Cerebral Blood Flow and Neuronal Damage During Progressive Cerebral Ischemia in Gerbils

Masayasu Matsumoto, MD, PhD, Takao Hatakeyama, MD, Kazuyoshi Morimoto, MD, PhD, and Takehiko Yanagihara, MD

A combined autoradiographic and immunohistochemical method was used to correlate the extent of focal cerebral ischemia and morphologic ischemic damage following unilateral carotid occlusion in 16 gerbils for 5–30 minutes. Immunohistochemical lesions detectable by the reaction for microtubule-associated proteins 1 and 2 were visible in the subiculum-CA1 and CA2 regions of the hippocampus and layer III/IV of the cerebral cortex after 5 minutes of ischemia (n=4). Local blood flow was promptly reduced but still heterogeneous after 10 minutes of ischemia (n=4); local blood flow in immunohistochemical lesions was <5 ml/100 g/min except in highly vulnerable regions, where flow values of 5–15 ml/100 g/min were observed. After 15 minutes of ischemia (n=4) local blood flow in less vulnerable regions including the thalamus and caudoputamen also declined to <5 ml/100 g/min, and immunohistochemical lesions became visible in those regions after 30 minutes of ischemia (n=4). On the other hand, many brain regions tolerated local blood flow of <5 ml/100 g/min without ischemic damage. The present study demonstrates that selective tissue vulnerability during progressive cerebral ischemia depends on the degree of hypoperfusion and on factors inherent to neurons in various brain regions. (Stroke 1990;21:1470–1477)

In cerebral ischemia, cerebral blood flow (CBF) is reduced and ischemic tissue damage occurs in various regions of the brain. While CBF measurement, particularly by quantitative autoradiography, has served well in demonstrating residual blood flow in ischemic brains, its contribution in elucidating the pathophysiologic mechanism of selective tissue vulnerability during progressive cerebral ischemia is relatively limited, partly because of the lack of a sensitive morphologic method for detecting early ischemic damage. Recently, we observed immunohistochemistry to be very sensitive in detecting ischemic damage after cervical or intracranial arterial occlusion in gerbils, and we have developed a method to evaluate the correlation between degree of ischemia and evolution of ischemic damage by combining quantitative autoradiography and immunohistochemical techniques. We report our observations on cerebral ischemia after unilateral common carotid artery (CCA) occlusion for 5–30 minutes in gerbils preselected for their susceptibility to the development of severe ischemic manifestations.

Materials and Methods

We used 20 Mongolian gerbils (Meriones unguiculatus) of either sex weighing 60–80 g. The animals had free access to water and food. To preselect gerbils that would develop typical signs of severe cerebral ischemia such as neck torsion, circling, and hemiparesis ≤5 minutes after unilateral CCA occlusion, each gerbil was anesthetized with 100 mg/kg i.p. ketamine hydrochloride and the right CCA was occluded for ≤30 seconds to observe the extent of collapse of this artery distal to the occlusion. Gerbils preselected to have severe cerebral ischemia had the tail artery and right saphenous vein cannulated with PE-10 polyethylene catheters as described previously and were allowed to recover from anesthesia.

Several hours later, each gerbil was lightly anesthetized with ether and the right CCA was permanently occluded by ligation. Typical neurologic signs of severe cerebral ischemia could be identified within 3 minutes. Just before CBF determination, each gerbil was anesthetized again with 50 mg/kg i.p. ketamine hydrochloride. After determination of the mean arterial blood pressure and collection of 100 μl...
arterial blood from the tail artery for blood gas analysis, 30 \( \mu \)Ci of 4-iodo[N-methyl-\( ^{14} \)C]antipyrine (Amersham, Arlington Heights, Ill.) dissolved in 0.8 ml physiological saline was infused intravenously for 1 minute; serial arterial blood samples were collected according to Sakurada et al.\(^{10}\) The gerbil was then decapitated, and the brain was frozen quickly in chilled isopentane (-50°C). Four gerbils were used for each ischemic period (5, 10, 15, or 30 minutes). Due to time constraints, mean arterial blood pressure and blood gases were not measured in gerbils subjected to 5 minutes of ischemia. Four gerbils were sham-operated as controls (the CCA was not ligated).

The combined method for quantitative autoradiography and immunohistochemistry has been described elsewhere.\(^{6}\) Briefly, 20-\( \mu \)m frozen brain sections for the measurement of local CBF by autoradiography were prepared in a cryostat microtome and processed according to Sakurada et al.\(^{10}\) For the immunohistochemical procedure, 10-\( \mu \)m frozen brain sections adjacent to the sections for autoradiography were fixed in cold acetone (-20° C) and processed according to the peroxidase-antiperoxidase method for light microscopy.\(^{11}\) For the detection of ischemic lesions, an antiserum for microtubule-associated proteins (MAPs) 1 and 2 was used. This polyclonal antibody (MAPI A, MAP2, or \( \alpha \)-tubulin) had been identified by immunoelectrophoresis\(^{11}\) as an antibody for tubulin, but it has been reidentified as being raised against MAPI and MAP2 by the more specific immunoblot procedure. However, this antibody is superior to a monoclonal antibody for \( \alpha \)-tubulin because of its sensitivity and reliability in detecting ischemic and postischemic lesions\(^{12}\) and remains very useful for immunohistochemical investigations. Loss of the peroxidase reaction from the neuropil, neuronal perikarya, and dendrites were used as criteria for ischemic damage.\(^{13}\)

For quantification of local CBF values, the brain sections corresponding to the stereotactic section 0.1–0.3 mm rostral and 1.4–1.6 mm caudal to the bregma\(^{14}\) were chosen. An autoradiogram and the corresponding immunohistochemical section were first superimposed under microscopic manipulation to identify the immunohistochemical lesions and, after removal of the immunohistochemical section, microdensitometric measurement of the autoradiogram was carried out using a densitometer (Model PPD, Sargent-Welch, Skokie, Ill.) with an aperture diameter of 0.2 mm for 34 presellected vulnerable and less vulnerable anatomic sites.\(^{6}\) Local CBF values were computed according to Sakurada et al.\(^{10}\)

Tukey's method was used to compare serial CBF values and physiological parameters while Student's paired \( t \) test was employed to compare the mean local CBF values between anatomic sites as indicated.

### Results

After occlusion of the right CCA, no significant changes were observed in mean arterial blood pressure or other physiological parameters (Table 1). Distribution of the immunohistochemical lesions after 5–30 minutes of ischemia (Table 2) was similar to that after paraffin-embedding of similar specimens.\(^{1,2}\)

After 5 minutes of ischemia, immunohistochemical lesions were present in the subiculum-CA1 region (between the subiculum and the medial CA1 region) and the CA2 region of the hippocampus and in layer III/IV (anatomic sites 12, 15, 18, and 21). Two layers are combined due to difficulties in separating them clearly of the cerebral cortex (Table 2). Autoradiography showed heterogeneous reduction of local CBF (Figure 1). Quantitatively, mean local CBF was generally reduced to <20 ml/100 g/min except in midline structures of the cerebral cortex, hippocampus, and thalamus (Figure 2). Local CBF values were >10 ml/100 g/min in many regions of the hippocampus and cerebral cortex regardless of the presence of immunohistochemical lesions. While most lesions were <0.2 mm in diameter, those >0.2 mm in diameter invariably had CBF values of <5 ml/100 g/min. Immunohistochemical lesions in the subiculum-CA1 region (anatomic site 2) resided at the transition zone between a region with substantial residual blood flow (anatomic site 1) and a region with severe ischemia (anatomic site 3), causing a steep CBF gradient. Mean±SD local CBF values were 31.8±15.4, 13.0±6.9, and 8.8±4.9 ml/100 g/min for anatomic sites 1, 2, and 3, respectively (\( p < 0.01 \) CBF for anatomic site 1 different from that for anatomic site 2 or 3). Local CBF values for the CA2 region (anatomic site 5) and its adjacent regions were similar; mean±SD local CBF values were 8.0±5.3, 8.3±5.2, and 9.8±6.9 ml/100 g/min for anatomic sites 4, 5, and 6, respectively. In the cerebral cortex, immunohistochemical lesions were observed in layer III/IV, with the columnar distribution of local CBF reduction seen on autoradiograms corresponding to

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### Table 1. Physiological Parameters During Progressive Cerebral Ischemia in Gerbils

<table>
<thead>
<tr>
<th>Ischemic period</th>
<th>( n )</th>
<th>Ht (%)</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>pH</th>
<th>( \text{Pao}_2 ) (mm Hg)</th>
<th>( \text{Paco}_2 ) (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>49.5±1.5</td>
<td>84.5±5.0</td>
<td>7.31±0.01</td>
<td>76.5±2.5</td>
<td>39.3±1.1</td>
</tr>
<tr>
<td>5 min</td>
<td>4</td>
<td>47.3±1.9</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>10 min</td>
<td>4</td>
<td>43.8±3.0</td>
<td>87.0±5.2</td>
<td>7.25±0.03</td>
<td>87.3±7.0</td>
<td>41.1±2.3</td>
</tr>
<tr>
<td>15 min</td>
<td>4</td>
<td>45.8±3.5</td>
<td>91.5±6.5</td>
<td>7.25±0.03</td>
<td>107.8±12.2</td>
<td>35.5±2.2</td>
</tr>
<tr>
<td>30 min</td>
<td>4</td>
<td>48.8±3.0</td>
<td>85.0±3.9</td>
<td>7.33±0.02</td>
<td>93.3±4.3</td>
<td>33.8±2.1</td>
</tr>
</tbody>
</table>

Results are mean±SEM. Ht, hematocrit.
TABLE 2. Frequency of Immunohistochemical Lesions at Various Anatomic Sites in Gerbils After Progressive Cerebral Ischemia

<table>
<thead>
<tr>
<th>Anatomic site</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subiculum-CAl</td>
<td>100*</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CA1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CA2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CA3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CA4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Frontoparietal cortex</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>100*</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Habenular nucleus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caudoputamen</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Septal nucleus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are percentage of gerbils with immunohistochemical lesions, n=4 for each ischemic period.

*Anatomic sites in which at least 75% of lesions were <0.2 mm in diameter.

FIGURE 1. Representative autoradiograms of brain sections of gerbils including cerebral cortex and caudoputamen (left column) and cerebral cortex, hippocampus, and thalamus (right column) during progressive cerebral ischemia for 5 (A and B), 10 (C and D), 15 (E and F), or 30 (G and H) minutes. Note heterogeneous motiled and columnar distribution of areas with hypoperfusion during ischemia for 5 and 10 minutes.

After 10 minutes of ischemia, the immunohistochemical lesions in the hippocampus and cerebral cortex expanded and became more distinct (Figures 3-5). Within laminar lesions in layer III/IV of the cerebral cortex, columnar distribution was clearly present with immunohistochemical procedure (Figure 5). Mean local CBF was further reduced in many regions but was not significantly different from values observed after ischemia for 5 minutes. Local CBF values for immunohistochemical lesions >0.2 mm in diameter were consistently <5 ml/100 g/min. In the hippocampus, the immunohistochemical lesion in anatomic site 2 had a mean±SD local CBF value of 5.0±4.1 ml/100 g/min, and the adjacent subfield (anatomic site 1) and the medial CA1 region (anatomic site 3) had mean±SD local CBF values of

the columnar distribution of immunohistochemical lesions. The mean±SD local CBF value for four regions with residual blood flow (corresponding to the unaffected cortex) was 14.0±7.8 ml/100 g/min and that for four regions corresponding to the ischemic cortex was 4.8±4.5 ml/100 g/min (p<0.01). On the nonocluded side, a slight to moderate but significant (p<0.05) reduction in local CBF was observed in midline structures (including the cingulate cortex, septal nucleus, and paramedian thalamic nuclei) without immunohistochemical lesions.
17.8±4.8 and 4.5±3.1 ml/100 g/min (p<0.01 CBF of anatomic site 1 different from that of anatomic site 2 or 3), respectively. Local CBF values for the CA2 region (anatomic site 5) and the adjacent regions were similar; mean±SD local CBF values were 5.3±4.5, 3.3±2.2, and 5.8±4.1 ml/100 g/min for anatomic sites 4, 5, and 6, respectively. In the cerebral cortex, a columnar distribution of local CBF reduction was also present on autoradiograms (Figure 1). Four pale areas corresponding to immunohistochemical lesions in layer III/IV had a mean±SD CBF value of 2.0±1.4 ml/100 g/min, while four dark areas corresponding to the unaffected cerebral cortex had a mean±SD CBF value of 6.8±1.5 ml/100 g/min (p<0.01).

After 15 minutes of ischemia, the immunohistochemical lesions in the subiculum-CA1 region extended laterally, involving the medial CA1 region. The CA3 region was also affected. In the cerebral cortex, the laminar lesions in layer III/IV became clearer and broader (Figure 5). In general, local CBF...
values declined further, especially in the CA3 region of the hippocampus, the ventral nucleus of the thalamus, and the lateral part of the caudoputamen, where local CBF values became 0 ml/100 g/min or close to it. The columnar pattern of CBF reduction in the cerebral cortex was no longer observed.

After 30 minutes of ischemia, immunohistochemical lesions became visible in the CA4 region, while other preexisting lesions expanded further in the hippocampus (Figures 3 and 4). In the cerebral cortex, lesions were clearly visible in layer III/IV and below layer V in all gerbils anteriorly and in 50% of them posteriorly. Lesions also became visible in the ventral nucleus and adjacent regions of the thalamus and the lateral part of the caudoputamen. The hypothalamus and habenular nucleus also developed new lesions (Table 2). Local CBF values were very low, often 0 ml/100 g/min or close to it, except for midline structures.
of the hippocampus, cerebral cortex, and thalamus. However, many regions with local CBF values of <5 ml/100 g/min had no immunohistochemical lesions (Table 2), notably the lateral part of the CA1 region of the hippocampus (anatomic site 4), granular cells of the dentate gyrus (anatomic site 10), layer V of the cerebral cortex (anatomic sites 14 and 20), and the medial part of the caudoputamen (anatomic sites 30 and 33). In the nonoccluded cerebral hemisphere, local CBF values declined further and significantly (p<0.05) compared with corresponding values from the sham-operated controls. Mean local CBF was close to 25 ml/100 g/min in the cingulate cortex, and it reached as low as 6.0 ml/100 g/min in the septal nucleus. However, no immunohistochemical lesions developed on the left side.

Discussion

The model of cerebral ischemia after unilateral CCA occlusion in gerbils resembles carotid occlusion in humans. Clinical manifestations depend on the collateral circulation. Our previous investigations showed the development of signs of severe cerebral ischemia and immunohistochemical evidence of neuronal damage ≤5 minutes after unilateral CCA occlusion. Although our present investigation did not include the postischemic period, our previous studies indicated that discrete lesions in the subiculum-CA1 and CA2 regions of the hippocampus were already irreversible after 5 minutes of ischemia and that laminar lesions in layer III/IV of the cerebral cortex were irreversible after 10 minutes of ischemia. While no ischemic lesion evolved in the caudoputamen or thalamus after 15 minutes of ischemia, postischemic lesions developed during reperfusion. The irreversibility was further documented by the presence of acute eosinophilic neuronal degeneration (neuronal death) in those areas with hematoxylin and eosin staining after reperfusion for ≥24 hours. Therefore, assessment of the correlation between reduction of cerebral perfusion and induction of irreversible neuronal damage required the measurement of local CBF soon after CCA occlusion, before any morphologic damage emerged.

A method to preselect uniformly symptomatic gerbils and a method combining quantitative autoradiography and immunohistochemistry enabled us to carry out this task.

Our present investigation not only demonstrated very prompt reduction of local CBF in many regions of the gerbil brain but also demonstrated gradual reduction of local CBF in other regions. In general, regions developing immunohistochemical lesions rapidly (such as the hippocampus and cerebral cortex) showed a more precipitous decline of CBF than regions in which lesions were detected only after 30 minutes of ischemia (such as the thalamus and caudoputamen). While this supports the contention that selective tissue vulnerability in cerebral ischemia depends largely on the degree of hypoperfusion, considerable discrepancies between regions with profound hypoperfusion and those with early lesions indicated that factors other than the degree of hypoperfusion also determine selective tissue vulnerability.

The perfusion threshold for induction of ischemic damage has been sought. A local CBF of 10 ml/100 g/min is generally considered to be the threshold for depletion of adenosine triphosphate (ATP), influx of Ca²⁺ and Na⁺, loss of intracellular K⁺, profound acidosis, and release of free fatty acids. In our present study, local CBF values of <10 ml/100 g/min
were observed widely regardless of the presence of immunohistochemical lesions, but local CBF values were invariably <5 ml/100 g/min in lesions >0.2 mm in diameter. In contrast, local CBF values in highly vulnerable regions, which frequently developed immunohistochemical lesions <0.2 mm in diameter within 5 minutes, were often 5–15 ml/100 g/min. While the higher residual blood flow in these regions, including the subiculum-CA1 region of the hippocampus, may reflect overlap with adjacent regions having higher blood flows, this is unlikely to explain the local CBF values of 5–15 ml/100 g/min in other regions, including the CA2 region. In less vulnerable regions (such as the caudoputamen and thalamus) moderate residual blood flow was retained for the first 10 minutes in most gerbils, but local CBF declined to ≤5 ml/100 g/min after 15 minutes, before immunohistochemical lesions developed. Therefore, the results of our present study demonstrate the causal relation between a residual blood flow of <5 ml/100 g/min and ischemic damage in general and the relation between a residual blood flow of 5–15 ml/100 g/min and ischemic damage in highly vulnerable regions. It should be noted, however, that some regions tolerated local CBF values of <5 ml/100 g/min for up to 30 minutes without developing immunohistochemical lesions. In addition to the extent of ischemia, the duration of hypoperfusion is likely to be an important factor in the development of ischemic damage. However, this question cannot be answered by our present study alone.

The pathophysiologic mechanism for selective tissue vulnerability remains unsettled, and information gathered during progressive cerebral ischemia without reperfusion is particularly limited. Since Lowry et al. observed a precipitous decline in the concentration of ATP and a rise in the concentration of lactic acid after decapitation, many biochemical processes have been considered to be responsible for ischemic damage. Among them, several mechanisms responsible for damage caused by severe incomplete ischemia such as free radical formation, tissue acidosis, influx of Ca\(^{2+}\), and release of excitatory amino acid neurotransmitters may operate in many vulnerable regions such as the subiculum-CA1 region of the hippocampus, where regions with extremely low local CBF values and regions with substantial residual blood flow merge.

Among those biochemical processes, influx of Ca\(^{2+}\) is of particular interest for our present investigation since the excessive accumulation of Ca\(^{2+}\) can be induced by a failure of the calcium pump due to ATP depletion or by membrane depolarization caused by excitatory amino acid neurotransmitters and since the breakdown of MAPs and the disintegration of microtubules can be induced by calcium-dependent proteolytic enzymes such as calpains. Prompt disintegration of microtubules and disappearance of electron-dense precipitates for the immunohistochemical reaction have been observed in the hippocampus as early as 5 minutes after the onset of cerebral ischemia by transmission electron microscopy and immunoelectron microscopy. Our recent study also suggested that MAPs that project from the central core of microtubules break down first, before the central core disintegrates. Since the subiculum-CA1 region is exposed to the cerebrospinal fluid space, ions, water, and glucose may also enter the brain parenchyma during progressive cerebral ischemia and exert deleterious effects. However, this mechanism is unlikely to operate during early ischemic damage in the CA2 region.

In the cerebral cortex, the columnar pattern of CBF reduction was observed corresponding to the columnar distribution of immunohistochemical lesions in layer III/IV. This appears to correspond to the columnar pattern of increased tissue nicotinamide adenine dinucleotide (reduced form) fluorescence in incomplete cerebral ischemia and was probably caused by residual blood flow in the small arteries descending perpendicularly from the leptomeninges after carotid occlusion. In the thalamus, local CBF declined gradually. A steep CBF gradient was also present in the thalamus between the midline structure (anatomic sites 26 and 27) and the adjacent region (anatomic site 25) after 15 minutes of ischemia and may explain the development of immunohistochemical lesions in the paramedian structure (anatomic site 25) after 30 minutes of ischemia. However, this does not explain the development of lesions in the ventral nucleus (anatomic site 24). In the caudoputamen, a steep CBF gradient was not observed in the regions with immunohistochemical lesions (anatomic sites 31 and 32).

Thus, our present investigation demonstrates selective tissue vulnerability in various regions of the gerbil brain during progressive cerebral ischemia that depends not only on the reduction of local perfusion but also on factors inherent to neurons in certain locations. The exact nature of microenvironmental and endogenous factors inherent to neurons that determine selective tissue vulnerability remain uncertain, and further investigations are necessary to elucidate these factors.

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