Neuroscience 142 (2006) 87–96

THE INTRA-ARTERIAL INJECTION OF MICROGLIA PROTECTS HIPPOCAMPAL CA1 NEURONS AGAINST GLOBAL ISCHEMIA-INDUCED FUNCTIONAL DEFICITS IN RATS

Y. HAYASHI,[a] Y. TOMIMATSU,[a] H. SUZUKI,[b] J. YAMADA,[a] Z. WU,[a] H. YAO,[c] Y. KAGAMIISHI,[d] N. TATEISHI,[d] M. SAWADA[b] AND H. NAKANISHI[a]*

[a]*Laboratory of Oral Aging Science, Faculty of Dental Sciences, Kyushu University, Fukuoka 812-8582, Japan*

[b]*Department of Brain Life Science, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan*

[c]*Laboratory for Neurochemistry, Hizen National Hospital, Saga 342-0192, Japan*

[d]*Minase Research Laboratories, Ono Pharmaceutical Co., Ltd., Osaka 618-8585, Japan*

Abstract—In the present study, we have attempted to elucidate the effects of the intra-arterial injection of microglia on the global ischemia-induced functional and morphological deficits of hippocampal CA1 neurons. When PKH26-labeled immortalized microglial cells, GMIR1, were injected into the subclavian artery, these exogenous microglia were found to accumulate in the hippocampus at 24 h after ischemia. In hippocampal slices prepared from medium-injected rats subjected to ischemia 48 h earlier, synaptic dysfunctions including a significant reduction of synaptic responses and a marked reduction of long-term potentiation (LTP) of the CA3–CA1 Schaffer collateral synapses were observed. At this stage, however, neither significant neuronal degeneration nor gliosis was observed in the hippocampus. At 96 h after ischemia, there was a total loss of the synaptic activity and a marked neuronal death in the CA1 subfield. In contrast, the basal synaptic transmission and LTP of the CA3–CA1 synapses were well preserved after ischemia in the slices prepared from the microglia-injected animals. We also found the microglial-conditioned medium (MCM) to significantly increase the frequency of the spontaneous postsynaptic currents of CA1 neurons without affecting the amplitude, thus indicating that MCM increased the provability of the neurotransmitter release. The protective effect of the intra-arterial injected microglia against the ischemia-induced neuronal degeneration in the hippocampus was substantiated by immunohistochemical and immunoblot analyses. Furthermore, the arterial-injected microglia prevented the ischemia-induced decline of the brain-derived neurotrophic factor (BDNF) levels in CA1 neurons. These observations strongly suggest that the arterial-injection of microglia protected CA1 neurons against the ischemia-induced neuronal degeneration. The restoration of the ischemia-induced synaptic deficits and the resultant reduction of the BDNF levels in CA1 neurons, possibly by the release of diffusible factor(s), might thus contribute to the protective effect of the arterial-injection of microglia against ischemia-induced neuronal degeneration.

Key words: microglia, intra-arterial injection, global ischemia, hippocampal CA1 neurons, brain-derived neurotrophic factor, electrophysiology.

In response to pathological conditions including cerebral ischemia, ramified microglia rapidly transform into activated states and accumulate in pathological sites. When neurons are severely injured, the microglia further transform into phagocytic cells. It remains controversial whether the microglia which accumulate at pathological sites are harmful or beneficial. There is growing evidence that the activated microglia are harmful for the injured neurons associated with ischemia, while the inhibition of microglial activation could reduce ischemic brain injury (Lees, 1993). Lipid-soluble tetracyclines, doxycycline and minocycline, have been reported to inhibit microglial activation, while they also have a neuroprotective effect against global brain ischemia (Yrjänheikki et al., 1998). On the other hand, some studies have suggested that microglia might have a beneficial effect on ischemic injured neurons. The proliferation of microglia after ischemia has also been suggested to contribute to the ischemic tolerance (Liu et al., 2001).

The intra-cerebral introduction of microglia is one of the direct approaches to elucidate the role of microglia in the ischemic brain. Recently, Kitamura et al. (2004, 2005) examined the effects of i.c.v.-injected microglia on neurodegeneration induced by the middle cerebral artery occlusion (MCAO) and reperfusion. After MCAO, i.c.v.-injected microglia were found to accumulate in the infracted core in rat brain parenchyma. They performed histological and functional analyses to show a neuroprotective effect of exogenous microglia on neuronal injury induced by MCAO. Unfortunately, i.c.v.-injected microglia may induce several undesirable events such as the entry of blood cells to the ischemic lesions and immunologic responses, which complicate the analysis of the role of microglia. We have previously reported that microglial cell lines as well as primary cultured microglia retain the ability to enter the normal brain from the circulation (Imai et al., 1997; Sawada et al., 1998; Imai et al., 1999). Furthermore, intra-arterial-injected microglia migraded to the ischemic hippocampal CA1 sub-

*Corresponding author. Tel: +81-92-642-6413; fax: +81-92-642-6415.
E-mail address: nakan@dent.kyushu-u.ac.jp (H. Nakanishi).

Abbreviations: BDNF, brain-derived neurotrophic factor; CD, cathepsin D; CLSM, confocal laser-scanning microscope; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; I–O, input–output; LTP, long-term potentiation; MAP2, microtubule-associated protein-2; MCAO, middle cerebral artery occlusion; MCM, microglial-conditioned medium; MEM, minimal essential medium; NeuN, neuronal nuclei; NMDA, *N*-methyl-D-aspartic acid; PBS, phosphate-buffered saline; pEPSP, population excitatory postsynaptic potential; PI, propidium iodide; rCBF, regional cerebral blood flow; SDS, sodium dodecyl sulfate.

0306-4522/06$30.00+0.00
doi:10.1016/j.neuroscience.2006.06.003

field, one of the most vulnerable neuronal populations against global forebrain ischemia (Imai et al., 1997, 1999). Therefore, the intra-arterial injection of microglia can be a suitable approach to address the question of whether microglia are harmful or beneficial to the ischemic neuronal injury.

In the present study, we have thus utilized this intra-arterial injection system and found that the arterial-injected microglia protected CA1 neurons against the ischemia-induced neuronal degeneration by a combination of electrophysiological and morphological analyses. The arterial-injected microglia ameliorated the ischemia-induced synaptic deficits and the resultant reduction of brain-derived neurotrophic factor (BDNF) levels in CA1 neurons possibly by releasing diffusible factor(s). These effects might contribute to the protective effect of the arterial-injection of microglia against the ischemia-induced neuronal degeneration.

EXPERIMENTAL PROCEDURES

Animals

This study was approved by the Animal Research Committee of the Kyushu University Faculty of Dental Sciences. The study was carried out using male Wistar rats weighing 180–210 g. The rats were housed in group cages under 12-h light/dark conditions and then were given free access to food and water. The experimental procedure was approved by the Animal Research Committee of the Kyushu University Faculty of Dental Sciences, and was conducted in accordance with the guidelines of the U.S. National Institutes of Health on the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Cell culture

GMIR1, an immortalized microglial clone, was established from a rat primary microglial culture which has been described previously (Morihata et al., 2000; Salimi et al., 2002). GMIR1 cells displayed strong wheat germ agglutinin and IB4-lectin binding and immunoreactivity for antibodies recognizing OX-42 and ED1, indicating that this clone had microglial properties (Salimi et al., 2002). GMIR1 cells were cultured in Petri dishes in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 0.2% glucose, 5 mg/l bovine insulin, pH 7.3 and supplemented with 1 ng/ml granulocyte-macrophage colony stimulating factor (Genzyme, Cambridge, MA, USA) at 37 °C in 5% CO_2. Before injection, the cells were tagged with a lipid-soluble fluorescent dye PKH-26 (Zynaxis, Malvern, PA, USA). PKH26-stained cells were harvested using a rubber policeman in 2 ml of ice-cold phosphate-buffered saline (PBS) which was centrifuged three times.

Preparation of peritoneal macrophages

Macrophages were collected by injecting 20 ml of cold (4 °C) PBS into the peritoneal cavities of male Wistar rats. Peritoneal fluid was withdrawn three times with a 21-gauge needle and a plastic syringe. The cells were kept at 4 °C, centrifuged at 1000×*g* for 5 min, and seeded onto plastic dishes containing MEM with 10% FCS. The non-adherent cells were removed after 2 h and the adherent macrophages were cultured for 24 h in MEM containing 10% FCS, where they were allowed for 1 h at 37 °C in 5% CO_2.

Microglial injection and forebrain ischemia

Male Wistar rats were anesthetized with halothane and the tagged microglial cells (2×10^6 cells in 500 μl of medium) were injected as a bolus over 30 s into the recipient's subclavian artery. In some experiments, microglia fixed with 4% paraformaldehyde or peripheral macrophages (2×10^6 cells in 500 μl of medium) were injected into the recipient's subclavian artery. Four to 7 days after injection, rats were subjected to transient forebrain ischemia by clamping the carotid arteries bilaterally according to the method of Pulsinelli and Brierley (1979). Briefly, the animals were anesthetized with the mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and bilateral vertebral arteries were electrocauterized at the level of the first vertebra. On the following day, the common carotid arteries were gently exposed and both arteries were occluded with a vascular clamp for 10 min. The rectal temperature was maintained at 36.5–37.5 °C. The rats that had lost their righting reflexes during the period of ischemia were used as the postischemic group. This procedure induced a total loss of neurons in the hippocampal CA1 subfield of medium-injected rats assessed by Nissl staining and microtubule-associated protein-2 (MAP2)-immunohistochemistry. In a parallel set of experiments, the changes in the regional cerebral blood flow (rCBF) (1 mm posterior and 2–4 mm lateral to the bregma) were continuously monitored with a laser-Doppler flowmeter (ALF 21D, Advance Co. Ltd., Tokyo, Japan). The changes in the rCBF were expressed as a percentage of the average of two to three baseline values. After bilateral carotid occlusion, the rCBF decreased to 19.9% (range: 12.0–25.0%, number of animals=3) and 14.7% (range: 9.1–21.4%, number of animals=4) of the control values in the medium- and microglia-injected animals, respectively. The extents of rCBF reduction after carotid occlusion did not differ between the two groups.

Quantitative analysis of infiltrated cells

PKH26-labeled microglia-injected sham and animals subjected to 10-min of four-vessel occlusion 24 h and 48 h earlier (number of animals=3, each) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and killed by intracardiac perfusion with isotonic saline followed by a chilled fixative consisting of 4% paraformaldehyde in 0.2 M PBS (pH 7.4). After perfusion, the brain was removed and further fixed by immersion in the same fixative overnight at 4 °C, and then immersed in 20% sucrose (pH 7.4) for 24 h at 4 °C. Serial parasagittal sections (10 μm thick) of the whole hippocampus were prepared by a cryostat. Three sections were randomly selected from each group. Images of PKH26-positive cells infiltrated in each section were taken as a stack at 1-μm step size along z-direction with a 20× objective by a confocal laser-scanning microscope (CLSM) (LSM510MET, Carl Zeiss, Jena, Germany) (Shimizu et al., 2005).

Electrophysiology

For electrophysiological analyses, postischemic slices were taken from either group subjected to 10-min of four-vessel occlusion 48 h (number of animals=5, each group), 96 h (number of animals=5, each group) and 1 week (number of animals=3) earlier. Sham slices were taken from medium- and microglia-injected animals subjected to identical surgical exposure without vessel occlusion 96 h (number of animals=3, each group) earlier. Animals of both the ischemic and sham groups were decapitated under light ether anesthesia and then were rapidly removed and placed in ice-cold Krebs Ringer solution of the following composition (in mM): NaCl 124.0, KCl 2.5, KH_2PO_4 1.24, $NaHCO_3$ 26.0, $CaCl_2$ 2.4, $MgSO_4$ 1.3 and glucose 10.0. Transverse hippocampal slices (thickness of 400 μm) ranging from 2.0–3.5 mm lateral to the midline were cut with a vibrating microtome (VT 1000S, Leica, Heidelberg, Germany) were used for electrophysiological studies. A single hippocampal slice was placed in an interface-type record-