possibility, we examined the responses of the microglia to KA at different stages of cellular proliferation using the GMIR1 rat microglial cell line, which proliferates in response to granulocyte–macrophage colony-stimulating factor (GM-CSF) [9]. In the present study, we therefore provide evidence that a cell cycle-dependent regulation of both the response to KA and the expression level of KA receptor subunits exist in the microglia.

Materials and methods

Cell culture. GMIR1, an immortalized microglial clone, was established from a rat primary culture using a nonenzymatic and non-virus-transformed procedure [9,10]. The medium to maintain the GMIR1 cells was Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Wako Pure Chemical Industries, Ins., Osaka, Japan), 5 μg/ml insulin, 0.17% $NaHCO_3$, 0.2% glucose, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. 0.2 ng/ml rat recombinant GM-CSF (Genzyme, Cambridge, MA, USA) was supplemented in the culture medium to maintain the GMIR1 cells because these cells stop proliferating without it. The GMIR1 cells were grown in an atmosphere of 10% CO_2 at 37 °C and the medium was changed every 2–3 days. Every 2 months, new cells were started to avoid transformation. Primary cultured rat microglia were isolated from mixed glial cell cultures from the cerebral cortex of 3-day-old Wistar rats on the 14 day as reported previously [11]. Cultures were maintained in Eagle's MEM containing 10% FBS, 0.2% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 25 μg/ml insulin, 5000 U/ml penicillin, and 5 mg/ml streptomycin.

Electrophysiology. Whole-cell recordings were made as reported previously [2,5]. Microglial cells were whole-cell clamped using a patch pipette containing (in mM): CsCl 100, Na_2ATP 3, HEPES 5, $CaCl_2$ 1, $MgCl_2$ 4, EGTA 5, and *N*-methyl-D-glucamine (NMDG) 10. The pH of the solution was adjusted to 7.2 with NMDG. The pipette resistance was 5–8 MΩ. The external solution contained (in mM): KCl 2.5, NaCl 110, $CaCl_2$ 3, $BaCl_2$ 6, glucose 15, and HEPES 5, and the pH were adjusted to 7.4 with NMDG. Patch clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in voltage clamp mode. Na^+ free solutions were substituted NaCl for NMDG. The external KA or drugs were applied rapidly using the Y tube technique, which allows for the complete exchange of the external solution surrounding a cell within 20 ms. pH of KA solution was adjusted to 7.4 with 1 N NaOH. The experiments were performed at room temperature. Many GMIR1 cells showed a small round shape under phase-contrast microscopy. We recorded small rounded cells for electrophysiological recordings. KA, Glu, and (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA) were purchased from Sigma (St. Louis, MO, USA). (*RS*)-3-(2-carboxybenzyl) willardiine (UBP296) was purchased from Wako Pure Chemical Industries.

BrdU incorporation. The GMIR1 cells were plated 10^4 per one cover glass coated with polyethyllenimine and then stained with a BrdU immunohistochemistry kit (EMD Biosciences, Inc., La Jolla, CA, USA) according to the manufacturer's protocol. The images were obtained with a microscope (Akisionscope, Carl Zeiss) using a 40× water immersion objective. Images were transferred to a personal computer and analyzed using the Image J software program (v 3.04). A statistical analysis was carried out using ANOVA. A value of $P < 0.05$ was considered to indicate statistical significance.

Real-time quantitative RT-PCR. Total RNAs were prepared from 2 to 2.5×10^6 cells with a RNeasy RNA purification kit (Qiagen, Hilden, Germany). To avoid any contamination of genomic DNA, cytoplasmic RNAs were isolated and DNase was added according to the manufacturer's protocol. RT minus controls were run to confirm the presence of genomic contamination. First strand cDNA synthesized from 1 mg of total RNA with random hexamer primers and oligo (dT) primers was used as template in each reaction. Syber green based real-time RT-PCR was performed with DyNAmo SYBER Green 2-step qRT-PCR kit (Finnzymes, Espoo, Finland). Rotor Gene 3000 (Corbett Research, Mortlake, Australia) was used for the signal detection. PCR was performed using 1× master mix, 0.5 μM of each primer. For standardization, rat GAPDH was used. The primers for detection of cyclin D1, GluR5–7, and KA1-2 cDNAs were as follows: cyclin D1 (232 bp), 5′-GCGTACCCTGACACCAATCT-3′ and 5′-GCTCCAGAGACAA GAAACGG-3′; GluR5 (208 bp), 5′-GCCCCTCTCACCATCACAT AC-3′ and 5′-ACCTCGCAATCACAAACAGTACA-3′; GluR6 (260 bp) 5′-TTCCTGAATCCTCTCTCCCCT-3′ and 5′-CACCAAATGCCTCC CACTATC-3′; GluR7 (423 bp) were 5′-TGGGCCTTCACCTTGAT CATCA-3′ and 5′-ACTCCACACCCCGACCTTCT-3′; KA1 (267 bp), 5′-GGATCGCTGCTATCTTGGATG-3′ and 5′-CCTTCTCTCCACA GATGTTGCT-3′; KA2 (291 bp), 5′-ACAGCCAGTACGAGACTAC-3′ and 5′-ACTCAGC TTTGGCGCAGAT-3′. PCR conditions were 95 °C for 15 min, followed by 35–40 cycles at 94 °C for 10 s, 49–56 °C for 20 s, and 72 °C for 20 s. After real-time RT-PCR, the reaction products were analyzed by electrophoresis on ethidium bromide, stained agarose gel. All of the PCR experiments were performed in duplicate to verify the results.

Transient forebrain ischemia. Male Wistar rats (8 weeks) were subjected to transient forebrain ischemia by clamping the carotid arteries bilaterally as reported previously [12,13]. Briefly, the animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), and bilateral vertebral arteries were electrocauterized at the level of the first vertebra. On the following day, the common carotid arteries were gently exposed and both arteries were occluded with a vascular clamp for 15 min. The rectal temperature was maintained at 36.5–37.5 °C. Rats that had lost their righting reflexes during the period of ischemia were used as the postischemic group.

Immunohistochemistry. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and killed by intracardiac perfusion with isotonic saline followed by a chilled fixative consisting of 4% paraformaldehyde in 0.1 M PB (pH 7.4). After perfusion, the brain was removed and further fixed by immersion in the same fixative overnight at 4 °C, and then immersed in 30% sucrose (pH 7.4) for 72 h at 4 °C. Floating parasagittal sections (30 μm thick) of the hippocampus were prepared by a cryostat (CM1850, Leica, Nussloch, Germany). After blocking with 10% normal goat serum overnight at 4 °C, they were stained with anti-GluR6/7 (10 μg/ml) (Upstate, Lake Placid, NY, USA), anti-CD11b (1: 100) (OX42, Serotec, Bichester, UK) for 3 days at 4 °C. After washing with phosphate-buffered saline (PBS), sections were stained with goat anti-rabbit IgG-conjugated Alexa488 (Jackson Immunoresearch, West Grove, PA, USA) or goat anti-mouse IgG-conjugated Cy3 (Jackson Immunoresearch) for 6 h at 4 °C. The sections were mounted in the anti-fading medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined by a confocal laser scanning microscope (CLSM) (LSM510MET, Carl Zeiss, Jena, Germany).

Statistics. The significance of the differences was examined by Student's *t*-test, using Kaleida Graph 3.6 J, and $P < 0.05$ was considered significant.

Results

Density-dependent inhibition of GM-CSF-induced cell proliferation of the GMIR1 cells

Most GMIR1 cells showed an ameboid-like morphology. When the GMIR1 cells were plated at a low cell density in the presence of 0.2 ng/ml GM-CSF, the number of cells markedly increased and reached a confluent state after 7 days in culture (Fig. 1A). The cellular proliferation of the GMIR1 cells was fully dependent on GM-CSF and the number of cells did not change without GM-CSF. The total number of cells increased by approximately 260%, 400%, and 500% after 2, 5, and 7 days in culture, respectively. On the other hand, the ratio of BrdU-incorpo-

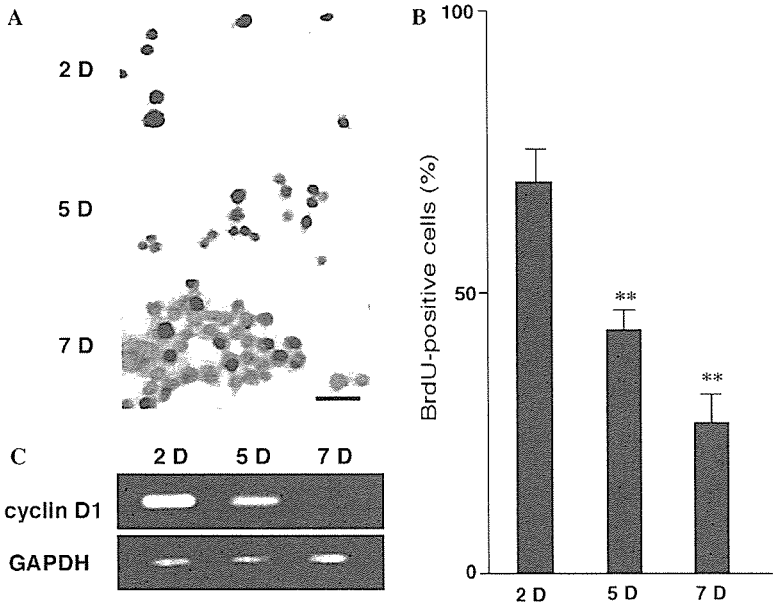

Fig. 1. Density-dependent inhibition of the proliferation of a microglia cell line, GMIR1. (A) BrdU incorporation into the GMIR1 cells after 2 (2D), 5 (5D), and 7 (7D) days in culture with the cell density of 1×10^4 cells/well. Scale bar, 15 μm. (B) The ratio of BrdU-positive cells to total cells at 2D, 5D, and 7D. Each column and vertical bar represents the mean and SE of four experiments, respectively. Asterisks indicate a significant difference from 2D (**$P < 0.01$, Student's t-test). (C) The expression of cyclin D1 mRNA in the GMIR1 cells at 2D, 5D, and 7D.

rated cells reached a peak after 2 days in culture and then gradually decreased (Fig. 1A and B). We next examined the expression of cyclin D1 in the GMIR1 cells. Relatively high expression levels of mRNA encoding cyclin D1 were seen in the GMIR1 cells after 2 days in culture (Fig. 1C). After 7 days in culture, the specific band of cyclin D1 could not be found. These observations indicate that the GMIR1 cells exhibited a cell density-dependent inhibition of proliferation in the presence of GM-CSF. Most cells proliferated after 2 days in culture, while most cells stopped cell division after 7 days in culture.

Cell cycle-dependent inhibition of responses for Glu and KA in the GMIR1 cells

We next examined effects of the cell cycle on the responses to Glu and KA in the GMIR1 cells. The application of Glu and KA induced inward currents under voltage clamp conditions at a holding potential of −60 mV. After 2 days in culture, 20 out of 25 cells (80%) exhibited relatively large inward currents after treatment with Glu and KA (Fig. 2A). The mean amplitudes of inward currents evoked by Glu and KA after 2 days in culture were 13.8 ± 3.0 and 8.4 ± 0.6 pA, respectively. After 5 days in culture, 7 out of 15 cells (46.7%) showed moderate responses to Glu and KA. After 7 days in culture, 18 out of 80 cells (22.5%) showed only small responses to Glu and KA. The mean amplitudes of inward currents evoked by Glu and KA after 7 days in culture were 4.7 ± 0.8 and 6.2 ± 1.2 pA, respectively. As shown in Fig. 2B, the mean normalized responses to Glu and KA in the GMIR1 cells after 7 days in culture were significantly smaller that those in GMIR1 cells after 2 days in culture. We also examined the effects of cell cycle on the responses to ATP in the GMIR1 cells. In contrast to the responses to Glu and KA, all cells exhibited large inward currents even after 7 days in culture and cell cycle-dependent inhibition of responses was not observed.

The KA-induced currents were concentration dependent between 10^{-5} M and 3×10^{-3} M (data not shown). The mean amplitudes of the inward currents induced by each concentration of KA between 10^{-5} M and 3×10^{-3} M were normalized to the one induced by 3×10^{-3} M KA in the same cell. The concentration–response relationship in the GMIR1 cells after 2 days in culture showed that the half-activation concentration, and the Hill coefficients were 6.8×10^{-5} M and 1.3, respectively. We further examined effects of a specific antagonist and agonist for KA receptors. In the GMIR1 cells after 2 days in culture, the KA-induced (100 μM) inward currents were markedly inhibited by UBP296 (30 μM), a selective antagonist for KA receptors [14] (Fig. 3A). The similar results with the GMIR1 cells in quiescent stage were obtained in the primary cultured rat microglia that do not increase in cell number in the absence of mitogens. In the primary cultured rat microglia, KA induced inward currents in 3 out of 35 cells examined (8.6%). In these KA-responsive cells, ATPA also induced inward currents (Fig. 3B). Furthermore, ATPA (30 μM), a selective agonist for GluR5 [15], induced inward currents in the GMIR1 cells. When extracellular Na^+ was replaced with NMDG, the ATPA-induced inward currents markedly decreased (Fig. 3C). On the other hand, the ATP-induced inward currents were not affected by the Na^+-free condition (Fig. 3C).

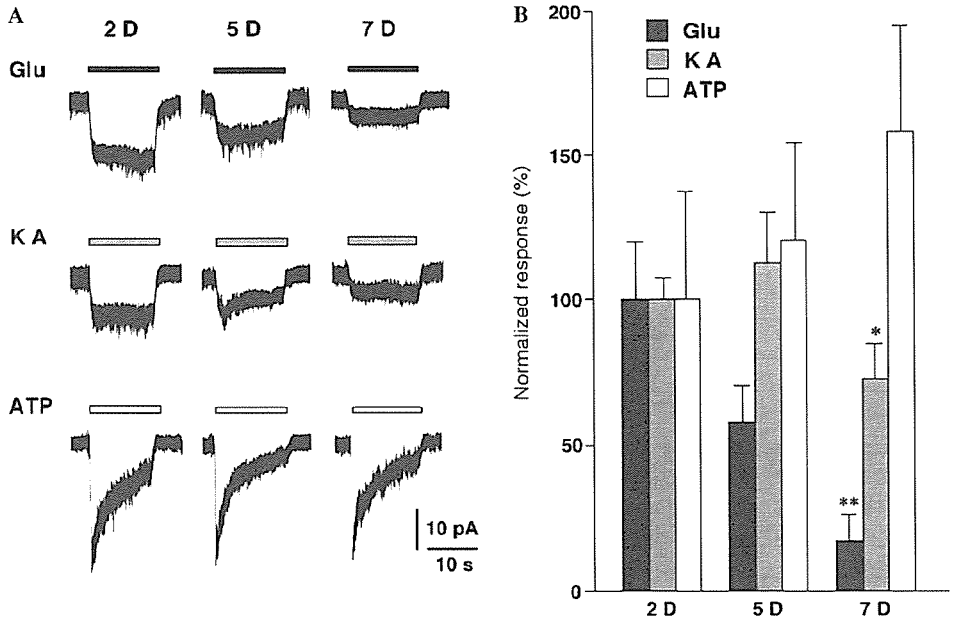

Fig. 2. Density-dependent reduction of the amplitude of the KA-induced inward currents in the GMIR1 cells. (A) KA (100 μM), Glu (100 μM), and ATP (100 μM)-induced inward currents in the GMIR1 cells at 2D, 5D, and 7D. The holding potential was −60 mV. (B) The mean normalized responses to Glu, KA, and ATP at 2D, 5D, and 7D. Each column and vertical bar represents the mean and SE of 7–15 experiments, respectively. Asterisks indicate a significant difference from 2D ($^{*}P < 0.05$, $^{**}P < 0.01$, Student's t-test).

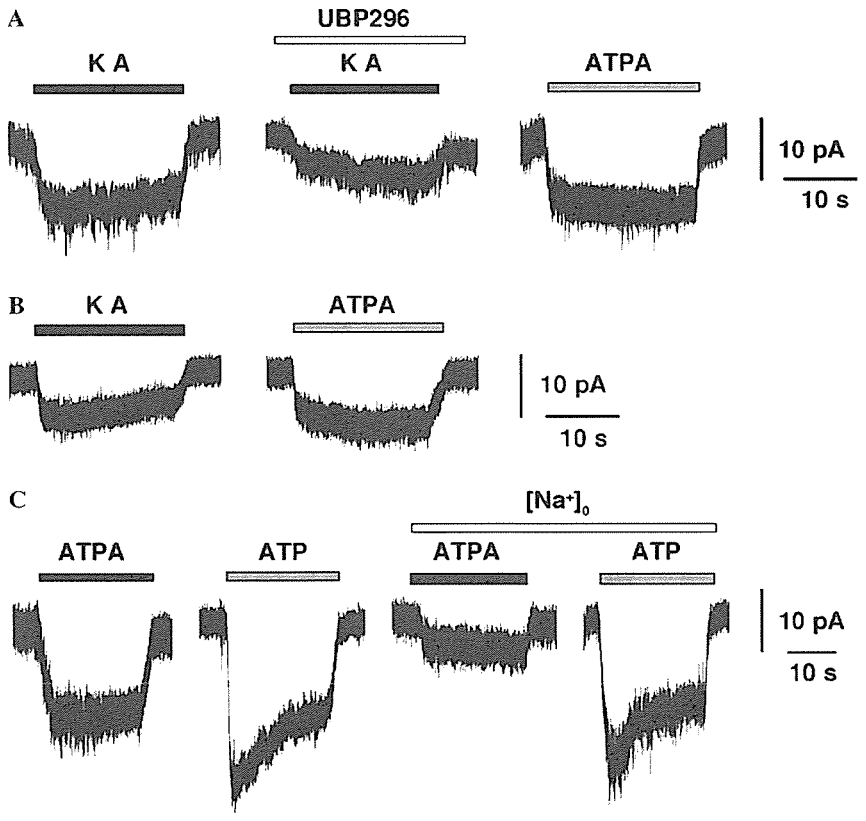

Fig. 3. The expression of functional KA receptors in the GMIR1 cell. (A) The inhibition of KA-induced inwards currents by 30 μM UBP296, a selective antagonist of KA receptor GluR5. ATPA (30 μM), a GluR5 selective agonist, also induced inward currents. (B) KA- and ATPA-induced inward currents in primary cultured rat microglia. (C) The effects of Na^{+}-free solution on the ATPA- and ATP-induced inward currents. When the external solution was replaced with Na^{+}-free ($[Na^{+}]_0$) solution, the amplitude of the ATPA-induced inward currents was markedly reduced.

Density-dependent downregulation of the expression levels of GluR5 and GluR6 in the GMIR1 cells

KA receptors are composed of five different subunits, namely GluR5, GluR6, GluR7, KA1, and KA2. GluR5, GluR6, and GluR7 assemble to form functional ion channels, whereas little is known about the functions of KA1 and KA2 [16]. Real-time quantitative RT-PCR was performed to examine the possible cell cycle-dependent alteration of KA receptor subunits, GluR5, GluR6, GluR7, KA1, and KA2. Our RT-PCR study showed that microglia expressed GluR5, GluR6, and KA2 mRNA, while GluR7 and KA1 mRNA were not detected (Fig. 4A). In comparison to the expression levels after 2 days in culture, the expression level of GluR5 mRNA significantly declined to $70.6 \pm 17.0\%$ and $47.8 \pm 16.0\%$ after 5 and 7 days in culture, respectively (Fig. 4B). Furthermore, the GluR6 mRNA expression levels also decreased to $41.2 \pm 2.0\%$ and $17.3 \pm 1.9\%$ after 5 and 7 days in culture, respectively. The expression levels of KA2 mRNA did not show any significant change. These observations were consistent with our electrophysiological findings.

We further examined the expression of KA receptor subunits in microglia in the hippocampus prepared from sham and post-ischemic rats. In the hippocampal sections prepared from sham rats, the strong immunoreactivity for GluR6/7 was observed in pyramidal cells (Fig. 4C). On the other hand, some glial cells in the stratum radiatum showed only a faint immunoreactivity for GluR6/7. In contrast, there was a strong immunoreactivity for GluR6/7 in the stratum radiatum of the hippocampal sections prepared from the animals subjected to ischemia 3 days earlier. GluR6/7-positive cells increased in the stratum radiatum were found to be mainly OX42-positive microglia (Fig. 4D, arrows).

Discussion

In the present study, we found that the responsiveness of microglia to Glu and KA was tightly regulated by the cell cycle mechanism. At the cell proliferation stage, approximately 80% of the cells exhibited Glu- and KA-induced inward currents with the mean amplitude of 13.8 ± 3.0 and 8.4 ± 0.6 pA, respectively. On the other hand, only 22.5% of the cells showed Glu- and KA-induced inward currents with the mean amplitude of 4.7 ± 0.8 and 6.2 ± 1.2 pA, respectively, at cell quiescent stage. These values were significantly smaller than those obtained at cell

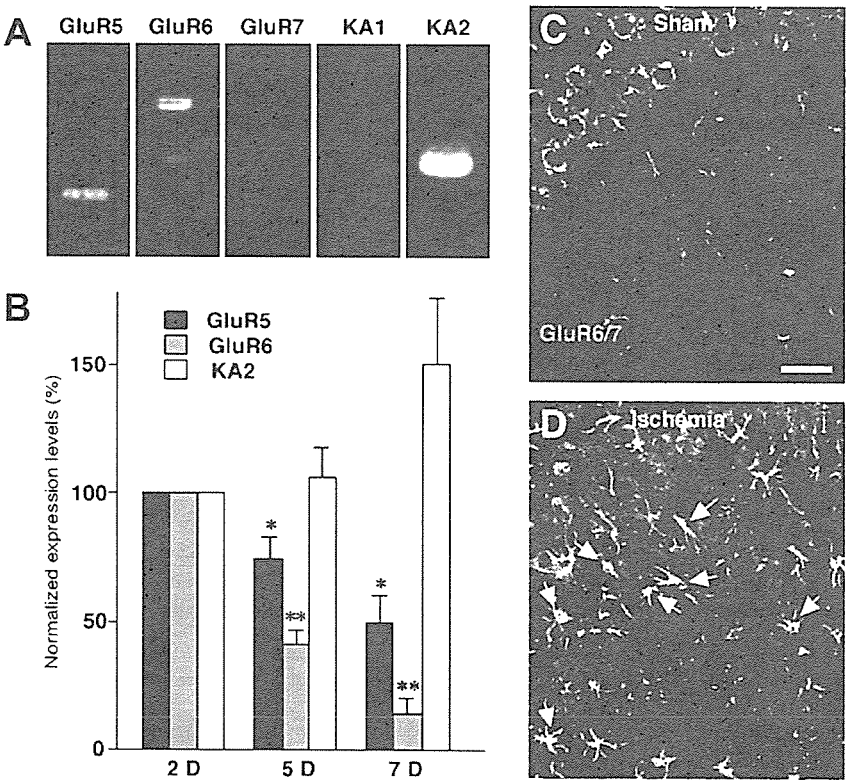

Fig. 4. Density-dependent reduction of the expression levels of KA in the GMIR1 cells. (A) RT-PCR was performed on mRNA from the GMIR1 cells to examine the expression of the KA receptor subunits mRNA. GluR5 (208 bp), GluR6 (260 bp) and KA2 (291 bp) were detected, whereas GluR7 and KA2 were not detected. (B) The normalized expression levels of GluR5. GluR6, and KA2 at 2D, 5D, and 7D by quantitative real-time RT-PCR. Each column and vertical bar represents the mean and SE of 3–4 experiments, respectively. Asterisks indicate a significant difference from 2D ($^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.) (C, D) Immunofluorescent CLSM images for GluR6/7 (green) and OX42 (red) in the hippocampus prepared from animals subjected to sham-operation (C) and global ischemia (D) 3 days earlier. It was noted that some OX42 positive microglia showed intense immunoreactivities for GluR6/7 in the post-ischemic hippocampus (arrows in D). Scale bar, 100 μm.

proliferation stage. These electrophysiological observations were substantiated by the mRNA expression levels of the KA receptor subunits, GluR5 and GluR6. The expression levels of both GluR5 and GluR6 were relatively high at the cell proliferation stage, while their expression levels stayed very low at the cell quiescent stage. We also found that the immunoreactivity for GluR6/7 markedly increased in activated microglia in the post-ischemic hippocampus. Although the precise roles of the Glu receptors located on the microglia remain unclear, this cell cycle-dependent responsiveness and expression of KA receptors may have functional implications. Recently, KA has been reported to induce a rapid rearrangement of F-actin which is required for migration and phagocytic abilities of activated microglia [17]. Therefore it is reasonable to consider that Glu may induce the rearrangement of the actin cytoskeleton through the upregulated GluR5 and GluR6 during cellular proliferation.

We have also shown the microglia to have functional KA receptors because the KA-induced currents were markedly inhibited by USB296, which is a selective antagonist for the KA receptors. Furthermore, KA-responsive cells also responded to both ATPA, which is a selective agonist for GluR5. The similar results were also obtained in primary cultured rat microglia that do not increase in cell number in the absence of mitogens. ATPA also induced inward currents in the KA-responsive small population of primary cultured microglia (approximately 10% of cells). We have previously reported that KA-induced currents were potentiated by concanavalin A, which blocks desensitization of KA receptors, in some primary cultured microglia (two out of seven cells) [5]. Furthermore, Eun et al. [18] have reported that the KA-induced c-fos gene expression in primary cultured rat microglia was not potentiated in the presence of cyclothiazide, which is known to potentiate AMPA-induced currents by inhibiting the desensitization of AMPA receptors. These observations further support our finding that the microglia have functional KA receptors. More recently, however, Hagino et al. [19] demonstrated that the KA-induced currents in primary cultured rat microglia were mostly AMPA receptor-mediated currents while the KA receptors were barely functional. This conclusion was mainly based on their finding that the KA-induced currents were completely inhibited by LY300164 at a concentration of 100 μM, a selective inhibitor for AMPA receptors. However, IC_{50} of LY300164 for AMPA and KA receptors were originally reported to be 1.7 μM and 2.6 μM, respectively [20]. Therefore, it is reasonable to consider that LY300164 at a concentration of 100 μM could therefore suppress the responses mediated by both AMPA and KA receptors.

In conclusion, microglia were thus found to have functional KA receptors, mainly consisting of GluR5 and GluR6, and the expression levels of these subunits are closely regulated by the cell cycle mechanism.

Acknowledgments

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Amyloid-β peptides induce several chemokine mRNA expressions in the primary microglia and Ra2 cell line via the PI3K/Akt and/or ERK pathway

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Abstract

Alzheimer's disease (AD) is characterized by the presence of senile plaques composed primarily of amyloid-β peptide (Aβ) in the brain. Microglia have been reported to surround these Aβ plaques, which have opposite roles, provoking a microglia-mediated inflammatory response that contributes to neuronal cell loss or the removal of Aβ and damaged neurons. To perform these tasks microglia migrate to the sites of Aβ secretion. We herein analyzed the process of chemokine expression induced by Aβ stimulation in primary murine microglia and Ra2 microglial cell line. We found that Aβ1-42 induced the expressions of CCL7, CCL2, CCL3, CCL4 and CXCL2 in the microglia. The signal transduction pathway for the expression of CCL2 and CCL7 mRNA induced by Aβ1-42 was found to depend on phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK), whereas the pathway for CCL4 depended only on PI3K/Akt. These inflammatory chemokine expressions by Aβ stimulation emphasize the contribution of neuroinflammatory mechanisms to the pathogenesis of AD.

Keywords: Microglia; Alzheimer's disease; Amyloid β; Chemokine; Akt; ERK

1. Introduction

Alzheimer's disease (AD) is characterized by the presence of senile plaques composed primarily of amyloid-β peptide (Aβ) in the brain. Microglia have been reported to surround Aβ plaques (Haga et al., 1989; Itagaki et al., 1989). Aβ-induced microglia have been shown to produce reactive oxygen species, TNFα, IL1β, which have been demonstrated to cause the degeneration of nervous cells (Akiyama et al., 2000; Ishii et al., 2000; Blasko et al., 2004). On the other hand, microglia play an important role in the clearance of Aβ by phagocytosis, primarily through scavenger receptor class A (SR-A, CD204), scavenger receptor-BI (SR-BI) and CD36 (El Khoury et al., 1996; Paresce et al., 1996; Husemann et al., 2002). Other receptors such as receptor for advanced glycosylation end-products (RAGE), integrins and heparan sulfate proteoglycans, have also been reported to bind with Aβ (Verdier and Penke, 2004). CD36 binds to Aβ *in vitro* (Bamberger et al., 2003), and it is physically associated with members of the Src family tyrosine kinase (Huang et al., 1991; Bull et al., 1994), which transduce signals from this receptor (Jimenez et al., 2000).

Microglia as a macrophage-lineage cell may produce several chemokines, which induce microglial chemotaxis. It has recently been reported that microglia produce MCP-1 (CCL2) and other chemokines by Aβ stimulation through CD36 receptor (El Khoury et al., 2003). Another report examined the production of chemokines in THP-1 monocytes

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase

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induced by Aβ (Giri et al., 2003). However, there have so far been no reports, which examined the whole series of inflammatory chemokine expression induced by Aβ in microglial cells. We herein extensively examined the whole series of inflammatory chemokine expression by Aβ stimulation using real-time PCR methods. By this examination we found that CCL2 (MCP-1), CCL3 (MIP-lα), CCL4 (MIP-1β), CCL7 (MCP-3) and CXCL2 were induced by Aβ1-42. Then we further analyzed the signaling pathway for CCL2, CCL4 and CCL7 mRNA expression induced by AB1-42 in the microglia.

2. Materials and methods

2.1. Materials

Synthetic human Aβ1-42 and Aβ1-40 were obtained from Peptide Institute Inc. (Osaka, Japan). Aβ1-42 and Aβ1-40 were dissolved in 0.1% NH_3 solution according to the manufacturer's instructions. Anti-phospho-Akt (Serine 473), anti-Akt, anti-phospho-ERKl/2 (Thr202/Tyr204) antibodies were from Cell Signaling (Beverly, MA). Anti-ERK antibody was from Santa Cruz (Santa Cruz, CA). Wortmannin and PD98059 were from Calbiochem (San Diego, CA). U0126 and SB203580 were from Promega (Madison, WI). All inhibitors were resolved in DMSO. Mouse recombinant granulocyte-macrophage colony-stimulating factor (mrGM-CSF) was from BD Pharmingen (San Diego, CA).

2.2. Cell culture

The microglial cell line Ra2 cells were established from neonatal C57BL/6J (H-2^b) mice using a non-enzymatic and non-virus-transformed procedure (Sawada et al., 1998). Ra2 cells proliferate in a culture medium containing GM-CSF. The medium for maintaining Ra2 cells was MGI medium [Eagle's MEM supplemented with 0.2% glucose, 5 μg/ml bovine Insulin (Sigma–Aldrich, St. Louis, MO), 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA)] and 0.8 ng/ml mrGM-CSF (BD Pharmingen). Before Aβ-treatment, the Ra2 cells were cultured in an MGI medium without mrGM-CSF for 16 h. The primary microglia was prepared using newborn C57BL/6 mice as described previously (Sawada et al., 1999), and then they were cultured in MGI medium containing 0.8 ng/ml mrGM-CSF. The purity of primary microglial cultures was estimated to >95% based on the expression of CD11b marker using flow cytometry.

2.3. Quantitative real-time RT-PCR

Ra2 cells and primary microglial cells were plated in 6 cm diameter dishes at 1×10^6 cells/dish and treated with Aβ or 0.1% NH_3 solution as a control. Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed to 20 μl cDNA using Superscript II Reverse Transcriptase (Invitrogen). Quantitative SYBR Green real-time PCR was performed on M×3000P (Promega) using the following program: 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 20 s at 60 °C. The reactions were carried out using 0.5 μl cDNA with SYBR Premix EX Taq (Takara, Shiga, Japan). The sequences of the primer for real-time PCR are depicted in Table 1. As an endogenous reference we used β-actin. Specificity of the PCR product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that single DNA sequence was amplified during PCR. In addition, end reaction products were visualized on ethdium bromide-stained 2.0% agarose gels. Appearance of a single band of the correct size confirmed specificity of the PCR. Quantitative analysis of gene expression was performed using the comparative cycle threshold (C_T) method, in which C_T is the threshold cycle number (Livak and Schmittgen, 2001). The target gene (target, i.e. CCL2) was normalized to an endogenous reference gene (β-actin). To indicate relative expression, we calculated using the expression $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_{T,target} - C_{T,actin})_{treated\ sample} - (C_{T,target} - C_{T,actin})_{control\ sample}$. Each sample was tested in triplicate with quantitative PCR, and samples obtained from three independent experiments were used to calculate the means ± S.D.

Table 1
The sequences of the primer for real-time PCR

Target (pruduct size)	Sequence (5′–3′)
CCL2 (127 bp)	Sense: TGAATGTGAAGTTGACCCGT Antisense: AAGGCATCACAGTCCGAGTC
CCL3 (163 bp)	Sense: CCTCTGTCACCTGCTCAACA Antisense: GATGAATTGGCGTGGAATCT
CCL4 (237 bp)	Sense: CCCACTTCCTGCTGTTTCTC Antisense: GAGGAGGCCTCTCCTGAAGT
CCL5 (242 bp)	Sense: ATATGGCTCGGACACCACTC Antisense: GGGAAGCGTATACAGGGTCA
CCL6 (185 bp)	Sense: GCCACACAGATCCCATGTAA Antisense: GCAATGACCTTGTTCCCAGA
CCL7 (157 bp)	Sense: GCATGGAAGTCTGTGCTGAA Antisense: AGAAAGAACAGCGGTGAGGA
CCL8 (282 bp)	Sense: TCAGCCCAGAGAAGCTGACT Antisense: TCCAGCTTTGGCTGTCTCTT
CCL9 (244 bp)	Sense: CAAAGGAGGGCATTATGAGC Antisense: CCTTGCTGTGCCTTCAGACT
CCL12 (181 bp)	Sense: GTCCTCAGGTATTGGCTGGA Antisense: GGGTCAGCACAGATCTCCTT
CCL19 (236 bp)	Sense: CAAGAACAAAGGCAACAGCA Antisense: CGGCTTTATTGGAAGCTCTG
CCL20 (154 bp)	Sense: CGTCTGCTCTTCCTTGCTTT Antisense: CTTCATCGGCCATCTGTCTT
CXCL2 (258 bp)	Sense: TCCAGAGCTTGAGTGTGACG Antisense: AGGCACATCAGGTACGATCC
β-Actin (298 bp)	Sense: AGTGTGACGTTGACATCCGT Antisense: GCAGCTCAGTAACAGTCCGC

2.4. Immunoblotting

The cells (1×10^6 cells/dish) were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 5% bromophenol blue). Protein concentrations were quantified by the Bradford assay using the Bio-Rad protein assay (Bio-Rad, Herucules, CA). Then total protein (50 μg per lane) was resolved by SDS-PAGE and then was transferred to PVDF membranes (Millipore, Billerica, CA). The blots were incubated with anti-phospho ERK or anti-phospho Akt overnight at 4 °C and then treated with HRP-conjugated secondary antibody. Signals were detected by ECL system (Amersham Biosciences, Little Chalfont, UK). The blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.8) and reprobed with anti-ERK or anti-Akt.

2.5. Statistical analysis

The results are expressed as the means ± S.D. Comparisons among means were performed using ANOVA, followed by the Scheffe's test. The two-tailed Student's *t*-test was used for comparisons between two means.

3. Results

3.1. Production of several chemokines by Aβ-induced microglia

We analyzed the effect of Aβ on the expression of chemokines, in the microglia, which has been shown to be secreted by inflammatory macrophages. The microglial cell

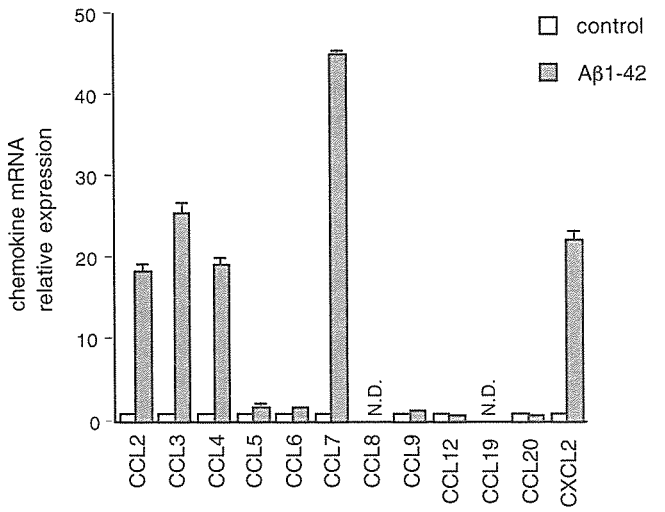

Fig. 1. Aβ1-42 induced the expression of chemokine. Ra2 cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Real-time PCR of a series of chemokine and β-actin mRNA were performed. The chemokine mRNA expression was normalized to β-actin. The results indicated relative expression of chemokine in Aβ-treated cells compared with control cells. The data represent the means ± S.D. of triplicate of three separate experiments. N.D.: the PCR signal was not detected.

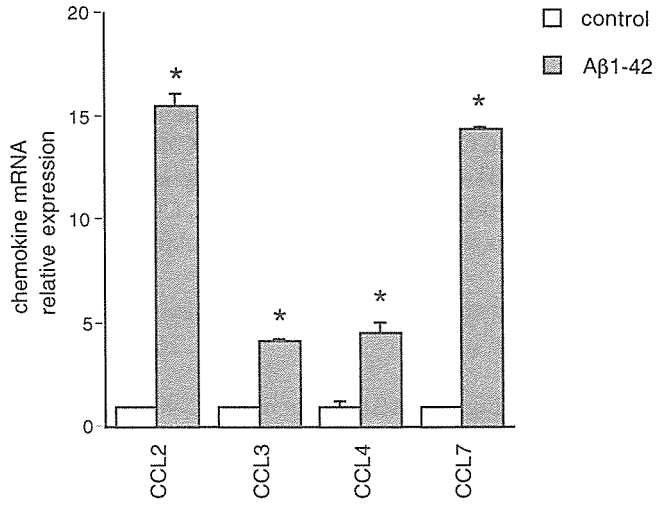

Fig. 2. Induction of the chemokine mRNA expression by Aβ1-42 in primary microglia. Primary cultured microglial cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Extracted mRNA was quantified by real-time PCR. The results indicated relative expression of CCL2, CCL3, CCL4 and CCL7 in Aβ-treated cells compared with control cells. The data represent the means ± S.D. of triplicate of three separate experiments. $^{*}p < 0.001$ vs. each control of chemokine.

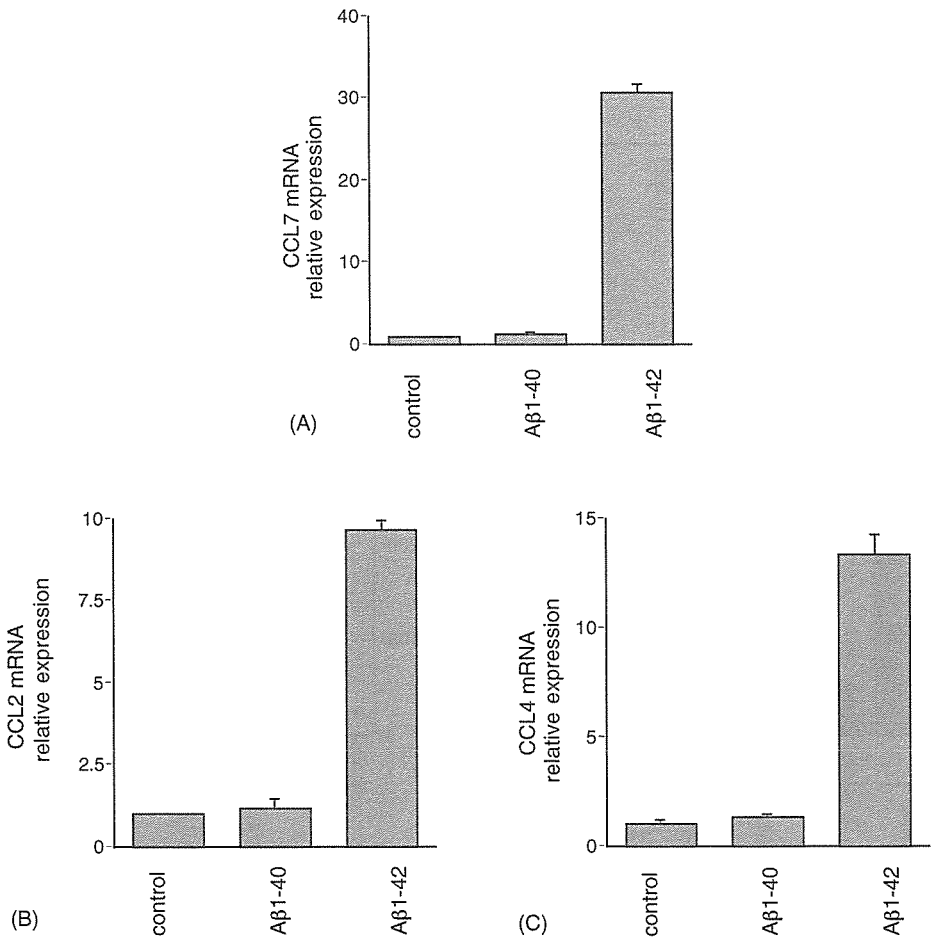

Fig. 3. The mRNA expression of chemokine induced with Aβ1-42 or Aβ1-40. Ra2 cells were stimulated 10 μM Aβ1-42 and 10 μM Aβ1-40 for 16 h. Extracted mRNA was quantified by real-time PCR. The results indicated relative expression of CCL7 (A), CCL2 (B), CCL4 (C) in Aβ-treated cells compared with control cells. The data represent the means ± S.D. of triplicate of three separate experiments.

line Ra2 was treated with 10 μM Aβ1-42 for 16 h, and the expression of chemokines was examined by quantitative real-time PCR. We found the expression of CCL7 mRNA to be highly increased by Aβ1-42 stimulation (Fig. 1). The expressions of CCL2, CCL3, CCL4 and CXCL2 were also increased by Aβ1-42. In the primary microglial cells cultured from C57BL/6 mouse newborn brain, CCL2, CCL3, CCL4 and CCL7 mRNA expression were induced by Aβ1-42 (Fig. 2).

3.2. Aβ1-42 but not Aβ1-40 induces chemokine production in the microglia

As shown in Fig. 3A, Aβ1-42 but not Aβ1-40 induces CCL7 mRNA. In our assay, the high induction of CCL7 mRNA expression was examined, when Aβ1-42 was added to the culture. Interestingly, Aβ1-40 was not found to induce CCL7 (Fig. 3A). The expression of CCL2 and CCL4 also were induced by Aβ1-42 but not Aβ1-40 (Fig. 3B and C).

3.3. Aβ induces CCL2 and CCL7 mRNA via the Erk and PI3-kinase signal cascade

Next, we examined the signal cascades for Aβ-induced CCL7 mRNA expression by using several chemical inhibitors (Fig. 4A). Wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, clearly inhibited the CCL7 mRNA expression induced by Aβ. Both U0126 and PD98059, MEK inhibitors, also inhibited the increase in CCL7 mRNA expression.

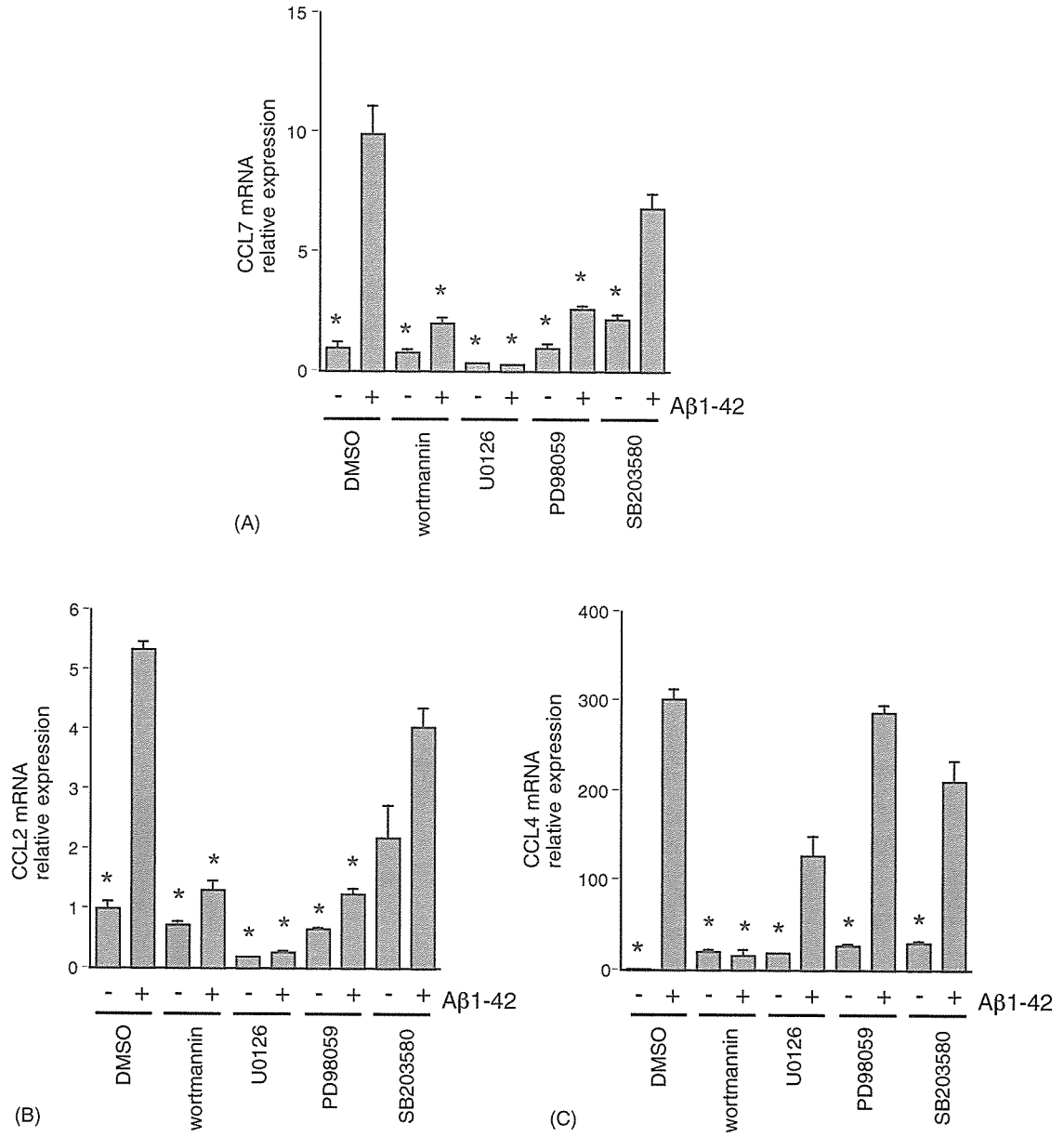

Fig. 4. Signal transduction for chemokine mRNA expression induced by Aβ1-42. Ra2 cells were preincubated with 200 nM wortmannin, 10 μM U0126, 10 μM PD98059 or 10 μM SB203580 for 30 min before the addition of 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 6 h. Extracted mRNA was quantified by real-time PCR. The results indicated relative expression of CCL7 (A), CCL2 (B) and CCL4 (C) in Aβ-treated cells compared with control cells. The data represent the means ± S.D. of triplicate of three separate experiments. $^{*}p < 0.001$ vs. Aβ-treated cells (DMSO).

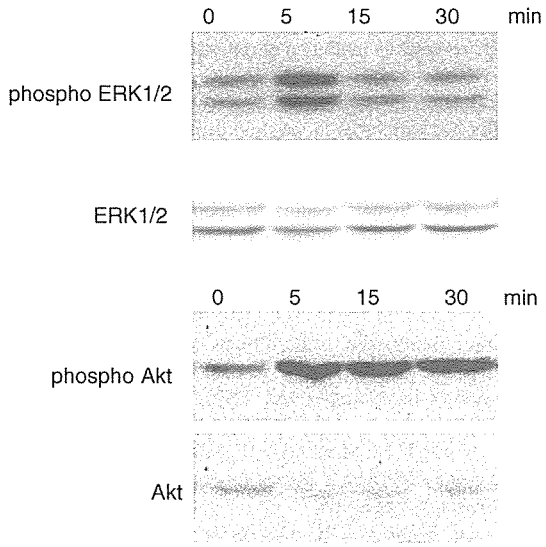

Fig. 5. Aβ1-42 induces the phosphorylation of ERK and Akt. Ra2 cells were treated with 10 μM Aβ1-42 for indicated times. Cell lysates were analyzed by immunoblotting using anti-phospho ERKl/2 or anti-phospho Akt antibody. The same blots were reprobed with anti-ERK or anti-Akt antibody.

However, SB203580, a p38 inhibitor, did not inhibit the CCL7 mRNA expression. These results indicate that Aβ induces increase in CCL7 mRNA via both the PI3K/Akt and ERK signaling pathways. For comparison, we also examined the signaling pathway of CCL2 and CCL4. The signaling pathway for the CCL2 mRNA was similar to that for CCL7, namely both the PI3K/Akt inhibitor and the MEK inhibitors blocked the increase in CCL2 mRNA expression (Fig. 4B). However, the CCL4 expression was clearly blocked by the PI3K/Akt inhibitor but not blocked by the MEK inhibitors (Fig. 4C). Immunoblotting analysis revealed that the phosphorylation of the ERK was induced by Aβ1-42 at a peak of 5 min (Fig. 5). In addition, we confirmed that Akt was phosphorylated at serine 473 by Aβ1-42.

4. Discussion

Tissue resident macrophage is activated by various stimuli such as infection, and secretes a range of cytokines and chemokines. Chemokines recruit leukocytes (macrophage and granulocyte) to sites of the tissue injury. In the case of AD, initial injurious stimulus is Aβ. Microglia as one of the macrophage-lineage cells might produce inflammatory chemokine induced by Aβ. The data presented in this paper demonstrate that the CCL2, CCL3, CCL4, CCL7 and CXCL2 mRNA expressions are induced by Aβ1-42 in microglia. Among these chemokines, the induction of CCL7 mRNA expression was the highest. MCP-1 (CCL2) and MIP-1β (CCL4) have been shown to be expressed in THP-1 monocyte stimulated by Aβ (Giri et al., 2003). However, it has not been reported that the induction of CCL7 mRNA by Aβ stimulation. In our results, Aβ1-42 but not Aβ1-40 induced the expression of CCLs. Because Aβ1-42 is easy to aggregate but Aβ1-40 tends to remain monomer, the difference of the induction of CCL mRNA expression may depend on the aggregation status of these peptides.

We have herein shown that the Aβ-induced CCL7 and CCL2 mRNA expressions correlated with the activation of the ERK and PI3K/Akt signaling cascades in the microglia. Several groups have examined that Aβ activated ERK pathway in microglia and THP-1 monocytes (McDonald et al., 1998; Combs et al., 1999). Giri et al. showed that Aβ induced the activation of MCP-1 (CCL2) via ERK pathway in THP-1 cells (Giri et al., 2003). Their results partially correlate with ours. We further showed that the PI3K/Akt pathway is also involved in CCL2 expression induced by Aβ. In our result, the induction of CCL7 and CCL2 mRNA need ERK and PI3K/Akt, but that of CCL4 correlated with only PI3K. A common transcriptional mechanism may therefore participate in CCL7 and CCL2 mRNA expression.

Although the function of chemokine in AD pathogenesis has not been clarified, the chemokine expression in AD patients has recently been studied. A Dutch-Italian Alzheimer Research group demonstrated the expressions of interferon-γ-inducible protein-10 (IP-10), CCL2, IL8 in CSF and serum of AD to be up-regulated in comparison to the control (Galimberti et al., 2003). A Swedish group reported an increase of CCL2 in the CSF and serum of AD patients (Sun et al., 2003). These studies suggest the importance of chemokine in AD pathogenesis. Our *in vitro* work will give some suggestions to the clinical studies of AD pathogenesis.

In conclusion, we have demonstrated that Aβ induces several chemokines (CCL2, CCL3, CCL4, CCL7 and CXCL2) in microglia. This study to our knowledge is the first to fully demonstrate the expression pattern of macrophage-lineage chemokine in microglia induced by Aβ. These chemokines may have important function in the AD pathogenesis. The signaling pathways from Aβ to chemokine mRNA expression should help us to develop therapeutic methods of AD.

Acknowledgements

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Role of cytokines in inflammatory process in Parkinson's disease

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Summary. We investigated whether the cytokines produced in activated microglia in the substantia nigra (SN) and putamen in sporadic Parkinson's disease (PD) are neuroprotective or neurotoxic. In autopsy brains of PD, the number of MHC class II (CR3/43)-positive activated microglia, which were also ICAM-1 (CD 54)-, LFA-1 (CD 11a)-, TNF-alpha-, and IL-6-positive, increased in the SN and putamen during progress of PD. At the early stage activated microglia were mainly associated with tyrosine hydroxylase (TH)-positive neurites in the putamen, and at the advanced stage with damaged TH-positive neurons in the SN. The activated microglia in PD were observed not only in the nigro-striatal region, but also in various brain regions such as the hippocampus and cerebral cortex. We examined the distribution of activated microglia and the expression of cytokines and neurotrophins in the hippocampus of PD and Lewy body disease (LBD). The levels of IL-6 and TNF-alpha mRNAs increased both in PD and LBD, but those of BDNF mRNA and protein drastically decreased specifically in LBD, in which neuronal loss was observed not only in the nigro-striatum but also in the hippocampus. The results suggest activated microglia in the hippocampus to be probably neuroprotective in PD, but those to be neurotoxic in LBD. As an evidence supporting this hypothesis, two subsets of microglia were isolated from mouse brain by cell sorting: one subset with high production of reactive oxygen species (ROS) and the other with no production of ROS. When co-cultured with neuronal cells, one microglia clone with high ROS production was neurotoxic, but another clone with no ROS production neuroprotective. On the other hand, Sawada with coworkers found that a neuroprotective microglial clone in a culture experiment converted to a toxic microglial clone by transduction of the HIV-1 Nef protein with increasing NADPH oxidase activity. Taken together, all these results suggest that activated microglia may change in vivo from neuroprotective to neurotoxic subtsets as degeneration of dopamine neurons in the SN progresses in PD. We conclude that the cytokines from activated microglia in the SN and putamen may be initially neuroprotective, but may later become neurotoxic during the progress of PD.

Toxic change of activated microglia may also occur in Alzheimer's disease and other neurodegenerative diseases in which inflammatory process is found.

Introduction

Parkinson's disease (PD) is characterized by specific degeneration of the dopamine

neurons in the substantia nigra (SN) pars compacta and the resulting loss of the nerve terminals in the striatum (the putamen and caudate nucleus), which is accompanied by a deficiency in the neurotransmitter dopamine in the striatum. This dopamine deficiency is responsible for most of the movement disorders called parkinsonism, i.e., muscle rigidity, akinesia, and resting tremor. The causative genes and their chromosomal locations of Familial PD (PARK) have been identified; PARK 1 (alpha-synuclein), PPRK 2 (parkin), PARK 5 (UCH-L1), PARK 6 (PINK 1), PARK 7 (DJ- 1), and PARK 8 (LRRK2) (Mizuno, 2005). However, most PD is sporadic without hereditary history. The pathogenesis of sporadic PD is still enigmatic (Foley and Riederer, 1999), but reactive free radicals produced by oxidative stress are speculated to play an important role (Youdim and Riederer, 1997).

We (Mogi and Nagatsu, 1999; Nagatsu et al., 1999, 2000; Nagatsu, 2002) previously reported by enzyme-linked immunosorbent assay (ELISA) increased levels of pro-inflammatory cytokines, decreased levels of neurotrophins, and changes in the levels of apoptosis-related factors in the nigro-striatal region of postmortem brain and/or ventricular or spinal cerebrospinal fluid in Parkinson's disease (PD) or in animal models of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or by 6-hydroxydopamine. Other workers (Hartmann et al., 2000; Hirsch et al., 1998, 1999, 2002) also reported changes in pro-inflammatory cytokines and their receptors, and apoptosis-related factors in the nigro-strialtal regions in PD, suggesting the presence of inflammatory process called neuroinflammation in parkinsonian brain. These changes in pro-inflammatory cytokines, neurotrophins and apoptosis-related factors in PD suggest apoptotic death of the nigro-striatal dopamine neurons in PD (Jellinger, 2000; Nagatsu et al., 1999, 2000; Hartmann et al., 2000; Hirsch et al., 1998, 1999, 2002).

Cytokines, neurotrophins, reactive oxygen species (ROS), and reactive nitrogen species (RNS) may be most probably produced by activated microglia in the brain with neuroinflammation. Although the causative relation with neuroinflammation is not clear, the presence of alpha-synuclein-positive intracellular inclusions, called Lewy bodies, in dopamine neurons in the substantia nigra is another feature of sporadic PD. In Lewy body disease (LBD) (Kosaka, 2000), also called dementia with Lewy bodies (DLB), both parkinsonian movement disorder and dimentia are observed, and Lewy bodies are widely distributed not only in the nigrostriatum but also in the cerebral cortex and hippocampus.

In the brain from patients with PD an increased number of major histocompatibility complex (MHC) class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive activated microglia were first reported by McGeer et al. (McGeer et al., 1998; McGeer and McGeer, 1995), which suggests inflammatory process to occur in the brain in PD patients and the origin of cytokines most probably to be activated microglia.

We (Imamura et al., 2003) first examined whether activated microglia in the brain in PD produce pro-inflammatory cytokines. Pro-inflammatory cytokines such as TNF-alpha and IL-6 are pleiotropic and can be either neuroprotective and neurotoxic. Therefore, we further aimed at asking the question whether increasing levels of cytokines and the presence of activated microglia in the brain in PD is neuroprotective rescuing dopamine neurons or neurotoxic causing dopamine cell loss.

Increasing levels of cytokines are produced from activated microglia in the putamen in PD

We (Imamura et al., 2003) identified by Western blot analysis TNF-alpha protein as a 21-kDa band, IL-6 protein as a 17-kDa

band, and MHC-II (CR3/43) protein as 34- and 28-kDa bands in homogenates of the putamen and peripheral blood mononuclear cells from PD patients, in agreement with our previous results by ELISA (Mogi and Nagatsu, 2000; Nagatsu et al., 1999). We then showed by immunohistochemistry that almost all activated microglia in the putamen from PD brains are positive for both ICAM-1 and LFA-1. We further proved by double immunofluorostaining the coexistence of TNF-alpha and IL-6 with MHC class II (CR3/43) in ICAM-1- and LFA-1-positive activated microglia in the putamen from PD patients. These results confirm that TNF-alpha and IL-6 are produced by activated microglia in the putamen in PD (Imamura et al., 2003).

Activated microglia are observed not only in the nigro-striatal region but also in various regions of the brain in PD

The presence of activated microglia and the absence of reactive astrocytosis in the substantia nigra of patients with PD suggest microglial involvement in the pathological process of dopamine neurons (McGeer et al., 1988; McGeer and McGeer, 1995; Mirza et al., 2000). We (Imamura et al., 2003) showed MHC class II (CR3/43)-positive activated microglia to be widely distributed not only in the substantia nigra and putamen, but also in various brain regions of PD patients, frequently in association with alpha-synuclein-positive Lewy neurites and monoaminergic neurons. In normal brains, many Ki-M1p-positive resting microglia, but only few MHC class II (CR3/43)-positive activated microglia were seen in the substantia nigra and putamen. In PD brains, however, MHC class II (CR3/43)-positive ramified microglia were seen in those regions. PD patients were shown to have a significantly higher number of MHC class II (CR3/43)-positive microglia compared with normal controls. The cell count of MHC class II (CR3/43)-positive microglia in PD increased as the neurodegeneration of pigmented cells in the substantia nigra advanced. Moreover, a significantly higher number of MHC class II (CR3/43)-positive microglia were also observed in the hippocampus (HC), transentorhinal cortex (TC), cingulate cortex (CC) and temporal cortex (TC) in PD compared with normal controls. In the early stages in PD, MHC class II (CR3/43)-positive microglia in the putamen were associated with intensively tyrosine hydroxylase (TH)-positive dopamine neurites without degeneration. In the advanced stage in PD, MHC class II (CR3/43)-positive microglia in the substantia nigra were associated with damaged TH-positive dopamine neurons and neurites. MHC class II (CR3/43)-positive microglia were also associated with non-degenerated serotonin (WH-3)-positive nerve terminals without degeneration in the substantia nigra. In the cingulate cortex in PD, activated microglia were frequently associated with alpha-synuclein-positive Lewy neurites. These immunohistochemical observations on PD brains suggest that activated microglia may act for either neuroprotection or neurotoxicity depending upon the brain regions and the stage of disease. We speculate that there may be neuroprotective and neurotoxic subtypes of microglia producing different kinds and different amounts of cytokines, neurotrophins, reactive oxygen species (ROS), and reactive nitrogen species (RNS), and that activated microglia in the nigro-striatal region in PD may be non-toxic subtype acting for neuroprotection at least in the early stage but may change to neurotoxic subtype causing neurodegeneration during the progression of the disease.

Our immunohistochemical results suggesting the neuroprotective or neurotoxic dual roles of activated microglia associated with healthy or damaged neurons and neurites in various brain regions in PD are schematically summarized in Fig. 1.

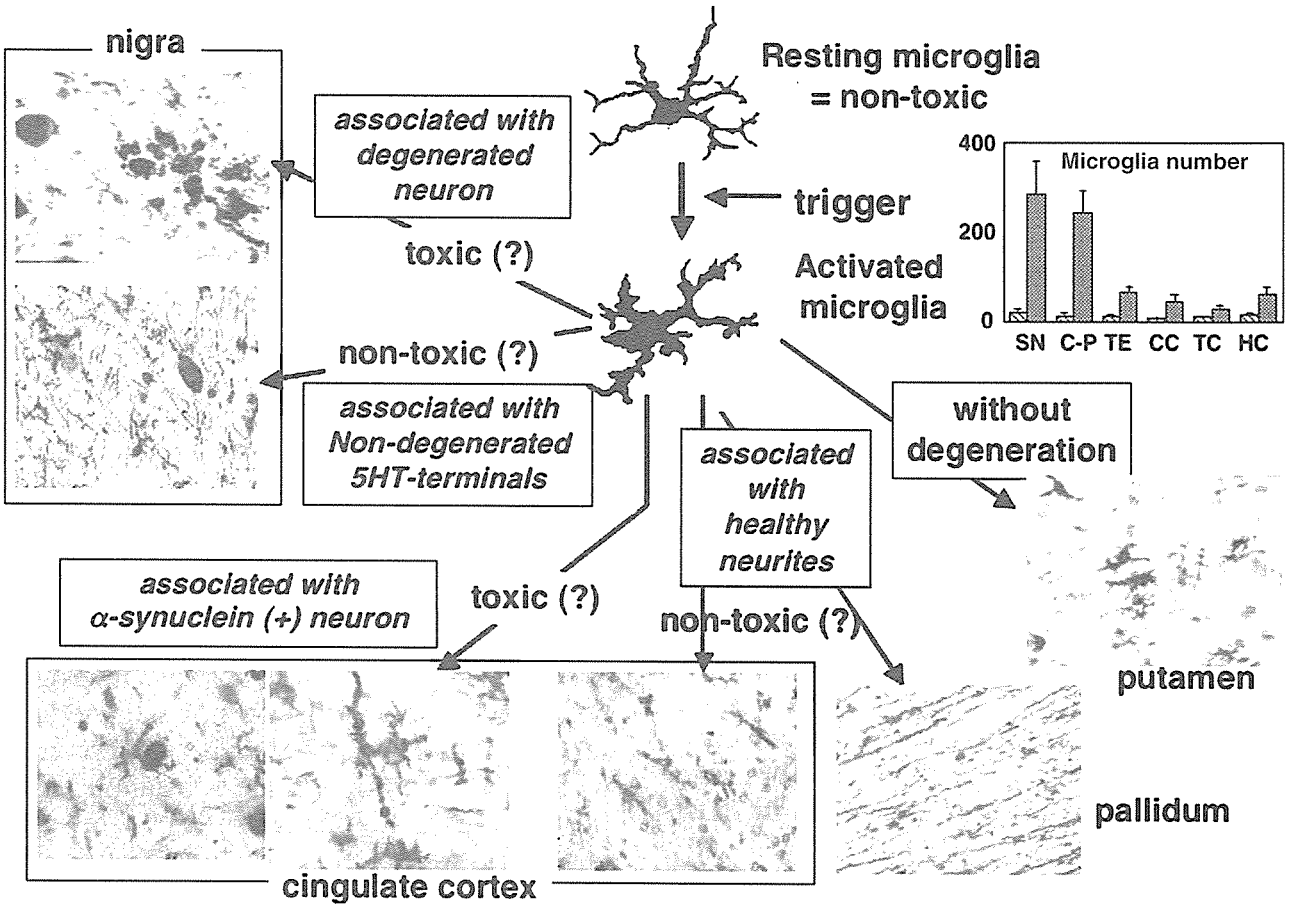

Fig. 1. Schematic diagram showing the dual potential roles of microglia in PD. In parkinsonian brains, activated microglia are observed not only in the substantia nigra (SN) and caudate-putamen (C–P) but also in other brain regions such as pallidum and cingulate cortex. Activated microglia associated with neurons or neurites without degeneration may be non-toxic and act for neuroprotection, whereas activated microglia associated with degenerated neurons and neurites may be neurotoxic and promote neurodegeneration. *TE* transenthorhinal cortex; *CC* cingulate cortex; *TC* temporal cortex; *HC* hippocampus

Expression of cytokines in the hippocampus and putamen in PD is different from that in LBD

Activated microglia have multiple roles. First, MHC II-positive activated microglia act for antigen presentation. Second, activated microglia phagocytose damaged cells. Third, activated microglia produce neurotoxic substances such as pro-inflammatory cytokines that are pleiotropic and either neurotoxic or neuroprotective like TNF-alpha, IL-1beta and IL-6, superoxide anions (ROS), nitric oxide (RNS), and glutamate. Fourth, activated microglia also produce neurotrophic substances such neurotrophins as BDNF, IL-6 and TNF-alpha that can also act for neuroprotection. As described above, we (Imamura et al., 2003) observed activated microglia in PD brain not only in the nigro-striatum but also in the hippocampus. We (Imamura et al., 2003, 2005) also observed in LBD activated microglia in the nigro-striatum and hippocampus. Neuronal degeneration in the putamen was observed in both PD and LBD, whereas neuronal loss in the hippocampus was observed in LBD, but not in PD without dementia. In order to examine whether activated microglia in the putamen and hippocampus in PD and LBD are neuro-

toxic or neuroprotective, we (Imamura et al., 2005) compared the expression of cytokines and neurotrophins in the hippocampus and putamen in postmortem brains in PD, LBD/DLB, and normal controls.

In normal controls, neuronal loss and activated microglia were not observed in the hippocampus CA 2/3 region, and neurons were strongly BDNF-positive. Immunohistochemical examination of the hippocampus CA 2/3 region in PD showed that the number of MHC II (R3/43)-positive microglia increased, which were also ICAM-1 (CD54)-, LFA-1 (CD11a)-, TNF-alpha-, and IL-6-positive. Alpha-synuclein-positive cells were also observed. BDNF-positive neurons were only slightly decreased in PD as compared with normal controls. In the hippocampus in LBD, the number of MHC-II (CR3/43)-positive microglia and alpha-synuclein-positive cells increased more than those in PD. Furthermore, in LBD all neurons were very weakly stained by anti-BDNF. These immunohistochemical data on the hippocampus CA 2/3 indicate that activated microglia increase both in PD and LBD, but that the content of neurotrophic BDNF drastically decreases specifically in LBD.

Expression of mRNAs of cytokines and neurotrophins was examined by RT-PCR in the hippocampus and putamen in normal controls, PD, and LBD. In the hippocampus, mRNA levels of IL-6 and TNF-alpha increased in both PD and LBD, but mRNA levels of BDNF greatly decreased in LBD, as compared with those of normal controls and PD. In the putamen, mRNA levels of IL-6 increased in both PD and LBD. In contrast, mRNA levels of BDNF increased in PD, but decreased in LBD. We (Mogi and Nagatsu, 2002) previously reported by ELISA that the content of BDNF protein decreased in the striatum in PD. Therefore, increased level of BDNF mRNA in PD is speculated to be a compensatory change probably by activated microglia. These different changes in mRNA levels of IL-6 and BDNF in PD and LBD suggest that activated microglia in the hippocampus and putamen in PD and LBD may be different in properties and may secrete different kinds and different amounts of cytokines and neurotrophins such as IL-6 and BDNF. We speculate that activated microglia in the hippocampus may be neuroprotective in PD and neuroltoxic in LBD (Imamura et al., 2005).

Two subsets of microglia and two clones of microglia with neurotoxic and neuroprotective properties are isolated in terms of intracellular ROS production induced by phorbol myristate acetate (PMA) stimulation

We separated two subsets of microglia from mouse brain by cell sorting based on profiles of intracellular ROS production induced by PMA. One subset of microglia produced greater amounts of ROS than another subset of microglia. The results suggest that there are at least two subsets of microglia in mouse brain; one active subset and another inactive subset in production of ROS upon stimulation by PMA. In supporting this hypothesis, two cell lines of microglia, Ra2 cells and 6-3 cells, were generated by spontaneous immortalization of primary mouse microglia. Both clones were dependent on granulocyte macrophage colony-stimulating factor (GM-CSF). The GM-CSF-dependent Ra2 microglia did not produce ROS by PMA stimulation. In contrast, the GM-CSF-dependent 6-3 microglia showed increasing ROS production upon stimulation by PMA. N18 neuronal cells were sensitive to oxidative stress by hydrogen peroxide, and showed dose-dependent apoptotic cell death by the addition of hydrogen peroxide. N18 cells cultured in the presence of 50 mM hydrogen peroxide died almost completely by apoptosis. When the N18 neuronal cells were co-cultured with macrophage RAW264.7 cells or 6-3 microglia cells and stimulated with PMA, cell viability of the neuronal cells decreased as determined by

cell viability assay (WST assay, PI dye exclusion assay, and TUNEL staining). On the contrary cell viability of the neuronal N18 cells increased by co-culture with Ra2 microglia. These results show 6-3 microglia to be neurotoxic and Ra2 microglia to be neuroprotective when these cells are co-cultured with neuronal cells, supporting our concept that there may exist neurotoxic and neuroprotective subsets of microglia in the brain.

In agreement with our hypothesis, Hirsch et al. (1998) also proposed separate populations of microglia; one subpopulation of glial cells may play a neuroprotecrive role by metabolizing dopamine and scavenging oxygen free radicals and another that may be deleterious to dopamine neurons by producing NO and toxic proinflammatory cytokins.

Lentiviral transduction of neuroprotective microglia with HIV-1 Nef protein induces toxic change

AIDS patients frequently develop an human immunodeficiency virus type 1 (HIV-1)-associated abnormalities in cognition and parkinsonian motor dysfunction. HIV-infected macrophages were observed in the striatum, and dopamine concentrations were significantly reduced in the striatum (Sarder et al., 1996).

Nef is the first viral protein detectable after human HIV-1 infection, enhances virus production and infectivity, and exerts pathologic effects independently of viral replication. Microglia are phagocytes of myeloid origin and the principal target of HIV infection in the brain. Microglia produce superoxide, and express all components of the superoxide generating phagocyte NADPH oxidase (Vilhardt et al., 2002). We transduced Nef protein using lenti virus vector into nontoxic Ra2 microglia. Both Ra2 and nefRa2 microglia were similar in GM-CSF dependency. Ra2 microglia did not produce ROS by stimulation with PMA. In contrast, nefRa2 robustly produced ROS owing to activation of NADPH oxidase. When N18 neuronal cells were co-cultured with Ra2 or nefRa2 microglia, Ra2 microglia were shown to be neuroprotective, but nefRa2 neurotoxic, indicating toxic change of Ra2 microglia by transduction of Nef protein. Addition of superoxide diamutase (SOD) partially recovered the neurotoxicity of nefRa2 to change the glial cell line to be neuroprotective. These results suggest that toxic change in nefRa2 microglia may be partially due to increased ROS production by increased NADPH oxidase. Another possibility of toxic change is increased production of myeloperoxidase (MPO).

The toxic change of reactive microglia suggests two step activation of microglia in PD

Based on these in vitro results suggesting the presence of neuroprotective or neurotoxic subsets of activated microglia, we propose a hypothesis of two-step activation of microglia in the brain in PD in vivo, as schematically shown in Fig. 2. Ramified resting microglia in the normal brain support neurons for control of neuronal activity, development, and homeostasis in the brain. The observation on activated microglia associated with intensely tyrosine hydroxylase (TH)-positive neurites in the striatum in the early stage of PD and with other non-degenerated neurons and neurites in various brain regions suggest that microglia activated by the first stimuli may act for neuroprotection by producing neurotrophins, neurotrophic cytokines, and antioxidant at the first step. The activated microglia at this first step may be neuroprotective. As described above, Sawada with coworkers found that microglia in a cell culture experiment are converted from the neuroprotective to neurotoxic forms upon expression of the HIV-1 Nef protein (Vilhardt et al., 2002). Similar toxic change of activated microglia may occur in PD brain as the second step by other factors such as in-

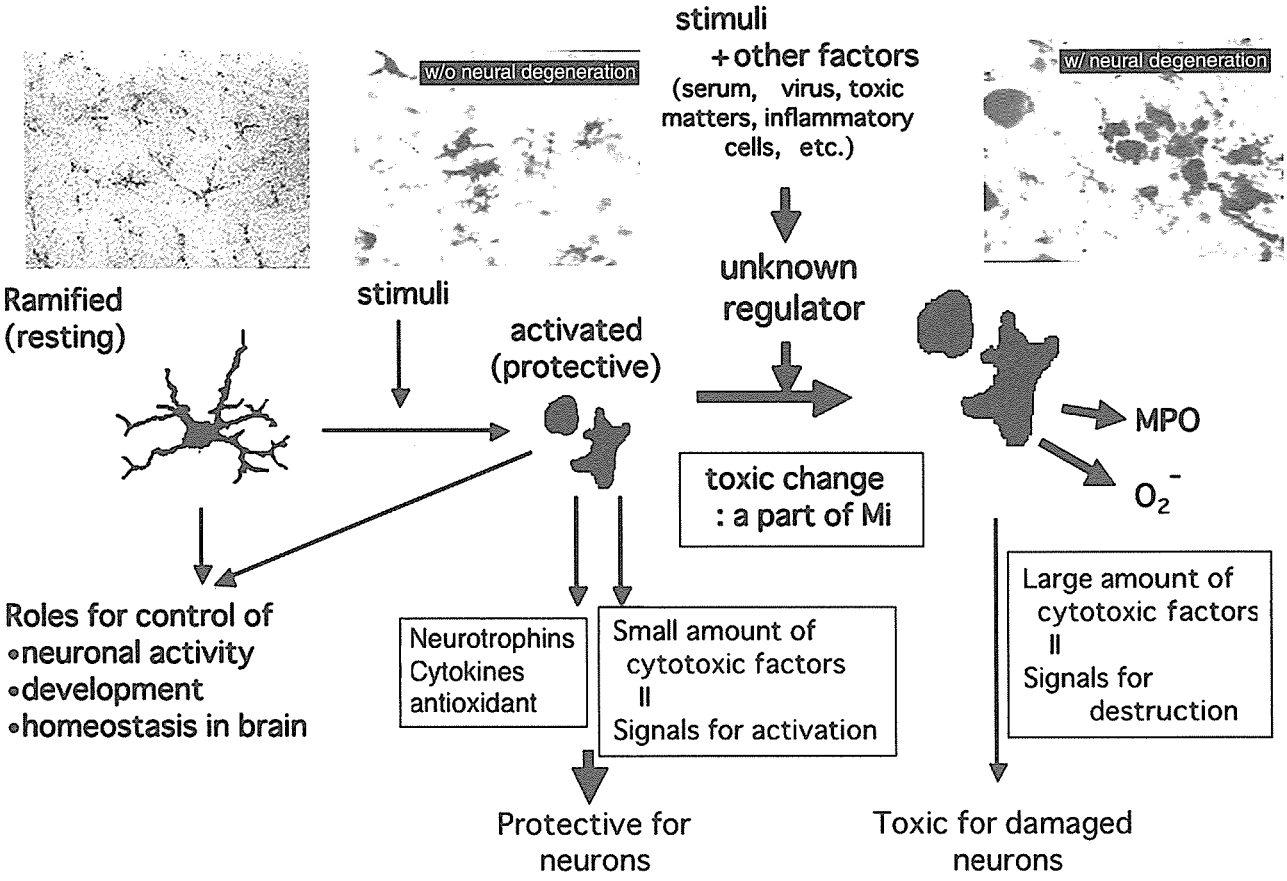

Fig. 2. Schematic diagram showing a hypothesis of two-step activation of microglia. We isolated neuroprotective and neurotoxic subsets of microglia, and also neuroprotective and neurotoxic clones from mouse brain. In addition, Sawada and coworkers (Vilhardt et al., 2002) found in a cell culture experiment that a neuroprotective microglial clone converted from the protective to toxic cells upon transduction of the HIV-1 Nef protein with activation of NADPH oxidase. Based on these results, we propose a hypothesis of two step activation of microglia. Activated microglia by the first stimuli may initially act for neuroprotection by producing protective neurotrophins, cytokines, and antioxidants, but by the second stimuli and unknown regulators may change to be neurotoxic by producing ROS and MPO. This toxic change of activated microglia may promote the progress of PD

vasion of serum, viruses, toxic matters, or inflammatory cells in a part of neuroprotective microglia in a specific brain regions such as the nigro-striatum in PD. As the results of toxic change of activated microglia, large amounts of cytotoxic factors such as ROS and RNS produced by NADPH oxidase or MPO may promote neuronal loss.

Conclusion and future prospects

Oxidative stress is thought to play a key role in sporadic PD (Youdim and Riederer, 1997). Presence of neuroinflammation and oxidative stress may have a causative link in PD. Oxidative stress may trigger microglia activation and neuroinflammation (Hald and Lutharius, 2005).

In the brain from patients with PD, activated microglia are observed not only in the nigro-striatum where cell loss of dopamine neurons occurs, but also in various brain regions such as the hippocampus. The activation of microglia may occur in tow steps. At the first step, the activated microglia produced by unknown stimuli may act for neuro-

protection at least in the early stages of PD. At the second stage by other unknown factors, neuroprotective microglia may be subjected to toxic change that convert microglia from neuroprotective to neurotoxic type to promote the progression of neurodegeneration.

There remain several points to confirm this hypothesis on the role of activated microglia and cytokines in PD. First, the presence of neuroprotective and neurotoxic microglia in the human brain should be confirmed. Second, in vivo evidences of toxic change of microglia are required in some experimental models of PD. Third, the stimuli to activate microglia at the first stage must be identified. Since the causative factors of sporadic PD are speculated to be multiple, the stimuli may also be multiple. Fourth, the factors and unknown regulators for the toxic change of activated microglia must be identified.

The present hypothesis is expected to be useful for developing drugs against PD. Anti-inflammatory drugs have been considered for the treatment of PD. However, such anti-inflammatory drugs should inhibit the toxic change of microglia or act only to toxic subtype of microglia.

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