Vascular Endothelial Growth Factor Promotes Pericyte Coverage of Brain Capillaries, Improves Cerebral Blood Flow During Subsequent Focal Cerebral Ischemia, and Preserves the Metabolic Penumbra

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Background and Purpose—Therapeutic angiogenesis aims at improving cerebral blood flow by amplification of vascular sprouting, thus promoting tissue survival under conditions of subsequent ischemia. It remains unknown whether induced angiogenesis leads to the formation of functional vessels that indeed result in hemodynamic improvements. Observations of hemodynamic steal phenomena and disturbed neurovascular integrity after vascular endothelial growth factor delivery questioned the concept of therapeutic angiogenesis.

Methods—Mice were treated with recombinant human vascular endothelial growth factor (0.02 μg/d; intracerebroventricular) for 3 to 21 days and subsequently exposed to 90-minute middle cerebral artery occlusion. Angiogenesis, histological brain injury, IgG extravasation, cerebral blood flow, protein synthesis and energy state, and pericyte coverage on brain capillaries were evaluated in a multiparametric approach combining histochemical, autoradiographic, and regional bioluminescence techniques.

Results—Vascular endothelial growth factor increased brain capillary density within 10 days and reduced infarct volume and inflammation after subsequent middle cerebral artery occlusion, and, when delivered for prolonged periods of 21 days, enhanced postischemic brain–barrier integrity. Increased cerebral blood flow was noted in ischemic brain areas exhibiting enhanced angiogenesis and was associated with preservation of the metabolic penumbra, defined as brain tissue in which protein synthesis has been suppressed but ATP preserved. Vascular endothelial growth factor enhanced pericyte coverage of brain endothelial cells via mechanisms involving increased N-cadherin expression on cerebral microvessels.

Conclusions—That cerebral blood flow is increased during subsequent ischemic episodes, leading to the stabilization of cerebral energy state, fosters hope that by promoting new vessel formation brain tissue survival may be improved.

Key Words: angiogenesis • autoradiography • bioluminescence imaging • energy metabolism

Patients with cerebrovascular diseases frequently exhibit chronic hemodynamic disturbances,1 predisposing them to severe and devastating strokes. There is a major need for revascularization strategies in such patients.1 Also in patients without hemodynamic deficits, vascular sprouting takes place after stroke.2,3 It has been proposed that subjects at vascular risk might benefit from the therapeutic amplification of angiogenesis.4 The underlying hypothesis is that vascular growth should increase blood flow and reduce the impact of future stroke events.3

Animal studies examining the effects of angiogenesis induced by vascular endothelial growth factor (VEGF) rapidly led to clinical trials in a variety of medical conditions, including coronary heart and peripheral occlusive artery disease.1,5 However, such trials have so far been of limited success.1,6 This raises questions about the viability of angiogenesis as a therapeutic approach. In animal models of ischemic stroke, VEGF-induced angiogenesis has been shown to result in structural neuroprotection and functional neurological recovery.7,8 Whether the preservation of ischemic tissue was a consequence of enhanced cerebral blood flow (CBF) or of neuroprotective effects of VEGF was unclear.9 VEGF promotes neuronal survival both directly via VEGF’s receptor VEGFR2 and indirectly by release of brain-derived neurotrophic factor.8

Although several studies have examined VEGF-induced angiogenesis in models of focal cerebral ischemia,1 only 2 have evaluated the extent to which VEGF influences regional

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CBF in the ischemic brain. In a MRI study, acute VEGF infusion was shown to induce a transient CBF increase in ischemic brain tissue lasting over 3 hours, which was interpreted as vasorelaxation induced by this growth factor. In mice expressing human VEGF chronically under a neuron-specific NSE promoter, increased CBF was observed in nonischemic brain areas alongside a reduction in CBF in ischemic brain areas, suggesting that the enhanced angiogenesis had induced a hemodynamic steal flow. The mice examined expressed human VEGF throughout the brain, resulting in globally increased vessel densities. CBF was scarcely altered in this mouse line under physiological conditions.9

Raising further questions whether newly formed blood vessels are functional, loss of pericyte coverage of endothelial cells has previously been noticed in Matrigel assays following VEGF treatment in a model of platelet-derived growth factor (PDGF)-BB–induced angiogenesis. Immunoprecipitation studies revealed a hitherto unknown VEGF-induced deactivation of PDGF-BB’s receptor PDGFRβ that is expressed on pericytes, which was mediated by the interaction of PDGFRβ with VEGF’s receptor VEGFR2. Inhibition of VEGFR2 prevented the formation of this receptor complex and restored pericyte coverage, thus stabilizing the newly formed blood vessels. Whether interactions between VEGFR2 and PDGFRβ take place in vivo in the brain and whether they are relevant for ischemic stroke were unknown.

To evaluate the concept of VEGF-induced therapeutic angiogenesis, we exposed mice that had been treated intracerebroventricularly with VEGF to focal cerebral ischemia. In a multiparametric imaging strategy, combining CBF and cerebral protein synthesis (CPS) autoradiography, regional ATP bioluminescence imaging and histochecmical techniques, we then assessed the effects of VEGF-induced angiogenesis on regional CBF, brain metabolism, and vascular integrity.

Materials and Methods

Experimental Groups

Experiments performed with government approval according to the National Institutes of Health guidelines for the care and use of laboratory animals. In the first set of studies, male C57BL/6j mice (20–25 g) were randomly assigned to 6 groups treated with vehicle (normal saline) or recombinant human VEGF165 (rhVEGF165) for 3, 10, or 21 days (6–7 animals per group). In these animals, no ischemia was induced. Brain capillary and arteriole densities and diameters were evaluated by immunohistochemistry or ex vivo angiography. In the second set of studies, male C57BL/6j mice were randomly assigned to 6 groups treated with vehicle (normal saline) or recombinant human VEGF165 (rhVEGF165). Three, 10, or 21 days later, 90 minutes of middle cerebral artery (MCA) was induced, followed by 90-minute reperfusion. These animals were used for CBF and CPS double autoradiography and regional ATP bioluminescence imaging.

Delivery of Recombinant Human VEGF

Cannulae linked to miniosmotic pumps (Alzet 2004; Palo Alto, CA) were implanted into the left lateral ventricle for administration of vehicle or rhVEGF165 (Peprotech, Hamburg, Germany; 0.02 μg/d), as described. For details, see Methods in the online-only Data Supplement.

Evaluation of Brain Microvessel Density

In 20-μm cryostat sections obtained from the rostrocaudal level of the midstratum, that is, the site of maximum extension of the MCA territory, brain microvessels were evaluated by fluorescence immunohistochemistry using a rabbit anti-CD31 antibody (BD Biosciences, San Diego, CA). The densities of capillaries (diameter, <10 μm) and arterioles (≥20 μm) were analyzed in 7 regions of interest (3 in parietal cortex and 4 in lateral striatum) in the MCA territory both ipsilateral and contralateral to the stroke using the Cell-F software (Olympus, Hamburg, Germany). For details, see Methods in the online-only Data Supplement.

Ex Vivo Angiography of Vessel Lumina

Microvessel lumina were visualized by injection of a mixture of carbon black dyes through the left ventricle. Photographs were taken from the ventral aspect of these brains, on which the mean diameter of the MCA was measured. Subsequently, cryostat sections were obtained that were stained using a rabbit anti-collagen-IV (1:500; Millipore, Billerica, MA) or rat anti-CD31 (BD Biosciences) antibody. The diameters of capillary and arteriole lumina were quantified in 7 regions of interest (see above) of the MCA territory both ipsilateral and contralateral to the stroke. For details, see Methods in the online-only Data Supplement.

Induction of Focal Cerebral Ischemia

Intraluminal MCA occlusion was induced during 1% isoflurane anesthesia using a silicon-coated microfilament. Animals were euthanized 24 hours later by transcardiac perfusion with normal saline or kept under anesthesia for delivery of radioactive tracers. For details, see Methods in the online-only Data Supplement.

Infarct Analysis

Cryostat sections 1 mm apart were stained with cresyl violet. The border between infarcted and healthy tissue was outlined using image analysis software (Image J; National Institutes of Health, Bethesda, MD) and the infarct volume quantified.

Serum IgG Extravasation Studies

Brain sections obtained from the midstratum were processed for serum IgG immunohistochemistry. Stained sections were scanned, converted into gray values, and densitometrically analyzed within the core of the MCA territory.

Western Blots

Protein lysates were obtained from extracted crude microvessels using samples collected from the ischemic and contralateral nonischemic MCA territory. Polyvinylidene fluoride membranes dissolved by SDS-PAGE were incubated with rabbit anti-collagen-IV antibody (4061; Millipore, Schwalbach, Germany). After secondary antibody exposure, membranes were exposed to photoluminescence solution. Protein loading was controlled using a mouse anti-β-actin antibody (4967; Millipore). Blots were repeated 3× to confirm reproducibility. Protein levels were densitometrically analyzed. For details, see Methods in the online-only Data Supplement.

Evaluation of Pericyte Coverage of Brain Capillaries

To evaluate endothelial proliferation and the pericyte coverage of brain capillaries, brain sections were stained with rat anti-CD31 (BD Biosciences; endothelial marker), rabbit anti-desmin (Abcam, Cambridge, UK; pericyte marker), goat-anti-PDGFRβ (R&D Systems; pericyte marker), rabbit anti-Ki67 (ab15580, Abcam, proliferation marker), goat anti-VEGFR2 (AF644; R&D Systems), rat anti-VEGFR1 (MAB471; R&D Systems), or rabbit anticleaved caspase-3 (AB3623, Millipore) antibody,11 which in some experiments was counterstained with terminal transferase dUTP nick-end labeling (kit 11684795910; Roche, Basel, Switzerland). In CD31/desmin, CD31/PDGFRβ, and
CD31/Ki67 double stainings, the percentage of pericyte+ (desmin, PDGFRβ) or proliferating (Ki67) microvessels was examined in 7 regions of interest (see above) ipsilateral and contralateral to the stroke, out of which mean values were formed. Confocal 3-dimensional (3D) stacks were also obtained using a laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Jena, Germany). In these stacks, CD31+ capillaries and surrounding desmin+ pericytes were outlined, allowing for the evaluation of capillary and pericyte volumes, respectively, which were integrated for all 2-dimensional images, resulting in capillary and pericyte volumes, from which volume ratios were formed. For details, see Methods in the online-only Data Supplement.

**CBF and CPS Double Autoradiography**

A total of 150 μCi L-[4,5-3H] leucine (specific activity 151 Ci/mmol; Amersham, Braunschweig, Germany) were administered intraperitoneally, followed by an intraperitoneal injection of 10 μCi 4-iodo-N-methyl-[14C] antipyrine (Amersham) 43 minutes later. After another 2 minutes, animals were instantly frozen in liquid nitrogen. Regional CBF and CPS were calculated as described previously. For details, see Methods in the online-only Data Supplement.

**Regional ATP Bioluminescence Imaging**

For ATP measurement, frozen sections were coated with a layer of frozen reaction mix, and light emissions were recorded using a CCD camera. The metabolic penumbra was calculated by subtracting the ATP-preserved area obtained from ATP bioluminescence images and the CPS-deficient area determined from CPS autoradiography. For details, see Methods in the online-only Data Supplement.

**Statistics**

Laser Doppler flow recordings were evaluated by repeated measurement ANOVA with values determined at 15-minute intervals during MCA occlusion and at 5-minute intervals after reperfusion. Capillary density, infarct volume, IgG extravasation, and all other histochemical studies comparing 4 or 6 groups were evaluated by 2-way ANOVA followed by 2-tailed t tests. Changes in CBF, ATP depletion, and the metabolic penumbra were analyzed by 2-tailed t tests, as were histochemical studies with comparisons between 2 groups. All data are presented as mean±SD. P<0.05 was considered significant.

**Figure 1.** Vascular endothelial growth factor (VEGF) enhances cerebral capillary formation. CD31 immunohistochemistry showing increased brain capillary densities starting 10 days after initiation of VEGF treatment (A) in the hemisphere in which VEGF was delivered, but not (B) in the contralateral hemisphere at distance to the infusion site. CD31 immunohistochemistry showing (C) increased survival of brain capillaries in ischemic tissue of VEGF–treated mice. (D) Again no effect was noticed in the contralateral nonischemic brain. Representative photographs from animals treated with vehicle or VEGF for 21 days are shown. Data are mean±SD (n=6–7 animals per group). *P<0.05/**P<0.01 compared with vehicle. Bars, 100 μm. ROI indicates regions of interest.
Results

Effects of VEGF on Brain Microvessels in Nonischemic Mice

Intraventricular VEGF delivery increased the density and diameter of CD31+ brain capillaries in the left-sided hemisphere, in which VEGF was infused. Capillary density increased starting on day 10 of VEGF treatment (Figure 1A; Table I in the online-only Data Supplement). In the right-sided contralateral hemisphere, capillary density and diameter did not differ between groups (Figure 1B; Table I in the online-only Data Supplement). Endothelial proliferation, assessed by CD31/Ki67 doublestaining, was increased by VEGF at 3 days after initiation of VEGF treatment, more strongly in the left-sided hemisphere receiving the VEGF infusion than contralateral to it (Figure I in the online-only Data Supplement).

In contrast to brain capillaries, the density of arterioles, evaluated in nonischemic animals, was not influenced by VEGF (Table I in the online-only Data Supplement). Notably, VEGF slightly increased the diameter of arterioles. This increase was significant in the contralateral hemisphere after 21 days of VEGF exposure (Table I in the online-only Data Supplement). The diameter of the MCA at its origin was not influenced by VEGF (ipsilateral MCA: vehicle, 0.12±0.01 mm/VEGF, 0.12±0.05 mm; contralateral MCA: vehicle, 0.13±0.01 mm/VEGF, 0.11±0.02 mm).

Effects of VEGF on Brain Capillary Survival in Ischemic Brain Tissue

In animals exposed to MCA occlusion, the survival of CD31+ brain capillaries was increased in ischemic tissue of mice that had been treated with VEGF for 10 or 21 days before (Figure 1C). In the contralateral hemisphere, capillary density again did not differ between groups (Figure 1D).

Effects of VEGF on Ischemic Injury and Blood–Brain Barrier Integrity

Laser Doppler flow recordings taken during and after MCA occlusion to control the reproducibility of ischemias did not reveal any differences between groups (Figure II in the online-only Data Supplement). On MCA occlusion, laser Doppler flow decreased to 10% to 20% of baseline values in all groups. Reperfusion was associated with a rapid restoration of blood flow.

Infarct measurements on cresyl violet staining 24 hours after reperfusion revealed reduced infarct volumes in animals treated with VEGF for 10 or 21 days, but not for 3 days (Figure 2A). The attenuation of brain injury was associated with reduced IgG extravasation in animals receiving VEGF for 21 days, but not for 3 or 10 days (Figure 2B), demonstrating the formation of intact vessels not associated with blood–brain barrier leakage.

VEGF Increases Regional CBF in Nonischemic and Ischemic Brain Tissue

VEGF treatment for 21 days enhanced regional CBF in both hemispheres in nonischemic brain tissue (79.7±63.5 versus 181.9±114.5 mL/100 g per minute in left-sided striatum/96.0±84.9 versus 199.6±107.0 mL/100 g per minute in right-sided striatum of vehicle-treated and VEGF-treated mice, respectively), demonstrating that new vessel formation did indeed translate into a functional improvement of blood flow. When additional MCA occlusion was imposed, increased CBF was noted in ischemic brain tissue (Figure 3A). Interestingly, increased CBF values were again also observed in the contralateral brain (Figure 3B), albeit capillary density was not increased by VEGF. These data suggested that mechanisms other than capillary density contributed to CBF changes.

VEGF-Induced Angiogenesis Stabilizes the Metabolic Penumbra and Prevents ATP Depletion

A stabilization of the metabolic penumbra, defined as brain tissue in which CPS is suppressed but ATP preserved (Figure 3C), and a reduction in the area of tissue exhibiting ATP depletion (Figure 3D) were found in VEGF but not in vehicle-treated animals, thus indicating that blood flow stabilization resulted in a preservation of energy metabolism.

Figure 2. Vascular endothelial growth factor (VEGF) attenuates brain injury after focal cerebral ischemia and reduces postischemic serum IgG extravasation. A, Infarct volumes exhibiting reduction of ischemic injury after VEGF treatment for 10 or 21 days, but not 3 days. B, IgG immunohistochemistry demonstrating reduced IgG extravasation after VEGF treatment for 21 days, but not 3 or 10 days. Data are means±SD (n=6–7 animals per group). *P<0.05/**P<0.01 compared with vehicle. MCA indicates middle cerebral artery.
VEGF Increases Pericyte Coverage of Brain Capillaries

In a conventional immunohistochemical analysis, the vast majority of capillaries (~90%) in the nonischemic brain tissue were surrounded by pericytes that were immunoreactive for desmin (Figure 4B). Thus, no effects of VEGF were noted (not shown). In the ischemic tissue, this value was lower in vehicle-treated mice (~60%; Figure 4A). Interestingly, VEGF did not affect the percentage of pericyte+capillaries, when initiated 3 or 10 days before stroke, but markedly increased pericyte coverage of ischemic capillaries, when started 21 days before (Figure 4A), indicating that VEGF induces the formation of mature vessels.

In view of the ceiling effect of pericyte coverage in the contralateral nonischemic tissue (Figure 4B), where an increase in CBF (Figure 3B), but not capillary density (Figure 1B), was found, a confocal data analysis was also performed, in which desmin+ pericyte volumes determined in 3D stacks were related to the volumes of CD31+cerebral capillaries. In this analysis, VEGF increased pericyte coverage in both hemispheres, when initiated 21 days before the stroke (Figure 4C and 4D). These data provided an explanation why VEGF increased CBF in the contralateral nonischemic brain tissue, although no increase in capillary density was seen.

Immunohistochemistry for PDGFRβ, a second pericyte marker, exhibited lower pericyte coverage rates than desmin in nonischemic brain tissue (~60%; Figure III in the online-only Data Supplement). Although VEGF did not affect the percentage of pericyte+capillaries when
delivered over 3 or 10 days before stroke, there was a trend toward increased pericyte coverage of ischemic brain capillaries in animals that received VEGF over 21 days (45.5±16.5% versus 62.6±12.2% in vehicle-treated versus VEGF-treated animals; nonsignificant in 2-way ANOVA; Figure III in the online-only Data Supplement). Taken together, our data indicated that VEGF enhances pericyte coverage on brain capillaries.

**VEGF Does Not Influence Apoptotic Death of Pericytes**

The number of desmin+/terminal transferase dUTP nick-end labeling+ and desmin+/cleaved caspase-3+ pericytes was not influenced by VEGF, indicating that VEGF neither influenced DNA fragmentation nor caspase-3 activation in pericytes (Figure IV in the online-only Data Supplement).

**VEGFR2 and VEGFR1 Are Not Expressed on Mouse Brain Pericytes**

Because VEGF has previously been shown to induce pericyte ablation in Matrigel assays, which was interpreted as consequence of VEGFR2/PDGFRβ interaction on pericytes,10 the question arose whether pericytes in C57BL6/j mice expressed VEGFR2 or VEGFR1. In immunostainings, we were unable to detect VEGFR2 and VEGFR1 on pericytes (Figure V in the online-only Data Supplement).

**VEGF Increases N-Cadherin on Cerebral Microvessels**

The alignment of pericytes to endothelial cells is mediated by the junctional protein N-cadherin. 20 To evaluate whether N-cadherin was influenced by VEGF, Western blots were prepared with capillary extracts which revealed increased...
reduced. These data were interpreted as hemodynamic steal of regional CBF within the ischemic MCA territory, whereas cerebral blood flow in the ischemic brain contrasts with earlier findings of blood flow redirected into nonischemic areas. In that mouse line, increased regional CBF, evaluated using endothelial cell expression of the junctional protein N-cadherin, N-cadherin expression on cerebral microvessels of animals treated with VEGF for 21 days followed by 90-minute middle cerebral artery (MCA) occlusion and 24-hour reperfusion. Representative blots are also shown. Data are mean±SD (n=6–7 animals per group). *P<0.05 compared with vehicle.

Figure 5. Vascular endothelial growth factor (VEGF) increases the expression of the junctional protein N-cadherin, N-cadherin expression in microvessels that had been treated with VEGF for 21 days (Figure 5).

Discussion

By applying prophylactic VEGF for up to 21 days, we have shown that VEGF induces the formation of mature, functional blood vessels, which enables the brain to cope better with subsequent ischemic strokes, thus enhancing regional CBF, stabilizing cerebral energy state, and reducing brain infarction. Increased brain capillary densities were noted within 10 days of initiation of VEGF treatment, which were accompanied by enhanced pericyte coverage of endothelial cells within 21 days. The induction of angiogenesis closely paralleled tissue survival, providing evidence of links among capillary formation, hemodynamic changes, and tissue preservation.

Our observation that induced angiogenesis increases blood flow in the ischemic brain contrasts with earlier findings of our group using a transgenic mouse line expressing human VEGF under control of a neuron-specific NSE promoter. In that mouse line, increased regional CBF, evaluated using 4-iodo-N-methyl-[14C] antipyrine, was described only in nonischemic brain areas outside the MCA territory, whereas regional CBF within the ischemic MCA territory was reduced. These data were interpreted as hemodynamic steal phenomenon induced by enhanced angiogenesis. Indeed, human VEGF-transgenic mice exhibited an increased capillary density throughout the brain, which might explain why blood flow was redirected into nonischemic areas. Although capillary density was increased by up to 100% or even more in human VEGF-transgenic mice, only mildly increased blood flow values were noted in this mouse line during hypercapnia. These findings raise doubts whether microvascular networks are adopted to tissue needs in this mouse line.

The capability of blood vessels to respond to tissue needs depends on proper interactions between endothelial cells with pericytes, which, as our data suggest, were enhanced by VEGF. Indeed, an increased pericyte coverage was noticed that was associated with enhanced N-cadherin expression. N-cadherin promotes the alignment of pericytes to endothelial cells. Our results are in contrast to observations after VEGF treatment in a model of PDGF-BB-induced angiogenesis, where a loss of pericyte coverage was reported, which was mediated by the deactivation of PDGFRβ by VEGFR2. We have not been able to detect VEGFR2 on brain pericytes. Differences of the growth factors used, combined VEGF/PDGF-BB delivery versus VEGF delivery only, together with differences in the experimental systems, Matrigel assay versus focal cerebral ischemia, may explain diverging results. We still do not know how VEGF increased pericyte coverage of endothelial cells. Because pericytes did not express VEGFR2 or VEGFR1 in our mouse model, indirect signals between endothelial cells and pericytes are likely to mediate pericyte proliferation.

A major concern regarding VEGF-induced angiogenesis is the increased blood–brain barrier permeability that is more pronounced after acute than prophylactic or postacute VEGF delivery, and more pronounced after intravenous than local exposure. In our study, blood–brain barrier integrity remained intact after VEGF delivery. A conceptual problem of therapeutic angiogenesis remains that it is impossible to predict where future ischemic episodes are likely to occur. Angiogenesis induced in 1 specific brain area may potentially have detrimental effects on brain hemodynamics, if subsequent ischemias occur elsewhere in the brain. Indicating that therapeutic angiogenesis may have beneficial effects beyond elevating capillary density, we observed an enhanced pericyte coverage after VEGF treatment also in areas not exhibiting vascular sprouting. In the contralateral striatum, at distance to the VEGF infusion, increased CBF values were noticed, despite lack of capillary growth. The diameter of arterioles was slightly but significantly increased, which might explain this finding. We herein evaluated therapeutic angiogenesis in hitherto healthy adolescent mice with intact vascular networks. The question remains whether the data obtained can be translated to atherosclerotic mice. Future studies will have to address this issue.

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Disclosures

None.
References

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SUPPLEMENTAL MATERIAL

Delivery of recombinant human VEGF$_{165}$

Cannulae linked to miniosmotic pumps (Alzet 2004; Palo Alto, CA, U.S.A.) were implanted under 1% isoflurane anaesthesia (30% O$_2$, remainder N$_2$O) into the left lateral ventricle (1 mm lateral to bregma/ 2.5 mm below brain surface) for administration of normal saline (used as vehicle) or rhVEGF$_{165}$ (Peprotech, Hamburg, Germany; 0.02 µg/day). This dose was chosen, since it has previously been shown to induce angiogenesis following intracerebroventricular administration. After implantation, wounds were carefully sutured, anaesthesia discontinued and animals returned to their cages.

Visualization of cerebral vessels using carbon black dyes

A combination of two commercially available carbon black dyes, i.e. CB1 (Stempelfarbe, Herlitz, Germany) and CB2 (Scribtol, Pelikan, Germany), were used as described, for the visualization of vasodilation induced by VEGF exposure. Briefly, the thoracic aorta was clipped and the right atrium was incised to allow venous outflow. A volume of 2 ml normal saline was injected into the left ventricle, followed by the infusion of the carbon black dyes. A total volume of 2 ml of prewarmed CB2 and CB1 mixture (90:10) was injected over 40-50s, with slight manual pressure. Ten minutes after injection the brains were removed and immersed in normal saline containing 4% paraformaldehyde.

Quantification of capillary and arteriole diameter
20µm brain cryostat sections were obtained from animals treated with saline or VEGF for 21 days and injected with carbon black dyes. Brain sections at the level of mid striatum were stained using a rabbit anti-collagen IV antibody (1:500, Millipore, Billerica, MA, U.S.A.) which was detected with 3,3’ diaminobenzidine (DAB). Microphotographs were then taken using a CCD camera attached to the microscope (Olympus, Hamburg, Germany) at 40X and 10X magnifications for the evaluation of capillaries and arterioles, respectively. The diameter of the dye filled lumen of the collagen IV stained vessels were quantified using the Cell-F software (Olympus). Cerebral vessels with a diameter <10 µm were included for evaluating capillary diameter, and vessels with a diameter ≥10 µm were included for evaluating the arteriole diameter. A total of seven ROI (four in the lateral striatum and three in the overlying parietal cortex) were analyzed for the evaluation of capillary diameter and six ROI were analyzed (two in the lateral striatum and four in the overlying parietal cortex) for the evaluation of the arteriole diameter with the investigator being blinded for experimental conditions at all stages of the data analysis.

**Quantification of the diameter of the proximal MCA**

Brains collected after carbon black injections were photographed on the ventral side using a camera attached to a surgery microscope (Zeiss, Jena, Germany) at 25X magnification. The diameter of the MCA was evaluated at three equidistant points from its offspring from the circle of Willis up to its most peripheral point, where it disappeared in the Sylvian fissure, out of which mean values were calculated, which were reported in the text.

**Induction of focal cerebral ischemia**
Intraluminal MCA occlusion was induced during 1% isoflurane anaesthesia by using a silicon-coated microfilament, as previously described. Laser Doppler flow (LDF) was measured during the experiments up to 30 minutes after reperfusion using a flexible fiberoptic probe attached to the skull overlying the core of the MCA territory. In the first set of experiments, anaesthesia was discontinued after this procedure. Wounds were sutured and animals returned to their cages. Animals were sacrificed 24 hours later by transcardiac perfusion with normal saline. In the second set of experiments, animals remained under anaesthesia for delivery of radioactive tracers.

**Evaluation of brain capillary and arteriole densities**

20 µm cryostat sections were prepared from brains of non-ischemic animals and of ischemic animals sacrificed 24 hours after reperfusion. In sections obtained from the rostrocaudal level of the mid-striatum, i.e., the site of maximum extension of the MCA territory, brain capillaries were evaluated by fluorescence immunohistochemistry using a rat anti-CD31 antibody (BD Biosciences, San Diego, CA, U.S.A.). Stainings were evaluated by counting the number of positive vessel profiles intersecting the 10 horizontal and 10 vertical lines of a 500 µm x 500 µm grid (used for capillaries) or of a 1 mm x 1 mm grid (used for arterioles). A total of three regions of interest (ROI) in the parietal cortex and four in the lateral striatum were evaluated within the MCA territory both ipsilateral and contralateral to the stroke. Means were calculated for all ROI that were used for further analysis.

**Evaluation of pericyte coverage of brain capillaries**

Cryostat sections from the level of the mid-striatum of ischemic animals sacrificed 24 hours after reperfusion were processed for double immunohistochemistry using
rabbit anti-desmin (Abcam, Cambridge, UK), rat anti-CD31 (BD Biosciences) and goat-anti PDGFRß (R&D systems) antibody. To evaluate pericyte coverage of brain capillaries, the percentage of pericyte positive microvessels was counted in seven ROI (see above) ipsilateral and contralateral to the MCA occlusion, out of which mean values were formed. Confocal 3D stacks were obtained using laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Jena, Germany) using 20 µm sections scanned at 2 µm intervals, which were viewed using Zeiss LSM image browser and analyzed using ImageJ. In these stacks, CD31+ capillaries and desmin+ pericytes were outlined, allowing for the evaluation of capillary and pericyte volumes, respectively, which were integrated for all levels, resulting in capillary and pericyte volumes, from which volume ratios were formed.

**CBF and CPS double-autoradiography**

Fifteen minutes after reperfusion, 150 µCi L-[4, 5-3H] leucine (specific activity 151 Ci/mmol; Amersham, Braunschweig, Germany) was administered intraperitoneally, followed by an intraperitoneal injection of 10 µCi 4-iodo-N-methyl-[14C] antipyrine (Amersham) 43 minutes later. After a further two minutes (i.e., one hour after reperfusion), animals were instantly frozen in liquid nitrogen. Blood samples were obtained from the heart, in which the activity of 4-iodo-N-methyl-[14C] antipyrine was measured. Brains were removed and cut into 20 µm thick sections that were mounted on poly-l-lysine coated slides. These were exposed for 14 days, together with 14C and 3H standards on 14C-Hyperfilm (Amersham) for CBF autoradiography. Brain slices were then incubated for 24 h in 10% trichloroacetic acid to remove labeled free leucine and metabolites other than proteins, and subsequently re-exposed for the same duration to perform 3H autoradiography of 3H-labeled proteins.
Zechariah et al: VEGF induced angiogenesis in focal cerebral ischemia/ 5

using Hyperfilm 3H (Amersham).\textsuperscript{7, 8} Regional CBF was calculated as described previously\textsuperscript{3, 7} by calibration with the $^{14}$C- and $^3$H-standards, and radioactivity values measured in the blood.

**Regional ATP bioluminescence imaging**

For ATP measurement, frozen sections were freeze-dried and coated with a layer of frozen reaction mix containing the enzymes, coenzymes and cofactors necessary for evoking ATP-specific bioluminescence.\textsuperscript{9, 10} The tissue/enzyme bilayer was thawed and light emissions were recorded using a CCD camera. The CPS-deficient and ATP-depleted area was determined on the CPS autoradiography and ATP bioluminescence images by outlining areas with preserved CPS and ATP in both hemispheres at the level of the mid-striatum. The metabolic penumbra was calculated from these results by subtracting the ATP preserved area from the CPS deficient area.\textsuperscript{10}

**References**


Suppl. Table 1. Effect of VEGF on microvessel density and diameter in non-ischemic mice

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<th>Brain hemisphere</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Vehicle</td>
<td>VEGF</td>
</tr>
<tr>
<td>Capillary density (vessels/ROI)</td>
<td>18.3±1.5</td>
<td>23.9±2.5**</td>
</tr>
<tr>
<td>Capillary diameter (µm)</td>
<td>4.1±0.5</td>
<td>5.4±0.3**</td>
</tr>
<tr>
<td>Arteriole density (vessels/ROI)</td>
<td>34.0±13.2</td>
<td>35.0±5.6</td>
</tr>
<tr>
<td>Arteriole diameter (µm)</td>
<td>13.6±0.5</td>
<td>14.2±0.4</td>
</tr>
</tbody>
</table>

Microvessel densities were evaluated by CD31 immunohistochemistry in non-ischemic mice exposed to VEGF for 21 days. Microvessel diameters were analyzed in *ex vivo* angiographies with carbon black, again in non-ischemic mice exposed to VEGF for 21 days. Capillaries were defined as microvessels with a diameter <10 µm, arterioles as microvessels with a diameter ≥10 µm. For capillary density, see also Figure 1. Ipsilateral, hemisphere with VEGF infusion; contralateral, hemisphere opposite to VEGF infusion. Data are means±S.D. **p<0.01 compared with vehicle (two-tailed t-tests).
Suppl. Figure 1. VEGF induces proliferation of brain endothelial cells. CD31/ Ki67 double immunohistochemistry showing increased proliferation of brain endothelial cells 3 days after initiation of VEGF treatment (A) in the hemisphere in which VEGF was delivered and to a lesser extent (B) in the contralateral hemisphere at distance to the infusion site. (C, D) This angiogenic response was augmented upon middle cerebral artery (MCA) occlusion followed by 24 hours reperfusion. Representative photographs from animals treated with vehicle or VEGF for 3 days are shown. Data are means±SD (n=6-7 animals/group). *p<0.05/**p<0.01/***p<0.001 compared with vehicle. Bar, 50 µm.
Suppl. Figure 2. Laser Doppler flow recordings during and after 90 minutes middle cerebral artery (MCA) occlusion. Note the reproducibility of MCA occlusion and reperfusion. Data are means ± SD (n=6-7 animals/ group). No differences between groups were found.
Supplementary figure 3

Effect of VEGF followed by MCA occlusion

Suppl. Figure 3. Effect of VEGF on pericyte coverage evaluated by CD31/ PDGFRβ immunohistochemistry. (A, B) Percentage of pericyte positive microvessels in the ischemic and contralateral non-ischemic brain tissue. Data are means ± SD (n=6-7 animals/group). No significant differences were found between groups. Bar, 100 µm.
Supplementary figure 4

**Effect of VEGF followed by MCA occlusion**

Suppl. Figure 4. VEGF does not influence pericyte injury. DNA-fragmented (i.e., TUNEL+) and apoptotic (i.e., cleaved caspase-3+) pericytes, evaluated using the pericyte marker desmin in animals treated with VEGF for 21 days. (TUNEL and cleaved caspase-3 in green/ desmin in red). Data are means ± SD (n=6-7 animals/ group). *p< 0.05/ **p< 0.01 compared with vehicle. Bar, 40 µm.
Suppl. Figure 5. Absence of VEGFR2 and VEGFR1 on pericytes. Immunofluorescence photographs revealing VEGFR2 (A) and VEGFR1 (B) (both in green) on ischemic endothelial cells (in case of VEGFR2 in A) or parenchymal cells (in case of VEGFR1 in B), but not on pericytes (in red). Bar, 20 µm.