Clinical Research

Vascular Endothelial Growth Factor<sub>165</sub> Gene Transfer Augments Circulating Endothelial Progenitor Cells in Human Subjects

Christoph Kalka, Haruchika Masuda, Tomono Takahashi, Rebecca Gordon, Oren Tepper, Edwin Gravereaux, Ann Pieczek, Hideki Iwaguro, Shin-Ichiro Hayashi, Jeffrey M. Isner, Takayuki Asahara

**Abstract**—Preclinical studies in animal models and early results of clinical trials in patients suggest that intramuscular injection of naked plasmid DNA encoding vascular endothelial growth factor (VEGF) can promote neovascularization of ischemic tissues. Such neovascularization has been attributed exclusively to sprout formation of endothelial cells derived from preexisting vessels. We investigated the hypothesis that VEGF gene transfer may also augment the population of circulating endothelial progenitor cells (EPCs). In patients with critical limb ischemia receiving VEGF gene transfer, gene expression was documented by a transient increase in plasma levels of VEGF. A culture assay documented a significant increase in EPCs (219%, \( P<0.001 \)), whereas patients who received an empty vector had no change in circulating EPCs, as was the case for volunteers who received saline injections (VEGF versus empty vector, \( P<0.001 \); VEGF versus saline, \( P<0.005 \)). Fluorescence-activated cell sorter analysis disclosed an overall increase of up to 30-fold in endothelial lineage markers KDR (VEGF receptor-2), VE-cadherin, CD34, \( \alpha_\beta_\text{il} \), and E-selectin after VEGF gene transfer. Constitutive overexpression of VEGF in patients with limb ischemia augments the population of circulating EPCs. These findings support the notion that neovascularization of human ischemic tissues after angiogenic growth factor therapy is not limited to angiogenesis but involves circulating endothelial precursors that may home to ischemic foci and differentiate in situ through a process of vasculogenesis. (Circ Res. 2000;86:1198-1202.)

**Key Words:** vascular endothelial growth factor ■ gene therapy ■ endothelial progenitor cells

Recent investigations have established the feasibility of using recombinant formulations or gene transfer of angiogenic growth factors to expedite and augment collateral artery development in patients with tissue ischemia.\(^1-4\) Such postnatal neovascularization was initially considered synonymous with proliferation and migration of preexisting, fully differentiated endothelial cells (ECs) resident within parent vessels, ie, angiogenesis.\(^5,6\) The demonstration, however, of postnatal circulating bone marrow–derived endothelial progenitor cells (EPCs) that may home to sites of neovascularization and differentiate into ECs in situ is consistent with “vasculogenesis,”\(^7\) a critical paradigm for establishment of the primordial vascular network in the embryo. Although the proportional contributions of angiogenesis and vasculogenesis to neovascularization of adult organisms remain to be clarified, the notion that growth and development of new blood vessels in the adult is not restricted to angiogenesis but encompasses both embryonic mechanisms has now been verified by several laboratories.\(^8-10\)

Among the mechanisms that may modulate the contribution of vasculogenesis to postnatal neovascularization, we considered that certain angiogenic growth factors, which are acknowledged to promote both angiogenesis and vasculogenesis in the embryo\(^11\) but have been assumed to promote neovascularization exclusively by angiogenesis in the adult,\(^12-14\) may in fact promote migration, proliferation, and mobilization of EPCs from adult bone marrow. Indeed, investigations performed in our laboratory using a bone marrow transplant model established that vascular endothelial growth factor (VEGF) may mobilize EPCs from murine bone marrow, resulting in augmented neovascularization.\(^15\)

Accordingly, the current study was designed to test the hypothesis that intramuscular gene transfer of naked plasmid DNA encoding human VEGF (phVEGF<sub>165</sub>) may increase the number of circulating human EPCs in patients with critical limb ischemia.

**Materials and Methods**

**Study Subjects**

The potential for VEGF to enhance the population of circulating EPCs was serially monitored in 20 patients (11 women and 9 men, age 59±16 years) undergoing intramuscular phVEGF<sub>165</sub> gene trans-
for critical limb ischemia. The same studies were performed on 9 patients who did not receive phVEGFind gene therapy. Four of these were healthy volunteers (4 men) ranging in age from 30 to 43 years (36±3), who were injected with normal saline. The remaining 5 patients included 2 women and 3 men with critical limb ischemia ages 48 to 78 years (67±12), who had been randomly assigned to receive a control (empty) vector.

**Intramuscular phVEGF165 Transfer**

The patients undergoing gene therapy received the eukaryotic expression vector pUC118 encoding VEGF165 transcriptionally regulated by the cytomegalovirus promoter/enhancer. A total of 4000 μg of DNA in 8 aliquots of 2.5 mL of sterile saline was administered at different sites into the ischemic limb by direct intramuscular injection.

**Plasma VEGF Levels**

Plasma levels of VEGF were measured by an ELISA assay in patients before intramuscular injection and weekly up to 4 weeks after the initial set of injections.3

**Isolation of Mononuclear Cells (MNCs)**

Blood samples were obtained from all individuals before and, weekly, up to 4 weeks after intramuscular injections. Peripheral blood MNCs were isolated in a cell preparation tube by density gradient centrifugation.

**EPC Culture Assay**

The culture system used in our laboratory to quantify circulating EPCs has been described elsewhere.15 MNCs from 500 μL of peripheral blood were cultured in EC basal medium-2 supplemented with endothelial cell growth medium (EGM-2) microvascular (MV) Single Quots. After 4 days, fluorescence staining of adherent cells was used to detect the binding of Ulex europaeus agglutinin I (UEA-1) and the uptake of acetylated LDL (acLDL). Dual-staining cells positive for both FITC-labeled UEA-1 and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)–labeled acLDL were judged as EPCs and counted per well.

**Flow Cytometry Analysis**

A total of 2 to 3×10^6 cells were incubated for 30 minutes at 4°C with monoclonal antibodies prepared against KDR, VE-cadherin, CD62E (E-selectin), CD51/61 (α,β), CD31, CD34, and CD14. Quantitative fluorescence-activated cell sorter (FACS) analysis was performed on a FACStar flow cytometer.

**Statistical Analysis**

All results are expressed as mean±SEM. Statistical significance was evaluated using unpaired Student t test and ANOVA. A value of *P*<0.05 was interpreted to denote statistical significance. The relationship between variables was determined by linear regression analysis.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

To evaluate the effect of VEGF on EPC kinetics, we obtained 100 samples of peripheral blood from patients with critical limb ischemia undergoing VEGF165 gene transfer. A total of 25 samples were obtained from patients in whom injection of empty DNA vector was performed. An additional 20 samples were obtained from healthy individuals who received saline injections.

**Transgene Expression After VEGF Gene Therapy**

Plasma levels of VEGF transiently increased in all patients after gene transfer. A mean 2.1±0.3-fold increase over baseline at day 7 (*P*<0.001) was followed by persistent elevation at day 14 (1.9±0.3-fold, *P*<0.001), day 21 (1.8±0.2-fold, *P*<0.01), and day 28 (1.5±0.2-fold, *P*<0.05, all versus baseline). The mean plasma VEGF concentrations increased from 33.1±5 pg/mL at baseline to a maximum value of 98.8±17 pg/mL after gene transfer (*P*<0.01). In contrast, no significant changes were documented in the control patients (Figure 1).

**EPC Culture Assay After phVEGF165 Gene Therapy**

Two independent criteria were used to assess the effect of phVEGF165 gene transfer on EPC kinetics. First, we applied a previously described13 culture assay in which EPCs were quantified by identification of cultured cells demonstrating both UEA-1 reactivity and uptake of acLDL (Figure 2). The impact of VEGF on circulating EPCs could be detected by culture assay from day 7 (80% increase versus baseline, *P*<0.0005) through day 14 (154% increase versus baseline, *P*<0.0005), day 21 (82% increase versus baseline, *P*<0.05), and day 28 after treatment (153% increase versus baseline, *P*<0.005). The increase in EPCs correlated with the rise in VEGF plasma levels (*R*^2^=0.83; *P*<0.0001) (Figure 1B online; available at http://www.circresaha.org). Patients injected with empty vector had comparable numbers of EPCs at baseline but failed to exhibit a significant change in cultured EPCs. The number of cultured EPCs was significantly different between the VEGF-treated patients and the control vector–injected group as early as day 7 (*P*<0.001) and over the following 3 weeks (day 14, *P*<0.005; day 21, *P*<0.05;
day 28, \(P<0.005\). Likewise, the saline-injected group showed no significant change in EPC counts over 4 weeks after gene transfer. There was no significant difference in EPC count between the saline- and empty vector–treated groups (Figure 1).

**FACS Analysis**

FACS analysis was used as a second independent measure to quantify the population of EPCs mobilized in response to VEGF gene transfer. Overall, we observed an average increase in the expression levels of the EC-specific antigens KDR (22.1±1.5-fold versus baseline, \(P<0.005\)), VE-cadherin (26±2-fold versus baseline, \(P<0.001\)), and CD34 (8±1.5-fold versus baseline, \(P<0.01\)). The number of KDR- and VE-cadherin–positive cells increased significantly over baseline values (KDR, 5.6±0.8×10^3/mL; VE-cadherin, 4.5±1.2×10^3/mL) as early as day 7 (KDR, 131.2±36 10^3/mL \([P<0.005]\); VE-cadherin, 134±45×10^3/mL \([P<0.005]\)) and continued to be elevated over the entire observation period (day 21, KDR, 112±35 10^3/mL \([P<0.01]\), and VE-cadherin, 103±19×10^3/mL \([P<0.0005]\); day 28, KDR, 148.9±48 10^3/mL \([P<0.05]\), and VE-cadherin, 132.6±38×10^3/mL \([P<0.02]\)) (Figure 3). In contrast, no significant changes were observed in the group injected with empty plasmid or in the group of healthy volunteers injected with saline. Likewise, the number of CD14-positive cells remained unchanged in all 3 study groups (data not shown). Representative of the measurements taken at the aforementioned time points, Figure 4 shows the expression values at day 14. The increase over baseline in VEGF plasma levels and in VE-cadherin–positive cells showed a high correlation at this time point (\(R^2=0.82\); \(P<0.0001\)) (Figure 1C online; available at http://www.circresaha.org). Findings in the VEGF-treated group differed significantly from both control groups.

To determine the relationship between the culture assay and the FACS analysis, we compared the individual increases in both assays at a representative time point (day 14), using the rise in the EPC count and in the number of VE-cadherin positive cells. The results revealed a positive correlation (\(R^2=0.77\); \(P<0.0005\)) (Figure 1A online; available at http://www.circresaha.org).

**Expression of EC Adhesion Molecules**

We also examined the effect of VEGF gene transfer on the expression of EC adhesion molecules. In patients subjected to VEGF gene transfer, the number of circulating MNCs with the surface expression of \(\alpha_v\beta_3\) (CD51/61) and E-selectin (CD62E) increased during the observation period on average 10^3/mL \([P<0.01]\), and VE-cadherin, 105±26×10^3/mL \([P<0.005]\); day 28, KDR, 148.9±48 10^3/mL \([P<0.05]\), and VE-cadherin, 132.6±38×10^3/mL \([P<0.02]\)) (Figure 3). In contrast, no significant changes were observed in the group injected with empty plasmid or in the group of healthy volunteers injected with saline. Likewise, the number of CD14-positive cells remained unchanged in all 3 study groups (data not shown). Representative of the measurements taken at the aforementioned time points, Figure 4 shows the expression values at day 14. The increase over baseline in VEGF plasma levels and in VE-cadherin–positive cells showed a high correlation at this time point (\(R^2=0.82\); \(P<0.0001\)) (Figure 1C online; available at http://www.circresaha.org). Findings in the VEGF-treated group differed significantly from both control groups.

To determine the relationship between the culture assay and the FACS analysis, we compared the individual increases in both assays at a representative time point (day 14), using the rise in the EPC count and in the number of VE-cadherin positive cells. The results revealed a positive correlation (\(R^2=0.77\); \(P<0.0005\)) (Figure 1A online; available at http://www.circresaha.org).

We also examined the effect of VEGF gene transfer on the expression of EC adhesion molecules. In patients subjected to VEGF gene transfer, the number of circulating MNCs with the surface expression of \(\alpha_v\beta_3\) (CD51/61) and E-selectin (CD62E) increased during the observation period on average...
by 5±1-fold and 25±1-fold, respectively. In contrast, expression values in individuals receiving empty vector and saline treatment remained unchanged. The expression pattern and levels of E-selectin were very similar to those of VE-cadherin and KDR, with maximal values at day 7 and day 28 after the treatment, whereas the levels for α,β were slightly different with a maximal expression at day 14. At the representative time point, day 14, levels of α,β, and E-selectin expression in the VEGF-treated group increased significantly compared with baseline (for α,β [CD51/61], 16.7±6 versus 1.9±0.7×10^3/mL [P<0.05], and for E-selectin [CD62E], 101±37 versus 3.6±1×10^3/mL [P<0.02]) and compared with both control groups (for α,β [CD51/61], VEGF versus empty plasmid, P<0.05, and VEGF versus saline, P<0.05; for E-selectin [CD62E], VEGF versus empty plasmid, P<0.02, and VEGF versus saline, P<0.02) (Figure 4).

**Discussion**

Preclinical studies in animal models and early studies performed in small numbers of patients with lower limb ischemia and myocardial ischemia support the notion that gene transfer of VEGF DNA may promote neovascularization of ischemic tissues. These previous reports established that direct injection of phVEGF into muscle of the ischemic limb, as well as into ischemic myocardium, transiently elevates plasma VEGF levels in the systemic circulation, a finding that is confirmed in the patients described above.

The current series of patients further establishes that the rise in plasma levels of VEGF is associated with modulation of EPC kinetics after VEGF gene transfer. The increase in EPCs was statistically significant as early as 1 week after gene transfer and remained statistically significant at 2, 3, and 4 weeks follow-up. By comparison, EPC kinetics in the control subjects—including patients with or without critical limb ischemia, injected with empty vector or saline—were unchanged.

Because of limitations in the types of analyses that may be performed in human subjects, the origin and fate of the augmented population of circulating EPCs in these patients must be inferred from experiments performed previously in live animal models. Daily intraperitoneal injection of recombinant human VEGF (rhVEGF) to C57BL/6J mice for 1 week increased the total number of circulating EPCs. These effects were abrogated by coincidental application of a neutralizing antibody prepared against rhVEGF.

When mice were pretreated with rhVEGF or control buffer for 7 days before cornea micropocket injury and then examined on day 7 after injury (ie, 7 days after the last dose of rhVEGF), in situ BS-1 lectin staining disclosed enhanced corneal neovascularization in the rhVEGF group compared with controls. These findings were reproduced in mice receiving bone marrow transplanted from transgenic mice constitutively expressing β-galactosidase encoded by lacZ under the transcriptional regulation of an EC-specific gene, tie-2, to establish direct evidence for incorporation of bone marrow–derived EPCs into capillaries and stromal tissue of the corneal neovascularization.

Like fully differentiated ECs, EPCs express specific endothelial antigens, including KDR (VEGF receptor-2), CD34, and VE-cadherin. Although KDR and VE-cadherin are generally considered to distinguish EPCs from hematopoietic stem cells, there exists no epitope of which the expression is restricted exclusively to EPCs versus fully differentiated ECs. There is, however, evidence that EPCs constitute the preponderance of such circulating, bone marrow–derived endothelial lineage cells. First, the present work indicates that the population of circulating EPCs in normal individuals (3 to 5×10^3/mL) far exceeds the number of differentiated ECs circulating in peripheral blood (2 to 3/mL). Second, animal experiments from our own laboratory have suggested that the majority of the cellular population mobilized into the circulation and then incorporated into neovascular foci after VEGF administration is most consistent with bone marrow–derived EPCs.

These clinical findings call into question certain fundamental concepts regarding the mechanisms by which VEGF promotes blood vessel growth and development in adult organisms. The role of VEGF in postnatal neovascularization has been previously considered synonymous with proliferation and migration of preexisting, fully differentiated ECs resident within parent vessels, ie, sprout formation or angiogenesis. The finding that VEGF augments the number of circulating EPCs in human patients, together with the aforementioned murine experiments, implies that its impact on postnatal neovascularization is the combined result of vasculogenesis as well as angiogenesis. The proportional contributions of angiogenesis and vasculogenesis to postnatal neovascularization, including the extent to which each is influenced by VEGF, remain to be clarified.

Finally, these findings have implications for the use of naked DNA in human gene therapy. Earlier studies suggested that the low transfection efficiency associated with the use of naked DNA might make it unsuitable for therapeutic appli-
cations in trials of human gene therapy. Subsequent experience in live animal models, however, demonstrated that transfer of genes encoding for secreted proteins, such as VEGF, could yield important biological effects due to the paracrine effects of the secreted gene product. The current demonstration that VEGF gene therapy augments the compartment of circulating EPCs constitutes further evidence that gene transfer of naked DNA may indeed be sufficient to modulate the biology of human subjects.

Acknowledgments
This work was supported in part by NIH Grants HL53354, HL57516, and HL60911 (to J.M.I.) and by a grant from Cologne Fortune Program, Cologne, Germany (to C.K.).

References
MATERIAL AND METHODS

Study subjects

The potential for VEGF to enhance the population of circulating EPCs was serially monitored in 20 patients (11 women and 9 men, age range 59±16 years) undergoing intramuscular phVEGF_{165} gene transfer for critical limb ischemia according to a protocol approved by the Human Institutional Review Board and Institutional Biosafety Committee of St. Elizabeth’s Medical Center, the Recombinant DNA Advisory Committee of the National Institutes of Health, and the U.S. Food and Drug Administration. These 20 patients satisfied eligibility criteria described previously \(^1\), including a) critical limb ischemia (rest pain and/or nonhealing ischemic ulcers) present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies, and b) no option for surgical or percutaneous revascularization. The patients ranged in age from 23 to 82 years (m±SEM= 60±34), and included 11 women and 9 men.

To determine the extent to which circulating EPCs might increase or decrease at random in human subjects, the same studies performed in patients undergoing phVEGF_{165} gene transfer were performed at the same serial timepoints on 9 patients who did not receive phVEGF_{165} gene therapy. Four of these patients were healthy volunteers (4 men) from our research laboratory ranging in age from 30 to 43 years (m±SEM=36±3); each of these four subjects was injected with normal saline. The remaining 5 patients included two women and 3 men with critical limb ischemia aged 48 to 78 (m±SEM= 67±12) who fulfilled the inclusion criteria described above, but who had been randomly assigned to a sequence of lower extremity intramuscular injections with a control (empty) vector as part of a parallel gene therapy investigation.
Intramuscular phVEGF$_{165}$ transfer

The 20 patients undergoing gene therapy received the eukaryotic expression vector pUC118 encoding VEGF$_{165}$ transcriptionally regulated by the cytomegalovirus promoter/enhancer. Preparation and purification of the plasmid from cultures of phVEGF$_{165}$-transformed E. coli were performed in the Human Gene Therapy Laboratory at St. Elizabeth's Medical Center using the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses. Aliquots of 500 µg of DNA were each diluted in 2.5ml sterile saline, and 8 aliquots (total 4000 µg/20 ml) were administered via a 27 gauge needle into the calf and/or distal thigh muscles of ischemic limb by direct intramuscular injection. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The 9 control subjects underwent an identical series of injections of either saline (four healthy volunteers) or empty vector diluted in saline (5 randomized patients).

Plasma VEGF levels

Plasma levels of VEGF were measured by an ELISA assay (R&D Systems) in patients prior to intramuscular injection (baseline) and weekly up to 4 weeks after the initial set of injections to detect evidence of gene expression at the protein level. Samples were stored as previously described$^1$. Results were compared to a standard curve constructed with rhVEGF. The lower limit of detection was 5 pg/ml. Samples were checked by serial dilution and were performed at least in duplicate.

Isolation of mononuclear cells

Blood samples were obtained from all individuals before and weekly up to 4 weeks following intramuscular injections. Approximately 6 ml of each blood sample was collected in a CPT-tube (Becton-Dickinson) and total peripheral blood mononuclear cells (MNCs) were isolated by density gradient centrifugation following the manufacturer’s protocol. Light density mononuclear cells were harvested, washed twice by Dublecco’s phosphate buffered saline (PBS,
no calcium or magnesium, BioWhittaker, Walkersville, MD) supplemented with 5mM EDTA (DPBS-E). Contaminating red blood cells were hemolyzed using ammonium chloride solution (Stem Cell Technologies). Viable cells were counted in a hemocytometer after Trypan Blue 0.4% (Gibco, Grand Island, NY) staining.

**Endothelial progenitor cell culture assay**

The culture system employed in our laboratory to quantify circulating EPCs has been described elsewhere. Immediately following isolation, MNCs from 500 µl peripheral blood (typically 7 x 10^5 to 1.4 x 10^6 cells) were plated on 4-well glass slides coated with human fibronectin (Sigma, St. Louis, MO) and maintained in endothelial cell basal medium-2 (EBM®-2) (Clonetics, San Diego, CA). The media was supplemented with EGM®-2 MV Single Quots® (Clonetics) including 5% fetal bovine serum (FBS), hEGF 0.5 ml, VEGF 0.5 ml, hFGF-B 2 ml, R3-IGF-1 0.5 ml and ascorbic acid 0.5 ml. After 4 days in culture, nonadherent cells were removed by thorough washing with PBS and adherent cells underwent cytochemical analysis. Direct chemical fluorescence staining was used to detect the binding of fluorescein isothiocyanate (FITC)-labeled Ulex europaeus (Sigma), and the uptake of acetylated low-density lipoprotein labeled with DiI (acLDL-DiI, Biomedical Technologies, Stoughton, MA) at a concentration of 10 µg/ml. After the staining procedures, the samples were viewed with an inverted fluorescence microscope (Nikon, Tokyo, Japan). Dual fluorescent staining cells positive for both FITC-labeled Ulex and acLDL-DiI (double positive cells) were judged as EPCs. Two independent investigators evaluated the number of EPCs per well by counting 20 randomly selected fields. The mean value of this calculation was then expressed as cells/mm².

**Flow cytometry analysis**

Approximately 5x10^6 cells were immediately fixed in 1% paraformaldehyde (PFA) and stored at 4°C in PBS in preparation for FACS analysis (see below). In 3 cases, flow cytometry
was performed without fixation to exclude effects of PFA on subsequent analyses. A total of 2 to 3x10^5 cells in PBS with 10% FBS were incubated for 30 min at 4°C with monoclonal antibodies (MoAb) prepared against human KDR (Sigma), and human VE-cadherin (clone BV 6, mouse IgG2a, gift from E. Dejana); the biotinylated MoAb against human CD62E (E-selectin, clone 68-5H11); the FITC-MoAb prepared against human CD51/61 (α,β3, clone 23C6, Pharmingen, CA); and the phycoerythrin (PE) conjugated MoAbs prepared against human CD31 (clone L133.1), human CD34 (clone 8G12), and human CD14 (clone ΦΦ9, mouse IgG2b, all from Becton-Dickinson). Unless otherwise stated, antibodies were mouse IgG1 isotype. Isotype-identical antibodies served as controls (IgG1 and IgG2, FITC/PE-conjugated, Pharmingen, CA). For analysis of KDR and VE-cadherin, cells were further incubated with a biotinylated anti-mouse IgG (H+L) antibody (Vector Laboratories, Burlingame, CA) and with FITC conjugated streptavidin, both for 30 additional min. Quantitative FACS analysis was performed on a FACStar flow cytometer (Becton Dickinson). Histograms of cell number versus logarithmic fluorescence intensity were recorded for 10,000 to 20,000 cells per sample. HUVECs and human dermal fibroblasts were used as positive and negative controls respectively. Both cell types were detached with cell dissociation buffer (Gibco, Grand Islands, N.Y.) and treated as described above.

**Statistical analysis**

All results are expressed as mean ± standard error (m±SEM). Statistical significance was evaluated using unpaired Student's t test for comparisons between two means, and using ANOVA for comparisons among 3 or more groups. A value of p<0.05 was interpreted to denote statistical significance. The relationship between variables was determined by linear regression analysis.
REFERENCES


Online-Only Supplementary Information:

Fig. 1 Online. Correlation between the rise in VEGF plasma levels and the increase in EPCs determined by the culture assay and FACS analysis.

A. The increase in the number of MNCs expressing the EC-specific antigen VE-cadherin measured by FACS analysis and in the number of EPCs determined by the culture assay at a representative timepoint, day 14 after VEGF gene transfer, correlated with a $R^2=0.77$ (p<0.0005). Evaluation between the increase of VEGF plasma levels over baseline versus the increase of EPCs in the culture assay (B) and the increase of VE-cadherin positive cells (C), respectively, revealed a high correlation (ELISA vs EPC culture assay, $R^2=0.83$, p<0.0001; ELISA vs FACS analysis: $R^2=0.82$, p<0.0001).
Figure 1 Online

**A**

FACS analysis: increase of VE-cadherin positive cells over baseline

\[ y = 25.194x - 30.443 \]

\[ R^2 = 0.7722 \]

EPC culture assay: increase of EPC number over baseline

**B**

ELISA: increase of VEGF plasma levels over baseline

\[ y = 0.8517x - 0.1406 \]

\[ R^2 = 0.8303 \]

EPC culture assay: increase of EPC number over baseline

**C**

ELISA: increase of VEGF plasma levels over baseline

\[ y = 0.0599x + 0.5676 \]

\[ R^2 = 0.819 \]

FACS analysis: increase of VE-cadherin positive cells over baseline