Immunohistochemical Investigation of Ischemic and Postischemic Damage After Bilateral Carotid Occlusion in Gerbils

Takao Hatakeyama, MD, Masayasu Matsumoto, MD, Joan M. Brengman, BA, and Takehiko Yanagihara, MD

We investigated progression and recovery of neuronal damage during and after global cerebral ischemia in gerbils after bilateral occlusion of the common carotid arteries, using the immunohistochemical method (reaction for tubulin and creatine kinase BB-isoenzyme). The earliest, but reversible, ischemic lesions occurred after 3 minutes' ischemia in the subiculum-CA1 and CA2 regions of the hippocampus. The lesions became irreversible after 4 minutes' ischemia. The ischemic and postischemic lesions in the cerebral cortex, thalamus, and caudoputamen were partially or completely reversible if the ischemic period was 5 minutes, whereas delayed degeneration occurred in the pyramidal cells of the medial CA1 region after reperfusion for 48 hours (delayed neuronal death). After 10 minutes' ischemia and subsequent reperfusion, delayed neuronal death extended from the medial to the lateral CA1 region; the ischemic and postischemic lesions in the cerebral cortex, thalamus, and caudoputamen also expanded during reperfusion. Our investigation demonstrates that selective vulnerability existed in global cerebral ischemia as in incomplete or regional ischemia and suggests that neurons in many areas of the brain possessed the potential for recovery, progressive deterioration, and even delayed neuronal death depending on the severity and duration of cerebral ischemia. 

Global cerebral ischemia has been produced experimentally in the past 2 decades in large animals such as cats,1 dogs,2 and monkeys3 by occlusion of major blood vessels in the thoracic cavity and in small animals such as rats by induction of intracranial hypertension4 or by occlusion of major cervical arteries.5 Because of poor development of the circle of Willis, unilateral or bilateral cerebral ischemia can be produced in gerbils by occluding one or both common carotid arteries (CCAs).6 Bilateral CCA occlusion in gerbils results in prompt and severe global cerebral ischemia.7 While the morphologic evidence of ischemic damage has been investigated vigorously in the past with various models of cerebral ischemia, the detection of early ischemic and postischemic damage has not been successful with conventional histologic methods. Since we previously demonstrated the feasibility of detecting such lesions immunohistochemically,8–10 we applied the same technique to investigate the selective tissue vulnerability by following the evolution, expansion, and disappearance of ischemic and postischemic lesions during and following severe global cerebral ischemia in gerbils. Our results have been reported in abstract form.11

Materials and Methods

We used Mongolian gerbils (Meriones unguiculatus) of both sexes weighing 60–80 g. They were kept in the animal quarter with free access to food and water before and after surgery. Under ether anesthesia, both CCAs of each gerbil were exposed through a midline incision in the neck and were occluded using miniature Mayfield aneurysm clips. For the investigation of progressive ischemia, the CCAs were occluded for 2, 3, 4, 5, 7, 10, 15, 30, and 60 minutes. For the evaluation of various postischemic periods, the clips were released after 2, 3, 4, 5, 7, and 10 minutes' ischemia and the gerbils were followed for 30 minutes, 3 or 12 hours, or 1, 2, 3, or 7 days (except that only 7 days' reperfusion was studied after 2 minutes' ischemia and that only 30 minutes', 3 hours', 12 hours', 1 day's, and 7 days' reperfusion were studied after 3 minutes' ischemia). At least one sham-operated gerbil (no arterial occlu-
The immunohistochemical reaction was accomplished using the peroxidase-antiperoxidase method as described. Briefly, each deparaffinized 5-μm coronal section was reacted with a primary antiserum (see below) and the corresponding secondary antiserum for 30 minutes before reaction with the peroxidase-antiperoxidase complex for 30 minutes. The immunohistochemical reaction was carried out by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The coronal section was counterstained with Harris’s hematoxylin to visualize cell nuclei. For comparison, an adjacent section was stained with hematoxylin and eosin (H-E). A control section was incubated with nonimmunized serum from the same species used to raise each primary antiserum. No control section showed a positive reaction. The antiserum for tubulin from gerbil brain was raised in a goat and diluted to 1:200. The antiserum for creatine kinase BB-isoenzyme (CK-BB) from human brain, a generous gift from Dr. Henry A. Homburger of our institution, was raised in a rabbit and diluted to 1:50. The antiserum for glial fibrillary acidic protein (GFAP) was raised in a rabbit and diluted to 1:200. The antiserum for glial fibrillary acidic protein (GFAP) from bovine spinal cord, raised in a rabbit, was used as the criterion for lesions. The lesions were mapped by a single investigator. No lesion was detected in any sham-gerbil. However, after 3 minutes’ ischemia (Table 1), loss of the immunohistochemical reactions in the neuropil, neuronal perikarya, and dendrites became apparent in the subiculum-CAl region of three gerbils and in the CA2 region in two. After 4 minutes’ ischemia, lesions were detected in the subiculum-CAl region in all four gerbils. After 5 minutes’ ischemia (Table 1), the earliest lesion in the cerebral cortex was observed as laminar loss of the immunohistochemical reactions in the layer III/IV, and one gerbil showed a second lesion below layer V. After 7 minutes’ ischemia, the described lesions expanded further and two gerbils showed lesions in the CA3 region. After 10 minutes’ ischemia (Table 1), new lesions evolved in the medial CA1 region adjacent to the subiculum-CAl region in one gerbil, in the CA4 region in three gerbils (Figures 1B and 2B), and in the septal nucleus in two gerbils; the laminar lesions in the cerebral cortex were clearly double-layered. After 15 minutes’ ischemia (Table 1), the reaction for tubulin and CK-BB demonstrated lesions in the subiculum-CAl to CA4 region of the hippocampus and the cerebral cortex in all four gerbils; with H-E staining, perineuronal spaces expanded in the subiculum-CAl to CA4 region of the hippocampus and the cerebral cortex in all four gerbils; with H-E staining, perineuronal spaces expanded in the subiculum-CAl region in one, in the CA4 region in two, and in the cerebral cortex in three gerbils. A new lesion also evolved in the ventral nucleus of the thalamus in one gerbil. After 30 minutes’ ischemia (Table 1), the subiculum-CAl to CA3 region became more widely affected. Lesions covered wide areas of the cerebral cortex anteriorly and posteriorly, and H-E staining revealed expanded perineuronal spaces in the cerebral cortex. Work is underway in our laboratory to identify them precisely. Results

Gerbils became unresponsive after occlusion of both CCAs and remained so unless cerebral reperfusion took place. Seizure activity (tonic-clonic seizures, rolling, jumping, or running) was observed during ischemia lasting >5 minutes, and 38% of the gerbils had seizures immediately after 10 minutes’ ischemia, most seizures subsiding within 30 minutes’ reperfusion. While no gerbil died during ischemia lasting ≤15 minutes, 33% and 43% of the gerbils died during 30 and 60 minutes’ ischemia, respectively. No gerbil died during reperfusion after ≤7 minutes’ ischemia, and the gerbils remained alert after recovery from seizure activity. However, after 10 minutes’ ischemia 20% of the gerbils died within 1 day and 43–50% died between 1 and 7 days.

Progressive Ischemia

The frequency and distribution of lesions in the coronal sections for five selected times demonstrated with the reaction for tubulin are shown in Figure 3, and immunohistochemical reactions and H-E staining for six selected times are compared in Table 1. After 2 minutes’ ischemia no lesion was detected in any gerbil. However, after 3 minutes’ ischemia (Table 1), loss of the immunohistochemical reactions in the neuropil, neuronal perikarya, and dendrites became apparent in the subiculum-CAl region of three gerbils and in the CA2 region in two. After 4 minutes’ ischemia, lesions were detected in the subiculum-CAl region in all four gerbils. After 5 minutes’ ischemia (Table 1), the earliest lesion in the cerebral cortex was observed as laminar loss of the immunohistochemical reactions in the layer III/IV, and one gerbil showed a second lesion below layer V. After 7 minutes’ ischemia, the described lesions expanded further and two gerbils showed lesions in the CA3 region. After 10 minutes’ ischemia (Table 1), new lesions evolved in the medial CA1 region adjacent to the subiculum-CAl region in one gerbil, in the CA4 region in three gerbils (Figures 1B and 2B), and in the septal nucleus in two gerbils; the laminar lesions in the cerebral cortex were clearly double-layered. After 15 minutes’ ischemia (Table 1), the reaction for tubulin and CK-BB demonstrated lesions in the subiculum-CAl to CA4 region of the hippocampus and the cerebral cortex in all four gerbils; with H-E staining, perineuronal spaces expanded in the subiculum-CAl region in one, in the CA4 region in two, and in the cerebral cortex in three gerbils. A new lesion also evolved in the ventral nucleus of the thalamus in one gerbil.
FIGURE 1. Photomicrographs of immunohistochemical reaction for tubulin in gerbil hippocampus in sham-operated control (A), and after 10 minutes' global cerebral ischemia without reperfusion (B), after 10 minutes' ischemia with 1 day's reperfusion (C), and after 10 minutes' ischemia with 7 days' reperfusion (D). Midline is on right and CA3 region is on left of each photomicrograph, ×20. Arrow on right in B, C, and D indicates subiculum-CA1 region; arrow on left in B, C, and D indicates CA2 region. Loss of reaction for tubulin in neuronal cell bodies and dendrites occurred in subiculum-CA1 region after 10 minutes' ischemia (B) and after subsequent 1 day's reperfusion (C). Loss of reaction for tubulin in neuronal cell bodies also occurred in CA2 region after 10 minutes' ischemia (B) and after 1 day's reperfusion with loss of reaction in apical dendrites (C). Medial and lateral CA1 region between arrows lost reaction for tubulin almost completely after 7 days' reperfusion (D), leaving scattered surviving nerve cell bodies and dendrites. Loss of reaction for tubulin also occurred in CA4 region within dentate gyrus after 10 minutes' ischemia with or without reperfusion.

affected hippocampal and cortical areas. Lesions in the thalamus rapidly expanded and involved the ventral and medial habenular nuclei, and H-E staining showed linear microvacuolation along the affected and intact areas close to the midline. In the lateral part of the caudoputamen, loss of the reaction for tubulin was observed in the neuronal perikarya in two gerbils. After 60 minutes' ischemia (Table 1), scattered loss of the reaction for tubulin occurred in the granular cells of the dentate gyrus in all four gerbils, and the caudoputamen became widely affected in all four gerbils.

Reperfusion

Following 2 or 3 minutes' ischemia. No abnormality was found after 7 days' reperfusion following 2 minutes' ischemia. The lesions in the subiculum-CA1 region, present after 3 minutes' ischemia, were still visible in all four gerbils after 30 minutes' reperfusion but the lesions gradually disappeared during 1 day's reperfusion; the lesions in the CA2 region similarly disappeared. After 1 day's reperfusion, a few reactive astrocytes were observed with the reaction for GFAP in these regions; the reactive astrocytes became darker and more numerous after 7 days' reperfusion. Reactive astrocytes were also scattered in the CA4 region and the caudoputamen.

Following 4 minutes' ischemia. The lesions in the subiculum-CA1 did not alter during 12 hours' reperfusion but began to extend to the medial part of the CA1 region after 2 days' reperfusion. After 7 days' reperfusion the lesions tended to be more extended laterally than those after 3 days' reperfusion. H-E staining began to demonstrate increased perineuronal spaces and pyknotic neurons with eosinophilic cytoplasm in the subiculum-CA1 region after 12 hours' reperfusion, corresponding to the immunohistochemical lesions; in the CA2 region, intensely
eosinophilic neurons were clearly visible after 2 days' reperfusion. In the CA3 region, a few pyramidal cells showed intracytoplasmic eosinophilic inclusion bodies after 12 hours' reperfusion; inclusion bodies were more definite after 1 day's reperfusion. However, only one gerbil showed eosinophilic neurons in the CA3 region after 3 and 7 days' reperfusion. Loss of the reactions for tubulin and CK-BB occurred in all four gerbils in the CA4 region after 12 hours' reperfusion, and these lesions did not disappear subsequently. In the cerebral cortex, laminar loss of the reactions for tubulin and CK-BB evolved in layer III/IV in all four gerbils after 30 minutes' reperfusion, but the lesions disappeared during 12 hours' recirculation. The reaction for GFAP revealed reactive astrocytes after 1 day's reperfusion in the areas with transient and permanent neuronal damage; reactive astrocytes were more notable after 7 days' reperfusion. Scattered reactive astrocytes were also observed in the dentate gyrus and the caudoputamen.

Following 5 minutes' ischemia. The frequency and distribution of postischemic lesions at selected times are shown in Table 2 and Figure 4. The lesions existing in the subiculum-CA1 region seen immediately after 5 minutes' ischemia expanded to the medial CA1 region after 2 days' reperfusion, involving the entire CA1 region in some gerbils after ≥3 days' reperfusion. While no expansion was observed in the CA2 region, eosinophilic degeneration of the pyramidal cells became clearly visible after 2 days' reperfusion. In the CA3 region, the lesions evolved in one gerbil after 3 hours' reperfusion and in all four gerbils after 2 days' reperfusion. With H-E staining, pyramidal cells with inclusion bodies became clearly visible after 12 hours' and 1 day's reperfusion, but only scattered eosinophilic neurons were visible after 7 days' reperfusion. In the CA4 region, loss of the immunohistochemical reaction evolved after 30 minutes' reperfusion and persisted afterward. Laminar lesions in the cerebral cortex receded gradually during reperfusion and even disappeared in some gerbils. In the thalamus, postischemic lesions evolved in the ventral nucleus after 30 minutes' reperfusion and expanded further after 3 hours' reperfusion. However, the lesions eventually receded and could not be identified after 3 days' reperfusion. Transient
lesions were also visible in the lateral part of the caudoputamen after 2 days' reperfusion. The reaction for GFAP showed reactive astrocytes in all those areas with transient or progressive postischemic lesions. Reactive astrocytes were also visible with the reaction for CK-BB in the CA1 region of the hippocampus of all four gerbils after 7 days' reperfusion, as described previously.10

**Following 7 minutes' ischemia.** Lateral extension of the postischemic lesions in the CA1 region was more obvious after 2 days' reperfusion than those seen immediately after 5 minutes' ischemia. With H-E staining, degraded eosinophilic neurons were more notable after 7 than after 2 days' reperfusion. In the CA3 region, many pyramidal cells possessed inclusion bodies or diffuse eosinophilic cytoplasm after 12 hours' and 1 day's reperfusion. Shrunken neurons with eosinophilic degeneration were clearly visible in the same area after 2 days' reperfusion, and further degradation was observed after 7 days' reperfusion. In the cerebral cortex, the laminar lesions persisted. Postischemic lesions evolved in the ventral nucleus of the thalamus after 30 minutes' reperfusion and were observed in all four gerbils after 7 hours' reperfusion but in only two gerbils after 7 days' reperfusion. Postischemic lesions also became visible in the caudoputamen after 12 hours' reperfusion in two gerbils, and the lesions persisted after 7 days' reperfusion. The reaction for GFAP revealed reactive astrocytes in all those areas after ≥1 day's reperfusion. The reaction for CK-BB also showed reactive astrocytes in the CA1 region of the hippocampus, sporadically after 3 days' reperfusion and more definitely after 7 days' reperfusion.

**Following 10 minutes' ischemia.** The frequency and distribution of the postischemic lesions at selected times are shown in Table 2 and Figure 5. While no major change was noted after 1 day's reperfusion (Figures 1C and 2C), the postischemic lesions in the CA1 region extended more laterally after 2 days' reperfusion than those seen immediately after 7 minutes' ischemia and covered the entire CA1 region after 3 and 7 days' reperfusion (Figures 1D and 2D). In the CA3 region, inclusion bodies in the pyramidal cells were abundant after 12 hours' reperfusion, but they were already intermingled with pyknotic pyramidal cells with eosinophilic cytoplasm. After 2 days' reperfusion pyknotic eosinophilic pyramidal cells were more numerous, and advanced neuronal degradation was observed after 7 days' reperfusion. No lesion was observed with the reactions for tubulin or CK-BB or H-E staining in the granular cells of the dentate gyrus for up to 7 days' reperfusion. In the cerebral cortex, laminar lesions persisted in double layers, one in layer III/IV and the other below layer V. After ≥1 day's reperfusion, the laminar lesions widened in some gerbils. In the same area, H-E staining revealed progressive eosinophilic neuronal degeneration. Postischemic lesions evolved in the ventral nucleus of the thalamus and its vicinity after 30 minutes' reperfusion and expanded thereafter; however, the anatomic structures close to the midline, including the habenular nucleus, were spared. Small postischemic lesions were observed in the lateral part of the caudoputamen in one gerbil after 3 hours' reperfusion; all four gerbils had postischemic lesions in the caudoputamen after 2 days' reperfusion, but the number of gerbils with such lesions declined afterward. In the septal nucleus, ischemic and postischemic lesions were observed in 25–75% of the four gerbils, with gradual expansion for up to 3 days' reperfusion. The reaction for GFAP revealed reactive astrocytes in those areas and scattered in the dentate gyrus. The reaction for CK-BB also revealed some reactive astrocytes in the CA1 region of the hippocampus after 3 days' reperfusion and more after 7 days' reperfusion.

**Discussion**

Bilateral CCA occlusion in gerbils results in residual blood flow as low as 5.0 ml/100 g/min after 5 minutes.7 The results of our preliminary investigation indicated residual blood flow even below 5.0 ml/100 g/min in wide areas as early as 3 minutes after bilateral CCA occlusion.16 While selective tissue vulnerability has been discussed with various...
TABLE 1. Frequency and Distribution of Immunohistochemical Lesions in Gerbil Brain During Progressive Global Cerebral Ischemia

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Data are % abnormal findings in groups of four gerbils. HE, hematoxylin and eosin staining; TB, immunohistochemical reaction for tubulin; CK, immunohistochemical reaction for creatine kinase BB-isoenzyme.

models of cerebral ischemia, it is not clear whether any difference exists between complete, global cerebral ischemia and incomplete or regional cerebral ischemia. Since we can produce not only unilateral and bilateral6 but also regional17 cerebral ischemia in gerbils, we were able to address this question in a single species. While the mechanism for loss of the immunohistochemical reactions from neuronal structures has not been clearly elucidated8,10 immunohistochemical reactions serve as suitable markers for the detection of ischemic damage and topographic mapping. Ideally, the extent of morphologic damage should be correlated with the degree of ischemia in the same brain. However, visualization of ischemic damage is better with the tissue embedded in paraffin, and this is necessary as the initial step for morphologic investigation to assess the exact extent of tissue damage.18

Compared with bilateral CCA occlusion, reduction of cerebral blood flow is slightly milder in severe unilateral ischemia,19 and unilateral occlusion of the posterior communicating or middle cerebral artery appears to cause an even milder reduction of cerebral blood flow.20 Immunohistochemically, evolution of the ischemic lesions was slightly slower after unilateral8,10 than after bilateral CCA occlusion. Although the midline structures, such as the cingulate cortex and the septal nucleus, were often spared after unilateral CCA occlusion, ischemic lesions in other areas developed in the same sequence after unilateral and bilateral CCA occlusion, indicating the presence of selective tissue vulnerability in the same anatomic locations in both models. After unilateral occlusion of the posterior communicating artery21 or the middle cerebral artery,22 the ischemic lesions progressed more slowly but they still occurred in the same anatomic locations (except for those in the midline structures). Postischemic lesions also evolved more rapidly after bilateral than after unilateral CCA occlusion9 or regional ischemia,21-22 but they still occurred in the same anatomic locations. Thus, a series of experiments demonstrated selective vulnerability of various anatomic sites in severe global cerebral ischemia as observed in incomplete or regional cerebral ischemia.

In 1982, Pulsinelli et al23 and Kirino24 independently reported histologic evidence of neuronal death that became obvious particularly after reperfusion over 24 hours in the CA1 (H1) region of rat and gerbil brains, and Kirino used the term "delayed neuronal death." The pathophysiologic mechanism remains unclear. Our close examination of the
subiculum-CA1 and the medial CA1 regions demonstrated the presence of delayed neuronal death in the medial CA1 region just lateral to but separate from the subiculum-CA1 region during reperfusion following ischemia for 4–10 minutes. The subiculum-CA1 region had persistent immunohistochemical lesions during progressive ischemia and reperfusion, indicating clear differences in the cellular vulnerability between these subfields. Since ischemic lesions in the subiculum-CA1 and CA2 regions were often not visible with H-E staining for 12–24 hours, they should not be mistaking as representing delayed neuronal death. The speed of evolution and the extent of delayed neuronal death in the CA1 region appear to depend on the duration of ischemia. After 4 minutes’ ischemia, clear evidence of neuronal degeneration did not occur until after 3 days’ reperfusion and the lateral part of the CA1 region was mostly spared, while neuronal degeneration was clearly present after 2 days’ reperfusion following 10 minutes’ ischemia and the entire CA1 region became affected after 3 days’ reperfusion. Delayed neuronal death is also likely to depend on the severity of ischemia since it was an inconsistent finding even after 15 minutes’ occlusion of a posterior communicating artery before reperfusion.21

Aside from the CA1 region of the hippocampus, delayed neuronal death is not widely known in other areas of the brain. Although Pulsinelli et al23 described delayed lesions in other areas of the hippocampus, cerebral cortex, and striatum, these lesions might have already existed at the end of an ischemic period of up to 30 minutes without reperfusion judging from the differences in the sensitivity of the immunohistochemical and the conventional histologic techniques we have observed. Previously, we have observed evolution of postischemic lesions in many areas of the brain during reperfusion for <24 hours.9,10,21,22 In this investigation, new lesions also developed in the cerebral cortex, thalamus, and caudoputamen during reperfusion for >24 hours. Therefore, our previous and present investigations suggest that delayed neuronal death may not be a phenomenon limited to the hippocampus and that delayed neuronal death can occur even within 24 hours after reperfusion. It appears that postischemic lesions develop more rapidly if

**Table 2.** Frequency and Distribution of Postischemic Lesions in Gerbil Brain After Recirculation Following 5 or 10 Minutes’ Global Cerebral Ischemia

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ischemia is severe and prolonged, while they can be reversible if ischemia is short or mild.

Intraneuronal eosinophilic inclusion bodies were first described by Ito et al. in the Sommer's H3 sector of the gerbil hippocampus during reperfusion. This phenomenon is believed to be a reversible process. However, our earlier investigations suggested that some neurons with inclusion bodies degraded further. In this investigation, after 4 minutes' ischemia we observed scattered inclusion bodies in the CA3 region following 12 hours' to 1 day's reperfusion, but the pyramidal cells in that area appeared normal in three of the four gerbils after 7 days' reperfusion. On the other hand, inclusion bodies were clearly observed after 7 minutes' ischemia followed by 12 hours' reperfusion and the pyramidal cells in the same area degraded to eosinophilic cell ghosts after 7 days' reperfusion. Our results suggest that neurons with inclusion bodies could recover if the ischemic period was short but that they would degenerate further if the ischemic period was long.

Thus, our present investigation, in combination with our previous studies, demonstrates that ischemic neuronal damage in many areas of the brain is dynamic and that recovery, progressive deterioration, and even delayed neuronal death can occur during reperfusion depending on the severity and duration of cerebral ischemia and the vulnerability of the neurons. The key metabolic factor(s) that determines the fate of individual neurons is not firmly established and must be investigated.

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**KEY WORDS** • cerebral ischemia • immunohistochemistry • gerbils
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