mitochondrial complexes I and II, presumably at the level of the 4Fe–4S clusters (Panov *et al.*, 2005).

All the properties of the afore-mentioned neurotoxins indicate that inhibition of complex I, resulting in ROS formation, oxidative stress, and ATP depletion in DA neurons may trigger the pathway of apoptotic cell death in these cells. In agreement with this neurotoxin-based hypothesis, a selective defect in complex I of the mitochondria in the nigro-striatal DA neurons from the post-mortem brain of sPD patients was reported by several laboratories (Youdim and Riederer, 1997; Mizuno *et al.*, 1998; Shapira *et al.*, 1998). However, it should be noted that the development of PD has been confirmed in humans only in the case of MPTP, a synthetic neurotoxin. That is, the toxicity of the afore-mentioned endogenously identified neurotoxin candidates has been proved only in animals.

6-Hydroxydopamine (6-OHDA) is a neurotoxin specific for DA neurons *in vitro* and *in vivo* (Kostrzewa and Jacobowitz, 1974). It is a very unstable compound and is easily oxidized to produce ROS, which may kill DA neurons via apoptosis. Although 6-OHDA is formed from DA *in vitro*, it is believed not to be formed *in vivo*. 6-OHDA does not cross the blood–brain barrier. Thus, it is widely used to produce hemiparkinsonian animal models by stereotaxic injection of it directly into the nigro-striatal region.

Since DA (or NA) is easily oxidized to form DA quinones and other reactive oxygen species, toxic metabolites of DA such as DA quinones (in the case of NA, NA quinones), which are intermediates of neuromelanin biosynthesis, or 3,4-dihydroxyphenylacetaldehyde (DOPAL; in the case of noradrenaline, 3,4-dihydroxyphenylethyleneglycolaldehyde, DOPEGAL) formed by MAO are postulated to play a role as endogenous neurotoxin candidates in PD (Eisenhofer *et al*, 2002). DA quinones may be formed either non-enzymatically or by the action of tyrosinase. Tyrosinase has been implicated in DA quinone and neuromelanin formation in the brain. However, we did not detect tyrosinase protein in the A9 nigral DA neurons in the human brain by using antibodies against human tyrosinase (Ikemoto *et al.*, 1998). DOPAL was identified in the PD brain, but not in the normal brain, by gas chromatography–mass spectrometry (Mattammal *et al.*, 1993), and was suggested to be toxic for DA neurons *in vitro* (Mattammal *et al.*, 1995). However, DA depletion in DA-deficient mice did not protect against acute MPTP toxicity *in vivo*, suggesting that DA does not contribute to this toxicity *in vivo* (Hasbani *et al.*, 2005).

Since iron exists in a high concentration in the basal ganglia and interacts with neuromelanin and DA, it is suggested to play a role in the formation of ROS and in the initiation of neurodegeneration of DA neurons by oxidative stress (Mochizuki *et al.*, 1993; Gerlach *et al.*, 1994).

All the effects of exogenous or endogenous neurotoxin candidates suggest that mitochondrial dysfunction and oxidative stress are important for the pathogenesis of sPD. Inhibition of complex I results in enhanced production of ROS, which in turn inhibits complex I. Thus, the vicious cycle resulting from even partial inhibition of complex I in DA neurons may lead to excessive stress and an ATP deficit that ends in cell death (Tretter *et al.*, 2004).

CAUSATIVE GENES OF FAMILIAL PD (PARK)

A small percentage (approximately 5%) of PD cases are familial with a hereditary history (fPD; Table I). Several causative genes of fPD, which mutations produce parkinsonism, and their chromosomal localization have recently been identified: PARK1 (*alpha-synuclein*), PARK2 (*parkin*), PARK4 (triplication of *alpha-synuclein*), PARK5 (*UCHL1*), PARK6 (*PINK1*), PARK7 (*DJ-1*), and PARK8 (*LRRK2*) (for reviews, see Chiba-Falek and Nussbaum, 2003; Feany, 2004; Forman *et al.*, 2004; Selkoe, 2004; Cookson, 2005; Grandhi and Wood, 2005; Lozano and Kalia, 2005; Mizuno, 2006).

In 1997, a causative mutation of fPD was first identified in the PARK1 gene, encoding the protein alpha-synuclein in autosomal dominant Italian and Greek families (Polimeropoulos *et al.*, 1997). Alpha-synuclein is a small (144 amino acids), presynaptic protein and probably plays a role in signaling between neurons (Goedert, 2001). As a finding on the physiological role of alpha-synuclein, Chandra *et al.* (2005) reported that alpha-synuclein in conjunction with CSP-alpha (cysteine-string protein alpha) has a powerful *in vivo* activity in protecting nerve terminals against injury in mice. CSP-alpha is a synaptic vesicle protein, essential for neural survival, has a cochaperon function, and may prevent the accumulation of nonnative, potentially toxic molecules during the continuous operation of a nerve terminal.

Not only mutations in alpha-synuclein such as a single amino acid substitution A30P or A53P in PARK1, but also triplication of the wild-type *alpha-synuclein* gene also causes autosomal dominant PARK4. Alpha-synuclein is a major protein component of Lewy bodies (Spillantini *et al.*, 1998), and thus may play an important role in both fPD and sPD. Alpha-synuclein is a natively unfolded soluble protein and has a central hydrophobic region and a high potency to aggregate to form oligomers or protofibrils and ultimately insoluble polymers or fibrils under certain conditions. The protofibrillar intermediates are toxic in neurons (Conway *et al.*, 2000). Alpha-synuclein fibrils cause mitochondrial complex I deficiency (Sherer *et al.*, 2003) and oxidative stress (Ischiropoulos and Beckman, 2003). Thus, alpha-synuclein may elicit a pathogenetic mechanism similar to that acting in sPD.

Drosophila (fruit fly) models based on overexpression of normal and mutant forms of the *alpha-synuclein* gene show selective loss of DA neurons and the formation of alpha-synuclein inclusions (Feany and Bender, 2000). Experiments using this model confirmed that phosphorylation at the Ser 129 residue is crucial to the toxicity of alpha-synuclein and that mutations of this serine residue abolishes the toxicity. The reduction in toxicity in this model is associated with increased inclusion body formation, which suggests that inclusion bodies may protect neurons by reducing the amount of diffusible toxic protein by sequestering it in inert bodies (Chen and Feany, 2005). All these findings on alpha-synuclein suggest that the misfolding of the protein is the key steps in mediating degeneration of DA neurons in both fPD and sPD. Alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration, which also suggests a close correlation between alpha-synuclein and the pathogenesis of sPD (Martin *et al.*, 2006).

In PARK2 the gene encoding parkin protein, which was discovered in a Japanese family, is the most common mutant gene in fPD (Kitada *et al.*, 1998). The *parkin*

gene is very large, about 1.4 Mb; and mutations of it are responsible for most of the cases of autosomal recessive juvenile (young-onset) PD. The parkin protein is an E3 ubiquitin ligase with two characteristic RING finger domains separated by an IBR (in-between ring) domain (Shimura *et al.*, 2000). There is a notable absence of Lewy bodies in patients with the homozygous deletion of parkin, although these bodies are present in patients with compound heterozygous parkin mutations. These findings suggest that parkin plays a significant role in Lewy body formation and that nigral cell loss and parkinsonism can occur in the absence of Lewy bodies. The identification of mutations of the *parkin* gene in PARK2 suggests that dysfunction of the UPS due to loss of function has an important role in PD and that the *parkin* gene may play a protective role. Ubiquitin is added to proteins by the action of E3 ubiquitin ligase to target them to the proteasome, a large multiprotein complex that functions to degrade most ubiquitin-marked cellular proteins. Parkin mutations cause the accumulation of parkin substrates, which probably contributes to the death of DA neurons. Many putative parkin substrates have been identified, including synphilin-1, 22-kDa O-glycosilated form of alpha-synuclein (alphaSp22; Shimura *et al.*, 2001), Pael-R (*p*arkin-*a*ssociated *e*ndothelin receptor-*l*ike *r*eceptor; Imai *et al.*, 2001), CHIP, Cdc-Rel1A, cyclin E, and synaptotagmin XI. Overexpression of the parkin substrate Pael-R produces DA cell death *in vitro*, which cells can be rescued by parkin overexpression (Yang *et al.*, 2003).

Parkin has been shown to be S-nitrosylated *in vitro* and *in vivo* in the MPTP mouse model of PD and in brains of patients with PD or LBD, and both neuron- and microglia-derived NO contributes to the S-nitrosylation of parkin in a biphasic fashion after MPTP intoxication. S-Nitrosylation inhibits the E3 ubiquitin ligase activity of parkin and thus its protective function (Chung *et al.*, 2004).

Synphilin-1, a substrate of parkin, was shown to interact with alpha-synuclein and to promote the formation of cytosolic inclusions. Synphilin-1 also interacts with E3 ubiquitin ligase SIAH (*s*even *i*n *a*bsentia *h*omologues)-1 and SIAH-2. SIAH proteins ubiquitinate synphilin-1, promoting its degradation by UPS, and may play a role in inclusion formation, since SIAH immunoreactivity was demonstrated in Lewy bodies in PD patients (Liani *et al.*, 2004). MAO inhibitor deprenyl (selegiline) was reported to act for protection of DA neurons by binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Tatton *et al.*, 2003). Snyder (2005) recently reported that an apoptotic stimulus turns on inducible NO synthase with its product NO causing S-nitrosylation of GAPDH in DA neurons. This modification allows GAPDH to bind to the E3 ubiquitin ligase SIAH, which transports GAPDH to the nucleus, leading to apoptotic cell death. They also showed that MAO B inhibitor deprenyl binds to SIAH protein, thereby preventing translocation of the GAPDH–SIAH complex from the cytoplasm to the nucleus and thus preventing apoptotic cell death.

PARK5 is another autosomal dominant fPD. The gene encodes UCHL1 (*u*biquitin *C*-terminal *h*ydrolase *L1*), which generates free ubiquitin and aids the recycling of polyubiquitin chains back to monomeric ubiquitin (Leroy *et al.*, 1998). UCLH1 can also exert a ubiquitin ligase activity. The discovery of the *UCHL1* gene further supports the importance of the role of UPS in the pathogenesis of PD.

The UPS may be important not only in fPD (PARK1, PARK2, PARK4, and PARK5) but also in sPD. Postmortem brain tissues from sPD patients show functional deficits in their 20S proteasome activity (Chung *et al.*, 2001). Also, systemic administration of inhibitors of the UPS to rodents produces selective nigral cell loss and Lewy body-like inclusions, which are accompanied by clinical signs of parkisonism (McNaught *et al.*, 2004).

PARK6 is another autosomal recessive fPD, and is caused by a mutation in a mitochondrial protein kinase called PINK1 [*P*TEN (phosphatase and *ten*sin Romolog deleted on chromosome *ten*)-*in*duced *k*inase-*1*; Valente *et al.*, 2004)]. PINK1 is the first nucleus-encoded mitochondrial protein to be implicated in the pathogenesis of fPD. The *PINK1* gene encodes a serine/threonine protein kinase with significant homology to the calcium/calmodulin-dependent protein kinase. Neuroblastoma cells transiently transfected with either wild-type or mutant *PINK1* do not show any detectable alterations in viability. In contrast, when these cells are challenged with a proteasome inhibitor, MG132, overexpression of the wild-type PINK1 mitigates cell death; whereas, overexpression of mutant PINK1 neither attenuates nor enhances MG132-mediated cytotoxicity (Valente *et al.*, 2004). These results suggest that the loss of PINK1 function renders DA neurons more vulnerable to injury. Transient knockdown of PINK1 renders cells susceptible to apoptosis on exposure to taxol (MacKeigan *et al.*, 2005). This neuroprotective function and the mitochondrial localization of PINK1 suggest its probable important role in mitochondria also in sPD.

DJ-1 is the causative gene of autosomal recessive early-onset PARK7 (Bonifati *et al.*, 2003). DJ-1 is a homodimeric and multifunctional protein, ubiquitously expressed in human tissues; and it plays essential roles in tissues with higher-order biological functions such as the testis and brain. DJ-1 is related to male fertility, and its level in sperm is decreased in response to exposure to sperm toxicants. DJ-1 was discovered as a novel mitogen-dependent oncogene product involved in a Ras-related signal transduction pathway (Nagakubo *et al.*, 1997). DJ-1 is up-regulated after oxidative stress and may play a role as an antioxidant protein and a sensor for oxidative stress. The crystal structure of DJ-1 indicates that the protein is structurally similar to a cysteine protease and may induce conformational changes to acquire its catalytic activity in response to oxidative stress (Honbou *et al.*, 2003). The function of DJ-1 as an anti-oxidant protein again suggests its pathogenetic role also in sPD.

The gene for the LRRK2 (*l*eucine-*r*ich *r*epeat *k*inase *2*) was identified as the PARK8 locus (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004). Mutations in *LRRK2* cause autosomal dominant PD with a broad spectrum of neuropathological features, such as neuronal loss in the substantia nigra either in the absence or in the widespread presence of Lewy bodies or in the presence of neurofibrillary tangles. The affected families originated from Italy, Portugal, and Brazil, indicating the presence of this mutation in different populations. The associated phenotype is broad, including early and late disease onset (Di Fonzo *et al.*, 2005). The *LRRK2* gene encodes a 286-kDa protein that is a member of a novel family of protein kinases called "dardarin" (meaning "tremor" in the Basque region,

from where some of the affected patients came). Dardarin contains leucine-rich repeats and a Ras/small GTPase superfamily domain, a tyrosine kinase-like domain, and the WD40 domains with sequence similarity to both tyrosine and serine/threonine kinases (Shen, 2004). The presence of these novel domains in dardarin suggests a unique and new function for this kinase in the survival of nigral DA neurons.

Mutations in *NR4A2* have also been found to be significantly associated with fPD (Le *et al.*, 2003). The *NR4A2* gene (also called *Nurr1*; i.e., *nu*clear *r*eceptor-*r*elated 1) encodes a transcription factor that belongs to the steroid/thyroid hormone receptor superfamily. Alternative splicing and selective use of transcription initiation sites control the expression of the human *Nurr1* gene (Ichinose *et al.*, 1999a,b). Interestingly, *Nurr1* is essential for the differentiation of the nigral DA neurons and is closely related to the expression and function of the DA system. The *Nurr1* gene activates expression of tyrosine hydroxylase (TH; Iwawaki *et al.*, 2000), and also enhances transcription of the DA transporter (Sacchetti *et al.*, 2001). NR4A2 (+/–) mice have a parkinsonian-like phenotype and are more susceptible than the wild type to MPTP (Warbt *et al.*, 2003). Although the mechanism is not yet clear, NR4A2 is thought to be a susceptibility gene for sPD.

DA deficiency due to mutations of the genes of the enzymes involved in DA biosynthesis, i.e., DA-synthesizing-enzyme TH or its cofactor tetrahydrobiopt erin (BH4)-synthesizing enzyme GTP cyclohydrolase I (GCH), causes DOPA-responsive dystonia, parkinsonism in infancy or progressive infantile encephalopathy with L-DOPA-nonresponsive dystonia, depending upon the degree of DA deficiency (Hoffmann *et al.*, 2003; Segawa *et al.*, 2003; Kobayashi and Nagatsu, 2005). Autosomal dominant GCH deficiency, which was first described by Segawa and thus called Segawa's disease, is a DOPA-responsive dystonia caused by a partial decrease of the activity of GCH due to a mutation of one of its alleles (Ichinose *et al.*, 1994, 1995, 1999; Nagatsu and Ichinose, 1999; Segawa *et al.*, 2003). Segawa's disease is a partial DA deficiency without any DA cell death and the symptom is completely controllable by L-DOPA administration. In contrast, PARK2 or autosomal recessive juvenile PD is initially similar to DOPA-responsive dystonia but progresses to parkinsonism, and is accompanied by DA cell death.

fPDs indicate the importance of the dysfunction of UPS and protein misfolding in the pathogenesis of PD. Overexpression of alpha-synuclein in mice and rats leads to the development of mitochondrial degeneration and produces DA cell death similarly as in sPD (Yamada *et al.*, 2004; Martin *et al.*, 2006). On the other hand, mitochondrial dysfunction in sPD also causes the dysfunction of UPS due to ATP deficiency. The above genes involved in fPD may be susceptibility genes for sPD. It has been reported that DA covalen1tly modifies and functionally inactivates parkin, suggesting a vulnerability of parkin to modification by DA and a mechanism for the progressive loss of the neuroprotective parkin function in DA neurons during aging and sPD (LaVoie *et al.*, 2005). Thus, fPD and sPD are different in their primary causes, but may ultimately produce nigral DA cell death by a final common pathway.

CHANGES IN CYTOKINES PRODUCED BY ACTIVATED MICROGLIA DURING THE NEUROINFLAMMATORY PROCESS IN PD

Elevated Pro-Inflammatory Cytokine Expression in the Presence of Activated Microglia in the Nigro-Striatal Region in sPD

We and others have reported increases and decreases in the levels of pro-inflammatory cytokines and neurotrophins, along with the appearance of activated microglia, in the brain of sPD patients, thus suggesting the presence of an inflammatory process (McGeer and McGeer, 1995; Anglade *et al.*, 1997; Hirsch *et al.*, 1999; Mogi and Nagatsu, 1999; Nagatsu *et al.*, 1999; Jellinger, 2000; Hartmann *et al.*, 2000; Nagatsu *et al.*, 2000a,b; Nagatsu, 2002a; Hayley, 2005; Herrera *et al.*, 2005; Nagatsu and Sawada, 2005). Based on the results of enzyme-linked immunosolvent assay (ELISAs; for a review, see Nagatsu, 2002a), we reported the changes in the levels of the following cytokines and neurotrophins in the postmortem brain (striatum) and/or ventricular or lumbar cerebrospinal fluid (CSF) in sPD patients as compared with their normal levels: (1) increased levels of TNF-alpha (Mogi *et al.*, 1994a), IL-1beta, IL-6 (Mogi *et al.*, 1994b), IL-2, IL-4, EGF, TGF-alpha, TGF-beta1, TGF-beta2, Bcl-2 (Mogi *et al.*, 1996), soluble FAS, TNF R1 (p55), caspase 1, and caspase 3 (Mogi *et al.*, 2000); and (2) decreased levels of neurotrophins BDNT and NGF. These data on changes in the levels of cytokines in human PD brains were also supported by the results obtained from animal models of PD. For example, MPTP-treated mice show an increased level of IL-1beta and a decreased level of NGF specifically in their striatum (Mogi *et al.*, 1998). As another model of PD, in hemiparkinsonian rats produced by injecting 6-OHDA into one side of the ventrotegmental bundle without or with L-DOPA treatment, the levels of TNF-alpha were significantly increased only in the substantia nigra and striatum of the injected side. L-DOPA administration did not produce any significant changes in TNF-alpha levels in either 6-OHDA-treated or control side of any of the brains (Mogi and Nagatsu, 1999). These results agree with the changes seen in the TNF-alpha levels in the striatum and lumbar CSF in PD patients and also suggest that the increased cytokine levels may not be due to the secondary effects of L-DOPA therapy in PD patients.

The increased levels of pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1beta and the decreased levels of neurotrophins such as BDNF and NGF, which changes are known to trigger the process of apoptosis, strongly suggest a pro-apoptotic environment in the striatum in PD. In fact, the levels of apoptosis-related factors such as Bcl-2 (Mogi *et al.*, 1996), soluble FAS, TNF R1 (p55), caspase 1 (IL-1-beta converting enzyme), and caspase 3 are increased in the PD brain (Mogi *et al.*, 2000). Fas antigen and 2 TNF receptors, p55 and p75, are implicated in triggering cell death upon stimulation by their natural ligands, i.e., TNF-alpha and Fas ligands (Nagata and Goldstein, 1995). Since TNF R1 and caspases 1 and 3 have been implicated as mediators of apoptotic cell death (Kumar, 1995), their increased levels support the presence of pro-apoptotic environment in the striatum in the PD brain. The increased levels of Bcl-2 (Mogi *et al.*, 1996) and sFAS, which are anti-apoptotic

factors, may suggest their compensatory production to cope with apoptosis. Marshall *et al.* (1997) also reported up-regulation of Bcl-2 in the basal ganglia in PD patients. We also found that the levels of two other factors trophic toward DA neurons, i.e., GDNF (*g*lial cell line-*d*erived *n*eurotrophic *f*actor) and bFGF (*b*asic *f*ibroblast *g*rowth *f*actor) were not decreased, although their concentrations were high in the striatum in control or PD brains. This is in contrast to the markedly reduced levels of BDNF or NGF specifically in that region in PD. The unchanged level of GDNF in PD could be due to compensatory production in glial cells, which occurs with neither BDNF nor NGF. In agreement with our results obtained by ELISA, Boka *et al.* (1994) found TNF-alpha immunoreactive glial cells in the substantia nigra in the PD brain, and other workers also reported increased cytokine levels in *de novo* PD without L-DOPA treatment: IL-1beta and IL-6 in lumbar CSF (Blum-Degan *et al.*, 1995) and TGF-beta1 and TGF-beta2 in ventricular CSF (Vawter *et al.*, 1996). Activated caspase 3 was also detected immunohistochemically and was proposed to be the final effector in the apoptotic cell death of DA neurons in PD (Hartmann *et al.*, 2002).

Pro-Inflammatory Cytokines in the PD Brain are Produced from Activated Microglia

The origin of pro-inflammatory cytokines in the PD brain is speculated to be activated microglia. McGeer *et al.* (1988) were the first to report an increase in the number of major histocompatibility complex class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive reactive microglia in the substantia nigra in PD patients. We also speculated that activated microglia are present in the PD brain to produce pro-apoptotic cytokines and neuroinflammation, ultimately promoting death of DA neurons in the substantia nigra. Imamura *et al.* (2003) proved that increased amounts of cytokines are produced by activated microglia in the putamen of sPD patients. Mogi *et al.* (1994a,b) had previously shown, by enzyme immunoassay, increased levels of TNF-alpha and IL-6 in the striatum in sPD. Imamura *et al.* (2003) identified by Western blot analysis TNF-alpha protein and IL-6 protein, along with MHC class II (CR3/43) protein, in homogenates of the putamen from sPD patients; and they further proved by an immunofluorescence technique the coexistence of TNF-alpha and IL-6 proteins in ICAM-I and LFA-1-positive MHC class II-bearing activated microglia in the putamen from sPD patients. These results confirmed that TNF-alpha and IL-6 proteins are produced from activated microglia in the putamen in sPD.

Activated Microglia may be Initially Non-Toxic/Neuroprotective and Then by a Toxic Change Become Neurotoxic to Cause Progression of PD

Activated microglia are known to produce either neuroprotective or neurotoxic factors. The question is whether these activated microglia are neuroprotective or neurotoxic toward the nigro-striatal DA neurons.

We aimed at elucidating the role of activated microglia in the postmortem brain of sPD at the cellular level. Activated microglia have multiple roles: (1) MHC class

II-positive ones act in antigen presentation; (2) activated microglia phagocytose damaged cells; (3) they produce substances such as the pro-inflammatory cytokines TNF-alpha and IL-6, which are pleiotropic and act either as neurotoxins or as neuroprotective agents, as well as neurotoxic substances, i.e., ROS, nitric oxide (reactive nitrogen species, RNS), and glutamate; and (4) they also produce neurotrophic substances such as the neurotrophin BDNF and cytokines that act neuroprotectively.

In the normal brain, many Kp1-positive resting microglia, which are non-toxic, are seen in the substantia nigra and putamen. In the PD ones, however, a large number of MHC class II-positive ramified, activated microglia are found in these regions compared with their number in normal controls. Furthermore, the number of MHC class II-positive microglia in the putamen in PD increases as the stage of PD advances. In the early stages of PD, MHC class II-positive microglia in the putamen and substantia nigra are associated with intensively TH-positive DA neurites showing no signs of degeneration. In the advanced stages, however, MHC class II-positive microglia in these areas are found with damaged TH-positive neurons and neurites. These results suggest that activated microglia in the substantia nigra and putamen may be non-toxic/neuroprotective or neurotoxic, depending on the stage of PD.

Imamura *et al.* (2003) observed that the number of MHC class II-positive activated microglia was significantly higher not only in the substantia nigra and putamen but also in various other brain regions such as the hippocampus, transentorhinal cortex, cingulate cortex, and temporal cortex in PD brains than in the control ones. Imamura *et al.* (2005) also observed activated microglia in the nigro-striatum and hippocampus in dementia with Lewy bodies (DLB), and compared them with those in PD. Neuronal degeneration in the putamen was observed in both PD and DLB, whereas neuronal loss in the hippocampus was observed in DLB but not in PD without dementia. In normal controls, neuronal loss, activated microglia, and alpha-synyclein-positive cells were not observed in the hippocampus (CA2/3 region), and neurons were strongly BDNF positive. In the hippocampus (CA2/3 region) in PD, the number of MHC class II-positive microglia was increased, which cells were also positive for ICAM-I (CD54), LFA-1, TNF-alpha, and IL-6. Alpha-synuclein-positive cells were also observed. BDNF-stained neurons were only slightly decreased in number in PD compared with those in controls. In the hippocampus (CA2/3 region) in DLB, the numbers of MHC class II (CR3/43)-positive microglia and alpha-synuclein-positive microglia, and alpha-synuclein-positive neurons were greater than those in PD, and the neurons were very weakly stained with anti-BDNF. These immunohistochemical data on the hippocampus (CA 2/3 region) indicate that the number of activated microglia increases in both PD and DLB and that the content of neurotrophic BDNF protein is markedly decreased in DLB but not in PD. Furthermore, in the hippocampus, mRNA levels of IL-6 and TNF-alpha were increased in both PD and DLB compared with the control levels; whereas the mRNA level of BDNF was greatly decreased in DLB, as compared with that in PD or normal controls. These different changes in the levels of mRNA and protein of BDNF, IL-6, or TNF-alpha in the hippocampus and putamen between PD and DLB suggest that activated microglia in these brain regions in PD and DLB are different in their properties and may secrete different kinds and different amounts of cytokines and neurotrophins such as BDNF and IL-6.

As other evidence supporting this concept of the presence of non-toxic/neuroprotective and neurotoxic microglia, we (Sawada *et al.*, 2006) separated two subsets of microglia from normal mouse brain by cell sorting based on profiles of intracellular ROS production induced by phorbol myristate acetate (PMA) stimulation: one subset of microglia producing a large amount of ROS and the other, just a minute amount of ROS. Furthermore, we obtained two cell lines of microglia, Ra2 cells and 6–3 cells, by spontaneous immortalization of mouse microglia in primary cultures. The Ra2 microglia cell line did not produce ROS upon PMA stimulation, whereas the 6–3 one produced ROS in large amounts in response to this stimulant. When co-cultured with N18 neuronal cells, Ra2 cells were neuroprotective, whereas 6–3 cells were neurotoxic. Furthermore, Sawada and co-workers found in a cell culture experiment a toxic change in activated microglia from neuroprotective to neurotoxic, caused by transduction of the cells with a lenti virus vector carrying HIV-1 Nef cDNA (Vilhardt *et al.*, 2002). It is speculated that a similar toxic change in activated microglia may occur *in vivo* in the PD brain as the second step, one caused by other factors such as invasion of serum, viruses, toxic substances, or inflammatory cells in some of the neuroprotective microglia in a specific brain regions, i.e., the nigro-striatum in PD. As a result of this toxic change, large amounts of cytotoxic factors such as ROS, NO, and RNS produced by NADPH oxidase, myeloperoxidase, cyclooxygenase 2 (COX 2), or nitric oxide synthase may promote the observed neuronal loss. The presence of reactive microglia in the substantia nigra years after MPTP exposure was detected in experimental monkeys (McGeer *et al.*, 2003) and in human patients (Langston *et al.*, 1999). These reactive microglia might have been produced by a toxic change in response to the exposure to MPTP. These results also suggest that a variety of causative agents of sPD, disappearing after having instituted long-lasting inflammatory changes, might cause progression of the disease.

Based on the results described earlier, Sawada *et al.* (2006) recently proposed a hypothesis of two-step activation of microglia *in vivo* in the PD brain. The observation on activated microglia associated with non-degenerating neurons and neurites in various brain regions such as the hippocampus in the early stage of PD suggests that microglia activated by the initiating factors of PD may be at first non-toxic and act for neuroprotection by producing neurotrophins, neurotrophic cytokines, and antioxidant substances in the first step. However, a toxic change in the activated microglia may occur as the second step to promote progression of the disease.

CONTRIBUTION OF JULIE AXELROD TO PD RESEARCH

Aside from his other numerous accomplishments, Dr. Julie Axelrod has made many great contributions also to PD research. One great contribution was his discovery of the reuptake of neurotransmitter catecholamines into the pre-synaptic nerve endings via membrane transporters and then from the cytoplasm to the synaptic vesicles via vesicular transporters (Axelrod *et al.*, 1959). This discovery provided a general principle for the termination of neurotransmission, and led to the identification of neurotransmitter transporters such as DA transporter (DAT) and NA transpoter (NAT), and vesicular monoamine transporters (VMAT), and to the

development of innovative drugs such as serotonin noradrenaline reuptake inhibitors as anti-depression drugs and of new diagnostic methods such as molecular imaging by PET (*p*ositron *e*mission *t*omography) or SPECT (*s*ingle *p*hoton *e*mission *c*omputed *t*omography) of synaptic function. Another great contribution is his discovery of catechol O-methyl transferase (COMT; Axelrod, 1957). Inhibitors of COMT are of great importance to the L-DOPA therapy of PD in combination with MAO B inhibitors such as deprenyl.

CONCLUSIONS AND FUTURE PROSPECTS

sPD is thought to be caused by the combination of a susceptible genetic background and various environmental factors. The biochemical analysis of postmortem brain from PD patients and neurotoxin-induced animal models indicates mitochondrial dysfunction and oxidative stress to be important. On the other hand, the causative genes of fPD indicate the accumulation of misfolded proteins due to UPS dysfunction to be important. It should be noted that both mitochondrial dysfunction and UPS dysfunction may be related to each other, and may trigger a common signal transduction pathway to programmed cell death. There are 2 types of programmed cell death, i.e., apoptosis and autophagy. Alpha-synuclein is degraded by both the ubiquitin–protesome pathway and the autophagy–lysosome pathway (Webb *et al.*, 2003). Much data on the pathogenesis of PD support the programmed cell death mechanism by apoptosis. However, this still remains controversial. The process of neuroinflammation may also be important, especially for the progression of PD. Sawada *et al.* (2006) has proposed a hypothesis of two-step activation of microglia in the brain and their toxic change in PD patients. In order to confirm this hypothesis, the following points remain to be proved: (1) the first and second causative stimuli must be identified; (2) a toxic change should be confirmed to occur *in vivo* in the nigro-striatum in PD models. A stimulus such as MPTP toxicity may directly produce degeneration of DA cells, and some signal from degenerating DA neurons may trigger activation of microglia, which may, due to the toxic change for producing neurotoxic cytokines, promote cell death of DA neurons, perpetuating a vicious circle. It is also possible that in fPD damaged DA neurons may send unknown signals to microglia to activate them. Thus, activated microglia producing neurotoxic cytokines may promote the progression of the disease in both sPD and fPD.

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Molecular Pathologies of and Enzyme Replacement Therapies for Lysosomal Diseases

Hitoshi Sakuraba[*,1,2], Makoto Sawada[1,3,4], Fumiko Matsuzawa[1,5], Sei-ichi Aikawa[1,5], Yasunori Chiba[1,6], Yoshifumi Jigami[1,6] and Kohji Itoh[1,7]

[1]*CREST, JST, Kawaguchi 332-0012, Japan*

[2]*Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo 113-8613, Japan*

[3]*Department of Brain Life Science, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan*

[4]*Tissue Targeting Japan, Inc., Nagoya 458-0039, Japan*

[5]*Celestar Lexico-Sciences, Inc., Chiba 261-8501, Japan*

[6]*Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8566, Japan*

[7]*Department of Medicinal Biotechnology, Institute for Medicinal Biotechnology, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770-8505, Japan*

Abstract: Lysosomal diseases comprise a group of inherited disorders resulting from defects of lysosomal enzymes and their cofactors, and in many of them the nervous system is affected. Recently, enzyme replacement therapy with recombinant lysosomal enzymes has been clinically available for several lysosomal diseases. Such enzyme replacement therapies can improve non-neurological disorders but is not effective for neurological ones. In this review, we discuss the molecular pathologies of lysosomal diseases from the protein structural aspect, current enzyme replacement therapies, and attempts to develop enzyme replacement therapies effective for lysosomal diseases associated with neurological disorders, i.e., production of enzymes, brain-specific delivery and incorporation of lysosomal enzymes into cells.

Keywords: Lysosomal enzyme, lysosomal disease, Tay-Sachs disease, Sandhoff disease, Fabry disease, protein structure, enzyme replacement therapy, drug delivery.

INTRODUCTION

Lysosomes are cytoplasmic vesicles that contain lysosomal enzymes and their cofactors including activators and stabilizing proteins. Many lysosomal enzymes are exohydrolases that are involved in the degradation of cellular materials including glycoconjugates. Gene defects of lysosomal enzymes and their cofactors cause "lysosomal diseases", which result in the accumulation of undegraded substrates in lysosomes as reviewed by Sakuraba [1]. Lysosomal diseases comprise a group of more than 40 different disorders as shown in Table **1**. Although the clinical presentations of lysosomal diseases are very heterogeneous, many of them involve neurological disorders with the exceptions of Gaucher disease type 1, Fabry disease cardiac type, Pompe disease, and so on [1,2].

Efforts have been made to develop therapies for lysosomal diseases. Although various experimental approaches including bone marrow transplantation, enzyme replacement, substrate-depletion and normal gene transfer have been made [3-7], enzyme replacement therapy is thought to be clinically effective for lysosomal diseases at present. Recombinant enzymes for enzyme replacement therapy have been produced in cultured mammalian cells including Chinese hamster ovary (CHO) cells and human fibroblasts, and some of them can be produced in the milk of mammals. They are clinically available for enzyme replacement therapy for Gaucher disease involving hepatosplenomegaly, anemia, thrombocytopenia, bone disorders (types 1, 2 and 3), psychomotor delay, muscular weakness, hypotonia, pseudo bulbar palsy, laryngeal spasm, supranuclear gaze palsy and strabismus (types 2 and 3) [8,9], Fabry disease involving pain, angiokeratoma, hypohidrosis, corneal opacities, vascular disorders, renal involvement (the classic type) and cardiac involvement (the classic type and the cardiac type) [10-13], mucopolysaccharidosis (MPS) I involving corneal opacities, dysostosis multiplex, organomegaly and mental retardation [14], Pompe disease involving cardiomegaly, hepatomegaly (the classic type) and muscular weakness and hypotonia (the classic type and the late onset type [15,16], and MPS VI involving corneal opacities and dysostosis mutiplex [17] as shown in Table **2**. Furthermore, an application for MPS II involving dysostosis multiplex (the severe type and the mild type) and mental retardation (the severe type) [18] has been submitted.

*Address correspondence to this author at the Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan; Tel: +81-3-3823-2105; Fax: +81-3-3823-6008; E-mail: sakuraba@rinshoken.or.jp

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Table 1. Lysosomal Diseases

Disease	Defect
GM1 gangliosidosis	β-galactosidase
GM2 gangliosidoses	
Tay-Sachs disease (B-variant)	β-hexosaminidase A
Sandhoff disease (O-variant)	β-hexosaminidases A and B
GM2 activator deficiency (AB-variant)	GM2 activator protein
Fabry disease	α-galactosidase
metachromatic leukodystrophy	arylsulfatase A
Krabbe disease (globoid-cell leukodystrophy)	galactocerebrosidase
Gaucher disease	glucocerebrosidase
Niemann-Pick disease types A and B	sphingomyelinase
Farber disease	ceramidase
Walman disease and cholesteryl ester storage disease	acid lipase
Pompe disease	α-glucosidase
fucosidosis	α-fucosidase
α-mannosidosis	α-mannosidase
β-mannosidosis	β-mannosidase
sialidosis	lysosomal sialidase
aspartylglucosaminuria	aspartylglucosaminidase
Schindler disease and Kanzaki disease	α-*N*-acetylgalactosaminidase
mucopolysaccharidoses	
type I (Hurler, Scheie)	α-L-iduronidase
type II (Hunter)	iduronate sulfatase
type IIIA (Sanfilippo A)	heparan *N*-sulfatase
type IIIB (Sanfilippo B)	α-*N*-acetylglucosaminidase
type IIIC (Sanfilippo C)	acetyl CoA:α-glucosaminide acetyltransferase
type IIID (Sanfilippo D)	*N*-acetylglucosamine 6-sulfatase
type IVA (Morquio A)	galactose 6-sulfatase
type IVB (Morquio B)	β-galactosidase
type VI (Maroteaux-Lamy)	arylsulfatase B
type VII (Sly)	β-glucuronidase
I-cell disease and pseudo-Hurler polydystrophy	UDP-*N*-acetylglucosamine: lysosomal enzyme *N*-acetylglucosamine 1-phophotransferase
prosaposin deficiency	prosaposin
metachromatic leukodystrophy-like storage disease (saposin B deficiency)	saposin B
Gaucher-like disease (saposin C deficiency)	saposin C
Galactosialidosis	protective protein/cathepsin A
Niemann-Pick disease type C	NPC1, NPC2
multiple sulfatase deficiency	arylsulfatases A, B and C
neuronal ceroid lipofuscinosis	
infantile type	lysosomal thioesterase
classical late infantile type	pepinase
juvenile type	lysosomal membrane protein
adult type	at least eight genes are involved
Salla disease and infantile free sialic acid storage disease	sialin

Table 2. Recombinant Lysosomal Enzymes for Enzyme Replacement Therapy

Enzyme	Drug	Targeted Disease	Company	Stage (Country)
glucocerebrosidase	imiglucerase	Gaucher disease	Genzyme	Approved (EU, USA, Japan)
glucocerebrosidase	GA-GCB	Gaucher disease	Shire	Phase I/II (-)
α-galactosidase	agalsidase beta	Fabry disease	Genzyme	Approved (EU, USA, Japan)
α-galactosidase	agalsidase alpha	Fabry disease	Shire	Approved (EU)
α-iduronidase	laronidase	MPS I	Biomarin/Genzyme	Approved (EU, USA)
α-glucosidase	algucosidase alpha	Pompe disease	Genzyme	Approved (EU, USA)
acid sphingomyelinase	-	Niemann-Pick disease B	Genzyme	Preclinical (-)
iduronate-2-sulfatase	idurosulfase	MPS II	Shire	Submitted (EU, USA)
arylsulfatase B	aryplase	MPS VI	BioMarin	Approved (EU, USA)

However, the present enzyme replacement therapies are effective for the improvement of non-neurological disorders but not for that of neurological disorders. Intravenously administered enzymes cannot be incorporated into the central nervous system because of the blood-brain barrier.

To develop enzyme replacement therapies for lysosomal diseases affecting the nervous system, understanding of their molecular pathologies and targeting of lysosomal enzymes to neuronal tissues are required.

In this review, we discuss the molecular pathologies of lysosomal diseases from the protein structural aspect and attempts to develop enzyme replacement therapies effective for lysosomal diseases associated with neurological disorders.

MOLECULAR PATHOLOGIES OF LYSOSOMAL DISEASES

We describe here the molecular pathologies of GM2 gangliosidoses including Tay-Sachs disease (B-Variant) and Sandhoff disease (O-Variant) as models of lysosomal diseases associated with neurological disorders. Lysosomal β-hexosaminidase (Hex, EC 3.2.1.52) is a glycosidase that catalyzes the hydrolysis of terminal *N*-acetylhexosamine residues at the non-reducing ends of oligosaccharides of glycoconjugates [19,20]. There are two major Hex isozymes in mammals including man, Hex A (αβ, a heterodimer of α- and β-subunits) and Hex B (ββ, a homodimer of β-subunits), and a minor unstable isozyme, Hex S (αα, a homodimer of α-subunits). All these Hex isozymes can cleave off terminal β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) residues, while only Hex A and Hex S prefer negatively charged substrates and cleave off the terminal *N*-acetylglucosamine 6-sulfate residues in keratan sulfate. Hex A is essential for cleavage of the GalNAc residue from GM2 ganglioside in co-operation with GM2 activator protein [19-21].

Tay-Sachs disease and Sandhoff disease are autosomal recessive GM2 gangliosidoses caused by mutations of *HEXA*, which encodes the Hex α-subunit on chromosome 15q 23-24, and *HEXB*, which encodes the Hex β-subunit on chromosome 5q13, respectively [19,20]. The genes exhibit sequence homology, and the gene products exhibit 57% similarity in amino acid sequence. In Tay-Sachs disease, a genetic defect of *HEXA* causes a deficiency of Hex A with excessive accumulation of GM2 ganglioside mainly in the nervous system including neurons of the cerebrum, cerebellum, spinal cord, dorsal root ganglion and visceral organs, resulting in progressive neurological disorders. In Sandhoff disease, an inherited defect of *HEXB* leads to simultaneous deficiencies of Hex A and Hex B with accumulation of GM2 ganglioside in the nervous system and of oligosaccharides carrying terminal GlcNAc residues at their non-reducing ends, resulting in systemic manifestations including hepatosplenomegaly as well as neurological manifestations.

Tay-Sachs disease and Sandhoff disease exhibit a spectrum of clinical phenotypes ranging from a severe infantile form to a milder late onset form, and many mutations have been identified for each gene [19,20]. Patients with the severe infantile form of GM2 gangliosidoses develop progressive psychomotor delay, muscular weakness, hypotonia, visual disturbance, cherry-red spot, seizures, and macrocephaly. Patients with the milder late-onset form of GM2 gangliosidoses develop dystonia, ataxia, incoordination, muscle wasting and weakness [19,20]. The incidence of Tay-Sachs disease is predicted to be 1 in 3,900 births in Jewish people and 1 in 320,000 births in non-Jewish people. On the other hand, the incidence of Sandhoff disease is deduced to be 1 in 1,000,000 births in Jewish people and 1 in 309,000 births in non-Jewish people [19]. Mutations in the GM2 activator protein gene *(GM2A)* result in a rare form of GM2 gangliosidosis, GM2 activator deficiency (AB-Variant) exhibiting the same clinical manifestations as the severe form of Tay-Sachs disease and Sandhoff disease [19,20].

Recently, the crystal structure of human Hex B was determined by Mark *et al.* [22] and then by Maier *et al.* [23]. This information prompted us to examine Tay-Sachs disease and Sandhoff disease from the protein structural aspect.

1. Three-Dimensional Structure of β-Hexosaminidase

According to the reports of Mark *et al.* [22] and Maier *et al.* [23], the β-subunit of Hex comprises two domains (domain I and domain II). Domain I has an α/β topology, and domain II is folded into a $(\beta/\alpha)_8$-barrel with the active site pocket at the *C*-termini of the β-strands. An extrahelix that follows the eighth helix of the $(\beta/\alpha)_8$-barrel is located between domain I and the barrel structure. A structural model of human Hex A (αβ heterodimer) has been constructed on

Table 3. Human β-Hexosaminidase α- and β-Subunits

	α-Subunit	β-Subunit
Signal peptide	Met1-Ala22	Met1-Ala42
Domain I	Leu23-Pro165	Ala43-Pro198
Domain II	Arg166-Thr529	Arg199-Met556
Processing site	Ser75-His88	Phe108-Lys121, Arg312-Lys315
Catalytic site	Asp207, His262, Glu323	Asp240, His294, Glu355
Disulfide bond	Cys58:Cys104, Cys277:Cys328, Cys505:Cys522	Cys91:Cys137, Cys309:Cys360, Cys534:Cys551
Glycosylation site	Asn115, Asn157, Asn295*	Asn84*, Asn142, Asn190, Asn327*

*Asn residue linked mannose undergoing glycosyl phosphorylation.

the structure of human Hex B by means of homology modeling method [24]. The human Hex α- and β-subunits have corresponding catalytic sites and three disulfide bonds. The α-subunit is predicted to have one processing site and three glycosylation sites, and the β-subunit two processing sites and four glycosylation sites (Table 3).

2. Structural Defect in Tay-Sachs Disease

The modeled structure of the wild-type Hex A (αβ heterodimer) and localization of representative amino acid substitutions (R170W, R178H, W420C, C458Y, L484P, R499C/H, and R504C/H) are shown in Fig. 1. Nine mutant structural models due to specific missense mutations were constructed, and compared with the wild-type model [24].

Among the mutations, R178H is deduced to affect the structure of the active site directly. The R178 residue is located close to the active site and is involved in substrate binding. R178H results in substitution of H for R178, which is an important residue for substrate binding. Moreover, R178H is thought to cause a conformational change of amino acid

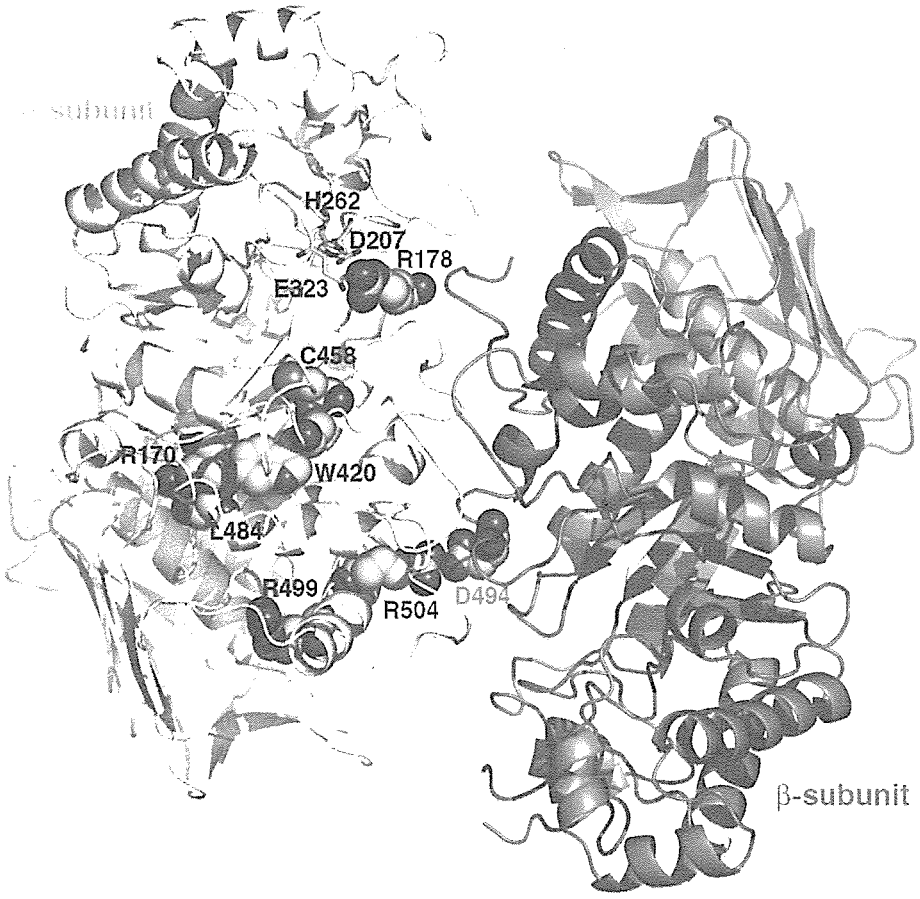

Fig. (1). Three-dimensional structure of human Hex A and residues involved in amino acid substitutions in the α-subunit.

A structural model of Hex A (αβ heterodimer) was constructed. Residues involved in the catalytic triad (D207, H262 and E323) are presented as ball-and-sticks models. Residues involved in amino acid substitutions in the α-subunit (R170, R178, W420, C458, L484, R499 and R504) and D494 in the β-subunit, which binds to R504 in the α-subunit, are presented as space-filling models.