Dynamic Changes in Cerebral Blood Flow and Angiogenesis After Transient Focal Cerebral Ischemia in Rats
Evaluation With Serial Magnetic Resonance Imaging

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Background and Purpose—Angiogenesis occurs after cerebral ischemia, but the relationship between angiogenesis and cerebral hemodynamic change is unknown. The aim of the present study was to investigate the relationship between ischemia-induced angiogenesis and hemodynamics in a well-defined 3-vessel occlusion model of the rat by using diffusion- (DWI), perfusion- , and T2-weighted MRI (T2WI).

Methods—Rats were subjected to 60 minutes of transient middle cerebral artery occlusion or sham operation. DWI and T2WI were used to characterize the extent of the ischemic lesion from 4.5 hours to 14 days after reperfusion. A flow-sensitive alternating inversion recovery method and dynamic susceptibility contrast MRI were used to evaluate the temporal changes in relative cerebral blood flow (CBF) and cerebral blood volume (CBV), respectively. Rats were randomly selected and killed at each time point for investigation of vascular density and for hematoxylin-eosin staining.

Results—Ischemic lesions developed in the ipsilateral cortex, as demonstrated by DWI and T2WI. CBF was significantly increased in the ipsilateral cortex, especially in the cortical outer layer from day 1 to day 14, and peaked on day 7 \( (P<0.05) \), while CBV was significantly increased on day 7 \( (P<0.01) \). The vascular density on the ipsilateral brain surface was gradually increased from day 1 to day 5, peaked on day 7, and then decreased on day 14. Histology study showed pannecrosis in the cortex from day 1 to day 5 and partial liquefaction of the necrotic tissues on days 7 and 14.

Conclusions—A delayed increase in both CBF and CBV is documented in the ipsilateral cortex after transient focal brain ischemia, and such an increase may be associated with angiogenesis. \( \text{Stroke. 2002;33:2985-2991.} \)

Key Words: angiogenesis ■ brain edema ■ cerebral ischemia, focal ■ infarct ■ reperfusion ■ stroke ■ rats

Ischemic brain injury is a consequence of a severe reduction in blood supply to the affected region. The resultant low tissue oxygen tension after ischemia often leads to compensatory neovascularization or angiogenesis in order to meet the metabolic demand.\(^1\) The extent of angiogenesis has been correlated with survival in stroke patients.\(^2\) Using an animal stroke model, previous studies have reported an increase both in expression of angiogenesis-related genes and in vascular density after ischemia/reperfusion in the rat.\(^3-5\) In recent years, there has been considerable interest in the measurement of cerebral hemodynamic changes in ischemic injury. With diffusion-weighted imaging (DWI) and perfusion-weighted imaging (PWI), the in vivo ischemic changes associated with cerebral injuries have been widely investigated.\(^6-8\) To our knowledge, however, there has been no report about the relationship between angiogenesis and cerebral hemodynamic changes after transient focal brain ischemia.

The purpose of the present study was to apply these new MRI techniques to investigate the relationship between ischemia-induced hemodynamic changes and vascular remodeling (angiogenesis) over time in a well-defined 3-vessel occlusion model in the rat. To achieve our goal, DWI and T2-weighted imaging (T2WI) were used to demonstrate the extent of brain injury from acute to chronic stages, while the PWI was used to measure regional relative cerebral blood flow (CBF) and cerebral blood volume (CBV) over time. The changes of CBF and CBV over time were then correlated with those in vascular density.

Materials and Methods
Induction of Focal Brain Ischemia
All procedures in the present study were approved by our institutional Animal Studies Committee and were in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals, United States Department of Agriculture regulations, and the Guidelines of the American Veterinary Medical Association Panel on Euthanasia.

Focal cerebral ischemia was induced with a previously described method.\(^9\) In brief, male Long-Evans rats weighing 250 to 300 g were

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anesthetized with chloral hydrate (360 mg/kg body wt IP), and the trunk of the right middle cerebral artery (MCA) above the rhinal fissure was identified under a stereomicroscope and ligated with a 10-0 suture. Interruption of blood flow distal to ligation was confirmed under the microscope. Both common carotid arteries were then occluded with the use of nontraumatic aneurysm clips. After 60 minutes of ischemia, the aneurysm clips and the suture were removed, and restoration of blood flow in all 3 arteries was verified. All DWI and T2WI measurements were performed in 1 group of rats without a contrast agent injection (n=6 for each time point). In a second group of rats, CBF values were determined initially (n=6 for each time point), and then CBV values were measured by injection with a contrast agent. After CBF and CBV measurements, the rats were killed at different time points (n=6 for each time point) for the assessment of vascular density and histology staining. Animals subjected to the same surgery without vascular occlusion served as sham-operated controls (n=6 for DWI and T2WI measurements and n=6 for CBF and CBV measurements). Under anesthesia, rectal temperature was monitored and maintained at 37.0±0.5°C with the use of a homeothermic blanket (Harvard). After each experiment, the rats were kept in an air-ventilated incubator at 24.0±0.5°C and provided with water and laboratory chow ad libitum until the end of the experiment, when they were killed for assessment of vascular density and histological analysis.

MRI Experiments
All MRI experiments were performed with the use of a 4.7-T Biospec 47/40 spectrometer with an active shielding gradient (5.6 G/cm in 500 μs). The rat, anesthetized with chloral hydrate (360 mg/kg body wt IP), was placed in a prone position and fitted with a custom-designed head holder inside the magnet, as previously described. Images were acquired with the use of a 20-cm volume coil as the transmitter coil and a separate 2-cm surface coil for signal detection. All MRI studies were performed at 4.5 hours and days 1, 2, 3, 5, 7, and 14 after 60 minutes of transient ischemia.

Evaluation of Ischemic Lesions by DWI and T2WI
Multislice axial T2WI and DWI were scanned in the same location with a field of view of 4 cm, a slice thickness of 2 mm, and a matrix size of 256×128. The offset was set corresponding to the section −0.3 mm from the bregma, as illustrated in a rat brain atlas. The T2WI was acquired with a repetition time (TR) of 4 seconds and 6 echo times (TE) (20, 40, 60, 80, 100, and 120 ms) to generate T2 maps. The DWI was acquired with the use of a diffusion-weighted stimulated echo sequence with a TR of 2 seconds, TE of 43.5 ms, mixing time (TM) of 84.8 ms, Δ of 100 ms, and δ of 10 ms. Four b values (0, 500, 1000, and 1400 s/mm²) were used to calculate the apparent diffusion coefficient (ADC) along each of the 3 orthogonal diffusion gradient axes (x, y, and z).

Assessment of Relative CBF and Relative CBV
Relative CBF was evaluated with flow-sensitive alternating inversion recovery (FAIR) techniques. The FAIR experiment was performed with the use of 2 inversion recovery–fast spin-echo (IR-FSE) sequences with and without a slice selective gradient during an inversion pulse. Two slice and nonslice IR-FSE images were collected with TR of 3 seconds, TE of 20 ms, effective TE of 50 ms, echo train length of 4, slice thickness of 2 mm, field of view of 4 cm, inversion time of 1.5 seconds, and matrix size of 256×128. A slab thickness of 5 mm was inverted for the slice IR-FSE images.

Relative CBV was studied with dynamic susceptibility contrast (DSC) MRL. A series of 40 gradient-echo, 2-mm-thick, transverse, single-slice images with TR of 30 ms, TE of 10 ms, pulse angle of 15°, and matrix size of 256×64 were acquired. The bolus of the susceptibility contrast agent gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA, 0.3 mmol/kg, Schering AG) was injected intravenously 10 seconds after the start of image acquisition.

Assessment of Vascular Density and Histology Staining
The rats subjected to 60 minutes of transient ischemia were killed at different time points (4.5 hours and days 1, 2, 3, 5, 7, and 14 after ischemia/reperfusion), and the brains were removed and photographed for visual evaluation of vascular density, as previously described. Briefly, the extent and intensity of the vessels on the surface of the ipsilateral hemisphere were compared with those on the surface of the contralateral hemisphere and the hemispheres of the sham-operated rats by a researcher who was unaware of the time point assigned, and the results at each time point were reported as decreased, unchanged, mildly increased, moderately increased, and heavily increased vascular density. The brains were then frozen, sliced, and fixed in 4% formalin and stained with hematoxylin-eosin, as described previously.

Data Analyses
Data were processed with the use of commercially available image analysis software (MRVision, MRVision Co). T2 and ADC maps, calculated from the multiecho T2WI and multi-b DWI, respectively, were produced on a pixel-by-pixel basis with the use of linear least-squares regression. The ADC maps were obtained by averaging the 3 orthogonal (x, y, and z) ADCs. The relative regional CBV maps were generated from the integrated area under the AR₆ transit curves on a pixel-by-pixel basis. The FAIR images were generated by the subtraction of the nonslice inversion images from their corresponding slice inversion images. The region of interest was determined by manually tracing the cortex of both ipsilateral and contralateral hemispheres after optimizing the contrast, and the area of the region of interest was used to represent the size of cortex to assess the volume effect induced by ischemia/reperfusion. The changes of T2 and ADC values at each time point were expressed as the ipsilateral/contralateral ratio. The CBF, CBV, and size of cortex at each time point were normalized by the corresponding CBF, CBV, and size of cortex acquired from the hemisphere of the sham-operated rat.

Statistical Analyses
Data are presented as mean±SD. One-way ANOVA was used to compare the relative CBV and CBF at different time points. The level of significance for differences between groups was further analyzed with post hoc Fisher’s protected t tests with the use of statistical software (GB-STAT 5.0.4, Dynamic Microsystems, Inc). A value of P<0.05 was considered significant. The association between a semiquantitative scale of vascular density and CBV was correlated by nonparametric (Spearman) analysis, and a value of P<0.05 was considered significant.

Results
Ischemic Lesions on DWI and T2WI Over Time
At acute stages, all rats subjected to 60 minutes of ischemia developed ischemic lesions in the ipsilateral cortex demonstrated by DWI after ischemia/reperfusion, and the full extent of lesions was seen on T2WI 24 hours after reperfusion and thereafter. No lesions were observed on either DWI (791.66±29.59 10⁻⁶ mm²/s contralateral and 804.5±30.08 10⁻⁶ mm²/s ipsilateral) or T2WI (60.28±1.29 ms contralateral and 59.18±1.05 ms ipsilateral) in the sham-operated rats. Figure 1 shows a representative set of ADC and T2 maps from a rat subjected to 60 minutes of transient MCA occlusion at different time points and from a sham-operated rat.

A quantitative analysis of the temporal changes in the ADC and T2 ratios in the region of interest within the ipsilateral cortex is shown in Figure 2A. As expected, the ADC values
decreased during the acute stage and reached a minimum of 60% of sham-operated control levels 24 hours after reperfusion, then slowly pseudonormalized, reaching a value higher than in the sham-operated control during the later period. The T2 values gradually increased and peaked 48 hours after reperfusion at a level 1.6-fold higher than in the sham-operated controls, then gradually decreased. Interestingly, a second increase was seen at chronic stage (from day 7 to day 14).

The ischemia/reperfusion-induced volume effect was examined by measuring the size of the ipsilateral and contralateral cortex. As shown in Figure 2B, the size of the ipsilateral cortex transiently increased and peaked at 48 hours after reperfusion, then slowly decreased to a level even lower than that in sham-operated controls during the late reperfusion periods. The size of the contralateral cortex did not change significantly in the early reperfusion periods but increased slightly during the late reperfusion periods compared with sham-operated controls.

Relative CBF and CBV Changes Over Time
After 60 minutes of transient ischemia, CBF in the ipsilateral cortex, especially in the outer cortical layers, increased gradually from day 1 to day 7 ($P<0.05$ from day 1 to day 5; $P<0.01$ on day 7 compared with the sham-operated control) and then slightly decreased but was still higher than the control value ($P<0.05$). A significant increase in CBF was observed on the contralateral side from day 3 to day 7 ($P<0.05$), although the increase was less marked, and a significant increase was seen for a narrow duration. Quantitative analysis indicated a 3-fold increase in the ipsilateral hemisphere and a 2-fold increase in the contralateral side on day 7 compared with the sham-operated control. The representative FAIR images and quantitative CBF obtained at various time points after 60 minutes of transient ischemia are shown in Figure 3B and 3C.

CBV showed a delayed and transient increase in the ipsilateral cerebral cortex, particularly in the outer cortical layers. Figure 4 shows an example of the changes in brain signal intensity in a single voxel within the ipsilateral cortex during the first-pass transit of the intravenously injected contrast agent Gd-DTPA. Quantitative analysis showed a significant increase (2.3-fold; $P<0.01$) only on day 7 compared with sham-operated controls in the ipsilateral hemisphere, whereas no significant CBV change was noted in the contralateral side ($P>0.05$). Representative CBV maps and quantitative CBV analyses at various time points are shown in Figure 5A and 5B.
Vascular Density and Hematoxylin-Eosin Staining at Each Time Point

Vascular density in the sham-operated rats was unchanged. Vascular density in the ipsilateral cortex of ischemic rats was mildly increased on day 1, moderately increased on days 3 and 5, heavily increased on day 7, and unchanged on day 14, as shown in Figure 3A. Nonparametric (Spearman) analysis was used to correlate the association between a semiquantitative scale of vascular density and CBF, and a significant correlation ($P\leq 0.0085$) was found. Compared with the contralateral side, the ipsilateral side of the brain was swollen on days 1 and 2 after reperfusion, but no swelling was noted on day 7 after reperfusion. A slight atrophy of the ipsilateral hemisphere was seen on day 14.

Histological examination showed clear pannecrosis in the cortex of the MCA territory on days 1, 2, 3, and 5. On days 7 and 14, some of the necrotic tissue was liquefied. No abnormalities were found in the sham-operated rats (Figure 1).

Discussion

In this study DWI and T2WI were used to document the temporal evolution of ischemic lesions over time. The ischemic lesions demonstrated by DWI after 60 minutes of transient ischemia were consistently confined to the right MCA cortex, which is in agreement with previous studies.\textsuperscript{13,14} The size of the ipsilateral cortex gradually increased, peaked at 48 hours after ischemia/reperfusion, and then gradually decreased, which is in agreement with a previous report that the ipsilateral water content peaks between 24 and 72 hours after reperfusion.\textsuperscript{9} This temporal profile of swelling showed a good match with that of the T2 changes in the ipsilateral cortex, indicating that T2 increase at this time point is related to vasogenic edema, a well-known phenomenon.\textsuperscript{15} Since ADC values were still lower than normal control at 48 hours after reperfusion, it is likely that there is a coexistence of cytotoxic edema and vasogenic edema. However, the exact mechanism responsible for the “mismatch” between the T2 and ADC values that is also demonstrated by Li and colleagues\textsuperscript{16,17} is not clear. The gradual decline in T2 may be related to a diminution of vasogenic edema, as suggested by the resolution of hemispheric swelling in this study, or to an increased iron deposition, as reported previously.\textsuperscript{18} The second increase in T2 is likely due to liquefaction of the necrotic tissues, as suggested by the microscopic examination.

Hemodynamic changes after transient ischemia at an acute stage have been widely studied with PWI. In transient ischemic models, the decreased CBF occurring during ischemia can reverse to normal or change to hyperemia after reperfusion, and the hyperemia may be followed by a period of hypoperfusion.\textsuperscript{19–24} The increased CBF early after reperfusion is believed to be related to the vascular dilatation caused by accumulated Pco$_2$, NO, acidosis, and/or other factors.\textsuperscript{25–27} To our knowledge, CBF and CBV changes at a chronic stage after transient ischemia have not been reported. The delayed gradual increase in CBF and CBV in this study is unlikely to be related to the hypoperfusion or “luxury perfusion” phenomenon. The increased CBF and CBV, tightly coupled with the increased vascular density in this study, may be a result of new collateral formation and angiogenesis, as suggested by previous studies.\textsuperscript{1,28–30} Angiogenesis, the new formation of blood vessels by sprouting from preexisting vessels, can occur after focal brain ischemia.\textsuperscript{1} It has been shown that active angiogenesis expressed as increased microvessel density develops in the penumbral area both in patients with cerebral stroke\textsuperscript{2,31} and in experimental stroke in
The new vessel formation is tightly regulated by several growth factors and their receptors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 and angiopoietin-2, and the endothelial tyrosine kinase receptors tie1 and tie2.\textsuperscript{1,2,8,34} Using an MCA occlusion rat model, Plate and colleagues\textsuperscript{35} found that many of VEGF-expressing cells are in the penumbra, suggesting the crucial role of growth factors in angiogenesis. Furthermore, several studies have demonstrated that there is a close correlation between the increased vascular density and angiogenesis-related gene expression,\textsuperscript{3-5} suggesting that the increased vascular density on the brain surface is likely due to the formation of new collateral circulation by angiogenesis. In this study we did not quantify the microvessels in the regions with increased CBF and CBV demonstrated by MRI. Instead, the vascular density on the brain surface area was semiquantitatively determined by Western blot\textsuperscript{5} and seems very well correlated with the changes of CBF and CBV. The increased CBF and CBV in the present study are predominantly in the outer cortical layer, which is consistent with the location of angiogenesis in previous studies.\textsuperscript{32,33} On the basis of this evidence, we believe that the increased CBF and CBV documented in this study are most likely related to angiogenesis after transient focal brain ischemia.

Interestingly, a significant increase in CBF was also noted in the contralateral cerebral cortex. This result is in agreement with a previous report that subtle changes in capillary density are noted in the contralateral cerebral cortex in this MCA occlusion model.\textsuperscript{4} A recent study has demonstrated that angiogenesis develops in the neurohypophysis after focal ischemia in the cerebral cortex.\textsuperscript{36} These results suggest that the increase in CBF in the contralateral cortex may be related to angiogenesis as well.

One possible mechanism causing reperfusion injury is the disturbance of the blood-brain barrier, which may potentially generate confounding effects of CBV measurement with the use of the DSC technique. Gd-DTPA, the contrast agent that we used, is a paramagnetic metal complex that can shorten both the T1 and T2 relaxation times and is compartmentalized within normal brain capillaries. In the presence of blood-brain barrier disruption, as the contrast agent leaves the intravascular space, the contrast agent concentration-time curves might vary with the contrast agent–induced transient changes of signal intensity. This effect can be minimized by using blood pool agents that prolong intravascular residence times. The second effect of the disruption of blood-brain barrier is the possible accumulation of the contrast agent in the extravascular space, which leads to T1 enhancement as a result of the large dipolar relaxivity of Gd-DTPA. Therefore, dynamic contrast-enhanced T1-weighted imaging has been used extensively to study the involvement of blood-brain barrier breakdown in various pathological lesions such as brain tumors and cerebral infarctions.\textsuperscript{37} Furthermore, the signal change in the DSC method for CBV measurement using heavy gradient- or spin-echo T2WI is caused predominantly by induced magnetic susceptibility differences between brain capillaries and the surrounding tissues, and this effect is much larger than that due to relaxivity changes. Numerous studies have validated this high-speed DSC technique to resolve the cerebral transit of contrast agent and to measure cerebral hemodynamics.

Although angiogenesis after brain ischemia has been well recognized, the clinical significance of angiogenesis after ischemic stroke is currently not conclusive. Several studies suggest that angiogenesis may have beneficial effects on ischemic stroke. First, Krupinski et al\textsuperscript{2} demonstrated that the higher microvessel density in the ischemic penumbra is correlated with longer survival in stroke patients. Second, using an animal model, Wei and colleagues\textsuperscript{33} found that angiogenesis in the ischemic border may permit neuronal

![Figure 5. Time course of regional CBV changes after 60 minutes of focal cerebral ischemia. A, Representative CBV maps at corresponding time points. There was a marked increase of CBV in the outer cortical layer on day 7. B, Quantitative analysis of CBV in the ipsilateral cortex showed a significant increase on day 7 ($P<0.01$), but no significant change was found on days 1 and 14 ($P>0.05$) (mean$\pm$SD; $n=6$).](http://stroke.ahajournals.org/)}
plasticity for functional recovery. Indeed, the expression of axonal sprouting protein, growth-associated protein 43, is increased after MCA occlusion in regions very similar to those that show increased angiogenesis in the peri-infarct region and in contralateral cortical regions that may contribute to the enhancement of the functional recovery effect by basic fibroblast growth factor. Third, recombinant VEGF can induce angiogenesis in rat brain, and the use of VEGF reduces ischemic injury in a rat stroke model. Further studies are needed to investigate the physiological significance of increased CBF and angiogenesis. Moreover, the mechanism for the eventual decline in angiogenesis at 2 weeks after ischemia is not clear. Since angiogenic activity reflects a balance between the angiogenic and angiostatic drives, it is likely that the expression of angiostatic factors may contribute to the resolution of posts ischemic angiogenesis.

In summary, this study documents a gradual increase and then decrease in both CBF and CBV in the ipsilateral cortex after 60 minutes of transient focal cerebral ischemia; the increase in both CBF and CBV is tightly coupled with increased vascular density, a possible sign of angiogenesis. The exact mechanisms and physiological significance of these observations remain to be studied.

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