Fate of Disseminated Dead Neurons in the Cortical Ischemic Penumbra
Ultrastructure Indicating a Novel Scavenger Mechanism of Microglia and Astrocytes

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Background and Purpose—Because the mechanism for scavenging acidophilic electron-dense dead neurons disseminated among the neuritic networks of surviving neurons in the ischemic penumbra of the cerebral cortex is still obscure, we investigated the fate of them up to 24 weeks after the ischemic insult.

Methods—Stroke-positive animals were selected according to their stroke index score during the first 10-minute left carotid occlusion done twice with a 5-hour interval. The animals were killed at various times after the second ischemic insult. Ultrathin sections including the second through fourth cortical layers were obtained from the neocortex coronally sectioned at the infundibular level in which the penumbra appeared and was observed by electron microscopy. We determined the percentages of resting, activated, and phagocytic microglia and astrocytes in the specimens obtained at various times postischemia.

Results—The electron-dense neurons had been fragmented into granular pieces by invading astrocytic processes from the periphery of the dead neurons and only the central portion remained. These granular pieces were dispersed along the extracellular spaces in the neuropil. By 8 to 24 weeks, the central core portion became a tiny vesicular particle (3.5 to 5.5 μm in diameter) with a central dot. Microglia and astrocytes phagocytized these dispersed granular pieces.

Conclusions—We found a novel scavenger mechanism in the ischemic penumbra, one by which dead neurons were fragmented by invading small astrocytic processes and only a thinned-out core portion remained, which finally became a tiny vesicular particle. The dispersed fragmented pieces were phagocytized by the microglia and astrocytes late, at 8 to 24 weeks postischemia. (Stroke. 2007;38:2577-2583.)

Key Words: cortical ischemic penumbra ■ phagocytosis ■ scavenging of dead neurons ■ transient cerebral ischemia

Previously, we investigated the neuronal remodeling process of the neurons that survived in the cortical ischemic penumbra after transient ischemia.1 We investigated the scavenger mechanism involved in removing the acidophilic dead neurons in the same penumbra. Regarding the maturation of the lesions in ischemic stroke,2 numerous studies investigating the delayed neuronal death have been performed during the past 3 decades.3–6 In the classical neuropathology seen after cerebral ischemia, the cytoplasm of the ischemic neuron becomes markedly eosinophilic, and the nucleus appears shrunken and darkly stained or develops clumped chromatin condensations. In the later stage, the cytoplasm is uniformly structureless, and the nucleus shows advanced degeneration and appears homogeneous. Activated microglial cells accumulate around the dying neurons and scaveng them by neuronophagia.3 However, as Rosenblum stated in his review article,6 the histopathologic literature reveals uncertainty concerning the fate of all the acidophilic neurons after ischemia/hypoxia. The morphological description of the “eosinophilic ghost cell” is very limited in several papers,7–10 a vague term used for morphological description at the light microscopic (LMS) level of necrotic neurons with a faintly stained nucleus and cytoplasm.

In previous studies, a clear separation of infarction and penumbra was not made. However, by giving a threshold amount of temporary ischemic insult to induce cerebral cortical infarction, we have developed a model in which a slowly developing large ischemic penumbra forms around a focal infarction in the cerebral cortex of Mongolian gerbils.11,12 In this model, by dividing the ischemic insult into 2 parts, the mortality rate of the animals attributable to epileptic seizure is decreased drastically,11 the infarction size becomes

Received February 6, 2007; final revision received March 9, 2007; accepted March 13, 2007.
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Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.107.484394
uniform so that focal infarction appears in face A and the penumbra in face B, and the cellular response to ischemic injury is similar to the model of less than 20-minute single ischemia but progresses more uniformly.1,2,12–15

In the present study, using this model and focusing on the scavenging mechanism, we investigated the fate of these disseminated dead neurons in the ischemic penumbra from 4 days to 24 weeks after the transient ischemic insult to answer the following questions: (1) Is such a neuron phagocytized by a single phagocytic cell or by many such cells? This question arises in light of the following facts: In the ischemic penumbra, where neurons die compacted in a disseminated fashion, the neuropil is still tight with narrow and complicated extracellular spaces, and the blood–brain barrier is not broken. Therefore, bloodborne macrophages cannot enter the neuropil to phagocytize them. These electron-dense dead neurons have long neuronal processes making a complicated network with the surviving neurons. (2) Are these structures phagocytized by the intrinsic resident microglia, in which cells are reported to respond to injury gradually, first becoming activated and later being transformed into macrophages?1,6,16,17

Materials and Methods

Under anesthesia with 2% halothane, 70% nitrous oxide, and 30% oxygen, a midline cervical incision was made and the left carotid artery of adult male Mongolian gerbils (60 to 80 g) was twice occluded with a Heifetz aneurysmal clip for 10 minutes each time with a 5-hour interval between the 2 occlusions. Anesthesia was discontinued immediately after each cervical surgery, and the behavior of the animals was carefully observed in the awake condition for 10 minutes after the first carotid occlusion.

Ischemia-positive animals were selected based on over 13 points of the stroke index score determined during the first occlusion.18 These animals were killed at 5, 12, 24, or 48 hours or 4 days or 1, 4, 8, 12, or 24 weeks after the last ischemic insult by intracardiac perfusion with diluted fixative (1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer) for 5 minutes followed by perfusion with concentrated fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.1 mol/L cacodylate buffer) for 20 minutes (3 animals in each time group for electron microscopy [EM]) or with 10% phosphate-buffered formaldehyde fixative for 30 minutes (5 animals in each time group for LMS).

In this model, after restoration of blood flow, an ischemic penumbra with progressing disseminated selective neuronal necrosis appears in the coronal face sectioned at the infundibular level (face B), and focal infarction evolves among the disseminated selective neuronal necrosis in the coronal face sectioned at the optic chiasma (face A).

We used 3 animals for each time point in the EM study, and from each of them, 2 adjacent ultrathin sections, including the second through fourth cortical layers, were obtained from the left cerebral cortex at the midpoint between the interhemispheric and rhinal fissures on face B. The sections were double-stained by uranyl acetate and lead solution and observed with an EM (H9000; Hitachi). Paraffin sections were separately stained with hematoxylin–eosin or periodic acid fuchsin Schiff (PAS).

Using an eyepiece micrometer (U-OCMSQ10/10), we calculated the percentage of the tiny PAS-positive vesicular particles among PAS-stained remnants of thinned-out dead neurons that appeared in all cortical layers on face B, counting all of the dead neurons in one section from each of 5 animals at 8 (1132±108) and at 24 weeks (618.3±51.9) postischemia.

Six ultrathin sections for each time group were observed by EM under 1500 times magnification, and the number of microglia and astrocytes at various time points were counted by sliding the specimen cursor from one area to another and increasing the magnification in the various areas for detailed observation. We determined the percentage of resting, activated, and phagocytic microglia in the specimens obtained from the control and from the ischemic animals at 1, 4, 8, and 12 weeks after the ischemic insult (total number of all 3 kinds of microglia counted in each time group was 211.6±26.2). Likewise, the percentage of astrocytes, activated astrocytes, and phagocytic astrocytes in the same specimens was determined (total number of all 3 kinds of astrocytes counted in each time group was 104.6±14.6). The statistical difference between each time group was analyzed by analysis of variance followed by Bonferroni-Dunn test. All data given in the text and in the figures were presented as the average±SEM and a statistically significant difference was accepted at the *P*<0.05 level.

Results

Temporal Profile of the Fate of the Dead Neurons

In the ischemic penumbra of the cerebral cortex in face B viewed by LMS, eosinophilic ischemic neurons appeared and spread in disseminated fashion among the normal-looking neurons in the second through sixth cortical layers approximately 5 hours after the ischemic insult. Some of these eosinophilic cell bodies were remarkably shrunken with condensed and clumped nuclear chromatin at 12 to 48 hours (Figure 1A). These shrunken eosinophilic neurons were observed by EM as disseminated electron-dense dark neurons that were homogeneously condensed and surrounded by remarkably swollen astrocytic processes (Figure 2A).

By 4 to 7 days after the ischemic insult, the shrunken dark neurons had become fragmented into an accumulation of electron-dense granules by invading slender astrocytic cell processes containing glycogen granules and fibrils (Figure 2B–C). Small spots of condensed chromatin were scattered in the nucleus (Figure 2C, inset). These fragmented dark neurons were scattered among the viable neurons, astrocytes, and microglia (Figure 2D). By LMS, these fragmented neurons were observed to have a faint form without nuclear staining, and they looked like ghost cells when stained with PAS (Figure 1B). In face A, focal infarction evolved and developed among the disseminated selective neuronal necrosis from 12 hours to 4 days, and foamy macrophages increased in number in the liquefaction necrosis from 4 to 7 days.

By 2 to 8 weeks after the ischemic insult, the fragmented electron-dense granular debris of the dead neurons had become dispersed throughout the narrow extracellular spaces in the neuropil and scattered eccentrically from the remainder of the dead neurons (Figure 3A). Thus, the peripheral cytoplasm was tattered and thinned out with only an electron-dense central portion remaining (Figure 3A). By LMS, the PAS-stained ghost cells gradually reduced their size attributable to decay of their peripheral cytoplasm. At 8 weeks, tiny PAS-positive vesicular particles (3.5 to 5.5 μm in diameter), each consisting of a PAS-positive thin circular frame and central dot, appeared occasionally among the thinned-out ghost cells (Figure 1C, inset). During 12 to 24 weeks, remarkably slimmed tiny remnants of the eosinophilic ghost cells and the tiny PAS-positive vesicular particles (Figure 1D, inset) were observed by LMS to be grouped in the third and occasionally in the fifth cortical layer (Figure 1D). These vesicular particles were also observed to be disseminated independent of the previously mentioned groups (Figure 1D, inset). The average number of
the dead neurons in the cerebral cortex of face B decreased from 1132±108 to 618.3±51.9 (P<0.05) at 8 and 24 weeks postischemia, whereas the average percentage of the tiny PAS-positive vesicular particles among the dead neurons increased from 4.5±0.11 to 57.1±0.11 (P<0.05).

Electron Microscopy Observations of the Tiny Periodic Acid-Schiff-Positive Vesicular Particles Revealed
Particles composed of a central electron-dense core and a peripheral thin circular electron-dense frame corresponding to the tiny PAS-positive vesicular particles with a central dot was observed by LMS. Monolayers of clear cube-shaped structures had invaded the space between the 2 electron-dense structures and surrounded the frame. These cube-shaped structures consisted of axons with degenerated large mitochondria and electron-dense particles, astrocytic processes, and occasionally presynaptic axon terminals (Figure 3B–D). Occasionally, an electron-dense core was surrounded by multiple layers of processes with the same cube-shaped structures (Figure 3E).

The neuropil surrounding them was densely packed with voluminously enlarged polygonal axon terminals filled with synaptic vesicles, spines, and neurites of surviving neurons and showed only a very few degenerated axons (Figure 3B–E).

Small spots of condensed chromatin, like those seen in the nucleus of dead neurons (Figure 2C, inset), were also observed in the electron-dense cores (Figure 3B–E).

Activation and Phagocytic Activities of Resident Microglia and Astrocytes
Rod-shaped resting microglia were observed along the capillaries, in the neuropil, and capping the surviving neurons (Figure 4A). Their nucleus was oval or elongated with chromatin clumps beneath the nuclear envelope. The chromatin clumps were more prominent and occupied a larger proportion of the nuclear volume compared with those of the oligodendrocytes. The cytoplasm formed a thin rim around the nucleus and often extended out in processes, and displayed long, narrow, stringy granular endoplasmic reticulum and fewer microtubules than that of the oligodendrocytes.

Microglia showed activation as evidenced by a globular cytoplasm having a large round dark nucleus with a large amount of chromatin condensation along the nuclear membrane and a large nucleolus. This round nucleus was surrounded by a narrow cytoplasm rich in ribosomes and rough-surfaced endoplasmic reticulum. Two cells that had fused together were often seen (Figure 4B). The percentage of these activated microglia in the control was 9.1±0.35%; and in the ischemic gerbils, 21.2±0.8% at 1 week, 23.5±3.5% at 4 weeks, 46.6±0.9% at 8 weeks, and 18.0±5.5% at 12 weeks after the ischemic insult (Figure 6A).

Astrocytes showed activation as evidenced by a large amount of chromatin condensation along the nuclear membrane and a large nucleolus (Figure 5A). Activated astrocytes were also observed along with fusion of 2 cells (Figure 5B). These activated astrocytes were 0% in the control; and in the ischemic gerbils, they constituted 9.60±3.1% at 1 week, 19.4±2.4% at 4 weeks, 45.9±2.1% at 8 weeks, and 11.1±1.8% at 12 weeks after the ischemic insult (Figure 6B).

From 8 to 24 weeks, by LMS observation, we frequently detected microglia filled with cytoplasmic PAS-positive materials (Figure 1D, inset). EM observation of their cytoplasm revealed phagosomes containing laminated membrane structures, electron-dense debris, and lysosomes (Figure 4C). These heterolysosomes containing ingested materials and lysosomes were surrounded by single-membrane-bound structures (Figure 4C, inset). These phagocytic microglia were first observed at 8 weeks (7.0±0.8%) and increased in percentage at 12 weeks (42.1±11.1%) after the ischemic insult (Figure 6A). Phagocytic astrocytes (Figure 5C) showed the same type of heterolysosomes as the microglia during these periods (Figure 5C, inset). They were first observed at 8 weeks (7.6±2.3%) and increased in percentage at 12 weeks (34.2±1.8%) after the ischemic insult (Figure 6B).
Throughout this study, no inflammatory cells or blood-borne macrophages appeared in the neuropil in the ischemic penumbra. Also, apoptotic bodies were not observed in the neurons or in the microglia.

Discussion

Temporal Profiles of the Fate of Dead Neurons

We found a novel scavenger mechanism for the removal of disseminated dead neurons in the ischemic penumbra. The electron-dense dead neurons in the ischemic penumbra were fragmented to electron-dense granular debris by the invasion of multiple astrocytic cell processes. These fragmented and pulverized debris of dead neurons became dispersed throughout the narrow extracellular space of the neuropil with the thinned-out central core portions of the dark neurons remaining. A recent ultrastructural study showed that sporadically distributed compacted dead dark neurons in the nonnecrotic tissue, in which death was induced by condenser-discharge electric shock, underwent cytoplasmic fragmentation. That report described that these fragments were engulfed by astrocytes and transported to the capillaries and removed through blood vessels and that neither proliferation of microglial cells nor infiltration of hematogenous macrophages was observed. In the present study on the ischemic penumbra, where tissue necrosis did not occur, the fragmented debris were not removed immediately by blood vessels or by phagocytosis by microglia, in which cells were in the process of becoming activated from 1 to 8 weeks after the ischemia, but these debris were later removed by microglia and astrocytes.

It has been thought that dead neurons and ischemically injured tissue are scavenged by activated resident microglia and/or macrophages that have invaded into the injured tissue from the bloodstream. However, in the cortical ischemic penumbra, compacted dead neurons were found in a disseminated fashion (disseminated selective neuronal necrosis) among the surviving neurons. The dendrites and axons of the neurons have been fragmented by invading slender astrocytic processes (arrows) (bar=1.6 μm). C, Dead neuron 4 days after ischemia. Glycogen granules (arrows) and glial fibrils (arrowheads) are noticed in these invading astrocytic cell processes. DN, dead neuron. Bar=0.71 μm. Inset, Small spots of condensed chromatin (arrows) are scattered in the nucleus (Nc). Cp, cytoplasm. Bar=0.67 μm. D, One week after the ischemic insult, fragmented shrunken electron-dense dead neurons (DN) are scattered among the viable neurons (N), astrocytes (A), activated astrocytes (AA), mitotic astrocytes (MA), microglia (M), and activated microglia (AM) (bar=19.3 μm).
Dying neurons were still connected to multiple axon terminals and dendrites of the surviving neurons. Thus, these dying neurons could not be phagocytized by a single microglia or macrophage. In this situation, it is not surprising that the shrunken dead neurons would become fragmented and pulverized into electron-dense granular debris.

Final Remnants of the Dead Neurons

The average number of the dead neurons in the cerebral cortex of Face B decreased from 1132 ± 108 to 618.3 ± 51.9 at 8 and 24 weeks postischemia, whereas the average percentage of the tiny PAS-positive vesicular particles among the dead neurons increased from 4.5 ± 0.11 to 57.1 ± 0.11. By 24 weeks, remarkably slimmed tiny eosinophilic dead neurons and the tiny PAS-positive vesicular particles remained in a group, and the latter were often scattered, in the third and fifth cortical layers of the cerebral cortex. These vesicular particles seem to be the final remnants of the dead neurons.

The neuropil surrounding these particles was densely packed with voluminously enlarged polygonal axon terminals filled with synaptic vesicles, spines, and neurites of surviving neurons and showed only a very few degenerated axons (Figure 3B–E). Therefore, we consider the cube-shaped structures with degenerated large mitochondria and electron-dense particles, astrocytic processes, and occasional presynaptic axon terminals, to be around the frame and have invaded (arrow) between these structures (bar=0.70 μm). Disarranged vesicular particle. Between the small electron-dense central core and disrupted electron-dense surrounding circular frame (arrowheads), multilayered clear cubic arrangements with large degenerated mitochondria have filled in the space. The circular frame is also surrounded by a multilayered cubic arrangement (bar=1.25 μm). The central electron-dense core (arrowhead) is surrounded by multiple layers of cubic arrangement (bar=0.82 μm).

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However, it is uncertain whether all slimmed ghost cells are destined to become these tiny vesicular particles. These particles have not been reported previously in the literature.
Further longer-term quantitative analysis of various ultrastructures showing the formation and fate of these tiny PAS-positive vesicular particles is necessary.

**Activation and Phagocytic Activities of Resident Microglia and Astrocytes**

Generally, the microglial response to various neuronal injuries occurs gradually. First, microglia proliferate and become activated; and second, they are transformed into intrinsic brain phagocytes. However, at the site of ischemic damage, microglial proliferation and activation has been reported to occur rapidly within the first 24 hours after an ischemic insult. Within the infarct, phagocytes with a foamy cell appearance, those derived from local resting microglia and/or from the bloodstream, are abundant within a few days. All of these studies were done on ischemic lesions where the infarction and penumbra were not clearly separated. In the ischemic penumbra, in the present study, microglial proliferation and activation occurred gradually from 1 to 12 weeks after the transient ischemic insult, and phagocytic microglia were first prominent at 8 to 12 weeks.

Microglial and astroglial activation often occurs in concert induced by interleukin-1 during central nervous system injury. Reactive astrocytes may contribute to neuronal remodeling of the surviving neurons by secreting neurotrophic factors. Phagocytic activity of astrocytes in adults after various brain injuries is still controversial. Astrocytic phagocytosis of carbon particles inserted around the needle injury of the adult rat cerebral cortex and uptake of Indian black particles by cultured human adult astrocytes have been reported. In the present study, astrocytic proliferation and activation occurred from 1 to 12 weeks postischemia; and heterolysosomes in which ingested materials and lysosomes were surrounded by a single membrane-bound structure were observed at 8 to 12 weeks after the ischemic insult, almost in accordance with the microglial changes.

Primarily, apoptosis was defined by EM. In the present study, our careful examination by EM revealed only necrotic findings in dying neurons, but no involvement of apoptosis in dying neurons or the mechanism responsible for scavenging them.

**Conclusions**

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in disseminated fashion among the surviving neurons were fragmented to become an accumulation of granular debris by the action of slender astrocytic cell processes that had invaded and separated the shrunken neurons into fragments. This debris was phagocytized by microglia and astrocytes late after the ischemic insult, i.e., at 8 to 24 weeks. The fragmented dead neurons became reduced in size attributable to decay of their peripheral cytoplasm. The tiny PAS-positive vesicular particles remained in groups, although the latter were often scattered, in the third and fifth cortical layers of the cerebral cortex.

Source of Funding

This work was supported in part by a grant from the Japanese Ministry of Health, Labor and Welfare, Research on Psychiatric and Neurological Diseases (H16-kokoro-017 to K.O.).

Disclosures

None.

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Stroke. 2007;38:2577-2583; originally published online August 2, 2007;
doi: 10.1161/STROKEAHA.107.484394

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