Immunohistochemical investigation of cerebral ischemia after middle cerebral artery occlusion in gerbils

KAZUMI YAMAMOTO, M.D., FUMIHARU AKAI, M.D., TOSHIKI YOSHIMINE, M.D., AND TAKEHIKO YANAGIHARA, M.D.

Department of Neurology and Cerebrovascular Research Center, Mayo Clinic, Rochester, Minnesota

Progression and recovery of ischemic and postischemic damage after occlusion of the middle cerebral artery and subsequent reperfusion were investigated in the gerbil. This study was performed by immunohistochemical reaction testing for tubulin and creatine kinase BB-isoenzyme to visualize the neuronal structure and by immunohistochemical reaction testing for astroprotein (an astrocyte-specific protein) to visualize reactive astrocytes. The earliest ischemic lesion became visible in the frontoparietal cortex after 7 minutes of ischemia as a laminar loss of the reaction for tubulin involving the neuropil, neuronal perikarya, and dendrites. The earliest lesion in the caudoputamen evolved after 30 minutes of ischemia. After reestablishment of cerebral circulation, the immunohistochemical ischemic lesions in the neuronal structure disappeared if the ischemic period was 10 minutes or less and partially disappeared even after ischemia for 15 minutes in the cerebral cortex, while the postischemic lesion in the caudoputamen disappeared even after ischemia for 15 minutes. Reactive astrocytes were detected in the cerebral cortex and caudoputamen as early as 24 hours after reperfusion, both in the areas with and without the neuronal lesions. No lesion was identified in the hippocampus or thalamus. This experimental model is suitable for investigation of rapidly progressive regional ischemia in the cerebral cortex and for comparison with other regional or global cerebral ischemia in the gerbil or other animal species.

KEY WORDS • cerebral ischemia • middle cerebral artery occlusion • immunohistochemistry • gerbil

Materials and Methods

Mongolian gerbils weighing 60 to 70 gm each were used for the present investigation. The surgical procedure has been described in detail. Each gerbil was anesthetized by intramuscular administration of ketamine hydrochloride (80 mg/kg), and a linear skin incision was made along the right zygomatic arch. After separation of the zygomatic arch and masseter muscles, a small hole was drilled at the base of the skull just behind the foramen ovale. Upon opening the dura, the right MCA was identified running vertically across the lateral olfactory tract. The MCA was occluded with a microclip constructed by modifying a miniature Mayfield aneurysm clip. The bone defect was covered with Oxyel and the skin incision was closed.

For investigation of progressive cerebral ischemia, each animal was reanesthetized (if awake) with inhalation of ether and subjected to an ischemic period ranging between 5 minutes and 48 hours. Each animal was then decapitated and the brain was promptly removed. After coronal section of the supratentorial structure, each tissue block was fixed in ethanol containing 5%
acetic acid at 4°C, dehydrated, and embedded in paraaffin.21 Five gerbils were examined for each ischemic period. In a study of the perfusion pattern, three gerbils were perfused with 20% India ink containing 0.5% agarose and 10% formalin through the left ventricle of the heart 30 minutes after occlusion of the right MCA.18 Each brain was immersed in glycerol and sectioned 1 mm thick.

The postischemic period was investigated in the following manner. Each gerbil was anesthetized with inhalation of ether after a predetermined ischemic period of 5 to 15 minutes, and the microclip was removed. Blood recirculation and absence of periarterial tissue damage were ascertained visually, and the bone defect and skin incision were closed. After reperfusion for periods between 1 hour and 7 days, each gerbil was reanesthetized with ether. In order to confirm the patency of the MCA and its branches, each gerbil was perfused through the left ventricle of the heart with 20% India ink in 0.9% saline solution containing 0.5% agarose and 1% formalin.16 Brain sections were fixed in ethanol containing 5% acetic acid and were embedded as before. Five gerbils were studied at each postischemic period.

The immunohistochemical procedure for light microscopic examination and the antiserum for tubulin has been described previously.21 The immunohistochemical use of antiserum for creatine kinase BB-isoenzyme (CK-BB)15 has been described elsewhere.21 The antiserum for astroprotein† was prepared in a rabbit against the antigen from human gliomas.16 Astroprotein is specific for astrocytes and immunoreactive to glial fibrillar acidic protein,1 which are probably identical.7 Each deparaffinized tissue section, 5 μm thick, was reacted with each primary and corresponding secondary antiserum, and then reacted with the peroxidase-antiperoxidase complex. The peroxidase reaction was accomplished by incubation in the presence of 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide. The control section for each reaction was treated with the nonimmunized serum from the same animal species as was used to raise the primary antiserum. No reaction was observed in any control section. Cell nuclei were visualized by counterstaining with Harris′ hematoxylin. An adjacent section was stained with hematoxylin and eosin (H & E) for comparison. The previously observed abnormal findings, including loss of the immunohistochemical reaction for tubulin or CK-BB in the neuropil, neuronal perikarya, and dendrites, and the presence of an enlarged perineuronal space, pyknosis, and eosinophilic neuronal cytoplasm revealed by H & E staining, were used as the criteria for ischemic and postischemic damage.19,20 The enhanced peroxidase reaction and expanded processes detected by the reaction for astroprotein were used as the criteria for reactive astrocytes.20 No abnormality was found in the nonoccluded side as compared to normal gerbil brains, and the reaction pattern of the nonoccluded side was used as a control for the occluded side.

The outline of the ischemic and postischemic lesions as visualized with the reaction for tubulin was traced with the aid of a camera lucida attached to a light microscope, and each tracing was superimposed to achieve a topographic presentation at each ischemic and postischemic period.

Results

Transcardiac perfusion with India ink after occlusion of the MCA for 30 minutes (Fig. 1) revealed marked hypoperfusion in the cerebral cortex from the frontal to the posterior parietal region with preservation of the cingulate cortex. Perfusion was moderately reduced in the piriform cortex. In the area with marked hypoperfusion, the branches from the leptomeningeal arteries were faintly visualized descending perpendicularly into the cerebral cortex. Perfusion was also reduced in the lateral half of the caudoputamen, but this was variable. The medial half of the caudoputamen retained perfusion or showed only mild to moderate reduction because of blood supply from the perforating arteries. After reestablishment of regional circulation, the pre-
TABLE 1
Occurrence of immunohistochemical lesions after occlusion of the middle cerebral artery*

<table>
<thead>
<tr>
<th>Region</th>
<th>Reaction†</th>
<th>Ischemic Period (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>cerebral cortex</td>
<td>HE</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>0</td>
</tr>
<tr>
<td>caudoputamen</td>
<td>HE</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>0</td>
</tr>
</tbody>
</table>

* The results are expressed as percent of abnormal findings based on five gerbils for each ischemic period.
† HE = hematoxylin and eosin staining; TB and CK = immunohistochemical reaction for tubulin and creatine kinase BB-isoenzyme, respectively.

Previously ischemic areas were filled with India ink under light microscopic examination regardless of the length of the postischemic period (from 1 hour to 7 days) and regardless of the presence or absence of the immunohistochemical lesions.

The distribution of the immunohistochemical lesions in the cerebral cortex and caudoputamen during progressive ischemia is shown in Table 1 and Fig. 2, and after various postischemic periods in Table 2 and Fig. 3. No lesion was found in the hippocampus or thalamus.

**Immunohistochemical Lesions During Progressive Ischemia**

**Cerebral Cortex.** No lesion was detected after ischemia for 5 minutes. However, a narrow laminar loss of the reaction for tubulin was noted in a neuronal layer (tentatively identified as layer IV) of the parietal region in 20% of gerbils after ischemia for 7 minutes (Table 1). No notable difference was observed after ischemia for 10 minutes (Fig. 2). The laminar lesion became more distinct after ischemia for 15 minutes (Figs. 3 and 4) and was observed in 60% of gerbils. Within this lesion, the neuropil, neuronal perikarya, and ascending dendrites lost the reaction for tubulin. The reaction for CK-BB and the H & E staining remained unaffected. After ischemia for 30 minutes, the laminar lesion seen with the reaction for tubulin became more distinct and larger (Figs. 2 and 4), and was observed in all gerbils. The pale zone extended from the frontal to the posterior parietal region, but was maximal in the parietal region. Within the pale zone, dark tortuous ascending dendrites were seen. In 20% of gerbils, the reaction for tubulin also became faint in the wider area just below a neuronal layer, tentatively identified as layer V (Fig. 4). With the reaction for CK-BB, a pale zone in layer IV became visible in 40% of gerbils but the H & E staining still showed no abnormality.

After ischemia for 1 hour, the reaction for tubulin showed a wide pale zone in all gerbils, mostly in double layers (Figs. 2 and 4). The reaction for CK-BB also showed as a pale zone in all gerbils. With H & E staining, increased perineuronal spaces and pyknotic neurons were observed in 80% of gerbils in the area corresponding to the pale zone mentioned above (Table 1). After ischemia for 2 to 3 hours, loss of the reaction for tubulin and CK-BB became diffuse, but scattered dark neuronal perikarya and tortuous dendrites were still observed in layer V. Staining with H & E showed increased perineuronal spaces and pyknotic neurons in extensive areas in all gerbils. After ischemia for 6 hours,

---

**Fig. 2.** Topographic presentation of the ischemic lesions demonstrated by the reaction for tubulin after occlusion of the right middle cerebral artery for 10, 30, 60, and 120 minutes (from left to right). The right cerebral hemisphere is shown on the left side of each coronal section. The upper row shows the coronal section that includes the frontoparietal cortex and caudoputamen, while the lower row shows the coronal section with the parietal cortex, hippocampus, and thalamus. The areas affected in 20%, 40% to 60%, and 80% to 100% of gerbils are shown by horizontal bars, cross-hatching, and solid black areas, respectively.
the cerebral cortex was diffusely affected except for the cingulate cortex and the piriform cortex. After ischemia for 12 to 24 hours, the positive immunohistochemical reaction was associated only with scattered shrunken neuronal perikarya. After ischemia for 48 hours, even those neuronal perikarya lost the peroxidase reaction; however, there was no further expansion of the distribution of the immunohistochemical lesions beyond the areas seen after ischemia for 6 hours.

Caudoputamen. After ischemia for 30 minutes, loss of the reaction for tubulin occurred in the neuropil and neuronal perikarya in 20% of gerbils in a small area in the superior lateral part of the caudoputamen (Fig. 5). After ischemia for 1 hour, patchy areas with loss of the reaction for tubulin became more distinct and were seen in 80% of gerbils (Figs. 2 and 5). While some neuronal perikarya lost the reaction, some others had a very dark reaction. Traversing axonal bundles were unaffected (Fig. 5). The neuropil also lost the reaction for CK-BB in the same area in 80% of gerbils but no abnormality was found with H & E staining (Table 1). After ischemia for 2 hours, diffuse or patchy loss of the reaction for tubulin and CK-BB was noted in all gerbils. Staining with H & E also revealed microvacuolation in the neuropil in all gerbils. After ischemia for 3 hours, loss of the reaction for tubulin and CK-BB was more extensive. Traversing axonal bundles became darker than the control brain, and the contour became irregular in some areas (Fig. 5). After ischemia for 6 to 48 hours, the architecture of the caudoputamen disintegrated as was observed in the cerebral cortex. However, there was no further expansion of the affected area.

Immunohistochemical Lesions During Reperfusion

Cerebral Cortex. After an ischemic period of 5 minutes and subsequent reperfusion for 7 days, no lesion was detected with the reaction for tubulin, CK-BB, or astroprotein, or with H & E staining. While there was no lesion after ischemia for 7 minutes and subsequent reperfusion for 7 days in the neuronal structure with the reaction for tubulin or CK-BB, or with H & E staining, mild to moderate proliferation of astrocytes was detected in 80% of gerbils with the reaction for astroprotein in layer IV and the area below layer V.

A laminar lesion was observed with a reaction for tubulin in 40% of gerbils after ischemia for 10 minutes and subsequent reperfusion between 3 and 48 hours (Table 2), but none was detected after reperfusion for 7 days (Fig. 3). No lesion was detected with the reaction for CK-BB or with H & E staining during the same reperfusion period. On the other hand, reaction for astroprotein revealed reactive astrocytes in layer IV and below layer V in all gerbils after reperfusion for 2 and 7 days (Fig. 6). Astrocytes were more reactive after reperfusion for 7 days than after 2 days.

In all specimens examined after ischemia for 15 minutes and reperfusion for 1 hour, the reaction for tubulin showed expansion of the laminar lesion. The lesion expanded slightly more after reperfusion for 3 hours (Fig. 3). These findings were observed in only 60% of gerbils after reperfusion for 7 days, and there

---

**TABLE 2**

Occurrence of immunohistochemical lesions during reperfusion*

<table>
<thead>
<tr>
<th>Ischemic Period</th>
<th>Reaction†</th>
<th>Postischemic Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>1 Hr</td>
</tr>
<tr>
<td>cerebral cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>HE 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB 20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CK 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AP 0</td>
<td>0</td>
</tr>
<tr>
<td>15 min</td>
<td>HE 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB 60</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CK 0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>AP 0</td>
<td>0</td>
</tr>
<tr>
<td>caudoputamen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>HE 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB 20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CK 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AP 0</td>
<td>0</td>
</tr>
<tr>
<td>15 min</td>
<td>HE 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TB 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CK 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AP 0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The results are expressed as percent of abnormal findings based on five gerbils for each postischemic period.
† HE = hematoxylin and eosin staining; TB, CK, and AP = immunohistochemical reaction for tubulin, creatine kinase BB-isoenzyme, and astroprotein, respectively.

---

\*J. Neurosurg. / Volume 67 / September, 1987 417
was reduction in the size of the laminar lesions during that period (Fig. 3). With the reaction for CK-BB, the laminar lesion was observed in only 20% of gerbils during reperfusion. While no abnormality was revealed with H & E staining 1 hour after reperfusion, enlarged perineuronal spaces were seen after reperfusion for 3 hours in 40% of gerbils in the area corresponding to the laminar lesion. After reperfusion for 1 to 2 days, neurons with shrunken nuclei and eosinophilic cytoplasm were observed in the same area in 80% of gerbils. Pyknotic neurons became less distinct and less frequent after reperfusion for 7 days. Reactive astrocytes were visible on immunohistochemical reaction for astroprotein after reperfusion for 2 days and were more reactive after 7 days. They were also more reactive during reperfusion after ischemia for 15 minutes than during reperfusion following ischemia for 10 minutes.

Caudoputamen. After ischemia for 5 minutes and subsequent reperfusion for 7 days, no lesion was detected with any immunohistochemical reactions, including the reaction for astroprotein. In specimens examined after ischemia for 7 minutes and subsequent reperfusion for 7 days, no lesion was detected in the neuronal structure with the reaction for tubulin or CK-BB, or with H & E staining, but mildly reactive astrocytes were visible in the lateral part of the caudoputamen in 60% of gerbils with the reaction for astroprotein. After ischemia for 10 minutes and subsequent reperfusion, the reaction for tubulin and CK-BB remained normal, but reactive astrocytes were more clearly ob-
Immunohistochemical study of ischemia in gerbils

FIG. 6. Photomicrographs showing astrocytic reaction in the cerebral cortex and caudoputamen after reperfusion. Immunohistochemical reaction for tubulin (A and C) and for astroprotein (B and D), × 40. In the cerebral cortex (B), reactive astrocytes were identified with the immunohistochemical reaction for astroprotein after ischemia for 10 minutes and reperfusion for 7 days, primarily in layer IV and the area below layer V. No lesion could be identified in the corresponding areas with the reaction for tubulin (A). Reactive astrocytes were also scattered in the lateral part of the caudoputamen (D) after ischemia for 10 minutes and reperfusion for 7 days. No lesion could be seen in the corresponding areas with the reaction for tubulin (C).

Discussion

The gerbil is unique in developing cerebral hemispheric ischemia after occlusion of a common carotid artery. This is because of incomplete or no anastomosis between the carotid and basilar circulation and between the anterior cerebral artery on each side. In this animal species, however, it is also possible to produce regional cerebral ischemia by occlusion of an MCA or PCoA. An experimental model with occlusion of an MCA offers an opportunity to compare regional and global cerebral ischemia and to compare those results with regional ischemia in other anatomical sites such as the hippocampus and thalamus after occlusion of a PCoA in the same animal species. Furthermore, the effects of regional ischemia after occlusion of an MCA in the gerbil can be compared with those of similar experimental models in other animal species such as the squirrel monkey, papoon, cat, rabbit, and rat.

Transcardiac perfusion with India ink revealed profound hypoperfusion in the frontal to posterior parietal cortex (Fig. 1), which was similar to the appearance of the cerebral cortex in global cerebral ischemia after occlusion of a common carotid artery. There was moderate hypoperfusion in the piriform cortex and variable hypoperfusion in the caudoputamen (Fig. 1). Hypoperfusion in the cerebral cortex was more severe and extensive than after transorbital occlusion of the MCA in the rabbit. The India ink perfusion method was useful in demonstrating the source and direction of residual blood flow. Cerebral blood flow measurement by quantitative autoradiography also revealed hypoperfusion in the same distribution. Quantitatively, there was profound hypoperfusion with the residual flow ranging only from 5 to 10 ml/100 gm/min in the frontal to the parietal cortex. This hypoperfusion was more profound than after occlusion of the MCA in rats. Thus, occlusion of an MCA in the gerbil caused severe ischemia in the cerebral cortex with little collateral circulation except for limited flow from the leptomeningeal arteries.

Because of prompt disappearance of the immunohistochemical reactions from the neuropil, neuronal perikarya, and dendrites, it was possible to present the affected areas topographically even from the early ischemic period. While the ischemic lesions were clearly demonstrated in the areas where profound hypoperfusion had been delineated by India ink perfusion or autoradiography, the timing for appearance of the immunohistochemical lesion was somewhat variable from one location to another and from one gerbil to another. While the earliest lesion was noted 7 minutes after arterial occlusion in the frontoparietal cortex, only after 15 minutes of ischemia did all gerbils develop cortical lesions, and only after 2 hours did all gerbils develop lesions in the caudoputamen. Following reestablish-
ment of regional blood circulation, no lesion was observed in the neuronal structure if the ischemic period had lasted for 7 minutes or less, but the reaction for astroprotein was useful in demonstrating subtle evidence of ischemic insult in the cerebral cortex after ischemia for 7 minutes or longer. Transient neuronal lesions were clearly visible during the early reperfusion period following an ischemic period of 10 minutes, and the topographic demonstration revealed the postischemic lesions in the cerebral cortex to be partly reversible even after an ischemic period of 15 minutes. The topographic presentation of early ischemic and postischemic lesions is very useful not only for demonstration of their progression and regression, but also for comparison with various imaging procedures including autoradiographic measurement of cerebral blood flow.

The mechanism for prompt disappearance of the immunohistochemical reaction within the neuronal structure remains uncertain. However, our recent immunoelectron microscopic investigation demonstrated disappearance of the peroxidase reaction within the dendrites coinciding with disintegration of microtubules, and this could be the cause for loss of the reaction observed under light microscopy. While loss of the reaction for CK-BB may represent loss of the protein from the neuronal structure into cerebrospinal fluid or bloodstream, as observed among patients with acute stroke, there may be other explanations.

The present investigation demonstrated that occlusion of an MCA in the gerbil resulted in prompt and profound regional cerebral ischemia in the cerebral cortex and caudoputamen. This experimental model can be compared with other experimental models of global or regional cerebral ischemia for further understanding of the pathophysiological mechanism of cerebral ischemia and infarction.

References


Manuscript received December 22, 1986. This work was supported by Research Grant NS-06663 from the National Institutes of Health. Address for Drs. Yamamoto and Yoshimine: Osaka University Medical School, Osaka, Japan.

Address reprint requests to: Takehiko Yanagihara, M.D., Department of Neurology, Mayo Clinic, Rochester, Minnesota 55905.