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Short Communication

Blockade of microglial glutamate release protects against ischemic brain injury

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ABSTRACT

Glutamate released by activated microglia induces excito-neurotoxicity and may contribute to neurodegeneration in numerous neurological diseases including ischemia, inflammation, epilepsy, and neurodegenerative diseases. We observed that the gap junction blocker carbenoxolone (CBX) or the glutaminase inhibitor 6-diazo-5-oxo-L-norleucine (DON) decreased glutamate release from activated microglia and rescued neuronal death in a dose-dependent manner *in vitro*. In gerbils, treatment with CBX or DON also prevented the delayed death of hippocampal neurons following transient global ischemia. Thus, blockade of microglial glutamate release may be an effective therapeutic strategy against neurodegeneration after ischemic injury.

Currently, thrombolysis is the main treatment for ischemic brain damage. Neuroprotective drugs are required to prevent subsequent neurodegeneration (Goldstein, 2007). Microglial activation is a putative cause of neurodegeneration in various neurological disorders including ischemia, inflammation, epilepsy, and neurodegenerative diseases (Block et al., 2007; Kreutzberg, 1996; Nelson et al., 2002). Activated microglia release large amounts of glutamate that induce excito-neurotoxicity (Barger and Basile, 2001; Piani et al., 1992; Takeuchi et al., 2005). Thus, inhibiting microglial activation is a candidate therapeutic approach for these diseases. However, microglia also exert neuroprotective effects including release of neurotrophins, uptake of glutamate, and sequestration of neurotoxic substances (Kempermann and Neumann, 2003). Therefore, any therapeutic approach should inhibit the deleterious effects of microglia without diminishing their protective role. Recently, we demonstrated that activated microglia produce glutamate via glutaminase and release it through hemichannels of gap junctions (Takeuchi et al., 2006). Here, we investigated whether the gap junction blocker or the glutaminase inhibitor that decrease microglial glutamate release also prevent neuronal death *in vitro* and *in vivo* in a rodent model of transient ischemic brain injury.

All protocols were approved by the Animal Experiment Committee of Nagoya University. All reagents except those otherwise specified were obtained from Sigma. Microglia were prepared from newborn C57BL/6J mice with the 'shaking off' method as described previously (Suzumura et al., 1987). Microglia were cultured in 24-well dishes at a density of 5×10^4 cells/well. Neuronal cultures were prepared from C57BL/6J mice at embryonic day 17 as described previously (Takeuchi et al., 2005). Activated microglia treated with 1 μg/ml lipopolysaccharide (LPS) were incubated with each drug at the following concentrations: NMDA receptor antagonist, 10 μM MK801 (Calbiochem); AMPA/kainate receptor antagonist, 20 μM CNQX (Calbiochem); P2X receptor antagonist, 100 μM PPADS; glutaminase inhibitor, 0.01–1 mM 6-diazo-5-oxo-L-norleucine (DON); gap junction inhibitor, 1–100 μM carbenoxolone disodium (CBX); Connexin (Cx) 32 mimetic peptide, 0.25 mg/l ^{32}gap 27 (Thermo Electron GmbH); Cx43 mimetic peptide, 0.25 mg/l ^{43}gap 27 (Thermo Electron GmbH). After a 24-h incubation, microglia conditioned medium was applied to each well containing 5×10^4 neurons at 10–13 days *in vitro*. Evaluations of neuronal damage were performed 24 h after medium exchange.

The neuritic beading, an early feature of neuronal damage, was assessed under a phase-contrast microscopy 24 h after stimulation as described previously (Takeuchi et al., 2005). Cell death was evaluated by dye-exclusion with propidium iodide (Molecular Probes) as described previously (Takeuchi et al., 2002). To measure extracellular glutamate concentrations, we used the Glutamate Assay Kit colorimetric assay (Yamasa Corporation, Tokyo, Japan) as described previously (Takeuchi et al., 2005). Each assay was carried out in six independent trials.

Adult male Mongolian gerbils (Kyudo Co., Ltd., Fukuoka, Japan), 10–12 weeks old and weighing approximately 70 g ($n=5$ per group), were

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anesthetized with sevoflurane. Global forebrain ischemia was produced transiently by occluding both common carotid arteries for 5 min using aneurysm clips as described previously (Imai et al., 1999). CBX or DON was administered intraperitoneally every other day from the day of ischemia at each dosage (CBX: 0.2, 2, or 20 mg/kg; DON: 0.016, 0.16, or 1.6 mg/kg). Control animals were injected with the equal volume of vehicle, phosphate-buffered saline (PBS). Seven days after ischemia, gerbils were anesthetized and transcardically perfused with 4% paraformaldehyde in PBS. The brains were removed, frozen in liquid nitrogen, and embedded in O.C.T. compound (Tissue Tek, Elkhart, IN). Sections (8 μm) were stained with haematoxylin and eosin. To assess the effect of drug treatment on delayed neuronal death, we counted the

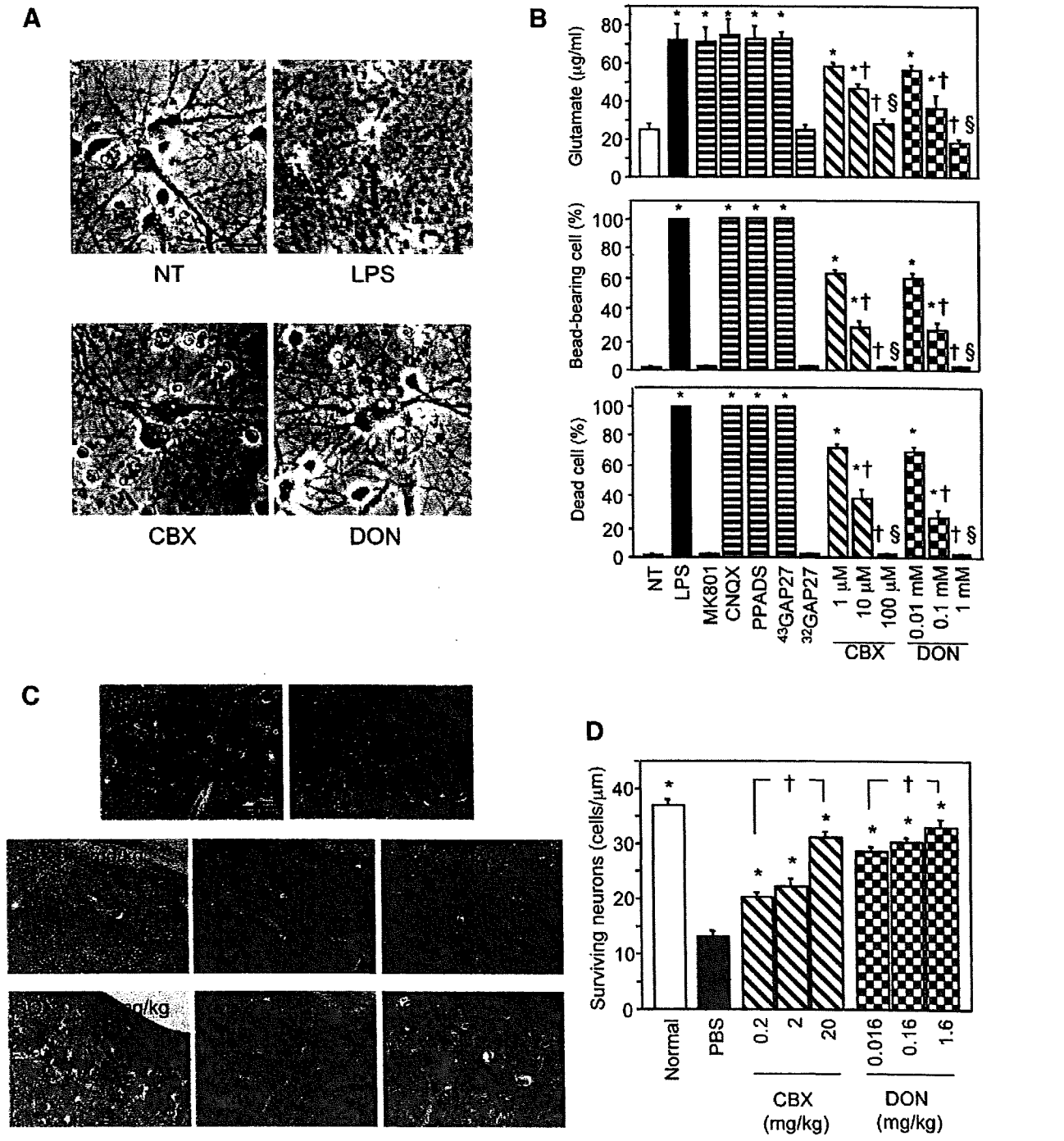

Fig. 1. CBX or DON reduced microglial glutamate release and subsequent excitotoxic neurodegeneration *in vitro* and *in vivo*. (A) Phase-contrast micrographs. NT, control neurons. LPS, neurons treated with LPS-stimulated microglial conditioned medium. CBX, neurons treated with LPS-stimulated microglial conditioned medium containing 100 μM CBX. DON, neurons treated with LPS-stimulated microglial conditioned medium containing 1 mM DON. Scale bar, 10 μm. (B) Extracellular glutamate concentration, frequency of bead-bearing neurons, and frequency of dead neurons. Microglial glutamate induced neuritic beading and subsequent neuronal death. Blockade of Cx32 hemichannel or NMDA receptor reduced microglial neurotoxicity, which revealed that activated microglia release glutamate from Cx32 hemichannels of gap junctions and microglial glutamate elicits neurotoxicity via NMDA receptor signaling. Both CBX and DON diminished microglial neurotoxicity in a dose-dependent manner. $^{*}p<0.05$ versus NT. $^{\dagger}p<0.05$ versus 1 μM. $^{\S}p<0.05$ versus 10 μM. (C) HE staining of gerbil hippocampal CA1. Normal, normal control. PBS, ischemic brain treated with PBS. CBX, ischemic brain treated with CBX at 0.2, 2, or 20 mg/kg. DON, ischemic brain treated with DON at 0.016, 0.16, or 1.6 mg/kg. Scale bar, 100 μm. (D) Density of surviving neurons in hippocampal CA1. Hippocampal CA1 neurons underwent delayed neuronal death 7 days after ischemia. Both CBX and DON decreased neuronal death in a dose-dependent manner. $^{*}p< 0.001$ versus PBS. $^{\dagger}p<0.001$.

number of surviving neurons per 100 μm in the hippocampal CA1 region under a deconvolution microscope system (Biozero, Keyence, Tokyo, Japan). All results were analyzed by one-way ANOVA with a Tukey–Kramer post-hoc test. The values represent the means±S.D.

We previously demonstrated that neuritic beading, focal bead-like swellings in dendrites and axons, is an early pathological feature of neuronal dysfunction that precedes neuronal death (Takeuchi et al., 2005). LPS-treated microglia released large amounts of glutamate and induced numerous beads in most neurites followed by neuronal death *in vitro* (Fig. 1A). This neuronal damage was almost completely abolished by the NMDA receptor antagonist MK801, not by AMPA/kainate antagonist CNQX nor P2X receptor antagonist PPADS (Fig. 1B). These findings were in consistent with our previous reports that microglial neurotoxicity is mediated primarily by NMDA receptor signaling (Takeuchi et al., 2005). Blockade of Cx32 with ^{32}gap27 strikingly diminished microglial glutamate release and subsequent neurotoxicity whereas blockade of Cx43 with ^{43}gap27 did not (Fig. 1B). These data were in accord with our previous report that Cx32 hemichannel of gap junction is a principal source of glutamate release from microglia (Takeuchi et al., 2006). Treatment with the gap junction blocker CBX or the glutaminase inhibitor DON effectively suppressed LPS-induced microglial glutamate release and subsequent neurotoxicity in a dose-dependent manner (Fig. 1B). These drugs did not suppress glutamate production in control neuronal cultures (data not shown). Subsequently, we assessed the neuroprotective effect of DON or CBX on delayed neuronal death in the hippocampal CA1 region of gerbils caused by transient global ischemia. As shown in Fig. 1C and D, CBX or DON significantly reduced neuronal death in a dose-dependent manner.

We previously reported that TNF-α is a key cytokine that induces microglial glutamate release by upregulating glutaminase and gap junctions (Takeuchi et al., 2006). Treatment with TNF-α neutralizing antibodies is an effective therapy for autoimmune diseases (Illei and Lipsky, 2000). However, this therapy has serious adverse side effects including an increased risk for infections and cancer. Thus, TNF-α neutralizing therapy is not readily applicable to chronic neurological diseases although TNF-α may play an important role in the progression of neurodegeneration. Blockade of NMDA receptors is another potent therapy for neurodegeneration. Unfortunately, adverse effects increase in a dose-dependent manner (Parsons et al., 1999). Cellular homeostasis of glutamate levels may be maintained primarily by the glutamate dehydrogenase pathway (Nissim, 1999), but activated microglia produce glutamate through a separate pathway involving the enzyme glutaminase and gap junctions (Takeuchi et al., 2006). Thus, CBX and DON represent promising therapeutic agents that may not perturb glutamate homeostasis. Moreover, blockade of gap junction may exert neuroprotection additively because neuronal and astrocytic gap junctions were also considered to contribute to ischemic neuronal damage (Lin et al., 1998; Thompson et al., 2006). Importantly, these drugs have been used clinically for other purposes (CBX, anti-inflammatory drug; DON, antitumor drug) but may also decrease neurodegeneration mediated by microglia following brain ischemia with minimum adverse side effects.

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Review

L-dopa therapy for Parkinson's disease: Past, present, and future

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Abstract

Dopamine (DA) supplementation therapy by L-dopa for Parkinson's disease (PD) was established around 1970. The dose of L-dopa can be reduced by the combined administration of inhibitors of peripheral L-amino acid decarboxylase (AADC), catechol *O*-methyltransferase (COMT), or monoamine oxidase B (MAO B). DA in the striatum may be produced from exogenously administered L-dopa by various AADC-containing cells, such as serotonin neurons. The long-term administration of L-dopa in PD patients may produce L-dopa–induced dyskinesia (LID), which may be due to chronic overstimulation of supersensitive DA D1 receptors. L-dopa may be used in combination with various new strategies such as gene therapy or transplantation in the future.

Keywords: L-dopa; Parkinson's disease; Dopamine; Dyskinesia; Selegiline

1. Historical overview of development of L-dopa therapy for Parkinson's disease

Parkinson's disease (PD) is an age-related movement disorder characterized by decreased levels of the neurotransmitter dopamine (DA) in the striatum of the brain, due to selective degeneration of the nigro-striatal DA neurons. In most instances PD appears to be sporadic, without any family history. While the etiology of PD has not been unequivocally determined, speculation has centered around complex mechanisms such as toxic environmental factors superimposed on susceptibility genes, oxidative stress caused by mitochondrial dysfunction, dysfunction of the ubiquitin proteasome system with resultant accumulation of misfolded proteins and endoplasmic reticulum stress, and formation of activated microglia accompanying an inflammatory process. These changes may lead to the programmed cell death (apoptosis or autophagy) of DA neurons [1,2]. DA in DA neurons is synthesized from L-3,4-dihydroxyphenylalanine (L-dopa), which is synthesized from tyrosine by tyrosine 3-monooxygenase (tyrosine hydroxylase, TH) [3]. Tyrosinase also produces L-dopa indirectly as an intermediate via dopaquinone and leucodopachrome from tyrosine in melanin-producing melanocytes [4], but tyrosinase protein is not detected in DA neurons [5]. In DA neurons, DA is synthesized from L-dopa as the first catecholamine (CA) neurotransmitter among three CAs (DA, noradrenaline/norepinephrine, and adrenaline/epinephrine) by the action of aromatic L-amino acid decarboxylase (AADC; dopa decarboxylase, DDC) [6]. These CAs are formed sequentially according to the following biosynthetic pathway: tyrosine → L-DOPA → DA → noradrenaline → adrenaline [7,8]. DA was discovered as a neurotransmitter in the animal brain based on its specific regional distribution. Depletion of DA by reserpine produces PD-like akinesia in animals, and supplementation of the deficient DA by L-dopa administration ameliorates the movement disorder. These results suggested that PD in humans is caused by DA deficiency in the nigro-striatum and led to the proposal that L-dopa might be effective for the treatment of PD by supplementing the deficient DA [9–11]. The presence and specific regional distribution of DA in the human brain as well as its deficiency in the striatum of PD patients were described in 1959 and 1960 [12–15]. L-dopa therapy to supplement the DA deficiency in the PD brain was tested by Sano and colleagues (1960) in Japan [13], by Birkmayer

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and Hornykiewicz (1961) in Austria [16], and by Barbeau and coworkers (1962) in Canada [17]. Positive and negative clinical results on the effects of L-dopa for PD were reported during the 1960s. Finally, in 1969 Cotzias and colleagues reported the achievement of remarkable clinical efficacy with L-dopa in PD patients by administering large doses to produce an adequate amount of DA in the PD brain [18]. These clinical studies established the efficacy of L-dopa therapy in PD.

The metabolism of orally administered L-dopa in PD patients was clarified in detail by gas-liquid chromatographic analysis of the metabolites in urine, which demonstrated increased output of the main DA metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and of a number of minor metablites, such as *p*- and *m*-hydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenyllactic acid [19]. These results indicate that metabolism of orally administered L-dopa in the periphery is catalyzed primarily by AADC, monoamine oxidase (MAO), and catechol *O*-methyltransferase (COMT).

Recognition of the role of peripheral AADC in the metabolism of L-dopa [20] led to the demonstration that the effective dose of L-dopa could be reduced by the co-administration of inhibitors (carbidopa, benzerazid) of peripheral AADC, which do not cross the blood–brain barrier and thus increase the blood level of L-dopa and its transfer into the brain. Co-administration of peripheral AADC inhibitors and L-dopa dramatically improved the bio-availability of L-dopa.

Subsequently, inhibitors of COMT [21] were found to inhibit the peripheral metabolism of L-dopa to 3-*O*-methyldopa and thus to increase the bio-availability of L-dopa. The COMT inhibitor entacapone does not cross the blood–brain barrier and increases the bio-availability of L-dopa by inhibiting peripheral COMT. Since COMT is also responsible for the metabolic inactivation of DA as well as L-dopa [22,23], another COMT inhibitor, tolcapone, which crosses the blood–brain barrier, reduces the effective dose of L-dopa and also affects the synthesis and metabolism of DA in the brain (see review by Bonifácio et al. [24]).

DA in the human brain is oxidized primarily by MAO B. Thus, inhibitors of MAO B are expected to protect DA against its degradation. Birkmayer and colleagues [25] reported that a specific inhibitor of MAO B, deprenyl (selegiline), is clinically effective in PD patients.

Furthermore, in the early stage of PD, various DA receptor agonists have been found to be effective either alone or in combination with smaller amounts of L-dopa. However, L-dopa is still "the gold standard" for PD drug therapy.

In this review, present problems and future prospects concerning L-dopa therapy for PD will be discussed.

2. In which cells in the brain is functionally effective DA produced from exogenous L-dopa?

In the PD brain, nigro-striatal DA neurons degenerate, and the activities and levels of mRNA and protein of TH and AADC that synthesize DA from tyrosine are reduced in the striatum (see review by Nagatsu and Sawada [26]). Therefore, exogenously administered L-dopa transferred from the blood into the brain cannot be efficiently converted to DA owing to the paucity of residual nigro-striatal DA neurons in the advanced stages of PD. However, exogenously administered L-dopa may still be decarboxylated to DA, primarily within AADC-containing cells present in the striatum besides the remaining DA nerve terminals, and thus reach postsynaptic DA receptors. There actually are several types of AADC-containing cells besides the residual DA neurons in the brain: NA neurons, serotonin neurons, AADC-containing "D" neurons, DA neurons intrinsic to the striatum [27], and glial cells.

The DA neurons intrinsic to the striatum are morphologically similar to the medium-sized aspiny striatal interneurons, and express TH, DA-transporter, and glutamic acid decarboxylase (GAD). These TH-positive neurons may act as a local source of DA in the striatum and may be part of a compensatory mechanism that can be artificially enhanced to alleviate or delay PD symptoms [27].

Kitahama and co-workers examined DA immunoreactive neuronal structures by using immunohistochemistry with an anti-DA antiserum, following injection of L-dopa with or without pargyline, an MAO inhibitor that inhibits both MAO A and B, in the cat brain [28]. As a result, they observed DA immunoreactivity in the cell bodies and axons of serotonin neurons and noradrenaline neurons, and also in AADC-containing "D neurons".

Glial cells are considered to be important for the conversion of L-dopa to DA, since the number of glial cells is much larger than that of AADC-containing neural cells.

In L-dopa therapy, serotonin neurons and glial cells are speculated to be the main cells that produce DA for stimulating DA receptors. In fact, L-dopa–derived DA within serotonin fibers in the striatum was identified immunohistochemically in 6-hydroxy-dopamine (6-OHDA)–induced PD model rats [29].

There are controversial opinions regarding the functional efficacy of DA produced in non-DA cells in compensating for the decreased DA neurotransmission in the striatum of PD patients. DA produced outside of DA terminals in the striatum may be taken up into residual DA neurons by DA transporters, and stored in DA synaptic vesicles by vesicular monoamine transporter 2 (VMAT2; SLC18A2) to be released for neurotransmission. However, pre-synaptic DA transporter and VMAT2 are also decreased in the DA terminals in the striatum during the progression of PD. Mice that expressed ~5% of the normal VMAT2 were found to display age-associated nigro-striatal DA dysfunction (i.e., increased toxic cysteinyl adducts to L-dopa and DOPAC, and decreased striatal DA and DA transporter levels) that ultimately resulted in neurodegeneration [30]. Therefore, the residual activity of DA transporters and VMAT 2 is speculated to be important for DA formed in AADC-containing non-DA cells to act for neurotransmission and neuroprotection. However, since in advanced stage PD, DA produced from exogenous L-dopa in

non-DA cells may not be able to be taken up into DA neurons and stored in DA synaptic vesicles owing to the reduction in DA transporters and VMAT2, DA formed from exogenous L-dopa may directly act on postsynaptic DA receptors for neurotransmission, as do DA agonist drugs.

2.1. Mechanisms responsible for dyskinesia in L-dopa therapy

L-Dopa therapy is highly effective during the early stages of treatment. However, with prolonged L-dopa treatment, many patients develop motor complications such as fluctuations in clinical response and abnormal involuntary movements (dyskinesia). L-dopa-induced dyskinesia (LID) is common and difficult to treat. The clinical aspects and mechanisms of LID have been extensively investigated [31,32]. Many DA and non-DA systems including noradrenaline, glutamate, GABA, histamine, and cannabinoid ones may be involved in the generation of LID. Thus, many neurotransmitter receptors may participate in LID, such as mGlu5 [33], α2 adrenaline, serotonin 5HT1A and 5HT2A, histamine H3, cannabinoid CB1, and transient receptor potential vanilloid type-1 (TRPV1) receptors [34,35].

Fluctuations in L-dopa concentrations in the blood and in the brain, produced by daily periodic L-dopa administration, may induce pulsatile stimulation of DA receptors in the striatum, which may in turn cause fluctuations in clinical response leading to LID. Consequently, altering L-dopa dosing and timing to hourly doses or even its continuous infusion into the bloodstream may abate motor complications. More continuous, rather than pulsatile, DA receptor stimulation may be provided by "triple therapy" with L-dopa/AADC inhibitor/COMT inhibitor due to less fluctuation in the blood level of DA [36]. Since DA agonists have a longer half life than L-dopa, DA agonists are less likely to cause motor complications by virtue of their continuous stimulation of DA receptors [37].

Carta and colleagues [38] reported that development of LID in rats with 6-OHDA–induced lesions of the nigrostriatal DA pathway is critically dependent on the integrity and function of the serotonin system. Removal of the serotonin afferents, or damping of serotonin neuron activity by selective agonist drugs for the presynaptic serotonin 5HT1A and 5HT1B auto-receptors that regulate the terminal serotonin release, resulted in a near-complete blockage of the LID. This suggests that dysregulated DA release from serotonin terminals is the prime trigger of dyskinesia in the rat PD model. In animals with complete DA lesions, the spared serotonin innervation was unable to sustain the therapeutic effect of L-dopa, suggesting that DA released as a "false neurotransmitter" from serotonin terminals is detrimental rather than beneficial. This concept is also supported by experiments indicating that serotonin neuron transplants exacerbate LID in a rat PD model [39].

Rommelfanger and co-workers reported that noradrenaline loss in the locus ceruleus in dopamine beta-hydroxylase knockout (*Dbh–/–*) mice produces more profound motor deficits than 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in mice and that the mice display spontaneous dyskinesia [40]. They proposed that since the locus ceruleus noradrenaline loss in PD may exacerbate the motor symptoms associated with DA-neuron loss by disabling the firing capabilities of surviving DA neurons, the loss of noradrenaline and DA may have a synergistic effect on the emergence of the PD phenotype, particularly in the early-to-mid stages of the disease when sufficient numbers of DA neurons are intact and attempting to compensate with elevated DA turnover and supersensitive DA receptors.

Misu and Goshima proved that L-dopa itself may be a biologically active substance, such as a neurotransmitter or neuromodulator, and that it may stimulate DA neurotransmission [41]. Thus L-dopa itself could also play a role in the genesis of dyskinesia in patients receiving chronic L-dopa therapy.

Supersensitivity of postsynaptic DA receptors in the striatum due to presynaptic DA deficiency in the striatum in PD may also be related to LID. DA D1 receptors are believed to become highly supersensitive. Supersensitivity of DA D3 receptors in PD primates and rodents with LID has also been shown to occur *in vivo* [42].

While many pathways within the basal ganglia may contribute to the expression of LID, a core component of the pathophysiology of LID is considered to be overactivity of the direct striatal output pathway [43]. This pathway is activated via DA D1 receptors, and it provides a direct GABAergic connection by which the striatum inhibits the output regions of the basal ganglia, the internal globus pallidus, and the substantia nigra pars reticulata. Studies performed in MPTP-lesioned monkeys show that LID appears concomitantly with augmented coupling of striatal DA D1 receptors to G-protein [44]. Supersensitive DA D1 receptors may enhance the activity of downstream pathways, such as the extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) [p44/42 mitogen-activated protein (MAP) kinases] pathway [45].

Santini and colleagues reported that attenuation of cAMP signaling in the medium spiny neurons of the striatum via DA D1 receptors, achieved by genetic inactivation of the DA and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), reduces LID in 6-OHDA–lesioned PD mice [46]. In such dyskinetic mice, sensitized cAMP/cAMP-dependent protein kinase-1 (PKA1)/DARPP-32 signaling leads to phosphorylation/activation of ERK1/2. The increase in ERK1/2 phosphorylation associated with dyskinesia results in activation of mitogen- and stress-activated kinase (MSK-1) and phosphorylation of histone H3, two downstream targets of ERK involved in transcriptional regulation. Based on these results, Santini and colleagues attributed a significant proportion of the abnormal involuntary movements developed in response to chronic L-dopa to hyperactivation of striatal medium spiny neurons, the major DA target, of a signaling pathway including sequential phosphorylation of DARPP-32, ERK1/2, MSK-1, and histone H3.

Bychkov and coworkers reported that DA depletion and

subsequent treatment with L-dopa, but not the long-lived DA agonist pergolide, enhances the Akt (serine/threonine protein kinase, protein kinase B) pathway in the rat striatum [47]. They formulated the hypothesis that the Akt pathway is involved in LID. The Akt-GSK3 (glycogen synthase kinase 3) cascade was shown to mediate signaling via DA D2 receptors in the striatum [48,49]. Therefore, Bychkov and colleagues suggested that either supersensitive DA D1 receptors, whose action may overweigh that of DA D2 or DA D3 receptors, may contribute to a different pattern of Akt/GSK3 activation in the DA-depleted striatum [47].

Putative therapies to reduce the problem of dyskinesia have been under extensive investigation based on the mechanisms described above. Fabbrini and co-workers, in their review of LID, reported that the only currently available drug with proven efficacy for the treatment of dyskinesia is amantadine, which is a glutamate antagonist acting on NMDA receptors [32].

3. Is L-dopa neurotoxic or neuroprotective for DA neurons?

There have been many reports indicating that L-dopa is neurotoxic to DA neurons, especially at the relatively high concentrations used in DA cell culture systems. L-dopa is easily oxidized non-enzymatically to produce highly reactive metabolites that are thought to attack subcellular organelles and cell membranes of DA neurons [50]. The association of motor fluctuations and dyskinesia with chronic L-dopa therapy in PD patients has been interpreted by some as a toxic effect of L-dopa rather than an adverse effect. However, clinical evidence suggests that L-dopa does not accelerate the progression of PD, but actually slows it [51,52].

The idea that L-dopa is not neurotoxic enough to cause degeneration of DA neurons, at least in the relatively low doses used in patients, is supported by the clinical evidence that administration of L-dopa for many years produces no side effects for patients with Segawa's disease, which is a GTP cyclohydrolase I deficiency/autosomal dominant dopa-responsive dystonia (see review by Segawa et al. [53]), in which reduced activity of TH due to deficiency of the tetrahydrobiopterin (BH4) cofactor causes a reduced DA level specifically in the nigro-striatal DA neurons [54]. However, in contrast to Segawa's disease, in which cell death of DA neurons does not occur, DA neurons slowly but progressively degenerate in PD and L-dopa is unable to protect against progression of the neurodegeneration. Thus, the clinical efficacy of L-dopa in PD is thought to be solely symptomatic by supplementing DA deficiency in the striatum.

In the ELLDOPA study, the Parkinson Study Group [55] (reviewed by Weiner [52]) reported that L-dopa, in a dose–response pattern, significantly reduced the worsening of symptoms compared to placebo as indicated by the changes in the total score on the Unified Parkinson's Disease Rating Scale (UPDRS) between the baseline visit and week 42 after 40 weeks of L-dopa treatment and a 2-week washout period. The ELLDOPA study compared the use of daily L-dopa doses of 150 mg, 300 mg, and 600 mg versus placebo in patients with very early PD. This study provides confirmatory evidence for a functional protective effect of L-dopa. Furthermore, by using models for disease progression and pharmacodynamic models for drug effects to quantify the relative symptomatic benefits and to distinguish symptomatic effects from protective effects, Halford and associates obtained evidence for the slowing of disease progression by L-dopa/carbidopa as well as by the DA agonists, bromocriptine and pergolide [56]. Their results support those obtained by the Parkinson Study Group [55]. Chan and co-workers further reported that L-dopa slows progression of PD based on the stochastic pharmacokinetic–pharmacodynamic and disease progress model [57].

4. Future prospects for L-dopa therapy

L-dopa is expected to remain the "gold standard" for PD drug therapy at least until the development of more potent and safer DA agonist drugs or complete neuroprotective or neurorestorative therapies. In considering both the possible neuroprotective effect of L-dopa, at least at low doses, and the potential side effects such as dyskinesia seen with long-term L-dopa administration, combination L-dopa therapy, either with other drugs to increase the bio-availability (i.e., MAO B or COMT inhibitors) and/or to produce neuroprotection (i.e., MAO B inhibitors), or with new strategies such as gene therapy or transplantation will be important to minimize the doses of L-dopa and to maintain the initial potent efficacy. The challenge is to find a highly effective therapy that halts disease progression [58].

Accumulating evidence indicates that the MAO B inhibitor selegiline may have not only a beneficial effect on symptoms by preventing oxidation of DA, but also neuroprotective effects (see review by Nagatsu and Sawada [59]). Youdim and associates reported that another MAO B inhibitor, rasagiline (*N*-propargyl-1*R*-aminoindan), also showed neuroprotective effects [60]. The possible mechanism of the neuroprotective effects of selegiline is thought to be complex: inhibition of DA degradation, resulting in reduced production of H_2O_2 and reactive oxygen species (ROS); the accumulation of neurotrophic substances such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor, the induction and activation of multiple anti-oxidative stress and anti-apoptotic factors such as catalase, superoxide dismutase 1 and 2, thioredoxin, and Bcl-2; cellular poly(ADP-ribosyl)ation; and binding to glyceroaldehyde-3-phosphate dehydrogenase (GAPDH). It has been recently reported that selegiline slows the progression of the symptoms of PD and that combined therapy with L-dopa is suitable to delay the progression of the signs and symptoms of PD [61].

Another new strategy for L-dopa therapy would be its combination with gene therapy or transplantation. Ozawa, Nakano and colleagues are now employing gene therapy for PD patients by introducing the human *AADC* gene [62] into the striatum via adeno-associated virus vector along

with administration of a small amount of L-dopa, based on their successful treatment of MPTP-induced PD monkeys for 6 years with this approach [63]. In this strategy, the expression of the *AADC* gene for the production of DA in the striatum can be controlled by the dose of L-dopa (Keiya Ozawa, personal communication). Others have also reported promising preliminary results in clinical trials with AADC gene therapy combined with L-dopa [64,65].

In conclusion, since L-dopa is the best available drug for PD at present, one important approach would be to develop neuroprotective strategies to prevent progression of PD with minimal doses of L-dopa without side effects such as motor fluctuations or dyskinesia.

Conflict of interest

The authors have no conflict of interest to report. No funding applicable.

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Neuroprotective and toxic changes in microglia in neurodegenerative disease

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Abstract

Microglia are macrophage-like cells in the CNS. As macrophages, activated microglia remove potentially deleterious debris and promote tissue repair. However, they can release potentially cytotoxic substances *in vitro*. So-called fully activated microglia, observed at the injury site in many neurodegenerative conditions, are neurotoxic. This suggests that some factor(s) may contribute to change microglial phenotype from protective to toxic, but details are not clear. Recently, we generated HIV-derived Nef protein-transduced microglia. They increase the potential to produce O_2^- and MPO-like peroxidase activity, resulting in neurotoxicity. Therefore, the target protein(s) of Nef might be involved in the control of microglial neurotoxicity.

Keywords: Microglia; Toxic transformation; HIV Nef; ROS

1. Microglia in Parkinson's brain

The presence of oxidative stress and inflammatory activity is one of the significant pathological features of Parkinson's disease (PD) [1,2]. It has been shown that the levels of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ are elevated in the substantia nigra of patients with PD [3]. Since microglia are a principal source of these cytokines, the data support microglial involvement in the pathogenesis of PD. However, the role of activated microglia is controversial. The characteristic pathological features of the PD brain are a selective and progressive loss of dopamine neurons of the substantia nigra and their terminals in the caudate-putamen, along with focal accumulation of activated microglia in the substantia nigra and caudate-putamen. The traditional view is that microglia act merely as scavengers and their activation is secondary to the neuronal damage. However, activated microglia have been observed in the limbic system and neocortex, where there are few or no degenerating neurons, in significantly higher numbers in PD brains than in brains from normal controls [4].

Activation of microglia has also been identified in the substantia nigra and/or striatum of parkinsonian animal models, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced parkinsonism [5–7]. The link between microglia activation and selective neuronal vulnerability has led many researchers to suggest that microglia activity participates in neuronal demise. In this respect, microglial cytotoxicity may contribute to, or even promote neuronal damage. Activated microglia are capable of releasing numerous cytotoxic agents, including proteolytic enzymes, cytokines, complement proteins, reactive oxygen intermediates, NMDA-like toxins, and nitric oxide [8]. In fact, β-amyloid, the senile plaque-derived component found in Alzheimer's disease, appears to elicit neurotoxic responses through the activation of microglia [9]. However, this suggestion relies solely on *in vitro* data and as yet, no evidence has been presented that indicates that activated microglia destroy neurons under *in vivo* conditions.

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2. Existence of non-toxic activated form of microglia in the brain

Recently we showed that highly activated microglia treated with lipopolysaccharide (LPS) may have neurotrophic potential toward dopamine neurons in neonatal mice administered MPTP [10]. Tyrosine hydroxylase activity and the levels of dopamine and dihydroxyphyenylacetic acid (DOPAC), as well as those of the pro-inflammatory cytokines IL-1β and IL-6, were elevated in the midbrain of LPS-MPTP-treated neonatal mice. The viability of dopamine (A9) neurons was preserved in neonatal mice of the LPS-MPTP group compared with the MPTP group. In contrast, the viability of these neurons in aged mice dropped significantly. These results may suggest that activated microglia show different phenotypes; i.e., microglia activated by LPS in the neonatal brain may be neuroprotective for dopaminergic neurons in MPTP-treated mice, whereas in aged mice they may be neurotoxic for dopaminergic neurons. The dissociation between injury-induced microglial activation and neuronal degeneration in TNF receptor and colony stimulating factor-1 (CSF-1) knockout mice suggests that microglial activation is not a determinate event in dopaminergic neuronal damage in brain. Furthermore, there is a growing body of evidence that microglia may play a beneficial role in ischemia by secreting factors (growth factors or cytokines) that promote survival of neurons. Therefore, activated microglia may produce not only neurotoxic effects, but also neuroprotective ones, depending upon their environmental situation.

3. Direct evidence of neuroprotection of microglia in the brain

We have reported that exogenous microglia enter the brain parenchyma through the blood–brain barrier and migrate to ischemic hippocampal lesions when they are injected into the circulation. By applying this brain-targeted delivery technique, we investigated the effect of exogenous microglia on ischemic pyramidal neurons [11]. To this end, we isolated microglia from neonatal mixed brain cultures, labeled them with a fluorescent dye PKH26, and injected them into the artery of Mongolian gerbils subjected to ischemia reperfusion neuronal injury. PKH26-labeled microglia migrated to the ischemic hippocampal lesion and increased the survival of neurons, even when the cells were injected 24 h after the ischemic insult. Stimulation of isolated microglia with IFN-γ enhanced the neuroprotective effect on the ischemic neurons. Microglia also protected ischemia-induced learning disability. As migratory microglia increased the expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in the ischemic hippocampus, they might induce neurotrophin-dependent protective activity in damaged neurons. These results represent the first experimental demonstration of neurotrophic effects of microglia on transient global ischemia *in vivo*. Since peripherally injected microglia exhibit specific affinity for ischemic brain lesions and protect against ischemic neuronal injury in the present model, we suggest that microglia may have the potential to be used as a candidate for cell therapy for central nervous system (CNS) repair following transitory global ischemia and other neurodegenerative diseases including PD.

4. Heterogeneity of microglia

Microglial cells show a rather uniform distribution of cell numbers throughout the brain with only minor preponderance in some regions. Their in situ morphologies, however, may vary markedly from elongated forms observed in apposition with neuronal fibers to spherical cell bodies with sometimes extremely elaborate branching. This heterogeneity gave rise to the hypothesis that these cells are differentially conditioned by their microenvironment and, therefore, also display specific patterns of differential gene expression. The distinct phenotypes in activated forms of microglia might result from this heterogeneity.

Recently, we identified two distinct sub-populations of microglia in the normal mouse brain: type I and type II. Both types had similar phagocytic activity, but they showed distinct phenotypes such as cell-surface markers, mRNA expression, and growth factor dependency. Type I, but not type II, microglia expressed the hallmarks of microglia such as CD14 and C3b receptors, and production of Il-1β. Type II expressed surface markers for immature bone marrow cells, such as ER-MP12 and 20, and one tenth the amount of those present on mature monocytes/macrophages, such as C3b and F4/80 antigen. Type I microglia exhibited M-CSF-dependent growth, while the type II were M-CSF-independent. Microglia produce superoxide [12] and express all components of the superoxide generating phagocyte NADPH oxidase [13]. Isolated microglia were analyzed for superoxide production by DCFH-DA fluorescence with FACS. Purified microglia indicated low and high superoxide production activity. Two-parameter analysis showed that these activities resulted from distinct cell populations of microglia. The high superoxide-producing population had a toxic effect on cholinergic neurons and induced cell death of cholinergic neurons in culture. Since this toxic effect was blocked by superoxide dismutase, it seemed to be caused by superoxide from activated microglia. The low superoxide-producing population did not have a toxic effect but rather a protective effect on cholinergic neurons. These observations indicate that there are at least two distinct populations of microglia in the brain, which are probably of different origins and have different functions.

5. Toxic transformation of microglia by Nef protein introduction

Nef, a multifunctional HIV protein, activates the Vav/Rac/p21-activated kinase (PAK) signaling pathway. Given the potential role of this pathway in the activation of the phagocyte NADPH oxidase, we have investigated the effect of the HIV-1 Nef protein on microglia superoxide production

and toxicity for neurons [13]. Microglia were transduced with lentiviral expression vectors to produce a high level of Nef protein. Expression of Nef did not activate the NADPH oxidase by itself, but led to a massive enhancement of the responses to a variety of stimuli (Ca^{2+} ionophore, formyl peptide, endotoxin) and induction to produce a large amount of superoxide. These effects were not caused by up-regulation of phagocyte NADPH oxidase subunits. Nef mutants lacking motifs involved in the interaction with plasma membrane and/or other cytosolic proteins failed to reproduce the effects of wild-type Nef, suggesting involvement of a certain signaling pathway in microglia for their trophic-toxic control. Our results suggest a key role for Rac activation in the priming for microglia toxic activation, which is enhanced by Nef introduction in the non-toxic form of microglia. Rac activation is not sufficient to induce the toxic form of microglia accompanied by stimulation of the phagocyte NADPH oxidase; however, it markedly enhances the NADPH oxidase response to other stimuli and might be involved in the trophic-toxic control of microglia.

6. Conclusion

Microglia, macrophage-like cells in the CNS, are multifunctional cells; they play an important role in the removal of dead cells or their remnants by phagocytosis in CNS degeneration, as well as being one of the important cell populations in the CNS cytokine network. They are thought to originate from mesoderm, and to be similar cells to other tissue-resident macrophages. As macrophages, activated microglia have been shown to remove potentially deleterious debris and promote tissue repair by secreting neurotrophic factors at the neuronal injury sites. However, they can release potentially cytotoxic substances *in vitro*, and at least the so-called fully activated form of microglia, which are observed at the injury site in many neurodegenerative conditions such as PD, Alzheimer's disease, and AIDS dementia, are neurotoxic. This suggests that some factor(s) may contribute to changing the microglial phenotype from protective to toxic, but details are not clear. Recently we generated HIV-derived Nef protein transduced microglia. They increase the potential to produce both O_2^- and MPO-like peroxidase activity, resulting in neurotoxicity. Therefore, the target protein(s) of Nef might be involved in the control of microglial neurotoxicity.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

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Marked induction of inducible nitric oxide synthase and tumor necrosis factor-α in rat CD40$^+$ microglia by comparison to CD40$^-$ microglia

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ABSTRACT

There may be two subtypes of microglia (MG) at least in the CNS. We separated the two types from rat mixed glial culture. mRNAs and proteins for inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNFα) were more induced in CD40$^+$ MG than CD40$^-$ MG after LPS stimulation. Although the expression level of LPS receptors showed a little difference between the subtypes, LPS-induced degradation of phosphorylated IκBα was marked in CD40$^+$ MG. These results strongly suggest that CD40$^+$ MG produce larger amount of NO and TNFα to exhibit neurotoxic action under certain pathological conditions in brains.

1. Introduction

Microglia (MG), which are distinguished from other glial cells by their origin and function, play important roles in inflammatory and immune responses in the central nervous system (CNS) (González-Scarano and Baltuch, 1999; Kreutzberg, 1996; Nakajima and Kohsaka, 2001). During brain injuries and neurodegenerative diseases, MG are activated and accumulated in affected areas (Dickson et al., 1993; Itagaki et al., 1989; McGeer and McGeer, 1995). MG activation leads to the production of many cytotoxic factors including nitric oxide (NO) and tumor necrosis factor-α (TNFα) (González-Scarano and Baltuch, 1999; McGeer and McGeer, 1995; Banati et al., 1993), which attack on infected microorganisms or neurodegenerative pathogens and result in protection of the brain. Therefore, like immune cells in other organs, MG play a dual role, amplifying the inflammation and protecting the CNS.

Recent reports suggest that there may be two or more subtypes of MG in the CNS. The work from Andjelkovic et al. (1998) suggested that MG have a dual origin, the first coming from the yolk sac macrophages during the nonvascularized prenatal stage followed by the second engraftment from circulating monocytes postnatally. This hypothesis has been also suggested by independent studies (for review, see Chan et al., 2007). Even apart from arguments on the MG origins, there are several papers reporting the MG heterogeneity. In the absence of pathological situations, human MG that are positive for MHC class II and CD4 are found preferentially in white matter as opposed to grey matter regions (Hayes et al., 1987). Rat hippocampal MG express higher levels of mRNAs for TNFα, CD4 and FcγRII than do MG from the diencephalons, tegmentum, cerebellum and cerebral cortex (Ren et al., 1999). Bulloch et al. (2008) showed the CD11c$^+$ dendritic cells residing among the heterogeneous MG population in normal mouse brain. In addition, studies on cell surface properties and immune functions in a panel of non-virus transformed cell lines derived from individual MG precursors revealed that there may be immunocompetent and noncompetent subpopulations of MG (Walker et al., 1995b; Suzumura et al., 1998; Kanzawa et al., 2000; Katoh et al., 2001; Okada et al., 2003). These results strongly suggest that the heterogeneity of MG populations exists in the CNS.

We recently showed that interleukin-4 (IL-4)-stimulated uptake and degradation of β-amyloid peptide were enhanced selectively in one subtype of MG, namely rat type-2 MG, but not in type-1 MG that express CD40 (Shimizu et al., 2008). This finding suggests that CD40$^-$ (type-2) MG are more responsive to IL-4, whereas CD40$^+$ (type-1) MG are less responsive, and that these two MG subtypes may play different neuro-immunomodulatory roles in the β-amyloid-overproducing brain. We preliminarily reported that lipopolysaccharide (LPS) or LPS/IFNγ-activated CD40$^+$ (type-1) MG, but not CD40$^-$ (type-2) MG, exhibit neurotoxic action, in the co-culture system of rat MG with primary hippocampal neurons (Kawahara et al., 2004). However,

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whether the MG subtypes produce different amounts of pro-inflammatory molecules, such as NO and TNFα under inflammatory conditions, still remains to be elucidated.

In the present study, we compared and showed different responses to LPS stimulation of two MG populations ($CD40^+$ and $CD40^-$) that were separated from rat primary mixed glial cell culture, and those of two mouse cell lines 6-3 and Ra2, which are $CD40^+$ and $CD40^-$ MG (Kanzawa et al., 2000), respectively. We also investigated what molecules are involved in the different inductions of iNOS and TNFα between the subtypes.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from Kyudo (Kumamoto, Japan). Animals were treated according to the guidelines of the Kumamoto University Animal Committee.

2.2. Cell culture

Primary $CD40^+$ and $CD40^-$ MG were harvested from primary mixed glial cell cultures prepared from neonatal Wistar rat pups as previously reported (Sawada et al., 1990; Suzumura et al., 1987). In brief, after the meninges were carefully removed, the neonatal brain was dissociated by pipetting. The cell suspension was plated in 75 cm^2 culture flasks at a density of five brains per 12 flasks in 10 ml Mi medium (Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), 5 μg/ml bovine insulin and 0.2% glucose). $CD40^+$ MG were isolated on days 14–16 by the "shaking off" method previously described (Sawada et al., 1990; Shimizu et al., 2008; Suzumura et al., 1987). $CD40^-$ MG were isolated on days 19–21 by harvesting with 5 mM EDTA in phosphate buffer solution using a modified "mild trypsinization" method (Saura et al., 2003; Shimizu et al., 2008). The murine MG cell lines, Ra2 and 6-3, were maintained as described previously (Kanzawa et al., 2000). Cells were treated with *Escherichia coli* LPS (serotype 0127:B8; Sigma-Aldrich Inc., St. Louis, MO) for the indicated periods. A NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) and a proteasome inhibitor MG-132 were purchased from Wako Pure Chemical Industries (Japan) and Peptide Institute Inc. (Japan), respectively.

2.3. RT-PCR analysis

Total RNA was isolated using the acid guanidium thiocyanate phenol–chloroform extraction procedure. cDNA was synthesized using reverse transcriptase XL (AMV, Takara, Tokyo, Japan) and Taq PCR Master mix kit (Qiagen Inc., Valencia, CA). The primers used were: sense primer, 5′-GACCAGGACGAGGAAAGTGT-3′ and antisense primer, 5′-AGCAGTATCCCGCAGTGAAT-3′ for CD14; sense primer, 5′-AAGAGCTGGAATACCTGGAC-3′ and antisense primer, 5′-GAAATGCTACAGTGGCTACC-3′ for TLR4; sense primer, 5′-CTTTTCGACGCTGCTTTCTC-3′ and antisense primer, 5′-CTGTGATGGCCCTTAGGAAA-3′ for MD-2; sense primer, 5′-GACAGGATGCAGAAGGAGAT-3′ and antisense primer, 5′-TTGCTGATCCACATCTGCTG-3′ for β-actin. The primer sets for CD14, TLR4, MD-2 and β-actin were expected to give PCR products with a size of 718, 684, 378 and 145 bp, respectively. PCR consisted of an initial denaturation cycle at 94 °C for 2 min, followed by the 29 cycles for TLR4, 27 cycles for MD-2, 23 cycles for CD14 and β-actin consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1.5 min. An additional cycle at 72 °C for 5 min completed the amplification process. Amplified PCR products were separated by 1.5% for CD14, TLR4, MD-2 or 2% for β-actin agarose gel electrophoresis and visualized with the use of ethidium bromide staining. Densitometric quantification was done

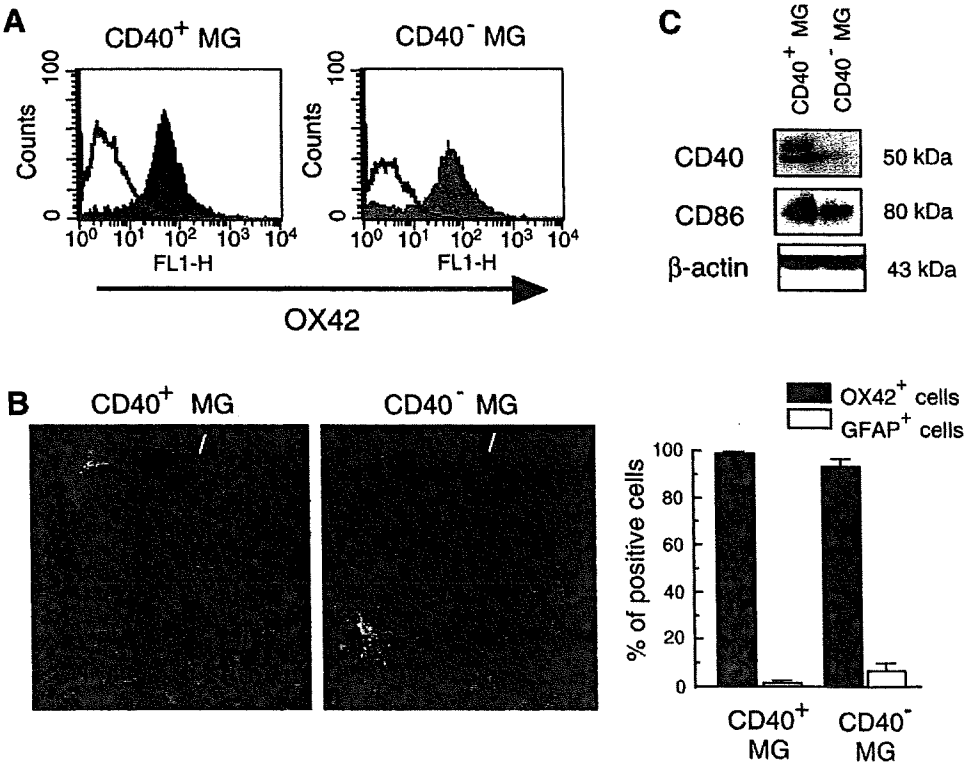

Fig. 1. Expression of some macrophage-associated markers on rat primary $CD40^+$ microglia (MG) and $CD40^-$ MG. (A) $CD40^+$ MG and $CD40^-$ MG were isolated as described in Materials and methods. Cells were stained with OX42 (mouse anti-CD11b monoclonal antibody) plus Alexa Fluor 488-labeled goat anti-mouse IgG. Cell populations observed are shown (filled histograms). Stained results with control IgG are also shown (open histograms). (B) Cells were incubated with either OX42 or rabbit anti-GFAP antiserum, and then with either Alexa Fluor 488-labeled goat anti-mouse IgG or Alexa Fluor 594-labeled goat anti-rabbit IgG, respectively. (Left panel) Fluorescent images are shown after merging. (Right panel) OX42-positive cells and GFAP-positive cells were counted independently, and the results were shown as means±SD ($n=4$). (C) Cell extracts (40 μg of protein) were subjected to immunoblot analysis with antibody against CD40, CD86, or β-actin.

using the Image Gauge software program (Fuji Photo Film Co., Tokyo, Japan).

2.4. RNA blot analysis

Total RNA from MG (5×10^6 cells/100-mm dish) was isolated using the acid guanidium thiocyanate phenol–chloroform extraction procedure. After performing electrophoresis through formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized using a transcription kit (Roche Molecular Biochemicals, Mannheim, Germany), from rat iNOS cDNA (Nagasaki et al., 1996), rat TNFα cDNA at nucleotide positions 401–858 (GenBank, accession no. BC107671) and rat β-actin cDNA at nucleotide positions 1012–1157 (GenBank, accession no. NM_031144). Hybridization, washing and chemiluminescent detection on X-ray films were carried out as recommended by Roche Diagnostics. Densitometric quantification was done using the Image Gauge software program (Fuji Photo Film Co., Tokyo, Japan).

2.5. Immunoblot analysis

MG (5×10^6 cells/100-mm dish) were homogenized in RIPA buffer (10 mM Hepes–NaOH (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 μg/ml soybean trypsin inhibitor). After centrifugation, the supernatants were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrotransferred to polyvinylidene difluoride membranes (PVDF). The membrane was then incubated with antibodies to iNOS (N32020; Transduction Laboratories, Lexington, KY, diluted 1:500), rat TNFα (R&D systems, Inc., Minneapolis, MN, 1 μg/ml), CD14 (M-305; Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:1000), CD40 (T-20; Santa Cruz Biotechnology, diluted 1:100), B7-2/CD86 (421340; Genezyme Techne, Minneapolis, MN, diluted 1:500), IκBα (Sigma-Aldrich Inc., diluted 1:2000), or β-actin (AC15; Sigma-Aldrich Inc., diluted 1:2000) followed by secondary antibody, HRP-linked antibodies against mouse and rabbit immunoglobulins (Zymed, San Francisco, CA, diluted 1:5000). Subsequently, bound HRP-labeled antibodies were detected by chemiluminescence (ECL kit, Pierce Biotechnology, Rockford, IL). Filters were analyzed using LAS-1000 Plus (Fuji Photo Film Co., Tokyo, Japan).

2.6. Immunofluorescent flow cytometry

Analysis of immunofluorescent flow cytometry was performed using a mouse monoclonal antibody against rat CD11b (OX42; Serotec Ltd., Oxford, UK). Cells (1×10^6 cells in 200 μl) were incubated with either OX42 (final concentration of 1 μg/ml), or mouse IgG2a (final concentration of 1 μg/ml) as negative control, at 4 °C for 30 min, followed by incubation with Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR, 1:500 dilution), and analyzed using a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ).

2.7. Immunocytochemical analysis

MG were plated onto 8-well LAB-TEK chamber slides and stimulated with LPS for the indicated periods. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and incubated with 0.05% Triton X-100 in PBS for 10 min and then blocked with 2.5% BSA in PBS for 30 min. The cells were then incubated with either mouse anti-NF-κB p65 monoclonal antibody (F-6, Santa Cruz Biotechnology, diluted 1:250), mouse monoclonal antibody OX42 (Serotec Ltd., diluted 1:100), or rabbit antiserum against glial fibrillary acidic protein (GFAP) (N1506; DakoCytomation, Kyoto, Japan). Thereafter, Alexa Flour 488- or 594-labeled antibody against mouse or rabbit immunoglobulin (Molecular Probes, 1:500 dilution), respectively, was used as secondary antibody. The stained cells were observed under a confocal laser scanning microscope (FLUOVIEW, Olympus, Tokyo, Japan).

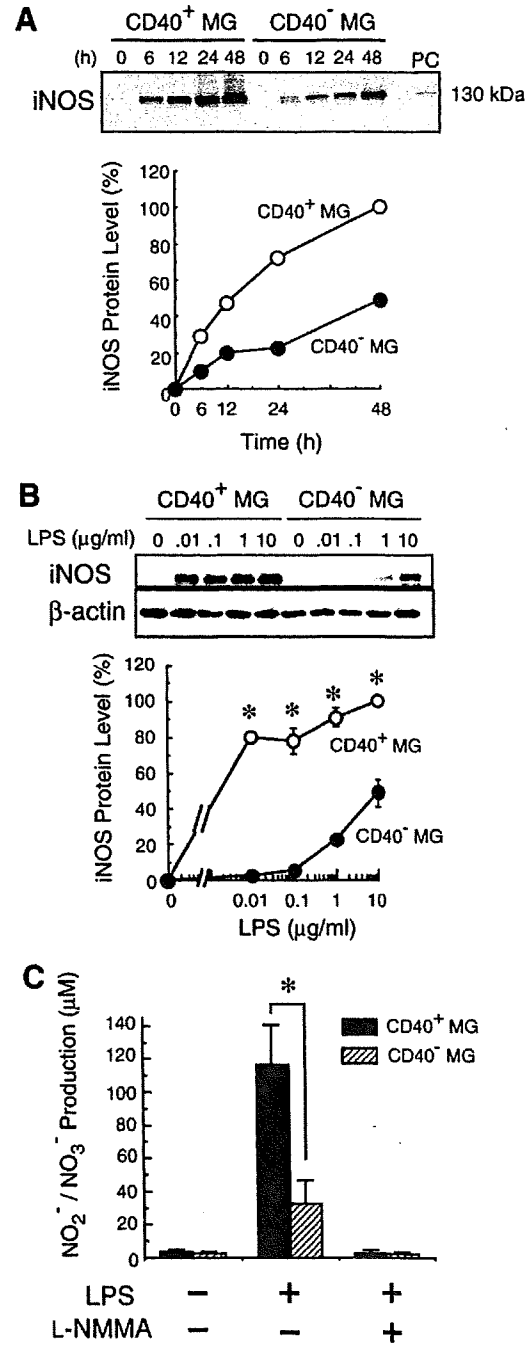

Fig. 2. Marked induction of iNOS by LPS in rat CD40⁺ MG compared to CD40⁻ MG. (A) The cells were treated with LPS (1 μg/ml) for the indicated periods. Cell extracts (30 μg of protein) were subjected to immunoblot analysis using the anti-mouse iNOS monoclonal antibody. Positive controls (PC) were extracts (10 μg of protein) from LPS-activated mouse microglial MG5 cells. The results were quantified. The maximal values were set at 100%. (B) Dose-dependent induction of iNOS in CD40⁺ MG and CD40⁻ MG treated with various concentrations of LPS for 24 h. Cell extracts (30 μg of protein) were subjected to immunoblot analysis using anti-mouse iNOS monoclonal antibody, or anti-β-actin monoclonal antibody. Results were quantified. The maximal values were set at 100%. Values were means±SD ($n=3$). *, $p<0.05$, significantly different from CD40⁻ MG. (C) CD40⁺ MG and CD40⁻ MG were treated with the indicated combinations of LPS (1 μg/ml) and a NOS inhibitor L-NMMA (2 mM) for 24 h. NO_2^-/NO_3^- in the medium were measured and results were shown as means±SD ($n=4$). *, $p<0.05$, significantly different from CD40⁻ MG.

2.8. Other methods

TNFα production was measured with the ELISA kit specific for rat TNFα (KRC3011, Biosource International, Camarillo, CA) and mouse TNFα (KMC3011, Biosource International). Concentration of NO_2^- plus NO_3^- in culture supernatant was measured with the Griss reagent using the NO_2^-/NO_3^- assay kit-C (Dojindo, Kumamoto, Japan). Protein concentrations were determined with the Bio-Rad protein assay regent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard.

3. Results

3.1. Marked induction of iNOS by LPS in rat CD40$^+$ MG compared to CD40$^-$ MG

More than one subtype of MG are considered to exist in the CNS and they may have different functions (Bulloch et al., 2008; Elkabes et al., 1996; Ren et al., 1999; Sriram et al., 2006). We separated CD40$^+$ MG and CD40$^-$ MG from a mixed glial cell culture by using a previously described method (Sawada et al., 1990; Suzumura et al., 1987; Shimizu et al., 2008). We evaluated the purity of both microglia preparations by flow cytometry and immunocytochemistry using OX42 as a microglia marker, and also determined the contamination of astrocytes by immunocytochemistry using GFAP as an astrocyte marker. Flow cytometric analysis revealed indistinguishable OX42 immunoreactivity (CD11b expression) between the two MG subtypes (Fig. 1A), as described previously (Kanzawa et al., 2000). Immunocytochemical staining with OX42 or anti-GFAP showed that the purity of microglia was 98.5% and 93.3%, and the astrocyte contamination was 1.5% and 6.7%, for CD40$^+$ MG and CD40$^-$ MG, respectively (Fig. 1B). Quality of two MG preparations was shown to be high and essentially similar. Successful separation of the subtypes was also confirmed, because the CD40$^+$ MG highly expressed CD40 and CD86, while CD40$^-$ MG did essentially no CD40 and CD86 weakly (Fig. 1C), as previously described (Kanzawa et al., 2000). Using the MG preparations, we compared the responses to inflammatory stimuli. After LPS stimulation, ramified MG morphology of both MG changed into amoeboid form (data not shown), and the morphological changes were not a determinant to distinguish the two MG. Time dependent iNOS protein expression was apparently observed after LPS treatment (Fig. 2A). iNOS protein was induced 6 h after treatment and continued to increase until 48 h in both subtypes (Fig. 2A). However, the induction levels of iNOS were different between the subtypes; the level in CD40$^+$ MG was about 3 times higher than that in CD40$^-$ MG between 6 and 48 h after LPS stimulation (Fig. 2A). Dose dependency of LPS on iNOS protein is shown in Fig. 2B. iNOS protein in CD40$^+$ MG was induced maximally at 0.01 μg/ml of LPS, whereas that in CD40$^-$ MG was barely induced at this concentration, but induced weakly at 0.1 μg/ml, and maximally induced at 10 μg/ml of LPS. The production of NO was measured by NO_2^- plus NO_3^- in the culture medium (Fig. 2C). The amounts of NO released from CD40$^+$ MG and CD40$^-$ MG by LPS (1 μg/ml) stimulation were 116.4 μM and 32.7 μM, respectively. The NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA, 2 mM) suppressed the LPS-stimulated NO production by close to the basal level. These results indicate that CD40$^+$ MG respond more sensitively to LPS for iNOS expression than CD40$^-$ MG.

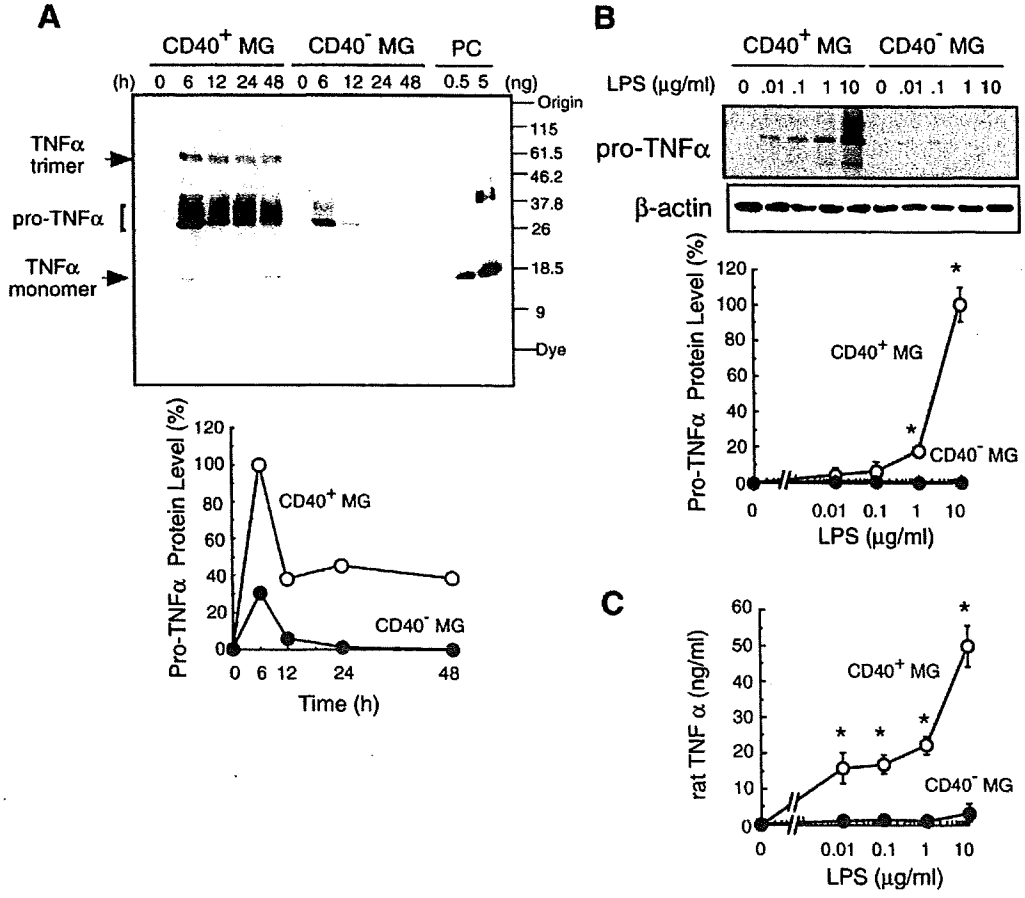

Fig. 3. Marked induction of TNFα by LPS in rat CD40$^+$ MG compared to CD40$^-$ MG. (A) The cells were treated with LPS (1 μg/ml) for the indicated periods. Cell extracts (30 μg of protein) were subjected to immunoblot analysis using the anti-rat TNFα monoclonal antibody. Positive controls (PC) are recombinant rat TNFα (0.5, 5 ng). Results were quantified. The maximal values were set at 100%. (B) The dose-dependent induction of precursor form of TNFα (pro-TNFα) in CD40$^+$ MG and CD40$^-$ MG treated with various concentrations of LPS for 24 h. The cell extracts (30 μg of protein) were subjected to an immunoblot analysis using the anti-rat TNFα monoclonal antibody, or anti-β-actin monoclonal antibody. The results were quantified. The maximal values were set at 100%. Values were means±SD (n=3). *, p< 0.05, significantly different from CD40$^-$ MG. (C) The cells were treated with various concentrations of LPS for 24 h. TNFα in the medium was measured with an ELISA kit. Values were means±SD (n=4). *, p<0.05, significantly different from CD40$^-$ MG.

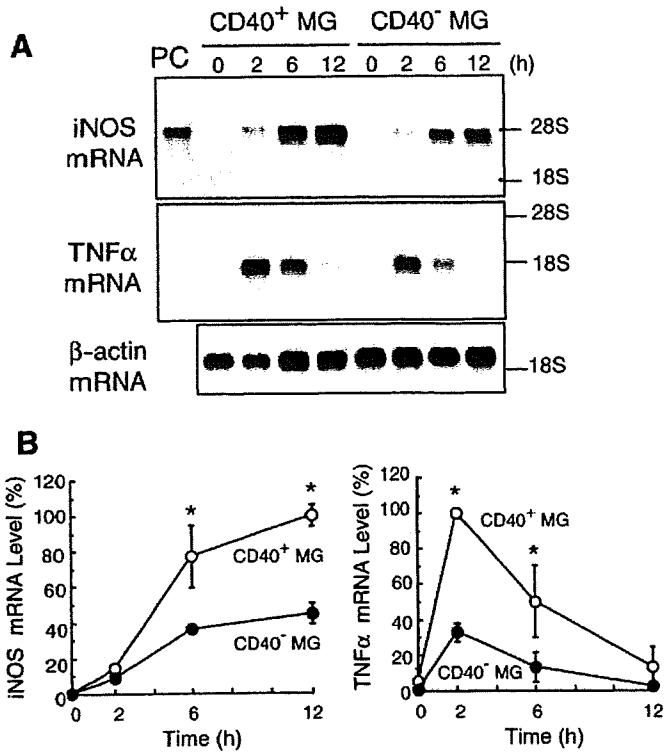

Fig. 4. More induction of iNOS and TNFα mRNAs by LPS in rat CD40⁺ MG than CD40⁻ MG. (A) The cells were treated with LPS (1 μg/ml) for the indicated periods. Total RNAs (2.0 μg) were subjected to blot analysis. Positive controls (PC) were total RNAs (2.0 μg) from LPS-activated mouse microglial MG5 cells. The bottom panel showed a Northern blot analysis for β-actin as a control for RNA loading. (B) Results in A were quantified. The maximal values were set at 100%. Values were means±SD (n=3). *, $p<0.05$, significantly different from CD40⁻ MG.

3.2. Marked induction of TNFα by LPS in rat CD40⁺ MG compared to CD40⁻ MG

Time course for the precursor form of TNFα protein (pro-TNFα) expression after LPS treatment was examined (Fig. 3A). The pro-TNFα expression in CD40⁺ MG was markedly induced 6 h after LPS treatment and thereafter remained high level until 48 h. In CD40⁻ MG, however, pro-TNFα was transiently induced at 6 h (0.3-fold in comparison to CD40⁺ MG) and decreased thereafter. The dose dependency of LPS on either pro-TNFα or TNFα release is shown in Fig. 3B and C, respectively. In CD40⁺ MG, pro-TNFα was induced by LPS in a dose dependent manner and reached maximum at 10 μg/ml LPS, whereas it was weakly induced in CD40⁻ MG at this concentration. On the amount of TNFα, CD40⁺ MG released 16.0 and 49.8 ng/ml by LPS of 0.01 and 10 μg/ml, while CD40⁻ MG did 1.3 and 3.2 ng/ml, respectively. These results indicate that CD40⁺ MG released more TNFα than CD40⁻ MG did in response to LPS.

3.3. More induction of iNOS and TNFα mRNAs by LPS in rat CD40⁺ MG than in CD40⁻ MG

An RNA blot analysis was used to determine whether the induction of iNOS and pro-TNFα proteins in rat CD40⁺ and CD40⁻ MG paralleled expression of their respective mRNAs. iNOS mRNA began to increase 2 h after stimulation and increased up to 12 h in both subtypes (Fig. 4). However, the induction level of iNOS mRNA in CD40⁺ MG was 2.5 times higher than that in CD40⁻ MG at 12 h after LPS stimulation (Fig. 4). TNFα mRNA was markedly induced 2 h after LPS treatment and thereafter it decreased in both subtypes (Fig. 4). However, the induction level of TNFα mRNA at 2 h after LPS treatment was 3 times higher in CD40⁺ MG than that CD40⁻ MG. These results correspond to the observed difference in protein levels between the subtypes.

3.4. Expression of LPS receptors in rat CD40⁺ and CD40⁻ MG

The role of LPS receptors on the different responses to LPS between the MG subtypes was next investigated (Fig. 5). In untreated CD40⁺ MG, the expression level of CD14 protein, an LPS receptor, was significantly higher than that in CD40⁻ MG by Western blot analysis (1.2-fold compared to CD40⁻ MG). When the cells were treated with LPS, the CD14 protein similarly increased in both cell types and the resultant expression level was still higher in CD40⁺ MG (1.3-fold) than in CD40⁻ MG. Next, the expression level of TLR4, a signal transducer for LPS, was compared by RT-PCR, because there were unavailable good antibodies against rat TLR4. mRNAs for CD14 and MD-2, which is an accessory molecule of TLR4, were also examined. The TLR4 mRNA was approximately at the same level between the two MG. CD14 mRNA of CD40⁺ MG was 2.4 times higher than that of CD40⁻ MG, while the difference of the protein level between the subtypes was only 1.2 times. The difference of MD-2 mRNA levels between two subtypes was also by 1.2 times. These results suggest that marked induction of iNOS and TNFα in rat CD40⁺ MG compared to CD40⁻ MG might be related partly to the higher expression of LPS receptors in CD40⁺ MG than that in CD40⁻ MG. However, there was a little difference in the expression level of LPS receptor (CD14 protein 1.2 times, TLR4 mRNA 1.1 times, MD-2 mRNA 1.2 times).

3.5. LPS-induced degradation of IκBα is highly observed in CD40⁺ MG

The difference in NF-κB activity between MG subtypes was examined on the basis of the report that NF-κB is required for induction of iNOS and TNFα mRNAs by LPS (Muller et al., 1993) (Fig. 6). NF-κB is a dimeric transcription factor that is composed of p50 (NF-κB1) and p65 (RelA) subunits (Hatada et al., 2000). To translocate NF-κB into the nucleus, the cytoplasmic NF-κB:IκBα complex needs to be disrupted (Hatada et al., 2000). A common pathway to achieve disruption of this complex is correlated to the specific phosphorylation of IκBα and the degradation of the phosphorylated IκBα protein by proteasomes. Inhibition of the proteasome activity by specific inhibitors, such as MG-132, prevents the IκBα degradation and thus prevents the nuclear translocation of NF-κB (Neish et al., 2000).

After stimulation of CD40⁺ MG with LPS (1 μg/ml), the IκBα phosphorylation became evident at 10 min, peaked at 30 min, and declined thereafter (Fig. 6A). Following the IκBα phosphorylation, LPS-induced degradation of IκBα reached maximum at 10 min (75% decrease compared to the initial level), remained at low level until 30 min, and then began to return to the control level at 60 min, although the level recovered at 120 min was still 60% of the initial level (Fig. 6A). In contrast, the LPS-induced degradation of IκBα occurred very slowly and at substantial level in CD40⁻ MG, although its phosphorylation was preceded similarly to CD40⁺ MG (Fig. 6A). The IκBα level in CD40⁻ MG decreased gradually between 10 and 30 min, reached bottom at 60 min (but only 40% decrease of the initial level), and then came back to the initial level at 120 min. In turn to the CD40⁺ MG, the efficient degradation of IκBα was observed even at low dose of LPS stimulation (0.01 μg/ml; Fig. 6B). These results may relate to the observed induction levels of iNOS and TNFα in CD40⁺ MG, which are prominent even at low dose of LPS (0.01 μg/ml) (Figs. 2B, and 3B, C).

Next, the effect of proteasome inhibitor MG-132 was assessed, whether proteasomal degradation of IκBα is directly required for LPS-induced NF-κB nuclear translocation and activation. As expected, MG-132 blocked the LPS-induced NF-κB translocation in a dose dependent manner in CD40⁺ MG (Fig. 6C). MG-132 also inhibited the LPS-induced iNOS expression in CD40⁺ MG and CD40⁻ MG as well (Fig. 6D). Collectively, these observations suggest that IκBα phosphorylation and proteasome-mediated degradation are involved in LPS-induced

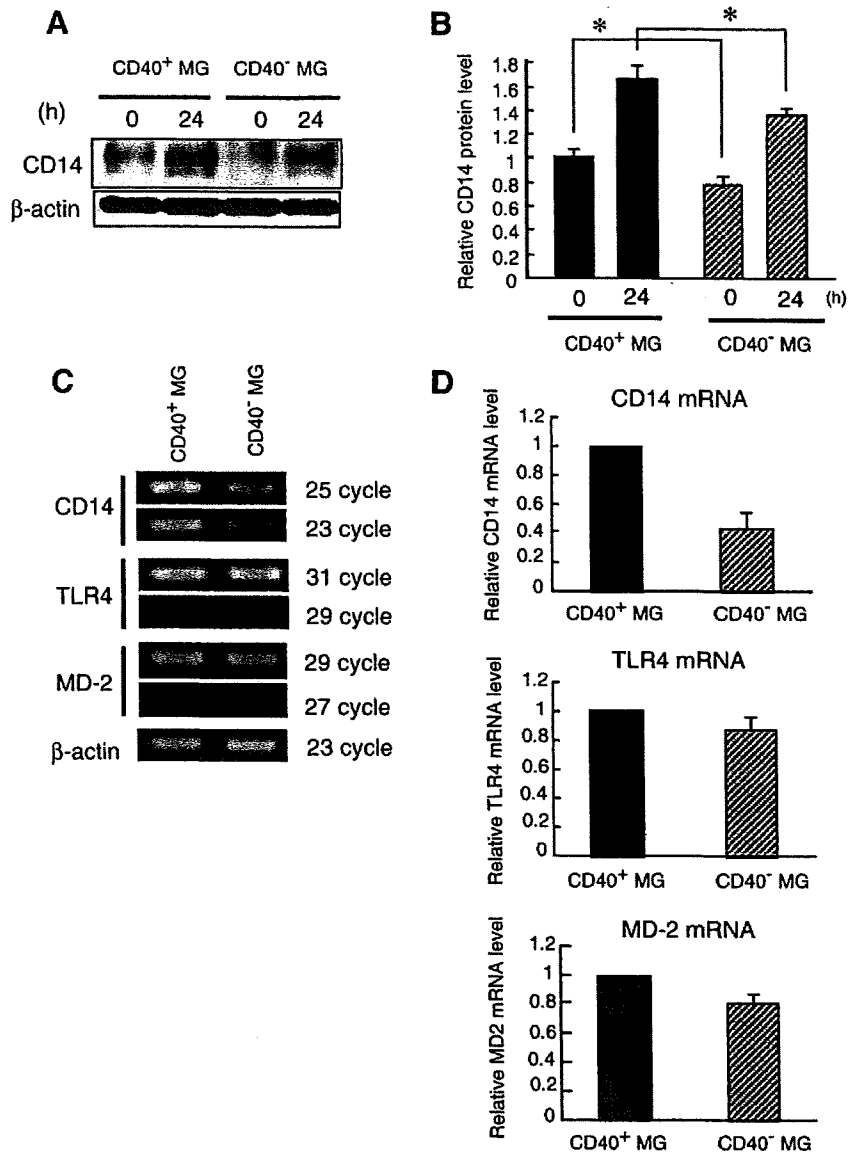

Fig. 5. The expression of LPS receptors in rat CD40⁺ MG and CD40⁻ MG. (A) Cells were treated with or without LPS (1 μg/ml) for 24 h. The cell extracts (30 μg of protein) were subjected to an immunoblot analysis using the anti-CD14 antibody or anti-β-actin monoclonal antibody. (B) The results in A were quantified. The value of unstimulated CD40⁺ MG was set at 1. Values were means±SD ($n=3$). *, $p<0.05$, significantly different from CD40⁻ MG. (C) Total RNA was extracted from CD40⁺ MG and CD40⁻ MG and amplified by PCR for CD14 (23, 25 cycles), TLR4 (29, 31 cycles), MD-2 (27, 29 cycles), or β-actin (23 cycles) using the respective primer pairs. (D) The results in C were quantified. The value of CD40⁺ MG was set at 1. Values were means±ranges ($n=2$).

NF-κB translocation and activation in both MG, but the LPS-induced NFκB translocation that is profoundly observed in CD40⁺ MG, is more closely related to the expression of iNOS and TNFα.

3.6. Induction of iNOS and TNFα by LPS was marked in 6-3 cells compared to Ra2 cells

Sawada et al. (2006) recently separated two subtypes of MG from mouse brains by cell sorting based on the profiles of intracellular reactive oxygen species (ROS) production. Mouse CD40⁺ MG produced greater amounts of ROS than CD40⁻ MG did upon phorbol myristate acetate stimulation. Previously, they generated two cell lines of MG, 6-3 and Ra2, by spontaneous immortalization of primary mouse MG (Kanzawa et al., 2000). In order to show that the observed different responses between CD40⁺ and CD40⁻ MG are due to MG subtypes themselves and not due to the astrocyte (or other cell) contamination in the MG preparation, we used 6-3 MG and Ra2 MG, as another set of CD40⁺ MG and CD40⁻ MG, respectively, and compared their responses to LPS stimulation.

After LPS (10 μg/ml) treatment, iNOS protein was induced only in 6-3 cells but not in Ra2 cells (Fig. 7A). The amount of TNFα released from 6-3 cells was much higher than that from Ra2 cells under similar treatment (Fig. 7B). Thus, LPS-induced induction of iNOS and release of TNFα were demonstrated more marked in 6-3 MG than in Ra2 MG, as similarly observed in rat CD40⁺ MG and CD40⁻ MG, respectively.

4. Discussion

During brain injuries and neurodegenerative diseases, the MG may play a dual role, namely, amplifying the effects of inflammation and mediating cellular degeneration, and protecting the CNS as well. Although iNOS derived NO acts beneficially as a cytotoxic agent on invading microorganisms or tumor cells (Hibbs et al., 1987; Ignarro, 2000), excess and long-lasting NO generation by activated MG has been considered to act harmfully on neighboring neurons and oligodendrocytes (Banati et al.,

1993; Merrill et al., 1993). TNFα produced from activated MG is also pleiotropic and acts for either neuroprotection or neurotoxicity (Suzuki et al., 2004; Sawada et al., 1995). A large amount of TNFα is thought to mediate neurodegenerative diseases (Barone et al., 1997). On the other hand, recent reports (Hayes et al., 1987; Ren et al., 1999; Bulloch et al., 2008) suggest that there may be at least two subtypes of MG in the CNS. However, it is not known whether the neurotrophic or toxic roles of MG are related to the heterogeneous cell population.

This study investigated the different responses of two MG subtypes against inflammatory stimuli and found that iNOS and TNFα were markedly induced in rat CD40$^+$ MG compared to CD40$^-$ MG after LPS stimulation. The distinct responses to LPS stimulation were also demonstrated between mouse MG cell lines of 6-3 (CD40$^+$) and Ra2 (CD40$^-$) (Fig. 7), supporting that marked induction of iNOS and TNFα is the characteristics of CD40$^+$ MG themselves and not due to the contamination of other cells like astrocytes. Sawada et al. (2006) showed recently that mouse CD40$^+$ MG-like cell line 6-3 was neurotoxic but mouse CD40$^-$ MG-like cell line Ra2 was neuroprotective, when they were co-cultured with neuronal cells. Similarly, using co-culture system of two subtypes of rat primary MG with rat primary hippocampal neurons, we preliminarily

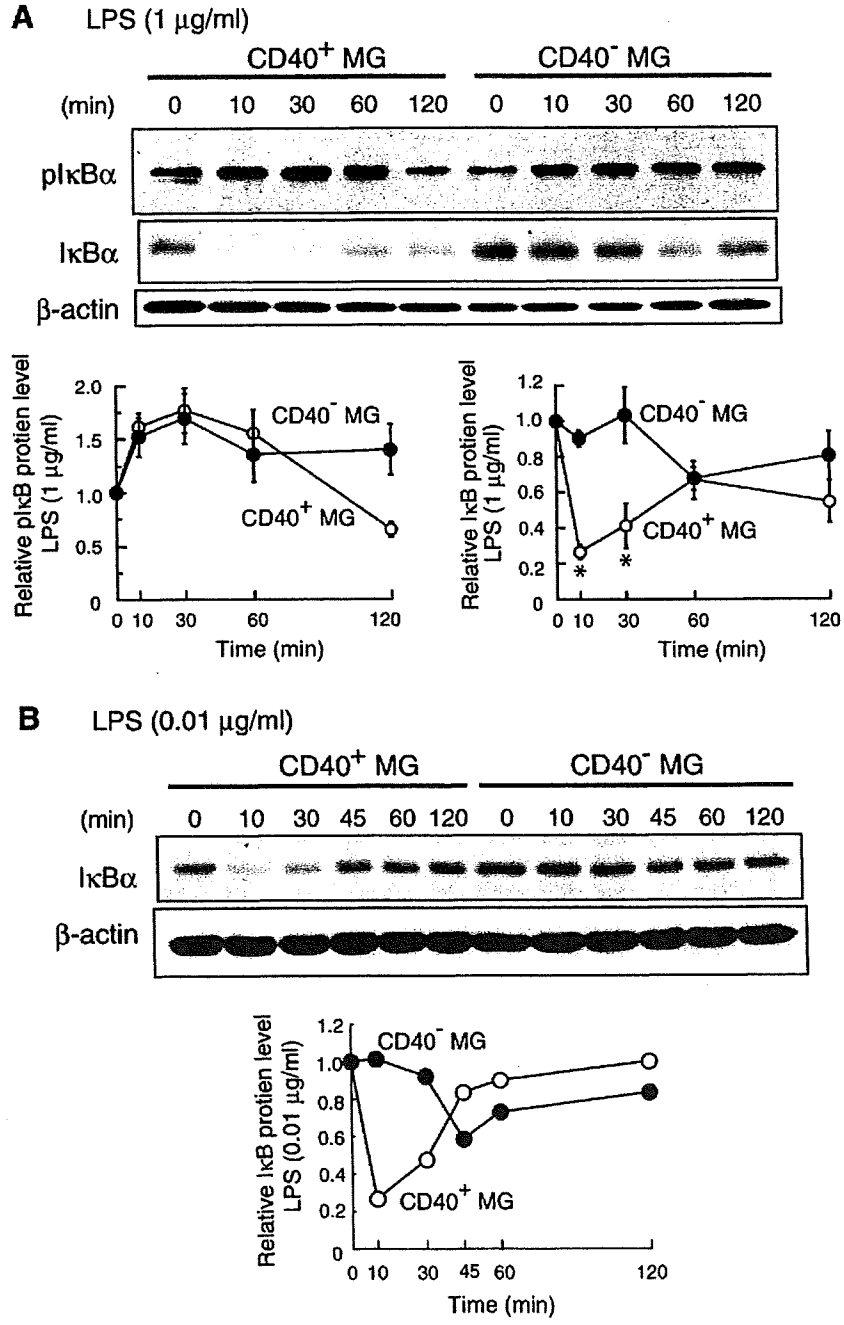

Fig. 6. LPS-induced degradation of IκBα is highly observed in rat CD40$^+$ MG. (A) The cells were treated with LPS (1 μg/ml) for the indicated periods. The cell extracts (30 μg of protein) were subjected to an immunoblot analysis using the anti-phosphorylated IκBα (pIκBα) antibody, anti-total IκBα antibody or anti-β-actin monoclonal antibody. The results were quantified. The maximal values were set at 1. Values were means±SD (n=3). *, p<0.05, significantly different from CD40$^-$ MG. (B) The cells were treated with LPS (0.01 μg/ml) for the indicated periods. The cell extracts (30 μg of protein) were subjected to an immunoblot analysis using the anti-total IκBα antibody or anti-β-actin monoclonal antibody. Results were quantified. The maximal values were set at 1. (C) Representative fields of NF-κB p65 fluorescence were shown in the CD40$^+$ MG that were pretreated with or without MG-132 for 1 h before treated with LPS treatment (1 μg/ml, 30 min) as indicated. The arrows indicated NF-κB in nucleus. (D) CD40$^+$ MG and CD40$^-$ MG were pretreated with or without MG-132 (1 μM) for 1 h before treated with LPS treatment (1 μg/ml, 6 h) as indicated. The cell extracts (30 μg of protein) were subjected to an immunoblot analysis using the anti-iNOS antibody. The results were quantified. Values were means±SD (n=3). *, p<0.05, significantly different from MG-132-treated cells.

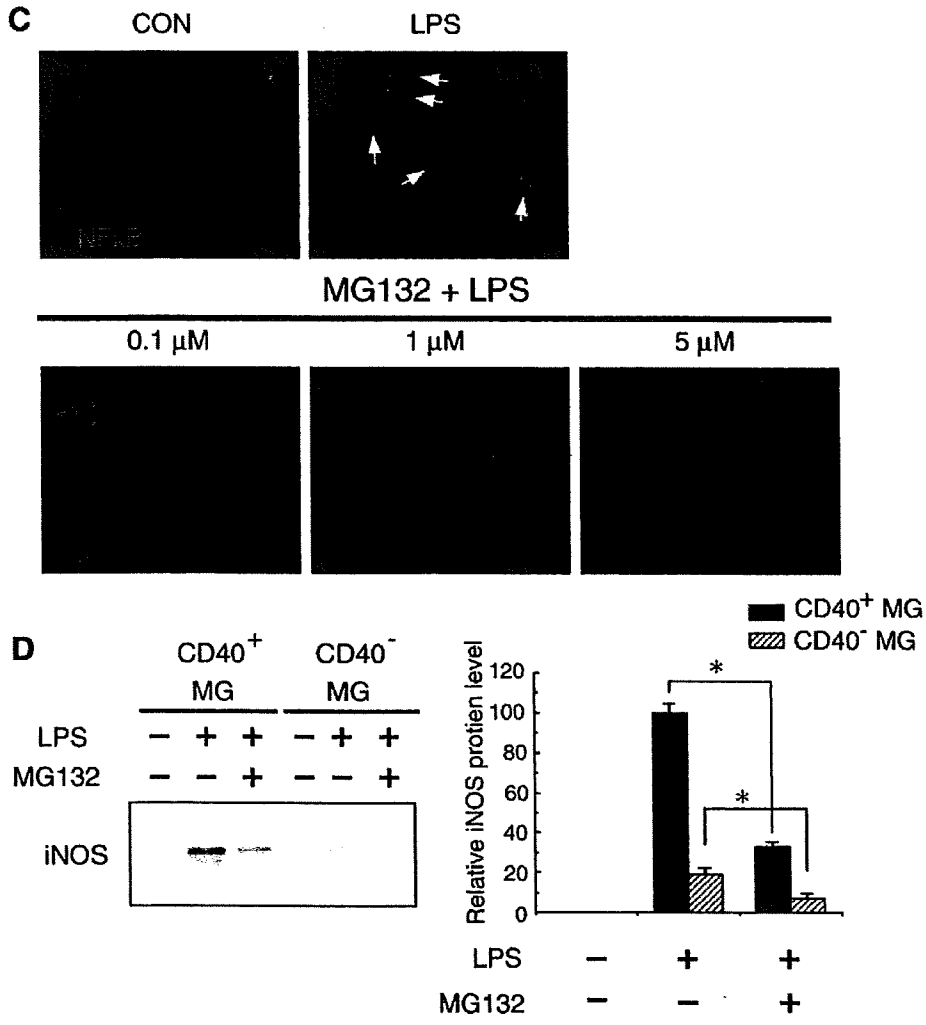

Fig. 6 (continued).

observed that LPS-activated CD40$^+$ MG exhibited neurotoxic action, whereas the similarly treated CD40$^-$ MG did little neurotoxicity (Kawahara et al., 2004). This suggests that the CD40$^+$ MG, which produced a large amount of NO and TNFα, may exhibit neurotoxic action under certain pathological conditions in the brain.

Paralleled to the protein levels, mRNAs for iNOS and pro-TNFα were expressed more highly in CD40$^+$ MG. The expression of CD14, a LPS receptor in CD40$^+$ MG was significantly higher than that in CD40$^-$ MG by Western blot analysis. It suggests that marked induction of iNOS and TNFα might be related partly to the higher expression of LPS receptor in CD40$^+$ MG (Fig. 4). However, the LPS-induced phosphorylation of IκBα as the subsequent signal step to LPS receptors was observed at essentially similar levels in both subtypes (Fig. 5A), suggesting that the different induction levels of iNOS and TNFα between the two cell types must be related to other signal transduction step(s) or mechanism that follows the IκBα phosphorylation.

We observed that degradation of IκBα after LPS stimulation was remarkably higher in CD40$^+$ MG than CD40$^-$ MG, at a low dose of LPS stimulation (0.01 μg/ml) in particular (Fig. 6B). Taken together with the preceding observation that iNOS and TNFα were enhanced profoundly in CD40$^+$ MG at low dose of LPS (0.01 μg/ml) (Figs. 2B and 3C), these results indicate that LPS-induced degradation of IκBα, and the following activation of NF-κB (p65 and p50) that is required for iNOS and TNFα transcription, are contributed more effectively in CD40$^+$ MG than CD40$^-$ MG. Although the ubiquitination of pIκBα and its proteasomal degradation in CD40$^+$ MG might be higher than those in CD40$^-$ MG, there is no data at present and future study will be needed.

A number of studies have shown that MG from mouse and rat effectively secrete large amounts of NO, while those from human and hamster generate very small or even no amounts of NO (Colton et al., 1996; Vodovotz et al., 1996; Walker et al., 1995a, for examples). Thus, the induction and regulation of iNOS in human glial cells seems substantially different from those in rodents. However, Ding et al. (1997) reported that human MG are capable of synthesizing iNOS and NO in response to cytokine stimulation. iNOS mRNA expression in MG has also been demonstrated in the brains of multiple sclerosis patients (Bagasra et al., 1995). Furthermore, immnunoreactive nitrotyrosine, a footprint of peroxynitrite formation, was found in MG within the necrotic foci in lesions of human white matter disorder periventricular leukomalacia (Haynes et al., 2003). Therefore, NO production has apparently demonstrated in activated human MG of MS and PVL patients, at least, and may be implicated in their pathogenesis.

Recent reports suggest that the functional diversity of activated MG is thought in the context that they are dependent on and derived from differing states of the same MG population; i.e. pro-inflammatory (classical activation) and anti-inflammatory (alternative activation) (Hanisch and Kettenmann, 2007; Schwartz et al., 2006). In this context, it might interpret that the different responses to LPS between CD40$^+$ MG and CD40$^-$ MG are dependent on the different cellular maturities (Kuwabara et al., 2003). On the other hand, several papers have been reported that there exist some subtypes of MG in the