Selective Cortical Neuronal Damage After Middle Cerebral Artery Occlusion in Rats

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We studied histopathologic changes in cerebral cortex of 20 rats after middle cerebral artery occlusion by using the Fink-Heimer suppressive silver impregnation method and conventional stains. At 6 hours after occlusion, Fink-Heimer-stained sections revealed abundant coarsely granular, intensely argyrophilic neurons in the ischemic cortex. These distinctive argyrophilic neurons could be clearly differentiated from neurons that suffered postmortem changes; argyrophilic neurons were present in all layers of the lateral parietal cortex but in only the superficial cortical layers II and III in the parasagittal area of the frontoparietal cortex and the temporo-occipital area. At 24 hours after occlusion as the ischemic region progressed to pannecrosis, argyrophilic neurons were still evident in peri-infarct regions, with more prominent neuritic silver deposits but no changes in number or spatial distribution. Over 2–7 days, the argyrophilic neurons gradually disappeared while many fine silver-impregnated degenerating terminals appeared in the peri-infarct regions. At 3–6 weeks after occlusion, no more argyrophilic neurons were seen in the cortex although degenerating axons were still present in the deep white matter. Our results indicate selective neuronal damage in the superficial cortical layers and massive axonal degeneration in the cerebrum surrounding infarcts. The neuronal damage does not appear to progress beyond 6 hours after middle cerebral artery occlusion. The Fink-Heimer method has many advantages over existing conventional stains for documenting selective neuronal damage in focal cerebral ischemia. (Stroke 1989;20:1516–1523)

In focal stroke, the border zone between an infarct and the surrounding brain is sometimes called the ischemic penumbra. Ischemic damage in the penumbra is believed to be reversible and has been a popular target of therapeutic intervention. Although the concept of an ischemic penumbra is widely cited and accepted, experimental evidence for it has come mainly from electrophysiological investigations. Relatively few studies of acute histologic change in the border zone have appeared in the literature. This is in part because histologic methods for detecting early neuronal damage have been capricious and prone to artifact.

Detection of early neuronal damage is a difficult and important problem in histopathologic studies of cerebral ischemia. Conventional stains of brain tissues can show neurons with abnormal appearances attributable to early ischemic damage; however, such changes are often difficult to distinguish from postmortem artifacts. Consistent fixation of tissues is essential for the interpretation of early neuronal injury. Uniform and reproducible perfusion fixation is difficult to carry out in brains that have suffered focal ischemia due to permanent arterial occlusion. Postmortem artifacts, such as the dark neurons of Cammermeyer, often appear in such brains and cannot be easily distinguished from evidence of early neuronal damage.

Suppressive silver stains, especially the Fink-Heimer method, have long been used to detect early axonal degeneration. Such methods have been reported to label the cell bodies, axons, and dendrites of degenerating neurons in some pathologic situations. Some investigators believe that suppressive silver stains can be used to distinguish degenerating neurons from dark neuron artifacts. The latter have nongranular silver deposits and relatively smooth contours, whereas degenerating neurons have a more granular pattern.

Materials and Methods

We studied 20 adult male Long-Evans hooded rats weighing 250–350 g. The rats were anesthetized with 40 mg/kg i.p. sodium pentobarbital. We occluded the right middle cerebral artery (MCA) using a modification of the method developed by Tamura et al and described previously. Briefly, a...
3-mm-diam. subtemporal craniotomy was made with a microdrill, avoiding the zygomatic joint. After opening the dura with a fine needle, we dissected the MCA free from pia-arachnoid and coagulated the artery 2 mm below the rhinal fissure by bipolar radiofrequency electrical current applied with forceps. The MCA was divided to ensure permanent occlusion.

At 6 (three rats), 24 (three rats), or 48 (two rats) hours, 3 (two rats), 5 (two rats), or 7 (three rats) days, 3 (three rats) or 6 (two rats) weeks after MCA occlusion (MCAo), the rats were again anesthetized with pentobarbital and perfused transcardially with 10% formalin in 0.01 M phosphate-buffered saline. The brains were removed and immersed in the same fixative solution overnight, then cut in the coronal plane to yield 1–2-mm-thick slices.

For Fink-Heimer staining, brain tissue containing ischemic lesions was immersed in 10% buffered formalin for ≥1 week. After postfixation, 40-μm-thick frozen coronal sections were serially cut and processed using the Fink-Heimer method. From the remaining tissue adjacent to those processed for Fink-Heimer staining, 40-μm-thick frozen sections or 10-μm-thick paraffin-embedded sections were made and were alternately stained using standard hematoxylin and eosin and/or cresyl violet and Luxol fast blue stains. In addition, we processed some frozen sections immunohistochemically for glial fibrillary acidic protein (GFAP) using a monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) and the avidin–biotin complex method. Brain areas were named according to the atlas of Paxinos and Watson.

After surgery, the rats were housed in boxes containing sterile hardwood chips and were fed ad libitum. Rats with MCAo usually showed evidence of focal ischemia for several hours after surgery, manifesting mild contralateral hemiparesis and a
tendency to circle toward the lesioned site; such symptoms usually did not persist for >24 hours. The rats were able to feed shortly after surgery and regained their preoperative weights within a week. No rat showed signs of distress or pain during the postoperative period.

Results

In the lateral parietal, insular, and piriform regions, where the infarct is typically seen after MCAo, abnormal-appearing neurons were present at 6 hours. On conventional hematoxylin and eosin-stained sections, these neurons were darkly stained and had mildly atrophied cell bodies and triangular, shrunken nuclei. Although these abnormal neurons were observed in all cortical layers, they were more concentrated in cortical layers II, III, and VI than in layers IV or V. These latter two layers did not appear damaged otherwise and had well-preserved tissue architecture at 6 hours after MCAo. In Fink-Heimer-stained sections, argyrophilic neurons, corresponding to abnormal darkly stained neurons in conventionally stained sections, were abundant. The cell bodies and dendritic processes of these argyrophilic neurons were intensely black with a granular texture, imparting a Golgi-like appearance (pseudo-Golgi) to the neurons (Figure 1). The silver-impregnated neurons stood out clearly against a golden-yellow background.

At 24 and 48 hours after MCAo, the infarct could be easily distinguished from surrounding regions on the basis of gross cell loss and the appearance of generally disrupted tissue. The infarcts had distinct borders and consistently involved the lateral pari-
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Although infarct borders were indistinct 6 hours after MCAo, on Fink-Heimer–stained sections argyrophilic neurons were found in the parasagittal frontoparietal cortex and the anterior temporoccipital area outside the zone that typically progresses to infarct. These neurons were distributed with a pattern different from that of the lateral parietal cortex at this time. Argyrophilic neurons were found primarily in the upper cortical layers (Figure 2). The cellular architecture of the cortex in these regions appeared relatively normal except for the selective neuronal damage. Fink-Heimer–stained sections demonstrated these degenerated neurons more clearly than conventionally stained sections (Figure 3). In particular, axodendritic abnormalities were clearly seen on Fink-Heimer–stained sections (Figure 3, top).

At 24 hours after MCAo, many argyrophilic neurons were present in the peri-infarct zones. Compared with sections from 6 hours after MCAo, the argyrophilic neurons were shrunken and had more prominent and extensive staining of neurites (Figure 4). These neurons were distributed in all layers of the cortex in regions adjacent to the infarct (Figure 5, left). At 1–2 mm from the infarct border, argyrophilic neurons were present in layers II, III, and VI. In more remote cortical areas, that is, 2–3 mm in the mediolateral and anteroposterior directions, argyrophilic neurons were found mostly in the superficial cortical layers (Figure 5, right).

At 48 hours after MCAo, silver-impregnated neurons had a similar spatial distribution as at 24 hours, but the neurons appeared more atrophic and fine silver grains representing terminal degeneration were evident on most sections (Figure 6). At 3, 5, and 7 days after MCAo, the number of argyrophilic neurons decreased and fine silver-impregnated degenerating terminals were prominent in all peri-infarct cortical regions. At 3 and 6 weeks after MCAo, degenerating axons were prominent in the deep white matter but no argyrophilic neurons were observed in the cortex. The number of fine silver grains was also markedly decreased in the cortex surrounding the infarct.

Table 1 summarizes the extent of selective neuronal damage suggested by the distribution of argyrophilic neurons in seven rats studied at 6, 24, and 48 hours after MCAo. Argyrophilic neurons were found in all areas of the MCA territory at 6 hours after MCAo, particularly in Par1 and Par2 and in the forelimb area of the parietal cortex. The extent of selective neuronal damage in the cortex at 6 hours did not differ from that at 24 and 48 hours, suggesting that neuronal damage did not progress in the cortex surrounding the infarct. In two rats, selective neuronal damage could not be estimated in Par1 because the infarct involved the entire area. In the two rats studied at 6 hours, infarct boundaries were not sufficiently distinct to allow assignment of cortical location.

Discussion

Our study confirms the usefulness of the Fink-Heimer method for detecting neuronal damage in
FIGURE 5. Photomicrographs comparing distribution of selective neuronal damage in peri-infarct cortical areas <1 mm and 1–2 mm from infarct border in rats at 24 hours after middle cerebral artery occlusion. Left: Degenerating neurons are distributed in all cortical layers in region close to infarct. Right: In cortex remote from infarct, argyrophilic neurons are limited to upper cortical layers. Fink-Heimer method. Bars indicate 150 μm.

rat cerebral cortex after focal ischemia. Mesulam had reported the characteristic appearance of degenerating neurons stained by the Fink-Heimer method in an experimental model of thiamine-deficient animals. Coarse silver grains were present in the cell bodies and extended into beaded dendrites and axons.

The Fink-Heimer method has several advantages over conventional histologic staining methods. During the acute stage of ischemia, damaged axons and dendrites can be discerned as well as cell bodies. During subacute stages, secondary axon and terminal degeneration are well documented by this method. Our results indicate massive degeneration of axon terminals in the cortex adjacent to an infarct, areas where conventional staining often shows no evidence of cell damage. We recently reported terminal degeneration in the brainstem reticular formation and trigeminal nuclei after MCAo. During chronic stages, for example, ≥7 days after MCAo, corticofugal axonal degeneration can be clearly seen in remote areas, particularly the brainstem and spinal cord. Finally, the distinctive appearance of argyrophilic neurons distinguishes degenerating cells from postmortem artifacts.

The dark neuron artifact is a central problem in neuropathologic studies of cerebral ischemia. Our experience suggests that the Fink-Heimer method is most useful in experiments with postoperative survival times of at least 6 hours. At shorter survival times (unpublished observations), we saw neurons with lighter and more dispersed silver deposits. While such cells may be degenerating and showing an early stage of argyrophilia, we cannot confidently rule out poor fixation and postmortem changes as the cause of this silver pattern in neurons. At >6 hours after MCAo, however, degenerating neurons show a strikingly different appearance, with coarse granular silver deposits. Figure 7 shows an example of a section that contains both types of neurons at 6
hours after MCAo. The degenerating neurons are quite distinctive.

Selective neuronal damage shows two characteristic distribution patterns in the peri-infarct area. In the cortex adjacent to an infarct, argyrophilic neurons were dispersed through entire cortical layers. In a study of monkeys after MCAo, DeGirolami et al. reported that transient occlusions (≤4 hours) produced selective neuronal necrosis in the cortex and basal ganglia, whereas permanent occlusion resulted in total tissue necrosis of the same areas. This finding suggests that selective neuronal damage can occur in association with transient ischemia. Partial ischemia in the areas immediately adjacent to an infarct may have caused the multilayer selective neuronal damage.

**TABLE 1. Extent of Selective Neuronal Damage Suggested by Distribution of Argyrophilic Neurons in Rats After Middle Cerebral Artery Occlusion**

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<tr>
<th>Rat no.</th>
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<th>FrM</th>
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FrM, frontal motor area; Par1, parietal 1 area; Par2, parietal 2 area; FL, forelimb area of sensorimotor cortex; HL, hindlimb area of sensorimotor cortex; Te, temporal cortex; Occ, occipital cortex; Grade 1, few neurons damaged in upper cortical layers; Grade 2, many neurons damaged in upper and/or deep cortical layers; Grade 3, neurons damaged in all cortical layers; +, selective neuronal damage could not be estimated because infarct involved entire region; ++, severe cortical infarct; +, partial involvement of infarct; -, no infarct; /, infarct boundaries could not be clearly defined.
On the other hand, in cortex >1 mm from the infarct border, argyrophilic neurons occurred primarily in the upper cortical layers. This pattern differs from the selective neuronal loss found in global forebrain ischemia or hypoxic brain injury; the lower cortical layers V and VI are most frequently involved in those models.18,19 Our finding of selective neuronal loss primarily in the upper cortical layers agrees with the observations of Nedergaard.4 Studies in the rat MCAo model suggest that blood flow is better preserved in the upper cortical layers after occlusion.20,21 Thus, events indirectly related to ischemia, but not ischemia per se, may be responsible for the degenerating neurons observed in remote peri-infarct regions.4

One possible cause of neuronal loss in the upper cortical layers surrounding an infarct is spreading depression.22 It has been reported to spread preferentially in the upper cortical layers23 and is believed to occur in the peri-infarct cortical regions after focal ischemia. Recurrent transient elevations of extracellular K+ activity have been observed in the cortex around an infarct.2,24 We recently observed that MCAo transiently abolished evoked potentials in the forelimb and hindlimb somatosensory cortex even though the infarct did not involve these areas.25 These findings suggest that MCAo causes widespread physiologic disruption of the cortex beyond the infarct site.

Repeated spreading depressions elicited by KCl application do not cause neuronal loss in normal rat brains.26 However, the cortical regions in which neuronal loss occurred are situated in the blood flow watershed zones between the MCA and the posterior and anterior cerebral arteries.27 Thus, these regions may have suffered transient ischemia and other blood flow abnormalities after MCAo. We would expect ischemia to produce neuronal loss preferentially in the deeper cortical layers or throughout cortical layers, as observed at the infarct edge. The combination of partial ischemic damage and spreading depression-like phenomena may account for the distribution of selective neuronal loss observed in the upper cortical layers 1–2 mm from the infarct edge.

We found no evidence of progressive neuronal damage in the peri-infarct cortical regions, at least not >6 hours after MCAo. Selective neuronal damage was present in the parasagittal frontoparietal cortex and the temporoparietal regions by 6 hours. The number of argyrophilic degenerating neurons did not appreciably increase in these peri-infarct areas between 6 and 24 hours even though the infarct progressed to pannecrosis in the interim. Later, the number of argyrophilic neurons decreased while secondary terminal degeneration increased. Nedergaard4 reported that neuronal damage at the infarct border is an acute nonprogressive phenomenon, and neuron counts indicated no delayed death. In earlier ionic studies, we found a sharp increase in tissue K+ loss and Ca2+ accumulation in the cortex between 4 and 6 hours after MCAo.28,29 The ischemic infarct boundaries, as well as the selective neuronal damage in peri-infarct zones, appear to be established by 6 hours after MCAo, and the therapeutic window, if it exists, probably occurs before this time.

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References


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