Long-Term Neuroblast Migration Along Blood Vessels in an Area With Transient Angiogenesis and Increased Vascularization After Stroke

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Background and Purpose—Stroke induced by middle cerebral artery occlusion (MCAO) causes long-term formation of new striatal neurons from stem/progenitor cells in the subventricular zone (SVZ). We explored whether MCAO leads to hypoxia, changes in vessel density, and angiogenesis in the ipsilateral SVZ and adjacent striatum, and determined the relation between the migrating neuroblasts and the vasculature.

Methods—Adult rats were subjected to 2 hours of MCAO. Hypoxia was studied by injecting Hypoxyprobe-1 during MCAO or 6 weeks later. Vessel density and length was estimated using stereology. New cells were labeled with 5’-bromo-2’-deoxyuridine (BrdU) during weeks 1 and 2 or 7 and 8 after MCAO, and angiogenesis was assessed immunohistochemically with antibodies against BrdU and endothelial cell markers. Distance from neuroblasts to nearest vessel was measured using confocal microscopy.

Results—The ischemic insult caused transient hypoxia and early, low-grade angiogenesis, but no damage or increase of vascular density in the SVZ. Angiogenesis was detected during the first 2 weeks in the dorsomedial striatum adjacent to the SVZ, which also showed long-lasting increase of vascularization. At 2, 6, and 16 weeks after MCAO, the majority of neuroblasts migrated through this area toward the damage, closely associated with blood vessels.

Conclusions—The vasculature plays an important role for long-term striatal neurogenesis after stroke. During several months, neuroblasts migrate close to blood vessels through an area exhibiting early vascular remodeling and persistently increased vessel density. Optimizing vascularization should be an important strategy to promote neurogenesis and repair after stroke. (Stroke. 2007;38:3032-3039.)

Key Words: angiogenesis ■ hypoxia ■ neurogenesis ■ stroke

Ischemic stroke, induced by middle cerebral artery occlusion (MCAO), leads to increased proliferation of neural stem/progenitor cells in the ipsilateral subventricular zone (SVZ)1,2 and migration of neuroblasts into the damaged striatum,3–5 a region where neurogenesis does not normally occur. Many stroke-generated neuroblasts differentiate into mature neurons with the phenotype of striatal projection neurons.3 Striatal neurogenesis continues for several months after stroke.6

Experimental evidence has indicated a close link between neurogenesis and angiogenesis in the adult brain. In the songbird, testosterone-induced angiogenesis leads to neurogenesis in the striatum.7 In the subgranular zone of the rat dentate gyrus, proliferating cells giving rise to granule cells are closely associated with the vasculature and dividing endothelial cells.8 After 1 electroconvulsive seizure, endothelial cell proliferation in the dentate gyrus occurs concomi-

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neurogenesis in the SVZ. Microarray analysis shows concomitant upregulation of genes associated with neurogenesis and angiogenesis in SVZ at 7 days after stroke in mice. Moreover, a cortical lesion that induces migration of neuroblasts into the striatum has been reported to trigger endothelial cell proliferation at 5 days and increased number of blood vessels 2 days later in ipsilateral SVZ.

Here we studied the relation between vasculature and new neurons during long-term neurogenesis after 2 hours of MCAO in rats. The objectives were 3-fold. First, the objective was to determine the distribution of hypoxia in SVZ and adjacent striatum through which neuroblasts migrate toward the damage. This seems highly warranted because hypoxia stimulates angiogenesis and proliferation and differentiation of neural progenitors in vitro and in vivo. The second objective was to explore whether MCAO causes changes in vascularization in SVZ and adjacent striatum, and whether these changes are attributable to angiogenesis. The third objective was to analyze the distribution of neuroblasts at various distances from blood vessels at different time points after MCAO.

Materials and Methods

Animals and Experimental Design
Male Wistar rats (280 to 320 grams; B&K Universal, Sollentuna, Sweden) were housed under 12-hour light/dark cycle with ad libitum access to food and water. Experimental procedures followed guidelines established by the Malmö-Lund Ethics Committee.

For analysis of hypoxia, animals were subjected to 2 hours of MCAO (n=10) or sham surgery (n=3) and injected with Hypoxyprobe-1 (60 mg/kg intraperitoneal; Chemicon) 30 minutes after the start of occlusion to have the probe (with half-life of 0.5 hour) present during the insult and avoid confounding effects of anesthesia or intubation. Rats were euthanized directly after the onset of reperfusion (n=6) or 4 days later (n=7). In a separate experiment, Hypoxyprobe-1 was injected 6 weeks after 2 hours of MCAO (n=3) or sham surgery (n=2), and rats were perfused 2 hours thereafter.

For analysis of angiogenesis, rats were injected with BrdU (50 mg/kg, intraperitoneal) twice daily during weeks 1 and 2 (n=5) or 7 and 8 (n=4) after 2 hours of MCAO. Sham-operated animals (n=3) were injected with BrdU during weeks 1 and 2. Rats were perfused directly after the end of injections. Another group of rats was injected with BrdU 3 times daily during days 3 and 4, 7 and 8, or 11 and 12 after 2 hours of MCAO, and perfused 48 hours after last injection (n=3 in each group).

For analysis of vessel density and length and distance between neuroblasts and blood vessels, animals were subjected to 2 hours of MCAO or sham surgery and perfused 2, 6, or 16 weeks later (n=4 in each group).

MCAO
Transient MCAO was induced by the intraluminal filament technique. After being fasted overnight, rats were anesthetized with \( N_2O \) and \( O_2 \) (70%-30%) and 1.5% isoflurane (Abbott Scandinavia) and intubated. A silicone rubber-coated nylon monofilament was inserted into the internal carotid artery and advanced past the origin of the middle cerebral artery. For sham surgery, the filament was advanced only a few millimeters inside the internal carotid artery. After 2 hours of occlusion, the animals were re-anesthetized and the filament was withdrawn. Physiological parameters were monitored during the entire procedure. Body temperature was regulated for 4 hours after MCAO. Only animals that showed no or incomplete forelimb placing with rotational asymmetry 24 hours after MCAO were included in the subsequent analysis.

Immunohistochemistry
After transcandial perfusion with 4% ice-cold paraformaldehyde, brains were post-fixed overnight in paraformaldehyde and sectioned coronally at 30 \( \mu \)m on dry ice. Before stainings using diaminobenzidine, sections were quenched for 20 minutes in 3% \( H_2O_2 \) and 10% methanol. Before BrdU-stainings, sections were incubated in HCl (1 mol/L) at 65°C for 10 minutes and at room temperature for 20 minutes. After preincubation with appropriate normal sera, sections were incubated overnight at 4°C with either of the following primary antibodies: rat anti-BrdU (1:100; Harlan Sera-Laboratory, Loughborough, UK), goat anti-doublecortin (Dcx; 1:400; Santa Cruz Biotecnology, Santa Cruz, Calif), mouse anti-NeuN (1:100; Chemicon), mouse anti-endothelial cell antigen (RECA; 1:400; AbD Serotec, Raleigh, NC), rabbit anti-laminin (1:100; Sigma; St Louis, Mo), or mouse anti-Ki67 (1:50; Novocastra Laboratories, Newcastle-on-Tyne, UK) in appropriate normal sera. Stainings were visualized by incubation for 2 hours with Cy3-conjugated (1:200; Jackson Immunoresearch, West Grove, Pa), Cy5-conjugated (1:200; Jackson), or biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, Calif), followed by Alexa 488-conjugated streptavidin (1:200; Molecular Probes, Eugene, Ore) for 2 hours for double stainings, or avidin-biotin-peroxidase complex for 1 hour followed by treatment with diaminobenzidine (0.5 mg/mL) and hydrogen peroxide for single stainings. For the latter, sections were dehydrated, mounted on glass slides, and cover-slipped.

For detection of hypoxia, sections were incubated with a primary monoclonal mouse antibody against Hypoxyprobe-1 (1:200; Chemicon) overnight at 4°C, followed by diaminobenzidine visualization and cresyl violet counter stain.

For Fluoro-Jade staining, sections were pretreated with 0.06% permanganate for 15 minutes, rinsed with distilled \( H_2O \), and immersed in Fluoro-Jade solution for 30 minutes (0.001% Fluoro-Jade in distilled \( H_2O \) with 0.1% acetic acid).

Morphometric Analysis

Estimation of Vessel Density and Length
Density of microvessels in SVZ and adjacent striatum was estimated with computer-generated isotropic virtual planes using computer-assisted stereology (version 2.3.5.1; Visiopharm). Taking into consideration the number of intersections between isotropic virtual planes and vessels, number of sampling box corners hitting the reference space, and total area of isotropic planes in each box, the computer-assisted stereology software calculates the vessel global spatial density.

By multiplying the global spatial density with the total volume of the reference space, measured with Cavalieri’s principle, an unbiased stereological assessment of global vessel length, \( L \), is yielded. Using these methods, vessel density and length were estimated in SVZ and in a 500-\( \mu \)m-wide column adjacent to SVZ (zone 1). This column was further divided in the dorso-ventral direction into 3 equally sized zones (zone 1a, zone 1b, and zone 1c).

The analysis was performed using a Nikon Eclipse 80i microscope with a motorized specimen stage controlling movements in the x-y axis. Sections were immunostained for RECA, counterstained with cresyl violet, and vessels analyzed at 40X magnification. A 3-dimensional sampling box (counting frame area 3329 \( \mu \)m\(^2\); sampling box height 13 \( \mu \)m; guard area 2 \( \mu \)m at the top of the section) was focused through the section. Within this box, virtual planes were generated with a fixed plane separation of 25 \( \mu \)m. In each animal and region analyzed, \( \sim \)150 to 200 blood vessel intersections with the virtual planes were counted in 4 evenly distributed sections.

Estimation of Angiogenesis
Sections were stained with antibodies against BrdU, RECA, and laminin. BrdU/RECA\(^+\) cells in SVZ and zone 1 were first counted in an epifluorescence microscope (Olympus BX-61), and double-labeled candidates were then analyzed for BrdU/RECA/laminin triple-labeling in a laser scanning confocal microscope (Leica).
Analysis of Neuroblast–Vessel Relationship
Number of Dcx+/H11001 neuroblasts was quantified in zones 1a, 1b, and 1c in 3 sections double-stained with antibodies against Dcx and RECA. Approximately 100 Dcx+/H11001 cells in each animal, randomly sampled in zone 1a, were then analyzed by generating 3-dimensional z-stacks in the confocal microscope. Within these z-stacks, the distance from each Dcx+/H11001 cell to nearest vessel was measured.

Statistical Analysis
All values are means±SEM. One-way ANOVA with Fischer post hoc test was used to assess differences between groups, and paired t test within the same animals. Differences were considered significant at P<0.05.

Results
Subventricular Zone Is Transiently Hypoxic After MCAO
We first assessed the distribution of hypoxia using Hypoxyprobe-1 staining. Hypoxyprobe-1 forms irreversible adducts with intracellular and extracellular proteins under hypoxic conditions (Po2 <10 mm Hg).24 These adducts can be visualized by immunohistochemistry. After 2 hours of MCAO, with Hypoxyprobe-1 injected during the occlusion and rats euthanized immediately thereafter, all animals showed stained cells and extracellular matrix ipsilaterally in the cerebral cortex, striatum, and SVZ (Figure 1a). Caudally, Hypoxyprobe-1 staining was detected in most of the ipsilateral hemisphere (Figure 1a). Staining was absent contralaterally and in sham-operated animals.

The distribution of Hypoxyprobe-1 staining in ipsilateral SVZ directly after 2 hours of MCAO was similar in all animals. Staining was absent rostrally but, moving caudally, became visible in lateral cell layers (Figure 1c). Typically, Hypoxyprobe-1 staining was uneven and less intense in the dorsal SVZ (Figure 1b) than in the more ventral parts (Figure 1c and 1d). More caudally, labeling extended from the middle SVZ to the ventral tip, with all cell layers being stained (Figure 1d).

Figure 1. Stroke induces transient hypoxia in SVZ. Distribution of Hypoxyprobe-1 staining (a) at 2 coronal levels from an animal perfused directly after 2 hours of MCAO. Photographs (b through d) of different parts of ipsilateral SVZ directly after 2 hours of MCAO. Sections were stained for Hypoxyprobe-1 (brown) and counterstained with cresyl-violet (blue). Note that Hypoxyprobe-1 labeling is more extensive in ventral SVZ. Photographs of Hypoxyprobe-1 (e), Fluoro-Jade (f), and NeuN (g) staining in dorso-lateral striatum 4 days after 2 hours of MCAO in sections from the same animal. Note that the striatal area showing neuronal degeneration (labeled with Fluoro-Jade and without NeuN staining) corresponds to the hypoxic area. Scalebar is 50 μm in b through d, and 500 μm in e through g. LV indicates lateral ventricle.

To determine whether the distribution of hypoxic areas correlated with those showing neuronal loss, we injected Hypoxyprobe-1 during 2 hours of MCAO or sham surgery and perfused the animals 4 days later. This time point was chosen for the stroke-induced neuronal degeneration to be virtually complete.25 All MCAO animals showed Hypoxyprobe-1 staining in the ipsilateral SVZ, striatum, and cerebral cortex (Figure 1e), whereas we observed no staining contralaterally or in sham-operated animals. Also, the dorsal SVZ was stained, indicating that the entire SVZ had been hypoxic as a result of the insult.

We then explored whether the Hypoxyprobe-1–labeled areas exhibited ischemic damage. Fluoro-Jade staining (degenerating neurons) and loss of NeuN-positive cells (mature neurons) were found in striatal and cortical areas labeled with Hypoxyprobe-1 (Figure 1e through 1g). Fluoro-Jade labeling and loss of NeuN-stained or cresyl violet-stained cells never extended into SVZ.

Neurogenesis continues for several months after stroke,6 and hypoxia can stimulate proliferation and differentiation of neural stem/progenitor cells.19 We hypothesized that SVZ hypoxia may extend beyond the acute phase. Hypoxyprobe-1 was injected 6 weeks after 2 hours of MCAO or sham surgery and animals perfused 2 hours thereafter. We found no Hypoxyprobe-1 labeling in the ipsilateral SVZ.

Subventricular Zone Exhibits Long-Term Increase of Volume and Transient Decrease of Vessel Density After Stroke
We have previously shown expansion of the ipsilateral SVZ at 2 and 6 weeks after 2 hours of MCAO. Here we found that the increased SVZ volume is maintained at the same level 16 weeks after the insult (Figure 2a). We hypothesized that this expansion may have been caused by increased vascularization. However, in comparison to sham-operated animals, vessel density in the SVZ of rats subjected to 2 hours of MCAO was decreased at 2 and 6 weeks but not different at 16 weeks.
weeks after the insult (Figure 2a). The total vessel length was higher than sham at 16 weeks (Figure 2a).

**Vessel Density Is Increased Long-Term in Striatum Adjacent to Subventricular Zone After Stroke**

We wanted to determine whether 2 hours of MCAO gave rise to changes in vascularization in striatum close to SVZ. The area of striatum encompassing the first 500 μm outside the SVZ was delineated zone 1 (Figure 2b). This area, which consists mostly of intact striatal tissue located between the SVZ and the ischemic core, comprises the majority of the neuroblasts generated after stroke.6 During 2 hours of MCAO, the ventro-caudal part of zone 1 was hypoxic, as indicated by Hypoxyprobe-1 staining (Figure 1d), and 4 days later exhibited neuronal degeneration. The dorso-rostral part of zone 1 was neither hypoxic nor damaged.

When the entire zone 1 was analyzed, no differences compared with sham-operated animals in vessel density or length were detected after MCAO. We then subdivided zone 1 in the dorso-ventral direction into 3 equally sized subzones (termed zone 1a, 1b, and 1c, respectively; Figure 2b), and found increased vessel density and length in zone 1a at both 6 and 16 weeks after 2 hours of MCAO (Figure 2c). Vessel density and length were higher in zone 1a than in the most ventral part, zone 1c, at all time points. Zone 1b did not exhibit any changes, nor did we observe any differences in vascularization between zone 1a, 1b, or 1c in sham-operated animals. Because of the variability in the extent of the ischemic lesion, analysis of vessel density in the striatal area between 750 and 1250 μm outside the SVZ was inconclusive.

**Angiogenesis Occurs in Striatum and Subventricular Zone Short-Term After Stroke**

To determine whether the ischemic insult and the associated hypoxia triggered angiogenesis, we counted proliferating endothelial cells in the ipsilateral SVZ and in zones 1a, 1b, and 1c of the adjacent striatum. Proliferating cells were labeled by BrdU injections during weeks 1 and 2 or weeks 7 and 8 after 2 hours of MCAO, and animals were euthanized

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**Figure 2.** Stroke induces transient decrease of vessel density in SVZ and long-term increase of SVZ volume and vessel density in adjacent striatum. SVZ (a) volume, vessel density, and length in sham animals and at 2, 6, and 16 weeks after 2 hours of MCAO. Schematic drawing (b) of zone 1 and its subzones. Vessel density and length (c) in zones 1a, 1b, and 1c in sham animals and at 2, 6, and 16 weeks after 2 hours of MCAO. Means±SEM, n=4 in each group. *P<0.05 compared with sham, one-way ANOVA with Fisher post-hoc test. †P<0.05 compared with corresponding zone 1a, paired t test. AC indicates anterior commissure; CC, corpus callosum.
directly thereafter. Endothelial cells were identified using antibodies against RECA, and BrdU+/RECA+ cells were counted in an epifluorescence microscope. To confirm that they were endothelial cells, BrdU+/RECA+ candidates were analyzed for triple-labeling also against laminin using confocal microscopy (Figure 3a through 3d). In the SVZ, there was a minor increase in the number of BrdU+ endothelial cells at 2 weeks but not at 8 weeks after the ischemic insult (Figure 3e). These findings argue against late endothelial cell proliferation as the mechanism underlying the increased vessel length at 16 weeks. Hypothetically, vessel length had increased through so-called intussusceptive angiogenesis, ie, the splitting of 1 vessel into 2 without endothelial cell proliferation.26 Endothelial cell proliferation in zone 1a was increased at 2 weeks after stroke (Figure 3e). Angiogenesis was also stimulated in zone 1b, but proliferating endothelial cells were fewer than in zone 1a, and angiogenesis was unchanged in zone 1c. In all zones, BrdU+ endothelial cells were found only in large, transversely cut arterioles or venules. At 8 weeks after 2 hours of MCAO, endothelial cell proliferation in zones 1a, 1b, and 1c was not different from that in sham-operated controls (Figure 3e).

We found that most of the stroke-induced angiogenesis occurred early after the insult. In animals injected with BrdU during days 3 and 4, 7 and 8, or 11 and 12 after 2 hours of MCAO and perfused 48 hours later, more BrdU+/RECA+/laminin+ cells were detected at 6 days after stroke, compared with at 10 and 14 days in both SVZ and zone 1a (Figure 3f). However, the angiogenic response was minor and transient, as indicated also by our findings using the cell cycle marker Ki67. We observed no Ki67+/RECA+ cells in the either zone 1a or SVZ of rats perfused at 6, 10, or 14 days after 2 hours of MCAO.
Neuroblasts Formed After Stroke are Associated With Blood Vessels

We wanted to determine the relation between neuroblasts migrating toward the damage and the vasculature in the striatal area adjacent to the SVZ. Consistent with our previous findings, we observed high numbers of Dcx neuroblasts in zone 1 at 2, 6, and 16 weeks after 2 hours of MCAO (Figure 4a). There was a clear gradient in the dorso-ventral direction within zone 1, with the majority of neuroblasts being distributed within zone 1a at all time points. Thus, the neuroblasts preferentially migrated in the striatal area exhibiting low-grade angiogenesis and increased vascular density (Figure 2c).

We measured the distance from each of 100 randomly sampled Dcx neuroblasts in zone 1 at 2, 6, and 16 weeks after 2 hours of MCAO (Figure 4a). There was a clear gradient in the dorso-ventral direction within zone 1, with the majority of neuroblasts being distributed within zone 1a at all time points. Thus, the neuroblasts preferentially migrated in the striatal area exhibiting low-grade angiogenesis and increased vascular density (Figure 2c).

Discussion

The ischemic insult also induces early angiogenesis and long-lasting increase of vessel density in dorso-medial striatum adjacent to SVZ. The majority of the stroke-generated neuroblasts migrate through this striatal area toward the ischemic damage and are closely associated with blood vessels.

Similar to what was recently described after 1 hour of MCAO in rats,24 we detected intense Hypoxyprobe-1 immunostaining in the parietal cortex and striatum after 2 hours of MCAO. In addition, we found that the SVZ expressed Hypoxyprobe-1 immunoreactivity both when animals were analyzed directly and 4 days after the insult. Our data indicate that hypoxia in SVZ induced by 2 hours of MCAO was transient since no Hypoxyprobe-1 immunoreactivity was found when the probe was injected 6 weeks after the insult. Interestingly, overall SVZ cell proliferation is increased 4 days to 2 weeks after MCAO 1–3, but has returned to baseline at 6 weeks. Our finding here raises the possibility that the SVZ hypoxia caused by MCAO stimulated cell proliferation in the early postischemic phase. Consistent with this interpretation, intermittent hypoxia in adult rats 19 and hypoxia/ischemia in perinatal rats 29 and neonatal mice 29 enhance proliferation of neural stem/progenitor cells in the SVZ.

Hypoxia is an important trigger also of angiogenesis.12 In the subgranular zone, there is a close association between
angiogenesis and neurogenesis. Approximately 37% of BrDU+ cells in this area in the intact brain were endothelial cells, indicating that the formation of new neurons occurs within an angiogenic niche. Our findings indicate that the situation in the SVZ is markedly different. We detected increased number of BrDU/RECA/laminin triple-labeled cells, validated with confocal microscopy, in the SVZ early after 2 hours of MCAO. However, the proliferating endothelial cells were few and represented <1% of all proliferating cells within the SVZ. Our data are at variance with those of Gotts and Chesselet, who reported that BrDU/RECA double-labeled cells were common in the ipsilateral SVZ after a cortical lesion. The reason for this discrepancy, apart from different injury models, is unclear. In agreement with Gotts and Chesselet, we found that BrDU/RECA/laminin triple-labeled cells were rare in sham-operated animals. Taken together, our data show only minor angiogenesis in the SVZ during the early phase after stroke, and provide little experimental support for the idea of a coregulation of neurogenesis and angiogenesis in the SVZ.

We found a close association between the neuroblasts and the striatal vasculature during long-term neurogenesis after stroke. First, at all time points, the majority of neuroblasts migrated toward the damage through the striatal area, which, compared with other areas adjacent to SVZ, exhibited long-lasting increase of vessel density and, during the first 2 weeks after the insult, endothelial cell proliferation. Second, the proximity of the neuroblasts to vessels was very similar at 2, 6, and 16 weeks, indicating that they migrate in the same way throughout long-term neurogenesis. In agreement with our findings, at 18 days after 30 minutes of MCAO in mice, chains of neuroblasts were wound around endothelial cells. In another model, at 7 days after cortical stroke in mice, neuroblasts were located in large numbers in physical proximity to endothelial cells in the peri-infarct cortex, where active vascular remodeling occurred. Our findings suggest that blood vessels may be important for the survival, migration, and differentiation of the closely located neuroblasts during long-term neurogenesis by endothelial release of factors such as brain-derived neurotrophic factor and stromal cell-derived factor 1. Consistent with this idea, the migration of neuroblasts that express the stromal cell-derived factor 1 receptor CXCR4, was inhibited by blocking stromal cell-derived factor 1/CXCR4 signaling at 4 to 6 weeks after 2 hours of MCAO. However, the widely different number of neuroblasts in areas with rather similar vascular density (comparing, eg, zones 1a, 1b, and 1c) indicates that migration is dependent also on nonvascular factors.

Our data support the notion that the vasculature plays an important role for striatal neurogenesis after stroke. Several studies have identified factors, which, when administered in the early postischemic phase, stimulate both angiogenesis and neurogenesis and lead to improved functional recovery after stroke including, eg, vascular endothelial growth factor and erythropoietin. Optimizing vascularization may be an important strategy to promote neurogenesis and repair in the stroke-damaged brain.

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Disclosures
None.

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