Expression and endocytosis of VEGF and its receptors in human colonic vascular endothelial cells

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1Department of Surgery, School of Medicine, University of California, San Francisco, California 94143-0790; and 2Department of Microbiology and Immunology, School of Medicine and the Walther Oncology Center, Indiana University, Indianapolis, Indiana 46202

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Wang, Dongfang, Richard E. Lehman, David B. Donner, Mary R. Matli, Robert S. Warren, and Mark L. Welton. Expression and endocytosis of VEGF and its receptors in human colonic vascular endothelial cells. Am J Physiol Gastrointest Liver Physiol 282: G1088–G1096, 2002.—Normal human colonic microvascular endothelial cells (HUCMEC) have been isolated from surgical specimens by their adherence to Ulex europaeus agglutinin bound to magnetic dynabeads that bind α-L-fucosyl residues on the endothelial cell membrane. Immunocytochemistry demonstrated the presence of a range of endothelial-specific markers on HUCMEC, including the von Willebrand factor, Ulex europaeus agglutinin, and platelet endothelial cell adhesion molecule-1. The growing cells form monolayers with the characteristic cobblestone morphology of endothelial cells and eventually form tube-like structures. HUCMEC produce vascular endothelial growth factor (VEGF), and express the receptors, kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase, through which VEGF mediates its actions in the endothelium. VEGF induces the tyrosine phosphorylation of KDR and a proliferative response from HUCMEC comparable to that elicited from human umbilical vein endothelial cells (HUVEC). On binding to HUCMEC or HUVEC, 125I-labeled VEGF internalizes or dissociates to the medium. Once internalized, 125I-labeled VEGF is degraded and no evidence of ligand recycling was observed. However, significantly less VEGF is internalized, and more is released to the medium from HUCMEC than HUVEC. Angiogenesis results from the proliferation, viability, and migration of endothelial cells in vitro and increases vascular permeability and angiogenesis in vivo (6, 11–12, 14–16, 46). In addition, VEGF stimulates glucose uptake and the production of tissue factor, collagenase, and plasminogen activators and inhibitors by endothelial cells. VEGF receptors are largely restricted to endothelial cells. Only a few nonendothelial cell types bind VEGF (1–3, 32, 33).

Two homologous, high-affinity VEGF receptors, kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase (Flt-1), have been identified on human endothelial cells (8, 34, 35, 38, 39, 47), including human umbilical vein endothelial cells (HUVEC) (56), gastric endothelial cells (30), and human dermal microvascular endothelial cells (HDMEC) (24). KDR and Flt-1 consist of ~1,300 amino acid residues. The receptors are composed of an extracellular region containing seven immunoglobulin-like loops, a short membrane-spanning sequence, and an intracellular, split tyrosine kinase (38, 44, 49). Binding of VEGF to KDR or Flt-1 induces receptor autophosphorylation, although activation of Flt-1 can be difficult to detect, probably due to its relative nonabundance and weak tyrosine kinase activity (38, 55). VEGF binding and receptor activation induce tyrosine phosphorylation of cytoplasmic signaling proteins that contain SH2 domains (22). These proteins allow KDR and Flt-1 to communicate with signaling pathways that promote responses to VEGF.

Numerous cell surface receptors undergo endocytosis after formation of a ligand-receptor complex. An acidic environment in endosomes promotes dissociation of ligand/receptor complexes. Subsequently, the ligand, the receptor, or both, can recycle to the cell surface or undergo degradation in lysosomes (7, 37, 53). Rela-
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...tively little is known of the mechanisms through which endothelial cells process VEGF. In one study, Dougher and Terman (9) found that VEGF is internalized by bovine aortic endothelial cells and human embryonic kidney epithelial cells transfected with KDR and that KDR activation is required not only for the induction of cellular responses but for receptor internalization and signal termination as well (9). However, considerable heterogeneity exists, both structurally and functionally, among endothelial cells from different organs and between the endothelium in large vessels and microvessels. Endothelial cells may differ in morphology, endothelial markers, response to growth factors, basal release of endothelial cell-derived factors, and even susceptibility to invasion by metastatic tumor cells (36). Thus it is important to identify the characteristics of the endothelial cells in specific organs that perform distinct processes and are subject to attack by distinct pathologies.

In a previous study with human large-vessel endothelial cells (HUVEC), we found that VEGF induced downregulation of both VEGF receptors (56). However, concomitant with downregulation of the receptor proteins, the mRNAs for both receptors were upregulated. These observations indicated that as VEGF action is important to endothelial cell survival (57), these cells are protected against inappropriate or prolonged loss of VEGF receptors (56). These results illustrate the importance of VEGF receptor expression to the endothelium and suggest that how endothelial cells treat VEGF would be comparably important.

As a first step in a long-term study of angiogenesis in colon cancer and inflammatory diseases, microvascular endothelial cells from human colon (HUCMEC) were isolated. HUCMEC express VEGF, KDR, and Flt-1 and are responsive to VEGF. Comparative studies show that HUCMEC and HUVEC process VEGF differently. Rather than internalizing and degrading most VEGF that binds the cells, HUCMEC release intact, active VEGF to the medium where it can support autocrine and paracrine mechanisms.

MATERIALS AND METHODS

Reagents. Collagenase type II was from Worthington Biochemical (Freehold, NJ). Ulex europaeus agglutinin-1 (UEA-1) unconjugated and conjugated with fluorescein isothiocyanate for 2 h at room temperature. The beads were collected with a magnet, washed three times for 15 min at 4°C with Hank’s balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS), and finally washed overnight. The beads were collected with a magnet and suspended at a 4 × 10⁸ beads/ml in HBSS containing 5% FBS.

Macroscopically normal colon was taken from the proximal margin of colectomies performed for malignancy. After mesentery was removed, the remaining tissue was placed in ice-cold PBS containing 10,000 U/ml of penicillin, 10,000 µg/ml streptomycin sulfate and 25 µg/ml of amphotericin B, and cut into ~5-mm cubes. The diced tissue was pelleted, suspended in 0.25% collagenase type II in PBS, and incubated at 37°C for 30–60 min while being shaken. Digested tissue was washed twice with HBSS/5% FBS, suspended in endothelial growth medium (EGM) (Clonetics, CA), and filtered through a 70-µm cell strainer (Falcon, Oxnard, CA). The mixed cell population was seeded into T-75 flasks coated with 2% gelatin and maintained at 37°C in 5% CO₂ for 24 h. Adherent cells were washed three times with HBSS/5% FBS and cultured in culture for 2–5 days. To isolate endothelial cells, the cells were trypsinized to allow resuspension and added to 180 µl HBSS/5% FBS, mixed with 20 µl of UEA-1 conjugated beads, and shaken for 15 min at room temperature. The beads were added in excess to give a ratio of ~20 beads per cell. The cell-bead mixture was suspended in 5 ml HBSS/5% FBS and gently agitated. Beads with attached endothelial cells were concentrated by adherence to a magnet during a collection process that proceeded for at least 1 min. The beads, with the attached endothelial cells, were washed with 5 ml HBSS/5% FBS, a process repeated at least three times. The beads were suspended in EGM, seeded into T-75 flasks coated with 2% gelatin, and grown in a 37°C 5% CO₂ humidified incubator. HUCMEC were studied between passages 2 and 5. HUVEC isolated by the method of Jaffe et al. (31) were cultured under the same conditions as HUCMEC.

Immunohistochemistry. Endothelial cells were cultured in PBS containing 5% normal goat serum and 0.1% saponin for 30 min, and then incubated for 16 h at 4°C with a mixture of antibodies to vWF (2 µg/ml), PECAM-1 (200 µg/ml), and CK5. The cells were washed and incubated for 2 h at room temperature with secondary antibodies conjugated to fluorescein isothiocyanate. To demonstrate Dil-Ac-LDL uptake, living cells were incubated for 16 h at 37°C with EGM containing 10 µg/ml Dil-Ac-LDL. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. To demonstrate UEA-1 binding, cells were fixed by 1% paraformaldehyde in PBS for 10 min at room temperature and incubated with 20 µg/ml fluorescein-labeled UEA-1 in PBS/5% normal goat serum for 16 h at 4°C. To identify endothelial cells in tissues, segments of human colon were fixed by incubation with 4% paraformaldehyde in PBS (pH 7.4) for 16 h at 4°C. Frozen sections of 10 µm were prepared. Slides were incubated with PBS/5% normal goat serum/0.3% Triton X-100 for 30 min and then incubated with anti-vWF in PBS/5% normal goat serum/0.3% Triton X-100 for 16 h at 4°C. Slides were washed and incubated with an anti-mouse IgG conjugated to fluorescein isothiocyanate for 2 h at room temperature. Endothelial cells were also detected by incubating colon sections with UEA-1 conjugated to rhodamine or fluorescein for 16 h at 4°C.

Assays for VEGF. For Northern blot analysis, total RNA was extracted from near-confluent HUCMEC using RNAzol (Tel-Test, Friendswood, TX). RNA was separated by electrophoresis through 1% agarose-formaldehyde and transferred to nylon membranes. Blots were hybridized to the full-length human VEGF cDNA labeled with [³²P]dCTP. For assay of...
VEGF by ELISA, cells were seeded into 10-cm dishes and cultured for 3 days in EGM. Media were collected, centrifuged at 1,200 rpm for 15 min at 4°C, and the supernatant was assayed for VEGF using a Quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, MN).

Assay of KDR and Flt-1 mRNA by RT-PCR. Total RNA was isolated from HUCMEC or HUVEC, as described above. SuperScript reverse transcriptase and random hexamers (GIBCO-BRL, Gaithersburg, MD) were used to synthesize cDNA. The PCR reaction was carried out in a Perkin-Elmer DNA thermal cycler. For KDR, the sense primer was 5'/H11032 AC(GTGA)ATG(TACGGTCTAT3' and the antisense primer was 5'/H11032 TTCCCAT(TTGTCGGCATCATA3'. For Flt-1, the sense primer was 5'/H11032 GCAAGGTGTGACTTTTGTTC3' and the antisense primer was 5'/H11032 AGGATTTCTTCCCCTGTGTA3'.

125I-labeled VEGF binding. Recombinant human VEGF was labeled to a specific activity of 167,000 counts·min⁻¹·ng⁻¹ using Iodo-Gen (17). For binding assays, duplicate nearly confluent cultures (3.8 cm²) in binding buffer (HBSS, 25 mM HEPES, pH 7.4, 1 mg/ml BSA) were incubated with 125I-labeled VEGF (1–400 pM) in the absence or presence of a 100-fold excess of VEGF for 6 h at 4°C. Binding of 125I-labeled VEGF by itself is defined as total binding. Binding of 125I-labeled VEGF in the presence of excess VEGF is defined as nonspecific binding. The difference between the total and nonspecific radioactive uptake is specific receptor binding. Cultures were washed with binding buffer and solubilized into 0.1 N NaOH; radioactivity was assayed in a gamma counter. Data were analyzed by the method of Scatchard (42).

KDR phosphorylation. HUCMEC were seeded into 6-cm tissue culture plates. Confluent cells were starved overnight in serum-free 1:1 F12-MEM, stimulated with 50 ng/ml human recombinant VEGF165, and lysed into 0.5 ml lysis buffer of (in mM): 50 HEPES, 150 NaCl, 1 EGTA, 50 NaF, 1 PMSF, 2 Na₃VO₄, plus 1% Triton X-100, 10% glycerol, 1 mg/ml aprotinin, and 1 mg/ml leupeptin. Lysates were centrifuged (10,000 g, 5 min), and the supernatants were incubated with 4 g/ml of rabbit anti-KDR (kindly provided by Dr. Harald App, Sugen, CA) for 16 h at 4°C and then with 20 µl of protein G Sepharose (Pharmacia) for 4 h. Sepharose/immune complex conjugates were collected by centrifugation, washed three times with lysis buffer, and boiled for 3 min in 40 µl of 2X sample buffer. After centrifugation, proteins in the supernatants were fractionated by electrophoresis on 7.5% polyacrylamide gels and transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham). The membranes were blocked with 3% milk powder-TBS [20 mM Tris·HCl (pH 7.6) and 137 mM NaCl] for 1 h at room temperature. Monoclonal phosphotyrosine antibody PY-20 (1:1,500) and goat anti-mouse IgG HRP conjugate (1:2,000) were used to detect phosphotyrosine.

VEGF trafficking. 125I-labeled VEGF165 internalization, release, degradation, and recycling were assayed as described (4, 13) with modifications. Briefly, 1 × 10⁵ HUVEC or...
HUCMEC were seeded into 24-well plates and cultured overnight. After washing with serum-free MEM, the cells were incubated with 400 pM 125I-labeled VEGF in serum-free MEM/0.1% BSA under the conditions indicated in the figure legends. The cells were washed three times with ice-cold PBS (pH 7.4) or MEM. Surface-bound 125I-labeled VEGF was dissociated by incubation of cells in ice-cold pH 2-PBS (2 times, 1 min for each dissociation cycle) and then assayed in a gamma counter. Internalized 125I-labeled VEGF was measured by dissociating cell surface-bound ligand and then assaying the remaining radioactivity in cells solubilized into 0.2 N NaOH. For assessing the integrity of 125I-labeled VEGF released from cells to the medium, cells were washed three times with ice-cold, serum-free MEM and then cultured in serum-free MEM at 37°C. At the indicated times, medium was collected, and trichloroacetic acid and BSA were added to final concentrations of 10 and 0.1%, respectively. Intact 125I-labeled VEGF precipitated in the trichloroacetic acid, whereas degraded 125I-labeled VEGF did not. After centrifugation (15,000 g, 10 min), degraded 125I-labeled VEGF was in the supernatant, and intact 125I-labeled VEGF was in the precipitate.

RESULTS

Isolation and characterization of colonic endothelial cells. We tested whether HUCMEC bind UEA-1 by analyzing sections of human colon incubated with fluorescein-labeled UEA-1. Fluorescein-labeled UEA-1 bound to endothelial cells of capillaries, venules, and arterioles throughout the submucosa, muscularis, and subserosa (Fig. 1A). To confirm that cells labeled with UEA-1 were endothelial cells, sections were incubated with vWF antibody. Fig. 1, B-D, illustrates that cells that interacted with UEA-1 also interacted with anti-vWF. Thus UEA-1 binds specifically to HUCMEC, suggesting the utility of UEA-1-coated magnetic beads for the isolation of such cells.

To isolate HUCMEC, cells from normal human colon were incubated with UEA-1-coated magnetic beads. The beads bound endothelial cells (Fig. 2A), allowing isolation of $1 \times 10^6$ endothelial cells/g of tissue. When these cells were cultured and grown to confluence, they displayed a “cobblestone” morphology characteristic of endothelial cells (Fig. 2B). The isolated cells were assayed for endothelial-specific properties. Fluorescein-labeled UEA-1 bound the plasma membrane of isolated HUCMEC (Fig. 2C). Strong surface fluorescence continued to be detected on HUCMEC through at least eight passages. Antibodies to vWF stained perinuclear vesicles in HUCMEC (Fig. 2D), and anti-PECAM-1, an integral membrane glycoprotein located at the intercellular junctions of endothelial cells (26), reacted with the cell surface (Fig. 2E). Ac-LDL labeled with 1,1’dioctadecyl-3,3,3’,3’-tetramethylindo-carbocyanine-perchlorate was taken up by HUCMEC and concentrated in discrete intracellular granules (Fig. 2F). After extended culture, HUCMEC spontaneously formed tube-like structures, a process that requires cell migration and proliferation (data not shown). Our observations show that HUCMEC retain various
endothelial cell properties after isolation from human colon.

HUVEC tested positively for each of the endothelial cell-specific properties displayed by HUCMEC and KNRK cells (rat kidney epithelial cells) negatively (data not shown). On the other hand, HUCMEC and HUVEC did not recognize an antibody to CK5, whereas KNRK cells were stained (data not shown). These results exclude the possibility that HUCMEC were contaminated with mesothelial or epithelial cells.

**VEGF expression.** We examined HUCMEC for expression of VEGF mRNA by Northern blot analysis and VEGF activity by ELISA. A transcript of 4.4 kb, which corresponds to the size of VEGF mRNA in colon carcinoma cell lines, was detected in HUCMEC from three patients (Fig. 3). VEGF mRNA was also detected in HUVEC. An ELISA of medium conditioned by HUCMEC for 72 h contained 401.4 ± 120.8 pg/ml (range 100–900 pg/ml) of VEGF. Thus cultured HUCMEC express and secrete VEGF.

**VEGF receptor expression.** VEGF receptors were detected by receptor binding assays with 125I-labeled VEGF. Scatchard analysis of saturation binding assays yielded curvilinear plots. The binding resolved into high affinity (K_D 130 ± 14 pM; 79,900 ± 6,350 sites/cell) and lower affinity (K_D 675 ± 66 pM; 231,300 ± 27,900 sites/cell) sites (Fig. 4A). These values are comparable to those obtained in studies with HUVEC (55).

RT-PCR was performed to determine whether the binding assays resulted from expression of KDR and Flt-1 by HUCMEC. Amplification with KDR and Flt-1-specific primers generated a 1,100 bp and a 510 bp product, respectively, from HUCMEC cDNA (Fig. 4B). The identity of the products from RT-PCR was confirmed by sequence analysis.

**Receptor function.** An MTT assay showed that VEGF augmented HUCMEC proliferation about twofold after 2 days of incubation (data not shown), a value comparable to that elicited from HUVEC by VEGF, which we reported previously (57). Incubation of HUCMEC with 50 ng/ml VEGF transiently augmented the tyrosine phosphorylation of KDR fourfold after a 10-min incubation at 37°C (Fig. 4C). As in our previous studies with HUVEC, Flt-1 tyrosine phosphorylation was undetectable. However, the antibodies to Flt-1 presently available are not recommended for immunoprecipitation, and Flt-1 is less well expressed by endothelial cells than KDR/Flik1.

**VEGF processing.** The time courses of 125I-labeled VEGF internalization, degradation, recycling, and release by HUCMEC, and HUVEC, were analyzed. In one method, VEGF endocytosis was determined by incubating 125I-labeled VEGF with cells at 4°C for 2 h (Fig. 5, A and C). At low temperature, internalization and degradation do not take place; consequently, 125I-
labeled VEGF accumulates at the cell surface. A shift of temperature to 37°C initiated internalization indicated by the inability of an acid wash to release 125I-labeled VEGF to the medium. Also, HUVEC or HUCMEC were incubated with 125I-labeled VEGF at 37°C (Fig. 5, B and D), allowing internalization to initiate immediately. Incubation of HUVEC with 125I-labeled VEGF at 4°C, followed by a temperature shift to 37°C, resulted in maximal internalization after 20 min. At this time, 59% of the 125I-labeled VEGF was internalized. Incubation of HUVEC with 125I-labeled VEGF at 37°C led to maximal internalization after 30 min, at which time 57% of the 125I-labeled VEGF was internalized (Fig. 5, top). In experiments with HUCMEC (Fig. 5, bottom), maximal internalization after a temperature shift to 37°C occurred after 20 min; incubation initiated at 37°C resulted in peak internalization after 30 min. Thus the time courses over which HUVEC and HUCMEC internalize 125I-labeled VEGF are comparable. However, HUCMEC internalized only about 15% of the bound 125I-labeled VEGF, a much smaller fraction than HUVEC (nearly 60%).

Release of 125I-labeled VEGF to the medium, and its integrity, assayed by SDS-PAGE, was determined. Cells were incubated with 125I-labeled VEGF for 1 h at 20°C to allow binding and internalization before the temperature was shifted to 37°C (Fig. 6, A and B). The rationale for incubation at 20°C during the first phase of incubation was based on the observation that after incubation at 20°C, internalized 125I-VEGF accumulates but is not degraded (13). After 90 min of incubation at 37°C, ~22% of 125I-labeled VEGF bound to HUVEC was released, 47% was internalized, and 20% was degraded. 125I-labeled VEGF on the surface of HUCMEC declined rapidly during incubation at 37°C. This resulted from dissociation of intact 125I-labeled VEGF to the medium rather than internalization and degradation. After 90 min, ~80% of the 125I-labeled VEGF was released, 7% was internalized, and 3% was degraded by HUCMEC. Thus HUVEC degrade more and release less 125I-labeled VEGF than HUCMEC.

In some cell types, the half time for recycling of most membrane components is 10–30 min (37). Thus 60 min of incubation with 125I-labeled VEGF would be sufficient to determine whether internalized 125I-labeled VEGF recycles. 125I-labeled VEGF was bound to HUVEC or HUCMEC at 4°C before the temperature was shifted to 37°C. Surface-bound or internalized 125I-labeled VEGF was then assayed (Fig. 6, C and D). HUVEC internalized a greater proportion of the 125I-labeled VEGF than HUCMEC. There was no evidence...
for recycling of internalized $^{125}$I-labeled VEGF back to the surface of HUVEC or HUCMEC.

**DISCUSSION**

Endothelium is heterogeneous, and cells isolated from this tissue can be distinguished based on the vessel size and organ within which the vessel resides. Biological characteristics of large-vessel vascular endothelial cells, such as HUVEC, may differ significantly from microvascular endothelial cells. Microvascular endothelial cells sprout from preexisting blood vessels during angiogenesis and are of particular importance to this process (41). We sought to isolate human microvascular colonic endothelial cells (HUCMEC), first for comparison with large-vessel endothelial cells and eventually for the study of angiogenesis in cancer and inflammatory diseases of the colon. It was not possible to isolate HUCMEC by Percoll gradient fractionation or weeding techniques. Magnetic beads coated with the lectin UEA-1, which specifically binds $\alpha$-L-fucosyl residues on the endothelial cell membrane, were successfully used for isolation. This method has been employed for isolation of endothelial cells from stomach, lung, decidua, and the mammary gland (18, 25, 27, 28, 30). Using this procedure, HUCMEC were isolated from specimens of human colon that maintained morphological characteristics of endothelial cells and endothelial-specific properties.

Expression of VEGF receptors and responsiveness to VEGF are important to endothelial function (11, 15, 16, 38). To understand how VEGF is processed in HUCMEC, we characterized the VEGF receptors of these cells. Scatchard analysis yielded a curvilinear plot that indicated that HUCMEC express two high-affinity VEGF binding sites. These were demonstrated to be KDR and Flt-1 by RT-PCR. Treatment of HUCMEC with VEGF promoted tyrosine phosphorylation of KDR (Fig. 4C), which promotes the growth response of HUVEC (57). Functionality of KDR on HUCMEC was further indicated by a comparable twofold increase of HUVEC (57) and HUCMEC proliferation induced by VEGF.

Flt-1 tyrosine phosphorylation could not be detected in control or VEGF-treated HUVEC or HUCMEC. This may be due to the different properties of the KDR and Flt-1 tyrosine kinases, different susceptibilities of these receptors to dephosphorylation by phosphotyrosine phosphatases, or the low level of Flt-1 expression by endothelial cells (23, 38, 55).
EGF, FGF, insulin, IGF-II/mannose-6-phosphate, and glucagon receptors (7, 13, 53, 54) mediate ligand internalization. Internalized ligand may be degraded or recycled to the cell surface, from which it may dissociate or translocate to the nucleus (37, 54). Endocytosis regulates receptor number and cellular responsiveness to ligand, the protein composition of the plasma membrane, remodeling of the cell surface, and the delivery of nutrients into cells (20, 52). We found that internalization and degradation were the predominant mechanisms used by HUVEC to process VEGF. HUCMEC internalize and degrade comparatively little VEGF and release a significantly greater fraction of the mitogen to the medium. The mechanism that underlies the different mode of VEGF processing by HUVEC and HUCMEC is undefined. One possibility is that more VEGF binds to low-affinity heparin sulfate proteoglycans (21) on HUCMEC than on HUVEC. Such interaction might preserve VEGF for release and subsequent interaction with the cells. In support of this possibility are observations that the extracellular domain of KDR contains a binding site for heparin and that Flt-1 shows affinity for heparin (5, 10). However, binding of VEGF-A to Flt-1 and KDR is oppositely affected by heparin; interaction with the former receptor is diminished but binding to the latter is augmented (48). Also, whereas VEGF-A binding to Flt-1 is diminished by heparin, the activity of the receptor is increased (29). Thus the effect of heparin on VEGF binding and function is complex and difficult to assess.

Although endothelial cells from different organs present many common functional and morphological features, they also display remarkable heterogeneity. Even in the same organ, the endothelium of large and small veins and arteries exhibits significant heterogeneity (19). For example, α-thrombin stimulates urokinase production and DNA synthesis in human cerebral microvascular endothelial cells but not in HUVEC (43). Human cerebral endothelial cells respond to vasoactive intestinal polypeptide and endothelin-1 with an increase in intracellular free calcium, whereas omental endothelial cells do not (50, 51). In our study, HUVEC and HUCMEC demonstrated marked differences in VEGF endocytosis. HUCMEC internalize and degrade less and release more VEGF from the cell surface to the medium than do HUVEC, and importantly, released VEGF can be reutilized. These results are consistent with observations showing that VEGF produced by microvascular cells in the neonatal dermis and eyes plays a role in activation of the endothelium (45, 58). It is interesting that microvascular cells, the endothelial cell type involved in angiogenesis, preserve VEGF such that it can again stimulate the cells and, thereby, neovascularization through autocrine and paracrine mechanisms.

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