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Short Communication

Intracerebroventricular Injection of Microglia Protects Against Focal Brain Ischemia

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Abstract. Microglia are macrophage-like phagocytic cells in the brain parenchyma. However, microglial function after neurodegeneration is not fully understood. In this study, occlusion of the middle cerebral artery (MCA) and reperfusion caused massive neuronal loss in the rat cerebral cortex and striatum after 3 days. When exogenous microglia were microinjected into the intracerebroventricle during MCA occlusion, neurodegenerative areas significantly decreased. At that time, migrated microglia were detected in the ischemic lesion. These results suggest that exogenous microglia can migrate into brain parenchyma and then protect against neurodegeneration induced by MCA occlusion and reperfusion.

Keywords: microglia, neuroprotection, focal brain ischemia

Glial cells, such as microglia and astrocytes, have been suggested to be physiologically involved in chaperoning neurons to neuronal synaptic sites, maintaining the functional integrity of the synapse and contributing to extracellular matrix proteins (1). On the other hand, it should be noted that microglia accumulate in the neighborhood of neurodegenerative sites in patients with Alzheimer's disease (AD), Parkinson's disease (PD), and focal cerebral ischemia (2). Although the in vitro study of cultured microglia indicates that microglial activation induces the production of both cytotoxic and neurotrophic molecules (1), microglial function in the in vivo brain is not fully understood. Since microglia are macrophage-like phagocytic cells in the brain (2, 3), it is thought that while microglia are mainly scavenger cells in the brain parenchyma, they also perform various other functions in tissue repair and neuronal regeneration (1, 2, 4). In the present study, we examined the effect of intracerebroventricular (i.c.v.) injection of microglia on neuronal death induced by 2-h occlusion of the middle cerebral artery (MCA) and reperfusion.

Mixed glial cells (a mixture of astrocytes and microglia) were prepared from the cerebral hemispheres, which were carefully removed from the meninges of newborn Wistar rats (Slc:Wistar/ST; SLC Inc., Hamamatsu). In addition, microglial culture (over 97%) was performed as described previously (3, 5). Cultured microglia were labeled by the fluorescent dye PKH26 (Sigma, St. Louis, MO, USA) (6) and then injected i.c.v. MCA occlusion was also performed in male Wistar rats (weighing approximately 300 g) as described previously (7). Selected rats were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) and then placed in a Kopf stereotaxic frame. The microinjection coordinates (0.2-mm caudal, 1.2-mm left lateral, 3.8-mm ventral to the bregma) were according to a rat brain atlas (8), and microinjection was achieved via a motor-driven 10-μl Hamilton syringe using a 22-gauge needle. At 1 h after the onset of MCA occlusion, operated rats received a microinjection of 2.0 μl of microglial cell suspension (approximately 4,000 or 40,000 cells) or vehicle control (sterilized phosphate-buffered saline, PBS). A 5-min waiting period allowed injected cells to settle before the needle was removed. In addition, the nylon thread was removed from the internal carotid artery under anesthesia at 2 h after the onset of occlusion to reperfuse the ischemic area. At 3 days after each brain insult, brain slices were prepared for an immunohistochemical analysis. Each group consisted of six or more rats. All animal experiments were carried out in accordance with Guide for the Care and Use of Laboratory Animals

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from the National Institutes of Health and the Japanese Pharmacological Society, and the protocols were approved by the Committee for Animal Research at Kyoto Pharmaceutical University.

Sections were incubated with primary antibody against microtubule-associated protein-2 (MAP2, diluted: 1:3,000; Sigma), cluster of differentiation antigen-11b (CD11b, 1:1,000; Harlan Sera-Lab, Loughbrough, UK) or glial fibrillary acidic protein (GFAP, 1:5,000; Chemicon, Temecula, CA, USA) for 3 days at 4°C. The antibody was detected by an ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine and nickel enhancement. Some of the sections were stained with hematoxylin and eosin, or 2,3,5-triphenyltetrazolium chloride to determine histological changes.

For the quantitative analysis, MAP2-immunostained sections were scanned using a camera (KY-F55MD; Victor, Tokyo) and then analyzed (WinRoof; Mitani, Fukui) (7). We analyzed coronal sections at 0.60-mm anterior from the bregma. For the quantitative analysis of neuronal death, the MAP2-immunoreactive areas in the cerebral cortex, striatum, and ipsilateral hemisphere (total) were measured (mm^2) and used as an index of neuronal survival (a decrease indicates neuronal loss). Data are presented as the mean ± S.E.M. of 6 – 9 rats. The significance of differences was determined by an analysis of variance (ANOVA) with Student's *t*-test.

We measured the MAP2-immunoreactive area in which neurons were normal and survived, since staining with 2,3,5-triphenyltetrazolium chloride was partially influenced by reactive glial cells such as microglia and astrocytes. MAP2-immunoreactivity gradually decreased in ipsilateral striatum after 2 h of MCA occlusion and reperfusion, and it was markedly reduced at 1 day. In addition, the loss of MAP2 immunoreactivity extended to the ipsilateral parietal cortex at 3 days (Fig. 1). Although GFAP-immunoreactive astrocytes were slightly activated in the border zones (perifocal regions), GFAP immunoreactivity also decreased in the infarcted core. On the other hand, CD11b-immunoreactive microglia were activated and accumulated in the infarcted core (Fig. 1).

Interestingly, when microglia were injected i.c.v. at 1 h after the onset of MCA occlusion (halfway through occlusion), exogenous microglia also migrated into the ischemic lesions. Briefly, several populations of microglia that were double-labeled with the fluorescent dye PKH26 and antibody against microglial membrane protein CD11b (also known as complement receptor-3) were detected (Fig. 2). In normal areas, few PKH-labeled microglia were observed around cerebroventricles or brain vessels (data not shown). Loss of MAP2 immunoreactivity induced by MCA occlusion and reperfusion was significantly suppressed by i.c.v.-injection of 4,000 and 40,000 microglial cells, and 4,000 cells were enough to exert effective neuroprotection (Fig. 3). On the other hand, we have preliminary results that an i.c.v. injection of undifferentiated mouse embryonic stem cells (about 4,000 cells)

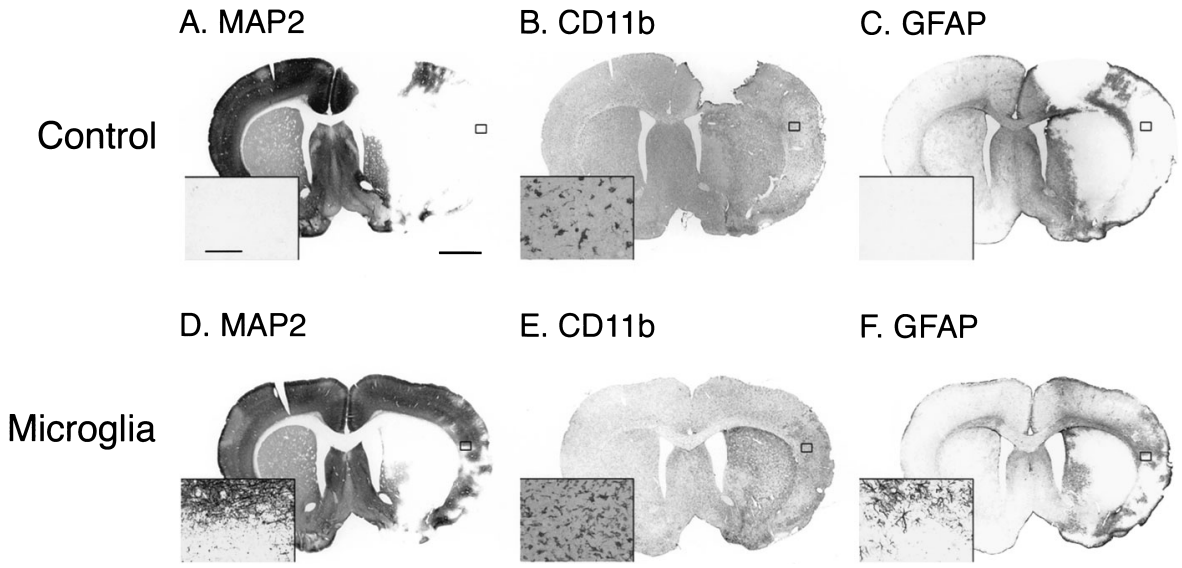

Fig. 1. Immunoreactivities for MAP2, CD11b, and GFAP. Midway through 2-h occlusion of MCA, rats were injected i.c.v. with vehicle (A – C) or 40,000 microglial cells (D – F). After 3 days, brain sections were immunostained by antibodies against MAP2 (for neurons, A and D), CD11b (for microglia, B and E), and GFAP (for astrocytes, C and F). Insets: corresponding regions of infarcted core or border zone (small boxes). Scale bars, 2 mm (A – F), 100 μm (insets).

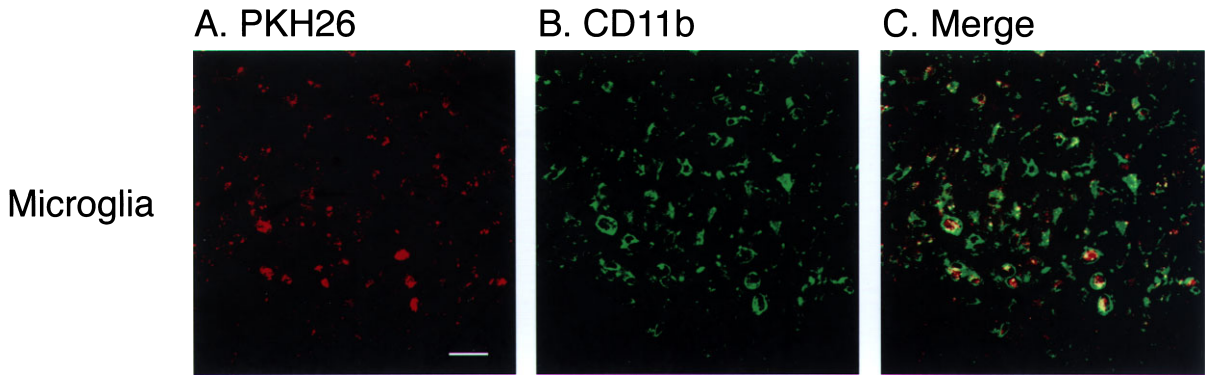

Fig. 2. Confocal microscopy analysis of microglia in the ischemic region. Isolated and cultured rat microglia were labeled by the fluorescent dye PKH26. Subsequently, 40,000 microglial cells were injected i.c.v. midway through MCA occlusion. A: PKH26 fluorescence for exogenous microglia (red). B: CD11b immunoreactivity for both endogenous and exogenous microglia (green). C: Merged image. Thus, exogenous microglia migrated into the ischemic region. Similar images were observed in three other rats. Scale bar, 40 μm.

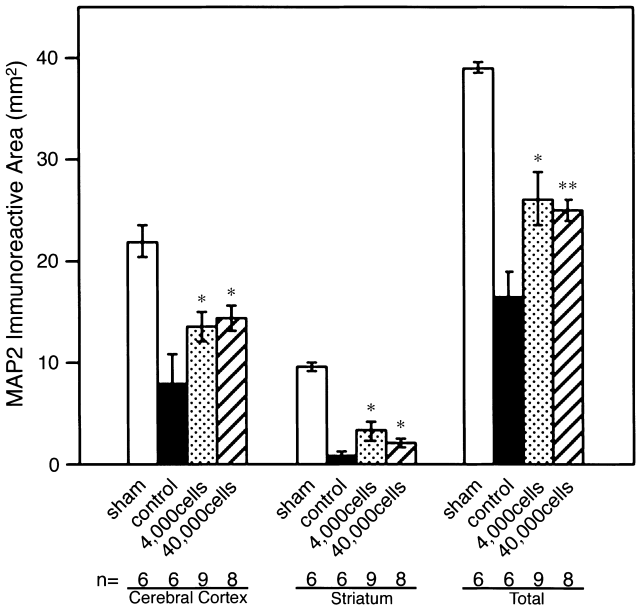

Fig. 3. Changes in MAP2-immunoreactive areas in the cerebral cortex and striatum. MAP2-immunoreactive area (mm^2) was measured in the cerebral cortex and striatum of coronal section (at 0.60 mm anterior from the bregma) in sham-operated rats (sham) and ischemic rats without (control) or with microinjection of 4,000 or 40,000 cells of microglia after 3 days. Total area (ipsilateral hemisphere) was also shown. n: number of operated rats. Each value is the mean ± S.E.M. **P*<0.05, ***P*<0.01 *vs* the control value.

did not show significant protective effect against ischemic injury (data not shown).

Recently, neural stem cells, neural progenitors, bone marrow cells, and embryonic stem cells have been widely discussed as optimal cell sources in cell therapy for neurodegenerative disorders such as PD, AD, and brain stroke (4, 9, 10). Although these multipotent stem cells can differentiate into glial cells (4, 10, 11), the usefulness of microglia in cell therapy is not fully understood.

Recent studies have suggested that when microglia ($1-2\times10^6$ cells), macrophages ($1-2\times10^6$ cells) or bone marrow cells (1×10^7 cells) were peripherally injected, microglia (but not macrophages) migrated specifically to the brain parenchyma (12), and some populations of immature hematopoietic cells also reached the brain (13). After transient ischemia in Mongolian gerbils, exogenous microglia accumulated in ischemic lesion sites (hippocampal CA1) and expressed glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor (12, 14). In the present study, although endogenous microglia accumulated in the ischemic core, massive neurodegeneration occurred, suggesting that the activation of only endogenous microglia may not be enough to protect against neurodegeneration. Exogenous rat microglia that had been injected i.c.v. with relatively lower doses (4,000 or 40,000 cells) also accumulated in the infarcted core in rat brain parenchyma. Unexpectedly, the neuroprotective effect of 4,000 cells was nearly equal to or may be higher than that of 40,000 cells of microglia. Therefore, 4,000 cells may be enough to produce such a neuroprotective effect. Thus, microglia exhibited specific affinity for neurodegenerative regions and had neuroprotective effects following peripheral and/or i.c.v. injection, without direct transplantation to the brain parenchyma. Glial cells produce neurotrophic factors, modify the response to injury, and assist in structural repair (1, 2, 4). In addition, it is thought that microglia may be able to deliver therapeutic genes (and/or drugs)

to the brain parenchyma (15). Based on these observations, we presume that migration of exogenous microglia may be induced by chemotactic factors or chemokines released from activated resident microglia in the ischemic core and subsequently exogenous microglia may scavenge dead neurons and may repair and protect damaged neurons through microglial phagocytosis and neurotrophic factor production. Thus, cell therapy using microglia may also be a potent strategy for the treatment of neurodegenerative disorders, such as brain stroke, in addition to neuron replacement therapy that involves the transplantation of neural stem cells or precursors (4, 9, 11).

In conclusion, i.c.v.-injected rat microglia migrated to regions of brain ischemia induced by MCA occlusion and reperfusion. In addition, neurodegenerative areas significantly decreased. These results suggest that the transplantation of glial cells may also be useful.

Acknowledgments

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