VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

A Recombinant Inhibitory Isoform of Vascular Endothelial Growth Factor_{164/165} Aggravates Ischemic Brain Damage in a Mouse Model of Focal Cerebral Ischemia

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Vascular endothelial growth factors (VEGF) are a Janus-faced family of growth factors exerting both neuroprotective and maladaptive effects on the blood–brain barrier. For example, VEGFs are beneficial in promoting postischemic brain angiogenesis, but the newly formed vessels are leaky. We investigated the role of the naturally occurring murine inhibitory VEGF isoform VEGF_{165b} in a mouse model of focal cerebral ischemia by middle cerebral artery occlusion and reperfusion (I/R) in male C57BL/6 mice. We investigated the roles of VEGF_{164/165} and VEGF_{165b} in both brain and nonbrain endothelial barrier, angiogenesis, and neutrophil migration using oxygen glucose deprivation and reoxygenation as *in vitro* model. We investigated the role of VEGF_{165b} in brain edema, neutrophil infiltration, ischemic brain damage, and neuronal death *in vivo* using an adenovirus encoding a recombinant VEGF_{164b} isoform. Neither VEGF_{164/165} nor VEGF_{165b} significantly altered brain endothelial barrier or angiogenesis *in vitro*. However, treatment of brain endothelial cells with VEGF_{165b} increased neutrophil migration *in vitro* and exacerbated stroke injury by aggravating neutrophil infiltration and neurodegeneration *in vivo*. Our results indicate that alterations in the delicate balance in the relative levels of pro- and antiangiogenic VEGF isoforms can result in either adaptive or detrimental effects, depending on the VEGF isoform levels and on the duration and extent of injury. (*Am J Pathol* 2013; 183: 1010–1024; <http://dx.doi.org/10.1016/j.ajpath.2013.06.009>)

Cerebral ischemia occurs when any blood vessel supplying blood to the brain is occluded by a thrombus or embolus. This usually results in the formation of an ischemic infarct comprising a dead necrotic core surrounded by a salvageable apoptotic penumbra.¹ The pathology of cerebral ischemia involves, but is not confined to, forms of apoptotic and/or necrotic cell death, severe inflammation, immune cell infiltration, and edema.^{1–6} Although several therapeutic options have been advanced for stroke treatment in recent decades, the tissue plasminogen activator protein tPA is the only drug shown to reduce the formation of ischemic infarcts. However, tPA is only modestly effective, and must be given within a 3-hour time window after stroke to be effective. Over the last few years, significant advances have

been made in rescuing ischemic brain tissue by promoting brain angiogenesis and neurogenesis through mobilization of *de novo* stem cells from the circulation and from the subventricular zone.^{7–10}

In addition to stem cell therapy, growth factor therapy aimed at protecting the neurovasculature and promoting ischemic brain angiogenesis is an area under active investigation.^{7,9,11,12} VEGFs are a family of growth factors currently under intense investigation in stroke research because of their involvement in neuronal survival and function, as

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well as in angiogenesis.^{11,13–16} Even though several VEGF isoforms with diverse functions exist, relatively few of them have received research attention, and some VEGF functions are still unknown. In stroke research, VEGF-A isoforms (VEGF_{164/165}, mouse VEGF-A isoform VEGF₁₆₄, human VEGF-A isoform VEGF₁₆₅) are among the best investigated as potential targets in clinical applications.

However, several reports point out that, although VEGF_{164/165} plays a major role in angiogenesis and neuroprotection, it also promotes endothelial barrier disturbances and hemorrhagic transformation.^{17–19} Inhibition of VEGF_{164/165} in nonbrain pathologies (eg, inflammatory bowel diseases) has been shown to be beneficial, and several therapeutic monoclonal antibodies targeting VEGF_{164/165} are being developed.^{20–22} In the intestine, the protection afforded by VEGF-A inhibition appears to mainly reflect inhibition of VEGF_{164/165}—mediated inflammatory angiogenesis.^{23,24} In the brain, however, poststroke angiogenesis and vasodilatation are valuable in providing an adequate blood supply to metabolically deprived regions. Currently, it is not clear whether inflammation associated with this process is beneficial or detrimental.²⁵ Given these mutually antagonistic functions of VEGF_{164/165}, it remains unclear whether either administration or inhibition of VEGF_{164/165} is likely to be of any therapeutic benefit in stroke.

We have recently shown that VEGF_{165b}, a naturally occurring isoform of VEGF_{164/165}, can effectively block VEGF_{164/165}—induced inflammatory angiogenesis and attenuate VEGF_{164/165}—induced endothelial barrier permeability in mouse colon endothelial cells.^{23,26} Importantly, VEGF_{165b}—mediated protection against barrier disturbances may mechanistically indicate suppression of VEGF_{164/165}—dependent vascular remodeling of junctional molecules.²⁶ The specific role of this inhibitory VEGF isoform in the pathophysiology of stroke is not known. More importantly, given that VEGF_{164/165} plays a critical role in ischemic protection of neurons, the outcome of VEGF_{164/165} antagonism by VEGF_{165b} is not easy to predict. Because VEGF_{165b} and VEGF_{164/165} can each exert beneficial effects in tissue injury, we hypothesized that VEGF_{165b} might be either helpful in stroke (by decreasing barrier permeability and immune cell infiltration) or detrimental (by interfering with VEGF_{164/165}—mediated angiogenesis after stroke).

To understand the roles of VEGF_{165b}, we initially characterized the cellular sources of VEGF₁₆₅ and VEGF_{165b} in the components of neurovascular unit after oxygen glucose deprivation and re-oxygenation (OGDR) *in vitro*. We next studied the effect of VEGF_{165b} on brain endothelial barrier function and *in vitro* capillary tube formation (angiogenesis) assay *in vitro*. Lastly, to under the functional role of VEGF_{165b} *in vivo*, we used an adenoviral vector to induce VEGF_{164b} before stroke and assessed specific functional roles of VEGF_{165b} in BBB damage, ischemic infarct sizes, behavioral deficit, neuronal survival, and immune responses in the pathophysiology of stroke.

Materials and Methods

Antibodies and Proteins

Human VEGF₁₆₅ (293-VE), human VEGF_{165b} (3045-VE), anti-human VEGF-A (MAB293), and anti-mouse VEGF₁₆₅ (MAB493) antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-human VEGF_{165b} was prepared as described previously.²⁷ Vascular growth factor receptor 1 (VEGFR-1) (sc-316) and VEGFR-2 (sc-505) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

The human brain endothelial cell line HCMEC-D3 was provided by Dr. Pierre Couraud (CNRS, Paris). The human brain endothelial cell line HBMEC-3 and ECV-304 cell lines were provided by Dr. Anat Erdreich-Epstein (University of Southern California). The astrocyte cell line HFA was provided by Dr. Danica Stanimirovic (University of Ottawa). Mouse brain endothelial cell line bEnd.3 (provided by Dr. Eugene Butcher, Department of Pathology, Stanford University) and the ECV-304 cell lines were cultured as described previously.²⁸ The human neuron-like cell line SHSY-5Y (ATCC, Manassas, VA) was cultured in 10% Dulbecco's modified Eagle's medium. Passage 1 to passage 3 primary human umbilical vein endothelial cells (HUVECs) isolated from normal umbilical veins were cultured in endothelial basal medium supplemented with a bulletkit (Cat No: CC-3124; Lonza, Basel, Switzerland).

In Vitro Model of Cerebral Ischemia

As an *in vitro* model of cerebral ischemia, HCMEC-D3, HFA, and SHSY-5Y cells were subjected to OGDR, as described previously.⁶ In brief, at confluency, cells were incubated in medium without glucose and serum (D5030; Sigma-Aldrich, St. Louis, MO) supplemented with 3.7 g/L NaHCO₃ and subjected to an atmosphere of approximately 95% N₂, 4% CO₂, and 1% O₂ in a hypoxia chamber for 6 hours. After 6 hours of oxygen glucose deprivation (OGD), buffer was replaced with regular culture medium and cells were incubated for 24 hours in 5% CO₂—enriched normoxic atmosphere, to reoxygenate.

Transendothelial Electrical Resistance

Human or mouse brain endothelial cells and HUVECs or ECV-304 (used as positive controls) were cultured in regular culture medium on 8.0-μm pore-size cell culture inserts (BD Falcon, San Jose, CA) with a surface area of 0.3cm² in the upper chamber. At confluency, normal (without OGDR) or OGDR-challenged brain endothelial cells (or HUVECs or ECV-304 cells as controls) were treated with 100 ng/mL VEGF_{164/165} or VEGF_{165b} applied on both apical and basal sides of the cell monolayer. Transendothelial electrical resistance (TEER) was then recorded as unit area resistance (Ω·cm²), using an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL), at baseline (0 hours) and for 3 days at 24-hour intervals.

Animals

C57BL/6 mice were bred and maintained in a specific-pathogen-free environment with a 12-hour light/dark cycle at the animal facility of Louisiana State University Health—Shreveport. All animal experiments were performed on male C57BL/6 mice at 12 to 18 weeks of age, weighing approximately 25 to 30 g, according to Institutional Animal Care and Use Committee guidelines under an approved animal protocol.

Adenoviral GFP and VEGF_{164b} Preparation and Treatment of Mice

The adenovirus encoding recombinant VEGF_{164b} (Ad-rVEGF_{164b}) and its higher potential, compared with naturally occurring VEGF_{165b}, have been described previously.²⁶ Ad5-CMV-GFP, consisting of an adenovirus serotype 5 expressing GFP under the CMV promoter, was used as a relevant control.

At 3 days before induction of cerebral ischemia, mice were injected intraperitoneally with 1×10^{11} viral particles, either Ad-GFP or Ad-rVEGF_{164b}. We had earlier determined that Ad-rVEGF_{164b} induces peak serum levels of VEGF_{164b} by 72 hours after adenoviral infection (data not shown).

In Vivo Animal Model of Cerebral Ischemia

Focal cerebral ischemia—reperfusion injury in mice was induced by transient middle cerebral artery occlusion. In brief, mice were anesthetized with a combination of 2.5 mg/kg xylazine and 50 mg/kg ketamine hydrochloride. After confirming that the animal was deeply anesthetized, a midline pharyngeal incision was made to expose the external and internal carotids and a 6-0 silicone-coated nylon monofilament (Doccol, Redlands, CA) was inserted from the cut end of the external carotid into the internal carotid and extended to a distance of 10 to 12 mm. The occlusion was detected by gentle resistance to further extension of the filament. The middle cerebral artery was occluded for 2 hours; the occluding filament was then removed and reperfusion was initiated. Sham animals were treated the same way as stroke animals except the nylon monofilament was not extended to occlude the middle cerebral artery.

Twenty-four hours after reperfusion, sham, Ad-rVEGF_{164b} treated, and Ad-GFP treated stroke mice were sacrificed by cardiac puncture or pneumothorax under deep xylazine—ketamine anesthesia and the brains were removed. Coronal slices (2 mm thick) were obtained from Ad-GFP—treated and Ad-rVEGF_{164b}—treated stroke mouse brains. Stroke mouse brain slices were incubated in 2% tetrazolium trichloride at 37°C until the development of chromophore (approximately 5 to 7 minutes). After color development, brain slices were fixed in 10% formalin and were photographed. Infarct size was measured directly by quantifying infarct volume in the ipsilateral hemisphere using

ImageJ analysis software version 1.46 (NIH, Bethesda, MD) as described previously.²⁹

Stroke-Induced Behavioral Deficits

Behavioral changes induced by stroke in mice were measured by grading behavioral deficits exhibited 24 hours after reperfusion.^{30–32} Scoring was on a six-point scale: 0, no noticeable behavioral deficit; 1, inability to extend forelimbs; 2, inability to move spontaneously; 3, contralateral spinning; 4, barrel rolling; 5, paralysis; and 6, dead.

Vasogenic Brain Edema

Twenty-four hours after ischemia—reperfusion injury, Ad-GFP—treated stroke animals and Ad-rVEGF_{164b}—treated stroke animals were sacrificed. The brains were isolated, and the wet weight was recorded. Later, the brains were desiccated in an incubator at 60°C for 72 hours, and the dry weight was recorded. The percentage of brain water content as a measure of edema was calculated as (brain wet weight – dry weight/wet weight) $\times$ 100.

Neutrophil Content

Brain neutrophil content in stroke brains was measured in terms of myeloperoxidase (MPO) activity. In brief, Ad-GFP—treated and Ad-rVEGF_{164b}—treated stroke mouse brains were isolated, homogenized in modified radioimmunoprecipitation assay buffer (0.25 mol/L sucrose, 50 mmol/L Tris base, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L MgCl₂, 10 mmol/L KCl, 1% NP-40, and 1% Tween 20 supplemented with protease and phosphatase inhibitor cocktail; Sigma-Aldrich), and sonicated three times with –80°C freeze—thaw cycles. Later, the homogenate was centrifuged and the supernatant was collected. Protein in the supernatants was estimated by a bicinchoninic acid assay (Bio-Rad Laboratories, Hercules, CA), and equal quantities of supernatant protein were used to quantify MPO by colorimetric conversion of ortho-dianisidine at 450 nm.

Western Blotting

Twenty-four hours after reperfusion, sham and stroke mice were sacrificed as described above, and the brains were removed. Ipsilateral hemispheric tissues were homogenized in modified radioimmunoprecipitation assay buffer and equal amounts of protein were separated on SDS-PAGE, followed by immunoblotting with VEGF₁₆₄ or VEGF_{165b} antibodies. Equal volumes of serum obtained from sham-treatment and stroke mice were resolved on SDS-PAGE and immunoblotted with either VEGF₁₆₄ or VEGF_{165b}. Normal and OGDR-challenged HCMEC-D3, HFA, and SHSY-5Y cell lysates were immunoblotted with VEGF₁₆₅ or VEGF_{165b}. The ratio of VEGF_{164/165} to VEGF_{165b} in mouse serum, brain lysates, and cell lysates was derived from scanning densitometry data.

The VEGF_{165b}/VEGF₁₆₅ ratio in tissue lysates and cell lysates was normalized to actin, and then the ratio of normalized VEGF₁₆₅ to VEGF_{165b} was calculated.

Capillary Tube Formation As an *in Vitro* Angiogenesis Assay

HCMEC-D3, bEnd.3, and HUVEC cells at confluency were subjected to OGD as described above for 6 hours (HCMEC-D3 and bEnd.3) or 3 hours (HUVEC). Control and OGD-treated cells were reoxygenated with treatment medium containing 100 ng of VEGF_{164/165} or VEGF_{165b} for 24 hours. Later, cells from each well were trypsinized and equal numbers of cells were plated on growth factor–reduced Matrigel (BD Biosciences, San Jose, CA). After 5 to 6 hours, capillary tube-like structures formed on Matrigel were photographed, and their numbers were counted in each field.

Mouse Neutrophil Isolation

Neutrophils were isolated from the bone marrow of the mice. In brief, bone marrow was flushed from hindlimbs and forelimbs of mice with ice-cold PBS and then incubated with phycoerythrin-conjugated Ly6 antibody. Neutrophils from bone marrow suspension were enriched using an EasySep Mouse PE Positive selection kit (18554; STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. The isolated neutrophils were used for transendothelial migration assay across bEnd.3 cells.

Human Neutrophil Isolation

Neutrophils from healthy volunteers were isolated from venous blood. In brief, venous blood was allowed to sediment in 6% dextran. Supernatant from the sedimented blood was spun down, and the red blood cells were lysed using RBC lysis buffer prepared by dissolving 8.3 g NH₄Cl, 1.0 g KHCO₃, and 1.8 mL 5% EDTA in 1 L H₂O. Later cells were layered on a Ficoll-Hypaque density gradient (1.077; GE Healthcare, Little Chalfont, UK), and the sedimented neutrophils were washed and used for neutrophil transendothelial migration across HCMEC-D3 cells. Neutrophils (1×10^7) isolated at 4°C were used as nonactivated controls, and 1×10^7 neutrophils were activated at 37°C for 24 hours with 100 nmol/L *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Later, cell lysates and culture supernatants were obtained and used for Western blotting with VEGF₁₆₅ and VEGF_{165b} antibodies.

Neutrophil Transendothelial Migration

Mouse or human brain endothelial cells were cultured on cell culture inserts as described above and at confluency were subjected to OGD or normal conditions for 6 hours as described above. After treatment, the medium was replaced with regular culture medium and allowed to reoxygenate for 24 hours. At the 24-hour time point, 100 ng of VEGF

(VEGF₁₆₄ for bEnd.3 and VEGF₁₆₅ for HCMEC-D3) and 100 ng of VEGF_{165b} was added to the lower chamber, and mouse or human neutrophils were added to the insert (1×10^6 /insert). FMLP (100 nmol/L) served as the positive control; PBS served as negative control. After 4 hours of incubation, cells migrated to the lower chamber were spun down and lysed in 0.5% hexadecylmethylammonium bromide. MPO was quantified by adding 3,3',5,5'-tetramethylbenzidine–H₂O₂. Reaction was stopped using H₂SO₄, and the absorbance of the end products was measured spectrophotometrically at 450 nm.

Statistical Analysis

Two-tailed unpaired *t*-test was used to test the significance between two groups. Repeated-measures analysis of variance with Dunnett's post hoc test was used to test for significance in endothelial barrier resistance studies.

Results

Differential Up-Regulation of VEGF₁₆₄ and VEGF_{165b} in Ischemic Brain Tissue and Serum of Stroke Mice

To evaluate the relative levels of VEGF₁₆₄ and VEGF_{165b} in mouse tissues after stroke, we performed Western blotting analysis on serum and brain lysates obtained from sham-treated and ischemia–reperfused stroke mice. Immunoblot analysis indicated a significant increase in levels of VEGF_{165b} in both serum and brain tissue after stroke, compared with sham-treatment controls (Figure 1, A and B). Interestingly, although VEGF₁₆₄ levels in stroke mouse brain were significantly lower, compared with control, serum VEGF₁₆₄ levels were significantly increased in stroke mice (Figure 1, A and B). More importantly, the relative level of VEGF_{165b} compared with total VEGF (as determined by the VEGF_{165b}/VEGF₁₆₄ ratio) was significantly higher in brains of stroke mice, compared with controls (Figure 1A). Furthermore, expression levels of VEGFR-1 and VEGFR-2 in the brain and of soluble VEGFR-1 and VEGFR-2 in serum of stroke mice were also significantly increased, compared with controls (Figure 1, C and D).

Differential Expression of VEGF₁₆₅ and VEGF_{165b} by Human Neurons, Astrocytes, and Brain Endothelial Cells

Because we observed a significant increase in the levels of VEGF_{165b} relative to VEGF₁₆₄ in the stroke brain, we investigated the cellular sources of VEGF_{165b} production in the brain neurovasculature.

Neurons

Although densitometric analysis indicated significantly decreased VEGF₁₆₅ levels in OGDR-challenged SHSY-5Y neurons ($P = 0.003$), the levels of VEGF_{165b} were significantly increased ($P = 0.04$), compared with normal. Importantly, the

Figure 1 Immunoblot analysis of brain and serum lysates. The VEGF monomer (23 kDa) is indicated by an **arrow**. **A:** Total VEGF₁₆₄ amino acid isoform (VEGF₁₆₄ and VEGF_{165b}) levels were significantly decreased in the brains of stroke mice, compared with sham-treated control mice; however, VEGF_{165b} levels were significantly increased. The VEGF_{165b}/total VEGF₁₆₄ ratio indicated significantly increased VEGF_{165b} levels. $n = 4$ (stroke); $n = 3$ (sham treatment). **B:** In the serum of stroke mice, both VEGF₁₆₄ and VEGF_{165b} levels were significantly elevated, compared with controls. The VEGF_{165b}/total VEGF₁₆₄ ratio indicated no statistical difference between stroke and control mice. $n = 4$ (stroke); $n = 3$ (sham treatment). **C:** A significant increase in brain VEGFR-1 and VEGFR-2 levels was observed in stroke mice. $n = 4$ (stroke); $n = 3$ (sham treatment). **D:** A significant increase in the soluble forms of VEGFR-1 and VEGFR-2 was also observed in the serum of stroke versus sham-treated mice. Data are expressed as means $\pm$ SEM. $n = 4$ (stroke); $n = 3$ (sham treatment). * $P < 0.05$, two-way analysis of variance.

VEGF_{165b}/VEGF₁₆₅ ratio was significantly increased, compared with the normal control, indicating that OGDR-challenged neurons have higher levels of VEGF_{165b} ($P = 0.004$) (Figure 2A and Supplemental Figure S1A).

Astrocytes

Densitometric analysis revealed no significant difference in VEGF₁₆₅ levels between control and OGDR-challenged

HFA cells ($P = 0.48$), but there was a significant increase in VEGF_{165b} levels ($P = 0.01$) and in the VEGF_{165b}/VEGF₁₆₅ ratio ($P = 0.005$) (Figure 2B and Supplemental Figure S1B). More importantly, in Western blot analysis of control and OGDR-challenged astrocytes, VEGF_{165b} antibody precipitated an additional isoform of VEGF_{165b} (Supplemental Figure S1B). Because VEGF_{165b} antibody is specific only to VEGF_{165b} protein (and therefore not to

Figure 2 Densitometric analyses of Western blotting for VEGF₁₆₅ and VEGF₁₆₅b in SHSY-5Y, HFA, and HCMEC-D3 cell lines. **A:** A significant difference was observed in levels of VEGF₁₆₅ and VEGF₁₆₅b between normal control (Con; without OGDR) and OGDR-challenged SHSY-5Y neurons. The VEGF₁₆₅b/VEGF₁₆₅ ratio in OGDR neurons was significantly increased, compared with control, indicating that VEGF₁₆₅b predominates over VEGF₁₆₅ in OGDR neurons. **B:** In HFA astrocytes, no significant difference in VEGF₁₆₅ levels was observed between control and OGDR conditions; however, both VEGF₁₆₅b levels and the VEGF₁₆₅b/VEGF₁₆₅ ratio were significantly increased in OGDR-challenged astrocytes, compared with normal control. **C:** Although a significant decrease in VEGF₁₆₅ levels was observed in OGDR-challenged HCMEC-D3 brain endothelial cells, compared with control, the levels of VEGF₁₆₅b did not differ significantly. The VEGF₁₆₅b/VEGF₁₆₅ ratio in OGDR HCMEC-D3 cells was significantly increased, compared with control, indicating that VEGF₁₆₅b predominates over VEGF₁₆₅ in OGDR HCMEC-D3 cells. Data are expressed as means $\pm$ SEM. $n = 6$. * $P < 0.05$, two-way analysis of variance.

VEGF₁₆₅), we hypothesize that the additional band observed in VEGF₁₆₅b-treated HFA cells corresponds to other isoforms of VEGF₁₆₅b with different molecular weights and sequences. The amino acid sequence and the functional roles of these isoforms may require further investigation.

Brain Endothelial Cells

Although a significant decrease in VEGF₁₆₅ levels was observed in OGDR-challenged HCMEC-D3 cells, compared with normal ($P = 0.02$), the densitometric analysis revealed no significant difference for VEGF₁₆₅b levels ($P = 0.502$). However, the VEGF₁₆₅b/VEGF₁₆₅ ratio indicated a significant increase in the levels of VEGF₁₆₅b, relative to VEGF₁₆₅, in HCMEC-D3 cells ($P = 0.04$) (Figure 2C and Supplemental Figure S1C).

Brain and Nonbrain Endothelial Barrier Permeability Is Differentially Influenced by VEGF₁₆₅ and VEGF₁₆₅b

TEER is a measure of barrier function that permits real-time studies of endothelial barrier over relatively long time frames and repeated measures, but without excessive alteration of cell-culture conditions. A lower TEER indicates lower barrier integrity which results in higher solute permeability and vice versa. We therefore used TEER to compare and correlate barrier integrity with endothelial solute permeability in our experimental models.

Human Brain HCMEC-D3 Cells

HCMEC-D3 cells at confluency were left untreated or were treated with 100 ng/mL of VEGF₁₆₅ or VEGF₁₆₅b, and barrier integrity was measured by recording TEER at baseline and at 24-hour intervals to day 3. Interestingly, we did not observe any difference in barrier resistance with either VEGF treatment under normal conditions (ie, without OGDR). A progressive decrease in barrier resistance of HCMEC-D3 cells was observed for all three treatments (ie, untreated, VEGF₁₆₅, VEGF₁₆₅b) over the 3 days ($P < 0.05$), but with no significant difference between treatment groups at any time point (Figure 3A). Day 3 values were $71.04 \pm 0.89\%$ of baseline for untreated control, $71.71 \pm 1.4\%$ for VEGF₁₆₅, and $67.92 \pm 1.8\%$ for VEGF₁₆₅b.

A significant decrease in VEGF₁₆₅b barrier resistance was observed between untreated and VEGF₁₆₅b-treated OGDR-challenged HCMEC-D3 cells at day 3 ($P = 0.015$) (Figure 3A and Supplemental Figure S2A). Day 3 values were $116.6 \pm 1.3\%$ of baseline for untreated control, $110.9 \pm 1.6\%$ for VEGF₁₆₅, and $108.1 \pm 1.6\%$ for VEGF₁₆₅b.

Human Brain HBMEC-3 Cells

In the absence of OGDR challenge, HBMEC-3 cells exhibited an increase in barrier resistance for all three treatments, compared with 0 hours. At day 3, the barrier resistance of VEGF₁₆₅-treated cells was slightly but significantly lower, compared with untreated control ($P = 0.003$). No significant difference in barrier resistance was observed between untreated and VEGF₁₆₅b-treated cultures

Figure 3 Effect of VEGF isoform treatment on barrier integrity with or without OGDR challenge in HMEC-D3 cells (A), HBMEC-3 cells (B), bEnd.3 cells (C), HUVEC cells (D), and the positive cell line control ECV-304 (E). Data are expressed as means $\pm$ SEM. $n = 9$ (A and B); $n = 6$ (C); $n = 3$ (D and E). * $P < 0.05$ versus 0 hours; $^{\dagger}P < 0.05$ versus untreated control at the same time point. $^{\ddagger}P < 0.05$ versus VEGF_{165b} at the same time point. Vertical scale differs among panels.

(Figure 3B). Day 3 values were $139.4 \pm 2.3\%$ of baseline for untreated control, $124.3 \pm 0.15\%$ for VEGF₁₆₅, and $130.6 \pm 3.3\%$ for VEGF_{165b}.

Untreated and VEGF₁₆₅ or VEGF_{165b} treated OGDR HBMEC-3 barrier showed a progressive increase (from day 0 to 1 to 2) in the barrier until 2d. However, at 3d the barrier of VEGF₁₆₅- and VEGF_{165b}-treated barrier showed a relative loss of barrier compared to untreated OGDR barrier. Although there was no significant difference in barrier resistance between the two VEGF treatments at day 3, a significant difference was observed between untreated and VEGF₁₆₅-treated cells ($P = 0.0109$) and between untreated and VEGF_{165b}-treated cells ($P = 0.001$) (Figure 3B and Supplemental Figure S2B). Day 3 values were $183.3 \pm 0.8\%$ of baseline for untreated control, $159.9 \pm 2.6\%$ for VEGF₁₆₅, and 160.6% for VEGF_{165b}.

Mouse Brain bEnd.3 Cells

In the absence of OGDR challenge, untreated and VEGF₁₆₄-treated but not VEGF_{165b}-treated bEnd.3 cells exhibited a progressive loss of barrier resistance until day 2. At day 3, there was significant decrease in barrier resistance

in all three treatment groups, but the resistance of VEGF_{165b}-treated cells was significantly higher than that of untreated cells ($P = 0.003$) or VEGF₁₆₄-treated cells ($P = 0.0012$). The difference barrier resistance among untreated, VEGF₁₆₄-treated, and VEGF_{165b}-treated cells was more pronounced at day 1. Although VEGF₁₆₄ significantly decreased the barrier resistance, compared with untreated control ($P = 0.011$), there was a significant difference between untreated and VEGF_{165b}-treated barrier resistance at day 1. More importantly, in VEGF_{165b}-treated cells barrier resistance was significantly higher than in VEGF₁₆₄-treated cells at day 1 ($P = 0.0012$) (Figure 3C). Day 3 values were $67.01 \pm 0.5\%$ of baseline for untreated control, $63.49 \pm 1.2\%$ for VEGF₁₆₅, and $73.77 \pm 0.1\%$ for VEGF_{165b}.

With OGDR challenge, although untreated and VEGF_{165b}-treated bEnd.3 barrier resistance exhibited a significant decrease at day 3, compared with 0 hours, no significant difference was observed at day 3 for the VEGF₁₆₄ treatment. Although at day 3 there was no significant difference in barrier resistance between untreated and VEGF_{165b}-treated cells or between VEGF₁₆₄-treated and VEGF_{165b}-treated cells, a significant increase was observed for VEGF₁₆₄-treated versus untreated cells ($P = 0.008$) (Figure 3C and Supplemental Figure S2C). Day 3 values were $81.76 \pm 1.8\%$ of baseline for untreated control, $91.34 \pm 0.6\%$ for VEGF₁₆₄, and $85.85 \pm 1.4\%$ for VEGF_{165b}.

Human Nonbrain Cells (HUVECs)

In the absence of OGDR challenge, HUVECs exhibited a progressive loss of barrier resistance under untreated and VEGF₁₆₅-treated and VEGF_{165b}-treated conditions until day 3, compared with 0 hours. Both VEGF₁₆₅ ($P = 0.0013$) and VEGF_{165b} ($P = 0.0015$) exhibited a significant decrease in barrier resistance at day 1, compared with control. Barrier resistance of VEGF₁₆₅-treated cultures was still significantly lower than that of untreated control at day 3 ($P = 0.001$). Barrier resistance of VEGF_{165b}-treated cultures was slightly but significantly lower, compared with controls, at day 3 ($P = 0.015$). However, barrier resistance of VEGF_{165b}-treated cultures was significantly higher than that of VEGF₁₆₅-treated cultures at day 3 ($P = 0.001$). (Figure 3D). Day 1 values were $82.88 \pm 0.4\%$ of baseline for untreated control, $63.7 \pm 0.7\%$ for VEGF₁₆₅, and $79.43 \pm 0.6\%$ for VEGF_{165b}; day 3 values were $87.39 \pm 2.7\%$ of baseline for untreated control, $72.08 \pm 1.4\%$ for VEGF₁₆₅, and $76.77 \pm 0.7\%$ for VEGF_{165b}.

With OGDR challenge, although untreated HUVECs did not exhibit significant difference in barrier resistance at any time point, compared with 0 hours, in VEGF₁₆₅-treated and VEGF_{165b}-treated cells barrier resistance was significantly decreased at all three time points, compared with 0 hours. However, barrier resistance of VEGF_{165b}-treated HUVECs was the same as that of untreated cells at day 3. Interestingly, although at day 1 barrier resistance of VEGF_{165b}-treated HUVECs was significantly lower than that of untreated cells ($P = 0.004$) and was lower (but not significantly) than that of VEGF₁₆₅-treated cells ($P = 0.07$), at day 3 barrier resistance

was significantly lower in VEGF₁₆₅—treated than in untreated cells ($P = 0.008$) or in VEGF_{165b}—treated cells ($P = 0.004$) (Figure 3D and Supplemental Figure S2D). Day 1 values were $100.8 \pm 0.8\%$ of baseline for untreated control, $90.46 \pm 1.6\%$ for VEGF₁₆₅, and $85.86 \pm 1.1\%$ VEGF_{165b}; day 3 values were $91.1 \pm 2.1\%$ of baseline for untreated control, $80.41 \pm 0.7\%$ for VEGF₁₆₅, and $91.41 \pm 1.1\%$ for VEGF_{165b}.

Mouse Brain ECV-304 Cells

In the absence of OGDR challenge, ECV-304 cells exhibited a significant decrease in barrier resistance in untreated and VEGF₁₆₅—treated cultures and a significant increase in VEGF_{165b}—treated cultures, compared with 0 hours. A significant increase in barrier resistance was observed in VEGF_{165b}—treated cultures at day 3, compared with untreated cultures ($P = 0.003$). VEGF₁₆₅—treated cultures had significantly decreased barrier resistance at day 3, compared with VEGF_{165b}—treated cultures ($P = 0.001$) or untreated cultures ($P = 0.02$). Even though no large difference in barrier resistance was observed in VEGF₁₆₅—treated ECV-304 cultures, compared with untreated control, VEGF_{165b} dramatically increased barrier resistance (Figure 3E). Day 3 values were $93.05 \pm 0.8\%$ of baseline for untreated control, $88.03 \pm 1.1\%$ for VEGF₁₆₅, and $107.9 \pm 2.3\%$ for VEGF_{165b}.

With OGDR challenge in ECV-304 cultures, VEGF₁₆₅ treatment induced barrier permeability, compared with untreated control ($P = 0.003$) or VEGF_{165b} treatment ($P = 0.036$). No difference was observed in barrier resistance between VEGF_{165b}—treated and untreated cells at day 3 (Figure 3E and Supplemental Figure S2E). Day 3 values were $121.3 \pm 1.2\%$ of baseline for untreated control, $116.1 \pm 0.8\%$ for VEGF₁₆₅, and $127.1 \pm 1.8\%$ for VEGF_{165b}.

Taken together, these results confirmed that the effects of VEGF isoforms are cell-type and insult specific.

VEGF_{165b} Differentially Regulates Brain Endothelial and Nonbrain Endothelial Angiogenesis *in Vitro*

Because VEGF_{165b} is an antiangiogenic isoform of VEGF₁₆₅, we investigated the effect of these isoforms using an *in vitro* angiogenesis assay.

HCMEC-D3

In the absence of OGDR challenge, there was no significant difference in the numbers of vessel-like structure formation on Matrigel either with VEGF₁₆₅ or VEGF_{165b}, compared with untreated controls. Surprisingly, although OGDR induced a significant increase in the formation of tube-like structures on Matrigel, compared with normal (absence of OGDR) controls, no significant difference was observed between untreated controls and VEGF₁₆₅ or VEGF_{165b} (Figure 4A). Because we used a growth factor—reduced Matrigel, the angiogenic activities of the cell lines used in this study reflect the effect of VEGF₁₆₅ or VEGF_{165b} treatment alone, without any effect of the growth factors present in regular growth factor—supplemented Matrigel.

bEnd.3

We observed similar results with the mouse brain endothelial cell line bEnd.3 and the human brain endothelial cell line HCMEC-D3, except that untreated OGDR-challenged bEnd.3 cells had significantly decreased numbers of vessel-like structures, compared with untreated bEnd.3 cells without OGDR challenge. No significant difference in the numbers of vessel-like structures formed was observed with either VEGF₁₆₅—treated or VEGF_{165b}—treated bEnd.3 under normal or OGDR conditions, compared with the respective untreated controls (Figure 4B).

HUVECs

Because HUVECs are extensively used to investigate angiogenic activity of various growth factors, we used HUVECs as a positive control. No significant difference in the numbers of vessel-like structure formation on Matrigel was observed between control and VEGF₁₆₅ or VEGF_{165b} under normal conditions. With OGDR challenge, however, VEGF_{165b} treatment induced a significant increase in the numbers of vessel-like structures formed, compared with untreated controls, but no significant difference was observed between untreated and VEGF₁₆₅—treated HUVECs (Figure 4C).

Ad-rVEGF_{164b} Increases Infarct Size, Behavioral Deficits, Neutrophil Infiltration, and Neuronal Damage but Not Brain Edema

Infarct volumes in Ad-rVEGF_{164b}—treated stroke mice were significantly increased, compared with recombinant (r) Ad-GFP—treated stroke controls. The infarct volume in Ad-rVEGF_{164b}—treated animals was almost doubled, compared with that in Ad-GFP—treated stroke mice, indicating a potentially deleterious role of VEGF_{165b} in ischemic brain pathology (Figure 5, A and B). Increased infarct size in Ad-rVEGF_{164b}—treated animals was also associated with a significant increase in behavioral deficits, with behavioral deficit scores almost double those of Ad-GFP—treated stroke mice (Figure 5C).

Ad-rVEGF_{164b} Did Not Influence Brain Edema

Because VEGF is well known to induce increased barrier permeability, we investigated whether recombinant (r)VEGF_{164b}, the inhibitory isoform of VEGF₁₆₅, can decrease brain edema after stroke. Brain edema estimates based on wet weight/dry weight ratios for Ad-rVEGF_{164b} and Ad-GFP were not significantly different (Figure 5D). Even though barrier integrity does not give direct information about permeability to solutes, it does reflect the possible status of solute permeability under several conditions. We consider that VEGF_{164b} treatment did not influence barrier integrity or permeability in this experiment.

Ad-rVEGF_{164b} Treatment Increased Poststroke Neuronal Death in the Ipsilateral Hemisphere

We also investigated the effect of VEGF_{165b} on neuronal death in stroke animals treated with Ad-rVEGF_{164b} and Ad-GFP.

Figure 4 Effect of VEGF₁₆₅ and VEGF_{165b} on *in vitro* capillary tube formation in HCMEC-D3 cells, bEnd.3 cells, and HUVECs. **A:** Under normal conditions (Con; without OGDR) or OGDR conditions, neither VEGF₁₆₅ nor VEGF_{165b} significantly increased or decreased the numbers of vessel-like tube structures formed by HCMEC-D3 cells on Matrigel, compared with respective untreated normal or OGDR controls. **B:** Under normal or OGDR conditions, neither VEGF₁₆₅ nor VEGF_{165b} significantly affected the number of vessel-like tube structures formed by bEnd.3 cells on Matrigel, compared with respective untreated normal or OGDR controls. **C:** Under normal conditions, neither VEGF₁₆₅ nor VEGF_{165b} significantly affected the numbers of vessel-like tube structures formed by HUVECs on Matrigel. However, OGDR challenged HUVEC treated with VEGF_{165b} significantly increased the numbers of these vessel-like structures on Matrigel, compared with untreated OGDR control. Data are expressed as means $\pm$ SEM. $n = 6$ (**A** and **B**); $n = 3$ (**C**). * $P < 0.05$, two-way analysis of variance between any two groups.

Cresyl violet staining of ischemic brain sections from Ad-rVEGF_{164b} and Ad-GFP-treated stroke mice revealed a significant loss of neurons in the ipsilateral hemisphere of Ad-rVEGF_{164b}-treated stroke mice at 24 hours, compared with Ad-GFP-treated stroke mice. Extensive neurodegeneration, tissue disruption, and increased necrosis were observed between Ad-rVEGF_{164b}-treated and Ad-GFP-treated stroke mice. No significant difference in neuronal death was observed in the contralateral hemisphere between Ad-GFP-treated and Ad-rVEGF_{164b}-treated stroke mice. The hippocampal region in which the difference in tissue damage was clearly evident was photographed and used as the representative image for comparing neurodegeneration after ischemia between Ad-rVEGF_{164b} and Ad-GFP mice (Figure 5E). At least four nonoverlapping images of cresyl violet-stained ischemic hippocampus, CA2 region, were obtained from each treatment group and were quantified by counting the number of degenerating neurons. The data are expressed as percentage neuronal death in Ad-GFP versus Ad-rVEGF_{164b} (Figure 5F).

Ad-rVEGF_{164b} Treatment Induced Neutrophil Infiltration into the Stroke Brain

To investigate the role of VEGF_{165b} in neutrophil infiltration into the ischemic brain, we quantified the amount of MPO in the infarcted area as an indicator of neutrophil infiltration in Ad-rVEGF_{164b}-treated and Ad-GFP-treated stroke mice. Ad-rVEGF_{164b}-treated stroke mice had greater neutrophil content in the infarct area, approximately double that of the control stroke mice (Figure 5G).

VEGF_{164/165} and VEGF_{165b} Induce Neutrophil Chemotaxis

To confirm that VEGF_{165b} can induce neutrophil chemotaxis, we performed neutrophil transendothelial migration across human or mouse brain endothelial cells. Although under the normal condition (without OGDR challenge) mouse bEnd.3 cells did not permit either

VEGF₁₆₄—mediated or VEGF₁₆₅b—mediated neutrophil transendothelial migration, in OGDR-challenged mouse bEnd.3 cells significant increase in VEGF₁₆₄—mediated and VEGF₁₆₅b—mediated neutrophil transendothelial migration was observed (Figure 6A). Both VEGF₁₆₅ and VEGF₁₆₅b significantly increased neutrophil transendothelial migration across both normal and OGDR-challenged human HCMEC-D3 cells (Figure 6B). FMLP, a well known neutrophil chemoattractant, was used as a positive control in these neutrophil-transendothelial migration experiments. As expected, FMLP significantly induced neutrophil-transendothelial migration across both human and mouse brain endothelial cells under normal or OGDR-challenged conditions. These results show that, apart from VEGF_{164/165}, VEGF₁₆₅b is also

a potent neutrophil chemoattractant and suggest that increased VEGF₁₆₅b levels in stroke brain can induce neutrophil chemoattraction and migration into the brain, thereby aggravating injury after ischemia–reperfusion. The *in vitro* transendothelial migration findings support our *in vivo* data showing that increased brain neutrophil content in Ad-rVEGF₁₆₄b—treated mouse stroke brains and also put forward a new role of VEGF₁₆₅b as a neutrophil chemoattractant.

Activated Neutrophils Secrete Significantly Higher Levels of VEGF₁₆₅b than of VEGF_{164/165}

To investigate whether increased neutrophil infiltration into the ischemic brain can further aggravate production of VEGF_{164/165} or VEGF₁₆₅b isoforms, we performed Western blot analysis on nonactivated (control) and FMLP-activated (100 nmol/L for 24 hours) mouse and human neutrophil lysates and culture supernatants. FMLP-activated mouse and human neutrophils produced significantly higher levels of VEGF_{164/165} and VEGF₁₆₅b; however, the VEGF₁₆₅b/

Figure 5 **A:** Effect of Ad-rVEGF₁₆₄b in mouse stroke. Ischemic brain slices from mice treated intraperitoneally with Ad-GFP ($n = 5$) or Ad-rVEGF₁₆₄b ($n = 6$) before experimentally induced stroke were stained with 2% tetrazolium trichloride. Ad-rVEGF₁₆₄b—treated stroke mice exhibited a dramatic increase in the infarct volume, compared with Ad-GFP—treated stroke mice. **B:** Quantification of the volume of ischemic brain infarcts demonstrated a significant increase in Ad-rVEGF₁₆₄b—treated versus Ad-GFP—treated stroke mice. Mean of infarct volumes in mm³ $\pm$ SEM. **C:** Behavioral deficit analysis based on a scoring of 0 (no deficit) to 5 (paralysis) demonstrated worsening of Ad-rVEGF₁₆₄b—treated stroke mice, compared with Ad-GFP—treated stroke mice. **D:** Effect of VEGF₁₆₄b on brain edema. No significant in brain edema, as indicated by the wet weight/dry weight ratio, was observed between Ad-GFP—treated ($n = 4$) and Ad-rVEGF₁₆₄b—treated ($n = 5$) stroke mice. **E:** Cresyl violet staining used to stain neurons in the brain revealed significant loss in the neuronal population in Ad-rVEGF₁₆₄b—treated stroke mice, compared with Ad-GFP—treated stroke mice ($n = 4$ per group). Representative images were captured of the CA2 region of ipsilateral and contralateral hippocampi of Ad-GFP—treated and Ad-rVEGF₁₆₄b—treated stroke animals. Although systemic Ad-rVEGF₁₆₄b did not have any detrimental effects in the contralateral hemisphere or in sham-treated animals, it greatly aggravated neuronal loss in the ipsilateral hemisphere. Regions with clear differences in necrosis and tissue damage and with extensive tissue disruption are indicated by arrows. **Insets:** CA2 hippocampal regions of Ad-rVEGF₁₆₄b and Ad-GFP—treated stroke mice show extensive neuronal loss and tissue disruption. **F:** Quantification of neuronal death in Ad-GFP versus Ad-rVEGF₁₆₄b at 24 hours. A significant increase in the numbers of degenerating neurons was observed in the ipsilateral hemisphere of both Ad-GFP—treated and Ad-rVEGF₁₆₄b—treated stroke mice, compared with the contralateral hemisphere. However, neuronal death in the ipsilateral hemisphere of Ad-rVEGF₁₆₄b—treated stroke mice was significantly higher than in that of Ad-GFP—treated stroke mice. No significant difference in neuronal death in the contralateral hemisphere was observed between Ad-GFP and Ad-rVEGF₁₆₄b—treated stroke mice. **G:** MPO assay revealed a significant increase in brain neutrophil content in Ad-rVEGF₁₆₄b—treated versus Ad-GFP—treated stroke mice ($n = 6$ per group). Neutrophil content was almost twofold greater, compared with Ad-GFP—treated stroke mice. Data are expressed as means $\pm$ SEM (with different sample sizes as given above). * $P < 0.05$, one-way analysis of variance with Bonferroni comparison between selected pairs of groups or two-way analysis of variance between two specific groups. Original magnification: $\times 2$; $\times 20$ (insets). Contra, contralateral; ipsi, ipsilateral; OD, optical density.

Figure 6 **A:** Mouse neutrophil transendothelial migration across bEnd.3 cells. Both VEGF₁₆₄ and VEGF_{165b} significantly increased neutrophil transendothelial migration across OGDR-challenged bEnd.3 cells, but not under normal conditions. **B:** Human neutrophil transendothelial migration across HCMEC-D3 cells. Both VEGF₁₆₅ and VEGF_{165b} significantly increased neutrophil transendothelial migration across normal and OGDR-challenged HCMEC-D3 cells. **C:** Western blot analysis of VEGF₁₆₄ and VEGF_{165b} in nonactivated (control) and FMLP-activated mouse neutrophils. A trend toward increased VEGF₁₆₄ level in activated neutrophil lysates was observed ($P = 0.057$), and a significant increase in the VEGF₁₆₄ was observed in activated neutrophil culture supernatants ($P = 0.04$). Levels of VEGF_{165b} were significantly increased in both activated neutrophil lysates ($P = 0.04$) and supernatants ($P = 0.01$). The VEGF_{165b}/VEGF₁₆₄ ratio indicated a significant increase in VEGF_{165b} relative to VEGF₁₆₄ in both lysates ($P = 0.005$) and supernatants ($P = 0.004$). **D:** Western blot analysis of VEGF₁₆₅ and VEGF_{165b} in nonactivated (control) and FMLP-activated human neutrophils. A significant increase in the VEGF₁₆₅ was observed in both activated neutrophil lysates ($P = 0.004$) and supernatants ($P = 0.003$). VEGF_{165b} levels were also significantly increased in both activated neutrophil lysates ($P = 0.006$) and supernatants ($P = 0.001$). The VEGF_{165b}/VEGF₁₆₅ ratio indicated a significant increase in VEGF_{165b} relative to VEGF₁₆₅ in lysates ($P = 0.04$) and supernatants ($P = 0.001$). Thus, activated neutrophils produce predominantly the VEGF_{165b} isoform, relative to VEGF_{164/165}. Data are expressed as means $\pm$ SEM. $n = 4$ per group. * $P < 0.05$ versus control, two-way analysis of variance between two groups. F, FMLP activated; Lys, cell lysates; Sup, culture supernatants; V, VEGF.

VEGF_{164/165} ratio indicated a significant increase in VEGF_{165b} levels, compared with VEGF_{164/165} levels, in activated neutrophil lysates and supernatants (Figure 6, C and D). This result indicates that neutrophils can promote brain tissue injury by secreting increased levels of VEGF_{165b}, in addition to producing reactive oxygen species and neutrophil elastase, which can aggravate tissue injury.³³ These results indicate that neutrophil infiltration into tissue might start a positive feedback cycle of increased VEGF_{165b} production and persistent neutrophil chemotaxis into the injured tissue, thereby amplifying the injury process.

Discussion

Although extensive research data are available on the role of VEGF-A₁₆₅ in stroke, the roles of other VEGF isoforms are not yet clear. For example, the role of VEGF_{165b}, an abundant

naturally occurring antiangiogenic isoform of VEGF_{164/165}, is not clear.²⁷ VEGF_{165b} is an antiangiogenic isoform of VEGF_{164/165} that shares similar structural properties with VEGF_{164/165} except that VEGF_{165b} is alternately spliced downstream of the exon-8 splice site, resulting in a frame shift of sequence CDKPRR to SLTRKD in humans. This change in the COOH terminus results in competition of VEGF_{165b} with VEGF_{164/165} in binding to VEGFR-1 and VEGFR-2, thereby decreasing VEGF₁₆₅ signaling.²⁷ Several studies have suggested a dramatic increase in VEGF₁₆₄ levels in rat models of cerebral ischemia using permanent middle cerebral artery occlusion, transient middle cerebral artery occlusion, and cardiac arrest.^{34–37} However, the relative differences between VEGF_{164/165} and its antiangiogenic isoform VEGF_{165b} are not clear. Interestingly, in the present study we found a decrease in VEGF_{164/165} levels in mouse model of focal cerebral ischemia.³⁸ Although we currently do not exactly understand this discrepancy with other data, we think it might

be due to species difference and other experimental conditions. However, in addition to altered VEGF_{164/165} expression, we observed differential VEGF_{165b} expression by neurons, astrocytes, and brain endothelial cells.^{39–42} More importantly, astrocytic and endothelial VEGF_{165b} levels predominate dramatically, even under normal conditions. Whether this indicates a mechanism by which brain angiogenesis is kept under control or whether it is an adaptive response to counteract any aberrant alterations in VEGF₁₆₅—induced barrier permeability is not known.

In previous studies, we had observed that VEGF_{165b} can effectively block VEGF_{164/165}—induced barrier permeability, inflammatory angiogenesis, neutrophil infiltration, junctional disintegration, and edema.^{23,26} In the present study, however, neither VEGF_{164/165} nor VEGF_{165b} induced barrier permeability or angiogenesis in brain endothelial cells as potently as in nonbrain endothelial cells. Furthermore, although several reports also suggest that VEGF_{164/165} affects barrier function, we did not observe any remarkable effects on brain endothelial barrier in bEnd.3, HBMEC-3, and HCEC-D3 cells. However, HUVECs and the endothelial-like cell line ECV-304, two recognized nonbrain endothelial models,^{43–47} exhibited decreased barrier in response to VEGF₁₆₅, compared with their respective controls and VEGF_{165b}—treated barrier under both normal and OGDR conditions. This indicates that brain endothelial cells respond differently to VEGFs than do nonbrain endothelial cells.

Consistent with the mouse brain endothelial *in vitro* barrier studies, our *in vivo* experiments did not demonstrate a significant difference in poststroke brain edema (an indicator of blood–brain barrier damage and increased permeability). Thus, the detrimental effect of VEGF_{165b} on cerebral vasculature does not appear to be mediated primarily by its effect on brain endothelium. Because different components of neurovascular units differentially produce VEGF_{165b} and VEGF₁₆₅, it is important to consider the role of neurovascular constituents and their interactions in mediating VEGF isoform-dependent barrier regulation.⁴⁸ Three-dimensional experiments with neurons or astrocytes cocultured with brain endothelial cells may provide more information on how neurovascular interactions can govern VEGF isoform-dependent barrier permeability.

To study functional roles of VEGF_{165b} after focal cerebral ischemia in mice, we induced systemic VEGF_{165b} by administering adenoviruses encoding either recombinant VEGF_{164b} or GFP (control) before experimental stroke. We have previously observed that Ad-rVEGF_{164b} significantly protects endothelial barrier against VEGF-induced permeability,^{23,26} and that VEGF_{164/165} increases leukocyte adhesion.⁴⁹ Although we expected an improvement in blood–brain barrier in Ad-rVEGF_{164b}—treated mice after stroke (compared with untreated or GFP controls), we found no significant differences in blood–brain barrier permeability between Ad-GFP—treated and Ad-rVEGF_{164b}—treated mice.

Because postischemic angiogenesis plays an important role in attenuation of ischemic brain injury,⁵⁰ we investigated

whether the Ad-rVEGF_{164b} treatment interfered with postischemic brain angiogenesis and thus contributed to aggravation of injury. However, our *in vitro* Matrigel angiogenesis assay revealed no significant differences in numbers of vascular structures with Ad-rVEGF_{164b} treatment, compared with controls. Interestingly, OGDR-challenged, VEGF_{165b}—treated HUVECs exhibited a significant increase in the numbers of capillary tubes formed on Matrigel, indicating that not only barrier integrity but also angiogenic responses to VEGFs differ by cell type and nature of insult. Consistent with these *in vitro* results, we did not observe significant differences in the numbers of cerebral blood vessels in VEGF_{164b}—treated stroke mice, compared with controls (data not shown). Taken together, our findings indicate that, rather than a cell-specific effect of VEGF isoforms, the relative levels of VEGF isoforms and their interactions with neurovascular units in stroke brain determine the final outcome of injury.

In the present study, although there was no significant effect of VEGF_{165b} on brain endothelial barrier or angiogenic potential *in vitro* or *in vivo*, ischemic brain damage in Ad-rVEGF_{164b}—treated stroke mice was significantly greater than in controls. One explanation might be interference with the VEGF_{164/165}—mediated neuroprotection in cerebral ischemia by increased VEGF_{165b} levels. Our results showing increased neurodegeneration in Ad-rVEGF_{164b}—treated stroke mice strongly support the notion that increased VEGF_{165b} levels might interfere with VEGF₁₆₅—mediated neuroprotection, thereby promoting neurodegeneration and ischemic brain injury. Although VEGF_{165b} has been shown to have a direct neuroprotective effect on hippocampal neurons,⁵¹ Lipinski et al⁵² found that VEGF_{165b} does not have any neuroprotective effect in a mouse model of end-stage retinitis pigmentosa. Because VEGF_{165b} has been shown to be a survival factor, the mechanism by which it counteracts VEGF_{164/165} function in barrier permeability and inflammatory angiogenesis but still functions in neuronal survival is of interest. Our current findings, however, suggest that, rather than having a direct effect on neurons during cerebral ischemia, VEGF_{165b} might instead interfere with VEGF_{164/165}—mediated neuroprotection and thus could have affected neuronal survival. These results indicate that the roles of VEGF isoforms cannot be generalized to all cell types and injuries, and that type and severity of disease and the cell or tissue types involved must be considered in greater specificity. This is important because in diseases outside the central nervous system (CNS), such as colitis or hindlimb ischemia, VEGF₁₆₅ induces both inflammatory angiogenesis and endothelial permeability, and increased VEGF_{165b} levels can provide significant protection by counteracting VEGF_{164/165} functions.^{20,23,26,49,53} However, because in the brain neuronal survival and ischemic brain angiogenesis are crucial for survival, interference with VEGF_{164/165}—mediated neuroprotection by VEGF_{165b} can aggravate stroke brain damage. This suggests a more complex cellular and biochemical signaling among VEGF_{164/165}, VEGF_{165b}, neurovascular units, and infiltrating immune cells. Nonetheless,

other possibilities exist. For example, increased circulating levels of VEGF_{165b}, as were seen here, have been demonstrated in pre-eclampsia (ie, pregnancy-related hypertension),^{54,55} and VEGF_{165b} has been shown to block VEGF₁₆₅—mediated vasodilatation.^{55,56} Thus, blocking VEGF₁₆₅—mediated vasodilatation and blood flow to the brain might in part explain the increase in infarct size. Further experiments to investigate the role of VEGF_{164b} in vasodilatation and blood flow during stroke, as well as VEGF isoform-dependent neurovascular interactions, are warranted.

Because VEGF_{164/165} plays a prominent role in neutrophil chemotaxis (in non-CNS injury models),⁴⁹ and because VEGF_{165b} can counteract VEGF_{164/165}—mediated neutrophil infiltration, we investigated the role of VEGF_{165b} in neutrophil infiltration after stroke. We observed a significant increase in brain neutrophil content in Ad-rVEGF_{164b}—treated stroke mice, compared with Ad-GFP controls. To investigate whether VEGF_{165b} can directly promote neutrophil infiltration, we assayed neutrophil transendothelial migration across mouse and human brain endothelial cells. Our results show that, apart from VEGF_{164/165}, VEGF_{165b} also functions as a potent chemoattractant for neutrophils, and elevated VEGF_{165b} levels can induce neutrophil chemoattraction into the tissue.

Neutrophils are known to secrete VEGF_{164/165}, which plays various roles in tissue and vascular remodeling.^{57–59} In the present study, activated neutrophils produced significantly higher levels of VEGF_{165b}, compared with VEGF_{164/165}. This result indicates that increased neutrophil infiltration can result in further increased levels of the VEGF_{165b} isoform in the brain of Ad-rVEGF_{164b}—treated stroke mice. Although both VEGF isoforms induced neutrophil migration across HCMEC-D3 cells under both control and OGDR conditions, we did not observe a significant difference in neutrophil migration between the two conditions. Cowan and Easton⁶⁰ showed that neutrophils block reversible barrier permeability induced by OGDR in HCMEC-D3 cells. It is well known that neutrophil migration across OGDR-challenged brain endothelial cells is dependent on OGDR-induced barrier permeability and chemokine gradient across the barrier. However, because neutrophils block OGDR-induced barrier permeability in HCMEC-D3 cells, the extent of neutrophil migration is dependent on chemokine gradients, but not on OGDR-induced barrier permeability. This might be the main reason behind the finding of no significant difference between control and OGDR-induced neutrophil migration for HCMEC-D3 cells. The observation that VEGF_{165b} treatment increased neutrophil migration and infiltration may indicate that VEGF_{165b} and VEGF₁₆₅ promote similar neutrophil infiltration in the CNS. Because VEGF₁₆₅ is neuroprotective, increased secretion of VEGF₁₆₅ by infiltrated neutrophils might be beneficial.^{57,60} However, VEGF_{165b} production by infiltrated neutrophils would further increase the VEGF_{165b}/VEGF₁₆₅ ratio and thus could tilt the balance from cerebrovascular protection to injury and stroke damage.

Further studies aimed at elucidating the specific role of neutrophil derived VEGF_{164/165} and VEGF_{165b} in modulating neuronal survival in stroke should help elucidate the cell-specific role of VEGF_{165b} in stroke pathology. Nevertheless, it can be understood that, rather than a direct effect on neurons, VEGF_{165b} might exert an indirect effect by interfering with VEGF₁₆₅ function and promoting neutrophil infiltration in stroke brain. The present study thus demonstrates an important role for the VEGF_{165b} isoform in immune system—CNS interactions after cerebral ischemia.

Conclusions

Even though VEGF is a potent inducer of systemic vascular leakage, in the brain the angiogenic VEGF isoforms are also powerful neuroprotective factors that may enhance repair and survival. Despite barrier permeability effects of VEGF_{164/165}, interference with VEGF_{164/165} signaling in the brain may aggravate postischemic brain damage and worsen neurological recovery and outcomes in stroke. The roles of VEGFs in the regulation of CNS—neurovascular and CNS—immune system interactions associated with ischemic brain angiogenesis are highly complex and merit further investigation.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.06.009>.

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