VEGF-induced mobilization of caveolae and increase in permeability of endothelial cells

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¹Departments of Medicine and Physiology, University Microscopy Center, State University of New York, Stony Brook, New York 11794-8152; ²Department of Cell Biology and Histology, Free University of Brussels, 1090 Brussels-Jette, Belgium; ³Department of Medicine, Tel Aviv University, Tel Aviv 49372, Israel; and ⁴Department of Medicine, The University of Tokyo, Tokyo 113, Japan

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Chen, Jun, Filip Braet, Sergey Brodsky, Talia Weinstein, Victor Romanov, Eisei Noiri, and Michael S. Goligorsky. VEGF-induced mobilization of caveolae and increase in permeability of endothelial cells. Am J Physiol Cell Physiol 282: C1053–C1063, 2002.—Glomerular epithelial cells (GEC) are a known site of vascular endothelial growth factor (VEGF) production. We established immortalized rat GEC, which retained the ability to produce VEGF. The isoforms expressed by GEC were defined as VEGF-205, -188, -120, and -164. The electrical resistance of endothelial cells cultured on GEC-conditioned matrix, an indicator of the permeability of monolayers to solutes, was significantly increased by the treatment with the neutralizing polyclonal antibodies to VEGF and decreased by VEGF-165. Transfection of endothelial cells with green fluorescence protein-caveolin construct and intravital confocal microscopy showed that VEGF results in a rapid appearance of transcellular elongated structures decorated with caveolin. Transmission electron microscopy of endothelial cells showed that caveolae undergo rapid internalization and fusion 30 min after application of VEGF-165. Later (36 h), endothelial cells pretreated with VEGF developed fenestrae and showed a decrease in electrical resistance. Immunoelectron microscopy of glomerular endothelium confirmed VEGF localization to podocytes and in the basement membrane. In summary, immortalized rat GEC, which retained the ability to produce VEGF, overproduce this growth factor and in normal vascular beds pretreated with VEGF. Feng et al. (8) and Vasile et al. (26) have demonstrated that VEGF increases vascular permeability by increasing the density of clustered caveolae, termed vesiculovacuolar organelles, in endothelial cells (8, 26). Glomerular epithelial cells (GEC) have recently been identified as the site of constitutive production of VEGF (4, 11). It has been suggested, therefore, that VEGF produced by GEC may be responsible for the maintenance of the fenestrated phenotype of glomerular endothelial cells (4, 24), thus facilitating the high rate of glomerular ultrafiltration. This view, however, requires reinforcement because of the fact that hydrodynamics of fluxes in the glomerular capillary wall are unfavorable for such an upstream paracrine action. The significance and potential implications of the above hypothesis warrant extensive investigations of VEGF production by GEC and its action on the endothelium. Unfortunately, cell culture models are scarce, and in vivo studies present difficulties in interpreting the results because of the circulating VEGF and other angiogenic/vasoactive substances. Several investigators have previously reported a successful isolation and culture of primary GEC (reviewed in Ref. 13); however, the procedure is tedious, cells rapidly dedifferentiate, and the properties of these primary cultures can fluctuate. Attempts to immortalize these cells have been reported (1). In the present study, we established and characterized a Simian virus (SV)-40-transformed GEC line and provide evidence of VEGF synthesis by GEC. Furthermore, a coculture model developed in this study yielded data on the effect of VEGF produced by GEC on the permeability of endothelial cells in vitro. In addition, the data obtained with green fluorescent protein (GFP)-caveolin and supplemented with electron microscopic analysis of renal microvascular and hu-
man umbilical vein endothelial cells (RMVEC and HUVEC, respectively) demonstrated that caveolin-decorated structures traverse endothelial cells and elongate after application of VEGF.

**MATERIALS AND METHODS**

**Cell cultures.** Primary rat GEC cultures were obtained and maintained according to the previously published procedure (15). Primary GEC were plated on collagen IV-coated 3-cm dishes and maintained in K-1 medium (Nipro, Osaka, Japan) supplemented with 2% NuSerum I (Collaborative Biomedical Products), 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO-BRL, Gaithersburg, MD). After colonies were formed, the medium was aspirated and high-titer (10⁶ virus/ml) wild-type SV-40 was added for 60 min as previously reported (25). GEC were isolated by limiting cloning. To validate the authenticity of the immortalized GEC, the expression of several markers of these cells and the lack of markers characteristic of endothelial and mesangial cells was examined. The selected clone of SV-40-transformed GEC expressed large-T antigen (data not shown; Noiri, unpublished observation), confirming the adequacy of transfection. Several markers of GEC were expressed by these cells. The Wilms’s tumor protein has been detected in situ in podocytes, and immunocytochemical staining also showed the presence of this marker in GEC (data not shown). Moreover, nephrin mRNA was detected in GEC. A monoclonal antibody (GSA3) recognizing a cell-specific surface antigen on podocytes (15) showed positive immunostaining of GEC (data not shown). Furthermore, puromycin-expressed GEC-endothelial cell coculture. To examine the effects of GEC on the phenotype of endothelial cells, GEC were grown on collagen IV-coated glass coverslips. At confluence, cells were overlayed with Vitrogen 100 or Matrigel, which formed a thin gel layer on the surface of GEC within 60 min of incubation at 37°C. Endothelial cells were seeded atop to form a “sandwich” and were allowed to coinubate for 4, 8, and 24 h. Cells were fixed, critical point-dried, and studied using scanning electron microscopy (EM). Alternatively, GEC cultured for 3 days on glass coverslips were thoroughly removed using repeated cycles of freezing-thawing, dishes were washed exhaustively with PBS until GEC remnants were undetectable by light microscopy, and endothelial cells were plated on GEC-conditioned extracellular matrix. In control experiments, the same endothelial cells served as a feeder layer to produce and condition the extracellular matrix.

**RT-PCR and identification of VEGF isoforms.** Oligonucleotide primers flanking the insertion/deletion site of VEGF-188 were designed to amplify VEGF mRNA from GEC-T cells to identify the unique VEGF isoforms. The sequence of sense primer was 5’-GGACATCTTCGACGTACC-3’, and the antisense primer was 5’- GTTCGAAAACCTGTAGG-3’. Total RNA was isolated from GEC-T cells with Trizol total RNA isolation reagent (GIBCO-BRL), and the mRNA was then reverse transcribed to cDNA and amplified with high-fidelity enzyme mix that was provided in the Titan One Tube RT-PCR System (Boehringer Mannheim, Indianapolis, IN).

10–100 ng of total RNA were used in a 50-μl reaction containing 1× RT-PCR reaction buffer, 0.2 mM dNTPs, 5 mM dithiothreitol, and 0.4 μM of each primer. The RT-PCR profile consisted of a 30-min incubation at 50°C, 2 min denaturation at 94°C, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 2 min elongation at 68°C, and finally a 6-min extension at 68°C. Products were analyzed by running 10% of the reaction mixture on a 2% agarose gel. The bands that have proper expected size were excised from the gel, recovered with a QiaQuick gel extraction kit (Qiagen, Valencia, CA), and then sequenced with an ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster, CA) directly or after cloning into the plasmid vector (Invitrogen, Carlsbad, CA). In the case of VEGF isoform 205, 20 cycles of secondary PCR reaction were carried out to enrich the cDNA fragment that has the predicted size.

**Immunoprecipitation and Western blot analysis.** After being washed with iced-cold PBS, cells were lysed in 200 μl of SDS gel-loading buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromphenol blue) containing 2.5% 2-mercaptoethanol. After being boiled for 10 min, samples were sonicated on ice and centrifuged for 10 min at 10,000 rpm. Supernatants were collected, and 20-μl samples were loaded on 8% SDS-polyacrylamide gels for Western blot analysis. The proteins were transferred to nitrocellulose membranes and then probed with primary antibodies. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham). The blots were developed with an enhanced chemiluminescence (ECL) detection kit (Amersham). The bands were visualized under UV light and photographed. The optical density of bands was measured with a Storm digital imaging system (Molecular Dynamics, Sunnyvale, CA).

Electrical resistance as an index of cell permeability to solutes. To examine the permeability of endothelial cells to solutes, endothelial cells were grown to confluence on the GEC-conditioned extracellular matrix or in the sandwich configuration (see above) on the microelectrodes of the epithelial cell permeability system. Each well contained a gold microelectrode and a reference electrode, both electroplated on the bottom of the well. Electrode units were placed in an incubator and connected to a lock-in amplifier interfaced to a computer registering electrical resistance and capacitance every second. The amplifier measured the in- and out-of-phase (real and imaginary) voltages across the small electrode, and these were converted by the computer into a resistance and capacitance in series, taking the external circuit into consideration. When cells are plated on this surface, the electrical resistance initially reflects the degree of cell adhesion and spreading and, upon reaching a confluent monolayer, reports the permeability of cells to solutes. Electrical impedance was monitored in real time for 10 h after addition of 1.0, 10.0, or 20.0 ng/ml human recombinant VEGF-165 (PeproTech, Rocky Hill, NJ) or rabbit polyclonal neutralizing antibodies to VEGF (PeproTech), as specified in Methods. To monitor the long-term permeability change, HUVEC were seeded at a higher density on a thin layer of matrigel-coated dishes, and electrical impedance measurements were performed for up to 40 h. To ensure that monolayers were unperturbed, only those wells showing high resistance (>16 kΩ) and displaying no “gaps” under light microscopy were selected for analyses.
electrophoresed on 4–20% SDS polyacrylamide gel. For detecting VEGF secreted in the culture medium, samples were subjected to immunoprecipitation. Briefly, 1.4 ml conditioned culture medium were kept overnight at 4°C on a rocker with the addition of 1 μg/ml rabbit anti-human VEGF polyclonal antibody (Santa Cruz). Next, 15 μl GammaBind plus Sepharose beads (Pharmacia, Uppsala, Sweden) were added for another 2 h. The Sepharose beads were then collected by centrifugation, washed two times in 0.01 M Tris (pH 8.0), 0.14 M NaCl, and 0.025% NaN₃ (TSA) containing 0.1% Triton X-100, one time in TSA buffer alone, and one additional time in 0.05 M Tris, pH 6.8. After being boiled for 5 min in 1× SDS gel-loading buffer, supernatant was transferred to two tubes with or without 2.5% 2-mercaptoethanol, boiled for an additional 5 min, and electrophoresed on a 4–20% SDS polyacrylamide gel. Separated proteins were blotted on polyvinylidene difluoride membranes (Millipore), blocked in PBS containing 1% casein for 60 min, and incubated overnight at 4°C in 1:100–200 diluted primary antibodies (rabbit anti-human VEGF polyclonal and mouse anti-human VEGF monoclonal antibodies). Cell lysate samples and mouse anti-human VEGF monoclonal antibody for immunoprecipitation samples (Santa Cruz). After intense washing, the membranes were incubated with 1:2,000 diluted secondary horseradish peroxidase-conjugated donkey anti-rabbit or sheep antimouse IgG (Amersham Life Sciences, Arlington Heights, IL) for 30 min at room temperature. Thereafter, the membranes were washed one time again and incubated in enhanced chemiluminescence substrate reagent (Amersham) for 1 min. The blots were exposed to X-ray film for 5–30 s, and the molecular weight of the immunodetected bands was compared with molecular weight standards (Novex).

Caveolin-1-GFP expression vector. In preliminary studies, the following two constructs were generated: caveolin-1-GFP and GFP-caveolin-1; studies presented herein utilized the first construct, as previously reported (12). The full open-reading frame of the human caveolin-1 (nucleotides 35·571) was cloned from the HUVEC cDNA library by PCR using appropriate primers containing XhoI and BamHI restriction sites at 5’ and 3’ with the stop codon mutated. cDNA was digested with XhoI and BamHI and ligated in sense orientation at the appropriate cloning site of the pEGFP-N1 plasmid (Clonetech) using a rapid DNA Ligation Kit (Boehringer Mannheim). Ligated plasmids were used to transform One Shot INValphaF′ Competent Cells (Invitrogen). Transformed cells were selected for kanamycin resistance, propagated, and isolated with Maxi-Prep (Qiagen). The construct was sequenced using a Dye Terminator kit and a 377 DNA automated sequencer (Applied Biosystems), and the authenticity of the product was confirmed.

HUVEC were incubated in endothelial basal medium (EBM)-2 basal medium (Clonetics) for 5 h. The caveolin-GFP fusion constructs (2 μg) were used in conjunction with the FuGENE 6 transfection reagent (Boehringer Mannheim), according to the manufacturer’s instructions. The transfection was carried out in EBM-2 media. The cells were used in the experiments 24–48 h after transfection. In a series of preliminary experiments, transfection of HUVEC with the caveolin cassette with GFP fluorescent tag resulted in an appropriately localized and functionally competent protein (12); therefore, this construct was used in all reported experiments.

Transmission electron microscopy and confocal fluorescence microscopy. The cultured cells were rinsed twice with PBS and fixed with 2% glutaraldehyde in sodium cacodylate buffer (0.1 M cacodylate and 0.1 M sucrose) at pH 7.4 for 12 h. Cells were subsequently postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate at pH 7.4 for 1 h. Samples were further dehydrated in graded alcohol solutions and embedded in epon. After hardening of the embedding medium, the culture dishes were broken using liquid nitrogen. Transverse sections of 60 nm were cut with a diamond knife, stained first with uranyl acetate and subsequently with lead citrate, and examined under a Philips Tecnai 10 transmission electron microscope at 80 kV. Morphometric analysis was performed on randomly acquired digitized images (MegaView II camera connected to the microscope operated with the analysis 3.0 software) at magnifications of ×2,900 or ×5,000, calibrated with a Polaron cross-grating replica (Polaron 54,800 lines/in. grating). Subsequently, the UTHSCSA Image Tool 2.0 software was used to trace the number and diameter of uncoated vesicular organelles. Caveolae and uncoated vesicles were discriminated from coated vesicles and vacuoles based on their morphology and size, as described previously (9). For each experiment, five cells were randomly selected, and images were obtained at both magnifications. All experiments were repeated three times, and data were expressed as means ± SE. Statistical analysis was performed with the Mann-Whitney two-tailed U-test.

In a separate series of experiments, immunoelectron microscopy of rat kidney sections was performed to visualize the distribution of VEGF. Slices of each kidney were fixed in 0.5% glutaraldehyde in PBS, pH 7.4. For immunohistochemistry using EM, 1-mm² tissue blocks of glutaraldehyde-fixed kidneys were washed with PBS, dehydrated in ethanol, and embedded in London Resin (LR)-white resin (Polysciences, Washington, PA). For EM morphology, similar tissue blocks were postfixed with 1% OsO₄ in veronal-acetate buffer, pH 7.4, for 1 h at 4°C, dehydrated in ethanol and propylene oxide, and embedded in araldite (Polysciences). For EM morphology, ultrathin araldite sections were mounted on naked 400-mesh grids, stained with uranyl acetate and lead citrate, and coated with carbon. For EM immunohistochemistry, ultrathin LR-white sections of ~60 nm were mounted on 200-mesh nickel grids, coated with Formvar film, and impregnated with carbon. The sections were treated with 1% BSA-0.05% Tween 20-BSA (blocking buffer) for 15 min, labeled with polyclonal anti-VEGF (Santa Cruz) diluted 1:50 in blocking buffer for 2 h, rinsed five times in PBS, and incubated for 1 h with goat anti-rabbit IgG conjugated to 15 nm gold (Biocell) diluted 1:50 in blocking buffer. This was followed by five rinses in PBS, a rinse with a stream of distilled water, and staining for 5 min with saturated uranyl acetate in 50% ethanol. Examination of all sections was carried out using a JEOL-100B electron microscope at 80 kV.

Fluorescence confocal microscopy was performed on fixed cells or intravitally with the distance between focal planes 0.2–0.5 μm, as specified in RESULTS, using a real-time laser system (Odyssey; Noran Instruments, Middleton, WI). Images were analyzed with MetaMorph software (Universal Imaging) using a Silicon Graphic system.

RESULTS

VEGF expression and distribution. The staining of GEC with polyclonal antibodies to VEGF (Santa Cruz) revealed that the cells expressed immunodetectable VEGF (Fig. 1, A–C). These data indicated that immortalized GEC preserved the ability to produce VEGF. Further confirmation was obtained in studies of splice variants of VEGF, as described below.
The VEGF gene contains eight exons, and several splicing variants exist. VEGF-188 has all eight exons, VEGF-205 has an insert, VEGF-164 lacks exon 6, and VEGF-120 lacks exons 6 and 7. Using primers that flank the common insertion and deletion site, we expected to amplify all VEGF isoforms with PCR and then distinguish them from one another by the predicted size of the amplified cDNA, as well as analysis of nucleic acid sequences. After 35 PCR cycles, four distinct cDNA bands were seen on agarose gels, which had the expected size for different rat VEGF isoforms as follows: 306 bp for VEGF-120, 438 bp for VEGF-164, 510 bp for VEGF-188 (these three are readily detectable after 20 PCR cycles), and 564 bp for VEGF-205 (Fig. 2, A and B). Sequence analysis confirmed the identity of the transcripts as representing VEGF-120, VEGF-164, VEGF-188, and VEGF-205. (An additional band above VEGF-205 is the result of a nonspecific amplification, and its sequence showed no homology with VEGF.) Hence, the established immortalized GEC cell line maintains the ability of producing four different VEGF isoforms, and among them VEGF-120 and -164 are the two abundant isoforms, and VEGF-188 and, especially, -205 are less expressed.

Western blot analysis of cell lysates and conditioned medium under reducing conditions (Fig. 2, C and D) revealed three bands at 17, 22, and 27 kDa, which correspond to the predicted molecular size for 120, 164, and 188 amino acid VEGF isoforms (using polyclonal and monoclonal antibodies). Under nonreducing conditions, we identified a band near 45 kDa present in the conditioned medium, which corresponds to the VEGF-164 homodimer. The lower molecular weight band seen under nonreducing conditions may represent a non-dimerized VEGF-164. VEGF-205 was undetectable with this technique.

Effects of VEGF, GEC, and GEC-conditioned extracellular matrix on the permeability of endothelial cells.

Fig. 1. Immunocytochemical detection of vascular endothelial growth factor (VEGF) distribution in glomerular epithelial cells (GEC). A–C: representative field of GEC stained with anti-VEGF. A: rhodamine phalloidin staining; B: anti-VEGF antibody; C: merged images.

Fig. 2. Agarose gel electrophoresis of RT-PCR products of VEGF mRNA in GEC cells. A: primers were designed based on the common sequences among different VEGF isoforms that flank the common insertion (between exons 6 and 7 for VEGF-205), full-length (VEGF-188), or deletion (exon 6 for VEGF-164, exons 6 and 7 for VEGF-120) sites in the VEGF mRNAs. B: sizes expected for VEGF-120, VEGF-164, VEGF-188, and VEGF-205 are 306, 438, 510, and 564 bp, respectively. After 35 cycles of amplification, four bands with the expected size were detected in GEC mRNA. Based on the electrophoretic mobility and additional sequencing results of each band (data not shown), it was confirmed that they represent VEGF-120, VEGF-164, VEGF-188, and VEGF-205, respectively. It appears that GEC in culture retain the ability to produce four different VEGF isoforms. Among them, VEGF-120 and -164 are the two abundant isoforms, and VEGF-188 and -205 are less expressed. C: Western blot analysis of GEC lysates immunoblotted with polyclonal antibodies (note the expression of three isoforms, except for VEGF-205). D: VEGF in GEC-conditioned culture medium, under reducing and nonreducing conditions, detected using immunoprecipitation (IP) with the polyclonal antibody, followed by blotting (IB) with a monoclonal antibody. Ab, antibody.
Transmission electron microscopy (TEM) of HUVEC and RMVEC treated with VEGF for various periods of time revealed an increase in the number of caveolae and an increase in the diameter of caveolae (Fig. 3 and Table 1). The increased number of uncoated vesicular organelles was detectable within 10–30 min, and these organelles exhibited multiple contacts, fused and formed vesiculovacuolar-like structures 10–30 min after VEGF application (Fig. 3, C-E). After 60 min, the burst of caveolae formation has subsided, and internalized uncoated vesicular structures were observed within the cytoplasm (Fig. 3F). Moreover, morphometry revealed a twofold increase in the number of uncoated vesicular organelles 60 min after application of VEGF, and the size of these uncoated vesicular structures, as measured by average diameters, was also enlarged (Fig. 3F and Table 1). Representative images in Fig. 3 were taken from HUVEC; similar results were also obtained with RMVEC (data not shown).

Next, we argued that, if VEGF induces the caveolae-enriched phenotype of endothelial cells, the electrical resistance of cell monolayers should serve as a convenient reporter of any changes in cell permeability, whereas changes in the electrical capacitance should reflect the state of the lipid membrane convolution. To accomplish this, cells were cultured in specially designed five-well plates with a miniature gold electrode and a large reference electrode electrosprayed on the bottom of the wells (14). As shown in Fig. 4A, HUVEC cultured on vitrogen-coated electrodes responded to blocking anti-VEGF antibodies with a rise in electrical resistance; in contrast, addition of VEGF decreased the electrical resistance, and this phenomenon did not occur when cells were pretreated with an inhibitor of endothelial nitric oxide synthase (nitro-L-arginine methyl ester), consistent with the previous data on nitric oxide production in response to VEGF (14). In coculture experiments, GEC, 48 h after plating, were removed by repeated cycles of freezing-thawing, and rat renal microvascular endothelial cells were plated on the GEC-conditioned extracellular matrix. In control experiments, endothelial cells were plated directly on the endothelial cell-conditioned matrix (Fig. 4B).

Application of 1–10 ng/ml VEGF-165 to the endothelial cell monolayers, kept in a VEGF-free medium for 12 h, resulted in the decline of the electrical resistance. Impedance analysis of endothelial cells grown on GEC-conditioned extracellular matrix (similar results were obtained in coculture) showed that, when neutralizing antibodies against VEGF were added and their effect was compared with that of VEGF-165, a sharp dissociation of curves occurred at 45 min and reached the plateau 2–4 h after application of these agents; the resistance of monolayers treated with the neutralizing antibody showed a gradual increase by 20 ± 4%, whereas that of VEGF-treated cultures showed a decrease in electrical resistance by 17 ± 5% (n = 3 each in triplicate; P < 0.05; Fig. 4B). Changes in the capac-

![Fig. 3. Transmission electron micrographs (TEM) of control (A–B) and VEGF-treated (C–F) human umbilical vein endothelial cells (HUVEC). A: low magnification showing the cell nucleus (N) and surrounding cytoplasm. Bar, 1 μm. B: high magnification showing caveolae at the basal plasma membrane (large arrowhead) and cytoplasm (small arrowhead). Bar, 200 nm. C: as early as 10 min after VEGF treatment, an increase in the number and accumulation of caveolae structures at the basal cytoplasm is observed (arrowheads). Bar, 200 nm. D: after 30 min of VEGF treatment, a higher number of caveolae at the basal plasma membrane (large arrowhead) and accumulation of fused caveolae within the cytoplasm (small arrowheads) are observed. Bar, 200 nm. E: moreover, around the perinuclear area, a group of high-density caveolae could be noticed, and some of these organelles make contacts, fuse, and form tubulovesicular-like structures (arrowheads). Bar, 200 nm. F: after 60 min of VEGF treatment, the burst of caveolae formation has subsided, and internalized uncoated vesicular structures are observed (arrowheads). Bar, 200 nm.](http://ajpcell.physiology.org/)
VEGF, CAVEOLAR DYNAMICS, AND ENDOTHELIAL PERMEABILITY

Table 1. Morphometry of uncoated vesicular organelles in VEGF-treated HUVEC and RMVEC

<table>
<thead>
<tr>
<th>VEGF, min</th>
<th>No. of Uncoated Vesicular Organelles/Area n/μm²</th>
<th>Diameter of Uncoated Vesicular Organelles, nm</th>
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<tbody>
<tr>
<td>HUVEC</td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>0.268 ± 0.024</td>
<td>69.78 ± 2.50</td>
</tr>
<tr>
<td>10</td>
<td>0.556 ± 0.032*</td>
<td>67.22 ± 2.04</td>
</tr>
<tr>
<td>30</td>
<td>0.598 ± 0.037*</td>
<td>70.92 ± 2.09</td>
</tr>
<tr>
<td>60</td>
<td>0.657 ± 0.036†</td>
<td>121.90 ± 3.17†</td>
</tr>
<tr>
<td>180</td>
<td>0.793 ± 0.054*</td>
<td>120.40 ± 5.04*</td>
</tr>
<tr>
<td>RMVEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.416 ± 0.036</td>
<td>73.85 ± 4.17</td>
</tr>
<tr>
<td>10</td>
<td>0.441 ± 0.030</td>
<td>70.38 ± 3.42</td>
</tr>
<tr>
<td>30</td>
<td>0.667 ± 0.032†</td>
<td>69.54 ± 3.40†</td>
</tr>
<tr>
<td>60</td>
<td>0.779 ± 0.041†</td>
<td>122.90 ± 4.06†</td>
</tr>
<tr>
<td>180</td>
<td>0.813 ± 0.049*</td>
<td>120.10 ± 6.89†</td>
</tr>
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</table>

Results are derived from 3 independent experiments and are expressed as means ± SE. Data on the no. and diameter of uncoated vesicular organelles obtained by transmission electron microscopy (TEM). Human umbilical vein endothelial cells (HUVEC) and renal microvascular vein endothelial cells (RMVEC) were treated with vascular endothelial growth factor (VEGF) for 0 (control), 10, 30, 60, and 180 min. Digitized TEM pictures were analyzed as described in MATERIALS AND METHODS. Uncoated vesicular organelles were defined as caveolae (see Fig. 3D) and larger uncoated, membrane-bounded vesicles (see Fig. 3F). For determining the no. of uncoated vesicular organelles (n)/area, 15 cells were photographed in randomly selected fields for each experimental variable, and from these cells 40 uncoated vesicular organelles were randomly selected and manually traced to determine the diameter. The diameter of the uncoated vesicular organelles (in nm) determined by the equation (length + breadth)/2. Notice the significant difference between the control (0 min) and treated cells, as indicated (*P < 0.01). †P < 0.05, significant difference at different time intervals. Regarding the number of uncoated vesicular organelles per area in HUVEC, a significant difference was observed between 30 and 60 min after treatment with VEGF. Significance determined with the Mann-Whitney two-sided U-test.

Intravital imaging of GFP-caveolin-1 and the distribution of caveolae. To visualize the dynamics of caveolae, we constructed a chimeric caveolin-1-green fluorescent protein vector and transiently transfected endothelial cells with this construct. Using this approach, treatment of HUVEC and RMVEC with VEGF-165 (10 ng/ml) did not affect the intensity of fluorescence and its planar distribution, but the three-dimensional distribution of GFP-caveolin underwent a striking reorganization. Z-reconstruction of confocal images showed that VEGF resulted in a reversible formation and elongation of cell-spanning structures oriented between the apical and basal cell surfaces, resembling previously described vesiculovacuolar organelles (Fig. 5, A–C). Cell-spanning GFP- and caveolin-1-decorated structures appeared hollow, and their average length increased twofold as early as 10 min after the application of VEGF-165. Incubation of transfected HUVEC with Texas red-conjugated horseradish peroxidase

Fig. 4. Electrical resistance and capacitance of endothelial cells. A: typical changes in the electrical resistance of HUVEC monolayers cultured on vitrogen-coated microelectrodes after application of blocking anti-VEGF antibody, VEGF-165, or nitro-L-arginine methyl ester (l-NAME). Arrow depicts the time of additions. A/b, antibody. B: normalized impedance of renal microvascular vein endothelial cells (RMVEC), cultured on GEC-conditioned matrix, increased after application of neutralizing VEGF antibodies and decreased after addition of 10 ng/ml VEGF-165. Untreated cells showed no changes in electrical resistance. Arrow depicts the time of VEGF additions. The same results were obtained in 3 separate experiments. C: changes in electrical capacitance of RMVEC treated with 10 ng/ml VEGF-165 (recorded simultaneously with electrical resistance, shown in B). Note the VEGF-induced increase in capacitance, consistent with the observed amplification of the cellular area of the lipid bilayers.
(HRP) showed no significant incorporation of the tagged probe into HUVEC (data not shown). After application of 10 ng/ml VEGF (30 min), this fluorescent probe was found entangled in the network of GFP-caveolin, as demonstrated by intravital confocal fluorescence microscopy of dual-labeled cells (Fig. 6, A and B). These data indicate that HRP is readily incorporated into the vesiculo-vacuolar structures decorated with GFP-caveolin after stimulation of endothelial cells with VEGF, strongly suggesting that these convoluted channel-like structures are permeable to the macromolecules.

When microscopic analysis of endothelial cells grown on matrigel was performed 36 h after VEGF-165 treatment or when endothelial cells were cocultured with GEC, two distinct patterns were observed, i.e., VEGF-treated HUVEC showed an elaborate capillary-like network at the light microscopic level (Fig. 7A), and TEM examination in these areas of attenuated cytoplasm revealed diaphragmed fenestrae (Fig. 7B). These findings were reproducible in RMVEC treated with VEGF-165 (data not shown). Long-term electrophysiological studies revealed that, 24–36 h after VEGF treatment, the electrical resistance of HUVEC grown on matrigel-coated microelectrodes decreases with the concomitant increase in the capacitance of confluent monolayers (Fig. 7, C and D), both findings consistent with the observed morphological changes. In the coculture system, vacuolar structures and diaphragmed fenestrae were detectable in RMVEC (Fig. 8).

Possible relevance to the ultrastructure of glomerular endothelial cells. To compare the above observations made in cell culture with the ultrastructure of normal glomerular endothelial cells, normal rat kidney sections were examined using EM in conjunction with immunogold labeling of VEGF. As shown in Fig. 9, gold-labeled VEGF was detectable in the podocytes and the glomerular basement membrane, further strengthening the idea of a paracrine action of the GEC-produced and matrix-deposited VEGF on glomerular endothelial cells.

**DISCUSSION**

Previous immunohistochemical studies have provided solid evidence of VEGF production by the podocytes (4, 7, 11, 24). The functional role of this phenomenon, however, remained obscure. Considering the intensity of ultrafiltration taking place in the glomer-
ular capillaries, it was difficult to reconcile it with the possible action of VEGF, produced by podocytes, on the target endothelial cells located upstream; the direction of flow should have made such a paracrine activity a futile one. Two sets of observations made in the cultured cells and in the rat kidney serve to reconcile this controversy. First, the production of all four splice variants of VEGF, three of which are heparan sulfate-binding, by cultured GEC suggests the possibility of VEGF deposition into the basement membrane. Second, immunoelectron microscopy of VEGF distribution in the rat glomerulus showed gold labeling in association with the podocytes and glomerular basement membrane.

Rat VEGF gene contains eight exons, and VEGF-188 incorporates all eight exons, VEGF-164 lacks exon 6, and VEGF-120 lacks exons 6 and 7. The major known functional difference among the various VEGF isoforms is their ability to bind to heparin and heparan sulfate proteoglycans distributed on cellular surfaces and within extracellular matrixes and basement membranes, and it is believed that this ability is imparted in the rat glomerulus showed gold labeling in association with the podocytes and glomerular basement membrane.

Fig. 6. Serial confocal images of a HUVEC expressing GFP-caveolin-1 in the presence of Texas red-horseradish peroxidase (HRP). Application of VEGF to HUVEC transfected with GFP-caveolin-1 results in the gradual incorporation of Texas red-HRP into tubulovesicular organelles. Confocal microscopy was performed as described in MATERIALS AND METHODS. Images were obtained from the same visual field 30 min after VEGF stimulation with 0.2-μm distances between each consecutive frame. No incorporation was detectable in the absence of VEGF (data not shown). Green, GFP-caveolin-1; red, HRP. A and B, planar and transverse optical sections, respectively. White line shows the site of transverse optical sectioning.

Fig. 7. Long-term effect of VEGF resulted in the formation of fenestration of endothelial cells. A: HUVEC were cultured on matrigel for 36 h in the presence of 10 ng/ml VEGF-165. Light microscopic image shows an elaborate capillary-like network formed under these conditions. B: TEM micrograph shows diaphragmed fenestrae (arrowheads). C and D: changes in electrical resistance and capacitance, respectively, in HUVEC treated with 10 ng/ml VEGF-165 (control cells were deprived of VEGF), demonstrating concomitant decrease in resistance and increase in capacitance compared with control. One-way ANOVA of experimental and control data in B and C showed that these curves are significantly different at P < 0.05.
mainly by exon 6. Addition of the highly cationic 24-amino-acid residue sequence encoded by this exon promotes even tighter binding of VEGF-188 to these endogenous polyanions. The fact that GEC express mRNA for the soluble secretory form VEGF-120, and for the soluble matrix-associable VEGF-164 and insoluble, heparin-binding matrix-associable VEGF-188 and VEGF-205 suggests that glomerular basement membrane may be the site of VEGF accumulation and storage. Furthermore, at least the VEGF-188 isoform requires urokinase for full activation, thus making the regulation of this potential paracrine mechanism even more complex (17). If VEGF-188 is indeed deposited in the glomerular basement membrane, its activation should occur in the vicinity of capillary endothelial cells producing urokinase, thus providing further spatial selectivity of VEGF action. The latter observation on the diversity of GEC-produced VEGF isoforms is not limited to the cell culture system; recent RT-PCR findings by Kretzler et al. (11) revealed the similar profile of VEGF splice variants in single aspirated podocytes obtained from microdissected mouse glomeruli. This imparts further benefits to the established immortalized GEC as a model to study VEGF production and its regulation.

To investigate the potential for paracrine VEGF signaling, we have analyzed the following two coculture systems: a sandwich GEC-collagen-RMVEC system and RMVEC plated on the GEC-deposited and conditioned extracellular matrix. Endothelial cell permeability was studied directly using a highly sensitive measurement of electrical resistance. These studies showed that the application of the neutralizing anti-VEGF antibodies increases the resistance of endothelial monolayers grown either in the sandwich configuration or on the surface of GEC-conditioned matrix, whereas the addition of VEGF to renal microvascular endothelial cells cultured in the absence of this growth factor resulted in the decline of electrical resistance. These data are consistent with VEGF or GEC-conditioned extracellular matrix serving to increase the permeability of endothelial cells.

Fig. 8. Long-term sandwich coculture of RMVEC with GEC: TEM characteristics. A: typical TEM composite image of RMVEC monolayer. B and C: RMVEC were treated with 10 ng/ml VEGF-165. Note that tight junctions are preserved (B), and rare fenestrae appear (arrowheads in C). D: RMVEC (EC) were cocultured with GEC (sandwich culture, see MATERIALS AND METHODS for details) for 36 h in the absence of exogenous VEGF. Note formation of vacuoles and fenestrae in endothelial cells.

The morphological route(s) for the VEGF-induced increase in endothelial permeability has been suggested (6, 8, 20, 21). Palade and colleagues (16) consider caveolae as plausible structures involved in the increase in endothelial permeability. Indeed, some investigators argued that caveolae, if studied by serial sectioning, extend far beyond the plasma-membrane vesicles (5) to form extensive invaginations. However, differences in techniques for serial sectioning and the choice of fixation protocols have been incriminated in the variability of findings (22, 23). In an attempt to resolve some of the existing problems in reconstructing the three-dimensional organiza-
tion of caveolae, we have generated a GFP-caveolin-1 vector to enable intravital microscopy of endothelial cells subjected to VEGF. Although fluorescence microscopy of transfected endothelial cells did not reveal significant changes in the distribution of GFP-caveolin, confocal microscopy disclosed that the probe is decorating transcellular channel-like structures that become conspicuous after exposure to VEGF. These data demonstrate, for the first time in vivo, that caveolin is organized into elongated cell-spanning structures in cells exposed to VEGF. EM studies confirmed and further extended these observations by demonstrating the enrichment in caveolae, their fission, and fusion after application of VEGF. An alternative route for increased permeability via fenestrae could not be detected in HUVEC or RMVEC at early times after application of VEGF. However, 36 h after addition of VEGF-165, HUVEC and RMVEC exhibited diaphragmed fenestrae. Furthermore, RMVEC cocultured with GEC (sandwich culture), in the absence of exogenous VEGF, showed vacuolation and fenestration, phenomena that have recently been associated with capillary remodeling and lumen formation (3). In a coculture model of adrenal capillary endothelial cells and choroid plexus epithelium, as well as in endothelial cells treated with 50–100 ng/ml VEGF-165, Esser and coworkers (7) were able to detect fenestrae only 24 h after the treatment. The same authors consistently observed fission and fusion of caveolae shortly after VEGF treatment. Vasilie and coauthors (26) have recently provided additional evidence of VEGF-induced clustering of caveolae, resulting in formation of vesiculovacuolar organelles in bovine microvascular endothelial cells cultured on floating matrigel-collagen gels. It is conceivable that VEGF elicits a rapid increase in vascular permeability via mobilization of caveolae, whereas the long-term effect requires formation of fenestrae. Recent demonstration of two VEGF receptors, neuropilin-1 and fetal liver kinase-1, in developing and mature glomerular capillaries further supports the idea of paracrine signaling from GEC to endothelial cells (18, 19). Collectively, the development of GEC-endothelial cell coculture systems and data obtained using intravital confocal microscopy techniques support the hypothesis that VEGF deposited in the basement membrane immediately acts upon endothelial cells by remodeling caveolae, elongating vesiculovacuolar structures and increasing endothelial permeability. The long-term effect of VEGF, however, results in the formation of fenestrae. The observed time course of VEGF action on endothelial cells may explain why caveolae are so sparse in glomerular endothelial cells in vivo. On the other hand, these data suggest that the unique ultrastructure of these cells is determined by their microenvironment rather than by the inherent propensity of the glomerular endothelial cells to form diaphragmed fenestrae. This particular feature of the endothelium may relate to changes in glomerular permeability after damage to podocytes (2, 10).

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