Temporal Profiles of Axon Terminals, Synapses and Spines in the Ischemic Penumbra of the Cerebral Cortex

Ultrastructure of Neuronal Remodeling

Umeo Ito, MD, PhD, FAHA; Toshihiko Kuroiwa, MD, PhD; Jun Nagasao, DVM; Emiko Kawakami, BS; Kiyomitsu Oyanagi, MD, PhD

Background and Purpose—Because the recovery process of axon terminals, synapses, and spine-dendrites in the ischemic penumbra of the cerebral cortex is obscure, we studied the temporal profile of these structures up to 12 weeks after the ischemic insult, using a gerbil model.

Methods—Stroke-positive animals were selected according to their stroke index score during the first 10-minute left carotid occlusion done twice with 5-hour interval. The animals were euthanized at various times after the second ischemic insult. Ultra-thin sections including the 2nd to 4th cortical layers were obtained from the neocortex coronally sectioned at the infundibular level, in which the penumbra appeared. We counted the number of synapses, spines and multiple synapse boutons, measured neurite thickness, and determined the percent volume of the axon terminals and spines by Weibel point counting method.

Results—The number of synapses, synaptic vesicles and spines and the total percent volume of the axon terminals and spines decreased until the 4th day. From 1 to 12 weeks after the ischemic insult, these values increased to or exceeded the control ones, and neuritic thickening and increase in number of multiple synapse boutons occurred.

Conclusions—In the ischemic penumbra, the above structures degenerated, with a reduction in their number and size, until 4 days and then recovered from 1 to 12 weeks after the ischemic insult. (Stroke. 2006;37:2134-2139.)

Key Words: axon terminal ■ dendrites ■ spine ■ synapses ■ transient cerebral ischemia

Cerebral infarction develops rapidly after a large ischemic insult. Earlier we developed a model of temporary ischemia in which a focal infarction surrounded by a large penumbra was produced in the cerebral cortex of Mongolian gerbils by giving a threshold amount of ischemic insult to induce focal infarction.1,2

Concerning the neuronal recovery, recent findings have revealed that synapses and their networks express a high degree of functional and structural plasticity.3 Ultrastructural changes in the postsynaptic density in hippocampal CA-1 were investigated after temporary ischemia.4-6 Degenerated boutons and multiple synapse boutons (MSBs) in this region were investigated by electron microscopy (EM) after temporary ischemia,7-9 as well as after temporary hypoxia/hypoglycemia in hippocampal slices.10 Changes in spines and dendrites were studied by time-lapse microscopy after temporary anoxia/hypoglycemia in cell culture.11,12 by light microscopy (LMS) of Golgi stain-impregnated sections of the 3rd to 5th cortical layers of the cerebral cortex after temporary ischemia,13 and by EM after temporary hypoxia/glycemia in hippocampal slice.10 Changes in CA-1 dendrites after temporary ischemia were investigated by light microscopy of horseradish peroxidase-injected specimens,14 by EM after temporary ischemia in CA-1,15,16 and by EM of Golgi stain-impregnated cerebral cortex after temporary ischemia for 20 minutes.17

Almost all of the above studies were performed in connection with delayed ischemia-induced injury to CA-1 neurons, and observations were made during a short period after ischemia. However, clinically, most patients show gradual recovery from behavioral dysfunctions after a stroke. The long-term integrated profile of axon terminals, synapses, spines, and dendrites during the recovery stage after an ischemic insult has remained obscure, especially in the ischemic penumbra of the cerebral cortex.

Because no functional recovery is anticipated in the infarction itself,18-20 we aimed to elucidate neuronal remodeling process in the ischemic penumbra in which neuronal death progresses in a disseminated fashion,2,18,20 by focusing on the temporal profiles of axon terminals, synapses, spines, and neurites.

Materials and Methods

Under anesthesia with 2% halothane, 70% nitrous oxide, and 30% oxygen, the left carotid artery of adult male Mongolian gerbils (60 to
80 g) was twice occluded with a Heifetz aneurismal clip for 10 minutes each time, with a 5-hour interval between the 2 occlusions, anesthesia was discontinued immediately after each cervical surgery, the animals soon became awake and moved spontaneously.

Ischemia-positive animals registering >13 points were selected based on the stroke index score determined during the first occlusion.21 The gerbils were euthanized at various times, i.e., at 5, 12, 24, 48-hour, 4 days, and 1, 5, 8, and 12 weeks after the ischemic insult. Anesthetization was followed 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 for EM (3 animals in each time group), or with 10% phosphate-buffered formaldehyde fixative for 30 minutes for LMS (5 animals in each time group).

In this model, after the restoration of blood flow, only ischemic pennumbra with progressing disseminated selective neuronal necrosis (DSNN) appeared in the coronal face sectioned at the infundibular level (Face B) and focal infarction evolved among the DSNN in the coronal face sectioned at the chiasm (Face A). Ultra-thin sections including the 2nd to 4th cortical layers were prepared from the left cerebral cortex at the mid-point between the interhemispheric and rhinal fissures on Face B, pennumbra >1 mm caudal to infarction edge. The sections were double stained with uranyl acetate and lead solution, and observed with an electron microscope (H9000, Hitachi). Paraffin sections of both faces were separately stained with hematoxylin-eosin (HE) or periodic acid fuchsin Schiff (PAS) or by Bodian silver impregnation or used for immunohistochemical detection of glial fibrillary acidic protein.

Placing 1.0 cm × 1.0 cm quadratic lattices of points on 5000 × 2.67 times enlarged EM photographs, we measured the number of synapses (synapses: consist of the pre- and postsynaptic densities associated with their cytoplasmic faces, and the synaptic cleft between them) and spines (spines: an ovoid bulb that is filled with a fluffy material and connected to dendrite directly or by a stalk) in each time group. We determined the thickness of 194 points (counting number varied according to the equation of the quadratic lattice in each time group. We measured the percentage of the axon terminals (axon terminals: presynaptic expansion of the axon that contains synaptic vesicles and mitochondria) and spines by using the point counting method22 in which the number of intersecting points touched by the axon terminals and/or spines were counted among 1000 to 15 000 points (counting number varied according to the equation of the relative error for different volumetric proportions) of the quadratic lattice in each time group. We measured the thickness of 194 ± 38 neurites (neurites: axons and dendrites those contain microtubules, neurofilaments and mitochondria; differentiation between them in transverse section is often difficult, especially in small ones) in each time group, as the maximal diameter perpendicular to their neurofilaments and/or microtubules. We also measured the percentage of MSBs by counting 327 ± 38 synapses in each time group, all on the same EM pictures. The statistical differences between each of the time groups were analyzed by ANOVA, followed by Bonferroni-Dunn test. All data in the Table and Figure 6 were presented as average ± SEM and a statistical difference was accepted at P < 0.05 level.

Results
In the ischemic penumbra of the cerebral cortex in Face B, eosinophilic ischemic neurons (HE staining) appeared in disseminated fashion among the normal-looking neurons in the 2nd to 6th cortical layers by LMS, around 5 hours after the ischemic insult. Some of these eosinophilic cell bodies became remarkably shrunken and died during the period of 12 to 48 hours, which indicates DSNN (Figure 1A). The eosinophilic ischemic neurons were found by EM to be disseminated electron-dense dark neurons that increased in number during the period of 12 to 48 hours after the ischemic insult.

From 4 days until 8 weeks, these condensed electron-dense dark neurons became fragmented into an accumulation of electron-dense granular fragments, which were observed by LMS as eosinophilic ghost cells of faintly formed cell bodies by HE and PAS staining (Figure 1B). During 2 to 12 weeks, these eosinophilic ghost cells accumulated in the 3rd and occasionally in the 5th cortical layer decreasing in size because of loss of their periphery (Figure 1B). In Face A, focal infarction evolved and developed among the DSNN from 12 hours to 4 days.

From 4 days to 12 weeks after the ischemic insult, the axon terminals of the surviving neurons were found being attached

<table>
<thead>
<tr>
<th>Percent of MSBs Among Synapses</th>
<th>Control</th>
<th>5 Hours</th>
<th>4 Days</th>
<th>1 Week</th>
<th>5 Weeks</th>
<th>8 Weeks</th>
<th>12 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>% MSBs</td>
<td>1.76 ± 0.82</td>
<td>0.85 ± 0.84</td>
<td>0.86 ± 0.67</td>
<td>1.18 ± 0.75</td>
<td>0.45 ± 0.45</td>
<td>3.14 ± 0.85</td>
<td>6.93 ± 2.24*</td>
</tr>
</tbody>
</table>

*Compared with control, 4 days, and 8 weeks; P < 0.05.
to the peripherally located electron-dense granular fragments and dendritic portions of the shrunken dark neurons. Some of these terminals appeared to have pinched off pieces of the dead neurons, and they bore a crust of the electron-dense granular fragments of the dead neurons (Figure 2A). Other axon terminals were found attached to the electron-dense thick neurites of dead neurons (Figure 2B). Some of the fragment-encrusted axon terminals were occasionally observed to have synapsed with the spines and neurites of the surviving neurons (Figure 3A). Some axons connected to the dying neurons showed globular and abnormal distensions of their terminals as seen by silver impregnation (Figure 4A). These structures appeared as degenerated axon by EM. The amplified degenerated axon contained degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules; the degenerated axon occasionally made synapses onto adjacent structures (inset of Figure 4A).

Such axons were often observed around the accumulations of the fragmented electron-dense granular pieces of the dead neurons (Figure 4B).

By 12 weeks after the ischemic insult, neuritic shafts and their branches were remarkably thickened (Figure 5B) compared with those at day 4 (Figure 5A), and they made synapses with voluminously enlarged and occasionally sprouting polygonal axon terminals filled with synaptic vesicles (Figure 5B). MSBs23 of the axon terminals (Figure 3B) increased in frequency compared with those of the control animals (Table).

The percent volume of the total axon terminals (Figure 6A) and spines (Figure 6B) in the neuropil decreased drastically to 30.9% and 24.8%, respectively, of the control value at that

**Figure 2.** Electron microscopy of 3rd layer of the cerebral cortex in Face B, 1 week after the ischemic insult. A, The axon terminals of the surviving neurons make contact with the periphery of the accumulated fragments of electron-dense granules of dead neurons (DN). Some of them appeared to have pinched off pieces of the dead neurons, and are encrusted by the electron-dense granular fragments of the dead neurons (arrows). Bar 2.3 μm. B, Some axon terminals are found attached to the electron dense thick neurites of dead neurons (arrows). Bar 2.6 μm.

**Figure 3.** A, Electron microscopy of the 3rd cortical layer in Face B, 1 week after the ischemic insult. Some axon terminals encrusted by the electron-dense granular fragments of the dead neurons are occasionally observed to have made synapses with the spines and neurites of the surviving neurons (arrow). Bar 3.1 μm. B, Electron microscopy of 3rd cortical layer of the cerebral cortex in Face B, 12 weeks after the ischemic insult. MSBs with >2 spines synapsed (arrows) to 1 axon terminal (a). A widened spine(s) with multiple synapses on axon terminal (a) is seen. Bar 3.2 μm.
time after the ischemic insult, with a decrease in frequency of synaptic vesicles especially those close to the synapses (Figure 5A). The number of synapses also decreased to 73.5% of the control value at 4 days, after a temporary increase up to 135% at 5 hours after the ischemic insult (Figure 6A). The number of spines also decreased to 35.8% of the control value, by 4 days. From 1 to 12 weeks after the ischemic insult, the percent volume of the total axon terminals (Figure 6A) and the percent volume of the total spines (Figure 6B) increased, being 162.8% and 86.7%, respectively, of the control value at 12 weeks. The number of synapses (Figure 6A) and spines (Figure 6B) also rose, becoming 113.2% and 91.9%, respectively, of it at that time.

The average thickness of neurites in the neuropil of the control animals were 0.607 μm. This value was unchanged at 0.587 μm at day 4, 0.604 μm at 1 week, and 0.665 μm at 8 weeks, and increased to 0.934 μm at 12 weeks after the ischemic insult (Figure 6C).

Discussion

In the neuropils of the ischemic penumbra in the cerebral cortex, we found a marked decrease in the number of the synapses and volume of the axon terminals from 5 hours to 4 days after the ischemic insult, along with a decrease in the number of synaptic vesicles. These changes may be attributed to a demolished synaptic neurotransmission attributable to calcium-dependent neuronal hyperexcitation and could be reduced by NMDA (N-methyl-D-aspartate) receptor antagonists as was reported in a morphological study recording excitatory postsynaptic potential from hippocampal slice cultures subjected to brief anoxia-hypoglycemia.

Almost in accordance with our present study, the LMS study of Golgi silver impregnated spine and dendrites showed that the number of spines and thickness of dendrites decreased maximally in 4 to 7 days after the temporary ischemia and recovered around 5 weeks in the 2nd to 3rd cortical layers of the rat cerebral cortex. Earlier studies on cultured

Figure 4. A, Light-microscopy. Four days after the ischemic insult, some axons attached to dying neurons show globular and abnormal distension of their terminals (arrow-heads). Bar 8.9 μm. Bodian’s silver impregnation. Inset: EM observation of the distended degenerated axon 3 weeks after the ischemic insult. It contains degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules and has synapses along its wall (arrows). Bar 1.3 μm. B, Electron microscopy of the 3rd layer of the cerebral cortex in Face B, 2 weeks after the ischemic insult. These amplified degenerated axons (arrows) are observed around accumulations of the fragmented electron-dense granular pieces of the dead neurons (DN). Bar 0.6 μm.

Figure 5. Electron microscopy of the 3rd layer of the cerebral cortex in Face B. A, Four days after the ischemic insult. The volume of axon-terminals and spines has decreased with a decrease in frequency of synaptic vesicles, especially those close to the synapse (arrows). The thickness of degenerated neurites has decreased slightly compared with that of the control (arrowheads). Bar 1.5 μm. B, Twelve weeks after the ischemic insult. The neuritic shafts and their branches are remarkably thickened (arrowheads) and make synapses with voluminously enlarged and occasionally sprouting polygonal axon terminals filled with synaptic vesicles (arrows). Bar 1.5 μm.
neurons showed a decreased spine number and segmental dendritic beading after temporary hypoxia/hypoglycemia followed by recovery,11,12 and a LMS study using horseradish protein injection showed beading of dendrites in the CA-1 of the hippocampus after temporary ischemia.14 Also an EM study on the CA-1 showed degeneration and shrinkage of dendrite around 3 to 4 days after temporary ischemia.15–17 In the present study, we found that the neurites degenerated around 4 days and that their thickness increased, in association with the recovery to normal of the number and percent volume of spines, at 12 weeks after the insult.

From 1 to 12 weeks after the ischemic insult, we found that the synaptic number increased gradually in association with an increase in the volume of axon terminals showing sprouting. The present study also showed an increase in the number of the MSBs from 8 to 12 weeks after the ischemic insult, which increase was associated with one in the number and volume of axon terminals and spines. The MSBs represent 2 independent dendritic spines contacting the same axon terminal.23 One spine branched to make synapses at >2 portions of 1 axon terminal is considered to facilitate neurotransmission.10 In another study, there was an increase in the number of MSBs in CA-1, paralleling the marked increase in the number of synaptic vesicles after temporary ischemia7 and after temporary hypoxia/hypoglycemia in hippocampal slices.10

From 4 days to 12 weeks after the ischemic insult, some axons attached to the dying neurons showed an abnormal distension of their terminals, which contained degenerated mitochondria, laminated dense bodies, and irregularly located neurofilaments and microtubules (degenerated axon).8,9 They were frequently observed around accumulations of the electron-dense granular fragments of the dead neurons.

Some axon terminals encrusted with the electron-dense granular fragments of the dead neurons became connected to the spines and neurites of the surviving neurons. These axon terminals, previously attached to the dying and/or dead neurons, seemed to become newly connected to the spines and to the thickened dendrites of the surviving neurons associated with synaptogenesis in the neuropil.24 However, it could also be that some of these axon terminals were originally contacting more than one dendrites or spines.

Clinically, most stroke survivors show recovery from behavioral dysfunctions. The short-term recovery may be attributable to the resolution of brain edema. A more gradual recovery, promoted by exercise for rehabilitation, may be attributed to the anatomical and functional recovery of the penumbra. Stroemer24 reported behavioral recovery after neocortical infarction in rats, which recovery was associated with neuronal sprouting followed by synaptogenesis, as demonstrated by immunohistochemical staining for GAP-43, a growth-associated protein expressed on axonal growth cones, and for synaptophysin. Functional remodeling of the cerebral cortex remote from the infarction was detected by intracortical microstimulation mapping of the hand of the squirrel monkey.25,26

Activation of the complement system was shown to promote neuronal survival and tissue remodeling.27 Postischemic treatment with brain-derived neurotrophic factor and physically exercised animals had better functional motor recovery, attributable to induction of widespread neuronal remodeling, as demonstrated by MAP1B and synaptophysin expression.19

Clinical introduction of novel agents and functional methods to promote synaptogenesis and neuronal networks in the ischemic penumbra, is highly anticipated.

Summary
In the penumbra around a focal infarction of the cerebral cortex, synapses, synaptic vesicles, axon terminals, spines...
degenerated, with a reduction in their number and size, until 4 days and then recovered from 1 to 12 weeks after the ischemic insult.

**Source of Funding**

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

**References**

Temporal Profiles of Axon Terminals, Synapses and Spines in the Ischemic Penumbra of the Cerebral Cortex: Ultrastructure of Neuronal Remodeling
Umeo Ito, Toshihiko Kuroiwa, Jun Nagasao, Emiko Kawakami and Kiyomitsu Oyanagi

Stroke. 2006;37:2134-2139; originally published online June 29, 2006;
doi: 10.1161/01.STR.0000231875.96714.b1
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/37/8/2134

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/