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Role of cytokines in inflammatory process in Parkinson's disease

M. Sawada[1], **K. Imamura**[2], and **T. Nagatsu**[1,3]

[1] Department of Brain Life Science, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Aichi,
[2] Department of Neurology, Okazaki City Hospital, Okazaki, Aichi, and
[3] Department of Pharmacology, School of Medicine, Fujita Health University, Toyoake, Aichi, Japan

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.

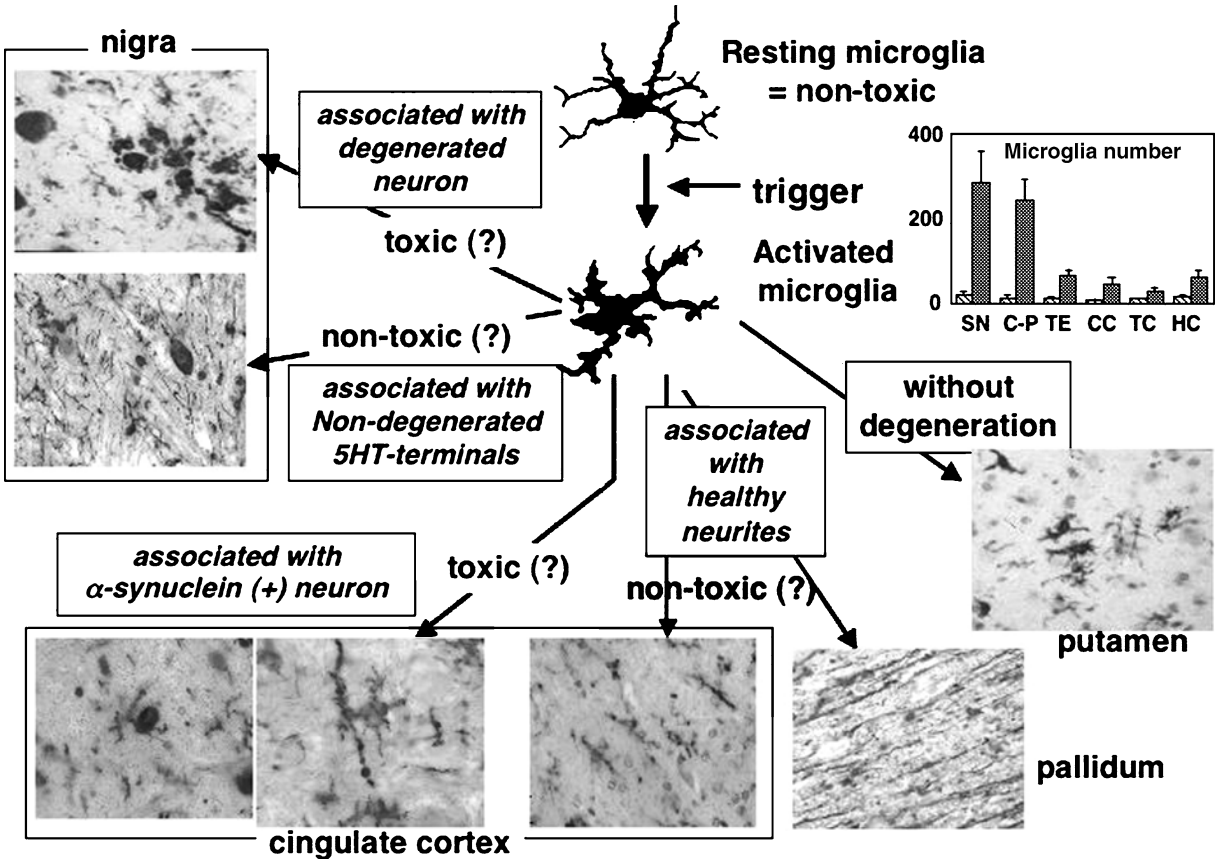

Fig. 1. Schematic diagram showing the dual potential roles of microglia in PD. In parkinsonian brains, activated microglia are observed not only in the substantia nigra (SN) and caudate-putamen (C–P) but also in other brain regions such as pallidum and cingulate cortex. Activated microglia associated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

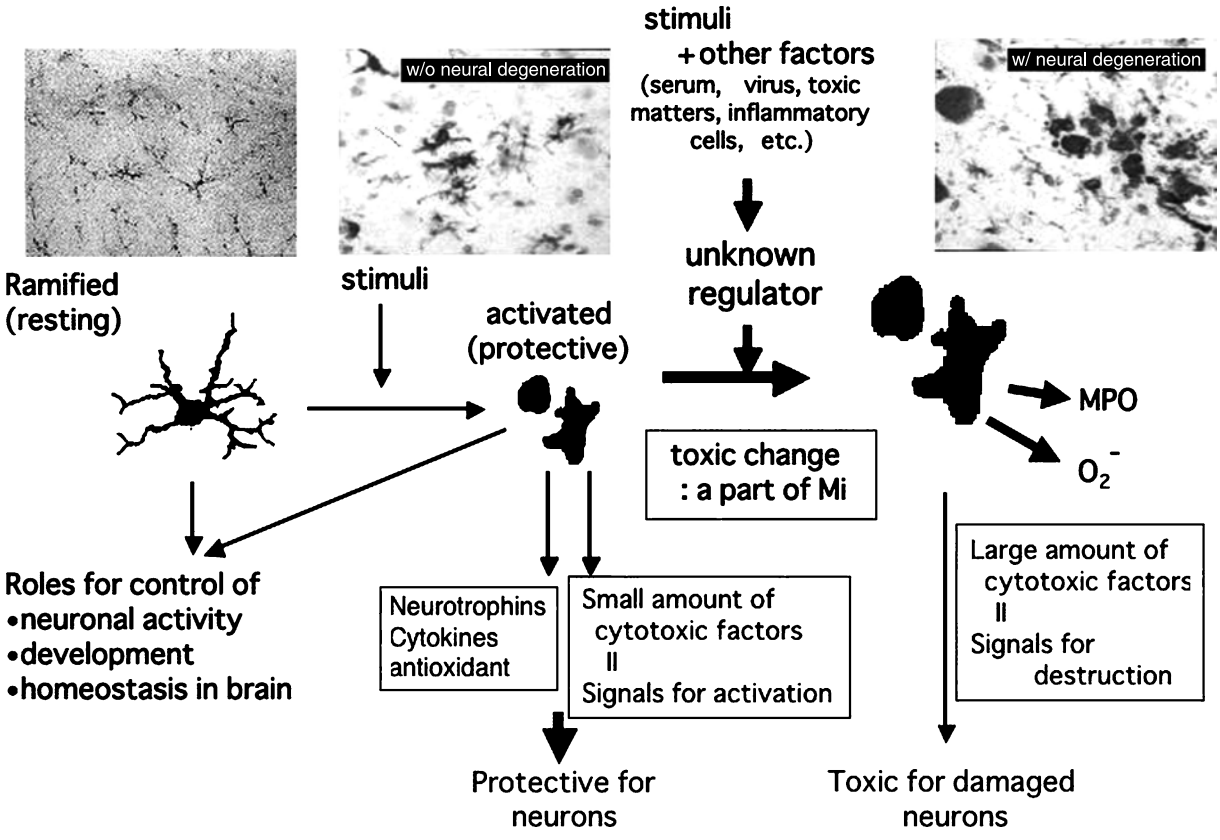

Fig. 2. Schematic diagram showing a hypothesis of two-step activation of microglia. We isolated neuroprotective and neurotoxic subsets of microglia, and also neuroprotective and neurotoxic clones from mouse brain. In addition, Sawada and coworkers (Vilhardt et al., 2002) found in a cell culture experiment 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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Author's address: Prof. T. Nagatsu, Department of Pharmacology, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan, e-mail: tnagatsu@fujita-hu.ac.jp