on coverslips at a density of 1.0×10^4 cells/ml and were fixed with 4% paraformaldehyde for 30 min at room temperature. Afterwards, the images were taken at an excitation of 470 nm and an emission of 530 nm by using a fluorescence microscopy (Leica Microsystems DMRB, Wetzlar, Germany).

2.5. ESR spectroscopy

ESR, together with the spin-trapping agent DEPMPO was employed to accurately detect the production of $^{\bullet}O_2^-$ radicals by activated microglia. The 6-3 microglial cells were plated on 12-well tissue culture plates at a density of 1.6×10^6 cells in 400 μl of serum free culture medium per well. The 6-3 cells were incubated 500 ng/ml LPS for 16 h in the presence or absence of pretreatment of PS/PC liposomes or PC liposomes for 1 h at 37 °C. Afterwards, the 6-3 cells were incubated at 37 °C with or without 400 ng/ml PMA for 30 min before beginning the detection of ESR spectra. Cell suspensions (4×10^6 cells/ml) in the culture medium containing 25 mM DEPMPO were transferred to a standard cell capillary, and the ESR measurements were performed at room temperature right after the incubation. The ESR spectra were obtained using a JES-RE1X ESR spectrometer (JEOL, Japan). The setting conditions of the instrument were as follows: magnetic field = 336.7 ± 7.5 mT, modulation amplitude = 2000, modulation width = 0.1 mT, modulation frequency = 100 kHz, time constant = 0.1 s, microwave power = 10 mW, microwave frequency = 9430 MHz and sweep time = 2 min.

2.6. Spin trapping in xanthine/xanthine oxidase sysytem

Xanthine oxidase (0.1 U/ml) was incubated with 0.4 mM xanthine in phosphate–buffer (PB) containing 2 mM DTPA and 20 mM DEPMPO in the presence or absence of 2 mM PS/PC liposomes or PC liposomes. Xanthine oxidase was added last to the mixture to start the reaction. The ESR spectra were recorded at room temperature on a JES-RE1X ESR spectrometer. The setting conditions of the instrument were as follows: magnetic field = 336.7 ± 7.5 mT, modulation amplitude = 500, modulation width = 0.1 mT, modulation frequency = 100 kHz, time constant = 0.03 s, microwave power = 10 mW, microwave frequency = 9430 MHz and sweep time = 1 min.

2.7. NO quantification

The accumulation of NO_2^-, a stable end-product, extensively used as an indicator of NO production by cultured cells, was assayed by the Griess reaction. The 6-3 microglial cells were plated on 24-well tissue culture plates at 9×10^5 per 200 μl per well and incubated in the presence or absence of pretreatment of PS/PC liposomes or PC liposomes for 1 h at 37 °C. Afterwards, the cells were incubated in the presence or absence of 500 ng/ml LPS and 400 ng/ml PMA at 37 °C. After 48 h, the cell-free supernatants were mixed with equal amounts of Griess reagent (Griess Reagent Kit; Dojindo, Kumamoto, Japan). Samples were incubated at room temperature for 15 min and subsequently absorbance was read at 540 nm using a plate reader (Multiskan MS; Labsystems, UK).

2.8. Statistics

The values were expressed as the means ± S.E.M. and analyzed by a one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. The significance was established at a level of $p < 0.05$.

3. Results

3.1. Confirmation of microglial phagocytosis of PS/PC liposomes

First, in order to confirm that the PS/PC liposomes are certainly engulfed by microglia, we treated primary cultured rat microglia with NBD-labeled PS/PC liposomes or NBD-labeled PC liposomes. After 3 h of treatment, microglia were fixed with 4% PFA and examined by fluorescence microscopy. The florescent images were merged with the corresponding phase contrast images. As shown in Fig. 1A, it was observed that well-defined microglial cytoplasm was filled with florescently labeled PS/PC liposomes (green). In contrast, few PC liposomes labeled with the florescence were observed in microglial cytoplasm (Fig. 1B). These findings indicate that PS/PC liposomes, but not PC liposomes, were phagocytosed by microglia.

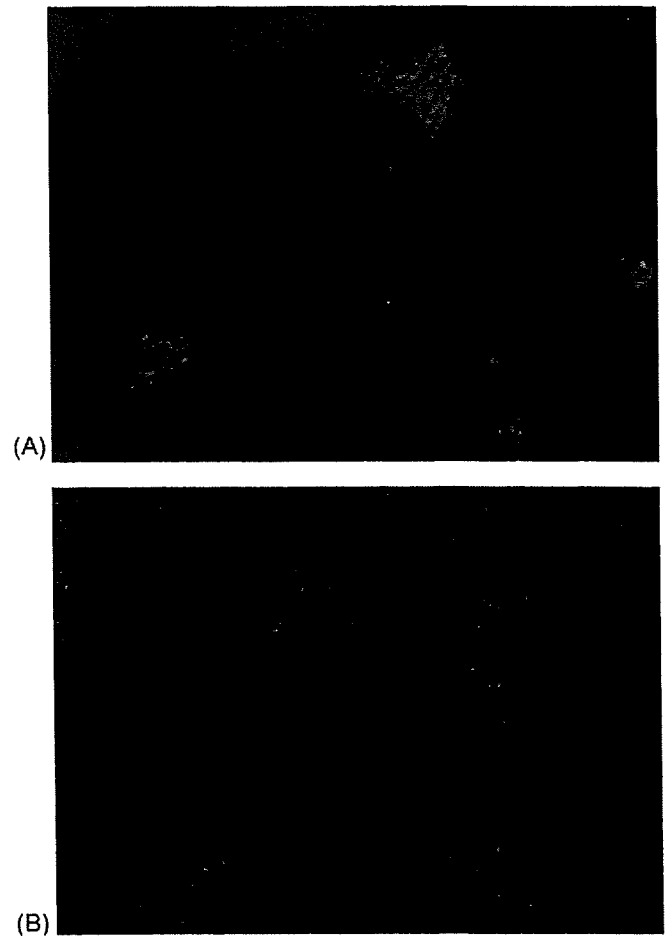

Fig. 1. Microglial phagocytosis of PS/PC liposomes. A typical fluorescence microphotograph showing (A) phagocytosis of NBD-labeled PS/PC liposomes (green) by primary cultured rat microglia and (B) little phagocytosis of NBD-labeled PC liposomes by primary cultured rat microglia.

3.2. Effect of the liposomes on the $^{\bullet}O_2^-$ production by LPS/PMA-activated microglia

We subsequently measured the generation of $^{\bullet}O_2^-$ associated with activated microglia by ESR monitoring with a spin trap DEPMPO. In the preparations of non-stimulated microglia (Fig. 2A) and 500 ng/ml LPS alone-stimulated microglia (Fig. 2B), no signals were obtained. Microglial cells stimulated by 500 ng/ml LPS combined with 400 ng/ml PMA in the presence of 25 mM DEPMPO showed the prominent signals whose spectrum consisting of a linear combination of a characteristic 12-line spectrum corresponding to $^{\bullet}O_2^-$ spin

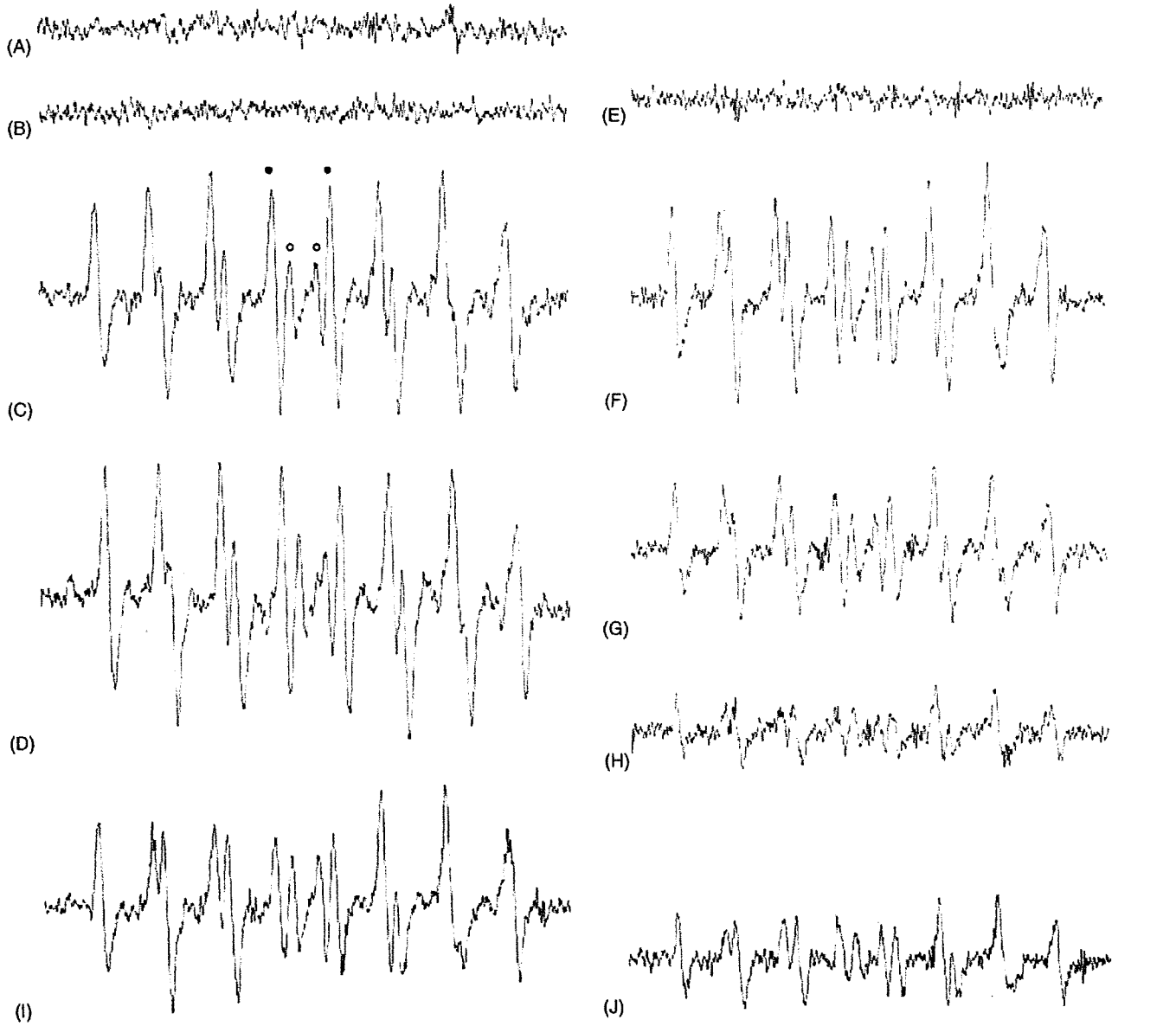

Fig. 2. Detection of $^{\bullet}O_2^-$ generation by LPS/PMA-activated microglia using ESR spin trap technique with DEPMPO. 6-3 microglial cells (4×10^6/ml) were incubated with LPS (500 ng/ml) for 16 h and PMA (400ng/ml) for 30 min at 37 °C with or without pretreatment of PS/PC liposomes for 1 h. The ESR spectra were then recorded in the presence of 25 mM DEPMPO at room temperature. (A) ESR spectra of DEPMPO adducts obtained from non-stimulated microglia. (B) ESR spectra of DEPMPO adducts obtained from microglia stimulated by LPS (500 ng/ml) alone. (C) ESR spectra of DEPMPO adducts obtained from LPS/PMA-activated microglia. Open and closed circles represent measured signal peaks of DEPMPO–OH and DEPMPO–OOH adducts, respectively. (D) ESR spectra of DEPMPO adducts obtained from microglia stimulated by PMA (400 ng/ml) alone. (E) the same as (C), but with the addition of SOD (160 μg/ml). (F) the same as (C), but with the addition of catalase (280 μg/ml). (G) the same as (C), but after pretreatment with PS/PC liposomes (0.1 mM) for 1 h. (H) the same as (C), but after pretreatment with PS/PC liposomes (1 mM) for 1 h. (I) the same as (C), but after pretreatment with PC liposomes (0.1 mM) for 1 h. (J) the same as (C), but after pretreatment with PC liposomes (1 mM) for 1 h.

adduct DEPMPO–OOH and an eight-line spectrum corresponding to $^{\bullet}OH$ spin adduct DEPMPO–OH (Fig. 2C). Computer simulation confirmed DEPMPO–OOH with hyperfine splittings $a_N = 13.15$ G, $a_H^\beta = 10.59$ G, $a_p = 49.73$ G, $a_H^\gamma = 0.72$ G and DEPMPO–OH with hyperfine splittings $a_N = 12.43$ G, $a_H = 13.49$ G, $a_p = 50.39$ G. These values are consistent with those described in a previous report (Sankarapandi et al., 1998). In addition, microglial cells stimulated by 400 ng/ml PMA alone showed essentially the same ESR spectra as those of LPS/PMA-activated microglia (Fig. 2D). To further confirm the original species of the spin adduct generated by LPS/PMA-activated microglia, SOD (160 μg/ml) or catalase (280 μg/ml) were also treated. The ESR signal intensity was substantially decreased by SOD (Fig. 2E), not by catalase (Fig. 2F). These results indicate that the spin adducts originated from $^{\bullet}O_2^-$ radical but not $^{\bullet}OH$ radical, which is derived from H_2O_2.

We next evaluated the effect of the liposomes on the generation of $^{\bullet}O_2^-$ associated with LPS/PMA-activated microglia. Pretreatment with PS/PC liposomes for 1 h considerably

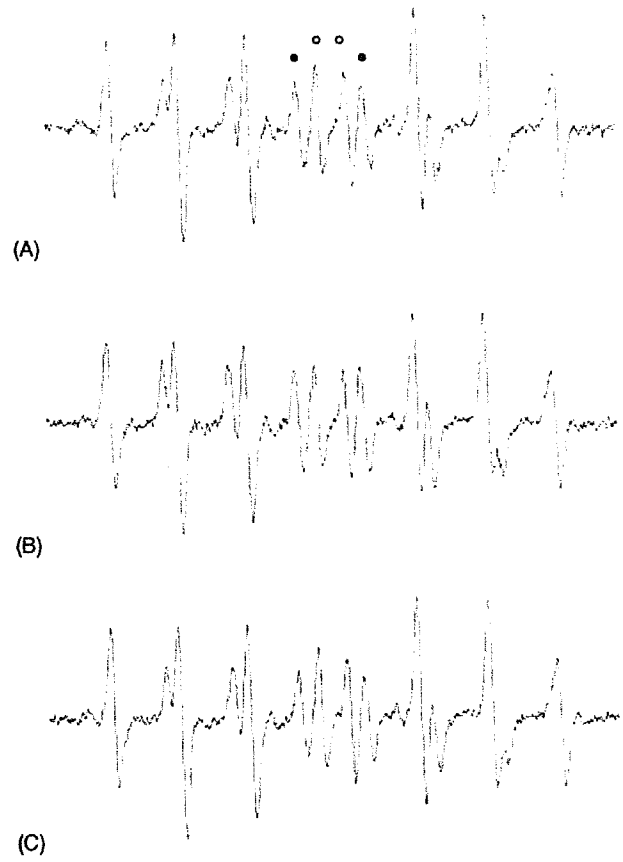

Fig. 3. Detection of $^{\bullet}O_2^-$ generation in xanthine/xanthine oxidase system using ESR spin trap technique with DEPMPO. The system contained 0.4 mM xanthine, 2 mM DTPA, and 20 mM DEPMPO in PB in the presence or absence of 2 mM PS/PC liposomes or PC liposomes. Xanthine oxidase (0.1 U/ml) was added last to the mixture to start the reaction. (A) ESR spectra of DEPMPO adducts obtained in the xanthine/xanthine oxidase system. Open and closed circles represent measured signal peaks of DEPMPO–OH and DEPMPO–OOH adducts, respectively. (B) the same as (A), but in the presence of 2 mM PS/PC liposomes. (C) the same as (A), but in the presence of 2 mM PC liposomes.

inhibited the signal intensity of the $^{\bullet}O_2^-$ adduct in a dose-dependent manner (Fig. 2G, H). In spite of few PC liposomes were phagocytosed by microglia, pretreatment with PC liposomes for 1 h also inhibited the signal intensity of the $^{\bullet}O_2^-$ adduct in a dose-dependent manner (Fig. 2I, J).

3.3. Effect of the liposomes on the $^{\bullet}O_2^-$ generation in xanthine/xanthine oxidase system

To confirm whether or not the liposomes *per se* scavenge $^{\bullet}O_2^-$, we measured the $^{\bullet}O_2^-$ production in xanthine/xanthine oxidase system in the presence or absence of the liposomes by ESR monitoring with a spin trap DEPMPO. Fig. 3A shows typical ESR spectra consisting of DEPMPO–OOH and DEPMPO–OH in xanthine/xanthine oxidase system. The formation of these spin adducts via trapping $^{\bullet}O_2^-$ was confirmed by experiments in which SOD (160 μg/ml) was added before xanthine oxidase and ESR signals were completely quenched (data not shown), while catalase

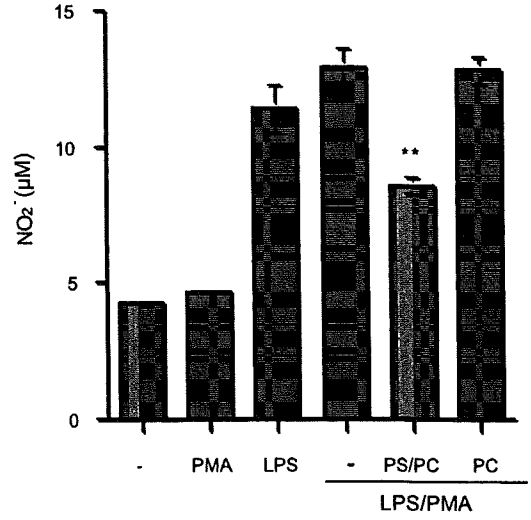

Fig. 4. Effect of the liposomes on the NO production by LPS/PMA-activated microglia. 6-3 microglial cells were incubated with LPS (500 ng/ml) and PMA (400 ng/ml) at 37 °C with or without pretreatment with 1 mM PS/PC liposomes or PC liposomes for 1 h. After 48 h, collected media were assayed for NO accumulation using the Griess reaction. $^{**}p < 0.01$, compared with LPS/PMA. The data are the mean values ± S.E.M. ($n = 3$).

(280 μg/ml) was added, in which ESR signals were not quenched at all (data not shown). The ESR spectra in the presence of either 2 mM PS/PC liposomes or PC liposomes showed essentially the same as those shown in Fig. 3A, thus indicating that neither PS/PC liposomes nor PC liposmes have scavenging effect on $^{\bullet}O_2^-$, but have the inhibitory effect on the $^{\bullet}O_2^-$-generating system in microglia (Fig. 3B, C).

3.4. Effect of the liposomes on the NO production by LPS/PMA-activated microglia

Using a Griess reaction assay, we investigated the effect of the liposomes on the microglial production of NO, a tally of $^{\bullet}O_2^-$ for forming $ONOO^-$. The incubation of microglial cells with 500 ng/ml LPS combined with 400 ng/ml PMA for 48 h resulted in a significant elevation in the accumulation of nitrite (Fig. 4). Five hundred nanograms per milliliters LPS alone, also, showed nearly the same amount of microglial production of NO, whereas, 400 ng/ml PMA alone could not induce a significant increase of the NO production. As expected, the LPS/PMA-induced microglial NO production significantly decreased after pretreatment with 1 mM PS/PC liposomes for 1 h (Fig. 4). In contrast, pretreatment with 1 mM PC liposomes for 1 h did not affect the microglial NO production at all (Fig. 4).

4. Discussion

In the present study, PS/PC liposomes considerably inhibited both $^{\bullet}O_2^-$ and NO production in LPS/PMA-activated microglia and thus presumably prevent the subsequent formation of $ONOO^-$, a powerful oxidant capable of inducing strong toxicity. $ONOO^-$ is formed from non-enzymatic reaction between $^{\bullet}O_2^-$ and NO at the near diffusion-limited

rate whose constant is three times faster than rate at which superoxide dismutase scavenges $^{\bullet}O_2^-$ (Beckman, 1994; Torreilles et al., 1999). $ONOO^-$, therefore, can be generated in several regions *in vivo* where $^{\bullet}O_2^-$ and NO are produced simultaneously, as it is presumed to occur in central nervous system. Indeed, the levels of nitrotyrosine, which is a product of the reaction of $ONOO^-$ with tyrosine residues and considered as a permanent footprint of $ONOO^-$, have been reported to increase in AD, PD and MS brains (Torreilles et al., 1999; Liu et al., 2001). Accordingly, the $ONOO^-$ forming blockers including PS/PC liposomes seem to be neuroprotective and a potentially useful treatment for these neurodegenerative diseases.

Due to the pivotal role of $^{\bullet}O_2^-$ in microglia-mediated neuroinflammation and oxidative stress, we employed ESR with the spin trap technique using DEPMPO to directly measure microglial $^{\bullet}O_2^-$ generation. The DEPMPO is an appropriate spin-trapping agent for cell-generated-$^{\bullet}O_2^-$ detection because of its stability and capability of differentiating between $^{\bullet}O_2^-$ and $^{\bullet}OH$ (Shi et al., 2005; Mojovic et al., 2005). LPS/PMA-activated microglia gave rise to ESR spectra consisting of a linear combination of $^{\bullet}O_2^-$ spin-adduct DEPMPO–OOH and spin-adduct DEPMPO–OH. Radical generation was totally quenched by SOD but not by catalase, thus indicating that H_2O_2, which is reduced to $^{\bullet}OH$ by Fenton reaction in the presence of Fe^{2+} or Zn^{2+}, was not a significant reactant in the formation of the observed radial signals. Moreover, the DEPMPO–OH appears to be generated by a spontaneous reduction of DEPMPO–OOH, not from H_2O_2-derived $^{\bullet}OH$ (Mojovic et al., 2005).

Colton et al. (1994) have reported that $^{\bullet}O_2^-$ and NO are apparently not produced by the same activating agent in rat primary cultured microglia. LPS is one of the most common stimulators used to activate microglia (O'Shea et al., 2006; Suuronen et al., 2006) both *in vivo* and *in vitro* models of neuroinflammation-mediated neurodegeneration and is known to activate protein–tyrosine kinases, mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), which have been implicated in the release of NO and various pro-inflammatory cytokines (Rivest, 2003; Suuronen et al., 2006; Szelenyi et al., 2006). On the other hand, PMA assembles NADPH oxidase via activation of protein kinase C (PKC) and thus is commonly used to induce abrupt and large amounts of microglial $^{\bullet}O_2^-$ production called as respiratory burst. Although several studies have demonstrated that microglia activated by LPS can form $^{\bullet}O_2^-$ (Qin et al., 2004, 2005), our study demonstrated that LPS alone did not affect the microglial $^{\bullet}O_2^-$ production but it did induce NO production. In contrast, PMA alone induced microglial $^{\bullet}O_2^-$ generation without affecting the NO production. In addition, according to our findings, and consistent with the study by Colton et al., it is suggested that $^{\bullet}O_2^-$ and NO production are differentially regulated in cultured murine microglial cells. Namely, LPS appears to be an inflammogen that provokes microglial production of NO rather than $^{\bullet}O_2^-$. We cannot, however, eliminate the possibility that LPS stimulation mediates the pathway(s) associated with $^{\bullet}O_2^-$ generation in microglia, because of differences on $^{\bullet}O_2^-$ detection sensitivity between the ESR assay and the other indirect methods.

We demonstrated that PS/PC liposomes considerably inhibited both the $^{\bullet}O_2^-$ and NO production in LPS/PMA-activated microglia, even though both of them appear to be regulated by distinct signal pathways. Furthermore, we also demonstrated that PS/PC liposomes did not scavenge $^{\bullet}O_2^-$, but instead act on the $^{\bullet}O_2^-$-generating system in microglia. The exact mechanism of PS/PC liposomes to suppress inflammatory activation of microglial has not yet been elucidated. Concerning $^{\bullet}O_2^-$ production, not only PS/PC liposomes but also PC liposomes inhibited the LPS/PMA-induced microglial production. Dipalmitoyl PC (DPPC), a kind of PC and the major component of pulmonary surfactant, has been shown to reduce monocyte respiratory burst via the downregulation of PKC associated with plasma membrane by the presumed mechanism that DPPC induces membrane perturbation (Tonks et al., 2005). In addition to that, according to our finding that few PC liposomes were phagocytosed by the micrglia, PC liposomes seem to act as membrane perturbers, thus reducing the LPS/PMA-induced and PKC-mediated $^{\bullet}O_2^-$ production. In contrast, PS has been reported to be engulfed by phagocytes through PS-recognizing receptor such as PS receptor (Fadok et al., 2000; De Simone et al., 2002) and scavenger receptor class B type 1 (Zhang et al., 2005). Indeed, it is confirmed that PS/PC liposomes could be phagocytosed by microglia in our study. However, we cannot rule out the possibility that PS/PC liposomes also induced the membrane perturbation under our experimental conditions because PS/PC liposomes contain 70 molar% PC. On the other hand, Ajmone-Cat et al. (2003) demonstrated that PS/PC-containing liposomes inhibited the phosphorylation of p38 MAPK and delayed that of cAMP responding element-binding protein in LPS-activated microglia. Because phosphorylation of p38 MAPK has been shown to mediate the signal pathway reacting for inflammatory stimulants and result in gene induction of NO synthase in microglia (Koistinaho and Koistinaho, 2002), the PS/PC liposomes-induced inhibition of p38 MAPK phosphorylation in activated microglia appears to suppress, at least partially, NO generation. In contrast, our finding that PC liposomes had no effect on LPS/PMA-induced NO production indicates that the presumed membrane perturbation induced by PC liposomes is not involved in NO-generating pathway(s) in the LPS/PMA-activated microglia.

Several neuroprotective compounds such as isoproterenol, dexamethasone, nicergoline, and naloxone have been shown to suppress microglial activation and thus decrease the ROS generation (Colton and Chernyshev, 1996; Yoshida et al., 2001; Qin et al., 2005). In comparison to the effective concentrations of these compounds (e.g. isoproterenol and nicergoline act at micromolar, naloxone acts even at femtomolar), the total lipids concentration such as millimolar of PS/PC liposomes used in this study seems to certainly be high. Borisenko et al. (2003), however, have suggested that phagocytes have a sensitivity threshold for PS externalized on the target cell surface, which thus provides for the reliable recognition and distinction between normal cells with low amounts of externalized PS and

apoptotic cells with remarkably elevated PS levels. They estimated that, using the liposomes containing PS and PC (1:1), the absolute amounts of PS required for phagocytosis by 5×10^4 macrophages (the threshold of macrophage sensitivity) was 7 pmol. This value of the PS amount for 10^6 macrophages (i.e. 140 pmol) approximates to the normalized value of the PS amount for 10^6 microglial cells, which was found to be considerably effective in our study (i.e. 75 pmol). Taken together, these findings suggest that microglia also require the relatively high phospholipids concentration to recognize PS/PC liposomes as apoptotic cells. Accordingly, new techniques to ameliorate the stability of PS/PC liposomes and thus reduce the effective phospholipids concentration are called for based on the findings of *in vivo* studies.

Acknowledgements

This study was supported by a grant from Inogashira Hospital (SH). We thank Prof. Yukihiro Shoyama and Dr. Satoshi Morimoto, Department of Plant Resources Regulation, Graduate School of Pharmaceutical Sciences, Kyushu University, for technical advice about preparing liposomes.

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Journal of Cerebral Blood Flow & Metabolism (2007) 27, 488–500

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Neuroprotective effect of exogenous microglia in global brain ischemia

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Exogenous microglia pass through the blood–brain barrier and migrate to ischemic hippocampal lesions when injected into the circulation. We investigated the effect of exogenous microglia on ischemic CA1 pyramidal neurons. Microglia were isolated from neonatal mixed brain cultures, labeled with the fluorescent dye PKH26, and injected into the subclavian artery of Mongolian gerbils subjected to ischemia reperfusion neuronal injury. PKH26-labeled microglia migrated to the ischemic hippocampal lesion, resulting in increased numbers of surviving neurons compared with control animals, even when injected 24 h after ischemia. Interferon-γ stimulation of isolated microglia enhanced the neuroprotective effect. Administration of exogenous microglia resulted in normal performance in a passive avoidance-learning task. Additionally, administration of exogenous microglia increased the expression of brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in the ischemic hippocampus, and thus might have induced neurotrophin-dependent protective activity in damaged neurons. Peripherally injected microglia exhibited a specific affinity for ischemic brain lesions, and protected against ischemic neuronal injury *in vivo*. It is possible that administration of exogenous microglia can be developed as a potential candidate therapy for central nervous system repair after transitory global ischemia.
Journal of Cerebral Blood Flow & Metabolism (2007) **27**, 488–500. doi:10.1038/sj.jcbfm.9600362; published online 5 July 2006

Keywords: blood–brain barrier; central nervous system; delayed neuronal death; drug delivery system; neurotrophic factor

Introduction

Microglia arise from the monocyte/macrophage lineage, and are ubiquitously distributed in the central nervous system, representing up to 20% of the total glial cell population in the brain (Lawson *et al*, 1990). In accordance with del Rio Hortega's early teaching (Hortega, 1932), the current view is that resident microglia are of mesodermal origin, derived from bone marrow precursor cells (Ling and Wong, 1993). These cells invade the central nervous system at an early embryonic stage to give rise to typical process-bearing microglia (Ling and Wong, 1993). Therefore, microglia may have high affinity for the brain.

Many reports describe neuron–microglia interactions after cerebral ischemia and mechanical injury (for review, see Gehrmann *et al*, 1995). It remains controversial, however, whether activated microglia promote neuronal death or neuronal survival. Many investigators maintain that microglia induce a neurotoxic effect by secreting nitric oxide (Chao *et al*, 1992) and toxic cytokines (Sawada *et al*, 1989). Moreover, microglia may contribute to the pathogenesis of neurodegeneration, such as in multiple sclerosis (Diemel *et al*, 1998), Alzheimer's disease (Barger and Harmon, 1997), and acquired immunodeficiency syndrome-associated dementia (Giulian *et al*, 1996). Conversely, other studies show that microglia protect neurons in the damaged brain by secreting cytokines, such as interleukin-1β (Giulian *et al*, 1986), interleukin-6 (Sawada *et al*, 1995), transforming growth factor-β (Suzumura *et al*, 1993),

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This work was supported by a Grant-in-Aid for Scientific Research from Fujita Health University and from the 'High-Tech Research Center' Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology, 2002 to 2006, Japan.
Received 13 December 2005; revised 22 May 2006; accepted 26 May 2006; published online 5 July 2006

basic fibroblast growth factor (Shimojo *et al*, 1991), hepatocyte growth factor (Hamanoue *et al*, 1996), nerve growth factor (Mallat *et al*, 1989), and tumor necrosis factor α (Sawada *et al*, 1989).

To determine whether microglia are neurotoxic or neuroprotective, they must be analyzed with *in vivo* models of neuronal injury. The role of microglia may be clarified by injecting isolated microglia at the site of neuronal injury in animal models. Direct injection of microglia into the brain, however, induces undesirable events, such as the entry of blood cells to the site of injury and immunologic responses, which complicate the analysis of the role of microglia.

Our previous studies showed that microglia isolated from rodent mixed-brain cultures retain the ability to enter the normal brain from the circulation (Imai *et al*, 1997, 1999; Sawada *et al*, 1998). Hence, microglia can be introduced into the central nervous system by injection into the bloodstream in animal models of neuronal injury. Using this system, the roles of microglia in various pathologic conditions can be analyzed.

The purpose of the present study was to evaluate whether microglia have neurotrophic or neurotoxic effects on *in vivo* brain ischemia, in which CA1 pyramidal neurons are specifically damaged. Microglia isolated from a primary culture of mixed glial cells from neonatal Mongolian gerbils were injected into the subclavian arteries of host animals subjected to ischemia reperfusion neuronal injury. We examined the number of CA1 pyramidal neurons in the brain at various time points up to 8 days. In another group of animals, we tested the effect of microglia injection on performance in a passive avoidance-learning task. We also investigated changes in the expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in the ischemic hippocampus after microglia injection.

Materials and methods

Animals and Induction of Global Forebrain Ischemia

Adult male Mongolian gerbils ($n = 300$; 7 to 8 weeks old; ≈60 g; Seac Yoshitomi, Ltd, Fukuoka, Japan) were used in the study. Animals were housed individually, with food and water available *ad libitum*. All procedures were performed in accordance with the Guidelines for Animal Experimentation of the Fujita Health University, School of Medicine.

Ischemia was induced as described by Tone *et al* (1987). In brief, anesthesia was induced and maintained with a mixture of 2.5% halothane and nitrous oxide/oxygen (1:1). Body temperature was maintained at 37°C with a heating pad. When the spinal reflex was absent, both common carotid arteries were exposed through a ventral cervical incision and occluded with aneurysm clips for 5 mins. The clips were then released to resume normal flow to the forebrain (ISCH). Gerbils operated on without occlusion of the carotid arteries were used as controls (sham ISCH). Postoperative rectal temperature was measured three times daily (at 0700, 1400, and 2200) immediately after the induction of anesthesia with a mixture of 2.5% halothane and nitrous oxide/oxygen (1:1).

Duration of Survival Period

Ischemia was confirmed by examination of Nissl-stained brain sections 7 days after ischemia. Delayed neuronal death was observed in CA1 pyramidal neurons in all animals ($n = 20$). Approximately 2% of the animals did not survive the ischemic episode. All animals that survived were included in the data analysis. There were no differences in survival between groups.

Cell Culture

Microglia were prepared using postnatal day 1 Mongolian gerbils ($n = 200$), as described previously (Suzumura *et al*, 1984). In brief, the meninges were removed, and the brain was dissociated by passing it through a 320-μm-pore nylon mesh. After washing with Hank's balanced salt solution, the cell suspension was triturated and plated in 75-cm^2 culture flasks (Falcon 3024, Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of one brain per flask in 10 mL Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, 5 μg/mL bovine insulin, and 0.2% glucose. Microglia were isolated on the 14th day by the shaking off method (Suzumura *et al*, 1984). In some experiments, purified microglia were stimulated by incubation with human interferon-γ (IFNγ; Shionogi Co., Osaka, Japan) at a concentration of 5×10^3 U in 10 mL in a plastic dish at 37°C for 24 h.

Macrophages were collected by injecting 10 mL of cold (4°C) phosphate-buffered saline into the peritoneal cavities of a separate group of gerbils. Peritoneal fluid was withdrawn three times with a 21-gauge needle attached to a plastic syringe. The cells were maintained at 4°C, centrifuged at 1,000 *g* for 5 mins, and seeded onto plastic dishes containing Eagle's minimal essential medium with 10% fetal bovine serum, where they adhered for 1 h at 37°C in 5% CO_2.

PKH26 Labeling

PKH staining provides stable, clear, intense, accurate, and reproducible fluorescent labeling of live cells for an extended period of time, with no apparent toxic effects (Horan and Slezak, 1989). PKH is an aliphatic reporter molecule that is inserted into the cell membrane lipid bilayer by selective partitioning. It is effective for a variety of cell types and exhibits no significant leaking or transfer between cells. We used PKH26 to label the exogenous microglia within the brain, using a PKH26 Red Fluorescent Cell Linker Kit for Phagocytic Cell Labeling (PKH26-PCL; Sigma Chemical Co., St Louis, MO, USA). PKH26-PCL labels phagocytic cells with excitation (551 nm) and emission (567 nm) wavelengths compatible

with rhodamine or phycoerythrin detection systems. The labeling occurs through the formation of aggregates of particles. The aggregation significantly inhibits the uptake of dye by nonphagocytic cells, such as lymphocytes, but facilitates dye uptake by phagocytic cells. Labeled cells appear patchy or spotted because the dye localizes in phagocytic compartments of the cells. The dye appears to be resistant to metabolic breakdown and labels the cells for at least 21 days *in vivo*. Use of PKH26 can be combined with immunocytochemistry (Silverman *et al*, 2000), but is not suitable for double staining, because it is easily abolished by the fixation required for immunocytochemistry.

Labeling of microglia and macrophages with PKH26 was performed using the standard phagocytosis procedure of microscopic particles, according to the manufacturer's instructions. In brief, cells in a 10-cm plastic dish were incubated with PKH26 staining solution containing 10 μmol/L PKH26, 50% Diluent B (a phagocytic cell-labeling solution, Sigma Chemical Co.), and 50% culture medium for 15 mins, then washed three times in 10 mL of serum-containing medium. PKH26-stained cells were harvested using a rubber policeman in 2 mL of ice-cold phosphate-buffered saline (pH 7.2) and washed three times with 5 mL of ice-cold phosphate-buffered saline (pH 7.2) by centrifugation at 1,000 *g* for 3 mins. The cells were then resuspended in the culture medium for injection into the host.

Injection of Cells

Intraarterial cell injection was performed as described previously (Ishihara *et al*, 1993). Cells were injected either 24 h before or 24 h after ischemia was induced. In a subset of experiments, microglia were injected 48 h after ischemia induction. In brief, sham ISCH and ISCH gerbils were anesthetized with a mixture of 2.5% halothane and nitrous oxide/oxygen (1:1). When the spinal reflex was absent, a transverse incision was made under the left clavicle. The left subclavian artery was exposed and dissected from the surrounding tissues. After temporary occlusion of the subclavian artery with an aneurysm clip, a small hole was made in the distal side of the artery with a 27-gauge needle, and a polyethylene tube (Becton Dickinson, Tokyo, Japan) was inserted proximally into the hole. After the aneurysm clip was released, PKH26-labeled microglia or macrophages (0.5 mL of final suspension media estimated to contain 1×10^6 cells) were injected as a bolus for 30 secs into the subclavian artery through the polyethylene tube.

Tissue Preparation

Gerbils were deeply anesthetized with ketamine hydrochloride (60 mg/kg, intraperitoneally) and killed by transcardial perfusion with isotonic saline solution. Brains were removed, frozen in liquid nitrogen, and embedded in OTC compound (Tissue Tek, Elkhart, IN, USA). Coronal sections (8 μm thick) at the hippocampal level were cut with a cryostat, mounted on slides, and dried. To preserve PKH26 labeling, the sections were lightly fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for 10 mins. To determine the location of exogenous microglia relative to ischemic CA1 pyramidal neurons, an immunofluorescent study was performed using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuracil nucleotides-biotin nick-end labeling (TUNEL), *Griffonia simplifolia* B4 isolectin (IB4), and an antibody to microtubule-associated protein 2 (MAP2). To localize BDNF and GDNF expression, immunohistochemistry using the avidin–biotin complex method was performed.

TUNEL staining was used to detect DNA fragmentation in cell nuclei. The hippocampal sections were pretreated with or without 20 μg/mL proteinase K (Sigma Chemical Co.) at room temperature (RT) for 15 mins. After treatment with 0.3% H_2O_2 in methanol for 30 mins, sections were incubated with 100 U/mL TdT and 10 nmol/L per mL biotinylated 16-2′-dUTP (Boehringer-Mannheim-Yamanouchi, Tokyo, Japan) in TdT buffer (100 mmol/L sodium cacodylate, pH 7.0, 1 mmol/L cobalt chloride, and 50 μg/mL gelatin) in a humid atmosphere at 37°C for 60 mins. Further incubation with fluorescein isothiocyanate-conjugated avidin (Nichirei, Tokyo, Japan) was performed for 60 mins at RT.

Microglial cells were identified by IB4 staining. Cryosections were incubated with 20 μg/mL biotinylated IB4-fluorescein isothiocyanate conjugate (Sigma Chemical Co.) in 0.01 mol/L phosphate-buffered 0.15 mol/L saline (pH 7.2) containing 0.1% Triton X-100 at RT for 2 h (Streit, 1990).

Hippocampal CA1 pyramidal neurons were visualized using a monoclonal antibody to MAP2 (Chemicon, Temecula, CA, USA). Sections were incubated with 10% normal goat serum for 30 mins at RT. They were then incubated with a mouse monoclonal anti-MAP2 antibody, diluted 1:100, for 1 day at 4°C. Further incubations were performed with fluorescein isothiocyanate-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:500, at RT for 60 mins.

We visualized BDNF epitopes using a rabbit polyclonal anti-BDNF antibody (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GDNF epitopes were visualized using a rabbit polyclonal anti-GDNF antibody (diluted 1:250; Santa Cruz Biotechnology). Sections were then incubated in secondary antibody, biotinylated goat anti-rabbit IgG, followed by avidin–biotin complex (Elite kit: Vector Laboratories, Burlingame, CA, USA), and visualized after chromogenic reaction with 3,3′-diaminobenzidine.

The sections were analyzed with an Olympus BX51 microscope equipped with bright-field and fluorescent light sources (Tokyo, Japan). Both bright-field and fluorescent images were captured from the same field using a Penguin 600 CL camera (Pixera Corporation, Los Gatos, CA, USA).

Cell Counts

Six serial, coronal frozen sections (8 μm thick) at the CA1 hippocampal level were examined. Cells were counted if

they were within a randomly selected 1-mm linear length of CA1. To determine the time course of exogenous microglia migration to the ischemic hippocampus, PKH26-labeled microglia were counted in animals injected with nonstimulated or IFNγ-stimulated microglia 24 h before ischemia, and at 2, 3, 5, and 7 days after ischemia. To determine whether exogenous microglia reduced ischemic damage caused by transient global ischemia, surviving pyramidal neurons were counted in ISCH or sham ISCH animals infused with vehicle (ISCH/VEH, SHAM/VEH), nonstimulated microglia (ISCH/Mi, SHAM/Mi), or IFNγ-stimulated microglia (ISCH/γMi, SHAM/γMi), 24 h before (PRE) or 24 h after (POST) ischemia. All neurons (cresyl violet-positive cells) with an intact morphologic appearance were counted in each animal either 7 or 14 days after ischemia. A total of 10 animals were examined for each of the 12 conditions.

Passive Avoidance Task

A step-through type passive avoidance apparatus was used to evaluate memory, as described previously (Nanri *et al*, 1998). The apparatus comprised two compartments: one light and one dark (each 16.5 × 16.0 × 14.7 cm), separated by a guillotine door. The dark compartment had a stainless-steel grid floor. A scrambled electric shock was delivered through the grid floor by a shock generator (Daiichi Kikai Inc., Tokushima, Japan). The passive avoidance learning trial was performed either 7 or 14 days after inducing ischemia. At the beginning of the training session, each animal was placed in the light compartment. When the animal stepped into the dark compartment, the door between compartments was closed and a foot shock (0.6 mA, 3 secs) was delivered through the floor. The door was then opened and the animal was allowed to cross back into the light compartment. Each time the animal stepped into the dark compartment, a foot shock was delivered. Eventually, the animal remained in the light compartment. The learning trial lasted 8 mins.

The retention test was performed 24 h after the learning trial. In the retention test, the gerbil was placed in the light compartment, and the latency to enter the dark compartment was measured. If the gerbil did not enter the dark compartment within 180 secs, a score of 180 secs was assigned. Performance on the passive avoidance task was evaluated in the SHAM/VEH-(PRE/POST), ISCH/VEH-(PRE/POST), ISCH/Mi-(PRE/POST), and ISCH/γMi-(PRE/POST) groups. Ten animals were used for each of the eight conditions.

Enzyme-Linked Immunosorbent Assay

Changes in BDNF and GDNF levels were measured in the hippocampus in SHAM/VEH, ISCH/VEH, ISCH/Mi, or ISCH/γMi animals using the BDNF and GDNF Emax Immunoassay System Kit (Promega, Madison, WI, USA). In the hippocampus of ISCH/VEH-PRE or ISCH/Mi-PRE gerbils, each neurotrophic factor level was measured 2 h, 1 day, 3 days, and 7 days after inducing ischemia. In the hippocampus of ISCH/VEH-POST and ISCH/Mi-POST animals, each neurotrophic factor level was measured 26 h, 2 days, 4 days, and 8 days after ischemia. As a control, we used the hippocampus from SHAM/VEH-PRE animals, which was dissected 6 h after the treatment (Marmigere *et al*, 2003). Neurotrophic factor levels were also measured in the rest of the brain after the hippocampus was removed.

Results

Recruitment of Exogenous Microglia into CA1 Hippocampal Lesions

Figure 1 shows fluorescent staining in the hippocampal CA1 field of ISCH animals injected with PKH26-labeled exogenous microglia 24 h before ischemia. PKH26-labeled cells accumulated near TUNEL-positive cells (Figures 1A–1D). PKH26-labeled cells were also located in the vicinity of MAP2-positive pyramidal neurons 3 days after ischemia (Figure 2A). IB4 staining of adjacent sections revealed that all PKH26-labeled cells were IB4-positive (Figures 2B–2D).

PKH26-labeled cells that reacted with IB4 were identified as microglia. Most of the microglia that accumulated in the CA1 field were both PKH26- and IB4-positive exogenous microglia, whereas there were only a few PKH26-negative and IB4-positive endogenous microglia (Figures 2B–2D). The TUNEL-positive cells were probably pyramidal neurons, because they were in the CA1 pyramidal cell layer and were not labeled with PKH26 (Figures 1A–1D). Neither TUNEL-positive pyramidal neurons nor PKH26-labeled microglia were detected in CA1 1 day after ischemia (data not shown). Two days after ischemia, only a few TUNEL-positive pyramidal neurons were detected in a portion of CA1, and PKH26-labeled microglia had accumulated in or around the TUNEL-positive neurons (Figures 1A and 1B). Three days after ischemia, a few TUNEL-positive pyramidal neurons were detected in CA1 (data not shown) and PKH26-labeled microglia were scattered throughout the CA1 field (Figure 2A). Five days after ischemia, many TUNEL-positive pyramidal neurons and PKH26-labeled microglia were distributed throughout CA1. Many of these cells had accumulated near the CA1–CA2 border (Figures 1C and 1D). The number of TUNEL-positive pyramidal neurons and PKH26-labeled microglia in the CA1 layer increased in a time-dependent manner until 7 days after ischemia (Figures 1A–1D and 3A). Stimulation with IFNγ increased the number of migrating exogenous microglia in the ischemic CA1 pyramidal cell layer (Figure 3A). The temporal profile of exogenous microglia recruitment was the same if the microglia were injected after ischemia onset (data not shown). In a separate set of experiments, PKH26-labeled macrophages did not enter the brain from the circulation (data not shown).

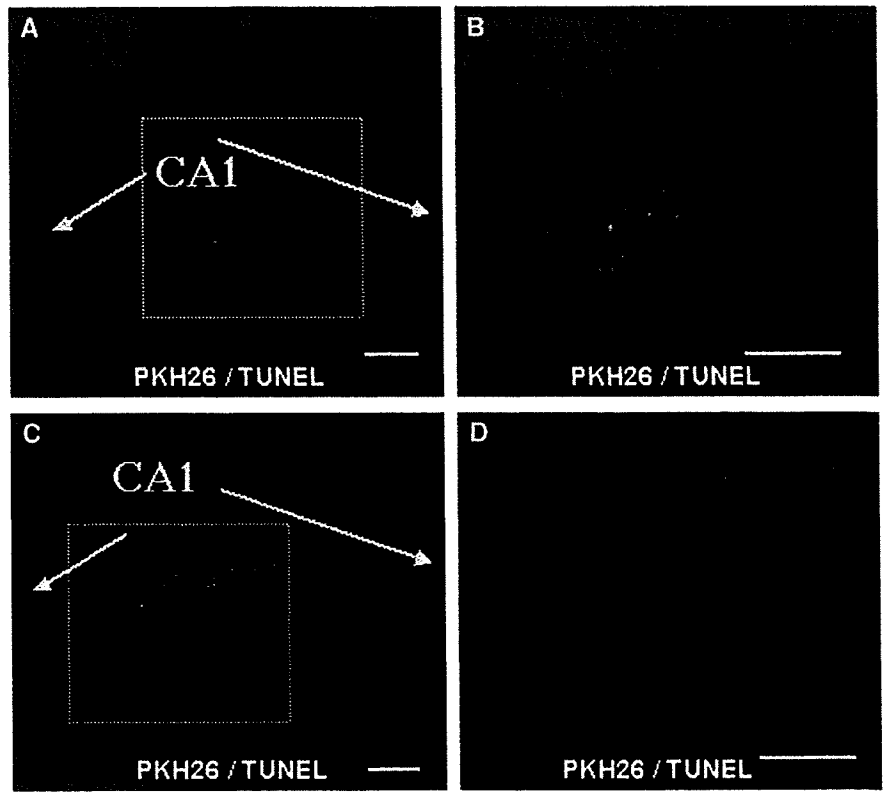

Figure 1 Migration of PKH26-labeled cells to TUNEL-positive CA1 pyramidal neurons after transient global ischemia. The panels show fluorescent staining in hippocampal CA1 sections obtained from ISCH animals injected with PKH26-labeled exogenous microglia 24 h before ischemia. (**A–D**) Double staining with PKH26 and TUNEL (PKH26/TUNEL). (**A**) Small numbers of TUNEL-positive pyramidal neurons (green) appeared in the CA1 field 2 days after ischemia. PKH26-labeled exogenous microglia (red) accumulated around TUNEL-positive pyramidal neurons (green). (**B**) Higher magnification of the boxed area in panel A. (**C**) Many TUNEL-positive pyramidal neurons (green) were present in the CA1 field 5 days after ischemia. Many PKH26-labeled exogenous microglia (red) accumulated in the ischemic CA1 field. (**D**) Higher magnification of the boxed area in panel C. (**A, C**) Arrows indicate the longitudinal extent of the CA1 field of the hippocampus. All scale bars = 10 μm.

Reduction of Ischemic Damage by Microglia Injection

Figure 3B shows the hippocampus from SHAM/VEH, ISCH/VEH, ISCH/Mi, and ISCH/γMi animals 7 days after ischemia. Most of the pyramidal neurons in the CA1 field had degenerated in ISCH/VEH animals. Microglial infusion (ISCH/Mi) increased the number of surviving pyramidal neurons, and IFNγ stimulation (ISCH/γMi) enhanced the microglial neuroprotective effect.

Figure 3C shows the number of surviving pyramidal neurons in the CA1 field 7 days after ischemia in SHAM/VEH, ISCH/VEH, ISCH/Mi, and ISCH/γMi animals. SHAM/VEH animals were used as controls ($n=10$). There was no effect of injection of nonstimulated microglia or IFNγ-stimulated microglia on the number of surviving CA1 pyramidal neurons in SHAM/VEH animals (data not shown). Ischemia significantly reduced the number of surviving pyramidal neurons. Intraarterial injection of microglia (either PRE or POST) significantly increased the number of surviving neurons ($P<0.001$). Stimulation of the microglia with IFNγ further enhanced neuron survival ($P<0.001$). The injection of vehicle, nonstimulated microglia, or IFNγ-stimulated microglia had no effect on postoperative body temperature throughout the survival period (data not shown). The number of surviving CA1 neurons at 14 days after ischemia was not different from that at 7 days after ischemia in each study (data not shown). Injection of microglia 48 h after ischemia had no significant effect on pyramidal neuron death (data not shown). The injection of PKH26-labeled macrophages into ISCH animals had no effect on the number of surviving CA1 pyramidal neurons (data not shown).

Prevention of Ischemia-Induced Learning Impairment by Microglia Injection

The passive avoidance task was used to determine whether exogenous microglia prevent ischemia-induced learning impairment. Figure 4 shows behavioral responses from gerbils 7 days after ischemia. The mean retention latency was shorter

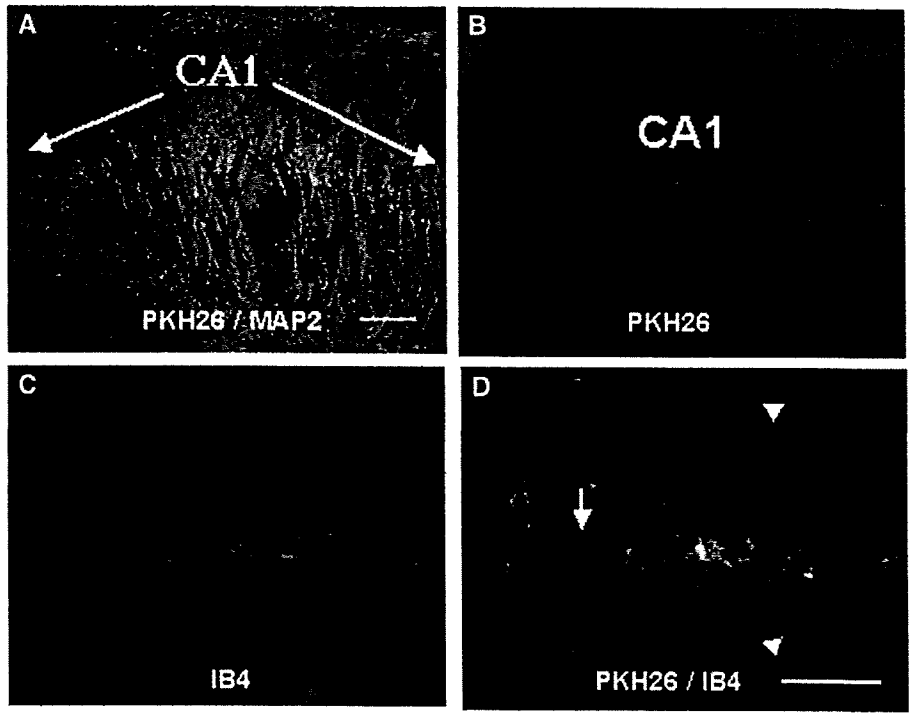

Figure 2 Migration of exogenous microglia to hippocampal CA1 lesions after transient global ischemia. The panels show fluorescent staining in hippocampal CA1 sections obtained from ISCH animals injected with PKH26-labeled exogenous microglia 24 h before ischemia. (**A**) Double staining with PKH26 and an anti-MAP2 antibody (PKH26/MAP2). Many PKH26-labeled exogenous microglia (red) were scattered around MAP2-positive pyramidal neurons (green) 3 days after ischemia. (**B**) PKH26 staining of the adjacent hippocampal section in panel A. (**C**) IB4 staining of the adjacent section. (**D**) Double staining of the adjacent section with PKH26 and IB4 (PKH26/IB4). All of the PKH26-labeled exogenous microglia (red) in the CA1 area expressed IB4 (green). A few IB4-positive and PKH26-negative endogenous microglia (arrow) were observed. Vascular endothelial cells around the hippocampus (arrowheads) also expressed IB4. Arrows in panel A indicate the longitudinal extent of the CA1 field of the hippocampus and the arrow in panel D indicates endogenous microglia. Arrowheads indicate endothelial cells. All scale bars = 10 μm.

for ISCH/VEH animals than for SHAM/VEH animals ($P<0.01$). The mean retention latency of the ISCH/Mi-PRE animals was longer than that of the ISCH/VEH-PRE animals ($P<0.01$), although the mean retention latency of the ISCH/Mi-POST animals was not different from the ISCH/VEH-POST animals. The mean retention latency was significantly longer in the ISCH/γMi-PRE ($P<0.001$) and ISCH/γMi-POST ($P<0.01$) animals (Figure 4) compared with the ISCH/VEH (PRE/POST) animals. The microglia-induced increase in the mean retention latency at 14 days after ischemia was not statistically different from the mean retention latency at 7 days after ischemia in each study (data not shown). The injection of macrophages into ISCH animals had no effect on retention latency (data not shown).

BDNF and GDNF Expression in the ISCH Hippocampus

The time course of hippocampal BDNF and GDNF expression was examined in SHAM and ISCH animals, and the effects of exogenous microglia on the expression of these neurotrophic factors was investigated. The time course of hippocampal BDNF and GDNF expression after ischemia was measured by enzyme-linked immunosorbent assay (Figure 5). In the hippocampus of ISCH animals, GDNF expression levels decreased significantly 2 h after ischemia and gradually increased during the next 7 days. In contrast, animals that received microglial injections 24 h before ischemia showed no decrease in BDNF or GDNF levels. Expression levels of BDNF remained constant until 3 days after ischemia, at which time they approximately doubled. Expression of GDNF in microglia-injected animals showed a rapid increase 2 h after ischemia and continued to increase during the course of 7 days to approximately 600% of the baseline level. There was no difference in BDNF or GDNF expression levels in animals injected with IFNγ-stimulated microglia, compared with nonstimulated microglia. Injection of microglia 24 h after ischemia induced a similar increase in BDNF and GDNF expression (Figure 5). Microglia-induced increases in neurotrophic factors were not observed except in the hippocampus (data not shown). The injection of macrophages into ISCH animals did not change BDNF or GDNF expression in the hippocampus (data not shown).

To determine which cells expressed BDNF and GDNF in our ischemia model, we examined the hippocampus using immunohistochemical analysis in SHAM/VEH, ISCH/VEH-PRE, and ISCH/Mi-PRE gerbils (Figure 6). In SHAM animals, hippocampal CA1 neurons and endogenous microglia-like cells expressed both GDNF and BDNF (Figures 6A and 6E). Two hours after ischemia, most of the CA1 neuron-like cells did not express BDNF and GDNF, and BDNF-positive endogenous microglia-like cells were scattered throughout CA1 (Figures 6B and 6F). Three days after ischemia, most CA1 neurons weakly expressed BDNF, whereas GDNF levels recovered (Figures 6C and 6G). Seven days after ischemia, BDNF immunoreactivity was detected in the CA1 neuron-like cell bodies, endogenous microglia, and also in some cell bodies and processes of reactive astrocyte-like cells near the ischemic CA1 field (Figure 6D).

At 7 days, damaged CA1 neuron-like cells expressed GDNF and many GDNF-positive, microglia-like cells were observed throughout CA1, whereas GDNF-positive, astrocyte-like cells were not detected (Figure 6H). Most of the CA1 neuron-like cells expressing BDNF or GDNF seemed to undergo degeneration because the cytoplasm was enlarged and the nucleus was not visible. Injection of microglia 24 h before ischemia greatly increased staining of BDNF- and GDNF-like immunoreactivity in CA1 neurons.

Discussion

In this study, we show that systemically injected microglia provide significant neuroprotection to the hippocampus after an ischemic insult. In addition to promoting CA1 cell survival, injection of microglia either before or 24 h after ischemia improved performance in a passive-avoidance learning task. There was also an increase in BDNF and GDNF expression in the hippocampus.

Previous studies indicate that endogenous microglia migrate to the CA1 pyramidal cell layer and protect ischemic neurons after reperfusion neuronal injury (Nitatori *et al*, 1995; Pinteaux *et al*, 2006; Neumann *et al*, 2006). In the present study, exogenous microglia migrated to areas that suffered ischemic damage, indicated by the presence of PKH26-positive cells around TUNEL-positive neurons in the CA1 pyramidal cell layer. Most of the cells were both PKH26-positive and IB4-positive exogenous microglia, although there were also a few

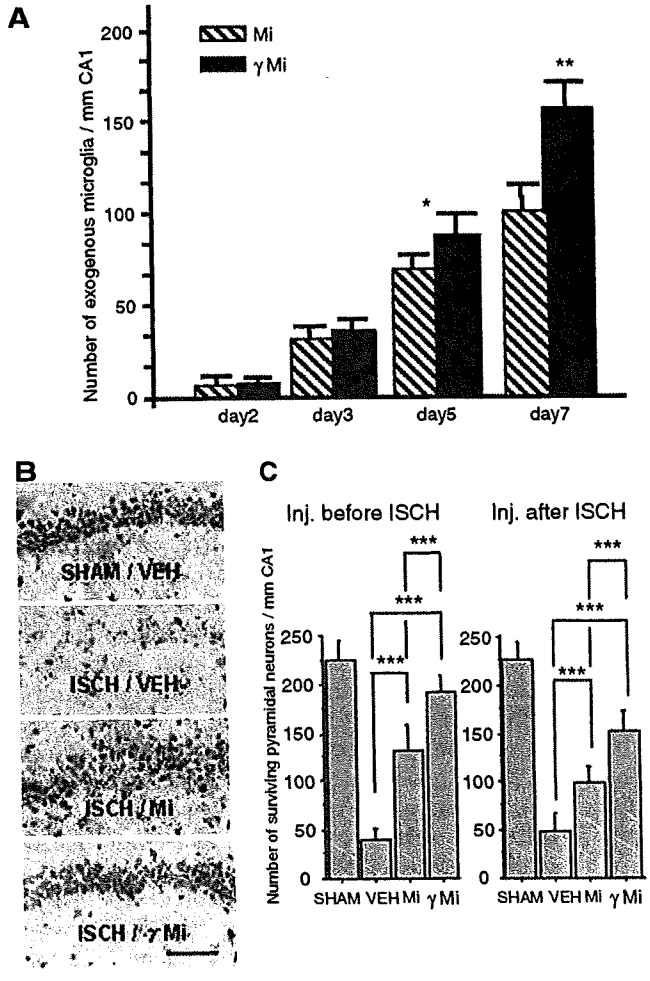

Figure 3 Neuroprotective effects of exogenous microglia. **(A)** Time course of the number of PKH26-labeled microglia migrating to the ischemic CA1 layer. Stimulation with IFNγ resulted in higher numbers of migrating microglia in the ischemic CA1 pyramidal than nonstimulated microglia. Vertical bars in each histogram indicate means ± s.d. ($n = 10$). Hatched columns (Mi) indicate the number of migrating nonstimulated microglia and black columns (γMi) indicate the number of IFNγ-stimulated microglia. $^*P < 0.05$ and $^{**}P < 0.01$, significantly different from the corresponding nonstimulated microglia-infused group (Student's *t*-test). **(B)** Photomicrographs of CA1 hippocampal sections 7 days after ischemic insult. SHAM/VEH: intraarterial injection of culture medium 24 h before sham-ischemia treatment; ISCH/VEH: intraarterial injection of culture medium 24 h before ischemia; ISCH/Mi: intraarterial injection of nonstimulated microglia 24 h before ischemia; ISCH/γMi: intraarterial injection of IFNγ-stimulated microglia 24 h before ischemia. Scale bar = 5 μm and applies to all four panels. **(C)** The number of surviving pyramidal neurons in the hippocampus 7 days after ischemia onset. Injection of microglia resulted in higher numbers of surviving pyramidal neurons after transient global ischemia (relative to ISCH/VEH), even when injections were placed 24 h after ischemia. Stimulation with IFNγ increased microglial neuroprotective effect. Vertical bars represent the mean ± s.d. ($n = 10$). $^{***}P < 0.001$, Analysis of variance with Bonferroni *post hoc* test for multiple pairwise comparisons. Inj. before ISCH: intraarterial injection 24 h before ischemia treatment; Inj. after ISCH: intraarterial injection 24 h after ischemia treatment; SHAM: injection of culture medium into sham-ischemia treated animals; VEH: injection of culture medium into ischemia-treated animals; Mi: injection of nonstimulated microglia into ischemia-treated animals; γMi: injection of IFNγ-stimulated microglia into ischemia-treated animals.

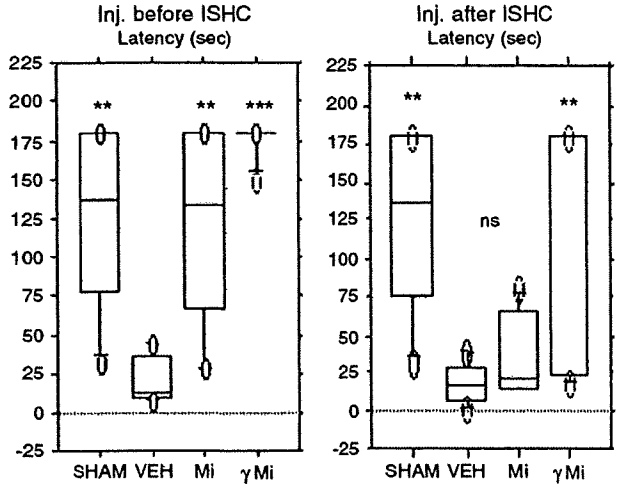

Figure 4 The effects of microglia on the latency of the transient ischemic gerbils in the step-through passive avoidance task. The graphs show the results of passive avoidance task 7 days after ischemia. The mean retention latency of ISCH/Mi-PRE and ISCH/γMi-PRE animals was longer than that of ISCH/VEH-PRE animals. The mean retention latency of ISCH/γMi-POST animals was significantly longer than that of ISCH/VEH-POST animals, whereas that of ISCH/Mi-POST was not significantly longer. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from ISCH/VEH with Scheffe's *post hoc* test for multiple pairwise comparisons ($n = 10$). Inj. before ISCH: intraarterial injection 24 h before ischemia treatment; Inj. after ISCH: intraarterial injection 24 h after ischemia treatment; SHAM: injection of culture medium into sham-ischemia treated animals; VEH: injection of culture medium into ischemia-treated animals; Mi: injection of nonstimulated microglia into ischemia-treated animals; γMi: injection of IFNγ-stimulated microglia into ischemia-treated animals.

PKH26-negative, IB4-positive cells. PKH26-negative, IB4-positive cells are either endogenous microglia/macrophage lineage cells or vascular endothelial cells.

Activated microglia share several characteristics with peripheral macrophages *in vitro*, but they display different phenotypes *in vivo* (Ling and Wong, 1993; Sawada *et al*, 1995, 1990, 1992). Several studies suggest a possible contribution of the invasion of blood-borne macrophages to delayed neuronal death after transient global ischemia (Lees, 1993). Our earlier studies showed that macrophages do not enter undamaged brain from the circulation (Imai *et al*, 1997). They might, however, enter the brain from the site of delayed neuronal death, where neovascularization and blood–brain barrier breakdown occur after ischemia (Kataoka *et al*, 2000). In our ischemia model, PKH26-labeled peritoneal macrophages did not enter the brain from the circulation. Therefore, IB4-positive, PKH26-negative cells migrating to the ischemic CA1 layer are likely to be endogenous microglia. Most of the migrating cells in the CA1 area were not endogenous microglia, but rather exogenous microglia. It is possible that isolated microglia from the mixed brain cultures are already activated (Streit, 1993) and so migrate to the ischemic lesion more rapidly than endogenous microglia.

Injection of exogenous microglia increased the number of surviving CA1 neurons after transient global ischemia, even when microglia were injected after the ischemic insult. The microglial neuroprotective effect also prevented an ischemia-induced learning impairment. In this and previous studies, we have shown that microglia isolated from a newborn mixed brain culture express both BDNF and GDNF, and intraarterial injection of microglia increases the presence of these neurotrophic factors in the ischemic hippocampus (Suzuki *et al*, 2001). Beyer *et al* (2000) reported that phagocytosis of Latex beads induced a microglial amoeboid morphology but did not increase immunologically relevant molecules. Microglia that phagocytose PKH26 would have similar properties as those that phagocytose Latex beads. PKH26 phagocytosis labeling did not affect microglial expression of neurotrophic factors and neuroprotection (data not shown). Upregulation of both neurotrophic factors in damaged brain is crucial for protection from neurodegeneration (Ebadi *et al*, 1997). The association between exogenous microglia at the lesion site and decreased cell death, increased neurotrophic factor expression, and improved learning ability after ischemic injury leads us to conclude that microglia have a protective effect rather than a toxic effect, on neurons under ischemic conditions.

We examined the time course of hippocampal BDNF and GDNF expression after transient global ischemia, and investigated the effect of exogenous microglia on the expression of these neurotrophic factors. In normal CA1 neurons, the expression of both neurotrophic factors was preserved; however, their immunoreactivity was reduced 2 h after ischemia and then increased in a time-dependent manner. Seven days after ischemia, the main sources of BDNF were damaged CA1 neurons, endogenous microglia, and reactive astrocytes. Reactive astrocytes were excluded as the source of GDNF because they do not express GDNF in our model. The BDNF results are consistent with previous studies (Kokaia *et al*, 1996; Lee *et al*, 2002), whereas the GDNF results are inconsistent with a study by Miyazaki *et al* (2001). They reported that GDNF expression in the hippocampal CA1 region increased between 3 and 24 h after ischemia, and then declined to baseline levels. In their model, in which rats are subjected to a transient global ischemia induced by four-vessel occlusion, reactive astrocytes express GDNF. The discrepancy in the results between the two studies is possibly because of the use of different animal models of ischemia. In the present study, injection of microglia prevented the decrease in BDNF and GDNF expression in the hippocampus immediately after ischemia, and later increased the expression of these trophic factors. CA1 neurons,

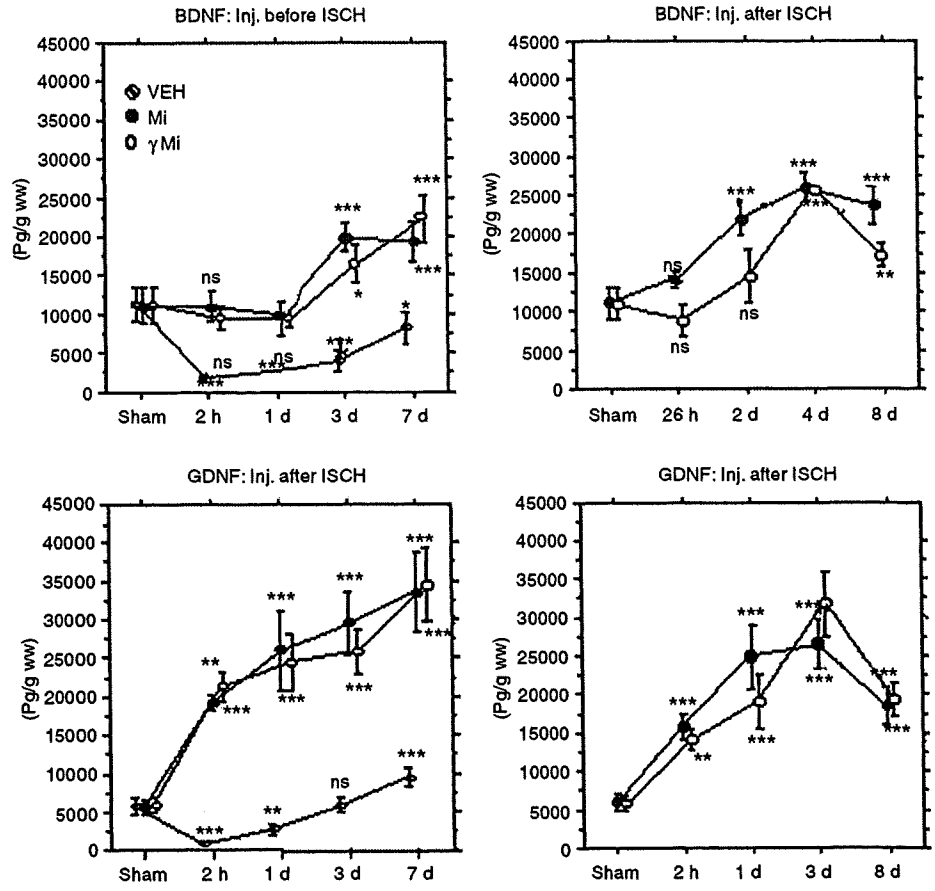

Figure 5 The effects of exogenous microglia on the time course of hippocampal BDNF and GDNF expression after transient global ischemia. Graphs indicate the BDNF and GDNF expression level in the hippocampus detected by enzyme-linked immunosorbent assay. Results are expressed as means of picograms of BDNF or GDNF normalized to gram of wet tissue weight (pg/g ww). As a control, we used SHAM/VEH hippocampus, which was dissected 6 h after the treatment. In SHAM/VEH hippocampus, BDNF and GDNF expression was observed. In ISCH/VEH-PRE animals, however, the levels were less than 20% of the baseline level (SHAM/VEH-PRE level) 2 h after ischemia. Injection of microglia prevented the decrease of both BDNF and GDNF and then induced an increase in both factors above baseline levels. In ISCH/Mi-PRE hippocampus, the neurotrophin levels were measured 2 h, 1 day, 3 days, and 7 days after ischemia. In ISCH/Mi-POST hippocampus, neurotrophin levels were measured 26 h, 2 days, 4 days, and 8 days after ischemia. Vertical bars represent the mean ± s.d. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from SHAM control with Bonferroni *post hoc* test. Abbreviations are as in Figure 2. Hatched circles (VEH) indicate values of BDNF or GDNF expression level in the ischemia-treated hippocampus injected with culture medium, black circles (Mi) indicate those injected with nonstimulated microglia, and open circles (γMi) indicate those injected with IFNγ-stimulated microglia.

rather than microglia or astrocytes, were the main source of the later high expression of BDNF and GDNF in the hippocampus observed 7 days after ischemia. Injections of microglia into SHAM animals did not significantly increase BDNF or GDNF expression in the brain, although a large number of PKH26-labeled microglia enter the brain from the circulation (Imai *et al*, 1997; Sawada *et al*, 1998). Astrocytes expressing these neurotrophic factors were not detected in ischemic hippocampus injected with microglia. The maintenance of BDNF and/or GDNF expression in injured neurons might be one mechanism of neuroprotection in the ischemic hippocampus.

Intraventricular administration of a Sendai virus vector carrying GDNF or nerve growth factor increases the expression of these neurotrophic factors in the hippocampus and prevents delayed neuronal death induced by transient global ischemia in gerbils, even when administered 6 h after ischemia (Shirakura *et al*, 2004). The time course for increased GDNF expression was similar to that in the present experiment, except that it took less time for exogenous microglia to express GDNF than it took for the Sendai virus vector. This might be one reason why we observed neuroprotection even when the microglia were injected 24 h after ischemia. Intrahippocampal administration of BDNF in adult rats improves performance in a spatial memory task (Cirulli *et al*, 2004). Administration of GDNF and BDNF to the site of ischemia might be one of the mechanisms underlying microglial neurotrophic effects on ischemic CA1 neurons.

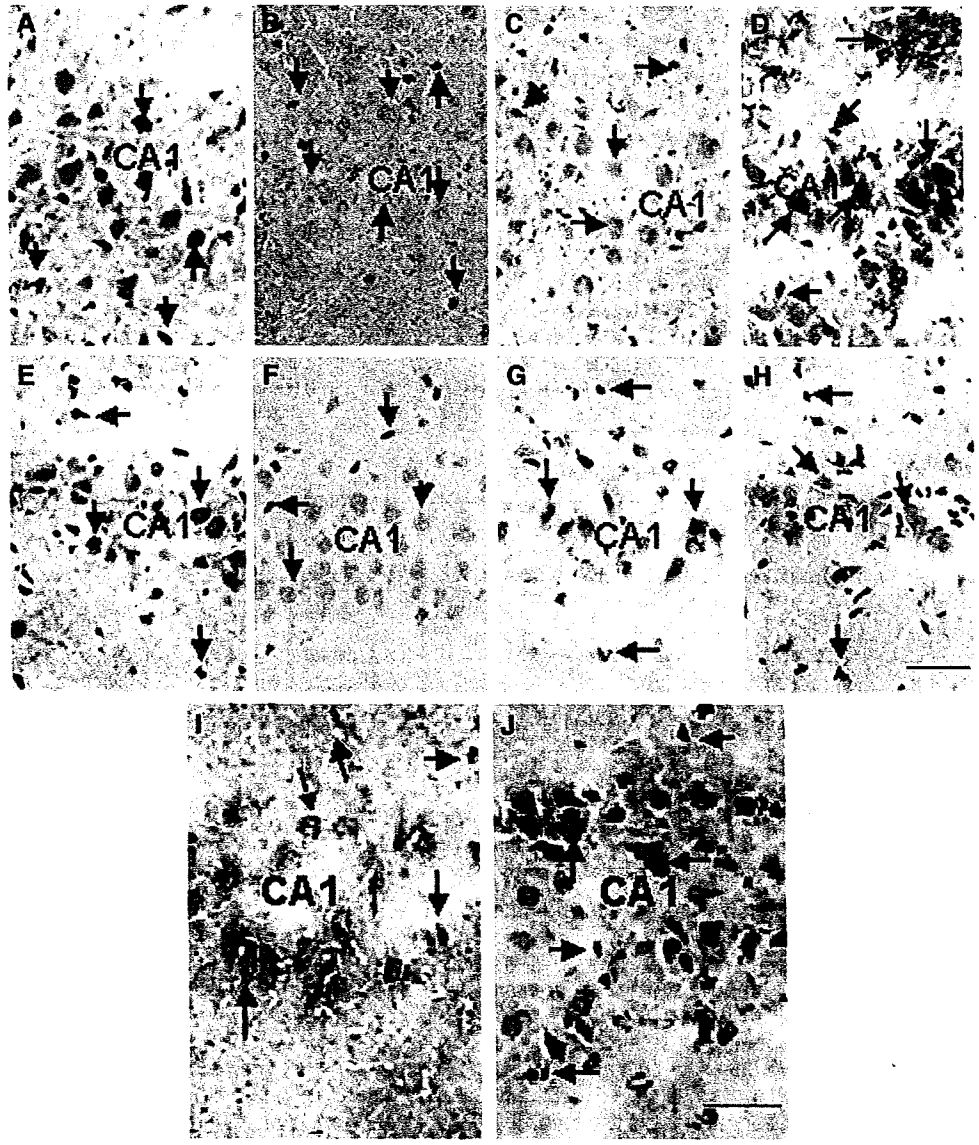

Figure 6 Immunohistochemical staining for BDNF and GDNF after transient global ischemia. (**A–H**) Time course of BDNF and GDNF expression in the hippocampus of SHAM and ischemia-treated animals injected with culture medium. (**A**) Most of the CA1 neurons expressed BDNF in the SHAM hippocampus. (**B**) Two hours after ischemia, there was little, if any, BDNF expression in CA1 neurons. BDNF-expressing endogenous microglia were observed around the CA1 field. (**C**) Three days after ischemia, most of the CA1 neurons weakly expressed BDNF. (**D**) Seven days after ischemia, a large number of damaged CA1 neurons and reactive astrocytes expressed BDNF. (**E**) Most of the CA1 neurons expressed GDNF in the SHAM hippocampus. (**F**) Two hours after ischemia, there was little, if any, GDNF expression in CA1 neurons, whereas there were a few GDNF-expressing endogenous microglia. (**G**) Three days after ischemia, most of the CA1 neurons expressed GDNF. (**H**) Seven days after ischemia, damaged CA1 neurons expressed GDNF and many GDNF-expressing exogenous microglia were scattered around them. (**I** and **J**) BDNF and GDNF expression, respectively, in the hippocampus 2 h after ischemia. Nonstimulated microglia were injected 24 h before ischemia treatment. Injection of microglia prevented the loss of both BDNF and GDNF expression in the hippocampus immediately after ischemia. Red arrows: CA1 neurons; black arrows: microglia; green arrows: reactive astrocytes. Scale bars = 5 μm.

Lee *et al* (2002) reported that BDNF mRNA increases 3 h after ischemia and returns to sham-operated level in the CA1 pyramidal neurons 1 day after ischemia, whereas BDNF protein decreases 3 h after ischemia and recovers to some extent at 7 days after ischemia. This expression disparity between neurotrophin mRNA and protein was reported to be because of a translational and/or posttranslational dysfunction of protein synthesis, release, or transport immediately after cerebral injury (Lee *et al*, 2002). In our study, the recovery of BDNF and GDNF expression was observed in degenerated neurons. The expression of neurotrophic factors might not enhance the survival of

ischemic pyramidal neurons, however, because most degenerating cells do not express BDNF or GDNF receptors after ischemic insult (Ferrer *et al*, 1998; Wang *et al*, 2004). Although pretreatment of microglia with IFNγ significantly enhanced microglial neuroprotective effects on ischemic CA1 pyramidal neurons, it did not increase hippocampal BDNF or GDNF expression, compared with nontreated microglia. Nevertheless, pretreatment with IFNγ increased the number of microglia migrating to the ischemic hippocampus. It is possible that microglia pretreated with IFNγ induce expression of neurotrophic factor receptors in ischemic pyramidal neurons, thus providing a neuroprotective effect. We are now investigating the mechanism.

Cytokines secreted by microglia might also affect ischemic CA1 neurons. We did not examine cytokine activity in this study because neurons and microglia interact with each other and produce complicated effects *in vivo*. For example, activated microglia release factors capable of generating oxidative damage, such as superoxide anions (O_2^-) and nitric oxide (Saud *et al*, 2005), whereas transient global ischemia increases transforming growth factor-β1 expression in CA1 pyramidal neurons (Zhu *et al*, 2000), which could eliminate microglial O_2^- and nitric oxide production (Herrera-Molina and von Bernhardi, 2005).

Microglial activation, after neuronal injury, appears to serve a neuroprotective function. A strong inflammatory response, however, could induce microglia to be hyperactive, which allows them to escape endogenous control and become toxic to neurons (Polazzi and Contenstabile, 2002). Interleukin-1β secreted by microglia activates p38 mitogen-associated protein kinase and cyclic adenosine monophosphate-response element binding protein to suppress long-term potentiation, which is thought to be an important underlying mechanism of learning and memory (Srinivasan *et al*, 2004). In our preliminary data, however, systemic injection of exogenous microglia in rabbits subjected to transient global ischemia prevented the ischemia-induced suppression of long-term potentiation (unpublished data). Microglia also produce interleukin-10 (Wu *et al*, 2005; Broderick *et al*, 2000), which reverses lipopolysaccharide-induced increases in signaling in the hippocampus (Lynch *et al*, 2004). Exogenous microglia introduced to the site of ischemia might reverse ischemia-induced disruption of endogenous control by secreting antiinflammatory cytokines such as interleukin-10 (Wu *et al*, 2005; Ooboshi *et al*, 2005; Broderick *et al*, 2000). Further studies are necessary to verify the mechanism of microglial neuroprotective effects on ischemic CA1 neurons.

It is also possible that injection of nonautologous microglia induced an immune reaction that is responsible for the present findings. We believe that this is unlikely because there is no evidence in the literature for a nonspecific immune response to be neuroprotective and/or reverse behavioral deficits. This will be examined in the future.

In conclusion, peripherally injected exogenous microglia exhibit a specific affinity for ischemic brain lesions and protect against neuronal damage in our ischemia model, suggesting that one role of microglia is to protect damaged neurons after transient global ischemic insult. To continue the exploration of the uses of exogenous microglia, we are currently isolating microglia from bone marrow (Ono *et al*, 1999; Tanaka *et al*, 2003). In the future, the administration of microglia might be a potential tool for cell or gene therapy in the treatment of brain disease.

Acknowledgements

We express our gratitude to Zlokovic Berislav, who belongs to the University of Rochester Medical Center, Rochester, NY, USA, for helpful discussion and critical review of the manuscript. The authors have no conflicting financial interests.

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www.elsevier.com/locate/freeradbiomed

Original Contribution

Phosphatidylserine and phosphatidylcholine-containing liposomes inhibit amyloid β and interferon-γ-induced microglial activation

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Received 21 September 2006; revised 16 November 2006; accepted 5 December 2006
Available online 9 December 2006

Abstract

There is increasing evidence that microglial activation is one of the major pathogenic factors for Alzheimer's disease (AD) and the inhibition of the inflammatory activation of the microglia thus appears to be neuroprotective and a potentially useful treatment for AD. Phospholipids such as phosphatidylserine (PS) and phosphatidylcholine (PC) have been reported to modulate the immune function of phagocytes. In addition, PS has been reported to be a nootropics that can be used as nonprescription memory or cognitive enhancers. We therefore evaluated the effects of liposomes, which comprise both PS and PC (PS/PC liposomes), on the microglial production of tumor necrosis factor-α (TNF-α), nitric oxide (NO), and superoxide ($^{\bullet}O_2^-$) induced by amyloid β (Aβ) and interferon-γ (IFN-γ). Pretreatment of microglia with PS/PC liposomes considerably inhibited the TNF-α, NO and $^{\bullet}O_2^-$ production induced by Aβ/IFN-γ. These results suggest that PS/PC liposomes have both neuroprotective and antioxidative properties through the inhibition of microglial activation, thus supporting the nootropic and antidementia effect of PS.

Keywords: Alzheimer's disease; Microglia; Phosphatidylserine; Phosphatidylcholine; Nitric oxide; Superoxide; Peroxynitrite; Electron spin resonance

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease known to cause progressive memory loss and confusion. Recently, much attention has been paid to microglial activation as a major pathogenic factor for AD. Senile plaque is the site of inflammatory processes, as evidenced by the presence of degenerating neurons and numerous reactive microglia and astrocytes associated with such plaques [1]. Microglial activation has been reported to be a relatively early pathogenetic event which precedes the process of neuropil destruction in AD patients [2]. According to in vitro studies, the microglia activated by amyloid β peptides (Aβ) have been well reported to damage or kill neurons by the excessive release of inflammatory and potentially neurotoxic molecules such as proinflammatory cytokines [e.g., tumor necrosis factor-α (TNF-α), intrleukin-1β], reactive oxygen species (ROS), and reactive nitrogen species

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyroline-*N*-oxide; ESR, electron spin resonance; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NADPH oxidase, nicotinamide adenine diphosphate reduced form oxidase; NO, nitric oxide; PS, phosphatidylserine; PC, phosphatidylcholine; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

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doi:10.1016/j.freeradbiomed.2006.12.003