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 ($H\text{-}2^b$) 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

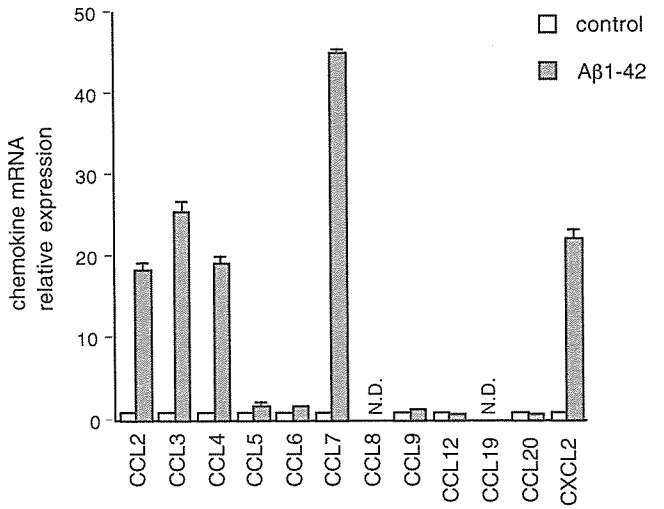

Fig. 1. Aβ1-42 induced the expression of chemokine. Ra2 cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Real-time PCR of a series of chemokine and β-actin mRNA were performed. The chemokine mRNA expression was normalized to β-actin. The results indicated relative expression 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.

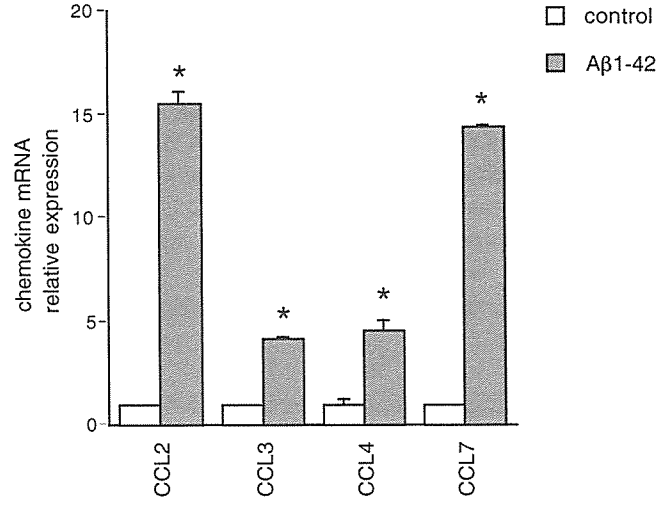

Fig. 2. Induction of the chemokine mRNA expression by Aβ1-42 in primary microglia. Primary cultured microglial cells were treated with 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 16 h. Extracted mRNA was quantified by real-time PCR. The results indicated relative expression of CCL2, CCL3, CCL4 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.

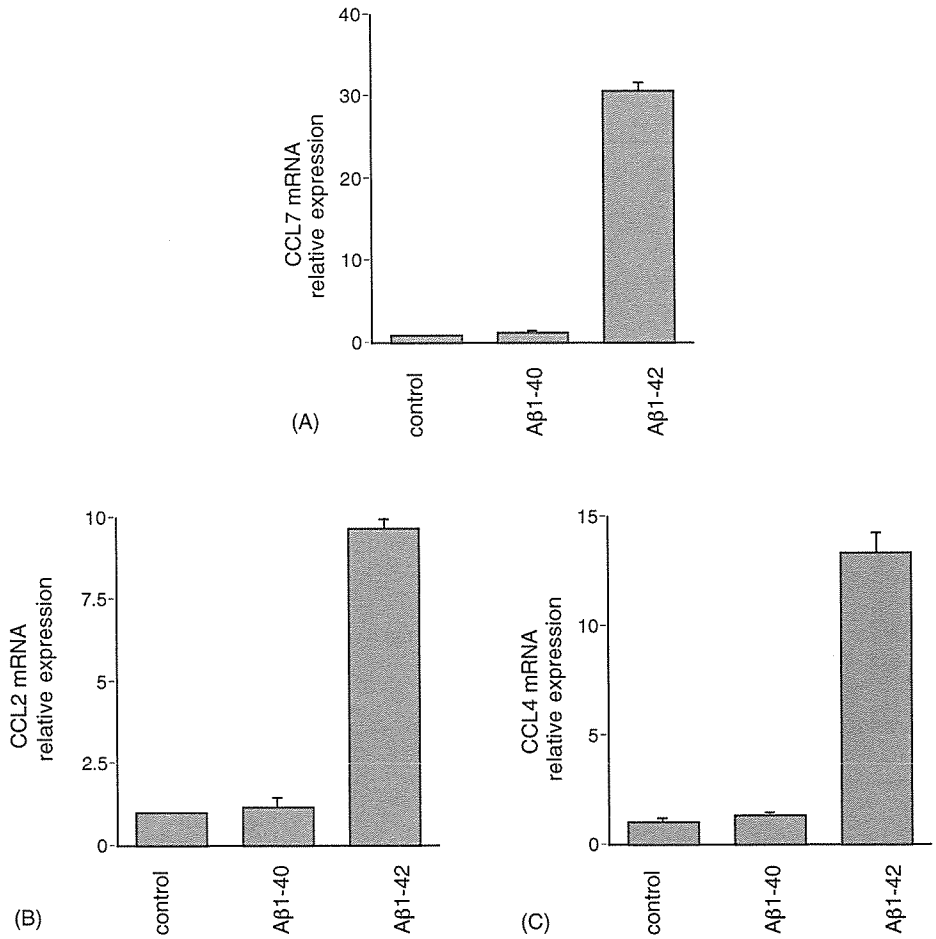

Fig. 3. The mRNA expression of chemokine induced with Aβ1-42 or Aβ1-40. Ra2 cells were stimulated 10 μM Aβ1-42 and 10 μM Aβ1-40 for 16 h. Extracted mRNA was quantified by real-time PCR. The results indicated relative expression of CCL7 (A), CCL2 (B), CCL4 (C) in Aβ-treated cells compared with control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

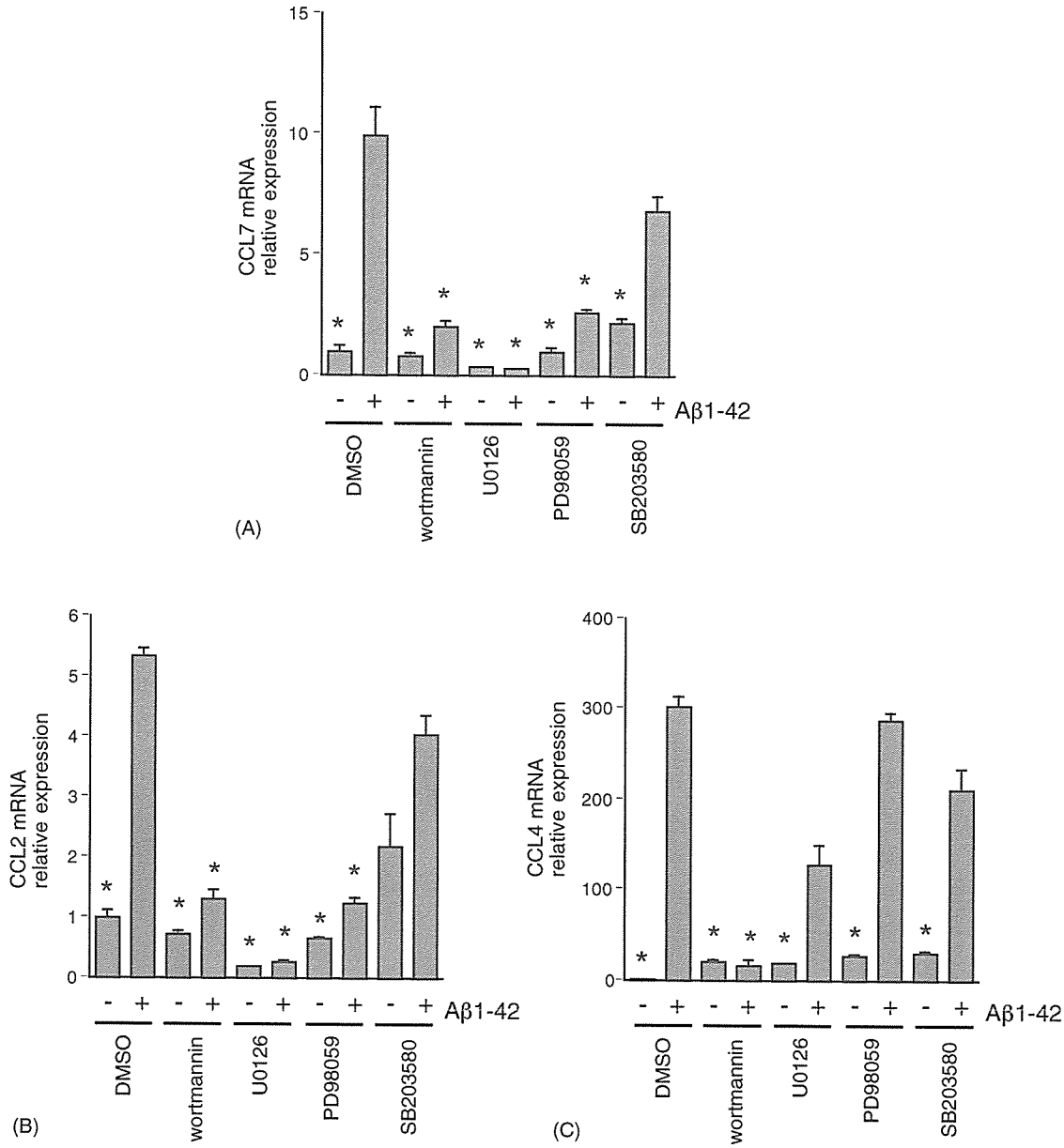

Fig. 4. Signal transduction for chemokine mRNA expression induced by Aβ1-42. Ra2 cells were preincubated with 200 nM wortmannin, 10 μM U0126, 10 μM PD98059 or 10 μM SB203580 for 30 min before the addition of 10 μM Aβ1-42 or 0.1% NH_3 solution as a control for 6 h. Extracted mRNA was quantified by real-time 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).

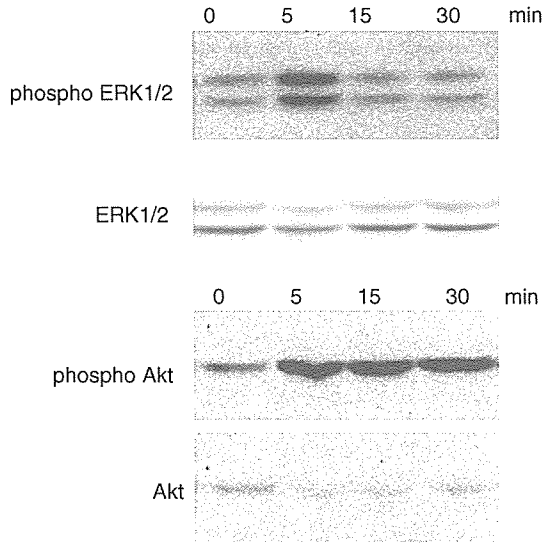

Fig. 5. Aβ1-42 induces the phosphorylation of ERK and Akt. Ra2 cells were treated with 10 μM Aβ1-42 for indicated times. Cell lysates were analyzed by immunoblotting using anti-phospho ERK1/2 or anti-phospho Akt antibody. The same blots were reprobed with anti-ERK or anti-Akt antibody.

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 nigrostriatum 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.

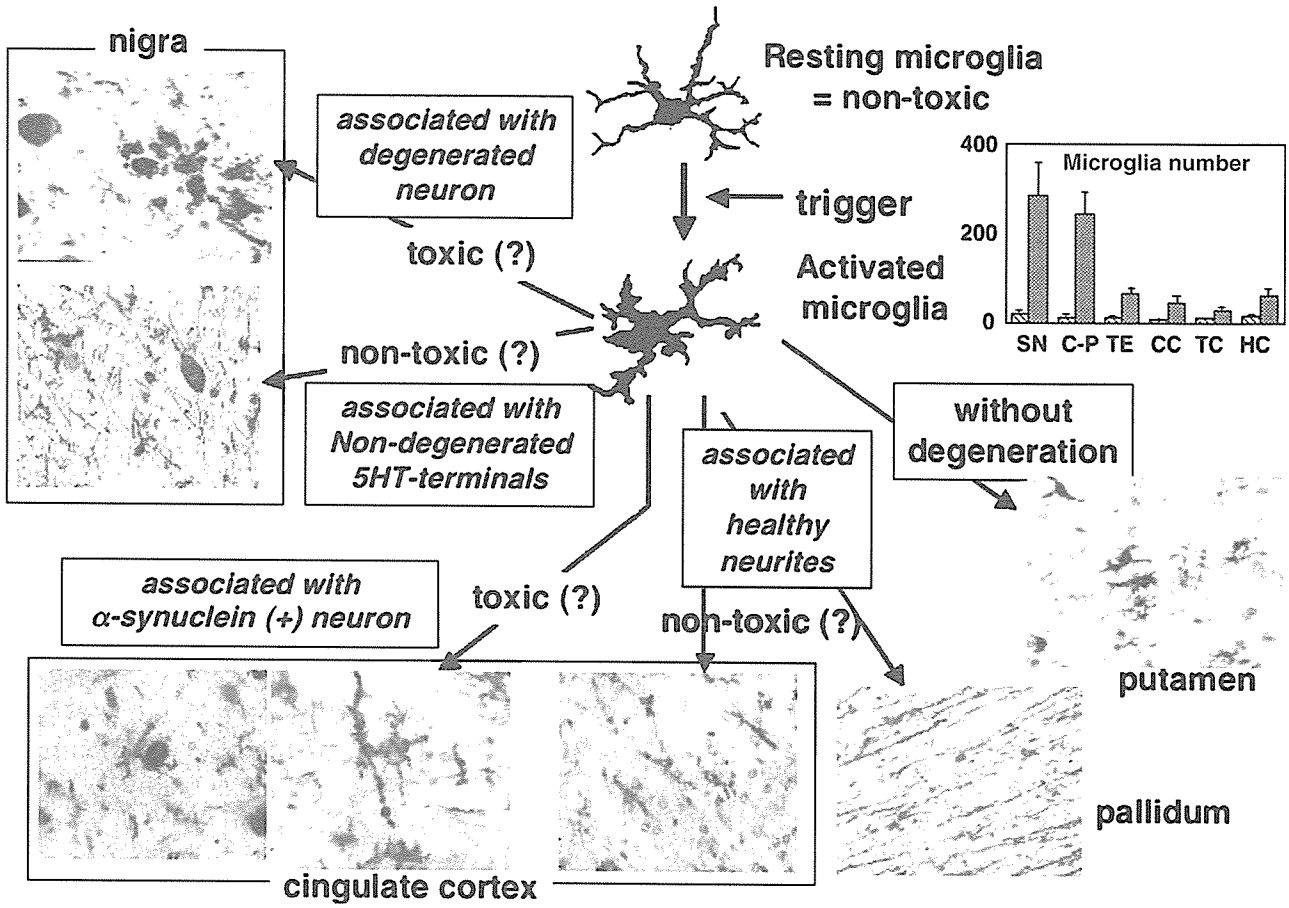

Fig. 1. Schematic diagram showing the dual potential roles of microglia in PD. In parkinsonian brains, activated microglia are observed not only in the substantia nigra (SN) and caudate-putamen (C–P) but also in other brain regions such as pallidum and cingulate cortex. Activated microglia associated 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-

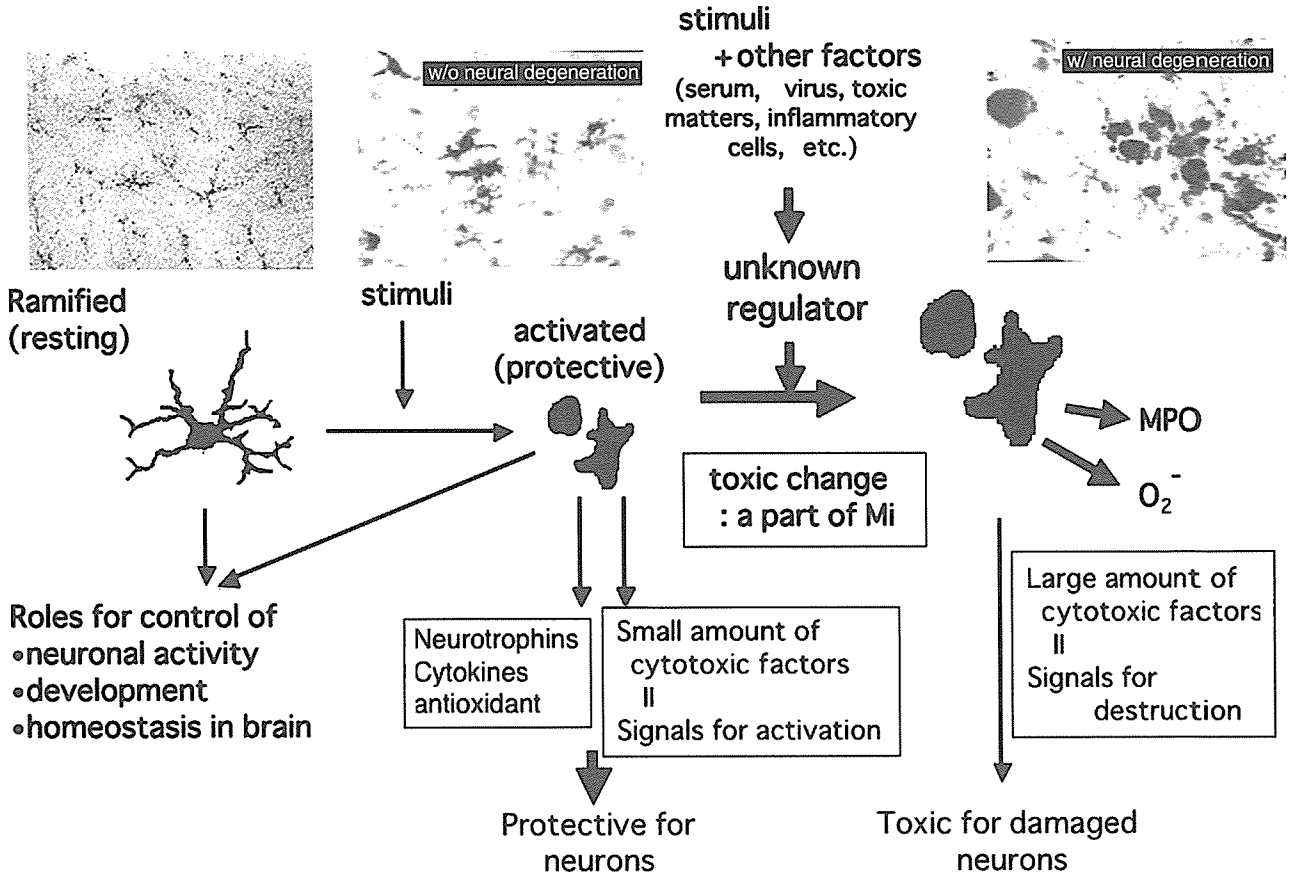

Fig. 2. Schematic diagram showing a hypothesis of two-step activation of microglia. We isolated neuroprotective and neurotoxic subsets of microglia, and also neuroprotective and neurotoxic clones from mouse brain. In addition, Sawada and coworkers (Vilhardt et al., 2002) found in a cell culture experiment 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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Molecular mechanism of the relation of monoamine oxidase B and its inhibitors to Parkinson's disease: possible implications of glial cells

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Summary Monoamine oxidases A and B (MAO A and MAO B) are the major enzymes that catalyze the oxidative deamination of monoamine neurotaransmitters such as dopamine (DA), noradrenaline, and serotonin in the central and peripheral nervous systems. MAO B is mainly localized in glial cells. MAO B also oxidizes the xenobiotic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to a parkinsonism-producing neurotoxin, 1-methyl-4-phenyl-pyridinium (MPP+). MAO B may be closely related to the pathogenesis of Parkinson's disease (PD), in which neuromelanin-containing DA neurons in the substantia nigra projecting to the striatum in the brain selectively degenerate. MAO B degrades the neurotransmitter DA that is deficient in the nigro-striatal region in PD, and forms H_2O_2 and toxic aldehyde metabolites of DA. H_2O_2 produces highly toxic reactive oxygen species (ROS) by Fenton reaction that is catalyzed by iron and neuromelanin. MAO B inhibitors such as L-(−)-deprenyl (selegiline) and rasagiline are effective for the treatment of PD. Concerning the mechanism of the clinical efficacy of MAO B inhibitors in PD, the inhibition of DA degradation (a symptomatic effect) and also the prevention of the formation of neurotoxic DA metabolites, i.e., ROS and dopamine derived aldehydes have been speculated. As another mechanism of clinical efficacy, MAO B inhibitors such as selegiline are speculated to have neuroprotective effects to prevent progress of PD. The possible mechanism of neuroprotection of MAO B inhibitors may be related not only to MAO B inhibition but also to induction and activation of multiple factors for anti-oxidative stress and anti-apoptosis: i.e., catalase, superoxide dismutase 1 and 2, thioredoxin, Bcl-2, the cellular poly(ADP-ribosyl)ation, and binding to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Furthermore, it should be noted that selegiline increases production of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrphic factor (GDNF), possibly from glial cells, to protect neurons from inflammatory process.

Abbreviations: *BDNF* brain-derived neurotrophic factor, *CSF* cerebrospinal fluid, *DA* dopamine, *GDNF* glial cell line-derived neurotrophic factor, *MAO A* monoamine oxidase A, *MAO B* monoamine oxidase B, *MPDP+* 1-methyl-4-phenyl-2,3-dihydro-pyridinium, *MPP+* 1-methyl-4-phenyl-piridinium, *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *NGF* nerve growth factor, *PD* Parkinson's disease, *ROS* reactive oxygen species, *TH* tyrosine hydroxylase

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Parkinson's disease (PD) is an aging-related movement disorder caused by a deficiency of the neurotransmitter dopamine (DA) in the striatum of the brain as a result of selective degeneration of the nigro-striatal DA neurons (A9 neurons). DA deficiency is due to decreased number of DA neurons in the substantia nigra, and the molecular activity (enzyme activity/enzyme protein) of tyrosine 3-monooxygenase (tyrosine hydroxylase, TH) in residual DA neurons increases resulting in compensation for DA deficiency (Mogi et al., 1988). Familial PD for which the causative genes have been identified constitutes a small percentage of PD, and most PD is sporadic (idiopathic) without hereditary history. The molecular mechanism of the DA cell death in sporadic PD is unknown, but monoamine oxidase (MAO), especially type B (MAO B), is speculated to play important roles. In the brain MAO B is mainly localized in glial cells. MAO B activity in the brain increases during aging probably due to increasing numbers of glial cells (Fowler et al., 1980), and aging is a high risk factor of PD. MAO B inhibitors, such as L-(−)-deprenyl (selegiline) and rasagiline, are proved to be clinically effective for the treatment of PD. In the present review, we will examine the molecular mechanism of PD in relation to the mechanism of probable neuroprotection by MAO B inhibitors, and to possible interrelationship between DA neurons and glial cells in the inflammatory process.

Monoamine oxidases A and B (MAO A and MAO B)

Monoamine oxidase (flavin-containing) [amine:oxygen oxidoreductase (deaminating) (flavin-containing); MAO; E.C. 1.4.3.4.] catalyzes the following reaction: $RCH_2NH_2 + H_2O + O_2 = RCHO + NH_3 + H_2O_2$. MAO acts on primary

amines, and also on some secondary and tertiary amines. The monoamine substrates for MAO include physiologically and pathologically important neurotransmitters and hormones, such as DA, noradrenaline, adrenaline, and serotonin, which are slow-acting neurotransmitters in the brain and function with rapidly-acting neurotransmitters, i.e., glutamic acid and gamma-aminobutyric acid (GABA), to regulate movement, emotion, reward, cognition, memory, and learning. Thus, MAO is closely related to higher brain functions by regulating the levels of monoamine neurotransmitters and also to the pathogenesis of PD (for reviews, see Nagatsu, 2004; Nicotora et al., 2004; Riederer and Youdim, 1990; Youdim and Riederer, 1997). In the brain, MAO is thought to be important together with catechol O-methyltransferase in regulating the level of DA. The DA level decreases specifically in the nigro-striatal region in PD, which is the characteristic biochemical change (for reviews, see Cookson, 2005; Hornykiewicz, 2001; Nagatsu, 1993).

MAO was first discovered as tyramine oxidase by Hare in 1928, since it catalyzed the oxidative deamination of tyramine. This enzyme was then found to oxidize various monoamines including catecholamines, i.e., DA, noradrenaline, and adrenaline, and serotonine, and was recognized as monoamine oxidase by Blashko, Zeller, Gorkin, and Quastel. The enzyme localizes in the outer membrane of mitochondria (Schneitman et al., 1967). MAO was purified from bovine liver (Gomes et al., 1969; Minamiura and Yasunobu, 1978) and bovine brain (Harada et al., 1971). The cofactor, flavin adenine dinucleotide (FAD), was identified in preparations of purified MAO (Harada and Nagatsu, 1969; Tipton, 1980). Purified MAO was discovered to contain FAD covalently bound as 8-a-cysteinyl-FAD (Walker et al., 1971).

Johnston (1968) pharmacologically discovered that the inhibitor clorgyline was able to distinguish two forms of MAO, i.e., MAO type A (MAO A) and MAO type B (MAO B). The presence of multiple forms of MAO in the human brain was also reported by Collins et al. (1970). The structures and functions of MAO A and MAO B have been elucidated by cDNA cloning, genomic DNA cloning, and genetic engineering (for review, see Shih, 2004; Shih et al., 1999).

Full-length cDNAs encoding human liver MAO A and MAO B and the genomic DNAs were cloned (Bach et al., 1988; Chen et al., 1991; Powell et al., 1991; Weyler et al., 1990). Human placental MAO A (Hsu et al., 1988), and rat liver MAO A and MAO B (Ito et al., 1988; Kwan and Abell, 1992) were also cloned and sequenced, and human and rat MAO A showed 86–88% identity. MAO B from human platelets and frontal cortex were found to have identical amino acid sequences, confirming that human MAO B is a single enzyme in various tissues (Chen et al., 1993).

Human MAO A and MAO B have subunit molecular weights of 59,700 and 58,000, respectively, consisting of 527 and 520 amino acids, respectively, and have a 70% amino acid sequence identity; and both sequences contain the pentapeptide Ser-Gly-Gly-Cys-Tyr, in which the obligatory cofactor FAD is covalently bound through a thio ether linkage to the cysteine (Bach et al., 1988; Chen et al., 1991). MAO is composed of two identical subunits (Minamiura and Yasunobu, 1978), and one FAD couples with each subunit of 60 kDa (Weyler, 1989). FAD is covalently linked to Cys-406 in MAO A and Cys-397 in MAO-B (Abell and Kwan, 2001; Edmondson et al., 2004).

The expression of functional enzymes by transfection of cells with cDNAs provides unequivocal evidence that the different catalytic activities of MAO A and MAO B reside in their primary amino acid sequences. Chimeric enzymes and site-directed mutagenesis studies contributed to elucidating the structure-function relationships of MAO A and MAO B. The enzymatic properties observed for the chimeric MAO enzymes suggest that the internal segment, but not the N- or C-terminal region, confers substrate and inhibitor specificities (Shih et al., 1998; Tsugeno and Ito, 1997; Tsugeno et al., 1995). The catalytic properties and specificity of MAO A were insensitive to substitution of both the NH_2- (up to position 112) and COOH-termini (from residue 395). The replacement of MAO A amino acids 161–375 by the corresponding region of MAO B converted MAO A catalytic properties to ones typical of MAO B; and the converted enzyme did not oxidize serotonin, a preferred substrate of MAO A, and was more sensitive to the MAO B-specific inhibitor, L-(−)-derenyl (selegiline), than to the MAO A-specific inhibitor clorgyline. These results demonstrated that amino acids 152–366 of MAO B contain a domain that confers substrate specificity and inhibitor selectivity on the enzyme (Chen and Shih, 1998; Cesura et al., 1998).

Because MAO A and MAO B are integrated proteins of the outer membrane of mitochondria, their crystallization has been difficult; and so their three-dimensional structure of human MAO B has been only recently elucidated (Binda et al., 2002a, b). Determination of the crystal structure of human MAO B allowed precise modeling of the structure of human MAO A, and preliminary models of human MAO A have been obtained by fold recognition and comparative modeling based on proteins sharing low sequence identity (Leonard et al., 2004). The 50-residue C-terminal tail of human MAO B forms an extended segment that traverses the protein surface and then folds into an alpha-helix, which protrudes from the basal face of the structure to anchor the

protein to the mitochondrial outer membrane (Binda et al., 2002a, b).

MAO A and *MAO B* genes are situated on the X chromosome, at Xp 11.23–22.1 (Chen et al., 1992; Kochersperger et al., 1986; Lan et al., 1989; Levy et al., 1989; Pintar et al., 1981). Both genes are closely located and are deleted in patients with Norrie's disease, a rare X-linked recessive neurological disorder characterized by blindness, hearing loss, and mental retardation. Human *MAO A* and *MAO B* genes consist of 15 exons and have an identical exon-intron organization. Exon 12 codes for the covalent FAD-binding site and is the most conserved exon, showing 93.9% amino acid identity between MAO A and MAO B (Chen et al., 1992; Grimsby et al., 1991).

The distribution of MAO in various tissues of various species has been investigated by use of specific inhibitors of MAO A and MAO B enzyme activities, immunohistochemistry, enzyme autoradiography, and in situ hybridization (for review, see Berry et al., 1994; Kitahama et al., 1994; Luque et al., 1998). MAO A and MAO B are distributed in various tissues including the brain of various species. Histochemical localization of MAO A and MAO B was examined in the rat brain (Willonghby et al., 1988). In the rat brain, MAO A was predominantly found in noradrenergic neurons; whereas MAO B was detected in serotonergic and histaminergic neurons and in glial cells (astrocytes) (Arai et al., 1997; Jahung et al., 1997; Levitt et al., 1982; Luque et al., 1995; Saura et al., 1994; Westlund et al., 1988a). However, DA neurons appear not to have MAO A or MAO B, in contrast to the fact that DA is a common substrate of both MAO A and MAO B activity (Arai et al., 1998; Hida et al., 1999). As another puzzling fact on the physiological role of MAO B, serotonin neurons contain only MAO B, but serotonin is a very poor substrate of MAO B (Arai et al., 1997; Levitt et al., 1982).

Most human tissues, including the brain, express both MAO A and MAO B (Konradi et al., 1988; Konradi et al., 1989; Westlund et al., 1988b). However, human placenta contains predominantly MAO A (Egashira and Yamanaka, 1981); and human platelets and lymphocytes express only MAO B (Bond and Cundall, 1997; Donnelly and Murphy, 1977). Thus platelet MAO B can be useful for estimation of brain MAO B (Oreland, 2004). mRNA transcripts of MAO A and MAO B were coexpressed in the same region in the adult human brain; and the relative concentrations of these transcripts were as follows in the decreasing order: frontal cortex, locus coeruleus, temporal cortex, posterior pensylvian cortex-supuramarginal gyri, anterior pensylvian cortex-opercular gyri, hippocampus and thalamus (Grimsby et al., 1990).

Cell-type specific expression of MAO A and MAO B were examined in cultured cells (Donnelly et al., 1976; Hawkins and Breakfield, 1978; Murphy et al., 1976; Nagatsu et al., 1981). The type of MAO activity did not vary through the stage of growth of mouse myoblast G8-1 cells, which contain mostly MAO A (95%) and a small amount of MAO B (5%) (Nagatsu et al., 1981). NG108-15 hybrid cells derived from mouse neuroblastoma × rat glioma showed both MAO A (65–90%) and MAO B (35–10%), and the total MAO A plus MAO B activity and the ratio of MAO B/MAO A activity increased as a function of time in culture. Prostaglandin E1 and theophylline, the best known combination of agents that increases the intracellular cyclic AMP content of NG-108-15 cells, caused similar increases in MAO and the MAO B/MAO A ratio in NG108-15 cells, suggesting that the activity and expression of MAO B are regulated in a cyclic AMP-dependent manner (Nakano et al., 1985). NCB 20 cells, which are a hybrid of mouse neuroblastoma N18TG-2 and Chinese hamster embryonic brain cells CHB C, had predominantly MAO B activity with a little MAO A activity (Nagatsu et al., 1981). MAO B and MAO A in hybrid NCB 20 cells were determined to be distinct enzyme molecules by peptide mapping (Nakano et al., 1986).

MAO B activity, but not MAO A activity in the brain increases during aging (Fowler et al., 1980). This increase may be due to the increase in the number of glial cells during aging. In the living human brain, MAO B can be detected by positron emission tomography (PET) using deuterium substituted [^{11}C] L-(−)-deprenyl (selegiline) (Fowler et al., 1998). The PET study indicated that MAO levels in the human brain were highest in the basal ganglia and the thalamus, intermediate in the frontal cortex and cingulate gyrus, and lowest in the parietal and temporal cortices and cerebellum. The results of PET confirm postmortem studies on increases in brain MAO B with age. The whole brain and the cortical regions and the basal ganglia, thalamus, pons, and cerebellum showed an average increase of 7.1 ± 1.3% per decade. There was also a large variability among subjects in the same age range. Interestingly, inhibition of MAO B was observed by PET study in the brain of smokers (Fowler et al., 1996). Smokers also showed low MAO B in platerets (Olerand, 2004), and are speculated to have a low incidence of PD.

MPTP-induced Parkinsonism and monoamine oxidase B (MAO B)

The discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as the first recognized synthetic neurotoxin

that is capable of inducing PD symptoms in humans has greatly contributed to the understanding of the molecular mechanism of sporadic PD. Calne and Langston (1983) reviewed the etiology of PD and pointed out the possibility of involvement of environmental toxic substances as being the important cause of PD, superimposed on a background of slow, sustained neuronal loss due to the process of aging. Humans are highly susceptible to MPTP, and non-human primates are also sensitive to the compound. Various non-primate animals including some strains of the mouse and even the fruit fly Drosophila also show PD-like movement disorder by administration of MPTP. The first human case of PD that appeared after intravenous injection of MPTP as a contaminant of 1-methyl-4-phenyl-piperidine-4-carboxulic acid ethyl ester (meperidine), which is a synthetic heroin, was a 23-year-old chemistry student at Bethesda, MD, USA. He synthesized that meperidine containing MPTP as a by-product and injected it intravenously into himself. L-3,4-Dihydroxyphenylalanine (L-DOPA) to supplement DA in the brain was effective in that patient as in PD patients. Kopin's group at the National Institutes of Health (NIH) identified MPTP in that meperidine preparation and reported the case in 1979 (Davis et al., 1979). Then, in 1983 in California, a group of young drug addicts acutely showed PD-like symptoms after self-administration of street batches of meperidine contaminated by MPTP. Like idiopathic PD, L-DOPA, which supplements DA in the brain as a substrate of aromatic L-amino acid decarboxylase, was an effective cure for the symptoms. These cases were reported by Langston et al. (1983), and since then the molecular mechanism of MPTP-elicited PD and investigation of similar neurotoxins in environment have been extensively studied (for review, see Nagatsu, 1997, 2002b). MPTP is highly lipophilic, and after its systemic injection, it rapidly crosses the blood-brain barrier to enter the brain. Once in the brain, MPTP, which is a pro-neurotoxin, is metabolized to 1-methyl-4-pheny-2,3-dihydro-pyridinium (MPDP+), by MAO B, which is localized in the outer membrane of mitochondria within glial cells. MPDP+ is then probably spontaneously oxidized to 1-methyl-4-phenyl-pyridinium (MPP+), the active PD-producing neurotoxin. MPP+ is then taken up via DA-transporter across the plasma membrane at the nerve terminals of the nigro-striatal DA neurons in the striatum. As acute reactions, MPP+ is taken up into synaptic vesicles from the cytoplasm by vesicular monoamine transporter type 2 (VMAT 2) to release DA from the nerve terminals; it also inhibits and inactivates tyrosine hydroxylase (TH) to decrease DA synthesis. In the chronic phase, MPP+ is transported from the nerve terminals of nigro-striatal DA neurons in the striatum to the cell bodies in the substantia nigra by retrograde axonal flow. MPP+ is also accumulated within the inner mitochondrial membrane, where it inhibits complex I (NADH ubiquinone oxidoreductase), one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation for ATP fromation, interrupts electron transport, releases reactive oxygen species (ROS) causing oxidative stress, and depletes ATP. Inhibition of mitochondrial complex I opens mitochondrial permeability transition pore, and subsequently triggers apoptotic cell death of the nigro-striatal DA neurons. Thus, MPP+ decreases DA acutely and chronically to produce PD-like symptoms. Oxidation of MPTP to MPP+ by mitochondrial MAO B in glial cells is essential for neurotoxicity, and selegiline as a specific MAO B inhibitor completely prevents the symptom of PD by MPTP. Mitochondrial dysfunction, especially decreased activity of complex I, is confirmed in the nigro-striatal region in the brain in sporadic PD (for review, see Mizuno et al., 1998). However, unlike sporadic PD, Lewy bodies are not observed in the remaining neurons in the substantia nigra in MPTP-induced PD.

Assuming that some MPTP-like neurotoxins in environment may trigger idiopathic PD, endogenous MPTP-like compounds have been investigated in postmortem brains and in the cerebrospinal fluid (CSF) from patients with PD. Two groups of MPTP-like compounds, isoquinolines (IQs) and beta-carbolines, were found in the human brains and CSF from patients with PD.

We found that MPP+ acutely inhibits the TH system in tissue slices of the rat striatum. In screening for various MPTP-like compounds that inhibit the striatal TH system, we found tetrahydroisoquinoline (TIQ) and its derivatives to be active inhibitors (Hirata et al., 1986). Tetrahydroisoquinoline alkaloids were first discovered in the brain as an in vivo metabolite of L-DOPA in humans by Sandler et al. (1973). Various TIQs were found in the brains of patients with PD and in those of non-parkinsonian control patients by gas chromatography/mass spectrometry: TIQ, 1-methyl-TIQ (1-Me-TIQ), N-Me-6,7-$(OH)_2$-TIQ, (N-Me-norsalsolinol), 1,N-$(Me)_2$-6,7-$(OH)_2$-TIQ (N-Me-salsolinol), 1-phenyl-TIQ, N-Me-1-phenyl-TIQ, and 1-benzyl-TIQ (1-Bn-TIQ) (for review, see Nagatsu, 1997, 2002b; Niwa et al., 1993). Exogenously administered TIQ easily crosses the blood-brain barrier and passes into the brain. However, endogenous TIQs in the brain are speculated to be enzymatically synthesized from precursor endogenous monoamines such as phenylethylamine or DA. Only the (R) enantiomer, (R)-N-Me-6,7-$(OH)_2$-TIQ (R-N-Me-salsolinol) is speculated to be enzymatically synthesized in the brain (Naoi et al., 1996). Among these TIQs in the brain, 1,N-$(Me)_2$-6,7-$(OH)_2$-TIQ (N-Me-salsolinol)

(Naoi et al., 1996), N-Me-6,7-$(OH)_2$-TIQ (N-Me-norsalsolinol) (Moser and Koempf, 1992), and 1-Bn-TIQ (Kotake et al., 1995, 1998) have been extensively investigated as probable neurotoxins to cause PD. It was also suggested that some cases of atypical PD in the French West Indies might have a link with the consumption as food of tropical plants that contain Bn-TIQs (Caparros-Lefebvre et al., 1999). Beta-carbolines have structures similar to those of MPTP/MPP+, and may be synthesized in vivo from tryptophan via tryptamine (Collins and Neafsey, 2000; Matsubara, 2000). A neurotoxic 2,9-dimethylated beta-carbonium, 2,9-dimethylated norharman, was found by gas chromatography/mass spectromrtry in CSF in half of the PD patients examined, but was not found in non-PD patients. 1-Trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) is another neurotoxic beta-carboline (Bringmann et al., 2000). TaClo can be synthesized in vivo from tryptamine and the synthetic chloral after application of the hypnotic chloral hydrate or after exposure to the widely used industrial solvent trichloroethylene, which is metabolized to chloral (Bringmann et al., 2000). However, since TaClo and the N-methylated derivative had no DA-transporter-mediated neurotoxicity in cultured cells transfected with the human DA-transporter gene, they may not cause neurotoxicity by a mechanism analogous to that of MPTP/MPP+ involving the uptake into DA neurons by DA-transporter.

Like MPTP, the neurotoxicity of 1-Bn-TIQ (Kotake et al., 1998), N-methyl-(R)-salsolinol (Naoi et al., 1996), and beta-carbolines (Collins and Neafsey, 2000; Matsubara, 2000) are suggested to be precursor neurotoxins, and to be protected by MAO B inhibitors. These compounds inhibit complex I to reduce ATP synthesis in agreement with low complex I activity in the brain in PD and may produce ROS.

Rotenone is a naturally occurring, lipophilic compound from the roots of certain plants (Derris species) with the structure not related to amines, and is used as the main component of many insecticides. Rotenone is a specific inhibitor of complex I, and in Lewis rats by the chronic systemic administration causes highly selective degeneration of the nigro-striatal DA neurons with behavioral PD symptoms of hypokinesia and rigidity and with formation of intracytoplasmic inclusions like Lewy bodies, which are mainly composed of alpha-synuclein and a characteristic feature of sporadic PD (Betarbet et al., 2000). The relation of rotenone to MAO B remains to be investigated.

Specific inhibitory activity towards complex I of IQs and beta-carbolines suggests that they might be the possible neurotoxins producing PD. However, the concentrations of IQs and beta-carbolines in postmortem brain and CSF are low (in the order of ng/g tissue), and their in vivo toxicity and clinical significance in human PD remain to be further examined. Also, the question remains; is there any relation between clinical efficacy of MAO B inhibitor L-deprenyl (selegiline) in PD patients, as describes below, and complete prevention of PD symptoms in animal PD-models produced by MPTP- or MPTP-like neurotoxins by the inhibitor?

Clinical efficacy of monoamine oxidase B inhibitors in Parkinson's disease

L-Deprenyl (R-(−)-deprenyl, the generic name selegiline) was the first discovered MAO B specific inhibitor (for review, see Knoll and Magyar, 1972; Knoll, 1980). Selegiline is a suicide inhibitor, i.e., the compound acts as a substrate for the target enzyme MAO B and results in irreversible inhibition (Riederer and Youdim, 1990). Clinical efficacy of the MAO B inhibitor, selegiline, for addition of L-DOPA that supplements deficient DA in PD was first reported by Birkmayer et al. (1985). In a long term (9 years) study of treatment of PD patients with L-DOPA alone or in combination with selegiline, a significant increase of life expectancy in L-DOPA-selegiline group was observed. The results were interpreted as indicating selegiline's ability to prevent or retard the degeneration of striatal DA neurons. This hypothesis was not far fetched since selegiline selectively prevents the degeneration of nigro-striatal DA neurons in animal PD models induced by MPTP, as described above. After the first work on the clinical efficacy of selegiline on Parkinson's disease (Birkmeyer et al., 1985), the Parkinson Study Group in USA (1989) preliminarily reported that the use of selegiline (10 mg per day) delays the onset of disability associated with early, other untreated cases of PD. The Parkinson Study Group (1993) further reported the results of the multicenter controlled clinical trial of Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (the "DATATOP" study). Selegiline and tocopherol (vitamin E as an antioxidant) clinical trial from 1987 for 5 years (the US DATATOP study, selegiline momotherapy) suggested that deprenyl (10 mg per day) but not tocopherol (2000 IU per day) dalays the onset of disability associated with early, otherwise untreated PD. However, this remains controversial (Lang and Lees, 2002). Further uncertainty arose in 1995, when a study by the Parkinson's Disease Research Group of the United Kingdom (UK-PDRG) found 57% higher mortality in patients receiving combined selegiline and L-DOPA treatment compared with patients on L-DOPA alone (Lees on behalf of the Parkinson's Disease Research

Group of the United Kingdom, 1995). Other clinical trials have, however, failed to show any increase in mortality and showed neuroprotective effects of selegiline (Counsell, 1998; Olanow and Riederer, 1996; Olanow et al., 1995). Furthermore, another MAO B inhibitor, rasagiline (N-propargyl-R-aminoindan) that is a selsective, irreversible, second-generation MAO B inhibitor, has shown effectiveness in early PD when given as once-daily treatment without dose titration (Parkinson Study Group, 2002). To clarify the role of MAO B inhibitors in the treatment of PD, Ives et al. (2004) did a meta-analysis of data from all published trials, and reported that MAO B inhibitors (selegiline, lazabemide, or rasagiline) with or without L-DOPA, versus placebo, L-DOPA, or both, reduce the need for L-DOPA, and the incidence of motor fluctuations, without substantial side effects or increased mortality. This study supported the efficacy and safety of monotherapy of early PD by MAO B inhibitors such as selegiline.

Molecular mechanism of neuroprotective effects of L-deprenyl (selegiline) against Parkinson's disease

Stimulated by clinical efficacy of selegiline as a MAO B inhibitor for the treatment of early PD as described above, mechanisms of possible neuroprotection by selegiline have been extensively studied. The early hypothesis on the mechanism of clinical efficacy of selegiline in the treatment without or with L-DOPA was the prevention of degradation of DA, which is produced endogenously from tyrosine by TH or from exogenously administered L-DOPA for treatment, by MAO B inhibition (symptomatic effect). However, accumulating results indicate that selegiline may also have neuroprotective effects by several mechanisms that are related or not related to MAO B inhibition.

Neuroprotection due to inhibition of dopamine degradation by MAO B inhibitor selegiline

DA is a common substrate of MAO B and MAO A. However, in PD only MAO B inhibitor is clinically effective. Selegiline may increase the level of DA in the synaptic cleft in the DA nerve endings in the striatum after release from presynaptic terminals by inhibiting MAO B. DA as a substrate of MAO B produces H_2O_2 and 3,4-dihydroxyphenylacetaldehyde as neurotoxic products. However, since presence of MAO activity is not observed in DA neurons (Arai et al., 1998), DA released from DA neurons or produced from exogenously administered L-DOPA in L-DOPA therapy may be oxidized in the outside of DA neurons possibly in glial cells that contain MAO B to produce cytotoxic H_2O_2 and the aldehyde metabolite. Then H_2O_2 may get into the nigro-striatal DA nerons, and may be oxidized to produce cytotoxic oxygen radicals (reactive oxygen species, ROS) by iron presumably catalytically with neuromelanine. Iron accumulates in the DA neurons in the substantia nigra in PD (Dexter et al., 1987; Hirsch et al., 1991; Jellinger et al., 1992; Sofic et al., 1988). ROS may cause lipid membrane peroxidation and finally cell death of DA neurons (Dexter et al., 1993; Youdim et al., 1993). MAO B inhibitors can prevent this neurotoxic process to protect DA neurons.

Another possible mechanism of selegiline related to MAO B inhibition is an amphetamine-like tonic effect due to increased accumulation of phenylethylamine. Phenylethylamine is a good substrate of MAO B and may be produced in glial cells. Phenylethylamine at high concentrations were found in the striatum in the postmortem brain from PD patients treated with selegiline, and may have an endogenous "amphetamine-like activity" to stimulate DA neurons (Gerlach et al., 1992).

Selegiline's neuroprotective mechanism that is not related to MAO B inhibition

It has been known for many years that neuroprotective effects of selegiline can be observed in cell culture experiments at lower concentrations than those for MAO B inhibition, suggesting that selegiline's neuroprotective effects may also be caused by some other mechanisms than MAO B inhibition.

Riederer and Lachenmayer (2003) pointed out the possibility of neuroprotection by selegiline independent from MAO B inhibition by re-examining the clinical studies such as the DATATOP study (1993) based on the half life of selegiline in vivo in humans. In those clinical studies, the efficacy of selegiline was evaluated at the end-point between baseline and the end of the study (14 months including a 2 months wash-out period). Reported data on the half life of seligiline were between about 2–10 days (Gerlach et al., 2003; Youdim and Tipton, 2002) and 40 days (Fowler et al., 1994). Even the slow recovery of MAO B activity as determined by Fowler et al. (1994) would indicate only a 20% recovery of MAO B activity after a 2-week wash-out period and less than 50% recovery after a 4-week period. However, a significant increase in amine neurotransmitter concentrations can only be demonstrated after the MAO activity has been inhibited by at least 80% (Green et al., 1977). Thus a recovery of only 20% of the MAO B activity is already sufficient to prevent an increase in the neurotransmitter concentration. These results would suggest that