Enhanced Expression of Iba1, Ionized Calcium-Binding Adapter Molecule 1, After Transient Focal Cerebral Ischemia In Rat Brain

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Background and Purpose—Iba1 is a novel calcium-binding protein and is specifically expressed in microglia in the brain. It has been suggested that Iba1 plays an important role in regulation of the function of microglia. In the present study we examined time-dependent Iba1 expression after transient middle cerebral artery occlusion and characterized microglial activation in various brain regions.

Methods—Rat middle cerebral artery occlusion was induced by the intraluminal filament technique. After 1.5 hours of transient ischemia, Iba1 expression was examined by immunohistochemical and immunoblot analyses. The microglial activation in association with ischemic severity was characterized by double immunostaining with other specific markers.

Results—In the peri-ischemic area, heavily Iba1 immunoreactive cells rapidly appeared at 3.5 hours after reperfusion. Immunoreactivity was further increased and peaked at 7 days. In the ischemic core, round Iba1-positive cells, which may be blood-borne monocytes, appeared from 24 hours and reached a peak at 4 to 7 days. Double immunostaining revealed that activated microglia in the peri-ischemic area upregulated Iba1 expression but were negative for the macrophage marker ED1. ED1-positive cells were clearly restricted to the ischemic core.

Conclusions—These findings suggest the following: (1) Iba1 expression may be associated with microglial activation in ischemic brain, and Iba1 immunostaining can be useful to evaluate the pathophysiological roles of activated microglia in ischemic injury. (2) Expression of ED1 antigen is strictly restricted to severe ischemic damage, whereas activated microglia in the peri-ischemic area showed Iba1 upregulation without ED1. Therefore, microglia may exhibit difference of antigenicity in the severity of ischemic brain injury. (Stroke. 2001;32:1208-1215.)

Key Words: gene expression • ischemia • macrophages • microglia • middle cerebral artery occlusion • stroke, ischemic

Microglia, 1 of 3 types of glial cells, constitute approximately 5% of all glial cells in the central nervous system (CNS). Activation of microglia commonly occurs in the early response of the CNS to a wide variety of pathological stimuli, including axotomy, trauma, inflammation, degeneration, and ischemia. Microglia can contribute to the elimination of microorganisms and deleterious debris and may be involved in neuroprotection by producing neurotrophic factors. On the other hand, microglia also exert a cytotoxic function by releasing reactive oxygen species, nitric oxide, or inflammatory cytokines, which trigger neuronal damage.

Several studies have suggested that microglia may be rapidly and time-dependently activated after ischemia, and microglial activation may reflect the extent of severity of ischemic injury. Some activated microglia may be transformed into phagocytic microglia in severe ischemic damage. In addition, leukocytes begin to infiltrate into the ischemic region 12 to 24 hours after ischemia and transform into brain macrophages. It has been suggested that phagocytic microglia and infiltrating leukocytes may mediate secondary injury to potentially viable tissue. However, these speculations are based only on morphological analyses, and the real function of microglia in ischemic brain still remains unclear. There have been inherent limitations with the experimental methods used in the previous studies because no specific markers for evaluating the activation of microglia have been available.

Recently, Imai et al have isolated a novel EF hand protein, Iba1 (ionized calcium-binding adapter molecule 1), which is highly and specifically expressed in monocytic cell lines and cultured microglia. By immunohistochemical analyses, anti-Iba1 antibody was found to specifically recognize ramified microglia in normal rat brain. An updated study has...
reported that Iba1 is involved in the Rho family of small GTPase, Rac, and calcium signaling pathways and may be required for cell mobility and phagocytosis,29 and Iba1 protein was strongly upregulated in activated microglia within the regenerating facial nucleus. 8 Therefore, Iba1 may serve as a novel marker for detecting the activation of microglia by double immunostaining with another monoclonal antibody. However, expression of Iba1 has never been examined in cerebral ischemia.

The aim of the present study was to determine the temporal profile of Iba1 expression after transient focal cerebral ischemia by immunohistochemical and immunoblot analyses in the rat brain. We have also characterized the microglial activation in association with severity of ischemic tissue damage.

Materials and Methods

Animals and Middle Cerebral Artery Occlusion

This protocol received prior approval as meeting the Animal Experimentation Guidelines of Keio University School of Medicine. Adult male Sprague-Dawley rats (weight, 280 to 350 g; n=42) (Japan Laboratory Animals, Tokyo, Japan) were used for all experiments. The animals were anesthetized with a mixture of 1.0% to 1.5% halothane and 30% oxygen/70% nitrogen during the operation. A temperature probe (TD-300, Shibaura Electronics) was inserted into the rectum, and a heating lamp was used to maintain the rectal temperature at 37.0°C to 37.5°C. Middle cerebral artery (MCA) occlusion was induced by the intraluminal filament technique of Belayev et al,30 as described previously. 31–33 In brief, a 3-0 nylon monofilament suture (Matsuda Ikkadoro) was inserted from the right external carotid artery into the internal carotid artery to obstruct the origin of the right MCA. After the intraluminal filament had been set in position, the neck incision was closed with a silk thread. The animals were then allowed to recover from the anesthesia and were evaluated 1 hour after the MCA occlusion to assess the extent of their neurological deficits. They were graded as having severe, moderate, mild, or no deficit on the basis of the neurological examination method devised by Bederson et al.34 In brief, animals consistently circling toward the left (paretic) side were classified grade 3. Rats consistently showing a reduced resistance to lateral push toward the left side, with no circling movement, were classified grade 2. Rats with any amount of consistent forelimb flexion and no other abnormalities were assigned to grade 1. Rats with no neurological deficits were assigned to grade 0. Only animals with grade $\geq 1$ were used for further study.

After 90 minutes of MCA occlusion, the rats were reanesthetized with the same anesthetic combination as described above, and the intraluminal suture was carefully removed. The animals were allowed to survive for 3.5 hours (n=6), 12 hours (n=6), 24 hours.
Immunohistochemistry was performed according to the method described previously. At the end of the recirculation period, the rats were given an injection of pentobarbital (100 mg/kg IP). They were then perfused through the left ventricle with 4% paraformaldehyde in PBS. The brains were subsequently removed and immersed in 20% sucrose in PBS until the tissue sank. These brains were rapidly frozen in dry ice powder and sliced into 20-μm sections with a cryostat. Cryosections were washed in PBS, and endogenous peroxidase activity was inhibited by incubation in 0.3% H2 O2 in methanol for 30 minutes. After it was washed in PBS, background was blocked in PBS containing 1% bovine serum albumin (BSA) (Sigma) and 1.5% normal goat serum for 2 hours at room temperature and then incubated with a 1:1000 dilution of rabbit anti-Iba1 polyclonal antibody overnight at 4°C. Sections were then washed in PBS and incubated with a 1:500 dilution of goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Bio-Rad) for 2 hours at room temperature. The presence of antibody was detected by reaction with 0.05% diaminobenzidine tetrahydrochloride and 0.01% H2 O2 in 50 mmol/L Tris-HCl (pH 7.2). Adjacent sections were stained with cresyl violet for conventional histological examination.

For double immunostaining, sections were incubated for 2 hours at room temperature in PBS containing 1% BSA and 1.5% normal goat serum to block the background, followed by incubation overnight at 4°C with a rabbit anti-Iba1 polyclonal antibody and either ED1 monoclonal antibody (Serotec) (1:50 dilution), mouse anti–gliarial fibrillary acidic protein (GFAP) monoclonal antibody (G-5-A; DAKO) (1:50 dilution), FITC-labeled GSA I-B4 (B4-isolectin from Griffonia Simplicifolia) (Sigma) (20 μg/mL), or mouse anti–neuronal nuclei (NeuN) monoclonal antibody (Chemicon) (1:250 dilution). Sections were then washed in PBS and incubated for 2 hours at room temperature with Texas Red-conjugated goat anti-rabbit IgG antibody (1:30 dilution) (Amersham) and/or FITC-conjugated anti-mouse IgG antibody (1:30 dilution) (Amersham).

Immunoblot Analysis
Immunoblot analysis was performed according to the method described previously. At the end of the predetermined period after ischemia, the brain was rapidly frozen and sectioned in a cryostat from the caudal end to the rostral end until ischemic tissue was reached. A couple of sections were stained with cresyl violet and examined by light microscopy for anatomic orientation of the ischemic lesion. On the basis of this staining, cortex was excised from the ischemic and contralateral hemispheres to obtain tissue samples of peri-ischemic area, ischemic core, and contralateral area. The topography of the excised cortical areas is illustrated in Figure 1. The tissue was immediately homogenized in 10 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid-NaOH, pH 7.4 containing 5 mmol/L EDTA, 50% glycerol, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 14 000 rpm for 10 minutes to remove insoluble debris. Protein concentrations were measured by Bradford’s method with a kit from Bio-Rad. Tissue extracts (30 μg) were loaded on a 12% SDS-PAGE gel, and separated proteins were transferred onto a clear blot membrane-P (Atto). The membrane was blocked and incubated with an anti-Iba1 antibody, as described previously. After it was washed, Iba1 protein was then visualized by using a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Amersham) and the enhanced chemiluminescence system (Amersham).

Results
The distribution of neuronal damage was evaluated by cresyl violet staining. The histological changes were confined to the region perfused by the occluded right MCA, ie, the right frontoparietal cortex and the dorsolateral part of the striatum (data not shown). The evolution of ischemic damage followed a profile similar to that previously reported in an identical model of MCA occlusion. Briefly, acute neuronal damage, shrunken cell bodies, and perineuronal vacuolations were detected in the striatum and the cortex at 3.5 hours after reperfusion. Neuronal necrosis was found in ischemic lesions over a period of 24 hours followed by infarct by day 7.
24 hours after reperfusion, leukocyte infiltration occurred in the infarct area and peaked on days 4 to 7. After 14 days, a cavity formation was found in the ischemic core.

**Immunostaining of Iba1 Protein 3.5 to 48 Hours After Reperfusion**

Figures 2 and 3 show representative microscopic pictures stained with anti-Iba1 antibody in the contralateral cortex, peri-ischemic area, and ischemic core at 3.5 to 48 hours after reperfusion.

In the peri-ischemic area, strongly stained cells with highly branched processes were observed on the operated side from 3.5 hours, suggesting that these immunopositive cells were activated microglia on the basis of immunoreactivity and morphology (Figure 2B and 2E). However, the shape of microglia in the ischemic core was shrunken and irregular, and their processes had been already fragmented by 3.5 hours (Figure 2C). Degeneration of microglia in the ischemic core had clearly emerged by 12 hours (Figure 2F). In the contralateral cortex, only resting microglia with ramified thin processes were stained, and their immunoreactivity was only weak (Figure 2A and 2D).

After 24 hours, several round stained cells appeared in the ischemic core (Figure 3C). On the basis of their morphology, population, and time course, the majority of these cells were thought to be infiltrating monocytes.

At 48 hours after reperfusion, Iba1 immunoreactivity was increased in the activated microglia in the peri-ischemic area (Figure 3E). In the ischemic core, the number of round stained cells had increased, and they were distributed throughout the ischemic core area. Some of these cells had a large cytoplasm and looked like macrophages (Figure 3F).

**Immunostaining of Iba1 Protein 96 Hours to 14 Days After Reperfusion**

At 96 hours and especially at 7 days after reperfusion, there was a significant increase in the intensity of staining as well as in the number of activated microglia in the peri-ischemic area (Figure 4B and 4E). The number of round stained cells dramatically increased within the ischemic core, and these...
cells were present throughout the entire ischemic lesion (Figure 4C and 4F).

After 14 days the number and intensity of staining of activated microglia in the peri-ischemic area were significantly decreased compared with those after 96 hours and 7 days (Figure 4H). The ischemic core became fully necrotic, and cavity formation was noted, but some round stained cells were still observed (Figure 4I). The immunoreactivity of these cells at this time appeared to be weaker than that at day 7.

Expression of Iba1 Protein by Immunoblot Analysis
The changes in expression of Iba1 after ischemia/reperfusion were further investigated by immunoblot analysis with the use of the cortex excised from the contralateral cortex, peri-ischemic area, and ischemic core (Figure 5). The peri-ischemic area showed a slightly increased level of Iba1 expression 12 hours after reperfusion and a continuous increase until 7 days, followed by a moderate decline at 14 days. In the ischemic core, expression of Iba1 was markedly increased at 96 hours and peaked at 7 days. No upregulation of Iba1 protein could be detected in the contralateral cortex. These findings were consistent with the results of immunohistochemical staining described above.

Double Staining With Anti-Iba1 Antibody and Other Molecular Markers
Figures 6 through 8 illustrate the boundary zone between the ischemic core (right side) and the peri-ischemic area (left side) 7 days after reperfusion. Representative double staining images obtained with anti-Iba1 antibody and GSA I-B4, which is known to bind to both microglia and brain macrophages, derived from blood-borne monocytes, are presented in Figure 6. The anti-Iba1 antibody and GSA I-B4 completely recognized the same cells in the ischemic lesion and peri-ischemic area. In addition, we confirmed that immunoreactivity of anti-Iba1 and anti-GFAP antibody was not colocalized in ischemic brain tissue (Figure 7). These findings indicated that Iba1 expression is upregulated specifically in activated microglia and brain macrophages during the period after ischemia/reperfusion.

Figure 8 demonstrates double staining with anti-Iba1 antibody and ED1, which is used as a rat macrophage marker, and neuron-specific marker NeuN staining on a consecutive section. Cells positive for both Iba1 and ED1 were widely distributed in the ischemic core. In the peri-ischemic area where NeuN-positive cells remain viable (Figure 8D), many Iba1-positive activated microglia were detected, whereas very few ED1-stained cells could be identified, so that the border zone was clearly visible by ED1 staining.

Discussion
In the present study we demonstrated time-dependent Iba1 expression in microglia reacting to ischemia after transient MCA occlusion by immunohistochemical and immunoblot analyses. In the peri-ischemic area, heavily Iba1-positive
cells with highly branched processes rapidly appeared at 3.5 hours after reperfusion, and this immunoreactivity peaked at 7 days (Figures 2 through 4). These findings were consistent with those of immunoblot analysis (Figure 5). We have previously reported similar time-dependent Iba1 expression in activated microglia after axotomy,8 suggesting that Iba1 protein plays an essential role in the activation of microglia after neuronal injury. The present findings imply that Iba1 can be used as a novel biochemical marker for detecting the activation of microglia in ischemic brain.

In the ischemic core, the majority of microglia may degenerate by 12 hours after reperfusion (Figure 2F). Round Iba1-stained cells then appeared from 24 hours and reached a peak at 4 to 7 days (Figures 3C and 3F and 4C, 4F, and 4I). We could not distinguish between blood-borne brain macrophages and the phagocytic microglia into which activated microglia transform because Iba1 protein was expressed not only in microglia but also in monocyctic lineage cells.28 Since we could not observe any morphological transitions from microglia to brain macrophages in the present study, we speculate that the majority of round stained cells in the ischemic core may be blood-borne monocytes, which transform into brain macrophages. However, we cannot exclude the possibility that some activated microglia may have invaded into the ischemic core and transformed into phagocytic microglia.

Recently, Ohsawa et al29 demonstrated that Iba1 protein, which is involved in Rac and the calcium signaling pathway, may be responsible for cell migration and phagocytic activity of microglia/macrophage. On the basis of these findings, upregulation of Iba1 in activated microglia in the peri-ischemic area can be assumed to contribute to cell migration, whereas Iba1 protein in brain macrophages in the ischemic core may be involved in phagocytic activity.

Another important finding in the present study was that activation of Iba1-positive microglia and brain macrophages in both the ischemic core and peri-ischemic area was clearly characterized. ED1-positive cells were apparently restricted to the ischemic core, where massive neuronal damage was observed (Figure 8). ED1 is known as a useful macrophage marker in rat.16 Its antigen is expressed on the membrane of cytoplasmic granules, like phagolysosomes, but its true function remains unclear.38 In the CNS, ED1 is not detected in resting microglia, whereas the previous studies using other experimental models have reported that activated microglia can express ED1 antigen under conditions of neuronal degeneration and may be associated with neuronal damage.39–41 It is thus possible that activated microglia migrate to the ischemic core and express ED1 antigen. Therefore, ED1-positive cells in the ischemic core may not be totally blood-borne monocytes. On the other hand, microglia in the peri-ischemic area, where most neurons remain viable (Figure 8D), showed upregulation of Iba1 protein expression without ED1. Therefore, expression of ED1 antigen can be considered strictly associated with severe ischemic damage.

Microglia have been known to exert dual effects on the CNS.7 Microglia are involved in neuroprotection by secretion of several neurotrophins and elimination of microorganisms and deleterious debris.13–15 Previous studies reported that activated microglia, in which Iba1 protein was upregulated without ED1 antigen, was involved in neuronal regeneration within axotomized motor nucleus.8,41 In contrast, microglia also exert neurotoxic effects by releasing reactive oxygen species, nitric oxide, or inflammatory cytokines, which may mediate neuronal damage.16–20 On basis of other experimental models, ED1-positive cells express inducible nitric oxide synthase and are a prominent source of nitric oxide.42,43 These ED1-positive cells were suggested to be associated with tissue damage, including in the CNS.39–41 Although there is no direct evidence for the function of microglia in ischemic brain, the aforementioned findings suggest that activated microglia may have dual effects on the severity of ischemic damage. Double staining with anti-Iba1 antibody and ED1 may be useful in characterizing microglial activation and assessing the severity of ischemic brain injury.
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