VEGF-Induced Permeability Increase Is Mediated by Caveolae

Yiyu Feng,1 Virginia J. Venema,1 Richard C. Venema,1,2 Nigel Tsai,1 M. Ali Bebzadian,1 and Ruth B. Caldwell1,3,4

PURPOSE. To determine the cellular route by which vascular endothelial cell growth factor (VEGF) increases the permeability of cultured retinal endothelial cells and to test whether nitric oxide (NO) production by NO synthase (NOS) is involved in signaling VEGF’s permeability enhancing effects.

METHODS. Cultured bovine retinal microvascular endothelial (BRE) cells were used for bioassay of permeability function and its ultrastructural correlates. The role of NOS activity in VEGF’s permeability enhancing effects was tested with the use of an NOS inhibitor. Because activity of endothelial NOS (eNOS) is thought to be regulated by its interaction with the caveolar protein caveolin-1, structural relationships between eNOS, caveolin-1, and the VEGF receptor Flk-1/KDR were analyzed with double-label immunofluorescence and cell fractionation procedures.

RESULTS. Bioassays of permeability function and structure demonstrated that VEGF increases permeability of cultured BRE cells by an NOS-dependent process of transcytotic transport in caveolae. Double-label analysis showed that Flk-1/KDR and eNOS colocalize with caveolin-1 in plasma membrane caveolae. Cell fractionation and immunoblot analysis confirmed this effect. Densitometry showed that Flk-1/KDR, eNOS, and caveolin-1 levels were highest in caveolar fractions. Similar results were obtained in studies with bovine aortic endothelial cells.

CONCLUSIONS. These results demonstrate that VEGF increases endothelial cell permeability by an eNOS-dependent mechanism of transcytosis in caveolae. Localization of Flk-1/KDR and eNOS with caveolin-1 suggests that VEGF signaling occurs within the caveolar compartment. (Invest Ophthalmol Vis Sci. 1999;40:157-167)

Visual endothelial cell growth factor (VEGF) was first discovered as vascular permeability factor, a tumor-secreted protein that induced a transient and reversible hyperpermeability.1 VEGF was subsequently recognized as an angiogenic factor and specific mitogen for vascular endothelial cells.2,3 VEGF consists of five different-sized polypeptides that are derived from alternative splicing of the same primary transcript.4,5 Each of the five proteins acts on endothelial cells through receptor tyrosine kinases known as Flk-1/KDR, Flt-1, and Flt-2. High levels of Flk-1/KDR mRNA are found in proliferating endothelial cells of growing capillaries, whereas levels are dramatically reduced in quiescent endothelial cells of adult capillaries, suggesting a role for this receptor in angiogenesis.6,7

From the 1Vascular Biology Center and the Departments of 2Pediatrics, 3Anatomy and Cellular Biology, and 4Ophthalmology, The Medical College of Georgia, Augusta.

Supported by grants from the National Institutes of Health (EY04618, EY11766 to RHC, and HL57201 to RCV), Bethesda, Maryland; the American Heart Association (National Center, to RCV), Dallas, Texas; and the Medical College of Georgia Research Institute Grants Program, Augusta, Georgia; and an unrestricted ophthalmology departmental award from Research to Prevent Blindness, New York, New York.

Submitted for publication February 13, 1998; revised May 28 and August 11, 1998; accepted September 21, 1998.

Proprietary interest category: N.

Reprint requests: Ruth B. Caldwell, CB1201, Vascular Biology Center, The Medical College of Georgia, Augusta, GA 30912.

The signaling mechanisms underlying VEGF actions on endothelial cells are not yet fully understood but probably involve production of nitric oxide (NO) by the endothelial isoform of NO synthase (eNOS). VEGF receptor stimulation has been found to lead to receptor autophosphorylation and tyrosine phosphorylation of various cellular proteins. These events result in the transient accumulation of intracellular Ca2+ and inositol 1,4,5-trisphosphate8 and eNOS activation by a mechanism that is inhibited by the tyrosine kinase inhibitor genistein.9 Morbidelli et al. have shown that VEGF’s activity in inducing growth of coronary vein endothelial cells requires NOS activity.10 They also demonstrated that NOS is persistently activated by VEGF and therefore that it could be involved in subsequent stages of VEGF-induced signaling of mitogenesis. Recently, NOS activity has also been shown to be required for VEGF’s permeability enhancing effects in an isolated microvesSEL preparation12 and also in the Miles assay model for determining permeability of the subcutaneous vasculature in vivo.13 NOS activity is also required for VEGF-induced neovascularization in the rabbit cornea model for angiogenesis in vivo.14

It has been suggested that VEGF’s effects in increasing permeability are mediated by increased transcytotic transport due to the formation of numerous interconnected membrane invaginations termed vesicular-vascular organelles (VVOs).15 Plasma membrane caveolae have recently been shown to have a similar elaborate structure, with a large percentage clustered around larger “vacuoles” in a rosette/bunch of grapes formation.16,17 It is likely, therefore, that VVOs and caveolae represent the same organelle. Caveolae were originally described as
intracellular compartments involved in endocytic and transcytotic transport, but now are recognized as having the additional functions of concentrating and internalizing small molecules by a process called cotranscytosis and serving as signal transduction organizing centers for segregating and concentrating membrane receptors with downstream effectors.

Recent work by our group and others has shown that eNOS is targeted to caveolae and that eNOS activity is regulated by protein-protein interactions with the caveolar structural protein caveolin-1. Thus, eNOS activity within the caveolae may have an important role in mediating VEGF's effects in increasing endothelial cell permeability. In this study, experiments were designed to develop and to test this concept by analysis in cell culture model systems. Our aims were to determine the cellular route by which VEGF increases the permeability of cultured retinal endothelial cells and to test whether NO production by eNOS is involved in signaling VEGF's permeability enhancing effects.

MATERIALS AND METHODS

Cell Culture
Freshly isolated cultures (passages 2 through 5) of bovine retinal endothelial (BRE) and bovine aorta endothelial (BAE) cells were used in these experiments. Retinal vessels were isolated from bovine eyes obtained from a local slaughterhouse with a sieving protocol to select for microvessels. Endothelial clonal colonies harvested by trypsinization were transferred to gelatin-coated dishes and amplified in serum-defined endothelial cell growth medium (Clonetics, San Diego, CA). Endothelial cultures were 98% pure as demonstrated by immunoreactivity for von Willebrand factor and by uptake of acetylated low-density lipoprotein. BAE cells were prepared as described previously.

Functional Assays
Cells (2 × 10^5) were plated in 0.33 cm² Transwell filter chambers with 0.4-μm pores (COSTAR, Cambridge, MA) that were coated with collagen and fibrinogen. Cultures were maintained for 5 to 7 days in endothelial cell growth medium and then were switched to serum-free endothelial basal medium (Clonetics) with 0.5% bovine serum albumin, for treatment with recombinant human VEGF165 (0.5 ng/ml to 25 ng/ml) and analyzed in cultures prepared routinely for either transmission or scanning electron microscopy (EM) after reaction with diaminobenzidine substrate buffer (0.1 M phosphate, 0.05 M citric acid, pH 5.0) for 15 minutes at 4°C. Then, 20-μl samples of cell extract were mixed with 150 μl o-phenylenediamine substrate buffer (0.1 M phosphate, 0.05 M citric acid, 0.012% H₂O₂, pH 5.0) and analyzed for HRP content as described. Molecules by a process called potocytosis and serving as signal transduction organizing centers for segregating and concentrating membrane receptors with downstream effectors.

The permeability values obtained from cultured BRE cells were always significantly lower than the background levels obtained from coated filters without cells, whereas TER values were always significantly higher. TER and permeability values were also consistent within batches of cells prepared at the same time. However, values for cultures prepared at different times occasionally varied. Therefore, all treatment effects were determined relative to values from control cultures prepared at the same time.

Intracellular uptake of HRP in VEGF-treated and control cultures was determined by a spectrophotometric method of measuring HRP. The cultures were solubilized by incubation in 1% Triton X-100 (in 0.1 M phosphate, 0.05 M citric acid, pH 5.0) for 15 minutes at 4°C. Then, 20-μl samples of cell extract were mixed with 150 μl o-phenylenediamine substrate buffer (0.1 M phosphate, 0.05 M citric acid, 0.012% H₂O₂, pH 5.0) and analyzed for HRP content as described.

A low-temperature transport block protocol was used to evaluate the role of endocytic transport in VEGF's effects on BRE cell permeability to HRP. BRE cultures were placed in HEPES-buffered medium containing HRP at 15°C and treated with or without VEGF (10 ng/ml). The cultures were cooled to 4°C over a 1-hour incubation interval and assayed for permeability.

Calcium Switch
Calcium dependency of the cultures' tight junctions was tested by switching cultures between medium containing normal calcium (1.8 mM) and low calcium (5 μM) as described in our previous analyses of epithelial cell barrier function. Low calcium medium was made with Dulbecco's modified Eagle's medium without calcium chloride (GIBCO-BRL, Grand Island, NY).

Morphologic Assays
Cultures labeled with rhodamine-phalloidin (Molecular Probes, Eugene, OR) were examined to detect cytoskeleton alterations and disruption of cell-to-cell attachments. Cell surface morphology and possible routes of the permeability increase were analyzed in cultures prepared routinely for either transmission electron microscopy (EM) after reaction with dianisobenzidine to visualize HRP as described previously or for scanning EM. Morphometric analysis of the density and diameter of membrane-attached caveolae was performed with scanning EM and computer-assisted morphometric procedures. Briefly, randomly selected areas in four VEGF-treated and four control
cultures were photographed and printed at a final magnification of ×83,300. Then, all caveolae present in the resultant images (total area, 14 mm²) were counted and measured to determine their average density and diameter.

**Immunocytochemistry**

For immunolocalization of caveolin-1, eNOS, and Flk-1/KDR, cells were plated on gelatin-coated glass chamber slides and serum-deprived overnight. The next day the cultures were washed with ice-cold PBS, fixed in acetone (−20°C for 30 minutes), rinsed in 0.01 N citrate buffer (pH 6.0), and then subjected to microwave treatment (for 6 minutes) to enhance antibody penetration. The cultures were then rinsed in PBS and processed routinely for immunocytochemistry. Subcellular localization of eNOS, Flk-1/KDR, and caveolin-1 was determined by indirect immunofluorescence microscopy with monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were incubated with the cells overnight and detected with fluorescein- or rhodamine-labeled secondary antibodies (1:200; Vector Laboratories, Burlingame, CA). In some experiments, Oregon green- and Texas red-labeled secondary antibodies (Molecular Probes, Eugene, OR) were used. Control experiments in which the primary antibodies were omitted were negative.

**Isolation of Caveolar Membranes**

These experiments were performed with confluent BRE and BAE cell cultures that had been serum-starved overnight and then treated with VEGF (10 ng/ml) for 0, 30, or 60 minutes. Caveolar membranes were isolated as described previously with the following minor modifications. All solutions used in the isolation were buffered at pH 7.5. In addition, caveolar fractions were trichloroacetic acid-precipitated before immunoblot analysis.

**RESULTS**

**VEGF-Induced Hyperpermeability**

Unless otherwise indicated all the following experiments were performed with 10 ng/ml VEGF. This concentration was chosen on the basis of two previous studies of VEGF ligand-receptor binding in BRE cells that had shown an effective concentration range that peaks between 10 ng/ml and 25 ng/ml. Many studies of VEGF's mitogenic effects have shown that rates of DNA synthesis or cell proliferation decrease slightly as concentration increases beyond the point of peak activity, suggesting possible inhibitory or cytotoxic effects of higher VEGF concentrations, or both. Therefore, we used 10 ng/ml VEGF to avoid the possibility of nonspecific effects. Initial experiments showed that 10 ng/ml VEGF stimulated a significant increase in BRE cell permeability to HRP and SF (140% to 160%) compared with the control cultures (Fig. 1A). This effect was rapid in onset and limited in duration as has been reported previously for experiments performed in vivo. Significant increases in the cultured cells' permeability were evident 30 minutes after VEGF addition and were resolved within 2 to 3 hours in different experimental preparations. The VEGF effect was concentration dependent, with an ED₅₀ of ~44 pM (2 ng/ml, Fig. 1B). BRE cell permeability was increased significantly by VEGF at 2 ng/ml, 5 ng/ml, and 10 ng/ml compared with 0.5 ng/ml, and VEGF at 10 ng/ml increased permeability significantly compared with 2 ng/ml. Other differences were not statistically significant. Control experiments testing the effects of another angiogenic factor, basic fibroblast growth factor (bFGF, 10 ng/ml), showed no change in endothelial cell permeability (Fig. 1C). This indicates that the response to VEGF is not secondary to its growth-stimulating effects and therefore represents a specific effect on permeability. To determine whether or not the permeability increase might be associated with VEGF-induced decreases in stability of the BRE cell tight junctions, VEGF effects on TER levels were also determined. The average TER levels reached by cultures prepared on different days varied somewhat, ranging from 20 ohm · cm² to 50 ohm · cm². VEGF treatment (10 ng/ml) had little effect on TER values. After VEGF treatment, average TER was slightly higher in some experiments and slightly lower in others, but group differences were always small, averaging less than 2 ohm · cm² (Fig. 1D). Moreover, TER remained substantially above background in all cultures. This shows that the cultures remained intact and stable during the experimental treatment. It also indicates that VEGF had little or no effect on tight junctions.

Experiments with a "calcium switch" protocol demonstrated the calcium dependency of the BRE cells' tight junctions. In a typical experiment, cultures maintained in medium containing normal calcium (1.8 mM) developed relatively high TER levels (51.9 ± 7.6 ohm · cm²). Within 5 minutes after being switched to medium with low calcium (<5 μM), their TER levels declined to near background levels (6.7 ± 1.6 ohm · cm²). This resistance decline was correlated with substantial increases in the culture's permeability to the paracellular tracer insulin and to SF (Fig. 2). These data are consistent with the well-known effects of calcium switch protocols in opening tight junctions of cultured epithelial cells and indicate that the effects of VEGF in stimulating an increase in BRE cell permeability are unlikely to involve a breakdown of the tight junctions.

**VEGF-Induced Endocytotic Transport in Caveolae**

Morphologic analyses with fluorescence microscopy and EM confirmed that the cell-to-cell junctions remained intact after VEGF treatment. When cultures prepared in the functional assays described above were reacted with rhodamine-phalloidin to label their actin microfilaments, fluorescence microscopy showed that they were organized as intact monolayers with actin staining localized preferentially at the cell borders (Fig. 3). The cells had numerous stress fibers, but areas of cell duplication were not observed. When the specimens were coded to conceal the treatment conditions and evaluated for possible differences in actin organization, in cell packing density, and in cell-to-cell contacts, the VEGF-treated cultures were found to be indistinguishable from the untreated controls. In parallel cultures prepared for transmission EM, control and VEGF-treated cultures were organized as confluent monolayers with continuous cell-to-cell contacts and well-developed junctions (Fig. 4A). Moreover, the interjunctional spaces were free of HRP reaction product, confirming the TER results and demonstrating that the tight junctions were not affected by VEGF.
Instead, the treated cultures contained numerous HRP-labeled vesicles deep within their cytoplasm, suggesting that endocytotic transport was increased. This latter effect was confirmed by biochemical analysis of intracellular HRP that showed a twofold increase in HRP uptake in VEGF-treated cultures compared with the control cultures (Fig. 4B).

To further evaluate the possible role of endocytotic transport in the VEGF-induced permeability increase, a low-temperature transport block protocol was used. Temperature-reduction protocols have been found to inhibit endocytotic transport in various cell types. Although the specific mechanism of this effect is not known, it has been demonstrated that the inhibitory effects are not a result of energy depletion and that protein synthesis takes place even at 15°C (for review, see Refs. 36 and 37). When the BRE cultures were treated with VEGF (10 ng/ml) at 15°C, and then cooled to 4°C during a 1-hour permeability assay, the increase in BRE cell permeability was completely blocked (Fig. 4C). These results are consistent with a vesicular pathway for the permeability effect.

Analysis by scanning EM confirmed a large increase in endocytotic transport activity in the VEGF-treated cultures as demonstrated by a 17-fold increase in the density of membrane-attached caveolae (Figs. 5A, 5B, 5C). These structures were significantly larger in the VEGF-treated cultures, with an average diameter of 50 nm compared with 39 nm in the control cultures (Fig. 5D). These data suggested that the permeability...
Caveolae Mediate VEGF-Induced Hyperpermeability

FIGURE 2. Experiments in which a "calcium switch" protocol was used demonstrate that the bovine retinal microvascular endothelial cell cultures form calcium-dependent tight junctions. Switching from medium with normal calcium levels (1.8 mM) to low calcium medium (<5 μM) decreased the cultures' transcellular electrical resistance levels from 51.9 ± 7.6 ohm * cm² to 6.7 ± 1.6 ohm * cm² and increased their permeability to sodium fluorescein (open bars) and inulin (horizontal striped bars; *P < 0.05, unpaired t-test). Data are mean ± SE.

increase in the VEGF-treated cultures is mediated by a mechanism of transcytotic transport in caveolae.

To further evaluate the role of caveolae-mediated endocytic transport in the VEGF-induced permeability increase, endocytosis of HRP was analyzed in relation to the intracellular distribution of caveolin-1, the principal protein component of caveolae. BRE cultures were first incubated with fluorescein isothiocyanate-labeled HRP in the presence or absence of VEGF (10 ng/ml) and then were prepared for immunofluorescence colocalization of caveolin-1 and HRP. The data showed that many HRP-containing vesicles were immunoreactive for caveolin-1, suggesting the involvement of this protein in endocytotic transport (Figs. 6A, 6B).

Role of NOS Activity in VEGF Signaling of Vascular Permeability

Previous studies with isolated and perfused coronary microvessels and the Miles assay for permeability in vivo strongly suggest that VEGF induces its effects in stimulating vascular permeability via a signaling cascade involving activity of NOS in producing NO.12,13 Because eNOS is specifically targeted to caveolae,22,23,25 it is thought that NO signaling in endothelial cells occurs within a caveolar compartment. Moreover, agonist-induced activation of eNOS in aortic endothelial cells has been found to involve changes in eNOS protein-protein interactions with caveolin-1.25 Therefore, we postulated that the VEGF induction of increased BRE cell permeability occurs by an eNOS dependent-signaling process involving the caveolar compartment. The next series of experiments was designed to test and to further develop this hypothesis and to examine the role of eNOS activity in the VEGF-signaling cascade in BRE cells. Experiments testing VEGF effects on cultures pretreated with the NOS inhibitor L-NAME (N^\-nitro-L-arginine methyl ester, 100 μM) showed that the VEGF effect on permeability was completely blocked by the inhibitor, indicating that NOS activity in producing NO is required for the BRE cell permeability response to VEGF (Table 1). Experiments measuring NO release from VEGF-treated BRE cultures with an NO electrode have confirmed that VEGF (10 ng/ml) induced NO release and that this effect is inhibitable by L-NAME.38 These observations are consistent with the previous data showing that VEGF effects on vascular growth, permeability, and vascular tone require NOS activity.10-12,14

We next tested whether the VEGF/NO signaling cascade could be localized to the caveolar compartment. These experiments used double immunolabeling and cell fractionation techniques to analyze the subcellular distribution of the VEGF.
receptor Flk-1/KDR and eNOS in relation to the caveolar protein caveolin-1. VEGF is known to stimulate at least two class III receptor tyrosine kinases in endothelial cells, Flt-1 and Flk-1/KDR. Flk-1/KDR is thought to be the relevant receptor tyrosine kinase for the VEGF-induced permeability increase because placenta growth factor, which binds only to Flt-1 and not to Flk-1/KDR, does not affect vascular permeability.15 These studies showed that Flk-1/KDR is localized in caveolae with caveolin-1. The caveolin-1 and Flk-1/KDR immunoreactive caveolae were concentrated in sites of cell-to-cell contact at the cell surface (Fig. 6C). Labeling for eNOS was also colocalized with caveolin-1 in particulate vesiclelike structures at the cell periphery (Fig. 6D). Immunoreactivity for eNOS and caveolin-1 was also concentrated in the Golgi area as observed previously in bovine lung microvascular endothelial cells.22 Cell fractionation studies in which sucrose density gradients were used to isolate the caveolar fraction support the immunolocalization data. The results of the cell fractionation experiments are illustrated in Figure 7. The caveolar fraction (containing >95% of the cellular caveolin-1) was always found in gradient fractions 3 and 4. These fractions were also significantly enriched in Flk-1/KDR and eNOS. Densitometry showed that 73% of Flk-1/KDR and 40% of eNOS immunoreactivity were contained within the caveolar fraction, indicating that there is substantial overlap in the distribution of all three proteins within caveolae. There was some variability in the relative levels of Flk-1/KDR and eNOS within fraction 3 compared with fraction 4. This may reflect the presence of different subpopulations of caveolae that differ in size, solubility properties, or both. These same patterns of protein distribution were observed when the experiments were performed with BAE cells.

**DISCUSSION**

**VEGF-Induced Hyperpermeability Mediated by Transcytotic Transport in Caveolae**

Our experiments demonstrate clearly that VEGF increases the permeability of cultured microvascular endothelial cells. Multiple lines of evidence from experiments in which a combination of functional, morphologic, and biochemical approaches were used demonstrate that this effect of VEGF in increasing permeability is mediated by a mechanism of transcytotic transport in caveolae. First, our functional assays showed that VEGF treatment stimulated a transient concentration-dependent increase in tracer flux across the monolayers, accompanied by an increased uptake of HRP within the endothelial cells' cytoplasm. This suggests that vesicular uptake and transport is increased by VEGF. Furthermore, the permeability effects of VEGF were entirely blocked by incubation of the cultures at 4°C, which is known to block endocytotic transport.56,57 Increases in tracer flux can also occur due to alterations in the paracellular permeability pathway associated with defective sealing properties of the tight junctions. However, the tight junctions appeared morphologically intact, and measurements of the cultures' TER levels showed little or no change after the VEGF treatment. These data indicate that the paracellular pathway through the tight junctions was not significantly altered by VEGF treatment. Finally, the large increase in numbers of plasma membrane caveolae observed in the VEGF-treated cells, together with the immunolocalization data showing that the HRP-labeled vesicles were positive for the caveolar protein.
Caveolae Mediate VEGF-Induced Hyperpermeability

Control VEGF

FIGURE 5. Vascular endothelial growth factor (VEGF; 10 ng/ml) stimulates formation of numerous large membrane-attached vesicles in microvascular bovine retinal microvascular endothelial (BRE) cells. Analysis of BRE cell-surface morphology with scanning electron microscopy revealed very few 40-nm to 60-nm membrane vesicles in control cultures (A) compared with the VEGF-treated cultures (B). Scale bar, 250 nm. Computer-assisted morphometry demonstrated significant increases in vesicle density (C) and size (D; \( *P < 0.01 \), unpaired t-test). Data are mean ± SE.

The morphologic pattern of the VEGF effect in our experiments is also similar to that described in vivo, in which the increase in permeability was found to be accompanied by the formation of grapelike clusters of plasma membrane-associated vesicles termed VVOs. The authors proposed that the VVOs are responsible for transcytotic transport. Because of the large size and complexity of these structures, it was felt that they would be unlikely to shuttle back and forth across endothelial cytoplasm as has been proposed for capillary caveolae. Rather, it was suggested that they are sessile structures whose permeability is regulated by diaphragms. However, our data showing the colocalization of HRP and caveolin-1 within the same cytoplasmic vesicles demonstrate that the VEGF-induced permeability increase in retinal endothelial cells is mediated by mobile caveolae that move freely between the plasma membrane and cytoplasmic compartments.

Our results differ from those reported in some experimental systems. In some in vivo studies, VEGF has been found to induce a rapid increase in permeability associated with the formation of interendothelial gaps and fenestrations. VEGF has also been found to induce fenestration formation in cultured bovine adrenal cortex endothelial cells. Esser et al.
Horseradish peroxidase (HRP), Flk-1/KDR, and the endothelial isoform of nitric oxide synthase (eNOS) are colocalized with caveolin-1 in cultured bovine retinal microvascular endothelial cells. (A) Cells from untreated cultures immunolabeled for caveolin-1 (red) show immunoreactivity in particulate, vesicle-like structures. (B) Cultures double-labeled for caveolin-1 (red) and fluorescein isothiocyanate-HRP (green) after vascular endothelial growth factor treatment (10 ng/ml) contain many HRP-labeled vesicles that are also immunoreactive for caveolin-1 (yellow). (C) Double immunolabeling of Flk-1/KDR (green) and caveolin-1 (red) in control cultures shows that the two proteins colocalize (yellow) in a compartment at or near the cell surface (arrows). (D) Double immunolabeling of untreated cultures for eNOS (green) and caveolin-1 (red) shows that the two proteins colocalize (yellow) at the periphery and in the perinuclear Golgi compartment. Scale bar, 5 μm.

showed that coculture of the endothelial cells together with epithelial cells that had been stably transfected with cDNA for VEGF120 or VEGF164 induced the formation of numerous fenestrations in the endothelial cells.11 The same effect was observed when the epithelial cells were replaced by an epithelial-derived basal lamina-type extracellular matrix, and the cultures were treated with purified VEGF. The VEGF-mediated induction of fenestrae was accompanied by an increase in the number of fused caveolae-like vesicles resembling the VVOs described previously in in vivo studies.10 These observations suggest that mechanisms other than an increase in transcytotic transport could be involved in VEGF's permeability enhancing effects. However, we were unable to detect any effects of VEGF in promoting the formation of either interendothelial cell gaps or fenestrations in cultured retinal endothelial cells. These differences may reflect differences be-
TABLE 1. L-NAME Effects on VEGF-Induced Permeability of BRE Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Permeability (µl/cm² per hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.36 ± 1.23</td>
</tr>
<tr>
<td>VEGF</td>
<td>20.23 ± 1.36</td>
</tr>
<tr>
<td>VEGF + L-NAME</td>
<td>14.32 ± 2.32</td>
</tr>
</tbody>
</table>

L-NAME, N^ω-nitro-l-arginine methyl ester; VEGF, vascular endothelial cell growth factor; BRE, bovine retinal microvascular endothelial. *Pretreatment of cultured BRE cells with L-NAME (100 µM, 30 min) blocked the effects of VEGF (10 ng/ml) on their permeability. Data are expressed as mean ± SE.

Localized VEGF Signaling within the Caveolar Compartment

The principal protein component of endothelial cell caveolae is caveolin-1, a 21-kDa to 24-kDa integral membrane protein. Because in various mammalian cells 90% of caveolin is localized in caveolae, with the remaining 10% found in the Golgi apparatus, redistribution of caveolin from the plasma membrane to the cellular interior has been used by many researchers to study the caveola-mediated transport processes of endocytosis and transcytosis. Our data showed that HRP-labeled endocytotic vesicles are also immunoreactive for caveolin-1. Taken together with the results of our functional and morphologic analyses showing that VEGF acts on BRE cells by promoting their transcytotic transport, these data demonstrate that the VEGF-stimulated increase in transcytosis is mediated by caveolae. Carrier-mediated transcytosis in endothelial cells has long been thought to occur in caveolae. For example, native albumin, acting as a carrier for fatty acids and other small ligands, has been shown to bind albondin for delivery in caveolae across the cell to the underlying tissue. However, to the best of our knowledge, our data provide the first direct evidence that the fluid phase uptake and intracellular transport of small molecules such as HRP is also mediated by caveolae.

Our observations add strong support to previous reports that VEGF binding of its receptors results in NOS activation. Our functional analyses showing that VEGF's effect on increasing BRE cell permeability is completely blocked by the NOS antagonist L-NAME confirm the specific requirement for NOS activity in signaling VEGF's effects on endothelial cell permeability, which was previously suggested in studies in which isolated-perfused coronary venules were used. We also have found that VEGF treatment induces NO release by cultured endothelial cells with a process that is blocked by pretreatment with L-NAME.

Our immunolocalization results showing that eNOS colocalizes with the VEGF receptor Flk-1/KDR and caveolin-1 suggest that interactions between the three proteins have an important role in the VEGF signal transduction processes. The results of our cell fractionation experiments strongly suggest that in addition to their role in VEGF-induced transcytosis that...
the caveolae function in VEGF signaling. The role of the caveolar compartment in signal transduction processes for other growth factors is already clearly established. A variety of tyrosine kinase receptors and effector molecules have also been shown to be localized in caveolae, including the platelet-derived growth factor receptor, the endothelial growth factor receptor, and protein kinase C. Subcellular targeting of eNOS to caveolae is thought to influence signal transduction mechanisms by restricting NO signaling to specific sites within the cell. Moreover, eNOS activity has been shown to depend on its transport in caveolae to a Golgi-associated perinuclear subcellular compartment and on Ca2+-calmodulin binding. Studies are now in progress to determine the role of caveolae in the VEGF signaling pathway.

The relationship between VEGF’s effects in increasing permeability and promoting angiogenesis is not understood. It has been suggested that the permeability increase facilitates angiogenesis by allowing plasma proteins to leak into the extravascular space in which they form a favorable matrix for new vessel growth. Thus, VEGF-induced transcytosis in the caveolar compartment could serve as a mechanism for the targeted delivery of molecules needed for the angiogenic process. Beyond such effects in facilitating the angiogenic process, caveolae-mediated transport may also serve a direct function in signaling VEGF’s effects. Furthermore, this process appears to be a general feature of VEGF signaling in endothelial cells because results similar to those reported here for BRE cells have also been obtained with aortic endothelial cells.

References

30. Song KS, Li S, Okamoto T, Quilliam LA, Sargsjoan M, Lisanti MP. Copurification and direct interaction of Ras with caveolin, and
integral membrane protein of caveolae microdomains: detergent-

stimulates retinal capillary endothelial cells through an autocrine

35. Thieme H, Aicillo LP, Takagi H, Ferraro N, King GI. Comparative
analysis of vascular endothelial growth factor receptors on retinal

36. Pastan I, Willingham MC. The pathway of endocytosis. In: I Pastan,
1–44.

37. Kuismanen E, Saraste J. Low temperature-induced transport blocks
as tools to manipulate membrane traffic. *Methods Cell Biol.* 1989;
32: 257–274.

38. He H, Venema VJ, Venema RC, Bichazian MA, Caldwell RB. VEGF
intracellular signal transduction pathway [ARVO Abstract]. *Invest

organelles and the regulation of venule permeability to macromol-
ecules by vascular permeability factor, histamine, and serotonin. *J

40. Roberts W, Palade G. Increased microvascular permeability and
endothelial fenestration induced by vascular endothelial growth

Vascular endothelial growth factor induces endothelial fenestra-

42. Wang W, Merrill MJ, Borchardt RT. Vascular endothelial growth
factor affects permeability of brain microvascular endothelial cells in

43. Criscuolo GR, Merrill MJ, Oldfield EH. Characterization of a protein
product of human malignant glial tumors that induces microvas-

44. Rothberg KG, Heuser JE, Donzell WC, Ying Y-S, Glenney JR.
Anderson RGW. Caveolin, a protein component of caveolae mem-

45. Conrad PA, Smart EJ, Ying Y-S, Anderson RGW, Bloom GS. Caveo-
lin cycles between plasma membrane caveolae and the Golgi
complex by microtubule-dependent and microtubule-independent

46. Kurzchalia TV, Parton RG. And still they are moving: dynamic

47. Smart EJ, Ying Y-S, Conrad PA, Anderson RGW. Caveolin moves
from caveolae to the Golgi apparatus in response to cholesterol

48. Milici AJ, Watrous NE, Stuckenbrock H, Palade GE. Transcytosis of
2612.

49. Schnitzer JE, Oh P. Albondin-mediated capillary permeability to
albumin: differential role of receptors in endothelial transcytosis
and endocytosis of native and modified albumins. *J Biol Chem.*

50. Liu P, Anderson RGW. Compartmentalized production of ceramide