Diverse effects of vascular endothelial growth factor on human pulmonary endothelial barrier and migration

T. Mirzapoiazova, I. Kolosova, P. V. Usatyuk, V. Natarajan, and A. D. Verin

Department of Medicine, the University of Chicago, Illinois

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Mirzapoiazova, T., I. Kolosova, P. V. Usatyuk, V. Natarajan, and A. D. Verin. Diverse effects of vascular endothelial growth factor on human pulmonary endothelial barrier and migration. Am J Physiol Lung Cell Mol Physiol 291: L718–L724, 2006. First published May 5, 2006; doi:10.1152/ajplung.0014.2006.—Increased endothelial permeability is involved in the pathogenesis of many cardiovascular and pulmonary diseases. Vascular endothelial growth factor (VEGF) is a permeability-increasing cytokine. At the same time, VEGF is known to have a beneficial effect on endothelial cells (EC), increasing their survival. Pulmonary endothelium, particularly, may be exposed to higher VEGF concentrations, since the VEGF level is the highest in the lungs than in any other organ. The purpose of this work was to evaluate the effects of VEGF on barrier function and motility of cultured human pulmonary EC. Using transendothelial resistance measurements as an indicator of permeability, we found that 10 ng/ml VEGF significantly improved barrier properties of cultured human pulmonary artery EC (118.6 ± 0.6% compared with 100% control, P < 0.001). In contrast, challenge with 100 ng/ml VEGF decreased endothelial barrier (71.6 ± 1.0% compared with 100% control, P < 0.001) and caused disruption of adherens junctions. VEGF at both concentrations increased cellular migration; however, 10 ng/ml VEGF had a significantly stronger effect. VEGF caused a dose-dependent increase in intracellular Ca2+ concentration; however, phosphorylation of myosin light chain was detectably elevated only after treatment with 100 ng/ml. In contrast, 10 ng/ml but not 100 ng/ml VEGF caused a significant increase in intracellular cAMP (known barrier-protective stimulus) compared with nonstimulated cells (1,096 ± 157 and 610 ± 86 fmol/mg, respectively; P < 0.024). Y576-specific phosphorylation of focal adhesion kinase was also stimulated by 10 ng/ml VEGF. Our data suggest that, depending on its concentration, VEGF may cause diverse effects on pulmonary endothelial permeability via different signaling pathways.

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is a potent endothelial cell mitogen and plays a critical role in angiogenesis and differentiation (13). VEGF was originally described as a tumor-derived vascular permeability factor that caused vascular leakage in skin tests (35). Increased vascular permeability in response to exogenous VEGF administration has also been reported in muscle (32) and gastrointestinal tract (37). Vascular permeability is defined mainly by the properties of an endothelial cell monolayer, which provides a barrier between blood and surrounding tissue. The mechanisms by which VEGF induces vascular permeability are not completely clear. Many studies using cultured endothelial cells (EC) from different sources demonstrated direct effects of VEGF on endothelial barrier function (4, 7, 9, 10, 24, 25, 32, 37, 45). Nevertheless, the role of VEGF in control of endothelial barrier in vivo is not completely understood.

Lung endothelium is of a particular interest with respect to VEGF-related regulation, since expression of the VEGF level is the highest, of all organs, in the lungs (6, 29, 36). VEGF production, however, is isolated from pulmonary endothelium and compartmentalized to the surface of alveolar epithelium. Kaner and Crystal (20) showed that human respiratory epithelial lining fluid contains 11 ± 5 ng/ml VEGF, a 500-fold greater concentration than plasma (22 ± 10 pg/ml). The authors speculate that this high level of VEGF protein on the respiratory epithelial surface plays a role in normal lung endothelial biology when slowly diffused through the alveolar epithelial barrier. However, VEGF may induce high lung endothelial permeability under conditions where the integrity of the epithelial barrier is disturbed. Until recently, VEGF was considered to be a factor contributing to the development of pulmonary edema by enhancing endothelial permeability during acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). It is evident, however, that the role of VEGF in ALI/ARDS is at least controversial (31). Clinical and animal data demonstrate that, in some cases, ALI is actually associated with a decreased level of VEGF in the lung (1, 26), while plasma VEGF becomes elevated in patients with ARDS (39). The accurate measurement of VEGF concentration at EC surface is impossible in clinical and animal settings. Surprisingly, there are only few existing studies on the mechanisms of VEGF action on cultured EC from pulmonary sources, none of them human (4, 19). The purpose of our work was to evaluate the effects of VEGF on functions of cultured human pulmonary EC. Two physiologically important properties, barrier function and migration, were studied. Both of them are interrelated and defined by actin cytoskeleton and cell–cell contacts.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant VEGF-165 was purchased from R&D Systems (Minneapolis, MN). The following antibodies were used: mouse monoclonal anti-FAK antibody (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-FAK [Y576] phosphospecific antibody (BioSource International, Camarillo, CA), rabbit polyclonal anti-ppMLC antibody (Cell Signaling Tech., Beverly, MA), mouse monoclonal anti-myosin (light chain 20K) antibody (Sigma, St. Louis, MO), rabbit polyclonal anti-VE-cadherin antibody (Cayman Chemical, Ann Arbor, MI), horseradish peroxidase (HRP)-linked anti-mouse and rabbit IgG antibodies and HRP Western blot detection kit (Cell Signaling, Beverly, MA). Fura-2 AM (cell permeate) was obtained from Molecular Probes (Eugene, OR).

Cell culture. Human pulmonary artery EC (HPAEC) were obtained from Clonetics (Walkersville, MD) and were used at passages 6–9. EC were grown in “EC growth medium,” consisting of EBM-2...
medium (Clonetics) with 10% FBS, 1% antibiotic-antimycotic mixture (K. C. Biologicals, Lenexa, KS), and 0.1% EC growth supplement (Collaborative Research, Bedford, MA).

**Immunofluorescent microscopy.** EC grown on glass coverslips were fixed in cold acetone for 10 min at 4°C and washed three times with PBS. The cells were permeabilized with 0.2% Triton X-100 in PBS supplemented with 0.1% Tween 20 for 5 min, washed three times with PBS, and blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 2% BSA (blocking buffer) for 30 min. Cells were thoroughly rinsed with TBST (3 times) and incubated with the appropriate primary antibody for 1 h and then with Alexa 488-labeled secondary antibody for 1 h at room temperature. Actin microfilaments were stained with Texas Red-labeled phalloidin for 1 h at room temperature. The cover slips were mounted and analyzed with the use of a Nikon Eclipse TE 300 inverted fluorescent microscope with a ×60 oil objective and a Hamamatsu digital camera.

**Measurement of transendothelial electrical resistance.** The cellular barrier properties were monitored using the highly sensitive biophysical assay with an electrical cell substrate impedance-sensing system (ECIS) (In Vitro Diagnostics, Troy, NY), described previously (15, 16, 41). Cells were cultured on small gold electrodes (10 mm², H9262) on a collagen-coated polycarbonate membrane. Migration cells on the bottom surface were stained with Diff-Quik set (EM Science, Gibbstown, NJ). The average number of cells per field was counted at ×20 magnification from five adjacent microscopic fields. In different sets of experiments, the number of the cells after 12-h migration was analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software to count total events.

**Cell migration assay using a wound-healing model.** HPAEC were subjected to scratched wound injury with a 200-μl pipet. Cells were washed with PBS and incubated in fresh Opti-MEM medium (Invitrogen) with 10 or 100 ng/ml VEGF. The original wound edge was determined by taking photographs after the cells were wounded. Cells that moved from the original wound edge within 18 h were counted by taking photographs of the same fields as the original wound. For each wound, the number of migrating cells was calculated from seven photographs of different fields.

**Measurement of intracellular Ca²⁺.** HPAEC were plated on glass coverslips (Hitachi Instruments) and grown to ~95% confluence, and intracellular Ca²⁺ concentration was monitored in the basic medium (in mM: 116 NaCl, 5.37 KCl, 26.2 NaHCO₃, 1.8 CaCl₂, 0.81 MgSO₄, 1.02 NaHPO₄, 5.5 glucose, and 10 HEPES-HCl, pH 7.4) as described earlier (42). Briefly, cells were loaded with 5 μM Fura-2 AM for 15 min at 37°C in 5% CO₂-95% air, rinsed twice with the above medium, and inserted into a 1.0-cm acrylic cuvette (Sarstedt, Newton, NC) filled with 3 ml of incubation media at 37°C. Fura-2 fluorescence was measured with an Aminco-Bowman Series 2 luminescence spectrophotometer (SLM/Aminco, Urbana, IL) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm with different concentrations of VEGF. Intracellular free calcium concentration ([Ca²⁺]; in nM) was calculated from the 340/380 ratio using the manufacturer’s software.

**Measurement of intracellular cAMP.** Cells were grown to subconfluent monolayer and stimulated with 10 and 100 ng/ml VEGF for 30 min. cAMP concentration in total EC lysates was determined with a Biotrak cAMP enzyme immunoassay system (Amersham, Piscataway, NJ) according to the instructions provided by the manufacturer.

**Cellular membrane preparation.** HPAEC were grown to confluence in 100-mm dishes and starved in M199 medium with 0.05% BSA for 1 h after stimulation with 10 or 100 ng/ml VEGF. The membrane fraction was isolated according to the method of Song et al. (38). Monolayers of HPAEC were washed with cold PBS and scraped into cold homogenization buffer (HB) containing 20 mM Tris·HCl (pH 7.4), 4 mM EDTA, 2 mM EGTA, 10% glycerol and protease inhibitor cocktail set III (1:200). All preparation steps were carried out at 4°C. The cells were lysed by sonication. The nuclei and cell debris were removed from homogenates by centrifugation at 900 g for 10 min. The resulting supernatant was ultracentrifuged at 100,000 g for 75 min. The membrane pellet was resuspended in HB with 1% Triton X-100 and incubated on ice for 30 min. Insoluble material was removed by centrifugation at 14,000 g for 10 min, while supernatant was designated as a membrane fraction. Total protein was measured with BCA protein assay kit (Pierce, Rockford, IL).

**Measurement of transendothelial electrical resistance.** Resistance was expressed as normalized resistance, and values from each microelectrode were pooled at discrete time points, calculated by the ECIS software program, and plotted as means ± SE.

**Transwell migration assay.** Transwell migration assay was carried out as described before (28). Costar transwell cell migration plates (Costar, Cambridge, MA) were used for this assay. The polycarbonate membranes with 8-μm pore size were covered with 0.2% gelatin. The experimental conditions were described in MATERIALS AND METHODS. Each error bar represents pooled values from 3 independent experiments. HPAEC were treated with vehicle or VEGF (10 or 100 ng/ml, as indicated), and TER was constantly monitored. Moment of VEGF addition was designated as zero. A: representative recordings of absolute values of electrical resistance. B: TER recording presented as normalized data (described in MATERIALS AND METHODS). Each error bar represents pooled values from 3 experiments (n = 6). *Time points at which the values become significantly different from vehicle control (P < 0.005). Maximal TER increase caused by 10 ng/ml VEGF (at 40 min) is 318 ± 6% compared with control (P < 0.001); maximal TER decrease caused by 100 ng/ml VEGF (at 160 min) is 71.6 ± 1.0% compared with control (P < 0.001).
protein assay reagent kit (Pierce, Rockford, IL). Membranes were resuspended in Laemmli buffer.

Statistics. For basic statistical analysis, the GraphPad Prism program was used. Data were compared by analysis of variance (ANOVA). *P* values of <0.05 were considered significant. Values are expressed as means ± SE. Experiments were repeated several times, as indicated in figures.

RESULTS

Effect of VEGF on EC barrier properties, cytoskeletal rearrangements, and migration. The TER was affected by VEGF in a concentration-dependent fashion. As shown in Fig. 1, treatment with 10 ng/ml VEGF caused sustained TER increase, indicating enhancement of endothelial barrier. At 100 ng/ml, VEGF caused a biphasic response with a brief (10 min) increase followed by a sustained decrease, indicating barrier disruption.

Immunofluorescence staining (Fig. 2) showed a reduced amount of actin stress fibers after 10 ng/ml VEGF treatment. VE-cadherin (a major component of adherens junctions) formed a broader and more continuous pattern around the cell periphery compared with control. This rearrangement of VE-cadherin may indicate increased overlapping of neighboring cells and tightening of cell-cell contacts. In contrast, treatment

Fig. 2. Effect of VEGF on actin cytoskeleton and adherens junctions in HPAEC. Cells were treated with indicated concentrations of VEGF for 120 min and then fixed and double stained for actin and VE-cadherin, as indicated.

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with 100 ng/ml VEGF resulted in an increased amount of actin stress fibers, formation of visible paracellular gaps, and disruption of adherens junctions.

Examination of the effect of different VEGF concentrations on EC migration (Fig. 3A) demonstrates that, although VEGF at both concentrations significantly increased migration, 10 ng/ml VEGF had a more pronounced effect. VEGF effects on migration were confirmed in the scratch wound assay, again with a stronger effect at 10 ng/ml (Fig. 3B).

**Effect of VEGF on intracellular Ca^{2+} and myosin light chain phosphorylation.** Activation of store-operated Ca^{2+} entry is coupled with myosin light chain (MLC) phosphorylation and is associated with intercellular gap formation and increased permeability (30). EC contraction driven by MLC phosphorylation is a key event in several models of agonist-induced barrier dysfunction (12). It is known that VEGF increases cytosolic Ca^{2+} concentrations through both Ca^{2+} influx and Ca^{2+} release from inositol trisphosphate-sensitive stores in EC (8). Our data demonstrate that VEGF caused a dose-dependent increase in [Ca^{2+}], (Fig. 4A). Only high VEGF concentration (100 ng/ml), however, produced a noticeable increase in MLC phosphorylation (Fig. 4B). Thus the barrier-disruptive effect of 100 ng/ml VEGF may be associated with increased MLC phosphorylation. It should be noted, however, that despite a sustained decrease in TER under these conditions (Fig. 1), only transient MLC phosphorylation was detected.

![Graph A](image1)

**Fig. 3.** Effect of VEGF on EC migration. A: chemotaxis assay. HPAEC were placed into gelatin-coated transwell migration plates and treated with VEGF or left untreated for 24 h. Cells on the bottom membrane surface were stained with Diff-Quik solutions and counted in 5 fields. Data are presented as means ± SE from 4 independent experiments (*P < 0.05 compared with control, **P < 0.05 compared with 100 ng/ml). B: scratched wounding assay. HPAEC monolayers were subjected to scratched wound injury. Cell migration was initiated by VEGF. Wound area is shown before and 24 h after VEGF stimulation.
Low concentration of VEGF increases cAMP in EC. Elevation of cAMP levels and activation of PKA are known to be associated with barrier enhancement (44). We found that only 10 ng/ml but not 100 ng/ml VEGF caused a small, but significant, rise in intracellular cAMP (Fig. 5). Therefore, the barrier-protective effect of low VEGF concentration may be mediated by cAMP/PKA signaling.

Differential effect of low and high concentration of VEGF on focal adhesion kinase phosphorylation. The assembly and disassembly of focal adhesions play a key role in the mechanism by which several extracellular stimuli regulate both cell morphology and movement. Focal adhesion kinase (FAK) is known to mediate EC adhesion and migration in response to VEGF. The activity of FAK is mainly regulated through phosphorylation (33). Phosphorylation of Y576 and Y577 within the kinase domain is required for maximal FAK catalytic activity (18). As shown in Fig. 6A, Y576-specific FAK phosphorylation was induced by 10 ng/ml VEGF but was unaffected by 100 ng/ml VEGF. Cellular fractionation revealed that content of the Y576-phosphorylated form of FAK in membrane fraction was increased after 10 ng/ml but not 100 ng/ml VEGF challenge (Fig. 6B). Accordingly, strong immunostaining of phosphorylated FAK was observed after 10 ng/ml VEGF treatment (Fig. 6C).

DISCUSSION

The major finding of this study is that, depending on its concentration, VEGF exhibits opposite effects on pulmonary endothelial barrier function. VEGF at 10 ng/ml has a barrier-protective effect, whereas it disrupts the barrier at 100 ng/ml. Barrier protection was accompanied by dissolution of stress fibers, whereas barrier disruption correlated with disarrangement of cell–cell junctions. Along with increasing TER, 10 ng/ml VEGF more potently stimulated EC migration than did 100 ng/ml.

We attempted to investigate the signaling mechanisms of VEGF action that lead to such different effects on permeability. It is known that inflammatory barrier-disruptive agonists such as histamine and thrombin induce robust MLC phosphorylation at Ser19/Thr18 in pulmonary EC (14, 43). Phosphorylation of MLC is a triggering mechanism of actomyosin contraction, leading to destabilization of cell–cell junctions and paracellular gap formation (12). VEGF at 100 ng/ml caused transient MLC phosphorylation in HPAEC at 10 min after the stimulation (Fig. 5B). However, at this time, TER was not increased (Fig. 1A), and no stress fibers were formed (Fig. 2). Furthermore, disruption of adherens junctions also occurred much later (Fig. 2). Therefore, the role of MLC phosphorylation in VEGF-induced barrier disruption in pulmonary EC remains unclear. Focal adhesion remodeling may be involved in VEGF-induced signal transduction, associated with barrier enhancement and migration. We found that 10 ng/ml but not 100 ng/ml VEGF induced FAK phosphorylation at Y576, which indicates FAK activation. Similar to our result, Abedi and Zachary (2) observed a maximal increase in FAK tyrosine phosphorylation at 10 ng/ml VEGF with a subsequent decline at higher VEGF concentrations. Tyrosine phosphorylation of FAK, associated with cell spreading and migration, occurs in brain EC (3) and human umbilical vein EC (2). Furthermore, sphingosine-1 phosphate, a strong barrier-improving agent, causes Tyr576 FAK phosphorylation in EC (34).

Our results also suggest that increased intracellular cAMP after 10 ng/ml VEGF challenge may play a role in VEGF-induced barrier enhancement. Interestingly, VEGF action on EC is mediated via the receptor tyrosine kinases vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2 (46), whereas activation of adenylyl cyclase and cAMP increase are triggered by Gαs protein-coupled signaling.

![Fig. 5. Effect of VEGF on intracellular cAMP. HPAEC were treated with different concentrations of VEGF for 30 min, and then intracellular cAMP was determined as described in MATERIALS AND METHODS. Treatment with 10 ng/ml but not 100 ng/ml VEGF caused a significant increase in cAMP level (each bar represents mean ± SE; n = 3, *P < 0.05).](http://ajplung.physiology.org/)

![Fig. 4. Effect of VEGF on intracellular Ca²⁺ and myosin light chain (MLC) phosphorylation. A: VEGF increases intracellular Ca²⁺ concentration ([Ca²⁺]i) in a dose-dependent manner. Inset: each bar represents maximal mean ± SE (n = 3, *P < 0.001). B: immunoblotting, using SDS-PAGE and a diphospho-specific MLC antibody, shows a time course of MLC phosphorylation after VEGF treatment. Total MLC staining shows equal loading.](http://ajplung.physiology.org/)
There is always a question as to what are physiologically relevant concentrations of VEGF, as applied to pulmonary EC. VEGF was registered in plasma only at the level of picograms per milliliter (20). However, the local concentration at the endothelial surface is unknown. Particularly, pulmonary endothelium may be exposed to a higher VEGF concentration, since it is adjacent to alveoli, where the VEGF level reaches tens of nanograms per milliliter (20). It is possible that even at normal conditions, VEGF diffusing across epithelium constantly affects microvascular EC. Our data show that VEGF at 10 ng/ml (which is a relatively high concentration compared with plasma VEGF) has a beneficial effect on pulmonary EC barrier function. A concentration one order of magnitude higher is required to disrupt the barrier. These diverse effects may explain the controversy surrounding the role of VEGF in the control of pulmonary vascular permeability. Until recently, increased VEGF concentration in lungs was considered to be associated with lung injury. However, clinical data reveal that, depending on the type of lung injury, the level of VEGF in lung may either increase (for example, in the case of LPS- or ischemia-reperfusion-induced ALI; Refs. 22, 23) or decrease (in the case of ALI induced by bacteria; Ref. 20). Ventilation-induced lung injury seems to have no effect on VEGF level (5, 17). In most cases, it is unclear whether altered VEGF level is a primary cause of injury. Adenovirus-mediated expression of VEGF in mouse lungs caused capillary leakage and edema, suggesting that the increased concentration of VEGF may be the mechanism of increased pulmonary vascular permeability during ALI. (21). However, expression of VEGF as mediated by adenoviral gene transfer may be higher than expression under any physiological condition, including ALI. Recent animal studies and clinical data support a protective role for VEGF in ALI and ARDS patients. For instance, VEGF production by alveolar type II cells was significantly increased during recovery from hyperoxic exposures (27). During the progression of ARDS, patients with increasing levels of VEGF in epithelial lining fluid had better recovery (40). The protective effect of IL-13 against hyperoxic ALI was mediated by VEGF (11). It has been suggested that VEGF may stimulate reparative responses by inducing proliferation and survival of endothelium (46). Our data indicate that VEGF at a certain concentration may also directly promote endothelial barrier enhancement.

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REFERENCES

VEGF AND HUMAN PULMONARY ENDOTHELIAL CELLS


