Cholinergic Deafferentation After Focal Cerebral Infarct in Rats

Kazuo Kataoka, MD; Toru Hayakawa, MD; Ryotaro Kuroda, MD; Takamichi Yuguchi, MD; and Kazuo Yamada, MD

Background and Purpose: For a better understanding of neuronal network disturbances after stroke, we investigated the changes in the cholinergic system after experimental focal infarct.

Methods: We quantitatively evaluated the highly sensitive acetylcholinesterase histochemistry and local glucose utilization 7 days after left middle cerebral artery occlusion in Wistar rats.

Results: In all rats with occlusion, the ipsilateral frontal cortex and the nucleus basalis Meynert developed no infarct, whereas the subcortical striatum did. In the frontal cortex on the occlusion side, the acetylcholinesterase-positive fiber density was significantly (p<0.05) reduced; a computer-assisted image-analyzing system quantified approximately 1.0 m/mm³ brain cortex acetylcholinesterase-positive fibers in the ipsilateral frontal cortex layers II–IV and approximately 9.7 m/mm³ brain cortex acetylcholinesterase-positive fibers in the contralateral frontal cortex layers II–IV. Local glucose utilization was also significantly (p<0.05) decreased in the ipsilateral frontal cortex compared to the contralateral side and sham-operated animals.

Conclusions: These results suggest that functional disturbances and disruption of the cholinergic pathway between the frontal cortex and the nucleus basalis Meynert occur after middle cerebral artery occlusion in rats. (Stroke 1991;22:1291–1296)

In the central nervous system, various neurotransmitter systems contribute to neuronal function. Of these, the cholinergic system between the neocortex and the nucleus basalis Meynert has been studied extensively, partly because of the possible relationship between Alzheimer's disease and pathology of the cholinergic system.1 The nucleus basalis Meynert is located near the globus pallidus in humans as well as in rats.2–4 In primates, the cholinergic pathway from the nucleus basalis Meynert to the neocortex runs in the ipsilateral external capsule and subcortical white matter.5 Similarly, fibers from cholinergic cells in the nucleus basalis Meynert fan out laterally through the striatum in rats. These fibers extend to and travel along the external capsule before turning into the cortex.6–8 Perforating arteries and branches of the middle cerebral artery (MCA) may supply this pathway in humans as well as in rats. Occlusion of the MCA may disrupt this pathway, resulting in unilateral cholinergic deafferentation in the neocortex.

In patients with subcortical infarct, the cholinergic deafferentation may be of importance because the neocortex survives, but the pathway is disrupted. Although the clinical implications of unilateral cholinergic deafferentation are not clearly understood, study of the cholinergic system after experimental MCA occlusion in rats contributes to a better understanding of neuronal network disturbances in stroke patients.

Materials and Methods

Eighteen mature female Wistar rats, each weighing approximately 300 g, were anesthetized with an intraperitoneal injection of ketamine hydrochloride (120–150 mg/kg). Lidocaine solution (0.5%) was applied topically to the surgical wounds. After enucleation of the left eye, the MCA was exposed via the transorbital approach and coagulated at the olfactory tract.9 In six sham-operated animals, the arachnoid surrounding the MCA was divided, but the artery was not coagulated.

We evaluated changes in the cholinergic pathway after MCA occlusion by highly sensitive acetylcholinesterase histochemistry described by Tago et al.10 Six rats were deeply anesthetized with sodium pentobarbital 7 days after MCA occlusion, and transcar-
FIGURE 1. Analysis of acetylcholinesterase histochemistry. Light microscopic image of the frontal cortex (Fr 1,3; layers II-IV) on the nonocclusion side was digitalized using an image-analyzing system. The original computerized image consisted of 256 gray tones (panel A). For further processing, the two-tone gray image of the neuronal fibers was extracted from the original 256-gray-tone image by binarization at an appropriate gray value threshold (panel B). After elimination of artifacts, the thinning process converted the two-tone gray image of fibers into a binary line image with 1-pixel width (panel C). Based on this final two-tone gray image, the length of fibers was calculated. The superimposed image shows a good relationship between the original 256-gray-tone image and the final two-tone gray image (panel D). Bar=10 μm.

Diace perfusion with 100 ml saline was performed, followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) solution. The brains then were taken out, and, after 1–2 days' storage in 4% paraformaldehyde in 0.1 M phosphate buffer, they were immersed overnight in a 30% sucrose solution for cryoprotection. Brain sections (20 μm) were cut with a cryostat. These sections were rinsed in 0.1 M maleate buffer (pH 6.0) and incubated for 30 minutes in a reagent solution containing 36 μM acetyltiocholine iodide, 5 μM potassium ferricyanide, 30 μM cupric sulfate, and 50 μM sodium citrate in 0.1 M maleate buffer. After rinsing in 0.05 M Tris buffer saline (pH 7.6), these sections were incubated for 5 minutes in a solution containing 0.04% 3,3-diaminobenzidine and 0.3% nickel ammonium sulfate in 0.05 M Tris buffer saline. Subsequently, H2O2 solution (total H2O2 concentration, 0.003%) was added to the solution. After the sections were incubated further for 10 minutes, they were washed and mounted. The acetylcholinesterase histochemistry described by Tago et al facilitates the identification of acetylcholinesterase-positive fibers in the cortex under appropriate magnification. It is possible to quantify the length of acetylcholinesterase-positive fibers by means of a computer-assisted image-analyzing system (Nexus-Qube, Nexus, Japan) (Figure 1). Half of the sections were counterstained by cresyl violet. The neuronal cells were counted, and areas of infarct were determined.

We evaluated neurofunctional changes after MCA occlusion by [14C]2-deoxyglucose autoradiography. The rats were fasted 1 day before [14C]2-deoxyglucose study. Seven days after operation, the rats were anesthetized with 100 mg/kg i.p. ketamine hydrochloride. A 1% lidocaine solution was applied topically to the surgical wound for additional anesthesia. Left femoral artery and vein catheters were placed, and the rats were restrained with a plaster cast on a board. When the animals became fully alert (approximately 6–8 hours after anesthesia), they received a bolus injection (100 μCi/kg) of [14C]2-deoxyglucose in the left femoral vein. Arterial blood pressure and arterial blood gas were measured just before the
isotope injection. Timed arterial blood samples were taken to determine plasma glucose and $^{14}C$ concentration. The rats were killed 45 minutes later with an intravenous overdose of sodium pentobarbital, and their brains were immediately removed and frozen. A cryostat was used to obtain 20-μm sections of the brain. These were placed on glass coverslips, dried on a hot plate (60°C), and exposed for 5–7 days to x-ray film (Kodak SB-5) together with calibrated $^{14}C$-embedded methacrylate standards. Local cerebral glucose utilization of the noninfarcted area was calculated using the equation of Sokoloff et al.12 In this deoxyglucose study, we used six rats for MCA occlusion and six rats as sham controls.

Statistical evaluation of the two groups was performed using the nonparametric Mann-Whitney U test. Interhemispheric differences were assessed for statistical significance by the Wilcoxon signed rank test. Values are given as mean±SD.

Results
In eight of 12 rats with MCA occlusion, the subcortical structure developed infarct, but the neocortex did not. In the other four rats, the neocortex and subcortical structure developed infarct of variable extension. In all 12 rats, the lateral part of the striatum and external capsule on the occlusion side consistently developed infarct, but the left globus pallidus and the left frontal cortex did not. We could not find histological differences in the magnocellular neurons of the nucleus basalis Meynert between the occlusion side and the nonocclusion side. Serial macroscopic coronal sections of acetylcholinesterase histochemistry showed gross reductions in acetylcholinesterase staining of the ipsilateral frontal cortex areas 1 and 3 (Fr 1,3), according to the comprehensive cortical parcellation described by Zilles11 (Figure 2).

Quantitative evaluation of acetylcholinesterase histochemistry showed approximately 9.7 m/mm² brain cortex acetylcholinesterase-positive fibers in the right frontal cortex (Fr 1,3) layers II–IV. Occlusion of the left MCA resulted in significant loss of acetylcholinesterase-positive fibers in the ipsilateral frontal cortex (Fr 1,3) (Figure 3, Table 1). The cell density in the frontal cortex on both sides was not significantly different (Table 1).

There were no significant differences in the physiological variables between the MCA occlusion and the sham-operated groups (Table 2). In the cortical structures, the left frontal cortex (Fr 1,3) showed a significant decrease in glucose utilization compared to the contralateral side and sham-operated rats (Table 3).

Discussion
There are only a few clinical reports concerning the pathology of the cholinergic system in relation to focal cerebral infarct. For example, Kato et al13 noted neurofibrillary tangle formation in the nucleus basalis Meynert ipsilateral to a massive, old cerebral infarct in the region of the unilateral MCA. Phillips et al14 reported that cerebrovascular lesions in the bilateral basal forebrain resulted in dementia. In humans, the nucleus basalis Meynert is located in the ventral part of the basal forebrain near the globus pallidus.23 If the cholinergic pathway from the nucleus basalis Meynert to the neocortex runs near the ipsilateral external capsule and subcortical white matter, then the subcortical cholinergic pathway may be affected by the subcortical infarct caused by MCA occlusion. It is reported that the cholinergic innervation of the neocortex plays a critical role in cognitive function in humans.1,15 At present, the clinical implications of
unilateral cholinergic deafferentation caused by subcortical stroke are not clearly understood. Therefore, we have begun to analyze the clinical symptoms of stroke patients in relation to the cholinergic pathway.

In experimental focal ischemia, the pathology of this cholinergic pathway has not been extensively investigated. Scremin and Jenden reported a close correlation between cortical choline concentra-

TABLE 1. Acetylcholinesterase-Positive Fiber Density and Cell Density After Left Middle Cerebral Artery Occlusion in Rats (n=6)

<table>
<thead>
<tr>
<th></th>
<th>AChE-positive fiber density (m/mm² cortex)</th>
<th>Cell density (x10⁷/mm² cortex)</th>
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<tbody>
<tr>
<td>Frontal cortex (Fr 1,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layers II-IV</td>
<td>Right</td>
<td>9.7±0.7</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1.0±0.4*</td>
</tr>
<tr>
<td>Layer V</td>
<td>Right</td>
<td>10.2±0.8</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>1.6±1.0*</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layers II-IV</td>
<td>Right</td>
<td>11.8±0.7</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>9.4±1.5*</td>
</tr>
<tr>
<td>Layer V</td>
<td>Right</td>
<td>11.1±2.1</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>9.7±2.2</td>
</tr>
</tbody>
</table>

Values are mean±SD. AChE, acetylcholinesterase; Fr 1,3, frontal cortex areas 1 and 3 according to the comprehensive cortical parcellation described by Zilles; NA, not analyzed.

*Significantly different from the contralateral side, p<0.05 (Wilcoxon test).
It has been reported that a unilateral excitotoxic or toxic lesion of the nucleus basalis Meynert produced 37–67% reductions in the activity of choline acetyltransferase-positive fibers. Duverger and MacKenzie reported that among five rat strains (Fisher 344, Sprague-Dawley, spontaneously hypertensive, stroke-prone hypertensive, and normotensive Wistar rats), a primary subcortical striatal infarct was most frequently seen in normotensive Wistar rats after MCA occlusion. In our study, approximately 67% of rats with occlusion developed primary subcortical infarct, and the others had cortical infarct. The frontal cortex and the globus pallidus including the nucleus basalis Meynert did not develop infarct after MCA occlusion in any rats included in this study. We previously reported that the cortical structure was preserved for 3 months after primary subcortical striatal infarct induced by MCA occlusion in Wistar rats. We chose Wistar rats for this study because we are interested in ischemic disturbance of the subcortical pathway rather than large cortical infarct.

It has been reported that a unilateral excitotoxic or electrolytic lesion of the nucleus basalis Meynert produced 37–67% reductions in the activity of acetylcholinesterase. Considered a biochemical marker of the cholinergic system, in the ipsilateral neocortex in rats. In our study, approximately 67% of rats with occlusion developed primary subcortical infarct, and the others had cortical infarct. The frontal cortex and the globus pallidus including the nucleus basalis Meynert did not develop infarct after MCA occlusion in any rats included in this study. We previously reported that the cortical structure was preserved for 3 months after primary subcortical striatal infarct induced by MCA occlusion in Wistar rats. We chose Wistar rats for this study because we are interested in ischemic disturbance of the subcortical pathway rather than large cortical infarct.

It has been reported that a unilateral excitotoxic or electrolytic lesion of the nucleus basalis Meynert produced 37–67% reductions in the activity of choline acetyltransferase. Considered a biochemical marker of the cholinergic system, in the ipsilateral frontal cortex in rats. Eckenstein et al. anatomically studied cholinergic innervation in the rat cerebral cortex by means of immunohistochemical localization of choline acetyltransferase and reported that the density of choline acetyltransferase–positive terminals in the ipsilateral parietal cortex was decreased by up to 80% after approximately 80% of the cholinergic neurons in the nucleus basalis Meynert were sacrificed by ibotenate injections. In our study, approximately 90% of acetylcholinesterase-positive fibers were eliminated in the ipsilateral frontal cortex after MCA occlusion. We mainly attribute these reductions in acetylcholinesterase-positive fibers to disruptions of cholinergic pathways produced by subcortical infarct. The differences between the results obtained from the neurochemical method and those obtained from the anatomical method are not clearly understood at present. Based on the present study, we cannot rule out a direct effect of mild to moderate cortical blood flow reductions in the ipsilateral frontal cortex on the acetylcholinesterase-positive fibers.

Although acetylcholinesterase is associated with the cholinergic system in the cerebrum, it is important to recognize that acetylcholinesterase is not a specific marker for the determination of the cholinergic pathway. Some noncholinergic neurons and neuronal fibers may contain acetylcholinesterase. Excitotoxic lesions of the basal forebrain cholinergic neurons, which should not affect other fibers of passage, eliminate a major proportion of acetylcholinesterase-positive fibers in rat neocortex. This suggests that the gross reduction in acetylcholinesterase-positive fibers we observed is indicative of cholinergic deafferentation.

In rats, the neocortex receives large afferents from the nucleus basalis Meynert located near the globus pallidus. In animal models, it is supposed that certain learning functions involve the cholinergic neurons in the nucleus basalis Meynert. In the conscious state, the value of glucose utilization evaluated by 2-deoxyglucose autoradiography may reflect neuronal function under nonpathological conditions. Unilateral ibotenate lesions of the nucleus basalis Meynert result in a decrease in glucose utilization in the ipsilateral neocortex in rats. In our study, glucose utilization in the ipsilateral frontal cortex was significantly lower than on the contralateral side and in sham-operated controls. Decreases in glucose utilization observed in the frontal cortex on the lesion side may reflect in part neurofunctional disturbances produced by ischemic cholinergic deafferentation. Obviously, functional disturbances of

<table>
<thead>
<tr>
<th>TABLE 2. Physiological Variables</th>
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<tbody>
<tr>
<td>MCA occlusion</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
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<tr>
<td>Plasma glucose (mg/dl)</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
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<td>pH</td>
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Values are mean±SD. MCA, middle cerebral artery.

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<tr>
<th>TABLE 3. Local Cerebral Glucose Utilization in 12 Rats Studied With [14C]2-Deoxyglucose Autoradiography</th>
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<tbody>
<tr>
<td>Cortical structure</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Frontal cortex (Fr 1,3)</td>
</tr>
<tr>
<td>Cingulate cortex</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
</tr>
<tr>
<td>Temporal cortex</td>
</tr>
</tbody>
</table>

Values are mean±SD (micromoles per 100 grams per minute). MCA, middle cerebral artery; Fr 1,3, frontal cortex areas 1 and 3 according to the comprehensive cortical parcellation described by Zilles.

*Significantly different from sham-operated controls, p<0.05 (Mann-Whitney U test).
†Significantly different from contralateral side, p<0.05 (Wilcoxon test).
many kinds of neuronal networks occur after focal cerebral infarct as documented in our rat MCA occlusion model.27

In this study, we quantitatively demonstrated the loss of acetylcholinesterase-positive fibers in the frontal cortex on the occlusion side. We propose that the mechanism underlying the loss of acetylcholinesterase-positive fibers is ischemic disruption of the cholinergic pathway between the neocortex and nucleus basalis Meynert. These fiber disruptions probably result in functional disturbances in the frontal cortex, as suggested by autoradiographic study.

References
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Key Words • acetylcholinesterase • cerebral ischemia • cholinergic fibers • rats
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