VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly

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VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. Am J Physiol Heart Circ Physiol 280: H434–H440, 2001.—Tight junctions between brain microvessel endothelial cells (BMECs) maintain the blood-brain barrier. Barrier breakdown is associated with brain tumors and central nervous system diseases. Tumor cell-secreted vascular endothelial growth factor (VEGF) increases microvasculature permeability in vivo and is correlated with the induction of clinically severe brain tumor edema. Here we investigated the permeability-increasing effect and tight junction formation of VEGF. By measuring [14C]sucrose flux and transendothelial electrical resistance (TER) across BMEC monolayer cultures, we found that VEGF increased sucrose permeability and decreased TER. VEGF also caused a loss of occludin and ZO-1 from the endothelial cell junctions and changed the staining pattern of the cell boundary. Western blot analysis of BMEC lysates revealed that the level of occludin but not of ZO-1 was lowered by VEGF treatment. These results suggest that VEGF increases BMEC monolayer permeability by reducing occludin expression and disrupting ZO-1 and occludin organization, which leads to tight junction disassembly. Occludin and ZO-1 appear to be downstream effectors of the VEGF signaling pathway.

Vascular permeability factor; blood-brain barrier; ZO-1; brain microvessel endothelial cell; vascular endothelial growth factor

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VEGF AFFECTS BRAIN ENDOTHELIUM PERMEABILITY

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METHODS

Materials. MEM and Ham’s F-12 medium were purchased from Mediatech (Washington, DC), collagenase-dispase and dispase from were Boehringer-Mannheim (Mannheim, Germany), [14C]sucrose was from New England Nuclear-DuPont (Chicago, IL), recombinant human VEGF was from Prepro Tech (Rocky Hill, NJ), endothelial cell growth supplements were from Collaborative Biomedical Products (Becton-Dickinson Labware, Bedford, MA), rhodamine-phalloidin was from Molecular Probes (Eugene, OR), and rabbit antibodies to ZO-1, occludin, and actin were from Zymed (South San Francisco, CA). Monoclonal anti-tubulin antibodies were a gift from R. Himes (University of Kansas). All other reagents were purchased from Sigma (St. Louis, MO).

BMEC isolation and cell culture. BMECs were isolated from bovine brain gray matter by enzymatic digestion as described previously (2, 3) and were seeded at 50,000 cells per cm² onto 24-mm-diameter Transwell filter inserts (with 0.4-µm pores) coated with collagen and fibronectin. Cells were cultured in 45% MEM, 45% F-12 medium, 10% platelet-poor horse serum, 50 µg/ml gentamicin, 125 µg/ml heparin, and 25 µg/ml endothelial cell growth supplements. Cells were grown in a 37°C incubator with 5% CO₂ and 95% humidity. BMECs formed a monolayer by day 7 and a tight monolayer 8–9 days after plating. The formation of the monolayer and its tightness were judged by staining filamentous actin with rhodamine-phalloidin or by measuring [14C]sucrose flux across the monolayer.

Paracellular permeability for sucrose and transendothelial electrical resistance measurement. Paracellular permeability for [14C]sucrose was determined as described previously (24). The amount of radiolabeled tracer that penetrated the BMEC monolayers was expressed (%/cm²) as follows

\[ \text{1 h sucrose flux} = 100 \times \left( \frac{C_i V_i}{C_f V_f A} \right) \]

where \( C_i \) is the count of the radiotracer appearing in the receiver chamber, \( C_f \) is the count of the initial tracer in the donor chamber, \( V_i \) is the volume (ml) of the medium in the receiver chamber, \( V_f \) is the volume (ml) of the medium in the donor chamber, and \( A \) is the surface area of the filter support (4.71 cm²).

Transendothelial electrical resistance (TER) was measured using Endohm-snap (World Precision Instruments, New Haven, CT). Resistances of blank filters were subtracted from those of filters with cells before final TER values (cm²) were calculated. All experiments were carried out in triplicate. The results were expressed as means ± SD of \( n = 3 \) experiments; \( P \) values were determined via ANOVA.

Immunocytochemistry. Immunofluorescent staining of occludin, ZO-1, and tubulin was performed using a modification of the protocol described by Stevenson and colleagues (20). BMECs grown on Transwell filters were washed once with PBS containing 1 mM CaCl₂ (PBS-Ca). Cells were fixed by immersion in 95% ethanol (at −20°C) followed by incubation in ethanol at 4°C for 30 min. Cells were permeabilized in −20°C acetone for 1–2 min. Alternatively, cells were fixed for 20 min in 3.7% formaldehyde in PBS and then permeabilized in PBS with 0.1% Triton X-100 for 10 min at room temperature. Cells were blocked for 1 h with PBS-Ca containing 10% horse serum and 0.1% Tween 20. Permeabilized cells were incubated overnight at 4°C with primary antibodies. Rabbit anti-occludin antibodies were combined with PBS containing 0.2% horse serum and 0.1% Tween 20 in a 1:200 dilution, anti-ZO-1 at a 1:100 dilution, and mouse anti-tubulin at a 1:2,000 dilution. Cells were washed with PBS-Tween (PBS with 0.1% Tween 20) and stained with FITC-labeled secondary antibodies. The results were expressed as means ± SD of \( n = 3 \) experiments; \( P \) values were determined via ANOVA.

RESULTS

Development of tight junctions during BMEC culture. To study the development and maintenance of tight junctions, BMECs were stained with antibodies against the tight junction proteins occludin and ZO-1. Because actin filaments play an important role in the organization of tight junctions, actin filaments were stained with rhodamine-phalloidin (Fig. 1). Five days after seeding was completed, the BMECs were subconfluent. Actin filaments generally were organized as cytoplasmic stress fibers, although some peripheral actin filament staining was observed (Fig. 1A). Occludin accumulated in the perinuclear cytoplasm, although it also was found at the periphery of some cells (Fig. 1D). Most ZO-1 was distributed in discontinuous lines along the cell periphery near the sites of cell-to-cell junction formation (Fig. 1G).

After 7 days in culture, the BMECs reached confluence and acquired the elongated shape typical of primary cultured BMECs (2). Cytoplasmic actin filaments disappeared, and actin filaments were predominantly found at the cell periphery (Fig. 1B). Perinuclear occludin disappeared, and most occludin and ZO-1 lined
the cell boundaries (Fig. 1, E and H). After 9 days of culture (2 days after reaching confluence) all cells exhibited peripheral actin filament staining (Fig. 1C). Occludin (Fig. 1F) and ZO-1 (Fig. 1I) lined the cell boundaries and appeared as continuous lines. Therefore, ZO-1 preceded the appearance of occludin at junction sites during tight junction formation. Occludin moved from the perinuclear region to the lateral junction sites of the plasma membrane as tight junctions formed.

Alteration of the permeability and TER of BMEC monolayers by VEGF. VEGF was added to the basolateral sides of BMEC confluent monolayers, cells were cultured for an additional 2 days, and cells were then processed for sucrose permeability and TER analysis. VEGF treatment caused a twofold increase in \[^{14}C\]sucrose flux (Fig. 2A) and a greater than threefold reduction in TER (Fig. 2B). Identical molar concentrations of basic fibroblast growth factor (bFGF), which is also an endothelial growth factor but not a permeability modulator, did not significantly alter \[^{14}C\]sucrose permeability or the TER (Fig. 2A and B). Treatment of control and VEGF-treated monolayers with 1 mM EGTA for 10 min disrupted the electrical barrier and reduced the electrical resistance to values near that of naked Transwell filters (Fig. 2B). When VEGF was added to the apical sides of the monolayers, there was no significant alteration of \[^{14}C\]sucrose permeability.

Effect of VEGF on tight junction proteins occludin and ZO-1 in BMEC monolayers. To determine the structural basis for the permeability-increasing activity of VEGF, the distribution of actin filaments and microtubules was compared with that of ZO-1 and occludin. VEGF was added to the basolateral side of confluent BMEC monolayers, and cells were cultured for an additional 2 days and then fixed and processed for immunolabeling. Two-day treatments of VEGF were chosen because preliminary results revealed that it took at least 10 h of VEGF treatment to induce a change in sucrose flux. Sucrose flux peaked 1–2 days after the start of treatment.

VEGF shifted the distribution of actin filaments from the cell cortex in untreated cells (Fig. 3A) to a less-organized pattern of cytoplasmic filaments in treated cells (Fig. 3B). Microtubule distribution was not altered by VEGF (compare Fig. 3, D and E). VEGF induced the redistribution of ZO-1 and occludin from the cell periphery to the cytoplasm. ZO-1, normally found along the cell boundaries (Fig. 3G), became clustered in patchy aggregates in the cytoplasm after VEGF treatment (Fig. 3H). Similarly, occludin, which was normally distributed along the periphery of cells (Fig. 3J), was either absent or was found in faintly stained cytoplasmic aggregates (Fig. 3K). Continuous lines of occludin and ZO-1 staining found along the cell-to-cell boundaries in control cells were rarely found after VEGF treatment. Cells treated with equimolar concentrations of bFGF looked identical to control cells and did not display altered patterns of actin, ZO-1, or occludin staining (Fig. 3, C, F, I, and L).
When VEGF was added to the apical sides of confluent BMEC monolayers, there were no significant changes in the distribution of either occludin or ZO-1 (not shown). This is consistent with earlier observations that VEGF receptors are located on the basolateral sides of BMECs and that VEGF does not readily cross the cell monolayer (22). These results indicate that VEGF specifically disrupted tight junction complexes in confluent BMEC monolayers.

Effect of VEGF on expression of cytoskeletal and tight junction proteins. VEGF-treated cells were further examined for the expression of tight junction proteins. VEGF was applied to the basolateral side of confluent BMEC monolayers, and cells were cultured for an additional 2 days. Control cells were cultured identically but were not treated with VEGF. Cells were then lysed, and equal amounts of control and VEGF-treated cells were analyzed on Western blots using antibodies to actin, tubulin, occludin, and ZO-1 (Fig. 4). There were no significant differences in actin and tubulin levels in control and VEGF-treated cells (Fig. 4, A and B).

Occludin antibodies recognized several 55- to 70-kDa polypeptides in the control cells (Fig. 4C, left), which is consistent with earlier observations that occludin appears as multiple bands between 62 and 82 kDa on Western blots (15, 16). The occludin antibodies also identified three additional polypeptides of 50, 85, and 105 kDa. The 50-kDa polypeptide may be an occludin breakdown product. ZO-1 antibodies recognized the two isoforms of ZO-1 that arise from alternative RNA splicing or to tyrosine phosphorylation concomitant with tight junction protein reorganization in A-431 cells (4, 23, 25). Densitometric analysis of Western blots revealed that VEGF caused a 0.2% increase in actin (not significant), an 0.8% decrease in tubulin, a 32% decrease in occludin, and a 16% increase in ZO-1 staining. Therefore, VEGF induced no significant changes in actin and tubulin levels but VEGF significantly decreased occludin levels and led to a slight increase in ZO-1.

The levels of ZO-1, occludin, and actin were further examined via ELISA (Fig. 5). These assays confirmed that VEGF had no significant effect on ZO-1 levels, but that it did significantly lower the levels of occludin and actin.

Taken together, these data suggest that VEGF induces permeability in BMEC monolayers by altering the organization of ZO-1 and occludin and by the subsequent loss of tight junctions between BMECs.

Fig. 2. Effects of vascular endothelial growth factor (VEGF) on 
\[^{14}C\]sucrose flux and transendothelial electrical resistance (TER) across BMEC monolayers. Confluent BMEC monolayers were incubated in culture medium containing 100 ng/ml VEGF or an equimolar concentration of 50 ng/ml basic fibroblast growth factor (bFGF) added to the basolateral sides of the monolayers for 2 days before fixation. A: permeability of BMEC monolayers to \[^{14}C\]sucrose. B: TER across BMEC monolayers. C-EGTA, control monolayers treated with 1 mM EGTA 10 min before TER measurement; V-EGTA, VEGF-treated monolayers treated with 1 mM EGTA 10 min before TER measurement. Results are expressed as means ± SD; n = 3 experiments; *P < 0.01 significantly different from control.

Fig. 3. Effect of VEGF and bFGF on the distribution of occludin, ZO-1, tubulin, and actin in BMEC monolayers. BMECs were cultured for 7 days to form confluent monolayers and were maintained in medium without VEGF (A, D, G, J), with 100 ng/ml VEGF (B, E, H, K), or with 50 ng/ml bFGF (C, F, I, L) added to the basolateral sides for 2 days. Cells were then fixed and stained for actin filaments with rhodamine-phalloidin (A–C) and antibodies against tubulin (D–F), ZO-1 (G–I), or occludin (J–L). Space between two vertical bars (L) is 10 μm.
DISCUSSION

Previously, we reported that VEGF increased the permeability of cultured BMECs to [14C]sucrose and suggested that VEGF may alter tight junction assembly in the BMEC monolayers (24). Here we report that VEGF treatment of BMEC monolayers disrupted the continuous pericellular distribution of the tight junction proteins ZO-1 and occludin as well as actin filament distribution. VEGF treatment also decreased occludin levels in BMECs. The protein levels of ZO-1, actin, or tubulin were not affected. These data suggest that VEGF induces tight junction disassembly and breakdown of the endothelial permeability barrier by altering the organization of tight junction proteins.

The mechanism by which VEGF disrupts tight junctions and increases permeability is not well understood. Several studies suggest that VEGF binding to endothelial cell receptors induces receptor dimerization, which then stimulates receptor autophosphorylation and the phosphorylation of downstream signal transduction proteins (22). VEGF-activated endothelial cell receptors are known to phosphorylate several cytoplasmic proteins, including ones that contain receptor phosphotyrosine-binding Src homology 2 (SH2) domains that may be involved in signal transduction (12). These signals may affect the expression and/or modification of proteins necessary for the maintenance of tight junctions. VEGF also induces rapid and transient elevation in cytosolic calcium in several types of cultured endothelial cells (8). Elevation of calcium levels may activate calcium-calmodulin-dependent protein kinases to alter protein phosphorylation and/or affect the actin cytoskeleton, which is important in organizing adhesion junctions and tight junctions.

Tight junctions are highly dynamic structures with permeability, assembly, and disassembly characteristics that can be altered by a variety of cellular and metabolic regulators. Studies using protein kinase activators and inhibitors have revealed that protein phosphorylation plays an important role in tight junction assembly, maintenance, and function in epithelial (5–8, 21) and endothelial (14) cells. Only recently have the phosphorylation levels of the tight junction proteins ZO-1 and occludin been correlated with tight junction assembly (6, 16). The role of protein phosphorylation in the structure of BMEC tight junctions is under investigation at this time. Preliminary results reveal several changes in protein phosphorylation after VEGF treatment but identification of specific occludin and ZO-1 bands have not been made (Wang and Borchardt, unpublished data).

Sakakibara and colleagues (16) reported that mammalian occludin migrates as a series of closely spaced bands between 62 and 82 kDa and that the apparent molecular weight increases with the degree of phosphorylation. They also reported that increased levels of occludin phosphorylation are correlated with increased TER and tight junction assembly. In our study, anti-occludin antibodies stained multiple bands between 55 and 70 kDa in control BMECs (with high TER). VEGF treatment decreases the high molecular weight bands, consistent with occludin dephosphorylation, and decreases TER values, consistent with tight junction disassembly.

The 105-kDa band recognized by occludin antibodies was not found in other endothelial cells and may be a factor that helps BMECs form tighter junctions than those in other tissues. The 85-kDa band may be a
break-down product of the 105-kDa polypeptide, because VEGF treatment reduced staining of the 105-kDa band and increased staining of the 85-kDa band.

A recent study conducted by Kevil and colleagues (13) using HUVECs and [14C]albumin demonstrated that VEGF treatment increased the permeability of HUVEC monolayers to [14C]albumin and disorganized endothelial junctional proteins. The VEGF-mediated permeability increase could be blocked by the MAPK inhibitors AG-126 and PD-98059, but G-6976 and staurosporine, protein kinase C antagonists, and wortmannin, a phosphatidylinositol 3-kinase blocker, did not block the effect of VEGF on HUVECs. Their study suggested that the MAPK signal transduction pathway is involved in the VEGF-induced permeability change.

At present, we do not understand the VEGF-mediated pathways in BMECs, but in the future we will carry out a more thorough study of the role of VEGF-stimulated protein kinases to elucidate the signal transduction pathway in BMECs.

We observed that occludin levels in BMECs decreased during VEGF treatment, which suggests that VEGF either downregulates occludin synthesis or increases its degradation. Further analysis of occludin mRNA levels will help clarify the mechanism by which VEGF decreases occludin levels. VEGF did not alter the levels of ZO-1, which suggests that ZO-1 organization is regulated by other mechanisms.

Immunoblot assays of whole cell lysates revealed that actin levels were similar in control and VEGF-treated cells but that actin levels were lower in VEGF-treated cells analyzed with an ELISA assay. This is most likely due to the fixation and extraction method employed for ELISA: whole cell lysates contained both soluble and filamentous actin, but the extraction of cells necessary for ELISA likely released soluble actin and preserved the filamentous actin. These data suggest that VEGF affects the assembly but not the total amount of cellular actin and are consistent with our immunofluorescence data showing rearrangement of actin in VEGF-treated cells.

In addition to its permeability-increasing activity, VEGF is a mitogen that stimulates endothelial cell growth. We observed 10–20% increases in protein level, cell number, and [3H]thymidine incorporation in VEGF-treated cells (Wang and Borchardt, unpublished data). Light and electron microscopic analysis of the cells revealed that some cell clusters grew on top of the BMEC monolayers. Even in regions of cell clusters and layers of two or more cells, the cell layer was intact and tight junctions blocked penetration of lanthanum through the layers (Wang and Dentler, unpublished data). Cell migration and proliferation may weaken the tight junctions and increase permeability. We tried to separate the mitogenic effect of VEGF from the permeability-increasing activity by using colchicine to inhibit cell division and cytochalasins B and D to inhibit cell migration, but each of these drugs induced cells to round up which disrupted the confluent monolayers. These results reveal the importance of the actin and microtubular cytoskeleton in the maintenance of cell shape and tight junctions.

The VEGF-induced permeability increase also could be caused by an increase in endocytotic activity or in the transcellular trafficking in BMECs. In a preliminary study we found that VEGF treatment increased the uptake of the endocytotic marker Lucifer yellow by BMECs (Wang and Borchardt, unpublished data), so VEGF does increase endocytic activity. The degree of the contribution of the paracellular and transcellular pathways to the permeability increase described here remains to be determined. Although the mechanism or signal transduction pathway for the permeability-increasing effect of VEGF is not well understood, it is certain that VEGF increases the permeability of the BMEC monolayers and modifies the localization of the tight junction proteins occludin and ZO-1. We expect that an understanding of the mechanisms of VEGF-induced permeability will enable the use of VEGF and related agents to facilitate drug delivery to the brain, as well as the development of therapeutic drugs to intervene in the signal transduction pathway of VEGF for the treatment of brain and related tumors.

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