Vascular Endothelial Growth Factor Receptor-2 Inhibition Promotes Cell Death and Limits Endothelial Cell Proliferation in a Neonatal Rodent Model of Stroke

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Background and Purpose—Recent studies in neonatal rodent stroke models suggest that recovery is due in part to upregulation of hypoxia-inducible factor-1-a and its downstream target, vascular endothelial growth factor. Vascular endothelial growth factor is upregulated after a hypoxic insult and is involved in neuronal survival, angiogenesis, and neurogenesis during the recovery process.

Methods—We performed a 1.5-hour transient middle cerebral artery occlusion in 10-day-old rats with injury verified by diffusion-weighted MRI during occlusion to determine the effects of vascular endothelial growth factor receptor-2 (VEGFR2) inhibition on injury, apoptosis, and angiogenesis. Two days after reperfusion, the pups received either the VEGFR inhibitor, SU5416 (10 mg/kg per dose) or vehicle (1% dimethyl sulfoxide) for 3 days.

Results—VEGFR2 inhibition worsened injury 7 days after injury when compared with the vehicle-treated and injury-alone groups ($P_{<0.01}$). Furthermore, receptor inhibition was associated with increased VEGFR2 expression 5 days after injury ($P_{<0.05}$) and increased spectrin cleavage with a shift in favor of the calpain-mediated, caspase-3-independent cleavage ($P_{<0.01}$). Increased areas of cleaved caspase-3 staining were seen in treated rats at 7 days ($P_{<0.01}$). There were no differences in gliosis or macrophage recruitment as measured by glial fibrillary acidic protein and Iba-1 expression at this time point. Lastly, VEGFR2 inhibition did not affect the overall vessel surface area but reduced endothelial cell proliferation in injured caudate.

Conclusions—Inhibition of VEGFR2 signaling worsens injury, affects cell death, and reduces endothelial cell proliferation after neonatal stroke. Injury exacerbation may be in part due to a shift of cell fate from apoptosis to necrosis on the continuum spectrum of cell death as well as effects on angiogenesis in the injured brain. (Stroke. 2010;41:343-349.)

Key Words: angiogenesis • apoptosis • cerebral ischemia • development • necrosis • postnatal

Neonatal stroke occurs in one in 4000 live births and leads to significant morbidity and mortality. Approximately half of the survivors have long-term sequelae, including seizures and neurological deficits. However, the pathophysiology of the injury sustained after stroke is not well understood and currently there are no preventive or treatment measures available clinically. We have previously described a nonhemorrhagic ischemia–reperfusion stroke model in the neonatal rat using transient right middle cerebral artery occlusion (MCAO). This model creates a reproducible injury verifiable by diffusion-weighted MRI in the ipsilateral cortex without affecting the contralateral hemisphere. Because the 10-day-old rat exhibits similar brain maturity to the human term newborn, this age was selected to study the pathophysiology of neonatal stroke.

Hypoxia-inducible factor-1 is a heterodimeric transcription factor with a constitutive β subunit and an inducible α subunit. After hypoxia, dimerization of the 2 subunits leads to upregulation of downstream targets thought to be protective, including erythropoietin, vascular endothelial growth factor (VEGF), glycolytic enzymes, and glucose transporters. These targets are thought to promote cell survival, angiogenesis, and neurogenesis leading to improved outcomes.

VEGF plays an essential role in angiogenesis. It is a potent mitogen for endothelial cells and plays a pivotal role in the regulation of normal and abnormal angiogenesis. VEGF-A is widely expressed throughout the developing fetus as well as in most major organs in the adult. It is thought that most biological signaling regulating cerebral angiogenesis occurs through VEGF receptor 2 (VEGFR2, also known as Flk-1). We have previously demonstrated an upregulation in both hypoxia-inducible factor-1a and its downstream target, VEGF, after MCAO.
In adult MCAO models, VEGF administration decreases infarct volume, improves injury scores and neurocognitive outcomes, and supports both neurogenesis and angiogenesis. Conversely, VEGFR2 inhibition worsens injury and increases cell death in an adult traumatic brain injury model. It has become apparent that, at least in adult animals, the time course of administration of VEGF is critical because early administration actually increases brain edema and blood-brain barrier breakdown, whereas later administration seems to induce neuroprotective effects.

The data in neonates are still limited. After hypoxia ischemia in neonatal mice, Laudenbach et al have found that VEGF plays an essential role in the beneficial and protective effects of hypoxic preconditioning in neonatal mice. Intra-cerebroventricular VEGF administration has been shown to decrease tissue loss and injury scores. However, the role of and effects of VEGF upregulation are not well described in the setting of neonatal stroke.

We hypothesize that VEGF signaling through VEGFR2 plays an essential role in the recovery and repair after neonatal stroke and that inhibiting this receptor will lead to increased tissue injury and will adversely affect angiogenesis and repair.

**Methods**

The University of California, San Francisco Institutional Animal Care and Use Committee approved all animal research and every effort was made to minimize animal suffering and reduce the number of animals used.

**Cerebral Focal Ischemia and Reperfusion and MRI**

Sprague-Dawley dams with a dated litter of pups were purchased from Charles River Laboratories (Wilmington, Mass). Transient 1.5-hour right MCAO was performed in 10-day-old pups (P10) as previously described.

Pups were examined by diffusion-weighted MRI spin echoplanar imaging at 75 to 80 minutes after MCAO. The entire brain was imaged with serial 2-mm thick coronal sections as previously described followed by reperfusion at 90 minutes. The incidence of the desired injury pattern—diffusion-weighted MRI abnormalities in the cortex and the caudate with no injury in brain stem—was seen on MRI in approximately 76%. Animals that exhibited ischemic injury in atypical regions such as the brain stem or with no cortical involvement were excluded from the study. The overall survival of injured animals was 71%. This nonsurgical mortality was potentially due to maternal neglect and poor suckling with weight loss despite gavage feedings.

**SU5416 Treatment and 5-Bromodeoxyuridine Injections**

Forty-eight hours after MCAO, pups were treated with either VEGFR2 inhibitor 3-[2,4-dimethylpyrroil-5-yl methylidenediy]-2-indolinone (SU5416) in 1% dimethyl sulfoxide (Calbiochem) at 10 mg/kg or 1% dimethyl sulfoxide as a once-daily intraperitoneal injection for 3 days (P12 to P14). Treatment was delayed to avoid possible adverse effects reported in adult brain injury models as well as prevent the effects of SU5416 on acutely occurring neuronal apoptosis. A subgroup of rats underwent MCAO but did not receive any treatment (MCAO only). A litter block design was used throughout the study. Starting at P15, 5-bromodeoxyuridine injections, 50 mg/kg per dose, were performed twice daily for 2 days.

**Histology and Injury Scoring**

At 7 days after surgery, animals were anesthetized and euthanized by transcardiac perfusion with cold 4% paraformaldehyde/phosphate-buffered saline (pH 7.4).

Representative 50-μm coronal Nissl-stained sections from the anterior and posterior caudate and cortex were analyzed for injury. Sections were graded on a scale of 0 to 3 with 0 = no injury, 1 = few small areas of focal injury, 2 = multiple areas of focal injury, and 3 = widespread injury with loss of architecture. These 4 regional scores were summed to provide a scale of 0 to 12.

**Immunohistochemistry**

Adjacent sections were immunostained using monoclonal anti-MAP2 antibody (1:500; Sigma), monoclonal antifibrillary acidic protein antibody (GFAP) (1:1000; ImmunO) or polyclonal rabbit anticleaved caspase 3 (1:200; Cell Signaling) as previously described. Areas of immunoreactivity were measured using Image J software (National Institutes of Health) and expressed as a percentage of the total area of the ipsilateral hemisphere.

**Immunofluorescence and Vessel Analysis**

In a subset of SU5416- and dimethyl sulfoxide-treated animals, sections were double-stained with rabbit polyclonal anti-PECAM (1:750; courtesy of Dr Peter Newman, Milwaukee, Wis) and mouse monoclonal anti-5-bromodeoxyuridyridine (Brdu) (1:750; BD Biosciences).

For analysis, stacks of 24 fluorescence images captured at 2-μm Z step (20× objective) were acquired (Axiointert 100; Zeiss, Inc) in a single section in injured anterior caudate regions and in similar contralateral regions using OpenLab Software (Improvision, Inc). Images were deconvolved and analyzed using Volocity Software (Improvision, Inc).

Mean vessel surface and total vessel surface were measured in 2 randomly chosen fields of view of identical volume, 1.5e+07 μm³ voxel. The total number of 5-BrdU-positive cells was measured in the same region of interest. In addition, percent of 5-BrdU-positive cells with >50% surface overlapping with PECAM-positive vessels was calculated in the same region of interest.

**Western Blot Analysis**

Western blot analysis was performed in lysates obtained from injured and matching contralateral brain tissue collected at P15 as previously described. Blots were probed with polyclonal rabbit anti-VEGF (1:400; Santa Cruz), rabbit anti-VEGFR2 (1:250; Santa Cruz), rabbit anti-Iba1 (1:1000; Wako Chemicals), mouse antispectrin (1:1000; Millipore), and mouse anti-GFAP (1:1000; ImmunO) antibody. The results were normalized to B-actin expression in the same samples.

**Statistical Analysis**

Data are shown as mean±SEM. Analysis of variance was performed with Bonferroni correction for multiple comparisons.

**Results**

**VEGFR2 Inhibition Worsens Injury**

MCAO resulted in consistent early injury, as evident from diffusion-weighted MRI during occlusion; injury occupied 66.9%±4.4% of the ipsilateral hemisphere.

Animals treated with SU5416 had increased injury score at 7 days compared with vehicle-treated or nontreated animals (P<0.01; Figure 1A).

**MCAO Affects VEGFR2 Expression**

VEGF expression was similar across the 3 treatment groups at 24 hours after the last SU5416 injection (5 days after injury; Fig 1B). However, there was a compensatory increase in VEGFR2 expression in the inhibitor-treated group when
compared with the MCAO-only group (*P*<0.05) but no significant difference between the inhibitor and vehicle-treated group (Figure 1C), possibly reflecting an effect of dimethyl sulfoxide on receptor expression.

**VEGFR2 Inhibition Enhances Cell Death After Unilateral MCAO**

We previously demonstrated that the structural protein spectrin is cleaved in acutely injured brain tissue after neonatal stroke and that cleavage occurs both by caspase-3-dependent and calpain-dependent mechanisms. At 5 days after injury, both caspase-3 and calpain-mediated spectrin cleavage were observed in injured tissue of all animals as was evident from the presence of the 120-kD (caspase-3-dependent) and 160-kD (calpain-dependent) products of spectrin degradation (Figure 2A). A 4.5-fold increase in calpain-dependent spectrin cleavage observed in ipsilateral compared with contralateral tissue in nontreated animals was not affected by vehicle treatment (4.1-fold increase) but was enhanced by VEGFR2 inhibition (7.5-fold increase, *P*=0.066; Figure 2B). A 6- to 10-fold increase in caspase-3-dependent spectrin cleavage in ipsilateral hemisphere was observed in all groups (Figure 2C, nonsignificant). Vehicle itself also shifted a sparse caspase-3- and calpain-dependent spectrin cleavage present in the contralateral hemisphere (Figure 2A). Seven days after injury, there was also increased area of cleaved caspase-3 immunostaining in the inhibitor group when compared with the MCAO-only and vehicle-treated groups (*P*<0.01; Figure 2D). At this time point, there were no differences in the area of loss of MAP2 staining across the 3 groups (Figure 3D).

**Glial Response to VEGFR2 Inhibition**

At 5 days after injury, there was an increase in Iba1 expression on Western blots in the SU5416-treated group when compared with the MCAO-only group (*P*<0.05; Figure 3A), suggesting increased microglial activation and/or macrophage accumulation after treatment, but not when compared with the vehicle-treated group. There was no significant difference in GFAP expression on Western blot (5 days) or area occupied by GFAP-positive cells (7 days) across the treatment groups (Figure 3B–C).

**VEGF Receptor Inhibition Affects Cell Proliferation in Injured Caudate**

Because VEGF signaling may contribute to angiogenesis after brain injury, endothelial proliferation and vessel surface area were determined after SU5416 treatment. Because the
caudate represents the core of the MCAO lesion, further studies were focused in this region.

In a subset of animals used (n = 4 to 5 per group), injury in the caudate was seen in all animals, and injury scores in the anterior caudate were 1.9 ± 0.4 in SU5416-treated group and 1.3 ± 0.8 in vehicle-treated group, respectively. A total PECAM-1-labeled vessel surface area in the contralateral caudate was $174 \times 10^{-3} \pm 29 \times 10^{-3}/\mu m^2$ per chosen voxel and $168 \times 10^{-3} \pm 36 \times 10^{-3}/\mu m^2$ per voxel in ipsilateral caudate in dimethyl sulfoxide- and SU5416-treated rats, respectively (nonsignificant). No significant changes in total vessel surface were seen in the ipsilateral hemisphere in any group.

The number of 5-Brdu-positive cells, which were determined in the same voxel in both injured and contralateral caudate, was not significantly different between hemispheres in the vehicle-treated group (Figure 4A, B; P > 0.1). SU5416 treatment tended to reduce the number of 5-Brdu-positive cells in injured caudate (Figure 4B; P = 0.10). Volumetric analysis of spatial distribution of 5-Brdu-positive cells within and outside (nontouching) of PECAM-1-positive vessels showed that 70% ± 20% of 5-Brdu-positive cells were seen within vessels in the contralateral caudate in the vehicle-treated group. In the inhibitor group, 80% ± 20% were seen within PECAM-1-positive vessels. The percentage of 5-Brdu-positive cells in vessels was significantly reduced by treatment (Figure 4C; P = 0.016), suggesting that VEGFR2 inhibition affects endothelial cell proliferation in injured regions within 1 week after MCAO at P10.

**Discussion**

This study demonstrates that delayed VEGFR2 inhibition after transient MCAO in P10 rats exacerbates injury in the neonatal brain, in part by increasing cell death and reducing endothelial cell proliferation. Importantly, VEGFR2 inhibition appeared to shift the balance of cell death toward necrosis on the apoptosis-necrosis continuum at the time point studied, suggesting that the VEGFR2 pathway mediates injury and cell death after neonatal stroke and that manipulation of this pathway may change the cell fate along the cell death continuum. Vehicle, dimethyl sulfoxide, a known neuroprotectant, appeared to affect cell death as well. The effects of VEGFR2 inhibition on inflammation and gliosis are unclear.

This is the first study of VEGFR2 inhibition in a neonatal transient MCAO model. Although the area with increased cleaved caspase-3 staining did correlate with the worsened injury scores, the associated spectrin cleavage data suggests that not all cell death is caspase-3-mediated. In fact, administration of SU5416 appeared to favor calpain-dependent cleavage yielding the presence of the 160-kD spectrin degradation product resulting in a shift from caspase-3-dependent to calpain-dependent cleavage. In the inhibitor group, increased calpain-mediated spectrin cleavage correlated with higher injury scores at 7 days. There also remains the question whether dimethyl sulfoxide in combination with MCAO is somehow inducing caspase-3-mediated spectrin cleavage, which is somewhat counterintuitive, because it has been shown that dimethyl sulfoxide, a free radical scavenger, is protective after ischemic injury in gerbils, at least in higher doses. In this study, in which we used a much smaller dimethyl sulfoxide concentration, 1%, there was no protection.

Inhibition of the receptor was associated with a compensatory increase in VEGFR2 expression. Our data concurs with adult data showing that receptor inhibition worsens injury and increases VEGFR2 expression. Furthermore, it complements previous studies showing that VEGF administration inhibits caspase-3 activation, decreases apoptosis, and improves infarct volumes. Inhibition of the VEGFR2 in an adult traumatic brain injury model results in increased VEGFR mRNA synthesis but a decrease in the levels of the active VEGFR2. Furthermore, receptor inhibition results in increased injury size and cell death as well as decreases capillary density.

VEGFR2 is known to mediate the majority of the downstream angiogenic effects of VEGF, including microvascular...
permeability, endothelial cell proliferation, migration, and survival. Binding of VEGF to its receptor on the surface of endothelial cells activates intracellular tyrosine kinases, triggering multiple downstream signals that promote angiogenesis. VEGFR2 knockout mice die in utero due to early defects in the development of hematopoietic and endothelial cells. The lack of a single VEGF allele results in abnormal vessel development and embryonic mortality.¹⁹

Figure 4. VEGFR2 inhibition reduces endothelial cell proliferation within 7 days after MCAO. A, A representative 3-dimensional reconstruction of an image (20× objective). Thin arrows point to proliferating cells; arrowheads point to parenchymal 5-bromodeoxyuridine (BrdU)-positive cells (nonassociated with vessels). Note that endothelial proliferation occurs in a subset but not all vessels. Red, PECAM; green, BrdU. B, Total number of BrdU-positive cells per region of interest. Percent of BrdU-positive cells associated with vessels. C, Percent of BrdU-positive cells associated with vessels.
Our previous results in the MCAO model in the P10 rat have shown that VEGF expression is increased early, within hours after MCAO in neurons and at 7 days after MCAO, predominantly in astrocytes, raising the possibility that VEGF-mediated angiogenic response is initiated within 1 week after injury. Therefore, we used several measures to see if postischemic angiogenesis occurs within this timeframe and whether it is affected by VEGFR2 inhibition. One week after injury, MCAO itself does not affect either the mean or the overall surface of PECAM-1-positive vessels in the injured caudate. Delayed inhibition of VEGFR2 does not affect vessel surface either. SU5416 treatment tended to decrease the total number of 5-bromodeoxyuridine-positive cells bilaterally in the caudate but more prominently in the injured caudate where the number of PECAM-1-positive/5-bromodeoxyuridine-positive cells was significantly decreased by treatment, indicating the negative effect of receptor inhibition on endothelial cell proliferation. It is unclear whether reduced endothelial cell proliferation or disrupted VEGF/VEGFR2 signaling in neurons contributes to increased necrosis and apoptosis. Angiogenesis can play opposite roles in long-term injury outcome. It can enhance the repair process by enabling and stimulating migration of progenitor cells and increasing neurogenesis, but it may be injurious as well by enabling excessive blood–brain barrier permeability and hemorrhagic transformation. Delayed SU5416 administration did not produce any hemorrhage in our experiments. Consistent with this finding, we observed essentially no substantial areas of lectin leakage in injured tissue after intravenous administration of fluorescently labeled lectin in a separate group of animals (unpublished data), suggesting that VEGFR2 inhibition days after injury does not substantially affect vessel permeability. Importantly, reduced endothelial cell proliferation in injured but not in contralateral tissue after VEGFR2 treatment revealed different susceptibility of endothelial cell proliferation to treatment between postischemic and unaffected tissue in the same animal. Taken together, these data suggest that 1 week after injury, angiogenesis is not increased in injured tissue beyond that seen in both naïve brain and in contralateral tissue of injured animals during this developmental stage and that postinjury angiogenesis is in its early stage at this time.

As noted previously, although VEGF was first identified as a promoter of angiogenesis, subsequent studies have concluded that its role in neuroprotection is not limited to the initiation and support of angiogenesis in the neurovascular niche. VEGF has been shown to support neurogenesis in vitro, completely independent of any vasculogenesis, stimulating axonal outgrowth and neuronal survival in dorsal root ganglia and superior cervical neurons as well as promoting the survival of mesencephalic explants. VEGF administration promotes neuronal survival after ischemia (oxygen glucose deprivation) in hippocampal and cortical neuron cultures as well as after glutamate administration. Conversely, VEGFR2 inhibition prevents endothelial cell capillary tube formation in vitro. In vivo, VEGF administration after MCAO in adult rodents is associated with improved infarct volumes, neurological outcomes, and specifically with increased numbers of 5-bromodeoxyuridine and double cortin or neuroD-coted labeled cells as far as 28 days postinjury, suggesting that the improved long-term outcome maybe due to improved survival of the recruited/stimulated precursors in the VEGF-treated animals compared with the injured controls. If indeed, VEGFR2 signaling promotes the long-term survival of stimulated neuronal precursors, receptor inhibition would lead to increased death of those precursors as well as other affected cells in the endothelial precursor lineage.

Our study has limitations. Although the injury volumes calculated from MRI were uniform shortly after MCAO, there was a fairly high mortality rate, which was unrelated to treatment, so the animals used for analysis 5 to 7 days later may have been, as a group, healthier animals. Animals were not analyzed by gender due to small numbers; therefore, it is impossible to know whether gender influences outcomes.

To summarize, these data show that VEGFR2 inhibition increases tissue injury and cell death in a neonatal MCAO model as well as reduces endothelial cell proliferation in injured caudate. In addition, it may affect recovery by shunting cells toward a necrotic pathway along the continuum pathway. Further assessing calpain versus caspase-3-mediated cell death, alterations in cell death phenotype, neurogenesis, and angiogenesis in the neurovascular niche as well as long-term cognitive and behavioral outcomes may uncover the role of VEGF signaling in recovery and regeneration after ischemia–reperfusion.

Acknowledgments
We thank Dr Peter Newman for his generous gift of anti-PECAM antibody, Ann Sheldon and Joel Faustino for technical assistance, and Kei Kaneshiro for administrative support.

Source of Funding
This work was funded by National Institutes of Health NS35902.

Disclosures
None.

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Stroke. 2010;41:343-349
doi: 10.1161/STROKEAHA.109.564229
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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