Filamentous Middle Cerebral Artery Occlusion Causes Ischemic Damage to the Retina in Mice

Ernest C. Steele, Jr, PhD; Qingmin Guo, PhD; Shobu Namura, MD, PhD

Background and Purpose—Filamentous middle cerebral artery occlusion (fMCAO) is the most frequently used focal cerebral ischemia model in rodents. The proximity of the ophthalmic artery to the middle cerebral artery suggests that fMCAO induces retinal ischemia. We therefore tested whether fMCAO induces ischemia/reperfusion damage in retina in mice.

Methods—SV129EV mice were subjected to transient (30 or 60 minutes) fMCAO followed by reperfusion under isoflurane anesthesia. Retinal perfusion was evaluated by intravenous injection of fluorescent microspheres combined with fluorescent microscopy using flat-mounted retinas. The fluorescent density of ipsilateral retina relative to contralateral retina was determined in each animal. Retinal injury was assessed by cresyl violet staining and in situ TUNEL.

Results—Microsphere analysis demonstrated perfusion defect in the ipsilateral retina after 60 minutes fMCAO and effective restoration after reperfusion. Thirty minutes fMCAO did not produce evident histological changes, even after 2 days of reperfusion. Sixty minutes fMCAO followed by 2 hours reperfusion resulted in extensive cell damage in the inner nuclear layer (30%) and ganglion cell layer (>50%) layers. TUNEL demonstrated very few positive cells, suggesting that damaged cells were mainly undergoing nonapoptotic cell death.

Conclusions—Sixty minutes fMCAO produces retinal injury in SV129EV mice. Potential visual dysfunction should be considered when a particular occlusion period is selected for studying neurological outcomes after fMCAO. Because visual disturbance is often associated with thrombotic/embolic stroke in humans, fMCAO represents an appropriate model for future studies aimed at understanding and ameliorating the changes that lead to retinal damage in these patients. (Stroke. 2008;39:2099-2104.)

Key Words: amaurosis fugax • focal ischemia • mice • middle cerebral artery occlusion • retina

Filamentous middle cerebral artery occlusion (fMCAO) in rodents is one of the most widely used experimental paradigms to induce focal cerebral ischemia.1 This model occludes arterial blood flow intraluminally and allows reperfusion by removing the inserted filament. In studies using focal cerebral ischemia, postural or movement laterality in the animals is used to assess neurological damage2 in addition to histological analyses. The corner test and adhesive removal test are sensitive to sensorimotor deficits and postural asymmetries.3 For the assessments of cognitive deficits after cerebral ischemia, the Morris water maze task4 and radial arm maze task5 have been used in rats and mice.6–9 Because these tasks require visual cues, visual function may impact the behavioral outcomes. A strong correlation between retinal degeneration and water maze performance was demonstrated in aged Sprague-Dawley rats.10 Therefore, it is important to investigate whether fMCAO that is used to study behavior outcomes after stroke causes retinal damage.

The ophthalmic artery that mainly supplies the inner retina originates from the internal carotid artery proximal to the origin of the middle cerebral artery (Figure 1A).11 We, therefore, expect that fMCAO simultaneously obstructs blood flow in the ipsilateral retina. Indeed, 2 previously published works using rats demonstrated that fMCAO induced retinal ischemia.12,13 Block et al12 demonstrated the first evidence of retinal ischemia by fMCAO. In their study, male Sprague-Dawley rats were subjected to 3 hours fMCAO and electroretinogram was recorded.12 During fMCAO, the amplitude of the a- and b-wave was markedly suppressed.12 The a-wave is associated with rod photoreceptor activity14 and b-wave reflects the combined activity of depolarizing bipolar cells and potassium currents of Müller cells.15 After reperfusion, the a-wave recovered completely but the amplitude of the b-wave recovered to approximately 50% of the pre-fMCAO level and remained at this level up to 2 days after reperfusion.12 The changes in electroretinogram were accompanied by increased glial fibrillary acidic protein immunoreactivity in Müller cells 3 days after reperfusion. However, no histological evidence for neuronal damage to any of the retinas after fMCAO was detected by cresyl violet staining. Kaja et

References

Temperature was set to 32°C. fMCAO was induced on the left side for 1 hour after discontinuing anesthesia, animals were kept in a chamber in which ambient thermostat-controlled heating pad. For 10 minutes after reperfusion using a flexible probe flowmeter (FLO-C1; Omegawave, Tokyo, Japan) throughout fMCAO and for 10 minutes after reperfusion. Regional cerebral blood flow was monitored using a laser-Doppler flowmeter (FLO-C1; Omegawave, Tokyo, Japan) throughout the procedures and for 10 minutes after reperfusion. In separate animals, 200 μL of FluoSpheres red fluorescent (580/605) polystyrene microspheres (Invitrogen) were injected into the left femoral circumflex artery, and left for 30 or 60 minutes (Figure 1A). For reperfusion, the filament was withdrawn and the CCA was reopened; however, the ECA remained permanently ligated. Animals underwent one of the following experimental paradigms: 30 minutes fMCAO with 2 hours (n=5), 24 hours (n=5), or 48 hours reperfusion (n=4); or 60 minutes fMCAO with 2 hours (n=4) or 24 hours reperfusion (n=4). Food and water was provided ad libitum. All procedures performed were reviewed and approved by the Morehouse School of Medicine Animal Care and Use Committee.

Assessment of Retinal Blood Flow
In separate animals, 200 μL of FluoSpheres red fluorescent (580/605) polystyrene microspheres (Invitrogen) were injected into the left femoral vein over a period of 1 minute. On initiation of injection, the left femoral circumflex artery was cut to equilibrate vascular pressure. Our preliminary studies demonstrated that the microsphere injection and phlebotomy procedures did not affect regional cerebral blood flow measured at the parietal area (data not shown). Immediately after injection, animals were euthanized and eyes were enucleated. The corneas of eyes were immediately pierced with a No. 11 surgical blade before immersion fixation in phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde for 30 minutes at room temperature. The corneas and lenses were then dissected from the fixed globes and the intact retinas were isolated from other ocular tissues. Retinas were mounted flat, vitreal face up, onto glass slides with glycerol and coverslipped for viewing with an epifluorescence microscope. Images of each entire retina was acquired using a 1.25× objective lens under a constant exposure setting with a Zeiss AxioCam HRc CCD camera. The mean fluorescent density of each entire retina was determined using the AxioVision measurement tools. The mean fluorescent density of the entire contralateral control retina for each animal was considered 100%, and the fluorescent density of the ipsilateral retina was expressed as a percentage of the control. Animals underwent one of the following treatments before injection: sham operation exposing the CCA, ECA, and internal carotid artery (n=3); 60 minutes permanent fMCAO.
### Table 1. Regional Cerebral Blood Flow

<table>
<thead>
<tr>
<th>Reperfusion Period</th>
<th>Basal CCA Ligation</th>
<th>During fMCAO</th>
<th>After Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes occlusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hours (n=5)</td>
<td>70.7±13.4</td>
<td>57.0±9.4</td>
<td>86.9±8.7</td>
</tr>
<tr>
<td>1 day (n=5)</td>
<td>87.0±9.4</td>
<td>15.6±3.3</td>
<td>93.0±8.7</td>
</tr>
<tr>
<td>2 days (n=4)</td>
<td>70.7±23.2</td>
<td>13.2±2.5</td>
<td>95.1±4.2</td>
</tr>
<tr>
<td>60 minutes occlusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hours (n=4)</td>
<td>77.8±16.2</td>
<td>14.0±5.2</td>
<td>81.8±17.1</td>
</tr>
<tr>
<td>1 day (n=4)</td>
<td>66.1±23.7</td>
<td>12.3±0.5</td>
<td>76.0±12.8</td>
</tr>
</tbody>
</table>

Regional cerebral blood flow was continuously measured through parietal skull by laser-Doppler flowmetry. Value before neck surgery was considered basal and average values during CCA ligation and fMCAO and after reperfusion were calculated as percent basal. Values are mean±SD.

### Nissl Staining

For histological analysis, animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and transcardially perfused with 10 mL of PBS containing 4% paraformaldehyde. Brains and eyecups were removed and further fixed as described previously but with lower eyelids left attached to retain superior-inferior orientation. Eyecups were cryoprotected in PBS containing 30% sucrose overnight at 4°C. Frozen blocks were prepared with Neg50 freezing medium (Richard Allen Scientific) and frozen in liquid nitrogen. A cryostat was used to serially cut transverse sections (12 μm), which were subsequently mounted onto glass slides and stained in 0.5% cresyl violet. Images were acquired using the Zeiss microscope system described previously. To quantitate the extent of retinal damage, pyknotic nuclei and total cellular nuclei were counted at 400× magnification by 2 independent observers. Two sections within 200 μm of the optic disc were randomly selected from each retina. In each section, 2 nonoverlapping views (250×250 μm) were randomly selected within both central (500 μm from the center bidirectionally to nasal and temporal) and peripheral (500 μm from the edges) areas from each section. Coronal brain sections (40 μm) were also made and stained with cresyl violet.

### TUNEL

TUNEL was performed as previously described. Briefly, sections were treated with an ascending ethanol series, chloroform (2 minutes), and rehydrated with a descending ethanol followed by PBS. The sections were incubated with terminal deoxynucleotidyl transferase buffer (30 mmol/L Trizma base, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) containing 2.5 μmol/L biotin-dUTP (Roche) and 500 U/mL terminal deoxynucleotidyl transferase (Roche) at 37°C for 70 minutes. The reaction was stopped with termination buffer (300 mmol/L NaCl, 30 mmol/L sodium citrate) for 15 minutes at room temperature. After blocking with PBS containing normal goat serum (10%), the sections were incubated with 4 μg/mL streptavidin-conjugated Alexa Fluor 488 (Invitrogen) and 1 μg/mL propidium iodide (Invitrogen) in PBS for 25 minutes at room temperature. Fluorescent images were acquired using a laser scanning confocal microscope system (Olympus).

### Statistical Analysis

Data are presented as mean±SD. Statistical analyses were made by one-way analysis of variance followed by Scheffe (retinal perfusion), 2-way repeated measures analysis of variance (regional cerebral blood flow), or unpaired Student t test (cell damage) using SPSS 15 (SPSS Inc). P<0.05 was considered statistically significant.

### Table 2. Retinal Perfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Percent Contralateral Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>3</td>
<td>118.5±26.4</td>
</tr>
<tr>
<td>60 minutes fMCAO</td>
<td>3</td>
<td>6.2±7.1</td>
</tr>
<tr>
<td>30 minutes fMCAO/30 minutes reperfusion</td>
<td>3</td>
<td>61.1±5.6†‡</td>
</tr>
<tr>
<td>ECA occlusion only</td>
<td>3</td>
<td>64.4±22.3†‡</td>
</tr>
</tbody>
</table>

Retinal perfusion was measured by systemic venous injection of fluorescent microspheres with an average diameter of 1 μm into the femoral vein and allowed sufficient time for the microspheres to distribute into the arterial bloodstream, dispersing throughout all tissues.

After sham operation, injected fluorescent microspheres were distributed throughout the vasculature of both left and right retina (Table 2). After 60 minutes of fMCAO, however, no fluorescent microspheres were observed in the ischemic retina (Figure 1B–C; Table 2). When fluorescent microspheres were injected at 30 minutes after reperfusion after 30 minutes fMCAO, we observed a reduction in the amount of fluorescent microspheres in the ischemic retina compared with the contralateral retina (Table 2). Because ocular structures are also supplied with ECA, some of the incomplete reperfusion might be attributable to the permanent ligation of the ECA in the current surgical protocol. We measured retinal perfusion in animals that received only permanent ligation of the ECA and observed a similar reduction as that after reperfusion (Table 2).

### Results

**Retinal Perfusion Reduction by Filamentous Middle Cerebral Artery Occlusion**

Laser-Doppler flowmetry allowed continuous and real-time assessment of regional cerebral perfusion status to confirm the efficiency of fMCAO and reperfusion (Table 1). To assess retinal perfusion status, we injected fluorescent microspheres with an average diameter of 1 μm into the femoral vein and allowed sufficient time for the microspheres to distribute into the arterial bloodstream, dispersing throughout all tissues.

After sham operation, injected fluorescent microspheres were distributed throughout the vasculature of both left and right retina (Table 2). After 60 minutes of fMCAO, however, no fluorescent microspheres were observed in the ischemic retina (Figure 1B–C; Table 2). When fluorescent microspheres were injected at 30 minutes after reperfusion after 30 minutes fMCAO, we observed a reduction in the amount of fluorescent microspheres in the ischemic retina compared with the contralateral retina (Table 2). Because ocular structures are also supplied with ECA, some of the incomplete reperfusion might be attributable to the permanent ligation of the ECA in the current surgical protocol. We measured retinal perfusion in animals that received only permanent ligation of the ECA and observed a similar reduction as that after reperfusion (Table 2).

### Retinal Damage by Filamentous Middle Cerebral Artery Occlusion

To assess histological damage, cells exhibiting a pyknotic nucleus were considered damaged. Thirty minutes fMCAO resulted in no obvious damage within any cell layer of the ischemic retina up to 2 days after reperfusion (data not shown). In contrast, after 60 minutes of fMCAO, many pyknotic cells were detected as early as at 2 hours after reperfusion. These pyknotic cells were within the inner nuclear layer (INL) and GCL (Figure 2A). In addition, tissue vacuolation was consistently observed in INL and GCL of ischemic retinas. This might be due to cell shrinkage and vessel dilatation. However, the contralateral retinas did not contain such pyknotic cells or vacuolation (Figure 2B). Disorganization of the outer nuclear layer was observed in some sections regardless of ischemic or nonischemic retina, which was likely due to the artifacts associated with the...
freezing/sectioning procedures (Figure 2A). It should be noted that minor cellular changes in outer nuclear layer might be overlooked because the small size, very little perinuclear cytoplasm, and densely stained nucleus of the normal photoreceptors did not allow conventional detection of morphological changes in these cells in outer nuclear layer. No loss of cells within the ischemic retina was observed as late as 1 day of reperfusion after 60 minutes fMCAO (Figure 2C). We did not observe a significant progression in cell damage from 2 hours to 1 day after reperfusion in either INL or GCL. Interestingly, INL demonstrated a greater vulnerability of peripheral retina to ischemia compared with central retina (67% ± 13% versus 37% ± 5% at 2 hours [P < 0.01] and 80% ± 11% versus 47% ± 18% at 1 day [P < 0.05] after reperfusion). However, such a topographical disparity in vulnerability was not observed in GCL.

Cresyl violet staining using brain sections demonstrated that all 4 animals after 1 day reperfusion after 60 minutes fMCAO exhibited extensive neuronal injury throughout the striatum (data not shown). Only one of them showed additional cell injury in the ipsilateral parietal cortex, lateral thalamus, and optic tract (data not shown). TUNEL-positive cells were detected mainly in the medial margin of the damaged area in the striatum (data not shown).

Filamentous Middle Cerebral Artery Occlusion-Induced Acute Retinal Damage Is Mediated by Nonapoptotic Mechanism

Because ischemic/reperfusion injury is often associated with apoptosis, we performed TUNEL to detect apoptosis in the retina after fMCAO/reperfusion. We rarely observed TUNEL-positive cells in the retina after 30 minutes fMCAO followed by 2 hours or 1 or 2 days of reperfusion (data not shown). Despite the high numbers of damaged cells in the ischemic retina after 60 minutes fMCAO and reperfusion for 2 hours and 1 day, very few TUNEL-positive cells were detected within INL and GCL at these 2 end points (Figure 3A–B). It thus appears that the ischemic damage in the retina after 60 minutes fMCAO with reperfusion occurs mainly by a rapid nonapoptotic mechanism.

Discussion

We demonstrated retinal injury after fMCAO in the SV129EV mouse. The injury was rapid and extensive in INL and GCL. Sixty minutes fMCAO/2 hours reperfusion was sufficient to induce injury detectable by Nissl staining in 30% to 50% of cells in these layers. However, TUNEL was rarely detectable in the ischemic retinas up to 1 day after reperfusion. In addition, no progression in cell injury from 2 hours to
1 day after reperfusion was evidenced. Therefore, at least during the acute phase after 60 minutes fMCAO, our findings suggest that nonapoptotic cell death is the major mode of retinal ischemic injury in the SV129 mouse. The current findings in mice contrast in both temporal and spatial aspects to the findings in the 2 previous studies that provided evidence of retinal ischemia by fMCAO in albino rats. Although functional evaluations of ischemic retina, including electroretinogram, need to be made in the mouse in the future, the retina of the SV129 mouse appears more susceptible to ischemia compared with that of the albino rat, at least evidenced by conventional histological methods. Nevertheless, our study demonstrated the first evidence of retinal ischemia by fMCAO in mice.

Because the Morris water maze task is guided by visual cues, visual deficits could confound the outcomes in this analysis. This possibility was suggested by previous studies using albino rats. Spencer et al demonstrated that aged Sprague-Dawley rats with more severe photoreceptor cell loss exhibited longer escape latencies. Davidson et al reported a similar relationship between retinal degeneration and performance outcome in Sprague-Dawley rats that underwent permanent bilateral CCA ligation. Yonemori et al investigated the water maze performance after chronic proximal permanent middle cerebral artery occlusion in Wistar rats. With this method, the ipsilateral eyes were damaged by the surgical procedure to expose the proximal portion of the middle cerebral artery. In this study, there was a clear tendency that sham-operated animals that underwent middle cerebral artery exposure without occlusion showed longer escape latencies compared with intact control animals. Thus, visual ability should be considered when the cognitive functions after cerebral ischemia are measured by the Morris water maze tasks.

We are aware of at least 4 published studies evaluating in mice post-fMCAO performance in the Morris water maze tasks. Two studies from one group demonstrated impairment compared with sham control in escape latency after 20 days after 60 minutes fMCAO in C57BL6 mice. In contrast, a work using Swiss mice did not detect such impairment compared with a sham group after 19 days after 60 minutes fMCAO. In these 3 studies, no information indicating visual function was described. Winters et al did not find effects of 30 minutes fMCAO in SV129 mice on the escape latency and path length in the routine hidden platform tasks performed at 7 weeks after reperfusion, suggesting that 30 minutes fMCAO is not likely to cause visual deficits that are detectable by the Morris water maze tasks. Consistent with this, our study did not demonstrate histological evidence of retinal injury up to 2 days after reperfusion after 30 minutes fMCAO in the same mouse strain.

In the mouse, over 97% of the total ganglion cell axons decussate at the optic chiasm, projecting to the contralateral lateral geniculate nucleus. Then, geniculo-cortical afferents project from lateral geniculate nucleus to the ipsilateral primary visual cortex. Thus, if fMCAO also impairs the ipsilateral optic tract, lateral geniculate nucleus, or visual cortex, vision on both sides would be affected. In our study, one of 4 animals that underwent 60 minutes fMCAO/1 day reperfusion demonstrated histological evidence of injury in the ipsilateral optic tract and lateral geniculate nucleus (data not shown). In addition, a previous study using autoradiography in SV129 mice demonstrated severe regional cerebral blood flow deficits in the ipsilateral optic tract, lateral geniculate nucleus, and occipital cortex at 4 hours after permanent fMCAO. In these animals, the visual ability relying on the contralateral eye might also be disturbed. Therefore, potential involvement of the ipsilateral optic pathway should be considered when a particular occlusion period is selected for evaluating vision-dependent tasks after fMCAO.
Although our study demonstrated the effect of fMCAO/reperfusion on retinal perfusion by using systemic injection of the fluorescent microspheres, the methodology does not allow continuous real-time monitoring in the same animals. Scanning laser ophthalmoscopy has been used for monitoring retinal vasculature in mice^25; however, this method does not measure dynamics of retinal perfusion. Laser speckle imaging^26 and MRF^27 are currently available for real-time and dynamic assessment of retinal perfusion in larger animals. However, the very small diameter of the pupil and limited spatial resolution currently prevent their application to mice. Future improvements in these technologies would make them applicable in mice, which allows more precise monitoring of the dynamics of retinal perfusion in mice.

As Kaja et al^13 indicated in their work, the presently described fMCAO/reperfusion model is more appropriate than other retinal ischemia models such as high intraocular pressure or optic nerve bundle ligation model to recapitulate the cellular and molecular changes in the retina after stroke. Acute thrombotic/embolic stroke and transient ischemic attack in humans are often associated with temporary diminishment (ie, amaurosis fugax) or even permanent loss of vision.28 Therefore, fMCAO is a more relevant model for studying these changes and testing the efficacy of therapeutic strategies to prevent or ameliorate these changes. Because vision loss resulting from retinal ischemia is often associated with stroke, it is important to demonstrate that a particular neuroprotective reagent or strategy effectively preserves vision by protecting the retina in addition to the brain. We also emphasize that the fMCAO model using transgenic mice will provide the unique opportunity to simultaneously study the role of specific gene products in ischemic damage within both the brain and retina, 2 similar but distinct neurovascular tissues.

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Disclosures

None.

References

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