Ischemic Damage and Subsequent Proliferation of Oligodendrocytes in Hippocampal CA1 Region following Repeated Brief Cerebral Ischemia

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Key Words

Oligodendrocyte · Astrocyte · Hippocampal CA1 ·
Repeated brief cerebral ischemia · Proliferating cell nuclear
antigen · Glial fibrillary acidic protein

Abstract

Changes in oligodendrocytes and astrocytes following repeated brief periods of ischemia were studied in gerbils. The repeated brief periods of cerebral ischemia were produced by occlusion of bilateral common arteries for 3 × 3 min at 1-hour intervals. Oligodendrocytes and astrocytes in hippocampal CA1 regions were examined on 1, 3, 7 and 14 days after ischemia. Seven days after ischemia reperfusion, astrocytes, large pale and small dark oligodendrocytes entered the necrotic pyramidal neuron layer and coexisted with microglia. The necrotic matter of neurons and phagolysosome was observed in cytoplasm of some large pale and small dark oligodendrocytes. Most oligodendrocytes also intermingled with reactive astrocytes. These reactive astrocytes showed glial fibrillary acidic protein-positive reaction. Furthermore, proliferating cell nuclear antigen-positive cells increased markedly 7 days after reperfusion. We conclude that the repeated brief cerebral ischemia caused proliferation of oligodendrocytes and astrocytes, and oligodendrocytes may play a phagocytic role for clearance of necrotic matter of neurons.

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Introduction

Oligodendrocytes form the myelin sheath of axons in the central nervous system (CNS). Recent studies have suggested further possible functions of oligodendrocytes during regeneration. Membrane protein fractions of myelinating oligodendrocytes have been found to have an inhibitory effect on nerve growth. Galactolipids, components of oligodendrocyte plasma membranes, have been found to transfer development signals, facilitate protein trafficking and stabilize membrane [1–3]. Oligodendrocytes have also been shown to regenerate in demyelinating diseases and participate in remyelination [4–6]. Although oligodendrocytes can respond to damage and proliferate in the brain, the role of oligodendrocytes in the repair process after brain injury such as trauma and ischemia has not been fully investigated. Xie et al. [7], after brain stab wounds, found that the number of reactive oligodendrocytes along the wound track increased until 28 days after operation. Mandai et al. [8] described subsequent proliferation of oligodendrocytes in focal cerebral ischemia. It has been established that repeated brief cerebral ischemia causes delayed neuronal death of hippocampal CA1 pyramidal cells in rodents [9–12]. Although reactions of astrocytes and microglia in delayed neuronal death were well studied [13–19], changes in oligodendrocytes are not well known. Studies of the prolifer-

erative and migratory reactivity of various glial cells in the adult hippocampus have indicated that after injury or ischemia, astrocytes might become reactive, change morphology and appear to multiply and/or migrate into the degenerating hippocampal subfields [14, 16, 19]. However, it is still unclear whether oligodendrocytes respond to neuronal damage and proliferate following brief cerebral ischemia. In addition, their exact role in the hippocampus in response to injury or regeneration is not fully understood. In the present investigation, we employed electron microscope and immunohistochemistry for glial fibrillary acidic protein (GFAP) and proliferating cell nuclear antigen (PCNA) to examine the changes in oligodendrocytes and astrocytes in hippocampal CA1 region following repeated brief cerebral ischemia.

Materials and Methods

Animals

A total of 27 male Mongolian gerbils, aged 12–14 weeks and weighing 55–70 g, were used: 3 animals for the control group and the rest for the experimental groups. They were allowed free access to food and tap water before and after surgery.

Repeated Cerebral Ischemia

Repeated cerebral ischemia was induced using a slightly modified method described by Chung et al. [10]. Body temperature was maintained at 37°C using a heating pad and a heating lamp during the operation and occlusion until the righting reflex reappeared. The gerbils were anesthetized with chloral hydrate. Bilateral common carotid arteries were exposed and occluded with aneurysm clips 3 × 3 min at 1-hour intervals. After removing the clips, restoration of blood flow was verified, the skin incision was sutured, and the animals were allowed to survive for 1, 3, 7 or 14 days. The gerbils in the control group underwent the same procedure as the experimental animals with the exception of arterial occlusion. The experiments were performed based on the Helsinki Guide for the care and use of laboratory animals.

Immunohistochemical Staining

The streptavidin biotin peroxidase complex method was employed for immunohistochemical staining of GFAP (astrocytes marker) and PCNA (cell proliferation). Briefly, deparaffinized sections were treated for 30 min with 0.3% H₂O₂ to block endogenous peroxidase and rinsed again 3 × 5 min in PBS. Sections were then incubated for 30 min with block of nonspecific reactive sites by applying 1:20 normal goat serum, and incubated with monoclonal anti-GFAP antibody (Sigma) or monoclonal anti-PCNA antibody (Dako) for 60 min. After washing in PBS, they were incubated with goat anti-mouse immunoglobulins for 30 min and then incubated with a mixture of streptavidin and biotinylated horseradish peroxidase for 30 min. After washing in PBS, they were reacted with 0.05% 3,3'-diaminobenzidine containing 0.01% H₂O₂. The PCNA-positive cells were quantitatively assessed using the WinROOF image analysis software (Mitani Corp.). The number of PCNA-positive nuclei was counted in a

total of 5 randomly selected microscopic fields at ×20 objective lens (0.56 mm²) and the mean number was determined. The statistical significance of data was assessed using paired-sample t test and a *p* < 0.05 was considered as significant.

Electron Microscopy

At 1, 3, 7 and 14 days after arterial occlusion, the animals were anesthetized with chloral hydrate, and in each case the brain was briefly washed by transcardiac perfusion with heparinized saline, followed by perfusion-fixation with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 37°C, at a pressure of 150 cm H₂O). The specimens were fixed with 2.5% glutaraldehyde for 1 day, postfixed with 1% osmium tetroxide for 90 min, dehydrated in graded alcohol series, and embedded in Epok 812. Thin sections were cut with an ultramicrotome, and then stained with uranyl acetate and lead citrate. The stained sections were examined under a transmission electron microscope (Hitachi, Japan).

Results

Light-Microscopic and Immunohistochemical Observations

In control animals, hippocampal CA1 region could be divided into 5 layers: stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum moleculare and stratum moleculare (fig. 1a). There were many glial cells in stratum lacunosum moleculare and stratum moleculare, but only scanty glial cells in stratum oriens, stratum pyramidale and stratum radiatum. A few GFAP-positive cells were seen within stratum oriens, stratum radiatum and stratum lacunosum moleculare (fig. 1d).

After 1 day of postischemic reperfusion, slightly shrunken neurons with a homogeneously eosinophilic cytoplasm and karyolytic nucleus were found intermingled with apparently normal neurons. The glial cells in all layers did not have a remarkable change. The immunoreactions for GFAP and PCNA did not differ from control.

After 3 days of reperfusion, hippocampal CA1 neurons in stratum pyramidale showed severe ischemic damage. Vacuolization, homogeneously eosinophilic cytoplasm and karyopyknosis were noted in almost all CA1 neurons (fig. 1b). Many GFAP-positive and a few PCNA-positive cells appeared in stratum oriens and stratum radiatum.

After 7 days of reperfusion, hippocampal CA1 neurons in stratum pyramidale were completely destroyed, and numbers of glial cells in stratum oriens, stratum pyramidale and stratum radiatum markedly increased (fig. 1c). Many strong GFAP-positive astrocytes, which had relatively fewer, thicker and shorter cell processes, were observed in all layers of hippocampal CA1 region

Fig. 1. Changes in light-microscopic features (**a–c**) as well as GFAP (**d, e**) and PCNA (**f**) immunohistochemical stainings in the hippocampal CA1 following repeated brief cerebral ischemia. Normal control hippocampal CA1 region (**a, d**), 3 days (**b**) and 7 days (**e, f**) after reperfusion. $\times 40$. At 3 days after reperfusion (**b**), the ischemic damage of pyramidal cells and increase in number of glial cells were noted. At 7 days after reperfusion, the pyramidal cells were completely destroyed, and the number of glial cells in

stratum oriens, stratum pyramidale and stratum radiatum were markedly increased (**c**). GFAP staining showed an abrupt increase in positive astrocytes (**e**). PCNA staining also showed many positive cells in stratum oriens, stratum pyramidale and stratum radiatum (**f**). or = Stratum oriens; p = stratum pyramidale; ra = stratum radiatum; lm = stratum lacunosum moleculare. Scale bar = 100 μm .

Fig. 2. Ultrastructural changes of oligodendrocytes in hippocampal CA1 regions. **a** Control: 2 different types of oligodendrocytes are shown: large size with pale cytoplasm (arrow) or small size with dark cytoplasm (arrowhead). **b** Three days after reperfusion: 2 small dark oligodendrocytes displayed dilations of the nuclear cleft, a thin rim of cytoplasm and dilatation in the endoplasmic reticulum. **c** Seven days after reperfusion: A large pale oligodendrocyte showed obvious nucleus, little heterochromatin and necrotic matter was the same as surrounding necrotic neurons in cytoplasm (arrows). **d** Fourteen days after reperfusion: Necrotic matters as the broken chromatin of neurons are shown in the cytoplasm of small dark oligodendrocyte (arrows). OL = Large pale oligodendrocyte; OS = small dark oligodendrocyte.

(fig. 1e). The reactive astrocytes were more numerous and close to the stratum pyramidale. Paired astrocytes were occasionally seen. Many PCNA-positive cells also appeared in hippocampal CA1 region and mainly presented in destroyed stratum pyramidale (fig. 1f). The mean PCNA-positive rate was 0 in control, 6.3 ± 1.2 in 3 days and 16 ± 3.7 in 7 days after reperfusion groups.

Electron-Microscopic Observation

In control gerbil hippocampal CA1 region, oligodendrocytes could be classified by size and staining pattern into large pale and small dark oligodendrocytes. The large pale oligodendrocytes possessed a narrow pale cytoplasmic rim and nuclear pores (fig. 2a). The small dark oligodendrocytes had many free ribosomes, thin cyto-

plasmic rim and narrow nuclear cleft (fig. 2a). Cytoplasm of astrocyte was relatively electron lucent compared with neuronal cytoplasm, and contained short cisternae of rough endoplasmic reticulum, scattered polyribosomes and microtubules, and rare glycogen rosettes (fig. 3a). The astrocytic nuclei were round to oval, with finely dispersed chromatin and a thin rim of marginated chromatin at the nuclear membrane. Rarely, glial filaments were seen in the perivascular processes.

After 3 days of reperfusion, the majority of neurons displayed severe signs of degeneration. At this stage, microglia, large pale and small dark oligodendrocytes were noted in hippocampal CA1 region. A thin rim of cytoplasm and dilatation in the endoplasmic reticulum were prominent in small dark oligodendrocytes (fig. 2b). As-

Fig. 3. Ultrastructural changes of astrocytes in hippocampal CA1 region. **a** Control: The cytoplasm of astrocyte was relatively electron lucent as compared with neuronal cytoplasm, and contains short cisternae of rough endoplasmic reticulum, scattered polyribosomes and microtubules, and rare glycogen rosettes. The astrocytic nucleus was oval, with finely dispersed chromatin and a thin rim of marginated chromatin at the nuclear membrane. **b** Three days after reperfusion: Glial filaments were increased in the cytoplasm of astrocyte. **c** Fourteen days after reperfusion: Many astrocytes appeared in the hippocampal CA1 region. These reactive astrocytes showed a marked increase in glial filaments.

trocytic processes were swollen compared to the controls. Glial filaments increased in the foot processes. Polyribosomes, microtubules and glycogen granules were rarely identified. The nuclei enlarged and had a slightly irregular outline. Chromatin was more dispersed. Glial filaments in perivascular foot processes were increased (fig. 3b).

After 7 days of reperfusion, almost all neurons were destroyed. Many pale oligodendrocytes appeared in such destroyed neurons in hippocampal CA1 region. As for the nuclei of these oligodendrocytes, heterochromatin was hardly found and the nucleolus was obvious. The necrotic matter in the cytoplasm, the same as the surrounding necrotic neurons, was observed (fig. 2c).

Many microglia and small dark oligodendrocytes were also observed in this region. Large pale and small dark oligodendrocyte took in the necrotic matter of neurons (fig. 2c). Many phagolysosomes were observed in the cytoplasm of oligodendrocytes and microglia (fig. 4). After 14 days of reperfusion, almost all pyramidal neurons disappeared, and the oligodendrocytes and microglia observed in a similar region as at 7 days (fig. 2d). Astrocytes had an enlarged euchromatic or round nuclei and plentiful cytoplasm containing many glial filaments, some microtubules and lipid droplet. Many glial filaments in pericapillary foot processes of astrocytes were observed (fig. 3c).

Fig. 4. At 14 days after reperfusion, there was a large phagolysosome in the cytoplasm of microglia.

Discussion

In this study, we observed that large pale and small dark oligodendrocytes after 7 days of postischemic reperfusion entered the necrotic pyramidal neurons layer and coexisted with microglia. The necrotic matter of neurons and phagolysosome were observed in the cytoplasm of some large pale and small dark oligodendrocytes. Most oligodendrocytes also intermingled with reactive astrocytes in stratum oriens and stratum radiatum. Many PCNA-positive cells also appeared in hippocampal CA1 region and mainly presented in destroyed stratum pyramidale. These results showed that oligodendroglial proliferation occurred in the hippocampal CA1 region after repeated brief cerebral ischemia.

The importance of regeneration of oligodendrocytes and their role in remyelination have recently been receiving increasing attention in view of the possible potential recovery from demyelinating disease. The relationship between oligodendrocyte proliferation and remyelination has been shown to be of significance [20]. Oligoden-

drocytes are responsible for myelin in the CNS. Full recovery of function following injury to the CNS requires oligodendroglial regeneration. Under some conditions, oligodendroglial proliferation is possible in adult brain such as virus-induced damage [21], cuprizone-derived lesions [22], physical trauma [7, 23], multiple sclerosis [5, 6] and focal cerebral ischemia [8]. Proliferation and hypertrophy of oligodendrocytes in the adult CNS under pathological conditions are presently of interest to neurobiologists. Herndon et al. [21] found that oligodendrocytes were responsible for remyelination in mice infected with JHE hepatitis virus. Ludwin and Sternberger [22] found that oligodendrocytes incorporated tritiated thymidine following cuprizone-induced demyelination. Xie et al. [7], after brain stab wounds, found that the number of reactive oligodendrocytes along the wound track increased until 28 days after operation, and then decreased so that very few were found at 70 days after operation. In contrast, the number of GFAP-positive cells continued to increase throughout the time period's studies. Maxwell and Kruger [24] described that the oligodendrocytes probably underwent morphological changes only in response to damage to neurons or their processes, since the initiation of the oligodendrocyte response was closely correlated with the earliest indications of neuronal damage. Within 48 h following irradiation, oligodendrocytes in the laminar lesion zone were found to change in appearance, and these altered cells will be considered reactive oligodendrocytes. Several studies indicated that oligodendrocytes rapidly responded to cerebral ischemia. Mandai et al. [8] described subsequent proliferation of oligodendrocytes in focal cerebral ischemia. They used a model of middle cerebral artery occlusion and found that the majority of oligodendrocytes detected near infarction were intermingled with reactive astrocytes, but some oligodendrocytes were found to be coexistent with macrophages identified as large cells positive for lectin histochemistry. In the present study, we used a model of repeated brief cerebral ischemia and found that after 7 days of ischemic reperfusion, oligodendrocytes entered necrotic stratum pyramidale and were coexistent with microglia. Some oligodendrocytes entered into the necrotic matter of neurons. This indicated proliferation of oligodendrocytes after repeated brief cerebral ischemia and suggested that oligodendrocytes might be employed to remove necrotic matter of neurons.

Although the phagocytic ability of microglia and astrocytes has been well established, the question of whether oligodendrocytes may become phagocytic remains controversial. From a light-microscopic study of the reac-

tion of oligodendrocyte following the removal of a portion of the cerebral hemispheres from cats, Ferraro and Davidoff [25] concluded that oligodendrocytes were involved in the degeneration of axons and that ultimately they transformed into the so-called compound granular corpuscles (or scavenger cells). Cook et al. [26] also found that, during Wallerian degeneration in the distal 9–13 mm of the optic nerve in cats and monkeys, phagocytosis of the degenerating axons and myelin sheaths was performed almost totally by the oligodendrocytes. The presence of myelin fragments in the cytoplasm of oligodendrocytes, which may result from the uptake of degenerating myelin from surrounding tissue or from the retraction of the cell's own myelin into the cell body following separation from its axons, has also been reported [27]. Our results of the present experiments showed necrotic matter of pyramidal neuron in cytoplasm of some large pale and small dark oligodendrocytes, and provided ultrastructural evidence for the phagocytic activity of oligodendrocytes.

Oligodendrocytes have been shown to regenerate in demyelinating diseases and participated in remyelination [21]. Although oligodendrocytes can respond to damage and proliferate in the brain following trauma [7, 23], and transplantation of 0-2A progenitor cells [28] or cultured oligodendrocytes [29] can repair demyelinating damage in the brain, the role of oligodendrocytes in the repair process after brain injury such as trauma and ischemia has not been fully investigated. Mandai et al. [8] demonstrated accumulation of oligodendrocytes in the ischemia-induced brain lesion with in situ hybridization histochemistry. Furthermore, a double simultaneous procedure with in situ hybridization and GFAP immunohistochemistry or with in situ hybridization and lectin histochemistry showed that most accumulating oligodendrocytes mingled with reactive astrocytes, but some oligodendrocytes also entered the infarcted tissue and coexisted with macrophages/microglia. While proliferating oligodendrocytes were considered to participate in remyelination, newly synthesized proteolipid protein at the margin of infarction may play a yet unidentified role for tissue repair other than repair of myelin structure [30]. In the present study, we demonstrated proliferation of oligodendrocytes in the repeated brief cerebral lesion with electron microscopy. Most proliferating oligodendrocytes mingled with reactive astrocytes, but some also entered the necrotic pyramidal layer and coexisted with microglia. Cytoplasm of some large pale and small dark oligodendrocytes contains necrotic matter of pyramidal neurons. Phagolysosome also appeared in some oligo-

dendroglial cytoplasm. This result suggested that oligodendrocytes proliferated, took up necrotic matter of neurons and repaired the neuronal damage in repeated brief cerebral ischemia.

In this study, we showed electron microscopic findings of proliferation of oligodendrocytes; however, immunohistochemical approaches such as for Olig2 could also have efficient capability to identify oligodendrocytes [31].

We also found astrocyte reactive changes. From 3 days recirculation, many filaments appeared in astrocytic cytoplasm. By 7 days, many astrocytes appeared in stratum oriens, stratum pyramidale and stratum radiatum. Many paired astrocytes also were seen. This result agrees with previous observations showing an astrocytic hypertrophy in the hippocampus after treatment with neurotoxins or after ischemia [15, 17, 32].

In this study, we also performed PCNA immunostaining. PCNA is a nuclear acidic protein necessary for DNA replication that is expressed through the cell cycle [33]. PCNA expression can indicate proliferation and DNA replication, and has been frequently used as a marker of cell proliferation [34]. We used this index to investigate the proliferative capability of glial cells in hippocampal CA1 region, although Ki-67, another marker of cell proliferation which is more cell-cycle restricted, could be applied with probably equal results [35]. The number and intensity of PCNA-positive cells increased markedly after 7 days reperfusion. Most PCNA-positive cells appeared in necrotic pyramidal layer. The expression of PCNA in CA1 region following brief ischemia showed an increase in proliferative capability of glial cells including oligodendrocytes and astrocytes.

In conclusion, we have shown that oligodendrocytes proliferate into the lesion site from 3 days after ischemia. Using electron microscope, these proliferative oligodendrocytes can be identified as large pale and small dark oligodendrocytes. The necrotic matter of neurons and phagolysosome were observed in the cytoplasm of some large pale and small dark oligodendrocytes. Most oligodendrocytes also intermingled with reactive astrocytes in stratum oriens and stratum radiatum. The present study shows that the repeated brief cerebral ischemia caused delayed neuronal death and a subsequent proliferation of oligodendrocytes. The proliferative oligodendrocytes may play a phagocytic role for clearance of neuronal necrotic matter.

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