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

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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 indeed 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 $VEGF_{165}$ (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 $249 \pm 13 \times 10^3$/ml; 265.5% increase, $p < 0.01$) than in the lymphocyte-size (LS) population (2164 ± 115 versus $1541 \pm 125 \times 10^3$/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 $105 \pm 11 \times 10^3$/ml; 355% increase, $p < 0.01$), Flk-1 (124 ± 29 versus $31 \pm 4 \times 10^3$/ml; 296% increase, $p < 0.05$) and VE cadherin (535 ± 47 versus $210 \pm 52 \times 10^3$/ml; 155% increase, $p < 0.01$), while macrophage antigen positivity for Moma2 was not significantly increased (123 ± 12 versus $88 \pm 5 \times 10^3$/ml, $p = $ not significant).

Blood mononuclear cells were divided into two groups based on acetylated low density lipoprotein (acLDL)
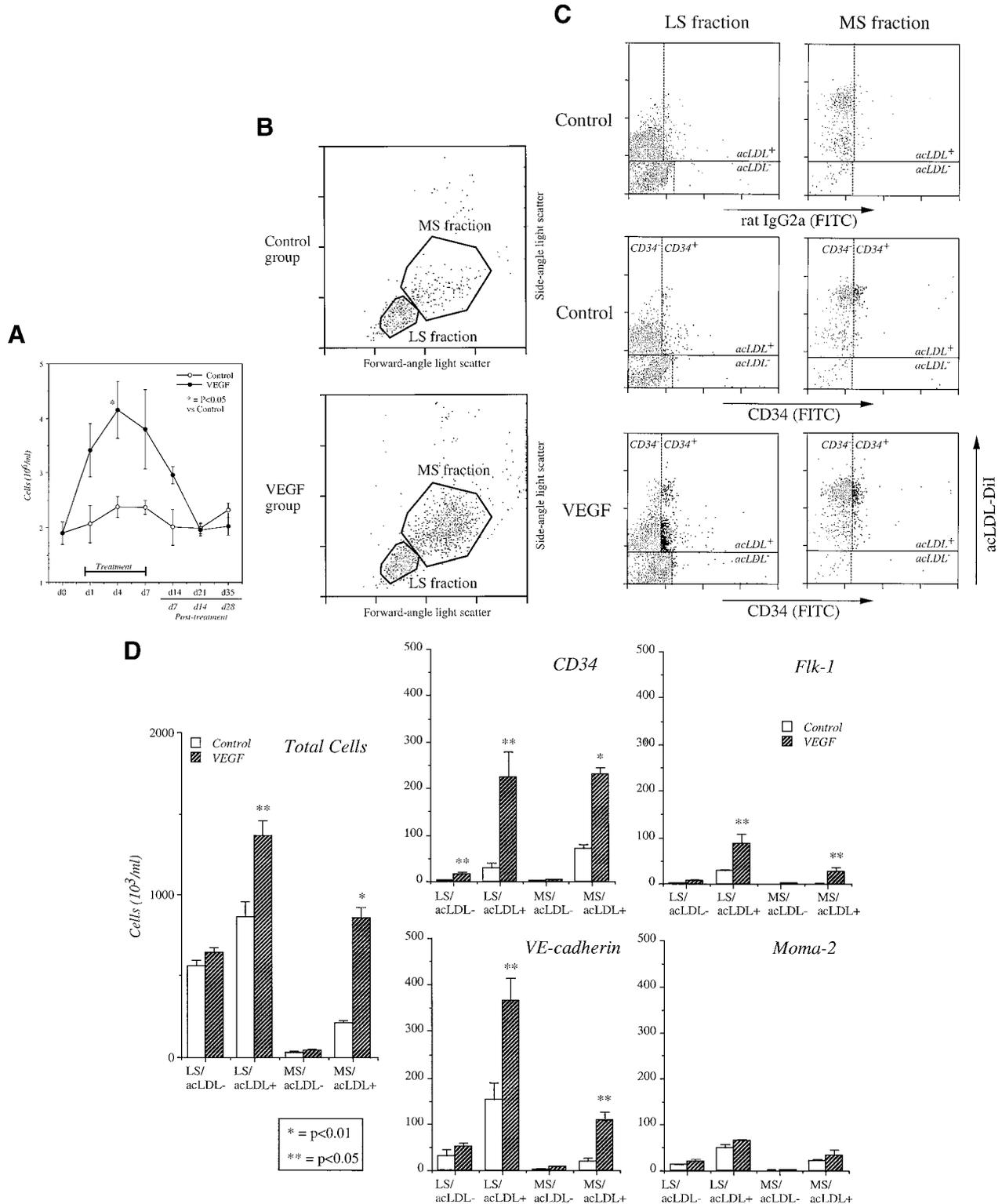
Fig. 1. (**A**) Change in number of PBMCs during and following administration of rhVEGF or control buffer. The total number of mononuclear cells increased beginning on treatment day 1 through day 7 post-treatment with a peak on treatment day 4 (174.8% of control). (**B**) Light scatter dot plots of monocyte-size (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.

uptake, using acLDL-DiI 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 Hirssch, 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 constitutively 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/LZ/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/LZ/BMT mice (Figure 4A). The Tie-2/LZ/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 ± 3.6×10^3, control = 7.9 ± 2.4×10^3, background activity in tissue: 0.2 ± 0.1×10^3, $p < 0.05$) (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) (Duhrsen *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
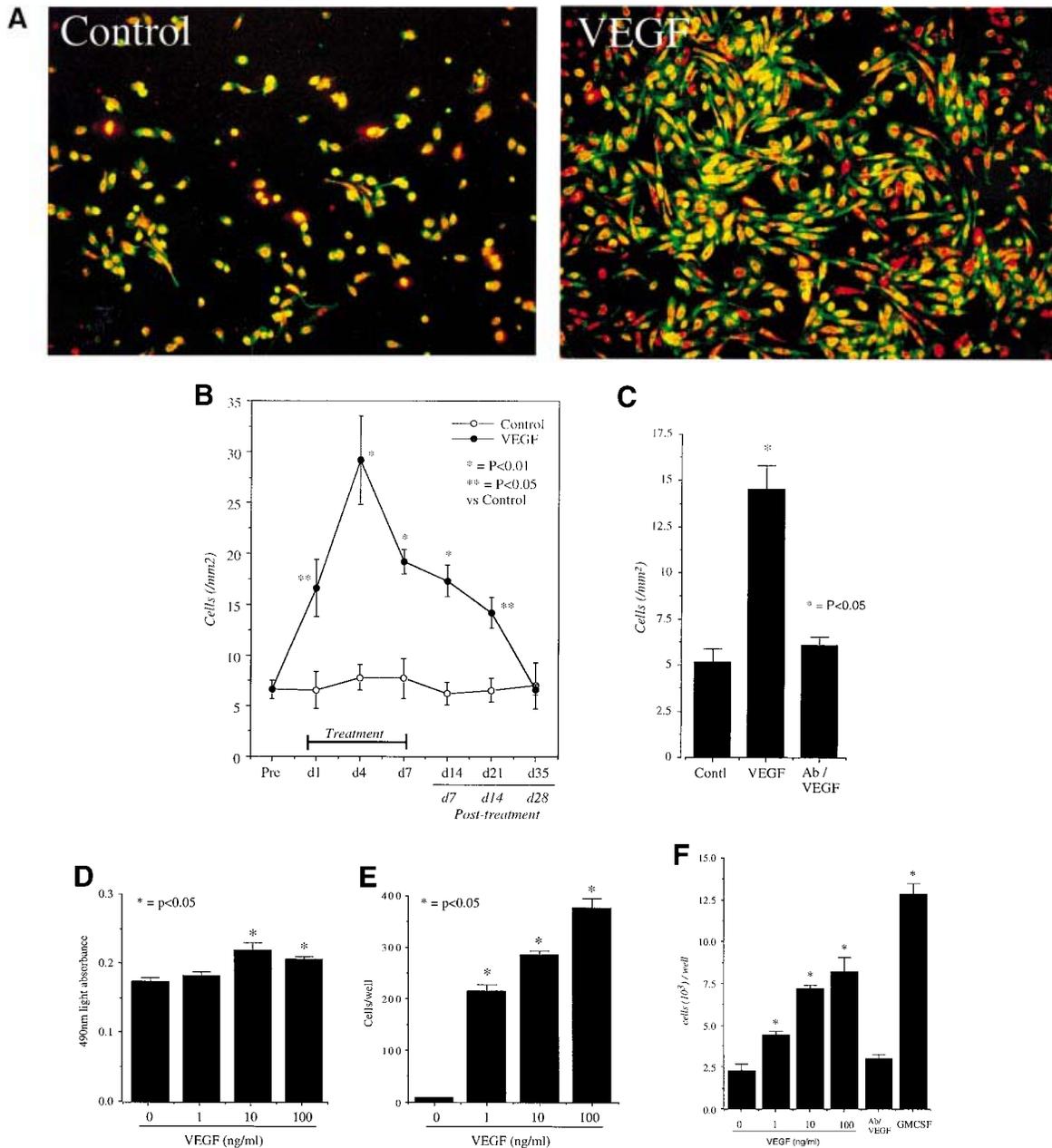
Fig. 2. (**A**) Representative fluorescence photomicrographs of EPCs cultured 4 days following isolation from peripheral blood. EPCs are identified as double-positive cells due to acLDL-DiI uptake (red) and BS-1 lectin reactivity (green). EPCs are more frequent and differentiated in VEGF (right) than 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

Control

VEGF
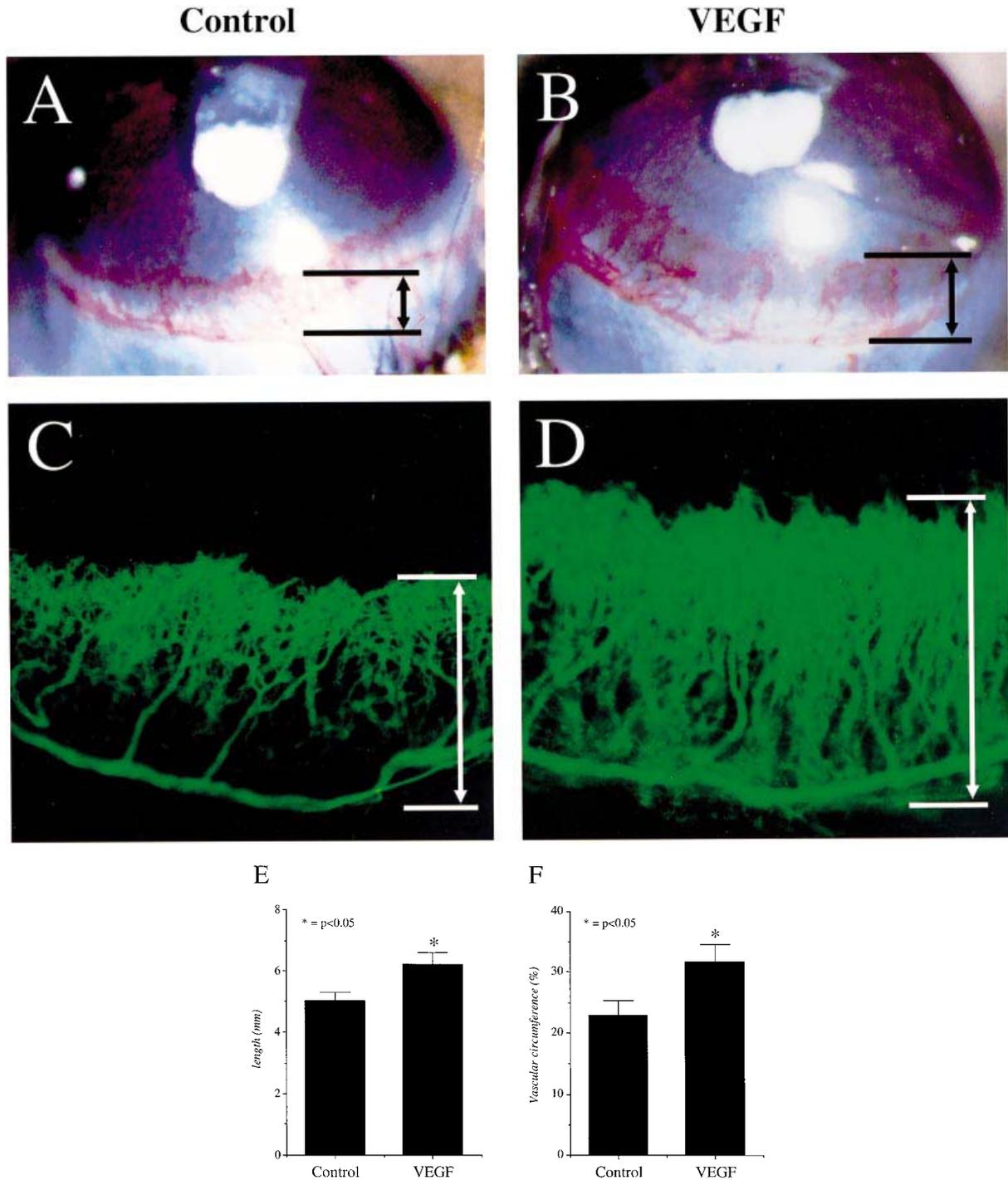
Fig. 3. Cornea neovascularization assay 6 days after micropocket implantation. Macroscopic photographs (**A** and **B**) and fluorescence photomicrographs following BS-1 lectin *in vivo* staining (**C** and **D**) demonstrate enhanced neovascularization in animals pre-treated with rhVEGF. (**E** and **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
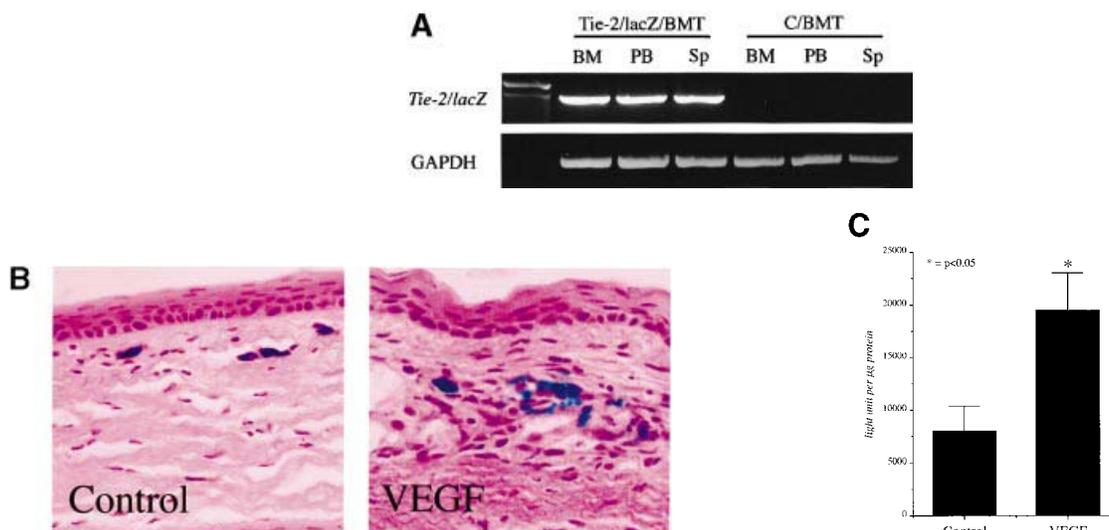
Fig. 4. Cornea neovascularization assay in bone marrow transplantation models, Tie-2/LZ/BMT. (**A**) Detection of *tie-2–lacZ* fusion transcripts by RT–PCR in hematopoietic organs confirmed the reconstitution of BM from the donor mice. (**B**) Representative photomicrographs following X-gal staining of corneas in the neovascularization assay of Tie-2/LZ/BMT mice. X-gal-stained cells (blue) indicate Tie-2-expressing BM-derived EPCs incorporated into the neovasculature. (**C**) The β-galactosidase activity assay demonstrates increased β-galactosidase activity in injured corneas of Tie-2/LZ/BMT mice pre-treated with rhVEGF versus controls.

effect of VEGF on EPC mobilization, probably related to prompt development of a chemoattractive 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 immunodeficient 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). Contaminated red blood cells were hemolyzed using ammonium chloride solution (Stem Cell Technologies).

Immediately following isolation, PBMCs from each animal were incubated in DPBS-E containing 10 µg/ml DiI-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 in light scatter analysis. 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 (generous gift of Dr Tak Mak). Moma-2 [fluorescein isothiocyanate (FITC)-conjugated rat IgG2b] (Serotec) was used as murine monocyte/macrophage lineage marker. An identical immunoglobulin control for each antibody was employed to detect non-specific binding of immunoglobulin.

EPC culture assay

Mononuclear cells isolated from 500 µl of peripheral blood from each mouse were cultured in EBM-2 (Clonetics) medium supplemented with 5% fetal bovine serum (FBS), antibiotics and growth factors (EPC medium) on 4-well glass slides coated with rat plasma vitronectin (Sigma) in 0.5% gelatin solution. Four days after culture, EPCs, recognized as attaching spindle-shaped cells, were assayed by co-staining with acLDL-DiI and BS-1 lectin, characteristic of the endothelial lineage, conjugated with FITC (Sigma). Fluorescent microscopy identified double-positive cells as EPCs.

Mitogenic activity and migration assay of cultured EPCs

Mitogenic activity (shown here as relative optical density) was assayed using a previously validated colorimetric MTS assay with the electron coupling reagent phenazine methosulfate (CellTiter 96 AQ; Promega) (Buttke *et al*., 1993). Mouse EPCs were harvested 7 days after culture and re-seeded into a 96-well plate in 0.1 ml of EBM-2 medium supplemented with 0.5% BSA overnight. rhVEGF was added to condition the wells for 48 h prior to the measurement.

EPC migration assays were performed using a 48-well microchemotaxis chamber (Neuroprobe) (Falk *et al*., 1980). rhVEGF was diluted to appropriate concentrations in EBM-2 medium supplemented with 0.5% BSA, and 25 µl of the final dilution was placed in the lower chamber of a modified Boyden chamber. Mouse EPCs cultured for 7 days were harvested and 2.5×10^5 cells suspended in 50 µl of EBM-2 medium supplemented with 0.5% BSA were re-seeded in the upper compartment. After incubation, the filter was removed and the upper side of the filter containing the non-migrating cells was scraped with a rubber policeman. The filters were fixed with methanol and stained with Giemsa solution (Diff-Quick, Baxter Diagnostics). Cells and those migrating into the lower chamber were counted manually in three random high-power fields ($\times 100$) in each well. All groups were studied at least in triplicate. The wells were incubated for 5 h at 37°C.

Chemotaxis assay of BM mononuclear cells

BM samples were obtained at each time point by first flushing the femurs and tibias, following which mononuclear cells were isolated by gradient centrifugation. Chemotaxis and chemokinesis in response to rhVEGF were assayed using a modified checkerboard assay. Chemotaxis buffer (100 µl of RPMI-1640 supplemented with 0.5% BSA) containing 1×10^6 BM cells was added to the upper chamber of a Coster Transwell

(6.5 mm diameter, 5 µm pore), and 600 µl of chemotaxis buffer (without cells) was added to the lower chamber. Chemoattractant concentration gradients of 1, 10 and 100 ng/ml rhVEGF were established by adding specific amounts of chemoattractants to the lower Transwell chamber. A positive chemoattractant concentration gradient (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 (+/+) or by not adding chemoattractant to either chamber (0/0). Murine GM-CSF (50 ng/ml) (R&D) was used for the positive chemoattractant response in this assay. Chambers were incubated at 37°C, 5% CO_2 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 on 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-TgN[TIE2L-acZ]182Sato, Jackson Lab). Low density BM mononuclear cells were isolated by density centrifugation over Histopaque-1083 (Sigma). The background mice, FVB/N (Jackson Laboratory), were lethally irradiated with 12.0 Gy and received i.v. infusion of ~2×10^6 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 PBMCs, BM and spleen of BMT recipients (versus control animals transplanted with normal, i.e. non-*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.

Primer pairs for the amplification of the *tie–2–lacZ* reverse transcribed product (Sato *et al*., 1995) were as follows: 5′-ggg Aag TCg CAA AgT TgT gAg TT-3′, 5′-CgT ggC CTg ATT CAT TCC-3′ (*tie–2–lacZ*).

A 12 µl aliquot of each PCR mixture was electrophoresed in 2 or 1% agarose gel containing ethidium bromide for *tie–2* and *lacZ* respectively. RT–PCR products were photographed under a UV transilluminator.

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, Galacto-Light 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.

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