Collateral Growth and Angiogenesis
Around Cortical Stroke

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**Background and Purpose**—We tested the hypothesis that there are significant long-term local vascular changes after ministroke that could form a basis for functional recovery.

**Methods**—A 6- to 8-mm cranial window was opened over the barrel cortex, which was identified by an intrinsic optical signal during mechanical stimulation of the whiskers in anesthetized female Wistar rats. Branches of the middle cerebral artery (MCA) to this region were ligated. Fluorescein isothiocyanate (FITC) transits were recorded by videomicroscopy in each rat just before, immediately after, and 30 days after ligation. Changes in surface vessels and parenchymal perfusion were measured. In similarly prepared rats, angiogenesis was identified by 5-bromo-2-deoxyuridine labeling and immunohistochemistry for the integrin family member α<sub>v</sub>β<sub>3</sub>.

**Results**—The intrinsic optical signal disappeared immediately after MCA ligations. FITC injection just after ligation demonstrated 3 concentric regions: 1 region of unchanged perfusion, surrounding 1 region of reduced perfusion (the ischemic border) surrounding a central core with little observable perfusion. At 30 days, the following had taken place: (1) diameters and lengths of surface collaterals in the ischemic border had grown significantly, but no new surface vessels were detected, (2) FITC entered occluded MCA segments, (3) arteriocapillary latencies in the ischemic border were shortened compared with latencies just after ligation, and (4) small infarcts were virtually identical to the poorly perfused core. Angiogenesis was confined to the ischemic border.

**Conclusions**—Arteriolar collateral growth and new capillaries support restored perfusion in the ischemic border after ministroke and could support long-term functional recovery. (Stroke. 2001;32:2179-2184.)

**Key Words:**
cerebral cortex ■ cerebral revascularization ■ microcirculation ■ stroke, experimental ■ rats

In the literature on stroke, much attention has been focused on the penumbra.¹ Excitotoxic damage, inflammation, and, ultimately, cell death in the penumbra are possible targets for therapy.² Reports from stroke models and patients document the proliferation of vascular cells and expression of markers for angiogenesis surrounding infarcts.³⁻⁶ Few, if any, new large surface vessels have been found in the brain after stroke,⁷ although there is evidence of arteriolar collateral enlargement.⁸⁻⁹ The relationship between angiogenesis and/or vessel growth and function in the brain after focal ischemia has not been characterized.

Within an hour after creating “ministrokes” in the barrel cortex, there are significant reductions of local cerebral blood flow.¹⁰ The goal of the present study was to evaluate the extent to which perfusion to small regions of ischemia recovers in the functionally identified rat whisker barrel cortex 30 days after ministroke. Specifically, we tested the hypothesis that enlargement and elongation of surface vessels is related to new vessels (angiogenesis) and recovery of flow. If altered surface collaterals can protect ischemic brain from death, they may ultimately support functional neuronal recovery just as angiogenesis may support postischemic myocardial recovery.¹¹ Normal, ischemic, and infarcted cortex was visualized simultaneously in the context of a functional area of the brain.

**Materials and Methods**

**Animal Preparation**

Adult female Wistar rats (250 to 300 g, Charles River, Wilmington, Mass) were studied according to approved protocols that meet or exceed National Institutes of Health and American Physiological Society guidelines. Animals were anesthetized with ketamine (87 mg/kg) and xylazine (13.5 mg/kg) intraperitoneally, supplemented as needed to abolish withdrawal reflexes. The right jugular vein was catheterized with heparinized PE-10 tubing. A 6- to 8-mm-diameter cranial window was opened on the right side over the whisker barrel cortex, keeping the dura intact. The window was rinsed with 37°C sterile buffered normal saline and coverslipped.

**Ligation of Arterioles Feeding Barrel Cortex**

An intrinsic optical signal (IOS), elicited by rostrocaudal stroking of the contralateral large whiskers (~3 Hz), was mapped by superimposition and subtraction of averaged control and stimulated images to find the barrel cortex.¹² Briefly, images were captured through
a Nikon Labophot microscope under a ×1.5 objective with white light and a 520- to 560-nm interference filter with a Dage-CCD-72 videocamera and recorded on VHS tape. Images were processed immediately or later from tape with a Power Macintosh for absorption changes with NIH Image 1.61. Typically, 3 to 6 proximal and distal branches of the middle cerebral artery (MCA) supplying the barrel cortex were identified, and a 10-0 suture was passed through the dura and under each arteriole and tied.13 Sham-operated control animals had the same surgery, and sutures were placed but not tied. Animals were kept in isolation until they had fully recovered from the anesthetic and then housed 3 rats per cage for 30 days.

FITC Angiography
Under epifluorescent illumination, 0.2 mL boluses of 1.3 mmol/L fluorescein isothiocyanate (FITC) were injected into a branch of the right jugular vein. Dye transits through the arteries, capillaries, and veins of the cortex were recorded through the microscope under a ×2 objective with a Quantex ICCD videocamera set to manual black and fixed gain settings on VHS tape. Taped images were processed on a Power Macintosh with NIH Image 1.61. After 30 days, the rats were anesthetized again, and the cranial windows were reopened for IOS and video microscopy of FITC transits and angiography. Of 15 animals res tudied at 30 days, 8 were successfully analyzed further. The other 7 animals could not be fully imaged because of dural scars or insufficient exposure at the time of ligation to view the cortex surrounding what became infarcts at 30 days.

Image Analysis
Vessel Dimensions
Collaterals provide alternate routes to the ischemic region before and after vessel ligation. Collateral and other “control” vessels were identified in angiograms made 30 days after the ligations. The same vessels were identified in video recordings made at the time of vessel ligation. Before and after measurements were then made with NIH Image 1.61 at identical points on the vessels, and after/before ratios were calculated. The average of 3 successive diameter measurements of FITC-filled vessels was determined. Vessel lengths were measured between branch points (nodes) in 2 ways: (1) straight lines from node to node and (2) the actual length along the segment. Similar measurements were made of vessels outside the ischemic border and in vessels from sham-operated rats.

Arteriocapillary Transits
Patterns of vessels were used to accurately relate images at 30 days to the initial images. Estimates of changes in blood flow were based on the timing of FITC transits over an identified small arterial branch and the neighboring parenchyma. Latencies between the first appearance of FITC in the artery and half-maximal fluorescence in the regions of interest were measured. Blood flow depends on blood volume in the pathway (flow = volume/time), but changes in arterio- lar and capillary volumes are small compared with large changes in timing. The ratio of latency after to latency before ischemia is an index of blood flow changes.10 Sham-operated control sites were near the center of the acute and reopened cranial window.

Histology
Proliferation
Four groups of rats (with 4 ministroke and 2 sham-operated controls each) were injected intraperitoneally with 50 to 70 mg/kg 5-bromo-2-deoxyuridine (BrdU, Sigma Chemical Co) as single doses at 2, 4, 6, or 9 days after placing ligatures. Rats were anesthetized 24 hours later and were fixed by perfusion with phosphate buffered (pH 7.4) 4% paraformaldehyde. Coronal sections were cut at 14 μm in a cryostat, and every fifth section was thawed onto Superfloat/Plus slides (Fisher Scientific) on a hot plate at 45°C, fixed in 4% paraformaldehyde for 10 minutes, rinsed in PBS 3 times, blocked in fish gel for 45 to 60 minutes, and rinsed in PBS.14 Mouse anti-BrdU (Chemicon) diluted 1:17 with PBS and a rabbit anti–glucose transporter (GLUT)-1 (a specific endothelial marker, Chemicon; 1:750) were puddled on each section at 4°C overnight in a closed container at 100% humidity. The sections were rinsed in PBS 3 times and stained for 30 minutes with secondary antibodies (anti-mouse cy3 and anti-rabbit FITC, Sigma). After rinsing in PBS 3 times, some sections were counterstained for 30 seconds with bis-benzamide (1:5000, Sigma) rinsed, and coverslipped with Vectashield (Vector Laboratories). At least ≥2 radial vessels 18 to 25 μm in diameter and 100 to 150 μm in length could be identified in the ischemic border that stained more intensely for GLUT-1 (see Results). Labeled nuclei in the 2 vessels closest to each side of the core/infarct were counted in 4 or 5 sections through the stroke for each animal.

Integrin αββ, Staining
Animals were euthanized at 3, 5, 7, or 10 days after the ligations (2 or 3 rats per time point), and the brains were removed quickly, blocked, frozen at −80°C, and sectioned in a cryostat. Mounted sections were fixed in acetone for 30 seconds and 70% ethanol for 3 to 5 minutes. After washing in PBS 3 times, the sections were blocked with 0.2% fish gel in PBS for 2 hours, incubated with 10 μg/mL of mouse anti-αββ, monoclonal antibody LM 60915 for 2 hours at 37.5°C, stained with rhodamine-conjugated goat anti-mouse IgG (1:150) for 1 hour, rinsed in PBS 3 times, counterstained with bis-benzamide, washed, and coverslipped.

There was no staining when any of the secondary antibodies were used alone. There was no staining for BrdU or αββ, in sham-operated control animals. Capillaries were GLUT-1 positive in the normal hemispheric sections and in the sham-operated control sections.

Statistical Analysis
Paired means were compared with the Student t test. The conservative Bonferroni correction was used for multiple pairwise comparisons.

Results
Changes in Angiograms of Particular Surface Vessels
Acutely after ligations, there were 3 qualitatively distinct zones: (1) regions in which there were no changes in the vessels or time course of the transits, (2) a relatively dark ischemic core surrounded by the ligatures that stayed dark during the angiogram, and (3) a border at which parenchymal perfusion slowed markedly. FITC trickled through acutely dilated arteriolar collaterals to the ischemic border, and the washout of dye that eventually entered the occluded arterial segments was slow (Figure 1A and 1B; see also Figure 2). Thirty days later, transits through enlarged collaterals were rapid and filled the acutely occluded segments of the MCA, and dye entered the parenchyma in the ischemic border more quickly (Figure 1C and 1D). Figure 1A and 1D (captured in 2 different animals 0.20 seconds after FITC injection) shows the remarkable changes in perfusion after 30 days.

Figure 1E shows an FITC angiogram just after the MCA ligations, and Figure 1F shows the same vessels 30 days later. Individual vessels were clearly recognized (before and after) and had changed (arrowheads). Alterations in collateral arterioles are obvious. After 30 days, there is substantial remodeling of surface vessels principally in the ischemic boarder, filling of capillaries in the ischemic region, and shorter transit of the dye.

Changes in Surface Vessel Dimensions
Collateral arterioles supplying the ischemic border were larger and more tortuous at 30 days (Figure 1F). Internal diameters nearly doubled (to ×1.91 ± 0.17 [mean ± SD],
cortical surface arterioles supplying collateral flow just after and 30 days after ligation in a rat. In panel E, 4 collaterals that filled with FITC immediately after ligating MCA branches are marked (arrowheads). Thirty days later (F), these same 4 vessels and many other unmarked vessels were enlarged and tortuous and filled more rapidly.

In the “peripheral” ischemic border, the ratio also increased significantly.

After 30 days, arteriocapillary latencies returned toward original values (Table). The ratio of latencies in the core decreased significantly compared with that in acute stage but was still higher (P<0.001) than that in 30-day sham-operated controls. The ratio of latencies in the peripheral ischemic border was shorter than at the acute stage but significantly slower than in 30-day sham-operated controls.

**Parenchymal Vessel Growth**

No BrdU label was observed in surface or intraparenchymal blood vessels in sham-operated controls, in the contralateral cerebral cortex, or in the normal cortex adjacent to the peripheral ischemic border (Figure 3A and 3C). BrdU and \(\alpha\beta_3\) immunostaining marked bands 100 to 200 \(\mu\)m wide separating the infarcted core from unstained “normal” cortex. Walls of surface vessels and endothelial cells of intraparenchymal microvessels had BrdU-labeled nuclei 3 to 10 days after ligations (Figure 3B). Endothelium of intraparenchymal vessels labeled with GLUT-1 (Figure 3C) and BrdU confirmed proliferation (Figure 3D). Endothelial labeling in radial vessels peaked at \(\approx 7\) days (Figure 4).

There was no detectable \(\alpha\beta_3\) staining in sham-operated control sections, in the nonischemic cortex (Figure 3E), or in the core. The integrin \(\alpha\beta_3\) was detected in the peripheral ischemic border after MCA ligations (Figure 3E and 3F). At the ischemic border, immunostaining for \(\alpha\beta_3\) was prevalent 7 days after the ischemic insult (Figure 3E) and was related to vessel walls (Figure 3F). \(\alpha\beta_3\) expression was particularly robust 10 days after ligation (Figure 3G).

**Discussion**

The present study demonstrates the growth of surface collaterals and angiogenesis in the vascular beds that they supply, which are related directly to increases in perfusion. We found dramatic localized changes in surface vessels and parenchymal changes in the ischemic capillary zone arterioles through surface collaterals (Figure 2E versus 2C).

Changes in Transits

Before ligations, the arterial network over the cortex filled uniformly (Figure 2C), and capillary fields were filled after short delays (Figure 2D). Just after the ligations, the ischemic region generally remained dark except for flow into some arterioles through surface collaterals (Figure 2E versus 2C). The time course of the transit in the ischemic capillary zone slowed by >10 (Figure 2F versus 2D). In this rat, arteriolar collaterals that supplied the surface arterial network isolated by the MCA ligations became enlarged and tortuous 30 days later (Figure 2G versus 2E). Perfusion improved in the capillaries in the acutely ischemic zone, and the arteriocapillary delay was shortened to 38% of that just after ligation (Figure 2H).

Transit latencies were measured in 2 locations in the ischemic border in this and 3 other rats and in 4 sham-operated control rats (Figure 2 and Table). Typical arteriocapillary latencies in rats before ligations and in sham-operated control rats were 0.5 to 1 second. No changes occurred in sham-operated control rats just after placing the sutures or after reopening the cranial windows 30 days later (Table). In the experimental rats, there were no statistically significant changes in latencies in cortical sites >1 mm peripheral to the ligations. Delays of FITC transits in the “central” ischemic border were prolonged to 2 to 4 seconds, and the ratio of latencies after/before increased significantly.

P<0.001; n=16). In sham-operated control rats, the arteriolar diameters in the cranial window were unchanged 30 days after the original surgery (\(\times 1.012 \pm 0.043, n=9\)). After 30 days, vessel segment lengths increased (\(\times 1.21 \pm 0.14, P<0.01; n=14\)), whereas the cord lengths between the ends of these segments remained unchanged (\(\times 1.04 \pm 0.04, P=0.12\)). There were no changes in true lengths (\(\times 1.04 \pm 0.03\) or cord lengths (\(\times 1.04 \pm 0.06\) of 9 vessel segments seen through the cranial window outside the ischemic border (P=0.17).

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mal perfusion correlating with poststroke angiogenesis. Surface collaterals in the ischemic border grew in the 30 days after ligation as perfusion of the peripheral ischemic border recovered. Patterns of BrdU labeling and $\alpha_v\beta_3$ expression indicate angiogenesis in the ischemic border. In particular, BrdU and GLUT-1 were colocalized in endothelial cells of new capillaries in the ischemic border. Collaterals not only feed areas deprived of their normal blood supply but also may exhibit increased flow as they supply new capillaries. By comparing penetrating vessels before and 30 days after ligation, there was little if any shift of the cerebral cortex around these small infarcts, indicating little or no loss of brain parenchyma in the ischemic border. Measurements of vessels to nonischemic cortex were similar to those in the sham-operated controls, in which no changes in diameter or length were observed after 30 days. To our knowledge, this is the first study to use multiple examinations of the same vessels in the cerebral microcirculation in the same animal, at the time of and after focal ischemia.

The ischemic border is not homogeneous (Table), yet the relative recovery in the central and peripheral ischemic borders is similar. This suggests a limit for angiogenesis and/or collateral growth, such as that described in other hypoxic/ischemic tissues. The term ischemic border is largely coextensive with the penumbra as originally de-

Figure 2. IOS images, FITC angiograms, and FITC transits just before, just after, and 30 days after ligating MCA branches. A, IOS after stimulation of all the large whiskers on the left face before ligations. Activated area represents larger whiskers (arrow, warm colors; midline is up and rostral to the right). Bar=0.5 mm (applies to B, C, E, and G). B, IOS 30 days later. The area activated by stimulation of the large whiskers was smaller and rostral to that in panel A (arrow). There is no activation of infarcted cortex (blue; compare with panel G). C, Arterial phase after FITC injection before ligations. Sites for the arterial time courses (a) and parenchymal fields in the central ('') or the peripheral ('†') ischemic border. D, Time courses of transits after FITC at the 3 locations. Arrowheads indicate half-maximal dye intensity values that were reached at short latencies after the dye first appeared in the arteriole (a) (<0.5 seconds). FITC recirculates after 4 seconds in the artery and the parenchyma. E, Transit 3 minutes after ligations (arrowheads) were placed. Collaterals provide delayed, less robust blood flow to the central ischemic border. F, FITC transit in the central ischemic border, which was considerably delayed and diminished acutely after ligations compared with that at the peripheral ischemic border, which was significantly slowed (compare with panel D). G, FITC transit 30 days later. Collaterals have increased in diameter, are more tortuous, and have more prominent branches. The infarct is at the lower left. H, FITC transit after 30 days, which was faster in the central ischemic border but delayed compared with that in the peripheral ischemic border. Transits were still slower than immediately before the ligations (D).

Figure 3. Specific labeling for DNA synthesis (BrdU), integrins ($\alpha_v\beta_3$), and capillary endothelium (GLUT-1). A, At 5 days after a cranial window without arteriolar ligations (sham-operated control), no microvessels (arrows) or surface vessels in the cortex were labeled with BrdU. B, BrdU-labeled nuclei in the ischemic border 5 days after ligations are shown. A surface arteriole at the upper right ('†') shows labeled nuclei in presumed perivascular smooth muscle (arrowheads). A radial intraparenchymal vessel below it has several labeled nuclei (arrows) that are likely endothelial. Other labeled nuclei are not associated with vessels. C, Endothelium of an intraparenchymal microvessel in a sham-operated control animal is labeled lightly for GLUT-1 (green). D, Double labeling of BrdU (yellow) and GLUT-1 demonstrates endothelial proliferation in an intraparenchymal capillary in the ischemic border. E, Antibody staining for $\alpha_v\beta_3$ shows a sharp division between the ischemic border (lower left) and normal unstained cortex (upper right) 7 days after ligation. A large and a small vessel are labeled (arrowheads). F, Higher power image of the field outlined in panel E shows association of $\alpha_v\beta_3$ with vessels in the ischemic border (bis-benzimide counterstain). Above the dashed line, there is no vascular staining for $\alpha_v\beta_3$ in the normal cortex ('†'). G, $\alpha_v\beta_3$ in the ischemic border 10 days after arteriolar ligation outlines vessels (arrowheads). Bars=100 $\mu$m (A, B, and E), 10 $\mu$m (G), and 50 $\mu$m (F and G).
Figure 4. BrdU labeling of endothelium in radial vessels through the ischemic border after arterial ligations. No endothelium was labeled in vessels from sham-operated control rats or in adjacent nonischemic cortex. Labeling peaked at ~7 days (mean ± SD). The increase between days 3 and 5 is significant (*P < 0.03), as is the decrease between days 7 and 10 (†P < 0.04).

In retrospect, when little dye was detected in the cortex acutely after the vessel ligations, even with imaging for long periods after these injections, that region was infarcted at 30 days. Collaterals provide alternate routes for circulation, both acutely and progressively when an artery is occluded physiologically or pathologically. Collaterals to the ischemic cortex dilated acutely (see Figure 2C versus 2E) without length changes. Thirty days later, diameters and lengths of these vessels had significantly increased, but we saw no new surface collateral vessels (see Figures 1E versus 1F and Figure 2C versus 2G). These findings directly support the previous proposals. The enlarged collaterals provided flow to occluded arterial segments lacking flow after the ligations, indicating recruitment of new flow pathways. Photographs of monkey cortex before and 3 months after small occlusive strokes show increased diameters of arterioles (collaterals) feeding regions around them without tortuosity. The growth of surface vessels could be stimulated by chronic elevation in wall shear stress, possibly by increasing shear stress. The enlarged collaterals imply increased conductances through them. Although “new” collaterals have been described, these are related to large regions of ischemia (major vessel territories of whole muscles). For instance, new vessels at the hip were demonstrated by x-ray angiography after ligation of the femoral artery. The limited resolution of radiograms precludes identification of smaller vessels that could support increased collateral flow through growth.

The GLUT-1 55-kDa isofrom, which is expressed in brain microvascular endothelium, was our marker for capillaries. GLUT-1 was expressed in capillaries in normal animals and sham-operated control animals. GLUT-1 expression increased in microvessels around infarcts at 2 days, peaking at 7 days after ischemia. Colocalized BrdU indicates that part of the increase in GLUT-1 capillary staining was a consequence of angiogenesis (Figure 3D). The integrin αβ3 is expressed specifically in young animals during angiogenesis and anti-αβ3 antibodies block cell attachment to collagen, preventing angiogenesis. In adults, αβ3 is not normally expressed but is upregulated after focal ischemia. αβ3 is upregulated in 30- to 50-μm-diameter arterioles around focal strokes. We found αβ3 expression associated with vessels in adults only in the ischemic border. These specific markers for angiogenesis correspond with increased microvascular staining, indicating significant new capillary growth in the ischemic border supplied by surface collaterals (Figure 3D).

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor play crucial roles in angiogenesis. Expression of VEGF is increased before angiogenesis. Exogenous VEGF produces significant cerebral angiogenesis and VEGF receptor-1 (flt-1) expression on adjacent reactive astrocytes in vivo. Basic fibroblast growth factor is upregulated in neurons and glia to stimulate angiogenesis in the ischemic border after stroke and stimulates neurite outgrowth. In rodents and in primates, recovery of sensorimotor function after unilateral cerebrocortical injury or stroke is accompanied by neuronal sprouting and synapse formation surrounding infarcts and in homotopic sites in the contralateral hemisphere.

A long-range goal of the present work is to understand the interactions between a defined region of the brain and the vessels that supply it before and after stroke. The present results indicate that imaging with intravascular dyes provides accurate predictions of the regions that progress to infarcts. Furthermore, the graded flow changes and recovery in the ischemic border allow evaluation of the perfusion necessary to prevent continuing cell death and to permit neural plasticity for functional recovery.

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