500 μg i.v. of BS-1 conjugated with FITC (Sigma) and were sacrificed 30 min later. The eyes were enucleated and fixed in 1% paraformaldehyde. After fixation, the corneas were placed on glass slides and studied by fluorescent microscopy.

**BMT model of Tie-2 transgenic mice**

BM cells were obtained by flushing the tibias and femurs of age-matched (4 weeks old), donor Tie-2 transgenic mice (FVB/N-TgTIE2L-acZ)182Sato, Jackson Lab) were lethally irradiated with 12.0 Gy and received i.v. infusion of ~2×10⁵ donor BM mononuclear cells each. BM mononuclear cells from normal FVB/N mice were transplanted into FVB/N mice as BMT controls. At 4 weeks post-BMT, by which time the BM of the recipient mice was reconstituted, VEGF or control BSA pre-treatment was administered for 7 days; 1 day later, surgery for cornea neovascularization assay was performed. Corneas of BMT animals were harvested at 6 days after surgery for histological or quantitative chemical detection of β-galactosidase expression.

To confirm reconstitution of BMT and the origin of the incorporated cells, we identified fusion transcripts of tie-2–lacZ in BMBCs, BM and spleen of BMT recipients (versus control animals transplanted with normal i.e. -tie-2–lacZ BM). RNA was extracted from each tissue sample using a total RNA isolation kit, Totally RNA (Ambion Inc). A 1 μg aliquot of total RNA from each sample was used for RT–PCR following DNase treatment. Reverse transcription and PCR were performed respectively using a Superscript Preamplification System kit (Gibco-BRL) and Ampli Taq (Perkin Elmer Co.), according to the supplemented protocol.

**β-galactosidase activity assay to quantify BM-derived EPCs**

For chemical detection of β-galactosidase activity, the enucleated eye was placed into liquid nitrogen, and stored at ~80°C. The assay was performed using the Chemiluminescence Reporter Gene Assay System, GalectoLight Plus™ (Tropix Inc.) according to the modified protocol. Briefly, the eye was placed in 1 ml of supplemented lysis buffer and, after adding 0.5 mM dithiothreitol, was homogenized with a Tissuemizer Mark II (Tekmar Co.). Homogenized lysis solution was centrifuged to remove debris. An aliquot of the supernatant from the homogenized lysis buffer was used for protein measurement using a BCA Protein Assay kit (Pierce). The supernatant was assayed after treatment with an
ion-exchange resin, Chelex100, and β-galactosidase activity was measured using a chemiluminometer (Lumat LB9501, Berthold). β-galactosidase activity was standardized according to protein concentration.

Acknowledgements

We thank M. Neely for secretarial assistance. Human recombinant VEGF was a generous gift from Genentech, Inc. This work was supported by grants (HL 40518, HL02824 and HL57516) from the National Institutes of Health, Bethesda, MD and the Weigand Foundation, Reno, NV.

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Received January 27, 1999; revised April 29, 1999; accepted May 19, 1999