VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain

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VEGF is a secreted mitogen associated with angiogenesis and is also a potent vascular permeability factor. The biological role of VEGF in the ischemic brain remains unknown. This study was undertaken to investigate whether VEGF enhances cerebral microvascular perfusion and increases blood-brain barrier (BBB) leakage in the ischemic brain. Using magnetic resonance imaging (MRI), three-dimensional laser-scanning confocal microscope, and functional neurological tests, we measured the effects of administering recombinant human VEGF165 (rhVEGF165) on angiogenesis, functional neurological outcome, and BBB leakage in a rat model of focal cerebral embolic ischemia. Late (48 hours) administration of rhVEGF165 to the ischemic rats enhanced angiogenesis in the ischemic penumbra and significantly improved neurological recovery. However, early postischemic (1 hour) administration of rhVEGF165 to ischemic rats significantly increased BBB leakage, hemorrhagic transformation, and ischemic lesions. Administration of rhVEGF165 to ischemic rats did not change BBB leakage and cerebral plasma perfusion in the contralateral hemisphere. Our results indicate that VEGF can markedly enhance angiogenesis in the ischemic brain and reduce neurological deficits during stroke recovery and that inhibition of VEGF at the acute stage of stroke may reduce the BBB permeability and the risk of hemorrhagic transformation after focal cerebral ischemia.


Introduction

Functional neurological recovery from stroke commonly occurs, but is often incomplete in patients who survive a stroke (1, 2). Stroke patients with a higher density of blood vessels appear to have reduced morbidity and survive longer (3). Functional imaging of stroke patients shows increased cerebral blood flow and metabolism in tissue surrounding focal brain infarcts (4, 5). These data suggest that restoration of cerebral microvascular circulation is important for functional recovery after a stroke. Late restoration of cerebral microcirculatory flow may involve increases in the collateral circulation and angiogenesis in the penumbra (6, 7). The substances responsible for inducing formation of new blood vessels in the ischemic brain have not been identified conclusively.

VEGF is an angiogenic growth factor that binds to two high-affinity receptors, fms-like tyrosine kinase (flt-1) and the kinase domain region (KDR) (8, 9). Administration of recombinant human VEGF165 (rhVEGF165) improves myocardial perfusion in patients with coronary ischemia and enhances angiogenesis in animal models of myocardial and limb ischemia (10–14). Transient and permanent middle cerebral artery (MCA) occlusion in rats evokes expression of VEGF in the ischemic brain (15–17), suggesting that after a stroke VEGF may be involved in angiogenesis. However, whether administration of rhVEGF165 to the ischemic brain has the potential to promote angiogenesis and thereby improve functional neurological outcome has not been tested. In addition, VEGF is a potent vascular permeability factor that increases microvascular permeability to blood plasma proteins within minutes after exposure (18). Administration of rhVEGF165 after focal cerebral ischemia may exacerbate blood-brain barrier (BBB) permeability. After a stroke, disruption of the BBB occurs acutely whereas regeneration of cerebral microvessels develops relatively late in ischemic brain (6, 19–24). Using a model of focal embolic cerebral ischemia in rats, we therefore tested the hypotheses that administration of VEGF at a late stage of stroke improves cerebral microvascular perfusion in the ischemic penumbra and that administration of VEGF early after a stroke increases BBB leakage.

Methods

All experimental procedures have been approved by the Care of Experimental Animals Committee of Henry Ford Health Sciences Center.

General experimental procedures. Male Wistar rats weighing 320–400 g were used in the experiments. Animals were anesthetized with 0.5–3.5% halothane in 70% N2O
and 30% O2 during the surgical procedure and magnetic resonance imaging (MRI) measurements. Rectal temperature was maintained at 37 ± 0.5°C throughout the experiments (25, 26). The right femoral artery and vein were cannulated with a PE-50 catheter for continuous monitoring of blood pressure, measurement of blood gases, and for drug administration, respectively.

Animal model. The MCA was occluded by placement of a single, intact, fibrin-rich, 24-hour-old, homologous clot (~1 µl) at the origin of the MCA (25). This model is most relevant to human stroke (25, 26).

Administration of rhVEGF_{165}. One hour or 48 hours after embolization, using a Harvard pump (Harvard Apparatus; South Natick, Massachusetts, USA), rhVEGF_{165} (Genentech Inc., San Francisco, California, USA) was infused intravenously to rats at a dose of 1 mg/kg over a 4-hour interval (5 µl/min) to reduce the hemodynamic side effects of VEGF (27, 28). The dose of rhVEGF_{165} was chosen based on previous studies in the same species (27, 28). An equal volume of 0.9% saline was administered to rats as a control group.

MRI measurements. MRI measurements were performed using a 3.0 T whole-body magnet (Magnex Scientific, Abingdon, United Kingdom) equipped with actively shielded gradients and a SMIS (Surrey, United Kingdom) console. MRI images were recorded using a birdcage radial frequency coil.

The apparent diffusion coefficient of water (ADC_{w}) was measured using the method described by LeBihan et al. (29). The diffusion-weighting gradient was increased, in a nonlinear manner, from 0 to approximately 2.8 gauss/cm to obtain a series of five images with gradient b values of 0, 200, 400, 600, 800 s/mm².

Perfusion estimates employed methods of Williams et al. that were further refined in our laboratory (30, 31). This technique is based on the selective inversion of blood-water protons at the level of the carotid arteries before 1H MRI measurement in the brain (31). Two images were obtained for perfusion measurement with parameters: repetition time (TR) = 1.055 seconds; echo time (TE) = 30 milliseconds, 64 × 64 image matrix, 2-mm slice thickness, and a 40-mm field of view (FOV). The duration of the inversion pulse was 1 second and 0.3 kHz in amplitude.

A gadolinium-chelate (Gd-DTPA) contrast material (Omniscan Gadodiamide; Nycomed Inc., Princeton, New Jersey, USA; 0.1 mmol/kg) was injected (intravenous bolus) at the end of the infusion of rhVEGF_{165} or saline to measure the BBB leakage. Contrast-enhanced MRIs were acquired in a multislice mode (nine slices) with a 2-mm slice thickness, a 40-µm FOV, and a 256 × 256 image matrix. The contrast-enhanced MRI images were obtained using multislice T_{1}-weighted images with spin-echo sequences (TR = 500 milliseconds, TE = 30 milliseconds).

MRI analyses of ADC_{w} and cerebral blood flow (CBF) maps were performed using Eigentool software (32). ADC_{w} maps were produced on a pixel-by-pixel basis using a least-squares fit to a straight-line plot of the natural logarithm of normalized image intensity versus gradient b value (ADC_{w}). CBF maps were calculated according to the method published previously (31).

Accelerating rotarod test. An accelerating rotarod test was employed to measure motor function of rats (33, 34). Each rat received a test trial on the accelerating rotarod at 2, 7, 14, and 28 days after embolic MCA occlusion, and the time the animal remained on the device was recorded. Data are presented as the time the rat remained on the rotating spindle normalized to the preschizophrenia duration.

Adhesive removal test. The adhesive removal test, which measures somatosensory deficit, was performed (35). Briefly, two small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The time required to remove the stimuli from each limb was recorded. Each animal received five trials at 2, 7, 14, and 28 days after embolic MCA occlusion, and the mean time required to remove both stimuli from limbs was recorded.

Tissue preparation. For paraffin sections, brains were fixed in 10% buffered formalin and embedded in paraffin. Six-micrometer-thick coronal sections were cut and stained with hematoxylin and eosin (H&E) for histopathological evaluation. For vibratome sections, brains were fixed in 4% paraformaldehyde at 4°C for 48 hours. Coronal sections (100 µm) were cut on a vibratome.

Immunohistochemistry. A mouse mAb against microtubule-associated protein 2 (MAP2; Serotec Ltd., Oxford, United Kingdom), the neuronal cytoskeletal protein, was used as a marker for early ischemic neuronal damage (36). For immunofluorescence, 100-µm thick vibratome sections were incubated with primary mAb’s against MAP2 (1:200) and with Cy 5–conjugated secondary Ab’s.

Three-dimensional image acquisition. The vibratome sections were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal imaging system (Bio-Rad, Cambridge, Massachusetts, USA). With the Evans blue- and FITC-perfused tissue samples, vibratome sections corresponding to the MRI coronal sections (interaural 6.20 mm to interaural 1.00 mm) (37) were scanned throughout the entire coronal section in 512 × 512 pixel (1042.4 × 1042.4 µm) format in the x-y direction using a 4× frame-scan average, and 16 optical sections along the z-axis with a 5-µm step-size under a ×10 objective were acquired with two separate photomultiplier tubes. Each image was assembled into a coronal section using Adobe Photoshop software (Adobe Systems Inc., Mountain View, California, USA). For FITC-dextran–perfused tissue stained with anti-MAP2 Ab (Cy5), green (FITC-dextran–perfused microvessels), and far red (Cy5, MAP2 immunoreactivity) fluorochromes on the sections were excited by a laser beam at 488 nm and 647 nm, respectively, and emissions were acquired simultaneously with two separate photomultiplier tubes through 522-nm and 680-nm emission filters, respectively.
Three-dimensional image analysis. Measurements of vascular diameters are limited in two dimensions and are based on the assumption that vessel contours are round (38, 39). However, this is not the case in vivo in which cerebral microangioarchitecture has a highly irregular pattern of tortuous, anastomosing capillaries. To overcome most limitations of previous methods, we developed a new computerized system, MIRAGE, to examine cerebral microvessels perfused in vivo by FITC-dextran (K. Davies et al., manuscript submitted for publication). The methodology used by MIRAGE for measurement of blood vessel diameter involves a linearly applied spherical inflation technique. For each vessel under analysis, the system follows the center of the vessel and determines the long-axis path of flow. Vessel diameter is continuously measured as the center of the vessel is traced by using the length of 24 different divergent vectors, each perpendicular to this axis of flow. In a preliminary study, we measured 8,930 microvascular diameters in a nonischemic rat cortex, and the average microvessel diameter was $4.15 \pm 1.24 \mu m$ ($3\text{–}8 \mu m$), which is comparable to cortical microvessel diameters of $4.4 \pm 1 \mu m$ ($2.5\text{–}8.7 \mu m$) obtained from intravital microscopy in vivo (39). $4.26 \pm 0.07 \mu m$ from manually measured fixed coronal sections stained with toluidine blue (38), and $3\text{–}8 \mu m$ measured by scanning electron microscopy on corrosion casts (40). We therefore employed this program to analyze cerebral microvessels in the present study. Images obtained under a $\times 40$ objective from a laser-scanning confocal microscope (LSCM) had a resolution of $0.5 \mu m$/pixel in the $x$ and $y$ dimensions and $1 \mu m$/pixel along the $z$ axis. All quantitative measurements were performed blindly.

Statistical analysis. Repeated measures analysis was conducted using a generalized estimating equation to test the treatment effect on the rotarod and the adhesive removal tests, respectively. Student’s $t$ tests were performed to compare cerebral microvascular plasma perfusion and vascular diameters in control and VEGF-treated groups. A paired $t$ test was used to analyze the effects of VEGF and saline on measurement of perfusion- and diffusion-weighted MRIs. All data are presented as mean plus or minus SE, and a $P$ value of 0.05 was considered to be statistically significant.

Results
The effects of administration of rhVEGF165 on cerebral microvascular perfusion and neurological outcome. To determine whether administration of rhVEGF165 enhances cerebral microvascular perfusion, embolic ischemic rats were treated with rhVEGF165 at a dose of $1 \text{mg/kg}$ 48 hours after a stroke to avoid the peak of brain edema in this stroke model and were sacrificed at 9 days after ischemia. The time point of 9 days after ischemia was chosen based on data from a previous study using a cranial window: newly formed vessels arising from mouse brain were first visible at 4 days and peaked at 12 days after VEGF administration (41). Cerebral microvascular plasma perfusion and vascular diameters were examined in three dimensions by LSCM and analyzed using the MIRAGE program. Compared with the saline-treated rats ($n = 6$, Figure 1, a and g), treatment with rhVEGF165 significantly ($P < 0.05, n = 5$) increased numbers of voxels of plasma-perfused vessels.

![Figure 1](image-url)

Figure 1 Composite images (through stacks of 14 optical sections of 5-\mu m thickness) of cerebral microvascular plasma perfusion obtained from the ischemic penumbra of the cortex 9 days after MCA occlusion. Late treatment with rhVEGF165 enhances cerebral microvascular plasma perfusion in the penumbra (b) compared with the control rat (a). Treatment with rhVEGF165 did not alter cerebral microvascular plasma perfusion in the contralateral hemisphere (d), compared with the contralateral hemisphere in the control rat (c). Bar, 100 \mu m. Quantitative data analyzed by the MIRAGE program (g) show a significant increase of plasma perfusion in vessels within 3 \mu m of diameters in the rhVEGF165–treated group (filled bars; $n = 5$) compared with the saline-treated group (open bars; $n = 6$; $^{*}P < 0.05$). Higher-magnification images show that cerebral microvessels in the ipsilateral hemisphere had an irregular pattern of tortuosity (e) compared with the vessels in the contralateral hemisphere (f). Dimension of the composite image is 260.6 $\times$ 260.6 $\times$ 20 \mu m$³. (h) The percentage of vessels with different diameters in the entire vessels perfused by FITC-dextran. The number of vessels with diameters within 2 \mu m is significantly ($P < 0.01$) higher in the ipsilateral hemisphere (filled squares; $n = 5$) than in the contralateral hemisphere (open squares; $n = 5$).
in the penumbra of the cortex (Figure 1b and 1g). However, treatment with rhVEGF165 did not alter cerebral microvascular plasma perfusion in the contralateral hemisphere (Figure 1d) compared with the contralateral hemisphere from the saline-treated rats (Figure 1c). We measured in three dimensions the diameters of plasma-perfused cerebral microvessels in the penumbra (Figure 1e) and in the contralateral homologous tissue (Figure 1f). Eighty-five percent of plasma-perfused vessels in the penumbra had diameters within 3 μm, whereas the majority of vessels in the contralateral tissue had diameters of 4 and 5 μm (Figure 1h), suggesting that increased plasma-perfused vessels in the penumbra are newly grown vessels.

To determine whether late administration of rhVEGF165 improves functional neurological recovery, embolic ischemic rats were treated with rhVEGF165 at a dose of 1 mg/kg (n = 7) or saline (n = 10) at 48 hours after a stroke, and the rotarod and adhesive removal tests were performed on these rats at 2, 7, 14, and 28 days after stroke. To avert potential effects of rhVEGF165 on the rotarod test due to the likelihood of hindlimb ischemia caused by cannulated femoral arteries and veins, rhVEGF165 or saline was administered in a tail vein, and mean artery blood pressure was not measured during infusion of rhVEGF165 or saline in these groups. Rats in the rhVEGF165 or the saline-treated groups showed marked motor and somatosensory impairments 2 days after a stroke. Partial recovery was detected in the rhVEGF165- and saline-treated groups at 7, 14, and 28 days after a stroke (Figure 2a). However, the ischemic rats treated with rhVEGF165 exhibited significant (P < 0.05) improvements on the rotarod test at 7, 14, and 28 days after the stroke compared with the saline-treated rats (Figure 2a). The adhesive removal test did not reveal significant differences between the rhVEGF165-treated and the saline-treated groups at 2 days (116 ± 3.6 vs. 115 ± 3.5 seconds), 7 days (66 ± 16.5 vs. 65.2 ± 11.1 seconds), and 14 days (40 ± 1.4 vs. 34.8 ± 3.2 seconds). However, a significant (P < 0.05) difference was detected at 28 days after stroke (11 ± 1.2 vs. 15 ± 1.1 seconds). In addition, brain tissue obtained at 28 days after ischemia exhibited more small vessels in the ipsilateral ischemic border zone (Figure 2, e and f) in rats treated with rhVEGF165 when compared with the vehicle-treated rats (Figure 2, b and c). An increase in microvessels in the ischemic border zone was confirmed by detecting more FITC-dextran-perfused microvessels (Figure 2g) in the rats treated with rhVEGF165 when compared with FITC-dextran-perfused microvessels in the penumbra from the saline-treated rats (Figure 2d).

**Figure 2**

(a) The percentage of time that rats persisted on the rotarod after ischemia compared with the preischemic value as a function of time of stroke. Rotarod test was performed at 2, 7, 14, and 28 days after stroke on the ischemic rats treated with rhVEGF165 or saline initiated at 48 hours after ischemia. Treatment with rhVEGF165 (triangles; n = 7) significantly (P < 0.05 or P < 0.01) increases time spent on the rotarod compared with the saline-treated group (circles; n = 10). Cerebral vessels and FITC-dextran-perfused cerebral microvessels from the saline-treated rat (b–d) and the rhVEGF165-treated rat (e–g) 28 days after the right MCA occlusion. Dorsal view of the right and left hemisphere vessels (b and e), a lateral view of the right hemisphere vessels (c and f), and FITC-dextran-perfused cerebral microvessels in the penumbra of the cortex (d and g). More vessels in the ipsilateral ischemic border zone (e, arrows, and f) and more FITC-dextran-perfused vessels (g) were observed in the rhVEGF165-treated rat compared with that in the saline-treated rat (b–d). Bar = 2 mm in f for b, c, e, and f; bar = 200 μm in g for d and g. Rats were decapitated one minute after injection of FITC-dextran and their brains were rapidly removed from the severed heads and digitized at 8X magnification (for panels b, c, e, and f) using an MCID image analysis system (Imaging Research, St. Catherines, Canada). After digitization the brains were placed in 4% paraformaldehyde at 4°C for 48 hours (for panels d and g).
ed ischemic rats, the hyperintense area localized to the ipsilateral MCA supplied the subcortex (Figure 3e). Administration of rhVEGF165 resulted in the hyperintense areas localized to the ipsilateral MCA-supplied cortex and subcortex (Figure 3b). The hyperintense areas on the MRI in VEGF-treated rats were significantly ($P < 0.05$) larger than the areas in the control animals (Figure 3i). LSCM images of coronal sections that matched MRI sections from the same animal revealed extensive Evans blue and FITC-dextran extravasation in rats treated with rhVEGF165 (Figure 3c), compared with the control animals (Figure 3f). Areas with leakage of fluorescent dyes localized only to the ipsilateral area supplied by the MCA (Figure 3c) and corresponded closely to hyperintense areas detected on Gd-DTPA MRI (Figure 3b). High-magnification images of LSCM show that the extravasation of Evans blue and FITC-dextran occurred in capillaries and in the lesion boundary areas with nonplasma and low-plasma perfusion (Figure 3g), and extravasation of fluorescent dyes was not detected in cerebral vessels in the contralateral homologous tissue (Figure 3h). These data demonstrate that administration of rhVEGF165 exacerbates BBB leakage. Disruption of the BBB was not detected in nonischemic rats treated with rhVEGF165 ($n = 3$, data not shown).

To examine the effect of exogenous VEGF on BBB leakage and the incidence of hemorrhagic transformation, rats were sacrificed at 24 hours after a stroke, since in this embolic stroke model hemorrhagic transformation primarily appears during this time period (25, 45). Administration of rhVEGF165 at the onset of 1 hour of MCA occlusion ($n = 6$) resulted in a significant ($P < 0.05$) increase in the incidence of gross hemorrhage (four out of six rats had gross hemorrhage, compared with none of the control rats ($n = 5$)).

**Figure 3**
Gd-DTPA contrast-enhanced MRI images of ischemic brains treated with rhVEGF165 at 1 hour after MCA occlusion (a and b) or 0.9% saline (d and e) and corresponding images of LSCM (c and f). Postcontrast images obtained at 30 minutes after injection of Gd-DTPA agent show the hyperintense areas in the ipsilateral MCA-supplied cortex and subcortex of a rat treated with rhVEGF165 (b) and in the ipsilateral MCA-supplied subcortex of a rat treated with 0.9% saline (e), which are not seen on the precontrast images (a and d). Composite images of LSCM (80 μm thick) from the same locations exhibit extravasation of Evans blue and FITC-dextran in the cortex and subcortex from the rat treated with rhVEGF165 (c) and extravasation of Evans blue in the subcortex from the rat treated with saline (f) and sacrificed 6 hours after MCA occlusion. High-magnification images of LSCM from the rat treated with rhVEGF165 show that the extravasation of Evans blue (g, red) and FITC-dextran (g, green) was present in capillaries with nonplasma and low-plasma perfusion in the ipsilateral hemisphere (g) and that cerebral microvessels in the contralateral hemisphere were well perfused with both dyes and did not exhibit any extravasation of fluorescent dyes (h). Bar, 40 μm for g and h. Hyperintense areas on Gd-DTPA-enhanced MRI are significantly ($P < 0.05$) larger in rhVEGF165-treated rats ($n = 5$) than the saline-treated rats ($n = 4$) (i). Hyperintense areas were measured on Gd-DTPA-enhanced images obtained at 30 minutes after injection of Gd-DTPA agent, which was injected after 4 hours of infusion of rhVEGF165 or saline.

**Figure 4**
Increase in the BBB leakage and hemorrhagic transformation. Large hyperintense areas on Gd-DTPA-enhanced MRI were observed 30 minutes after injection of Gd-DTPA in a rat treated with rhVEGF165 (b), compared with the precontrast image (a). A coronal section corresponding to the MRI section obtained at 24 hours after a stroke shows extensive Evans blue leakage and hemorrhage (c and d). Bar, 1 mm in d.
80%) compared with non-rhVEGF165–treated rats (two out of ten, 20%; n = 10). Areas with gross hemorrhage detected on the coronal sections at 24 hours after a stroke (Figure 4, c and d) match the anatomic hyperintense areas detected on Gd-DTPA–enhanced MRI measurements at 5 hours after a stroke (Figure 4b), suggesting that the early increase in the BBB leakage to large molecules may be related to the incidence of hemorrhage in this model.

To assess the effects of rhVEGF165 given 1 hour after onset of ischemia on the evolution of the tissue damage and ultimate lesion volume, we performed diffusion-weighted MRIs to measure the evolution of the ischemic lesion in vivo, MAP2 immunohistochemistry to examine neuronal damage, and H&E staining to measure ischemic areas. The ischemic areas defined as the hyperintensity on the diffusion-weighted images (DWI) were relatively similar at 1 hour of ischemia for rats treated with saline (Figure 5a, 1 hour) or with rhVEGF165 (Figure 5b, 1 hour), suggesting the same basal level of ischemic severity for rats in both groups. However, areas of hyperintensity in rhVEGF165–treated rats increased substantially during and after infusion of rhVEGF165 (Figure 5b, 6 hours and 24 hours), compared with the vehicle-treated ischemic rats at the same time points (Figure 5a, 6 hours and 24 hours). Brain tissue obtained from the same animal immediately after the last MRI measurements at 24 hours after ischemia, shows a larger ischemic lesion on H&E-stained coronal sections in the ipsilateral hemisphere (d) compared with the homologous tissue in the contralateral hemisphere (e) in a rat treated with rhVEGF165 (1 hour) and sacrificed at 6 hours after MCA occlusion. Bar, 20 μm for d and e.

Figure 5
Evolution of the ischemic lesion from 1 hour to 24 hours after MCA occlusion in a rat treated with 0.9% saline (a) or rhVEGF165 (b). Although areas of hyperintensity on the MRI were relatively similar at 1 hour of MCA occlusion in saline-treated (a) or rhVEGF165–treated (b) rat, an area of hyperintensity on MRI was larger at 6 hours and 24 hours of ischemia in the rhVEGF-treated (b) rat compared with an area of hyperintensity at the same time points in the saline-treated rat (a). Differences of ischemic lesions observed on MRI between these two rats are confirmed on H&E-stained coronal sections obtained at 24 hours of ischemia (H&E) (a and b). Quantitative analysis of the ischemic area as a percentage of hemisphere measured on DWI at 6 hours and 24 hours of MCA occlusion (c) shows that ischemic areas are significantly (\(P < 0.05\)) larger in rats treated with rhVEGF165 (solid line, n = 6) than in the control rats (dotted line, n = 7) at 6 hours and 24 hours of embolic MCA occlusion. Composite images (135 × 135 × 20 μm³) of microvessels (FITC-dextran, green) and MAP2 immunoreactivity (Cy5, red) show much less MAP2 immunoreactivity (red) in low plasma-perfused areas with extravasation of FITC-dextran (green) in the ipsilateral hemisphere (d) compared with the homologous tissue in the contralateral hemisphere (e) in a rat treated with rhVEGF165 (1 hour) and sacrificed at 6 hours after MCA occlusion. Bar, 20 μm for d and e.
ischemia compared with homologous tissue in the contralateral hemisphere (Figure 5e) for rats treated with rhVEGF165 indicating acute ischemic neuronal damage. Taken together, these data suggest that exogenous VEGF accelerates ischemic damage.

To examine whether administration of rhVEGF165 48 hours after ischemia increased leakage of Gd-DTPA across the BBB, Gd-DTPA–enhanced MRI measurements were made on a subset of rats (n = 6) immediately after infusion of rhVEGF165. These rats were sacrificed after the last MRI measurements, and cerebral microvascular leakage and plasma perfusion were measured by LSCM on these brains. Late administration of rhVEGF165 did not result in a significant increase in the BBB leakage measured on Gd-DTPA–enhanced MRIs (Figure 6, a and b) and on LSCM (Figure 6c).

The effects of rhVEGF165 administration on CBF. To examine the effects of early administration of rhVEGF165 on CBF, we performed perfusion-weighted MRI measurements using an arterial spin-tagging technique (31). CBF increased in the ischemic hemisphere during the 1–3 hour infusion of rhVEGF165 (Figure 7a, 2 hours) and returned to the preinfusion level at 4 hours of infusion of rhVEGF165 (Figure 7a, 4 hours). Transient increases in CBF were not detected in the ischemic hemisphere in the control rats (Figure 7b), and the differences of changes in CBF between the VEGF (n = 6) and the control groups (n = 6) were statistically significant (P < 0.05; Figure 7d), suggesting that early administration of rhVEGF165 induces a transient increase of CBF in the ischemic brain.

Hyperemia was detected by a perfusion-weighted MRI in the ischemic lesion at 48 hours after the stroke before infusion of rhVEGF165 (Figure 7c). Administration of rhVEGF165 at 48 hours after ischemia induced changes in the distribution of CBF within the ischemic lesion (Figure 7c). CBF was reduced in hyperemic areas during and after infusion of rhVEGF165 (Figure 7c), suggesting that exogenous VEGF at 48 hours after ischemia causes a redistribution of CBF within the ischemic lesion. 

**Discussion**

The present study demonstrates that rhVEGF165 significantly enhances cerebral microvascular plasma perfusion and improves functional neurological recovery when rhVEGF165 is administered to ischemic rats at 48 hours after a stroke. However, administration of rhVEGF165 at 1 hour after stroke exacerbates BBB leakage, increases hemorrhagic transformation, and promotes ischemic cell damage. These findings provide the first evidence of a dual role of VEGF for enhancing cerebral microvascular perfusion and for increasing the BBB leakage in the ischemic brain.

Studies from human and experimental strokes indicate that angiogenesis is present in the focal ischemic brain (3, 6, 46). Development of angiogenesis in the ischemic brain is incompletely understood. The spatial and temporal profiles of VEGF and its receptor mRNA correspond closely with angiogenesis during embryonic development in the mouse brain, suggesting that VEGF and its receptors regulate brain angiogenesis (47, 48). Our data show that late treatment of ischemic rats with rhVEGF165 resulted in a significant increase of cerebral microvascular plasma perfusion in the penumbra of the cortex. A significant increase in microvascular plasma perfusion may be attributed to newly formed capillaries, based on the morphology in which diameters (≤5 μm) of plasma-perfused vessels in the penumbra are significantly smaller than diameters (5 μm) in the contralateral homologous areas (49). In addition, microvessels in the ipsilateral border of ischemia in the VEGF-treated rats exceeded that observed in the saline-treated rats at 28 days after ischemia (Figure 2, b–g). Taken together, our data indicate that late treatment with rhVEGF165 enhances angiogenesis in ischemic brain. Our observation is consistent with results obtained from a phase-1 trial, where administration of rhVEGF to patients with severe coronal artery disease significantly increased myocardial perfusion (10).

Our data also demonstrate that late administration of rhVEGF165 to ischemic rats significantly improves neurological function. The rotarod test is a sensitive index
for assessing motor impairment after traumatic brain injury and focal cerebral ischemia (33, 34). In the present study, rats exhibited marked motor impairment at 2 days after a stroke, and partial recovery on the rotarod test was detected in the vehicle or the VEGF-treated groups at 7 and 28 days after a stroke, which is consistent with the fact that partial functional neurological recovery from a stroke occurs commonly (1, 2). However, VEGF-treated rats show a significant improvement on the rotarod test compared with vehicle-treated animals. An early significant improvement of the rotarod test (which primarily detects motor impairment in the cortex), but late modest improvement on the adhesive removal test (which detects somatosensory impairment in the cortex and the striatum that is the ischemic core), is consistent with a significant increase in plasma perfusion in the penumbra of the cortex, suggesting that exogenous VEGF may promote therapeutic angiogenesis in the ischemic rats (49). Our data are consistent with findings that functional imaging of stroke patients shows increased cerebral blood flow and metabolism in tissue surrounding focal brain infarcts (4, 5) and that stroke patients with a higher blood vessel density in the ischemic penumbra appear to have better progress and longer survival (3), indicating that restoration of cerebral microvascular circulation is important in the ischemic brain for functional recovery after a stroke. Therefore, treatment with VEGF to potentiate new vessel formation may be an important therapy for functional neurological recovery during the repair process after a stroke.

Mechanisms by which exogenously administered VEGF enhances cerebral microvascular perfusion and improves functional neurological outcome cannot be deduced from the present study. Increases in microvascular perfusion may be attributed to angiogenesis. Regeneration of cerebral microvessels requires a vascular basement membrane upon which endothelial cells migrate and proliferate to reform vascular channels after brain injury (50). VEGF is a specific mitogen for endothelial cells (8). VEGF may bind to the vascular basement membrane and cause proliferation and migration of endothelial cells (51, 52). Angiogenesis may promote ischemic brain plasticity and thereby improve functional neurological outcome (53). In addition to its effect on angiogenesis, VEGF may have direct effects on neuronal plasticity by its neurotrophic activity and by stimulating axonal outgrowth (54–56).

Based on the acute appearance of disruption of the BBB and early upregulation of VEGF in the ischemic core (our unpublished observation), we tested the hypothesis that an early increase of endogenous VEGF increases BBB leakage. We administered rhVEGF165 to the ischemic rats at 1 hour after onset of MCA occlusion and found a significant increase in BBB leakage in the ischemic lesion, but not in nonischemic areas, as measured by Gd-DTPA–enhanced MRI in the ischemic rats treated with rhVEGF165 compared with the saline-treated animals. An increase in BBB leakage was further confirmed by LSCM. Furthermore, intravenous administration of rhVEGF165 did not increase extravasation of FITC and 125I-BSA from normal brain microvessels (59). However, topical application of VEGF to the parietal cortex in nonischemic rats resulted in increases of BBB leakage to FITC-labeled dextran of molecular mass 10,000 d (58). Studies in vitro show that when it was applied to the abluminal plasma membrane of the endothelial cells, but not to luminal side, VEGF

Figure 7
CBF maps of coronal sections measured by perfusion-weighted MRIs. Images of CBF were obtained before, during, and at the end of infusion of rhVEGF165 (a) and saline (b) initiated at 1 hour after embolization. Increase of CBF was observed in the ischemic lesion during infusion of rhVEGF165 (2 hours) (a). The increase in CBF in the rhVEGF165–treated rats (triangles; n = 6) (d) was significant (P < 0.05) compared with that in the saline-treated animals (circles; n = 6). Images of CBF were obtained before, during, and at the end of infusion of rhVEGF165 initiated at 48 hours after embolization (c). Hyperemia was evident in the ischemic lesion before infusion of rhVEGF165 (48 hours) (c). Infusion of rhVEGF165 decreases hyperemic areas in the ischemic lesion (50 hours and 52 hours) (e).
increased permeability of cultured brain microvessel endothelial cells (58, 60, 61). These data indicate that the receptors for VEGF are located on the abluminal side of vessels (58, 60, 61). However, our findings extend previous observations by demonstrating that intravenously administered VEGF increases BBB leakage only in the ischemic lesion. An acute induction of VEGF receptors (Flt-1 and Flk-1) in the ischemic vessels and upregulation of neuropilin-1 (our unpublished observation) (62) may be responsible for the effect of exogenous VEGF on increasing BBB leakage only within the ischemic lesion. In contrast to early administration, late administration of VEGF does not increase the BBB leakage in the ischemic lesion, suggesting that there are some molecules that may inhibit VEGF-induced BBB leakage. Angiopoietin-1 has been demonstrated to counter peripheral vascular leakage induced by VEGF (63, 64). Expression of angiopoietin-1 is upregulated in this model (our unpublished observation). Future experiments to examine whether upregulation of angiopoietin-1 blocks ischemic cerebral vascular plasma leakage induced by VEGF are warranted.

Disruption of BBB was concomitant with increases in hemorrhagic transformation and ischemic lesions in rats treated with VEGF, suggesting that the early increase in the BBB leakage to large molecules causes subsequent hemorrhage and promotes ischemic cell damage. Overexpression of VEGF165 causes tumor-associated intracranial hemorrhage in mice (65). Furthermore, astrocytes play an important role in BBB integrity (66). Early upregulation of endogenous VEGF on astrocytes in the ischemic core may interact with receptors for VEGF on the ischemic vessels and contribute to the disruption of BBB leakage, thereby promoting hemorrhagic transformation and ischemic cell damage. Indeed, a recent study shows that administration of soluble VEGF-receptor chimera protein Flt-(1-3)-IgG, inactivating VEGF to transient MCA-occluded mice, significantly reduced ischemic lesion volume measured by T2-weighted MRI (67). In contrast, local application of VEGF to the ischemic rats subjected to 90 minutes of transient MCA occlusion resulted in a reduction of infarct volume and brain edema (68).

Mechanisms that contribute to increase of leakage of the BBB in response to VEGF administration are unknown. Some effects of exogenous VEGF on endothelial cells in vitro are partially attributed to the generation of nitric oxide (NO) from these cells (69, 70). In vivo, NO increases CBF (71). In the present study, a transient augmentation and redistribution of CBF were observed in the ischemic lesion after early and late administration of VEGF, respectively, suggesting that exogenous VEGF generates NO in ischemic brain. Therefore, a significant increase in BBB leakage after administration of rhVEGF165 may result, in part, from action of NO, as others have demonstrated that exogenous VEGF administration increases permeability of the BBB through a NO synthase/cGMP-dependent pathway (57). In addition to generating NO, VEGF acutely induces microvascular hyperpermeability through functional activation of vesicular-vaculargular organelles in the cytoplasm of endothelial cells (18, 72). In contrast to many vesicular-vaculargular organelles in endothelial cells of peripheral tissues, endothelial cells in brain capillaries contain few vesicular-vaculargular organelles under physiological conditions (73, 74). However, cerebral ischemia and brain trauma increase endothelial vesicles and vacuoles in cerebral capillaries (73, 74), which may contribute to increased leakage of the BBB in response to VEGF.

In summary, our data demonstrate that late administration of VEGF enhances angiogenesis in the ischemic brain, improving neurological recovery, and that early administration of VEGF exacerbates BBB leakage. This suggests a potentially effective therapeutic strategy aimed at administration of exogenous VEGF to promote therapeutic angiogenesis during the repair process after a stroke and inhibition of VEGF at the acute stage of stroke to reduce the BBB permeability and the risk of hemorrhagic transformation after cerebral ischemia.

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