outliers, a gradual exponential drift of the baseline was removed by using an exponential regression. Matlab was used for the data analysis.

2.4. Microdialysis

A guide cannula (outer diameter: 0.60 mm and inner diameter: 0.48 mm) was inserted stereotaxically into the rat striatum 1 week before the perfusion ($n = 5$). The probe was purchased from EICOM (Kyoto, Japan). The stereotaxic coordinates used were: (1) guide cannula: AP: 0, ML: +3, DV: +3.5 from the surface of the dura mater (Paxinos and Watson, 1986) and (2) microdialysis probe (into the striatum): AP: 0, ML: +3, DV: +6.5 from the surface of the dura mater. The length of the dialysis membrane was 3 mm, and the diameter was 0.22 mm.

The probes were perfused with artificial cerebrospinal fluid (in mM: 149 NaCl, 2.8 KCl, 1.2 $MgCl_2$, 1.2 $CaCl_2$, and 5.4 glucose; pH 7.3) at a flow rate of 1 μl/min. The dialysates were collected at 30 min intervals. The first five fractions were discarded, and the next one fraction (30 min) was collected for basal value determination, and the dialysates were then collected over a 2 h period after PEA injection. These were analyzed on line by using a high-performance liquid chromatography with the electrochemical detector (EICOM, Kyoto, Japan). The applied potential was 700 mV.

Monoamines and metabolites were separated on a C18 reverse-phase column (EICOMPAK SC-5ODS) (Nakamura et al., 1998). The flow rate was 2.3 ml/min. The mobile phase consisted of citric acid–sodium acetate buffer (pH 3.9), 5 mg/l EDTA, 160 mg/l sodium 1-octanesulphate and 18% methanol. Our HPLC method for monoamine detection was modified to measure ascorbate according to the previous studies (Miele et al., 2000; Serra et al., 2001). The sensitivity of the electrochemical detector was lowered temporally to quantitate a large peak with a short retention time (4 min) which was identical to ascorbate.

2.5. Histology

After the end of experiments (2 months after the implantation), three animals were deeply anesthetised with pentbarbital, and perfused transcardially with saline followed by perfusion with 10 mM phosphate-buffered 4% paraformaldehyde (pH 7.4). After postfixation, coronal sections of 30 μm in thickness were prepared with a cryostat. Serial sections of estimated position of the carbon fibre electrode were immunostained with glial fibrillary acidic protein (GFAP) for an enhanced view of glial response. The sections were treated with sheep anti-tyrosine hydroxylase (anti-TH) antibody (1:2000; Calbiochem, San Diego, CA) and rabbit anti-GFAP antibody (1:5000; DAKO Cambridgeshire, UK) in PBS with 0.05% Triton X-100 overnight. These sections were incubated in fluorescent secondary antibodies of FITC-conjugated anti-sheep IgG and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Low-poser fluorescence view of the section was obtained with a digital-fluorescence microscope (BIOREVO BZ-9000, KEYENCE, Osaka, Japan).

3. Results

3.1. In vitro measurements

The performance of the wireless system for electrochemical detection was evaluated *in vitro*. Dopamine (10^{-7} M) was added five times consecutively into PBS in the recording chamber (final concentration: 5.0×10^{-7} M). Dopamine signal current increased rapidly after each injection in a step-wise manner (Fig. 4). The limit of detection for dopamine in a single measurement was 2.7×10^{-7} M (S/N = 3).

3.2. In vivo measurement in the rat striatum

3.2.1. Changes in dopamine signal current induced by PEA

To validate dopamine detection by our wireless system *in vivo*, we examined the effect of PEA injection, well known to

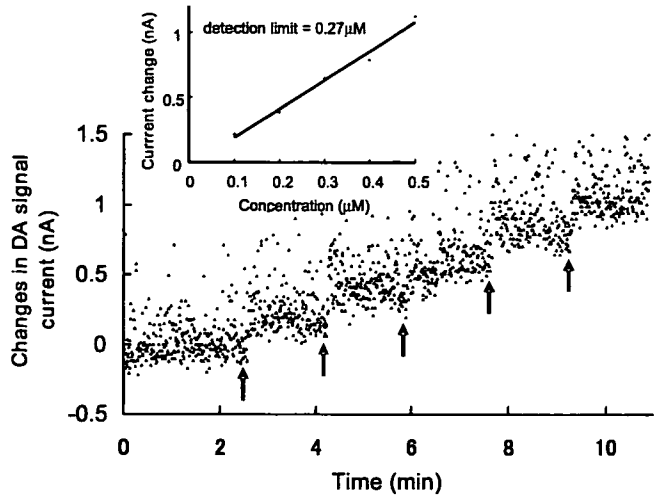

Fig. 4. Dopamine (DA) signal current responses *in vitro*. Dopamine solutions (10^{-4} M, 100 μl) were added five times (arrows) into 100 ml PBS. The final concentration increased with a step of 0.1 μM after each injection. Inset shows dose–response relationship. The detection limit in a single measurement was 0.27 μM (S/N = 3).

increase extracellular dopamine. The dopamine signal current in a representative case (Fig. 5A) increased soon after subcutaneous injection of PEA (100 mg/kg), reached a plateau at ~50 min, and began to decrease at ~100 min after the injection. Mean changes in dopamine signal current, averaged over five animals, showed a similar time course (Fig. 5B).

To compare the performance of the wireless system with our conventional wired voltammetry system, changes in dopamine signal current were also measured in the same rat as that of Fig. 5A using this conventional system after administration of PEA (Fig. 5C). The time course of the changes in dopamine signal current was very similar between the two voltammetry systems.

Using microdialysis, changes in dopamine concentration were also examined (Fig. 5D). Increase in dopamine concentration was observed (open circles), but its metabolite, DOPAC (open triangles) did not increase. It is worth noting that changes in dopamine concentration (Fig. 5D) were very similar to those in dopamine signal current measured with the wireless voltammetry system (Fig. 5B). Ascorbate (filled squares) showed a small increase but with a much slower time course.

To quantitatively evaluate whether the change in dopamine signal currents largely reflected changes of dopamine or those of ascorbate, we integrated the voltammetry signal over six consecutive 30 min periods up to 0, 30, 60, 90, 120 and 150 min. These integrated values were correlated with the concentrations of dopamine, and ascorbate measured with microdialysis at the six time points. Although the absolute concentration of dopamine (3.7 nM) was much smaller than that of ascorbate (4.6 μM), the correlation was close to one for dopamine concentration ($r = 0.95$, $p = 0.0033$) and 0.82 for ascorbate ($p = 0.045$). If the current signal exclusively reflects changes in ascorbate, but not dopamine at all, the correlation should have been smaller for dopamine, but this was not the case. Thus the present results suggest that the current signal dominantly reflected changes in dopamine concentration, at

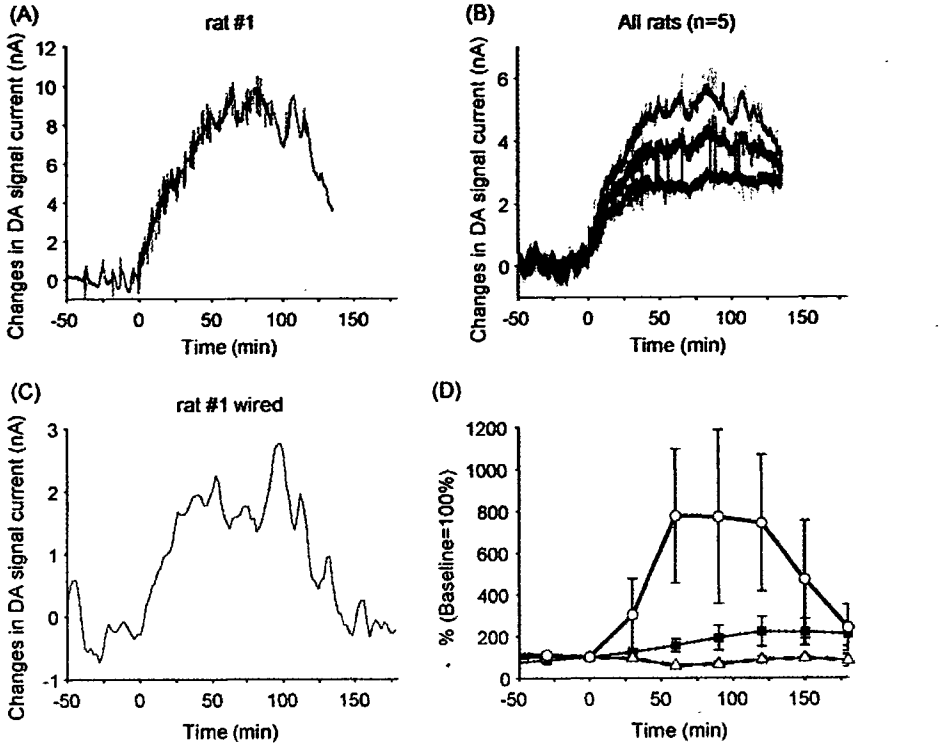

Fig. 5. In vivo measurements in the rat striatum after subcutaneous PEA administration (100 mg/kg). (A and B) Changes in dopamine (DA) signal current measured with the wireless system every 0.25 s. Typical data in a rat (140 days after operation), and the mean responses of five rats (7–140 days) are shown in A and B (red trace), respectively. Time zero shows the time of injection. Black traces in B shows the standard error of the mean. (C) Changes in dopamine signal current measured in the same rat as in A (120 days after operation) with a conventional wired system (every 2 min). (D) Changes in extracellular concentration of dopamine (○), DOPAC (△) and ascorbate (■) measured with microdialysis. Increase in dopamine concentration was observed, but DOPAC did not increase. Ascorbate showed a small increase. The absolute concentrations of these substances before injection were 3.7 nM, 1.38 μM, 4.63 μM, respectively. Note similar changes in dopamine signals across the four panels.

least during the initial period of rapid increase (0–30 min after the PEA injection) during which little increase of ascorbate was observed.

After the end of experiments (2 months after the implantation), we made serial sections of the striatum in three rats and double-stained the sections with an anti-GFAP antibody (astrocyte marker) and anti-tyrosine hydroxylase (anti-TH) antibody (dopamine neuron marker). Since the track of the carbon fibre electrode was not traceable exactly under microscopic observation, the location of the tip of the electrode was inferred from the deepest part of linearly stained GFAP positive cells (Fig. 6A). Although a small activation of glial cells along the track of the electrode was found (Fig. 6A and C), there was no change in the TH activity (Fig. 6B and D). The results clearly show that there was little, if any, damage to dopamine neurons after months of chronic measurements with the carbon fibre electrode.

3.2.2. Changes in dopamine signal current during the resident–intruder test

Changes in striatal dopamine signal current were measured in four resident rats while an intruder rat reared in a different home cage was introduced into the home cage of each resident rat for approximately 5 min (Fig. 2C). The intruder rat that carried no load often approached the resident rat, but both remained non-aggressive during the interaction. The dopamine signal current started to increase soon after introducing the intruder rat (Fig. 7). It reached a plateau approximately 2 min later, decreased gradually after the removal of the intruder, and returned to basal levels in approximately 5 min.

4. Discussion

The present report describes the construction of a wireless voltammetry system and its performance in high-speed electrochemical measurement, not only *in vitro*, but also in the striatum of unanesthetised behaving rats. We confirmed in both conditions that the performance of the wireless system was basically comparable with our conventional wired voltammetry system (Nakazato and Akiyama, 1999). We further showed that changes in the dopamine signal current after PEA injection correlated well with changes in dopamine concentration measured with microdialysis, but not or less with changes in those of DOPAC or ascorbate. The results of microdialysis were consistent with the previous report (Nakamura et al., 1998). We further demonstrated increase in the extracellular dopamine level after introducing an intruder to the resident rat. Although we admit that DOPAC may contribute to the measured signal current, it should be remarked that relative sensitivity of dopamine is 100 times as large as that of DOPAC (Nakazato and Akiyama, 1999). Therefore, we infer that increase of the signal current that occurred after introducing the intruder

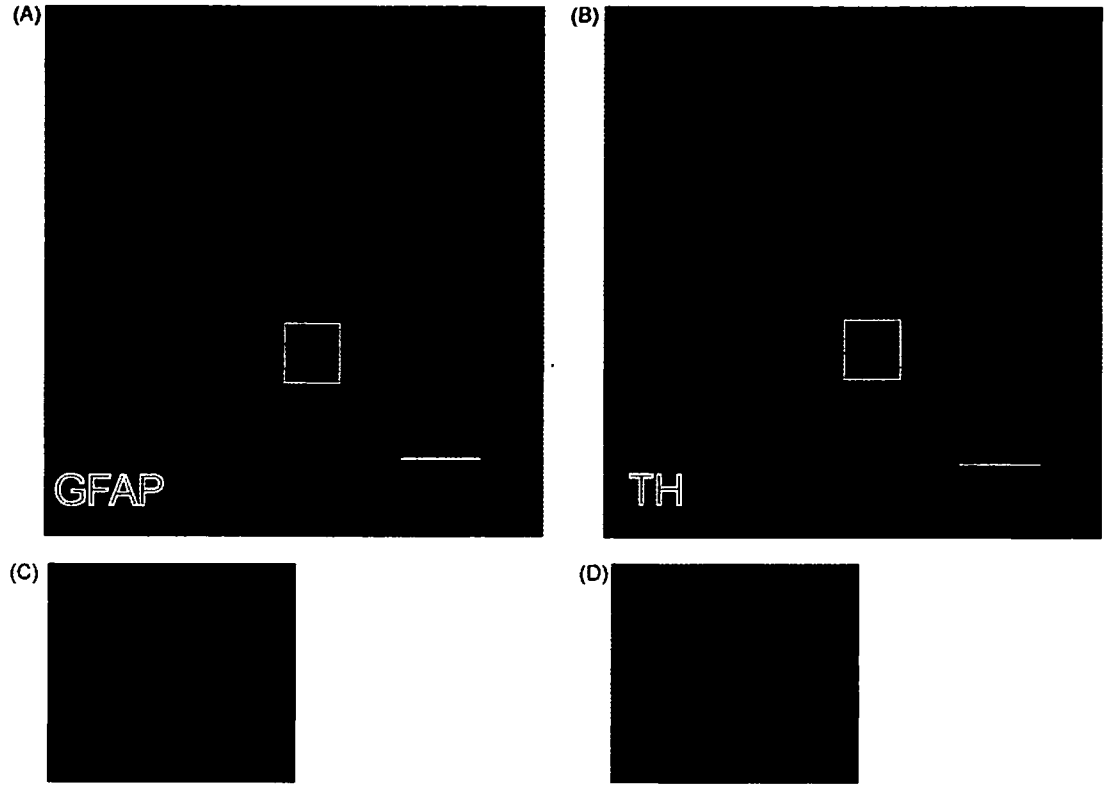

Fig. 6. Microphotograph of recording site in the striatum. The section was double immunofluorescence stained with anti-GFAP antibody (A and C) and anti-tyrosine hydroxylase (anti-TH) antibody (B and D). Higher magnification view of the area enclosed by the square (A and B) was shown in C and D. Although slight gliosis along the track of the carbon fibre recording electrode was found (A and C), there is no apparent decrease in the TH immunoreactivity (B and D). There was little, if any, damage to dopamine terminals after 2 months of chronic implantation. Bar = 1 mm (A and B).

largely reflected dopamine release, even if all released dopamine was converted into DOPAC thereafter. The results in resident–intruder test were in agreement with previous studies that reported increase in the extracellular dopamine levels in the dorsal (Robinson et al., 2002) and ventral (Louilot et al., 1986; Robinson et al., 2002; De Leonibus et al., 2006) striatum in response to the introduction of a conspecific. The

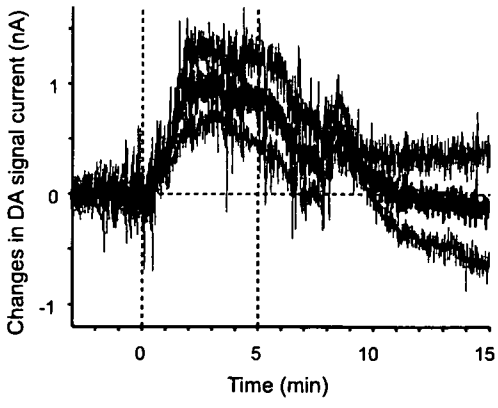

Fig. 7. Changes in dopamine (DA) signal current during a resident–intruder test (four rats). An intruder rat was introduced into the home cage of the resident rat at time zero and removed at 5 min (vertical broken lines). The red trace shows the mean dopamine signal current in the resident rat. Black lines show the standard error of the mean. The second peak around 9 min was contributed by a single rat and possibly reflects an artifact due to an abrupt movement of the rat. These experiments were performed 13–280 days after operations.

results show that the wireless system was able to detect increase in extracellular dopamine concentration.

4.1. Advantages of the present system

Comparing the present system with the two recently developed wireless voltammetry systems (Crespi et al., 2004; Garris et al., 2004), it is worth noting that ours is the only one that was actually applied for behaving animals. In addition, consistent responses were obtained from different animals over a period ranging from a week to several months after implantation of the recording electrodes. The results show that the present wireless system is especially suited for long-term repeated measurements over days or months, though recordings can be made intermittently due to the life-time of the battery. This long-term measurement became possible with the help of an activation pulses which were delivered before each measurement pulse. Presumably, continual activation pulses stabilize the carbon fibre response (Nakazato and Akiyama, 1999), although the physical effect on the carbon surface has not been well elucidated yet. This would be a great advantage for the experiment over several days or months.

Our wireless system was fully functional when it was applied on the behaving rats. Before our study, De Simoni et al. (1990) applied their wireless system on behaving rats, and the same system was successfully applied for examining the sleep–wake cycle in the serotonergic system (Imeri et al., 1994). While their

system used infrared radiation to achieve bidirectional wireless interconnections between the main unit and the satellite unit, we adopted a single-way transmission using radio waves. The infrared transmission is free from electric noise and is most advantageous in measuring concurrent electrophysiological signals like action potentials of single neurons (Imeri et al., 1994). However, the infrared transmission is more susceptible to interruptions with obstacles, like the other animal and the hand of the experimenter, than radio waves. In addition, the two-way design would cause not only the loss of data but also loss of functionality of the recording electrode as pointed out by Crespi et al. (2004). By using a single-way radio wave transmission, we were able to achieve measurement at 4 Hz, as compared to 0.01 Hz (once per 2 min) in the bidirectional system (Imeri et al., 1994), even in the existence of other animals. This advantage enabled us to detect rapid increase in the dopamine current that occurred within 2 min in the resident animal after introduction of an intruder.

4.2. Directions of improvement

Although we could successfully detect the changes in dopamine current in the striatum of behaving animals, there are still several limitations for its practical use. The present wireless system was still susceptible to interference from mechanical noise especially when the animals made vigorous movements like jumping. In addition, this system was a little too heavy (90 g) to allow the animal (350–400 g) to move as freely as when there is no load. Reduction of the weight is our remaining challenge because the continual activation pulses for long-term measurement requires a rather big battery. We are constructing a smaller model (Fig. 3B). The dimensions of the new model are 5.5 cm × 3 cm × 1.5 cm, and it weighs 44 g with a 4 h rechargeable battery, fitting easily onto the back of rats. We are expecting that the smaller model is more robust to the noise due to mechanical perturbations.

In conclusion, the present wireless voltammetry system should allow real-time examination of changes in dopamine in unanesthetised behaving and interacting animals repeatedly over periods as long as several months or more. This technology may have a key role in the study of acute responses in the brain social interaction and, moreover, the long-term changes in these responses.

Acknowledgements

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Parkin is expressed in vascular endothelial cells

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Abstract

Mutations in the parkin gene are related with early-onset Parkinson's disease. Parkin is identified as an E3-ligase that combines target proteins with ubiquitin. α-Synuclein and synphilin-1 are substrates for E3-ligase activity of parkin and considered to be involved in the pathogenesis of Parkinson's disease. We previously demonstrated both α-synuclein and synphilin-1 are expressed in vascular endothelial cells (VEC). In the present study, we addressed possible expression of parkin in VEC. Parkin immunoreactivity was detected in vascular endothelial cells in postmortem human brain. Expressions of parkin mRNA and protein in human umbilical vein endothelial cells (HUVEC) were demonstrated by reverse-transcription polymerase-chain reaction (RT-PCR) and western blotting. Expression of parkin in HUVEC was not altered with tunicamycin treatment, which exerts unfolded protein stress on cells. We conclude that parkin is expressed in VEC, and that unfolded protein stress may not regulate its expression.

Keywords: Parkin; Vascular endothelial cells; Tunicamycin

Mutations in parkin gene are implicated in autosomal recessive early-onset Parkinson's disease [4]. Parkin serves as E3 ubiquitin ligase [8] and thereby may regulate the proteasomal degradation of its target proteins. It has been reported that parkin ubiquitinates multiple proteins including parkin-associated endothelin-like receptor [2], a modified form of α-synuclein [9] and synphilin-1 [1]. Parkin is considered to be involved in the responses against unfolded protein stress through the quality control of target proteins [3]. Mutations of parkin gene may result in the accumulation of target proteins and lead to cell death of dopaminergic neurons in the substantia nigra.

Expression and function of parkin in cells of non-nervous tissues are not studied well. It has been reported that parkin is expressed in skeletal muscles [6] and in peripheral leukocytes [10]. However, there is no information about the expression of parkin in vascular endothelial cells. We have previously shown that α-synuclein [11] and synphilin-1 [12] are expressed in vascular endothelial cells. Both of these molecules are implicated in the pathogenesis of Parkinson's disease and potential targets of E3-ligase activity of the parkin protein. The present study was undertaken to address the expression of parkin in vascular endothelial cells.

We examined the cerebrum, cerebellum and brainstem from four autopsied patients, aged 53–73 years, who had no neurological diseases. Tissues were fixed with 10% formalin for 3 weeks and embedded in paraffin. Four-μm-thick sections were subjected to immunohistochemical procedure according to avidin–biotin–peroxidase complex method. The primary antibody was an anti-parkin polyclonal antibody, AB5112 (Chemicon, Temecula, CA, USA; diluted 1:100). The immunolabeled sections were counterstained with hematoxylin.

Consistent with previous studies [7,14], parkin immunoreactivity was observed in the neuronal cell bodies and processes in normal human brain (Fig. 1A). Parkin immunoreactivity was also detected in the walls of leptomeningeal vessels, and both endothelial and smooth muscle cells were intensely stained (Fig. 1B–D). Intraparenchymal vessels were stained less intensely and the capillary walls were negative for the immunoreactivity. Control experiments, using the secondary antibody only, did not show non-specific staining.

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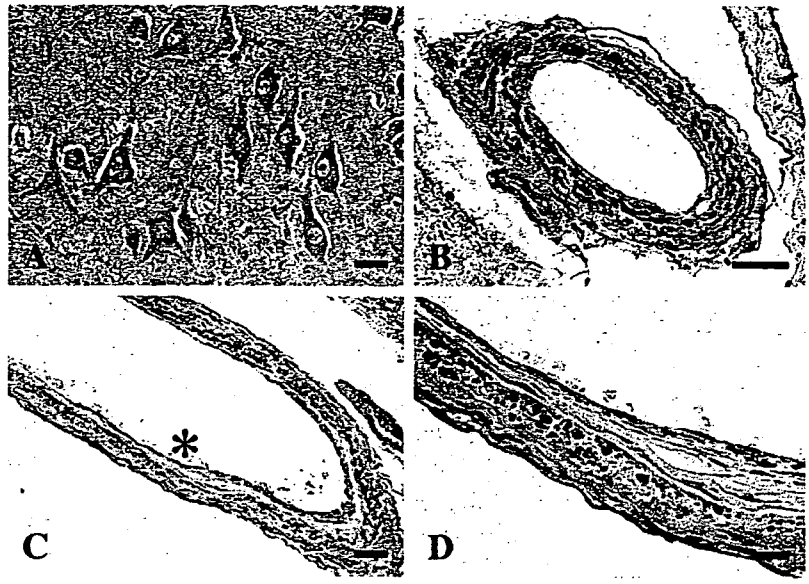

Fig. 1. Immunohistochemical identification of parkin in the brain. (A) The hippocampus with parkin immunoreactivity in the neuronal perikarya and proximal neurites. (B) An arterial blood vessel in the subarachnoid space showing parkin immunoreactivity. (C) A leptomeningeal artery showing parkin immunoreactivity. (D) A higher-magnification view of the area indicated by the asterisk in (C). The arterial wall shows intense immunoreactivity in the endothelium and smooth muscle layer. Scale bars, 20 μm for (A) and (D); and 50 μm for (B) and (C).

Next we examined the expression of parkin in cultured human umbilical vein endothelial cells (HUVEC). HUVEC were obtained from Cambrex (Walksville, MD, USA), and cultured as described previously [11,12]. The expression of parkin protein was examined by western blot analysis [3]. In untreated HUVEC, substantial amount of parkin protein, with molecular weight of about 51 K, was detected (Fig. 2A). It is reported that parkin is upregulated in response to unfolded protein stress [3]; however, there is controversy as to whether tunicamycin, which exerts unfolded protein stress to cells, alters parkin expression [5,13]. HUVEC were treated with 10 μg/ml of tunicamycin (BioMol, Plymouth Meeting, PA, USA) for up to 24 h. We found that treatment of HUVEC with tunicamycin did not affect the expression of parkin protein (Fig. 2A). The band with similar molecular weight was detected in the rat brain used as a positive control but not in BEAS-2B cells, a cell line derived form bronchial epithelial cells. BEAS-2B cells were transfected with full-length cDNA for parkin using an Effectene reagent (Qiagen, Hilden Germany), and a band of 51 K molecular mass was detected in parkin-transfected cells. We also examined the expression of parkin mRNA in HUVEC using semi-quantitative reverse-transcription polymerase-chain reaction (RT-PCR). The cDNA for a PCR template was synthesized, from 1 μg of total RNA isolated from cultures, using oligo(dT)$_{12-18}$ primer and M-Mulv reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA for parkin was amplified at the annealing temperature of 55 °C using *Taq* DNA polymerase (Qiagen) and the following two sets of oligonucleotide primers:

parkin-F1 (5′-AAGGAGGTGGTTGCTAAGCGAC-3′),
parkin-R1 (5′-CTCCCCTTCATGGTACGCTTC-3′),
parkin-F2 (5′-CCGGCTGACCAGTTGCGTG-3′),
and parkin-R2 (5′-CACCATACTGCTGGTACCGGTTG-3′).

First PCR was performed using primers F1 and R1 (30 cycles), and second nested PCR was with primers F2 and R2 (15 cycles). The products were analyzed by electrophoresis on a 1.2% agarose gel containing ethidium bromide. The

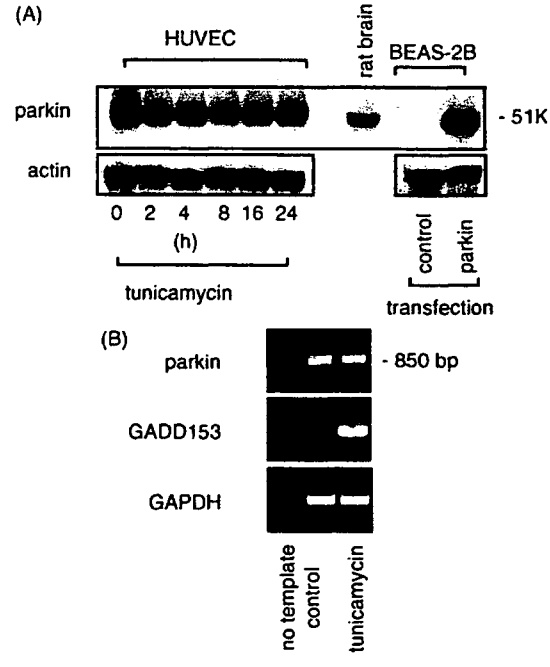

Fig. 2. (A) HUVEC were treated with 10 μg/ml tunicamycin for up to 24 h and the cells were lysed. BEAS-2B cells were transfected with full-length parkin cDNA, and the cells were lysed after 24 h of incubation. Lysates were subjected for the Western blot analysis for parkin or actin. As a positive control, rat brain homogenate was analyzed in a similar manner. (B) HUVEC were treated with 10 μg/ml tunicamycin for 8 h and RNA was extracted. RT-PCR analysis for parkin, GADD153 and GAPDH were performed.

850 bp product, which corresponds to full-length parkin, was obtained from HUVEC, and the expression of parkin mRNA was not altered by tunicamycin (Fig. 2B). The specific cDNA for growth arrest DNA damage 153 (GADD 153) was also amplified as a positive control for unfolded protein stress induced by tunicamycin. The cDNA for GADD153 or glyceraldehide-3-phosphate dehydrogenase (GAPDH) was amplified 30 times at the annealing temperature of 55 °C. The sequences of the primers used were as follows:

GADD153-F (5′-GCACCTCCCAGAGCCCTCACTCTCC-3′),
GADD153-R (5′-GTCTACTCCAAGCCTTCCCCCTGCG-3′),
GAPDH-F (5′-CCACCCATGGCAAATTCCATGGCA-3′),
and GAPDH-R (5′-TCTAGACGGCAGGTCAGGTCCACC-3′).

The size of amplified cDNA products for GADD153 and GAPDH was 422 and 598 bp, respectively. The expression of GADD 153 mRNA was enhanced by tunicamycin treatment (Fig. 2B). Thus parkin is expressed in HUVEC, and unfolded protein stress may not affect, at least in vascular endothelial cells, the expression of parkin. This may agree with the fact that parkin mutations are not associated with any particular vascular disorders. We also examined the effect of hypoxia or proinflammatory cytokines on the parkin expression in HUVEC; however, parkin expression was not affected by these treatments (not shown). In skeletal muscle cells, parkin may play a role in maintaining mitochondrial homeostasis and regulating β-amyloid accumulation [6]. There is no reason to excrude the similar role for parkin in vascular endothelial cells. Vascular endothelial cells also express α-synuclein [11] and synphilin-1 [12]; and parkin may play a role, as E3 ubiquitin ligase, in controlling the intracellular accumulation of these proteins. However, Lewy bodies have not been found in endothelial cells, and the role of parkin in Parkinson's disease might not directly relevant to endothelial parkin. Lewy body formation may necessitate some mechanisms specific to nervous tissues, or parkin may play unknown roles in endothelial cells. In order to examine the function of endothelial cell parkin, we tried to transfect HUVEC with full-length parkin cDNA or siRNA against parkin; but none of these were successful so far. Although the present study does not define the role of endothelial parkin, understanding of regulation and function of its expression in endothelial cells may provide important insights about the biological significance of parkin not only in vascular disorders but also in nervous diseases.

In conclusion, we found that parkin is constitutively expressed in vascular endothelial cells, and the expression of parkin may not be regulated by unfolded protein stress in this cell type.

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Review

Mitochondrial dysfunction in Parkinson's disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders characterized by resting tremor, rigidity, and bradykinesia. The primary cause of PD is still unknown, but oxidative stress and mitochondrial dysfunction have been implicated as important contributors to neuronal death in substantia nigra (SN) of PD. Considering neurons as post-mitotic cells, neurons could have error-avoiding mechanism against oxidative DNA damage. Indeed, several DNA repairing enzymes such as MTH1, OGG1, and MUTYH express in human brain. All the three enzymes up-regulated in the SN of PD patients, suggesting these three enzymes cooperate in mitochondrial DNA repairing in PD brain.

Keywords: Parkinson's disease; Oxidative stress; Mitochondrial dysfunction; MPTP; MTH1; OGG1; MUTYH

1. Introduction

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders with a prevalence of 2% in the population over the age of 65 (de Rijk et al., 1997). Neuropathologically, PD is characterized by loss of dopaminergic neurons in the substantia nigra (SN) and appearance of Lewy bodies in the remaining neurons. Although the primary pathogenesis of PD remains unknown, there has been growing environmental and genetic evidences that mitochondrial dysfunction and oxidative stress contribute to pathogenesis of PD. Since discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as environmental factor, a number of studies have revealed that mitochondrial dysfunction(s) play important roles in the selective dopaminagic neuronal loss (Langston et al., 1983; Heikkila et al., 1984). In addition, the identification of genetic mutations in several familial forms of PD has given new insights into the mechanism of neuronal loss in the SN. Interestingly, DJ-1 and PINK1, which have been identified from autosomal recessive form of PD, are considered to affect mitochondrial function and oxidative stress (Bonifati et al., 2003a,b; Valente et al., 2004).

2. Environmental evidences of mitochondrial dyfunction in PD

Mitochondria are intracellular organelles in which ATP is synthesized and such synthesis requires oxygen. In the mid 1980s, accidental injection of MPTP by heroin addicts led to find that MPTP causes parkinsonism in human (Davies et al., 1979; Langston et al., 1983). MPTP toxicity produces loss of dopaminegic neurons in a similar distribution to the pathology of PD. This discovery has given to great insight into research of mitochondrial dysfunction in the PD patients. MPTP is converted to 1-methyl-4-phenyl-pyridinium ion (MPP^+) by glial monoamine oxidase B in brain and then MPP^+ is selectively taken up into neurons via dopamine transporter. Within neurons, MPP^+ enters mitochondria and inhibits complex I of mitochondrial respirator chain (Heikkila et al., 1984; Mizuno et al., 1987). Furthermore, other environmental neurotoxins such as rotenone and paraquart are also inhibitors of complex I and able to induce dopaminergic loss. The inhibition of complex I induce energy crisis and generation of free

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radicals, resulting in neuronal death in the SN. Actually, complex I deficiency was identified in the SN of postmortem sporadic PD brain (Schapira et al., 1989).

3. Genetical evidences of mitochondrial dysfunction in PD

Mitochondria are under the control of two genomes: nuclear and mitochondrial DNA (mtDNA). Since mtDNA encodes 22 transfer RNA, 12S and 16S ribosomal RNA, and several subunits of electron transport chains, the genetic variation at the mitochondrial genome may contribute to the risk of developing PD. Previously, Shoffner and coworker reported that frequency of A4336G mutation in the tRNA(Gln) gene was higher in patients with PD and Alzheimer's disease than in age-matched controls (Shoffner et al., 1993). Recent polymorphism study also demonstrated that mtDNA belong to haplotypes J and K reduced the incidence of PD (van der Walt et al., 2003). Indeed, ρ^0 cells fused with mtDNA from PD patients have been showed 25% decreased complex I activity (Gu et al., 1998). It is one of evidences that suggest mtDNA affect mitochondrial function.

In nuclear gene, PINK1 (PARK6) and DJ-1 (PARK7), that are associated with mitochondrial protein and oxidative stress, have been identified from autosomal recessive PD (Bonifati et al., 2003a; Valente et al., 2004). Firstly, PINK1 gene encodes a 581-amino acids protein, consisted of mitochondrial targeting motif in the N-terminal (Valente et al., 2004). Indeed, transfected PINK1 located in mitochondria of cultured cells (Valente et al., 2004). The exact function of PINK1 protein is unknown, but PINK1 has high degree of homologous domain to the serine/threonine of the Ca^{2+}/calmodulin family. Since loss of PINK1 function cause PD, PINK1 may protect nigral neurons from mitochondrial dysfunction and apoptosis. In fact, PINK1 inhibited by siRNA induces apoptosis in SH-SH5Y cells (Deng et al., 2005). In vivo studies, a drosophila model with inactivated orthologue of human PINK1 showed apoptotic muscle degeneration and male sterility because of mitochondrial dysfunction (Clark et al., 2006; Park et al., 2006). Secondary, the human DJ-1 protein consists of 189 amino acids and belongs to the Thi/PfpI family (Bonifati et al., 2003b). Previous studies revealed that overexpression of DJ-1 protects against oxidative injury and DJ-1 knockdown enhances the susceptibility to oxidative stress, suggesting DJ-1 may have a role as antioxidant and redoxsensor (Bonifati et al., 2003a). Furthermore, DJ-1-deficient mice showed hypersensitivity to MPTP and oxidative stress (Kim et al., 2005). Further findings of these two gene products indicate the hypothesis that mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of PD.

4. Oxidative stress in PD

Since over 95% of all the oxygen taken up in the lung used in mitochondria, major source of free radicals is electron transport chain in mitochondria. Recent analysis estimates that about 1–2% of total molecular oxygen converted into reactive oxygen species (ROS) (Cadenas and Davies, 2000). Moreover, one of characteristic ROS origins in the nigral neurons is from dopamine metabolism. Cytosolic dopamine produces electrophilic semiquinones and quinones which themselves can act as oxidants by forming ROS (Sulzer and Zecca, 2000). In this point of view, antioxidant system is important for nigral neurons to keep nigral function.

Oxidative stress is another important factor in the pathogenesis of PD. The ROS are highly reactive and induce oxidative damage to various cellular components, such as proteins, lipids, and nucleic acids. The several postmortem studies suggest that presence of oxidative damage in the nigral neurons of PD patients. In particular, increased iron, oxidation of proteins, and lipid peroxidation in the SN appear to be important findings of oxidative stress (Dexter et al., 1987; Yoritaka et al., 1996; Alam et al., 1997b). In human tissue including brain, there are several antioxidant systems. For examples, superoxide dismutase (SOD), responsible for converting superoxide into H_2O_2 (Fig. 1A) is one of antioxidant systems. Interestingly, MnSOD, which contributes to metabolism of superoxide in mitochondria, have also been increased in PD (Saggu et al., 1989). In addition, glutathione peroxidase (GPX) catalyzes H_2O_2 to H_2O using glutathione (GSH) (Fig. 1A). Reduced levels of GSH in the midbrain may be indicative of increased free radical levels (Sian et al., 1991). These findings suggest that oxidative stress is increased in PD patients.

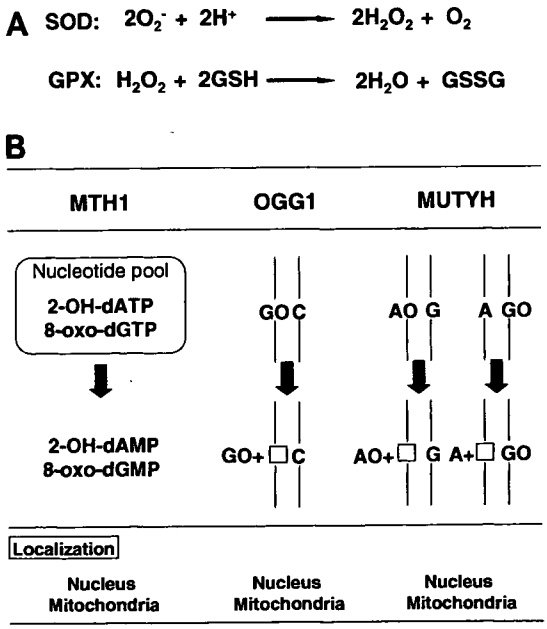

Fig. 1. (A) Antioxidant systems. SOD, superoxide dismutase; GPX, glutathione perxidase; GSH, glutathione; GSSG, oxidized glutathione. (B) Repair enzymes in oxidized DNA. G, guanine; A, adenine; GO, 8-oxoguanine; AO, 2-hydroxyadenine; see text for details.

5. DNA repairing enzymes in PD

Oxidized DNA such as 8-oxoguanine is so mutagenesis that induce G:C to T:A transversion mutation. MtDNA is more vulnerable to oxidative stress than nuclear DNA, because mtDNA is directly exposed to ROS leaked from electron transport chain and the lack of a histone coat for mtDNA. Since mtDNA encodes several components of respiratory chain, accumulation of mtDNA mutation could induce mitochondrial dysfunction(s). Indeed, the levels of 8-hydroxyguanine and 8-hydroxy-2-deoxyguanosine, one of the oxidized forms of guanine, are increased in the SN of PD brains (Alam et al., 1997a; Shimura-Miura et al., 1999; Zhang et al., 1999). Therefore, mtDNA repair enzymes are very important to maintain mitochondrial function(s). There are three major enzymes associated with error-avoiding mechanisms against oxidative DNA damage: MTH1, OGG1, and MUTYH (Fig. 1B). We previously investigated the expression levels of these three enzymes in the PD patients.

First enzyme is MTH1, which maintains lower spontaneous mutation rate by sanitizing the intracellular nucleotide pools. MTH1 hydrolyzes oxidized purine neucleoside triphosphates such as 8-oxo-7,8-dihydro-2′-deoxyguanosine triphosphate (8-oxo-dGTP), thereby protects cell damage caused by their misincorporation into DNA (Fig. 1B) (Fujikawa et al., 1999). Previously, we performed immunohistochemical examination for MTH1 in the SN of PD patients and control subjects. The results demonstrated that MTH1 was markedly increased in the SN of patients with PD, especially within mitochondria (Fig. 2A and B) (Shimura-Miura et al., 1999). Recent report revealed that MTH1-null mice exhibited a greater accumulation of 8-oxoG in mitochondrial DNA accompanied by a more significant decrease TH and dopamine transporter (DAT) immunoreactivities in striatal terminal fibers of dopamine neurons after chronic exposure to MPTP (Yamaguchi et al., 2006). These findings indicate that MTH1 protects the dopamine neurons from oxidative damage in the nucleic acids, especially in the mitochondrial DNAs of striatal nerve terminals of dopamine neurons.

Second enzyme is 8-oxoguanine DNA glycosylase (OGG1), which removes 8-oxoguanine paired with cytosine in DNA to avoid accumulation of oxidative damage in DNA (Fig. 1B). In human tissue, there are two major isoforms of OGG1 (Nishioka et al., 1999). While OGG1-1a contributes to repair DNA in nucleus, OGG1-2a is associated with repairing mitochondrial DNAs. We examined immunohistochemical and biochemical study for OGG1-2a in the PD patients and found that higher expression levels of OGG1-2a in the SN of PD patients compared with aged-matched control subjects (Fig. 2C and D) (Fukae et al., 2005). The most plausible explanation is that OGG1-2a is up-regulated in the PD patients secondary to mtDNA oxidative damage to neurons to protect neurons from mutagenesis. Indeed, overexpression of OGG1 within the mitochondria enhances the repair of mtDNA errors and rescues the cells from oxidative stress in the cultured cell experiments (Dobson et al., 2000; Rachek et al., 2002). Furthermore, expression of OGG1-2a was greatly increased in early stage but not in advance stage of PD patients. These findings indicate that a different time course of compensatory mechanism of mtDNA oxidation.

Third enzyme is human MutY homolog (hMUTYH), which has been identified as adenine DNA glycosylase. MUTYH excises adenine misincorporated opposite 8-oxoG during replication (Fig. 1B) (Ohtsubo et al., 2000). The hMUTYH was up-regulated in the mitochondria of the SN of PD patients (Arai et al., 2006) (Fig. 2E and F). The levels of all three enzymes were increased in the SN

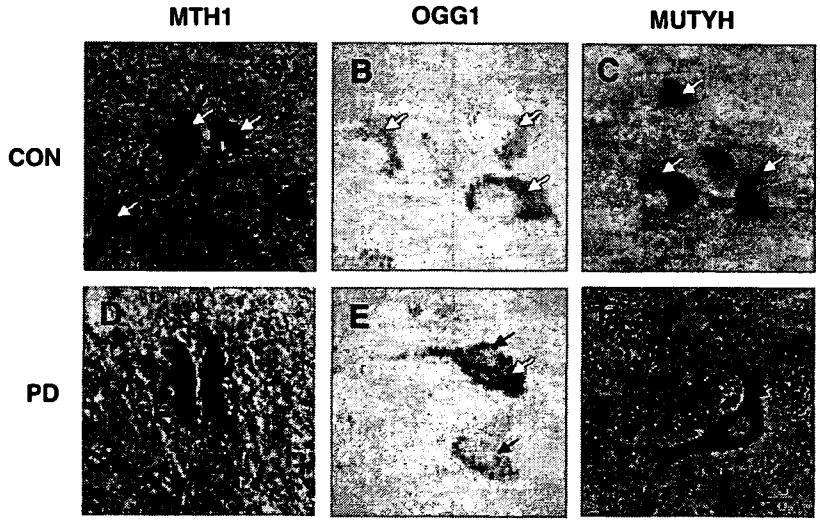

Fig. 2. Immunohistochemistry for MTH (A and D), OGG1 (B and E), and MUTYH (C and F) in the SN. A–C Control subjects; D–F PD; Note homogeneous staining for MTH1 and MUTYH (black arrows) and the granular staining for OGG1 (black arrows) in the cytoplasm of SN neurons in PD but control shows no staining. Neuromelanin (white arrows). Bar 10 μm.

of PD patients, suggesting that the DNA repair enzymes could play the protective roles against oxidative stress within the mitochondria of the SN in PD.

6. Conclusion

Although primary cause of sporadic PD is still unknown, multiple factors could be associated with nigral neurons degeneration in the PD. Mitochondrial dysfunction and oxidative stress may contribute to protein aggregation and impairment in protein degradation. To avoid accumulation of mtDNA somatic mutations could maintain mitochondrial function(s). The levels of the enzymes, hMTH1 and hOGG1, and hMUTYH, were increased in the SN of PD patients, suggesting that these three enzymes cooperate in the mitochondrial DNA repairing and beneficial targets for gene therapies aiming at neuroprotecion in PD.

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p53 protein, interferon-γ, and NF-κB levels are elevated in the parkinsonian brain

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Abstract

We and other workers found markedly increased levels of proinflammatory cytokines and apoptosis-related proteins in parkinsonian brain. Although the pathogenesis of Parkinson's disease (PD) remains enigmatic, apoptosis might be involved in the degeneration of dopaminergic neurons in PD. To investigate the possible presence of other inflammatory cytokines and/or apoptosis-related protein, the levels of p53 protein, interferon-γ, and NF-κB were measured for the first time in the brain (substantia nigra, caudate nucleus, putamen, cerebellum, and frontal cortex) from control and parkinsonian patients by a highly sensitive sandwich enzyme-linked immunosorbent assay. The p53 protein level in the caudate nucleus was significantly higher in parkinsonian patients than in controls ($P < 0.05$), whereas this protein in the substantia nigra, putamen, and cerebral cortex showed no significant difference between parkinsonian and control subjects. The interferon-γ level was significantly higher in the nigrostriatal dopaminergic regions (substantia nigra, caudate nucleus, and putamen) in parkinsonian patients than in the controls ($P < 0.05$), but was not significantly different in the cerebellum or frontal cortex between the two groups. In accordance with previous immunohistochemical analysis, the NF-κB level in the nigrostriatal dopaminergic regions was significantly higher in parkinsonian patients than in the controls ($P < 0.05$). These data suggest that the significant increase in the levels of p53 protein, interferon-γ, and NF-κB reflect apoptosis and the inflammatory state in the parkinsonian brain and that their elevation is involved in the degeneration of the nigrostriatal dopaminergic neurons.

Keywords: Parkinson's disease; Brain; p53 protein; Interferon-γ; NF-κB

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the nigrostriatal pathway. Although various treatments are successfully used to alleviate the symptoms of PD, none of them prevents or halts the neurodegenerative process of the disease. Neuronal death in PD may originate from the reciprocal interactions of a restricted number of conditions, such as mitochondrial defects, oxidative stress, and protein mishandling in the nigrostriatal pathway. In PD, nigrostriatal dopaminergic neurons are progressively depleted, but the surrounding astrocytes and microglia remain intact. Since these cells, especially activated microglia, have the potential to produce proinflammatory cytokines, several neurotrophic factors, and apoptosis-related proteins, changes in the activity and/or expression of them may explain both the loss of dopaminergic neurons and the compensatory mechanisms at play in the progression of PD [27].

One hypothesis concerning the cause of degeneration of the nigrostriatal dopaminergic neurons is that PD is caused by programmed cell death (apoptosis) due to increased levels of cytokines, apoptosis-related proteins and/or to decreased levels of neurotrophins [12,26]. Several findings obtained recently indicate that neuroinflammation and apoptosis may contribute to the pathogenesis in PD [25–27]. We and other workers found markedly increased levels of cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, transforming growth factor (TGF)-α, and TGF-β1, and decreased levels of neurotrophins such as brain-derived neurotrophic factor and nerve growth factor, but not glial-derived neurotrophic factor, in the nigrostriatal dopamine regions and

Abbreviations: CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; PD, Parkinson's disease; PMSF, phenylmethylsulfonylfluoride; TGF, transforming growth factor; TNF, tumor necrosis factor

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ventricular and lumbar cerebrospinal fluid of PD patients [7,16–19,20,22,23]. In addition, the levels of apoptosis-related proteins such as TNF-α receptor R1 (p55), bcl-2, soluble Fas, and the activities of caspase-1 and caspase-3 were also elevated in the nigrostriatal dopaminergic regions in PD [13,14,23].

p53 protein is a transcription factor that has a major role in determining cell fate in response to DNA damage at a relatively early stage. Several studies have indicated an alteration of the p53 protein level in the dopaminergic region of animals of a parkinsonian model produced by 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [1,4,5,26]. We speculate that negative modulation of the p53 protein system, which promotes apoptotic neuronal death [1,4], may have a protective effect against the toxicity of these factors and associated oxidative stress in the parkinsonian brain in vivo.

Furthermore, changes in lymphocyte populations in the cerebrospinal fluid and blood, in immunoglobulin synthesis, and in the production of cytokines and acute-phase proteins have been observed in patients with PD. In this regard, PD patients were reported to exhibit a lower frequency of infections and cancer, suggesting that stimulation of the immune system may occur in them [3]. Cell-surface expression of MHC class I molecules depends on the production of β2-microglobulin, which is noncovalently bound to MHC class I heavy chain for functional presentation of antigenic peptides to CD8 T-cells. The most potent inducer of MHC antigens is interferon-γ, which is produced by activated T-cells and NK cells. Although we previously reported elevated levels of β2-microglobulin in the dopaminergic region of parkinsonian patients [15], which is in accordance with a report on its expression in nigral dopaminergic neurons in the rat [11], only a single report on the levels of interferon-γ in parkinsonian brains has appeared [7]. Interestingly, Hunot et al. [6] demonstrated by use of immunohistochemical analysis that nuclear translocation of NF-κB, as a key factor in immuno-responses and inflammation also increased in the nigral dopaminergic region of the parkinsonian brain.

To investigate further possible presence of apoptosis-related proteins and inflammation-related proteins in the nigrostriatal dopaminergic region of PD patients, we determined immunochemically the contents of p53 protein, interferon-γ, and NF-κB in the postmortem brain tissues (substantia nigra, caudate nucleus, putamen, cerebellum, and frontal cortex) from such patients.

The present study was approved by the ethics committees of Aichi-Gakuin University, School of Pharmacy, and was conducted in accordance with the declaration of Helsinki on Biomedical Studies Involving Human Subjects (WMA, 1997). All PD cases were clinically and pathologically diagnosed. The controls had no known history of neurological illness and no pathological abnormalities. Control human brains (14 cases) and parkinsonian brains (15 cases) were obtained at autopsy, as described in our previous report [21–23]. Controls were age- and sex-matched with the patients. The control group consisted of six males and eight females with a mean age of 60.8 (range, 24–82) years. The parkinsonian group included 12 males and 3 females with a mean age of 69.7 (range, 51–84) years. The mean duration of PD was 16.9 years (5–29 years). The postmortem time at collection was 9.5 h (range, 3–18 h) for the control, and 6.5 h (4–13 h) for the parkinsonian group. The causes of death were as follows: pneumonia (11 cases), pyothorax (1 case), cirrhosis (1 case), cancer of the esophagus (1 case), and gastric cancer (1 case) in PD patients; and pneumonia (9 cases), gastric cancer (1 case), renal failure (1 case), rectal cancer (1 case), bacterial meningitis (1 case), and carcinoma of the small intestine (1 case) in the controls. The substantia nigra, caudate nucleus, putamen, cerebellum, and frontal cortex were dissected and stored frozen at −80 °C. Due to limited tissue, not all brain areas were available for each patients; therefore, sample numbers were not always the same for each brain area examined. Brain tissues were homogenized in 0.32 M sucrose containing protease inhibitors (100 μM phenylmethylsulfonylfluoride [PMSF] and 50 μg/ml of each of leupeptin, pepstatin, and antipain), and were lysed with lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, 2 mM PMSF, and 10 mM N-2-hydroxyethypiperadine-N-2-ethanesulfonic acid; pH 7.5).

The contents of p53 protein and interferon-γ in aliquots of the extracts were determined by use of highly sensitive enzyme-linked immunosorbent assays (ELISA) from Nichirei (Tokyo, Japan) and Endogen (MA, USA), respectively. According to the protocol for the p53 protein ELISA, the data were shown as the mean ± S.E.M. (A.U./mg protein). The NF-κB content in the extracts was also determined by use of a commercially available assay kit (Active Motif North America, CA, USA). Nuclear protein extract was prepared by a standard protocol. The protein concentration of the extracts was estimated by the method of Bradford, with bovine serum albumin used as a standard [2]. According to the protocol, the data on NF-κB were presented as the mean ± S.E.M. ($OD_{450\ nm}$/mg protein in the nuclear extract). Each experiment was repeated three times. Differences between control and parkinsonian patients were evaluated by the Mann–Whitney *U*-test.

The levels of apoptosis-related protein p53, protein in the brains from control and parkinsonian patients are shown in Table 1. The level in the caudate nucleus of the parkinsonian brains was significantly higher than that in the control subject ($P < 0.05$). On the other hand, p53 protein level in other dopaminergic regions (putamen and substantia nigra) and non-dopaminergic regions (cerebellum, and frontal cortex) was similar between the two groups.

The level of the inflammatory cytokine interferon-γ in the brains is shown in Table 2. The interferon-γ level in

Table 1
p53 protein content in the brains from control and parkinsonian patients

Brain region	p53 protein concentration (A.U./mg protein)	
	Control patients	Parkinsonian patients
Caudate (17)	ND	3.34 ± 2.35*
Putamen (6)	ND	0.20 ± 0.13
Substantia nigra (19)	0.47 ± 0.31	0.23 ± 0.09
Cerebellum (5)	2.58 ± 1.52	0.72 ± 0.57
Frontal cortex (5)	0.15 ± 0.11	ND

Each value represents the mean ± S .E.M. Significantly different from controls, *$P < 0.05$. ND, not detectable.

Table 2
Interferon-γ content in the brains from control and parkinsonian patients

Brain region	Interferon-γ concentration (pg/mg protein)	
	Control patients	Parkinsonian patients
Caudate (17)	138.2 ± 17.1 (100)	255.6 ± 21.6 (185)*
Putamen (6)	152.5 ± 23.1 (100)	295.7 ± 55.8 (194)*
Substantia nigra (19)	170.2 ± 19.5 (100)	266.3 ± 19.0 (156)*
Cerebellum (5)	146.5 ± 27.9 (100)	138.7 ± 23.1 (95)
Frontal cortex (5)	128.5 ± 20.4 (100)	123.3 ± 14.7 (96)

Each value represents the mean ± S.E.M. and percent of controls are shown in parentheses. Significantly different from controls, $^{*}P<0.05$.

the postmortem brains was relatively high (pg/mg protein) in comparison with that of other cytokines and growth factors [16–18,20–22], and the level in the dopaminergic regions (caudate nucleus, putamen, and substantia nigra) of the parkinsonian brains was significantly higher than that in those of the control brains ($P<0.05$). On the other hand, the level in the non-dopaminergic regions was similar in both groups.

Finally, the levels of NF-κB, which is a key factor for inflammation and apoptosis, in the brains from control and parkinsonian patients are shown in Table 3. NF-κB protein is abundantly expressed in various cell types of the nervous system, including neurons [10]. In accordance with a previous report [6], the NF-κB level in the dopaminergic regions (substantia nigra, caudate nucleus, and putamen) of the parkinsonian brains was significantly higher than that in those of the control ones ($P<0.05$). On the other hand, the level in non-dopaminergic regions was lower than the limit of sensitivity in either group.

Although we have no direct evidence that the increased level of interferon-γ is connected with the increased level of p53 protein (correlation coefficient: $r=0.078$) and/or NF-κB ($r=0.105$) in the parkinsonian brain, the cumulative interaction of cytokines with both factors may be involved in the pathogenesis of PD. In fact, we found a significant positive correlation between interferon-γ and NF-κB levels ($r=0.752$).

Although the results of previous studies suggested the involvement of inflammation-related and apoptosis-related factors in the pathophysiology of PD in animal models and in cultured cells, none of them demonstrated the presence of p53 protein, interferon-γ or NF-κB in parkinsonian brains by use of ELISA. In the present study, we demonstrated for the first time significant increases in the levels of p53 protein, interferon-γ and NF-κB in the nigrostriatal dopaminergic regions of the PD brain. Pathological findings indicated that the Braak stages of controls and PD subjects were 0 (and no incidental cases (stage 1–2)) and 3–5. We did not compare the contents of p53, interferon-γ, and NF-κB with the Braak stages of the brain samples, because our Braak stage classification was not quantitatively accurate enough to carry out the analysis.

Table 3
NF-κB content in the brains from control and parkinsonian patients

Brain region	NF-κB concentration ($OD_{450\,nm}$/mg protein in nuclear extract)	
	Control patients	Parkinsonian patients
Caudate (17)	12.5 ± 9.5	54.0 ± 20.2*
Putamen (6)	ND	32.6 ± 12.1*
Substantia nigra (19)	17.9 ± 10.1	108.4 ± 17.8**
Cerebellum (5)	ND	ND
Frontal cortex (5)	ND	ND

Each value represents the mean ± S.E.M. Significantly different from controls, $^{*}P<0.05$, $^{**}P<0.01$. ND, not detectable.

Immune responses in the central nervous system (CNS) are not fully understood, but may involve the action of several cytokines that contribute to the development and/or differentiation of cells in the CNS. It has been suggested that patients with idiopathic PD have altered functions of their immune system [3]. The pleiotropic cytokine interferon-γ may play an important role in this altered immunological process in PD. Interferon-γ induces the expression of MHC class-I/β_2-microglobulin antigen on astrocytes and microglia. Class I major MHC-I antigens compose the parts of the structure displayed on target cells for stimulation of and lysis by cytolytic T lymphocytes [11]. In accordance with these results, we previously showed that the content of β_2-microglobulin was increased in the PD brain [15]. In the substantia nigra of PD patients, a significant increase in the density of glial cells expressing interferon-γ was observed by use of immunohistochemical analysis [7]. Furthermore, the present ELISA study demonstrated that the level of interferon-γ in the dopaminergic regions was elevated specifically in the parkinsonian brain.

Notable differences were found in the levels of p53 protein, interferon-γ and NF-κB in the nigrostriatal region in the PD brain. Whereas the p53 protein level was increased only in the caudate nucleus, the levels of both interferon-γ and NF-κB were increased in the substantia nigra, caudate nucleus, and putamen in parkinsonian brains. Although we have no definite evidence on the origin of interferon-γ, p53 protein, and NF-κB in parkinsonian brains, we suspect that microglia and astrocytes may produce these factors in the dopaminergic regions. Recent histochemical studies indicate that activated microglia actively produce proinflammatory cytokines such as TNF-α and IL-6 [8,9,25]. Thus, activated microglia may also produce interferon-γ. Another possibility is that interferon-γ may be produced by macrophages that have invaded into the brain due to the assumed dysfunction of the blood–brain barrier in PD [24]. As described, the parkinsonian group included 12 males and 3 females. Therefore, the uneven number of male versus females in the PD group might cause some biases in the results. We might have missed the change in the p53 protein level in postmortem PD subjects, especially in the substantia nigra, since the alteration of its level there may occur at a relatively early stage of neurodegeneration.

The increased levels of interferon-γ, p53 protein, and NF-κB in the nigrostriatal dopaminergic region in PD patients suggest increases both in immune reactivity (inflammation) and in programmed cell death (apoptosis) of neuronal and/or glial cells. Inflammation initiated by neuronal damage in the striatum and the substantia nigra in PD may promote progression of the disease. Since we have no definitely answer whether inflammatory and apoptotic reactions via the neuronal death and increase of microglia might actually be a result and not the cause of the dopaminergic neuronal cell death in PD or

not, that remains to be elucidated. In light of our present findings and previous reports, increased cytokine levels, decreased neurotrophin levels, and a possible immune response in the nigrostriatal region in PD suggest new neuroprotective therapy such as non-steroidal anti-inflammatory drugs, immunosuppressive or immunophilin-binding drugs, anti-apoptotic drugs, and drugs increasing neurotrophin production.

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＜教育講演3＞

ここまでわかったパーキンソン病（PD）の成因
—遺伝性PDの病態からわかったこと

服部　信孝　　久保紳一郎

要旨：パーキンソン病（PD）のほとんどは，遺伝歴のない孤発型が主体となっている．しかしながら，近年の分子生物学の進歩により単一遺伝子異常にともなう遺伝性PD（FPD）が存在することがわかり，現在では単一遺伝子異常にともなうFPDの遺伝子産物の機能解析から孤発型PDの病態解明へ繋げようとする戦略が盛んにおこなわれるようになった．1983年の1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine（MPTP）によるパーキンソニズムの報告以来，PDの病態解明は加速的に進んでいる．一方で，依然真の要因については解明にいたっていないといわざるをえない．しかしながら，FPDの研究からLewy小体の主要構成成分であるα-synuclein分子の発見，parkin分子の発見によりubiquitin-proteasome pathwayの関与，更にはPark9のATP13A2の発見でautophagy-lysosomal pathwayも黒質ドパミン神経変性に関与していることがわかった．少なくとも二大蛋白分解系が神経変性に重要な役割をなしていることが推定される．更に劣性遺伝性FPDの遺伝子産物PINk1，DJ-1はミトコンドリア機能にかかわっていることが推定されており，parkinもふくめて神経変性の機序にミトコンドリア機能が関与していることが考えられている．おそらくFPDの遺伝子産物は，共通カスケードを形成していると推定される．黒質神経変性の機序は，まさしくFPDの機能解明から今解き明かされようとしている．

（臨床神経，47：774—778, 2007）

Key words：ユビキチン・プロテアソーム系，オートファジー・リソソーム系，レビー小体，遺伝性パーキンソン病，遺伝子産物機能

はじめに

パーキンソン病（PD）は，アルツハイマー病（AD）に次いで頻度の高い神経変性疾患である．1817年にJames Parkinsonが，その著書「Shaking Palsy」の中ですでにPDの臨床症状のほとんどを詳細に記載している．しかしながら，最近の臨床研究では，少なからずのPD患者が認知症を合併することが明らかにされ，単にドーパミン神経のみならずセロトンやコリン作動性神経にも病変の広がりがあることがわかった．現在では，単にmovement disordersの疾患とせずneuropsychiatric disordersと捉える必要が強調されている．PDのほとんどは遺伝歴のない孤発型とされてきた．ところが最近のデータでは，環境因子と遺伝的素因の相互作用により発症することが推定されており，遺伝子の関与は少ないことが予想されている．また，疫学調査によれば，全年齢過程を考慮すると同胞発症者がいれば更に発症者が出現するリスクは1.7倍といわれている．66歳以下にすると2.6倍にリスクが増えるとの報告がある[1]．FPDの最新のデータでは，13の遺伝子座，そして7つの原因遺伝子が単離・同定されている（Table 1）．このFPDの遺伝子産物の機能から，黒質神経脱落の共通機構が明らかにされようとしている．本稿では，FPDの機能解明から推定される神経変性の機序について解説したい．

1. 封入体形成について：優性遺伝性パーキンソン病を中心に（α-シヌクレインとLRRK2）

優性遺伝性パーキンソン病（ADFPD），代表的な原因遺伝子としてα-シヌクレインやLRRK2（leucine-rich repeat kinase[2]）が上げられる．現在では，PDに特徴的な病理所見であるLewy小体の主要成分としてα-シヌクレイン蛋白が同定され，以後，α-シヌクレインに関する知見は大幅に前進を遂げ，遺伝子変異や遺伝子コピー数が増えたmultiplicationの機序によりα-シヌクレイン蛋白の異常凝集がおこり，PDが発症することが証明されている．この遺伝子変異による病理所見は，脳内に広範囲にLewy小体が出現し，認知症を呈し若年発症で急激に進行するケースもみとめられる．その臨床症状は孤発型よりも，より重篤なばあいがある．点変異型は，きわめてまれな変異であるが，多重遺伝子コピー数によるFPDは，わが国でもその存在が確認された[2]．おそらく世界的に分布していると考えられる．この遺伝子コピー数の増えたタイプは，遺伝子の高発現が，発症の鍵となることが推定される．更に病理学的検討もなされ，グリア細胞にもα-シヌクレイン陽性の封入体の存在が確認されている．これら所見は，multiple system atrophy（MSA）の病態にもα-シヌクレインの過剰発現が関与している可能性を示している．更に2006年，

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Table 1　遺伝性パーキンソン病の一覧

Genetic Symbol	Mode of Inheritance	Chromosomal Location	Gene Name	Frequency of Familial PD	Frequency of Sporadic PD
SNCA（PARK1, PARK4）	AD	4q	SNCA	< 0.5%	—
PARK2	AR	6q	Parkin	10-20%	Rare
PARK3	AD	2p	?	?	—
PARK5	AD	4p	UCHL-1	Rare	—
PARK6	AR	1p	PINK1	2-7%	Rare
PARK7	AR	1p	DJ1	1-2%	Rare
PARK8	AD	12p-q	LRRK2	5-10%	2%
PARK9	AR	1p	ATP13A2	?	—
PARK10	Susceptible gene	1p	?	?	—
PARK11	AD	2q	?	?	—
PARK12	Susceptible gene	Xq21-25	?	—	—
PARK13	AD	2p	NRAT2	Rare	

Mizuta らより，日本人孤発型 PDE の大規模 SNPs 解析がおこなわれ，α-シヌクレイン蛋白をコードする遺伝子領域 SNCA の intron 4 に，$p=5.0\times10^{-10}$ という統計学的有意差をもって危険因子となる遺伝子多型が同定された[3]．この報告からも，α-シヌクレインは家族性パーキンソン病のみならず，孤発型においても何らかの有力な発病因子となっている可能性が推測できる．そしてこの SNP を持つと mRNA の発現が増加することが確認されている．α-シヌクレイン遺伝子の発現増加が，発症の危険因子となることが孤発型でも証明されたことになる．

LRRK2（leucine-rich repeat kinase 2）遺伝子変異は，我が国の常染色体優生遺伝パーキンソン病の大家系（相模原家系）により候補領域が絞りこまれ，近年二つのグループから原因遺伝子が同定された．LRRK2 遺伝子は 51 の exon をふくみ，2,527 個のアミノ酸からなる LRRK2 蛋白をコードする．発現は ubiquitous であるが，その量は脳よりも心臓，肝臓，肺で比較的多い．われわれの最新のデータでは，脂質ラフトに局在していることが判明しており，変異型の局在は正常型と変化がないことから，gain-of-function 型効果が発症にかかわっていることが想定される[4]．機能面から考えると，キナーゼ活性の関与が注目される．現在では世界中の多くの家系で 20 種類以上の変異型が確認されており，高齢発症の FPD においてもっとも多い変異である．代表的な変異としては，MAPKKK ドメイン内に G2019S，I2020T が存在する．G2019S 変異は，家族性パーキンソン病の中で 3～40% 程度の頻度でみとめている．LRRK2 遺伝子変異による臨床症状は，L-Dopa に対する反応性の良好な典型的なパーキンソン症状を呈し，経過は比較的緩徐であり，孤発性 PD との区別が困難であるばあいも多い．特徴的な病理所見として，臨床像は比較的均一であるにもかかわらず，多様な病理像を呈することが上げられる．孤発型 PD でもみとめられる中脳黒質の神経細胞脱落に加え，Lewy 小体をみとめないものから広範囲にみとめられるものまでその出現は多様である．さらに AD や進行性核上性麻痺などでみとめられるタウ蛋白の異常沈着もみとめられる症例もあり，種々の神経疾患の病理像が混在した所見を呈している．このため，LRRK2 はシヌクレイノパチーに加え，タウオパチーにおいても重要な蛋白であることが推測され，PD をふくめた神経変性疾患全般の病態を解明する上で重要な蛋白である．

最近，アジア人（台湾人，中国漢民族，日本人）のみで LRRK2 の exon48 内に孤発型 PD の危険因子となる G2385R という SNP が報告されている．$p=1.24\times10^{-4}$ という有意差で，危険因子としては変異型が同定されている．この SNP を所有したばあい，約 2.6 倍の危険率で発症しやすくなる[5]．未だ，LRRK 2 遺伝子変異や SNP が PD の発症過程においてどのように関与するのかは不明であるが，LRRK2 遺伝子の臨床および基礎的研究が FPD のみならず，孤発型パーキンソン病の発症メカニズムの解明，治療の開発につながる可能性を示唆している．

2. 黒質神経脱落における蛋白分解系の関与 —劣性遺伝性 PD を中心に

優性遺伝性 PD では，遺伝子産物が封入体形成に直接的に関与していることが推定されている．一方，parkin を代表とする劣性型 PD では封入体形成が一般にみとめられないことが報告されている．そしてその parkin は，蛋白分解系の一反応系ユビキチン・プロテアソーム系に関与していることがわかっている．蛋白分解系のユビキチン化にはリジン 48 番，63 番そしてモノユビキチン化がある（Fig. 1）．最近のデータで

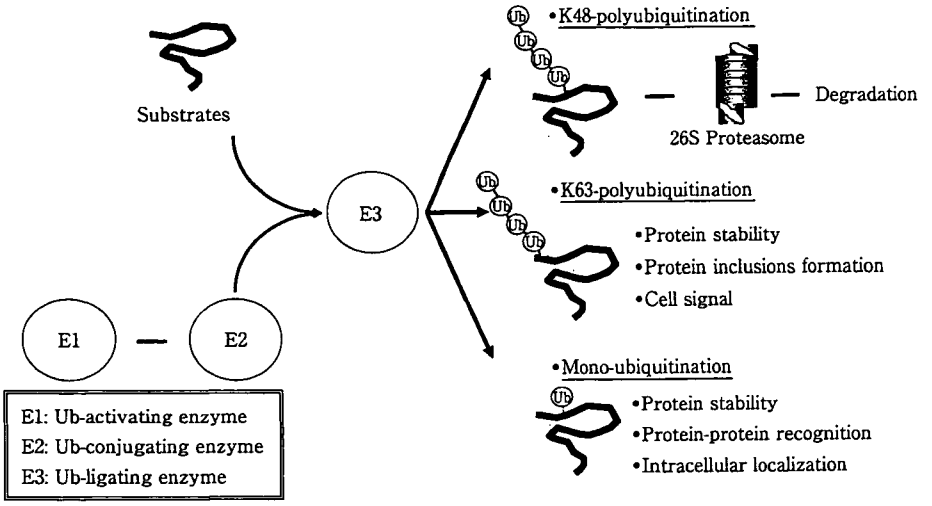

Fig. 1　蛋白分解系には，ユビキチンのリジン残基48番，63番，モノユビキチン化の三種類が存在する．48番目のリジン残基のポリユビキチン化は，蛋白分解系のシグナルになる．一方，63番のポリユビキチン化は，封入体形成に関与している可能性が指摘されている．そしてモノユビキチン化は，細胞内局在にかかわっている可能性が指摘されている．Parkinは，すべてのユビキチン化機能を持つとされている．

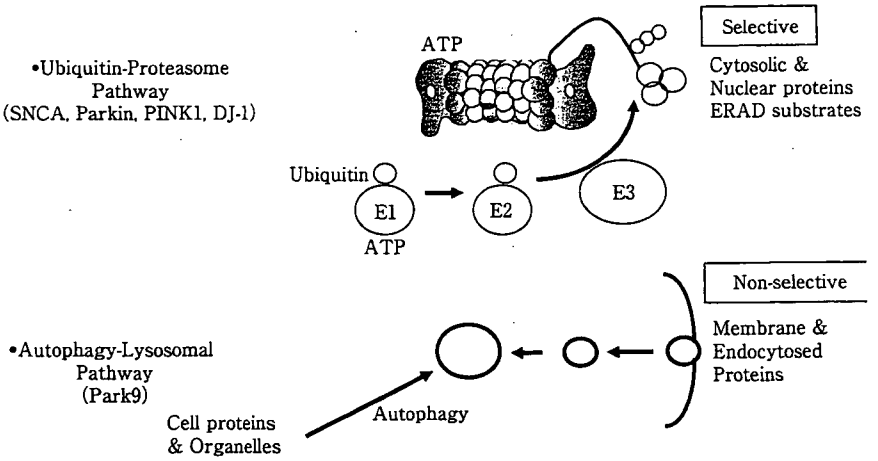

Fig. 2　2大蛋白分解系．ユビキチン・プロテアソーム系とリソソーム・オートファジー系が存在する．この蛋白分解系は，相互に作用していることが推定されている．

は，parkinは，蛋白分解系のシグナルだけでなく，リジン残基63番目にもポリユビキチン鎖が付加されることが報告されている．K63のポリユビキチン鎖は，蛋白分解系のシグナルには成りえず，レビー小体形成にかかわっていることが推定されている[6)7)]．更に細胞内局在の変化や蛋白安定性にかかわっているとされているモノユビキチン化の作用もparkinは，保持しているとされている[8)]．またリガーゼ活性に関しては，すべての変異型で消失しているわけでなく[8)]，parkin変異型のもたらすメカニズムについては，依然不明な点が多い．

一方で，parkinと他の劣性遺伝性PDの遺伝子産物の結合が報告されている．Parkinは変異型DJ-1と結合することが報告されている[9)]．Parkinは，DJ-1と結合してその安定性に影響を与えている可能性が指摘されている．興味深いことに，その結合は酸化ストレスにより誘導されることである．

蛋白分解系には，大きくオートファジー・リソソーム系とparkinが関与しているユビキチン・プロテアソーム系が存在する(Fig. 2)．蛋白分解系を考えたばあい，オートファジー系も神経変性で重要な機能をなしていることが報告された．臨床研の田中，水嶋の両グループは，別々のオートファジーの分子を潰した遺伝子改変マウスモデルを作製した．その結果，オートファジーのノックアウトマウスは，ユビキチン陽性の封入体を細胞内に形成することがわかった．更にこのノックアウトマウスの解析では，封入体形成の多い部位では神経脱落が少なく，封入体形成の少ない部位では，細胞死の脱落が多