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Feasibility and Validity of a Modified Finger-Nose-Finger Test

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Abstract: In essential tremor (ET) research, it is important to obtain standardized, objective data on tremor severity. Often, it is not possible to carry out in-person or videotaped neurological examinations. In place of these, handwriting samples can be collected, but they do not capture all of the variance in tremor severity. Although additional tests of tremor severity (finger-nose-finger [FNF] test) might be of use, these would need to be modified to allow ET patients to mail their results to the study investigator for rating. We modified the standard FNF test (sFNF) by asking subjects to hold a pen during this activity and mark a paper target. The purpose of this report was to determine whether the modified FNF (mFNF) test was feasible and valid. Of 70 subjects, 65 (92.9%) were able to complete the mFNF, demonstrating that it was feasible. The scores of the mFNF correlated highly with those of the sFNF ($r = 0.56–0.85$; all $P < 0.001$), indicating the mFNF is a valid measure of tremor severity. In addition, using the regression equation, sFNF = 0.174(mFNF) + 0.743, a sFNF score can be derived easily from the mFNF score. The mFNF may be used to collect valuable data on tremor severity in pathological, genetic, and epidemiological field studies of ET, in which in-person or videotaped neurological examinations are not possible. © 2005 Movement Disorder Society

Key words: essential tremor; epidemiology; validity; brain bank; screen

Essential tremor (ET) patients from throughout the United States currently are being recruited prospectively as potential brain donors to the Essential Tremor Centralized Brain Repository at Columbia University.[1] Over-arching goals of the repository are to identify the pathology of ET and to study the clinical correlates of that pathology (i.e., comparing the severity and laterality of clinical with pathological findings). It is imperative, therefore, to obtain standardized, objective data on tremor severity. Although an in-person examination or a visual record (videotaped examination) is ideal for this purpose, ET patients are scattered geographically throughout the United States and most do not have access to a videocamera. This problem is also encountered commonly in epidemiological and family studies of ET, and this provides the impetus to explore alternative objective methods. One approach is for the ET patients to mail handwriting samples to the investigators at the Brain Bank. Although these would be of some use, they would not capture all of the variance in tremor severity; tremor while writing can be minimal when compared with tremor during other actions (e.g., finger-nose-finger [FNF] test)[2]; alternatively, in cases of primary writing tremor, the converse is true.[3] In an attempt to devise additional methods for assessing tremor severity, we modified the FNF test by asking the subject to hold a pen during this activity and to mark a paper target. The purpose of this report was to determine the feasibility and validity of this method. If the method were valid, it could be used in this and other brain bank studies as well as in epidemiological and genetic studies to collect objective data on tremor severity.

Subjects and Methods

Subjects

We assessed the modified FNF (mFNF) test in individuals with a broad representation of tremor severities, ranging from severe action tremor to minimal or no action tremor. We enrolled individuals in three diagnostic groups, including individuals who were expected to have: (1) moderate to severe action tremor (patients with ET); (2) minimal or no action tremor (normal controls); and (3) intermediate amounts of action tremor, i.e., patients with Parkinson's disease (PD).[4] Our goal was to enroll 20 to 30 subjects per group (total sample size = 75), which would provide adequate power to address our aims.

As described previously,[5,6] ET patients and controls were participants in an epidemiological study. These ET patients were referred to the study by neurologists at the Neurological Institute of New York, Columbia University Medical Center (CUMC) and all controls were identified from the New York Metropolitan area using random digit telephone dialing as described previously.[5,6] Each subject had an in-person assessment, which was carried out by a trained research assistant. The assessment included a videotaped neurological examination with the standard FNF (sFNF) test. The data used in the current report were derived from ET patients and controls who were

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Phosphorylated IκBα is a component of Lewy body of Parkinson's disease

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Abstract

Ubiquitin is one of the major components of Lewy bodies (LB), the pathological hallmark of Parkinson's disease (PD). Here, we identified that a phosphorylated form of IκBα (pIκBα), an inhibitor of NF-κB, and $SCF^{\beta\text{-}TrCP}$, the ubiquitin ligase of pIκBα, are components of LB in brains of PD patients. In vitro studies identified those proteins in the ubiquitin- and α-synuclein (known as the major component of LB)-positive LB-like inclusions generated in dopaminergic SH-SY5Y cells treated with MG132, a proteasome inhibitor. Intriguingly, IκBα migration into such ubiquitinated inclusions in cells treated with MG132 was inhibited by a cell-permeable peptide known to block phosphorylation of IκBα, although this peptide did not influence cell viability under proteasomal inhibition. Our results indicate that phosphorylation of IκBα plays a role in the formation of IκBα-containing inclusions caused by proteasomal dysfunction, and that the generation of such inclusion is independent of cell death caused by impairment of proteasome.

Keywords: Parkinson's disease; Lewy bodies; Ubiquitin; Proteasome; IκBα; IκB-kinase

Parkinson's disease (PD) is one of the most common neurodegenerative disorders among the aged and its pathological hallmark is the preferential degeneration of dopaminergic neurons in the substantia nigra (SN) and the appearance of intracytoplasmic inclusions known as Lewy bodies (LB). The process of LB formation could provide important clues regarding the pathogenesis of PD because important proteins, such as α-synuclein (another familial PD gene product) and ubiquitin (Ub), are components of these inclusions [1,2].

The ubiquitin–proteasome pathway (UPP) is the major non-lysosomal degradation system for various proteins, such as short-lived, misfolded, and damaged polypeptides [3]. In this system, ubiquitin is conjugated to lysine residue of the target protein by a cascade of enzymatic reactions catalyzed by the E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes in an ATP-dependent manner, and polyubiquitination marks the proteins for degradation by the proteasome. Several lines of evidence suggest that derangements in the UPP play an important role in the pathogenesis of PD and describe inhibited hydrolytic activities of the proteasome in PD [4,5].

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On the other hand, the concentration of tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is increased in PD and this cytokine plays a role in the pathogenesis of PD [6,7]. TNF-α stimulates the multisubunit IκB-kinase (IKK), which is composed of IKKα, IKKβ and a non-catalytic regulatory component named NF-κB essential modifier (NEMO), which allows IκBα to be subsequently phosphorylated. The phosphorylated form, pIκBα, is polyubiquitinated by the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase [8–10]. Subsequently, they are rapidly degraded by 26S proteasomes, and NF-κB enters the nucleus, binds to DNA, and activates transcription of target genes [11]. In this scenario, the nuclear translocation of NF-κB is masked by physical association with IκBα, resulting in retention of NF-κB in the cytoplasm.

Intriguingly, a previous report implicated the involvement of NF-κB in the LB of PD [12], but the role of IκBα in the pathogenesis of PD remains unclear. The aims of the present study were the following: (1) to determine the presence or absence of pIκBα and components of the $SCF^{\beta\text{-}TrCP}$ complex in LB of PD, (2) whether pIκBα is colocalized in the cytoplasmic inclusions formed in MG132-treated human dopaminergic neuroblastoma cells (SH-SY5Y), and (3) to determine the effect of inhibition of IκBα phosphorylation on the formation of cytoplasmic inclusions and viability of cells treated with a proteasome inhibitor.

Materials and methods

Human neuroblastoma cell lines. Human dopaminergic neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) fetal bovine serum and penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. To induce differentiation, the cells were treated with 10 μM retinoic acid (Sigma Chemical, St. Louis, MO) in the dark for 4–6 days as described previously [13–15].

Application of agents. TNF-α (R&D Systems, Minneapolis, MN) and MG132 (Peptide Institute, Osaka, Japan) were prepared at 10 μg/ml (in H_2O) and 10 mM (in dimethyl sulfoxide, DMSO) stock solutions, respectively. The specific antibodies used were rabbit anti-ubiquitin (Dako, Carpinteria, CA), mouse anti-ubiquitin 1510 (Chemicon International, Temecula, CA), anti-synuclein-1 (Transduction Laboratories, Lexington, KY), sheep anti-α-synuclein [16], anti-pIκBα (Ser32), and anti-IκBα antibodies (from Cell Signaling Technology, Beverly, MA, and Calbiochem, La Jolla, CA). Anti-NF-κB p65 (sc-372) and anti-β-TrCP (N-15) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Note that anti-IκBα antibody reacts with both phosphorylated and unphosphorylated forms of IκBα. Anti-ROC1 and Cul-1 antibodies were prepared as described previously [17]. The secondary antibodies used were goat anti-mouse IgG coupled with Alexa Fluor 488, goat anti-mouse IgG coupled with Alexa Fluor 594, anti-rabbit IgG coupled with Alexa Fluor 594 (Molecular Probes, Eugene, OR), and fluorescein goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

Immunohistochemistry. For the LB staining experiments using paraffin embedded samples, autopsied brains from seven patients with PD (age, 51–78 years), one patient with dementia with Lewy bodies (DLB, age, 64 years), and five control subjects (age, 20–65 years) were examined. The five control samples were obtained from patients free of neurological diseases and confirmed to have no neuropathological changes in sections of the substantia nigra pars compacta. Immunohistochemistry was performed as described previously [18].

Immunolabeling of isolated Lewy bodies. Immunomagnetic isolation and immunostaining of LB and Lewy neuritis from fresh frozen brains of patients with DLB were performed as described previously [19]. Smears were prepared on gelatin-coated glass slides, from homogenates, factions of each washing step, and LB-enriched Percoll fractions. The smears were air-dried overnight, fixed for 10 min in 4% formaldehyde–2% picric acid–0.1 M phosphate-buffered saline (pH 7.4), and then incubated for 10 min in 3% H_2O_2 in 50% methanol in Tris-buffered saline (TBS, pH 7.4). Following three rinses in 0.1 M TBS containing 0.05% sodium azide and 1 mM phenylmethylsulfonyl fluoride (PMSF) (TBS), the smears were incubated with 20% normal horse serum in TBS for 30 min to block non-specific antibody-binding sites, and incubated overnight with sheep antibody against α-synuclein and pIκBα, ROC1, Cul-1, and β-TrCP in TBS containing 0.5% bovine serum albumin. Control sections were stained by incubating the smears with TBS containing either or neither antibodies. Following three rinses in TBS, the smears were incubated for 1 h with donkey anti-sheep IgG or donkey anti-rabbit IgG conjugated with Cy2, Cy3 or Cy5 in TBS (all from Jackson Immunoresearch Laboratories, West Grove, PA), with the fluorochromes either singly or in combinations of Cy2/Cy3 or Cy2/Cy5.

Immunocytochemistry. For double-labeling immunofluorescence staining, fixed cells were permeabilized with 0.25% Triton X-100 for 20 min. The cells were blocked with 5% normal goat serum for 1 h, followed by incubation of antibodies to ubiquitin (Chemicon; diluted 1:100), pIκBα Ser32 (Cell Signaling; diluted 1:100), ROC1 (diluted 1:100), or Cullin-1 (diluted 1:100) for 1 h at room temperature. After washing in TBS, the cells were incubated in anti-mouse, anti-rabbit fluorochrome-linked secondary antibodies. After washing in TBS, the cells were covered with glass slides using mounting medium with 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei (Vector Laboratories). To assess the colocalization of ubiquitin and α-synuclein, we used anti-ubiquitin (Dako; diluted 1:100) and anti-synuclein-1 (diluted 1:100) antibodies. Signal was observed under a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, NY).

Western blotting. Neuronally differentiated SH-SY5Y cells were treated for 24 h with 10 μM MG132 or 20 ng/ml TNF-α. The cells were simultaneously preincubated for 2 h with 10 μM MG132, followed by treatment for 22 h with 10 μM MG132 in the presence of 20 ng/ml TNF-α. The cells were collected and washed in ice-cold phosphate-buffered saline (PBS) and lysis buffer as described previously [20]. Detergent-insoluble material was pelleted by centrifugation at 100,000*g* for 20 min and resuspended in 50 mM Tris–HCl (pH 7.4) and 6 M urea. Equal amounts of protein from both fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% or 10–20%) and transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in 5% non-fat milk for 1 h at room temperature, and incubated at 4 °C with specific antibodies.

Treatment of IKK inhibiting peptide. Recent studies have reported that an NH_2-terminal α-helical region of NEMO associates with a hexapeptide sequence within the extreme carboxyl terminus of IKKβ and IKKα, termed NEMO-binding domain (NBD). Importantly, a short cell-permeable peptide spanning the IKKβ NBD was found to disrupt the association of NEMO with IKKβ and blocked the activity of IKK [21]. We synthesized two peptides at Juntendo University School of Medicine, a functional wild-type NBD with a sequence derived from the Antennapedia homeodomain that mediates membrane translocation without loss of cell viability, and a negative control mutant NBD, as described previously [21–24]. To determine how inhibition of phosphorylation of IκBα affects the formation of such inclusions, the differentiated SH-SY5Y cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 10 μM MG132 in the presence of 40 μM of each NBD peptide. The cells were simultaneously preincubated for 3 h

with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. These cells with ubiquitin-positive inclusions were co-stained with antibodies against pIκBα or IκBα.

Cell viability assay. Cell viability assay was performed using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt assay, as described previously [25]. The differentiated SH-SY5Y cells were plated on a 96-well plate and incubated for 24 h at 37 °C in 5% CO_2. The cells were preincubated for 3 h with 40 μM of either wild-type or mutant NBD peptide, followed by treatment for 24 h with 40 μM of each NBD peptide in the presence or absence of 10 μM MG132. The cells were simultaneously preincubated for 3 h with no peptide, followed by treatment for 24 h with 10 μM MG132 alone. The live cell count was assayed using Cell Counting Kit-8 according to the instructions provided by Doujin (Cell Counting Kit-8; Kumamoto, Japan).

Statistical analysis. All data are expressed as means ± SEM. Comparisons between groups were performed using analysis of variance (Tukey's multiple *t* test).

A p value <0.05 indicated statistically significant differences.

Results

Phosphorylated IκBα and $SCF^{\beta\text{-}TrCP}$ complex are novel components of Lewy bodies

We first examined whether LB contain pIκBα and the components of $SCF^{\beta\text{-}TrCP}$ complex, which are major downstream components of the TNF-α signaling pathway. Immunohistochemical analysis revealed that anti-pIκBα and ROC1 antibodies predominantly recognized the LB in PD cases (Figs. 1A and B). Immunostaining with anti-NF-κB p65 antibody also showed the staining of LB (Fig. 1C). Anti-pIκBα and anti-ROC1 signals were strongly present in the halo region of LB, and the anti-NF-κB-p65 signal was present in the core region. In contrast, such immunoreactivities for pIκBα ROC1 and NF-κB were not observed in the control brains and when the primary antibody was omitted in PD and DLB brains (data not shown).

In the next step, the isolated LB were used to investigate whether these proteins are associated with LB. Confocal laser-scanning microscopic examination of sections prepared from freshly isolated LB from postmortem brains of DLB showed immunoreactivities for pIκBα, ROC1, Cul-1, and β-TrCP (Figs. 1D, G, J, and M). LB were identifiable by their strong α-synuclein staining in smears of isolated LB from DLB cortex (Figs. 1E, H, K, and N), but not in sections from a normal control cortex (data not shown). These immunoreactivities for the indicated antibodies in LB were distributed across or sometimes more concentrated in the central region of LB (Figs. 1F, I, L, and O). Counting of α-synuclein-positive LB indicated that 80–90% of the cortical LB ($n = 300$, pooled from three DLB cases) were also positive for ROC1 and Cul-1. A similar staining pattern was also observed in LB isolated from the substantia nigra (SN) of PD (data not shown).

Localization of pIκBα, ROC1, and Cul-1 in cytoplasmic inclusions of SH-SY5Y cells

As a model for the formation of cytoplasmic inclusions, we used SH-SY5Y cell lines treated with MG132 [26]. Localization of pIκBα, ROC1, and Cul-1 was investigated after the addition of 10 μM MG132 for 24 h in differentiated SH-SY5Y cells. Proteasomal dysfunction caused typical cytoplasmic inclusions that were stained with anti-ubiquitin (Ub) antibodies, and interestingly many, if not all, ubiquitinated inclusions were also positive for pIκBα, ROC1, and Cul-1 (Figs. 2A–C). Under normal conditions without MG132, the cells displayed low-level cytoplasmic staining for the indicated proteins (data not shown). Although we examined the effect of TNF-α on the formation of the inclusions, no inclusions that contained ubiquitin and pIκBα were observed after treatment with TNF-α alone. In addition, the effect of simultaneous treatment with TNF-α and MG132 was not significantly different from the results of MG132 treatment alone (data not shown).

We next examined whether these cells also contained α-synuclein in such cytoplasmic ubiquitinated inclusions. Following proteasomal inhibition with 10 μM MG132, some of the ubiquitinated cytoplasmic inclusions also exhibited α-synuclein immunoreactivity (Fig. 2D). Moreover, we examined whether pIκBα and components of the SCF complex colocalize with α-synuclein in the presence of 10 μM MG132. The α-synuclein-positive inclusions were also immunoreactive for pIκBα ROC1 and Cul-1 following treatment with 10 μM MG132 (Figs. 2E–G). The proportion of cells treated with 10 μM MG132 that contained aggregates immunoreactive for both pIκBα and α-synuclein was 7.98 ± 1.14%. In contrast, the proportion of 10 μM MG132-treated cells containing inclusions positive for both pIκBα and ubiquitin was 23.19 ± 3.84%, suggesting the relative low frequency of α-synuclein/pIκBα-containing inclusions (see Fig. 5B). Inclusions containing only ubiquitin, α-synuclein, or pIκBα were also noted, and their size was also comparatively heterogeneous (data not shown).

Inhibition of proteasomes increases phosphorylated IκBα level in SH-SY5Y cells

We examined the migration pattern of endogenous ubiquitin or pIκBα by SDS–PAGE in differentiated SH-SY5Y cells following proteasomal inhibition with MG132 and/or TNF-α for 24 h. Cells were treated as indicated in Fig. 3, and then the resulting cell extracts were separated into detergent-soluble and detergent-insoluble fractions. Treatment with 10 μM MG132 resulted in accumulation of high-molecular weight ubiquitin–protein conjugates particularly within the insoluble fractions but not in TNF-α alone and control

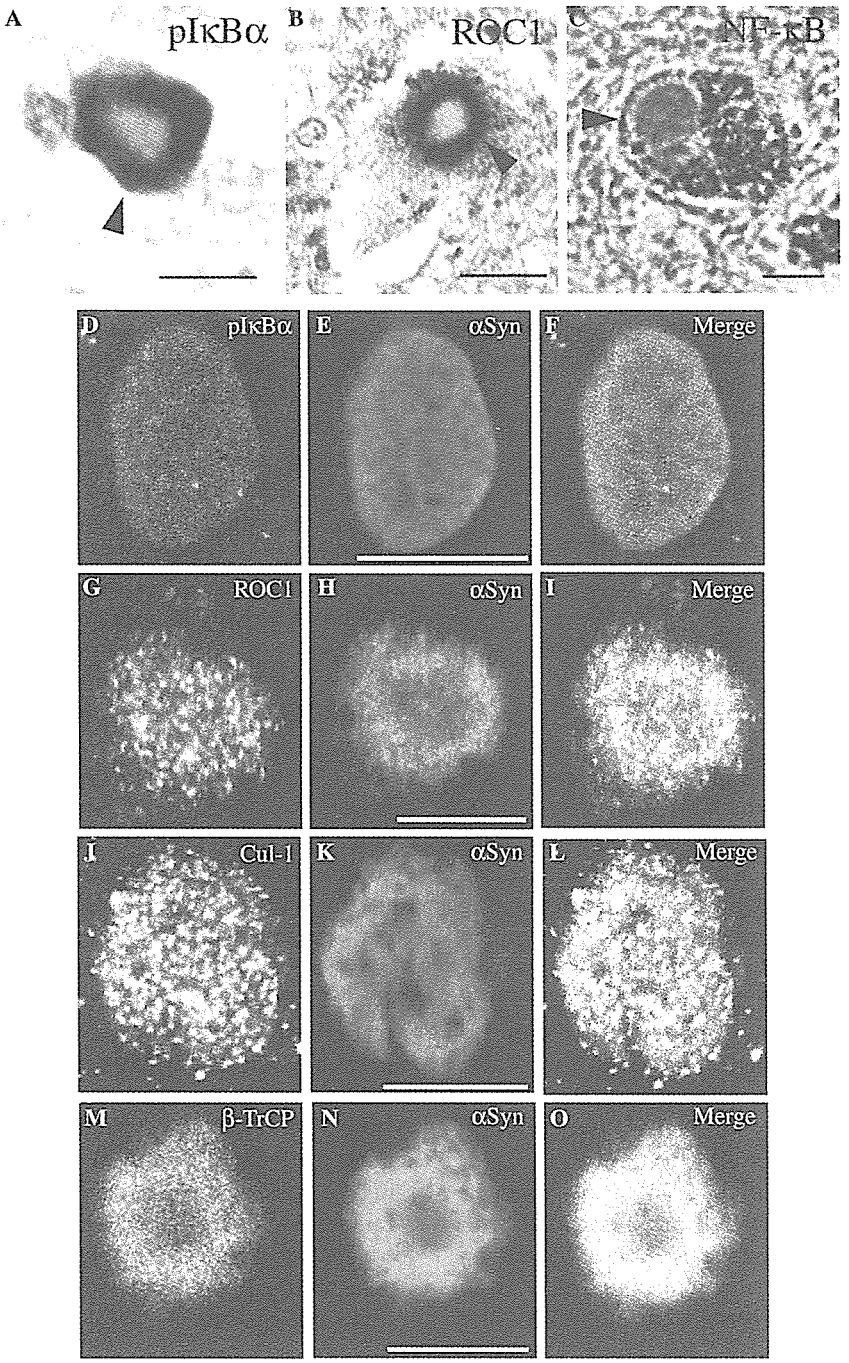

Fig. 1. Identification of phosphorylated IκBα and components of $SCF^{\beta\text{-}TrCP}$ in Lewy bodies. (Upper panel) Paraffin sections of autopsied human brain samples with PD were immunostained with antibodies against pIκBα (A), ROC1 (B), and NF-κB p65 (C). Lewy bodies are marked by arrowheads. Scale bars = 20 μm (A–C). (Lower panel) Colocalization of α-synuclein (αSyn), pIκBα, and components of $SCF^{\beta\text{-}TrCP}$ in isolated LB from DLB (Dementia with LB) cases. LB were identified by α-synuclein staining. Each preparation was doubly stained with sheep anti-α-synuclein (E, H, K, and N) and various antibodies against pIκBα (D), ROC1 (G), Cul-1 (J), and β-TrCP (M), and analyzed with a laser-scanning confocal microscope. Panels (F, I, L, and O) at right correspond to merged images; yellow-colored structures indicate colocalization. Scale bars = 10 μm (D–O).

cells (Fig. 3A). Unexpectedly, the effect of TNF-α was very weak in SH-SY5Y cells, because no massive reduction of IκBα was observed upon treatment with TNF-α for 1, 12, or 24 h (Fig. 3C and data not shown). This finding was in marked contrast to the almost complete disappearance of IκBα in HeLa cells treated with 20 ng/ml TNF-α within 1 h (data not shown). However, TNF-α significantly increased the pIκBα level (Fig. 3B), indicating the existence of TNF-α response to a lesser extent in SH-SY5Y cells. It is of note that MG132 alone

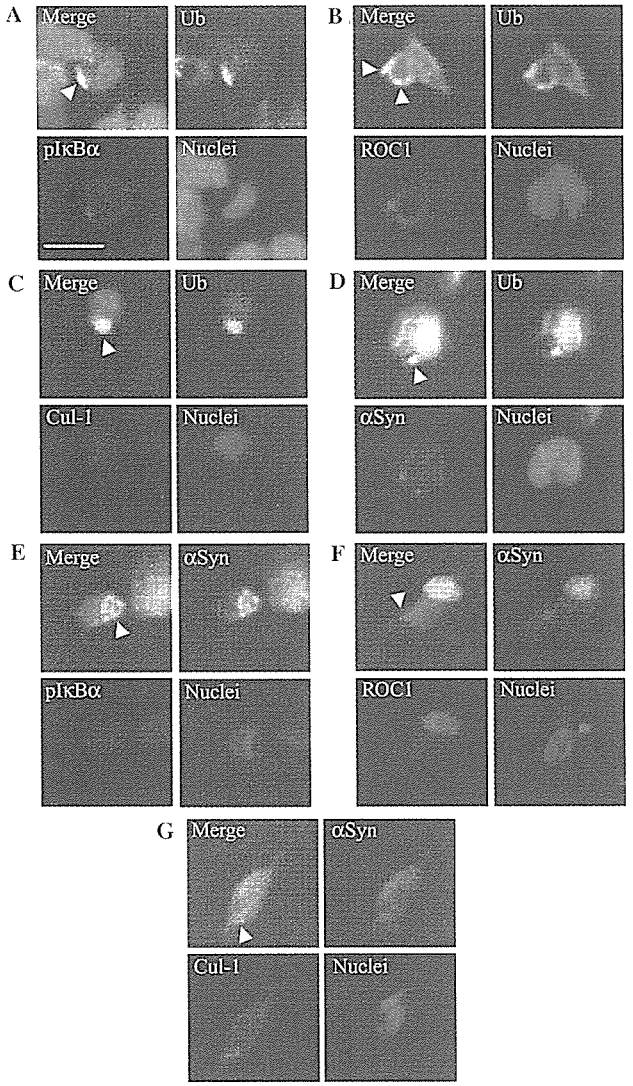

Fig. 2. Proteasomal inhibition leads to formation of pIκBα-positive cytoplasmic inclusions in SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with 10 μM MG132 for 24 h, fixed and then double-stained with various combinations of antibodies as indicated. (A–D) Cytoplasmic inclusions positive for ubiquitin (Ub) were co-stained for pIκBα (A), ROC1 (B), Cul-1 (C), and α-synuclein (D). Arrowheads indicate the inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. (E–G) α-Synuclein (αSyn)-positive cytoplasmic inclusions were co-stained for pIκBα (E), ROC1 (F), and Cul-1 (G). Regions of overlap between α-synuclein (green) and immunoreactivities of the indicated proteins (red) appear in yellow color. Scale bar = 10 μm.

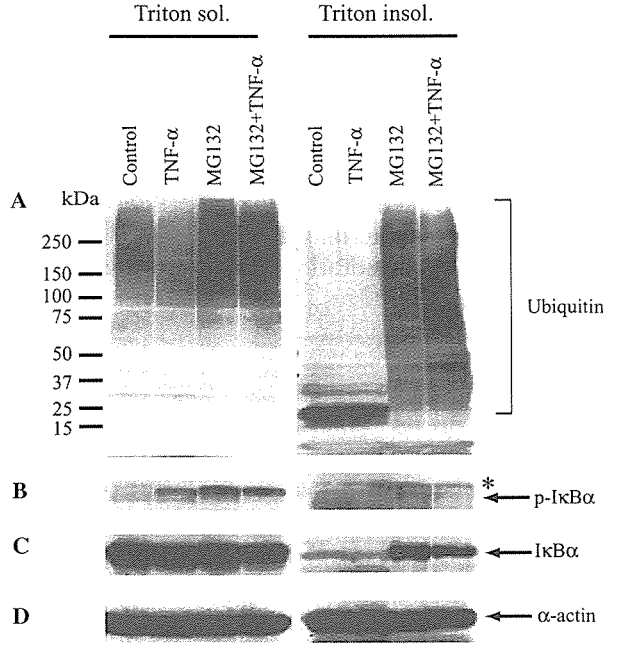

Fig. 3. Inhibition of the proteasome results in accumulation of pIκBα within the detergent-insoluble fraction of SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 and/or 20 ng/ml TNF-α, and the cell lysates were processed for Western blotting, as described in Materials and methods. The protein was blotted onto PVDF membranes and probed with antibodies against ubiquitin (A), pIκBα (B), and IκBα (C). Note that anti-IκBα antibody reacted both phosphorylated and unphosphorylated forms. α-Actin served as a loading control (D). Asterisk indicates a non-specific band.

increased the pIκBα level in the cells (Fig. 3B), although additive effects of TNF-α and MG132 were not observed for phosphorylation of IκBα. Intriguingly, when detergent-soluble and -insoluble fractions were immunoblotted with anti-pIκBα or anti-IκBα antibody, both proteins were clearly detected in the detergent-insoluble fraction after treatment with 10 μM MG132 but not in TNF-α alone and control cells (Figs. 3B and C). In addition, simultaneous treatment with TNF-α and MG132 had no significant effects in comparison with MG132 treatment alone. Thus, it is clear that IκBα, perhaps its phosphorylated form, is incorporated into the detergent-insoluble fraction under the conditions of proteasome inhibition.

The NBD peptide inhibits pIκBα entry into cytoplasmic ubiquitin-positive inclusions

The presence of pIκBα in LB of autopsied brains of PD patients and ubiquitinated inclusions in SH-SY5Y in the present study led us to examine whether inhibition of IKK, which phosphorylates IκBα, alters the processes of inclusion formation and cell death. First, we determined the optimal concentration of the cell-permeable NBD peptide, which is known to block the activity of IKK. To study the effect of NBD, SH-SY5Y cells were pre-treated with various concentrations of wild-type NBD for 3 h and then stimulated by 20 ng/ml TNF-α. In the present study, we used 40 μM NBD as the optimal concentration to block phosphorylation of IκBα. We also examined the effect of high concentrations of the NBD peptide (about 1000 μM), as described previously [21], but peptide toxicity was observed in our cell lines.

We next treated the cells with MG132 in the presence or absence of NBD peptide and then performed double

staining using antibodies for pIκBα, IκBα, and ubiquitin. Ubiquitinated inclusions containing pIκBα were identified in cells treated with MG132 alone or with MG132 in the presence of mutant NBD lacking inhibitory activity for IKK. On the other hand, while ubiquitinated inclusions were observed in cells treated with MG132 in the presence of wild-type NBD, only a few cells contained ubiquitinated inclusions positive for pIκBα (Fig. 4A). In addition, the use of an antibody for IκBα in the presence of wild-type NBD was also associated with reduced number of cells with ubiquitinated inclusions positive for IκBα, compared with those treated with MG132 or MG132 in the presence of mutant NBD (Fig. 4B), indicating that phosphorylation of IκBα may be required for its incorporation into cytoplasmic inclusions generated by proteasome inhibition.

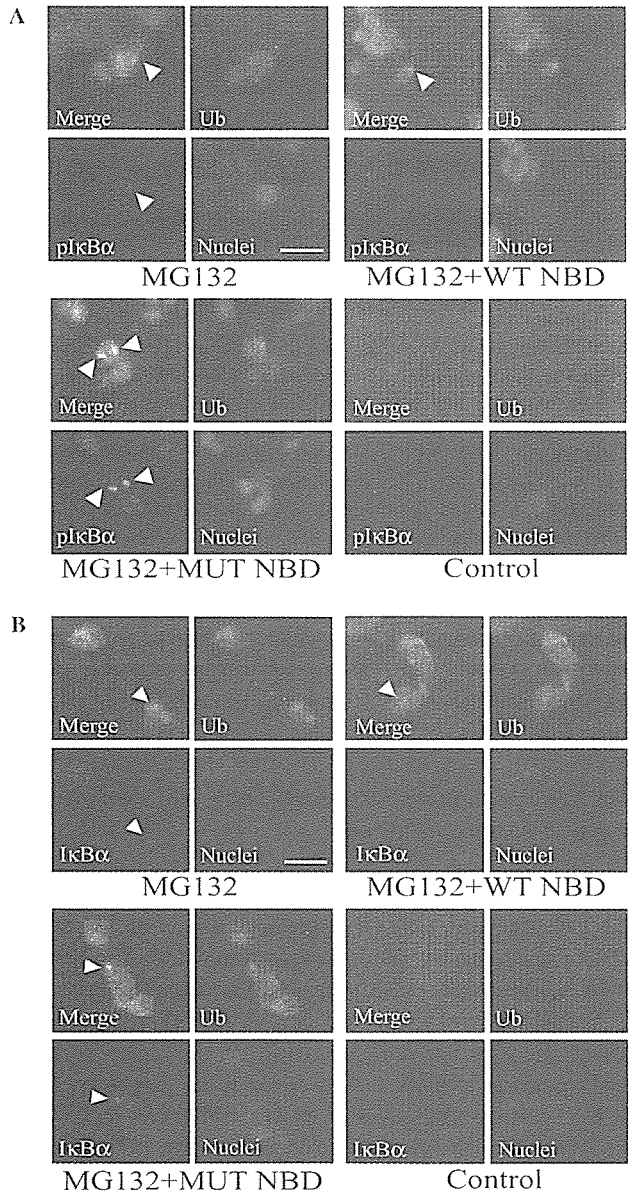

Fig. 4. Wild-type NBD decreases pIκBα level within the ubiquitinated inclusions in SH-SY5Y cells. The cells were treated for 24 h with 10 μM MG132 alone, or with 10 μM MG132 in the presence of 40 μM of either wild-type (WT NBD) or mutant NBD peptide (MUT NBD) as indicated in Materials and methods. Cells with ubiquitinated inclusions were co-stained with pIκBα (A) and IκBα (B). Arrowheads indicate the ubiquitinated inclusions. Regions of overlap between ubiquitin (green) and immunoreactivities of the indicated proteins (red) are shown in yellow color. Scale bar = 20 μm.

We then counted the number of cells with aggregated immunoreactivity for both ubiquitin and pIκBα antibodies under basal condition and following treatment with 10 μM MG132 with or without NBD peptide. It is worth noting that whereas approximately 50% of total cells contained ubiquitin-positive inclusions, pIκBα-positive inclusions were below 25% (Figs. 5A and B), suggesting that pIκBα is not incorporated into all inclusions. Wild-type NBD significantly decreased the number of cells with ubiquitinated inclusions (Fig. 5A, $p < 0.05$), and cells with cytoplasmic inclusions positive for pIκBα and ubiquitin, compared with cells treated with MG132 alone (Fig. 5B, $p < 0.001$). In comparison, mutant NBD did not show the same effects on phosphorylation of IκBα as wild-type NBD. Finally, we examined the toxicity of 10 μM MG132 on these cell lines. Treatment with 10 μM MG132 reduced cell viability to $37.84 \pm 1.46\%$. In contrast, wild-type NBD did not influence cell viability under proteasomal inhibition (Fig. 5C).

Discussion

The appearance of LB in SN is a prominent feature in PD, but the pathogenic role of such inclusions remains elusive. In this study, we identified novel components including pIκBα and components of $SCF^{\beta\text{-}TrCP}$ ligase in LB. To date, several studies have reported that the UPP-related proteins (such as ubiquitin, the 20S proteasome subunit, and HSP70) are localized in LB of PD [27,28]. These findings indicate that there appears to be an important correlation between some pathological alteration in UPP and the formation of LB in PD. In this regard, the pathogenic nature of proteasomal dysfunction has been studied in experimental models using a proteasome inhibitor. It has been demonstrated that inhibition of proteasomal function induces the formation of cytoplasmic inclusions immunoreactive for ubiquitin and α-synuclein in PC12 cells and mesencephalic cultures [20,29]. These observations suggest that proteasomal dysfunction is associated with the development of cytoplasmic inclusions that may have features similar to those of LB, in terms of containing two proteins; i.e., α-synuclein and ubiquitin, described as the major components of LB [2].

pIκBα and SCF ligase are also involved in UPP-related proteins, and these molecules have not been adequately studied in PD. Therefore, to explore how these

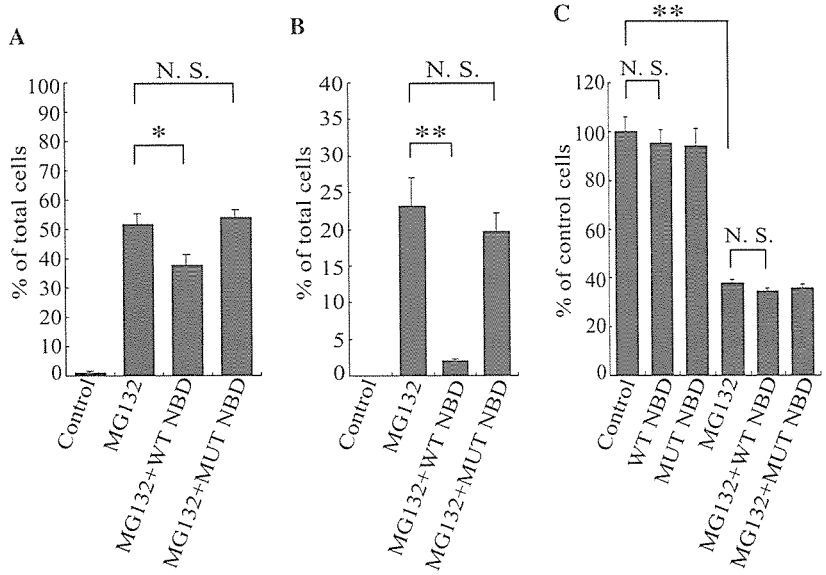

Fig. 5. Effects of NBD on formation of pIκBα-containing inclusions and cell death caused by proteasomal inhibition. After the cells were treated as explained in Fig. 4, the proportions of cells with cytoplasmic inclusions were determined. (A) The proportion of cells with ubiquitin-positive inclusions was calculated relative to total cells. (B) The proportion of cells with ubiquitinated inclusions containing pIκBα was calculated relative to total cells. In each experiment, 10 fields of 50 cells were counted. Similar effects of NBD were seen in two or more independent experiments. Data are means ± SEM. $*p < 0.05$; $**p < 0.001$ for differences between cell lines (Tukey's multiple t test). NS, not significant. (C) Cell viability was assessed as described in Materials and methods, and is expressed as the percentage of untreated cells. Similar results were seen in three independent experiments. Values are means ± SEM, each $n = 8$. $**p < 0.001$ for differences between cell lines (Tukey's multiple t test).

molecules are present in LB would be important in considering the process of LB formation. In our cell culture model, inhibition of normal proteasomal function by MG132 also induced the formation of ubiquitinated cytoplasmic inclusions containing α-synuclein, and this finding is consistent with previous reports [20,29], as described above. Intriguingly, our results showed that these inclusions were positive for pIκBα and some components of its ligase that are found in the LB. These findings suggest that the existence of pIκBα in LB is more likely and proteasomal dysfunction is an important factor in the formation of cytoplasmic inclusions.

Using SDS–PAGE analysis of detergent-soluble and -insoluble fractions, we found high-molecular weight ubiquitinated proteins particularly in the detergent-insoluble fraction, and pIκBα in the insoluble fraction following proteasomal inhibition with MG132. In contrast, after incubation with TNF-α alone, neither high-molecular weight ubiquitinated bands nor pIκBα was detected in the insoluble fractions, and cytoplasmic inclusions containing ubiquitin and pIκBα were not observed. These findings suggest that phosphorylation of IκBα alone is insufficient for the formation of cytoplasmic inclusions, and there appears to be a strong causal link between the accumulation of poorly degraded proteins, resulting from proteasomal dysfunction, and the formation of cytoplasmic inclusions.

We also showed that the presence of pIκBα in the ubiquitinated inclusions was markedly inhibited by a specific IKK inhibitor, under the conditions of MG132 treatment. This finding also supports the above-mentioned data that pIκBα is involved in the cytoplasmic inclusions resulting from proteasomal inhibition in our SH-SY5Y cells. In addition, this finding provides us a further possibility. In some neurodegenerative disorders, the ubiquitin-positive inclusions are considered to involve the ubiquitin–protein conjugates [28,30]. However, it is not clear which types of proteins are directly polyubiquitinated in LB. IκBα is phosphorylated by IKK, and pIκBα is polyubiquitinated by the $SCF^{\beta\text{-}TrCP}$, then degraded by the 26S proteasome. Thus, it is conceivable that once phosphorylation of IκBα is inhibited, neither polyubiquitination after its phosphorylation nor accumulation of IκBα into inclusion bodies is observed. We demonstrated that wild-type NBD peptide reduced the proportion of not only ubiquitin-positive inclusions, but also ubiquitinated inclusions containing pIκBα. Based on our finding, it is possible that the polyubiquitination of pIκBα resulting from proteasomal dysfunction triggers its entry into ubiquitinated cytoplasmic inclusions.

It is still not clear whether LB are cytoprotective or cytotoxic for neurons in the SN of PD. Recent studies suggest that the formation of protein aggregates or intracellular inclusions may be beneficial for cell survival rather than enhance cell death [31,32]. In the present study, exposure to MG132 alone or MG132 in the presence of wild-type NBD peptide did not alter cell viability whereas the same conditions decreased the ubiquitinated cytoplasmic inclusions. This finding at least supports the

conclusion of the above studies [31,32], i.e., the formation of cytoplasmic inclusions is not a toxic response against cell survival. Viewed from a different angle, our finding may suggest that inclusion bodies formed following proteasomal inhibition are independent of cell death.

In conclusion, we demonstrated the presence of pIκBα in LB of PD, and that similar inclusion bodies are produced in the presence of significant proteasomal dysfunction in cultured cells. Our observations in cultured cells may reflect, at least in part, the formation of LB in dopaminergic neurons of PD.

Acknowledgments

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Common anti-apoptotic roles of parkin and α-synuclein in human dopaminergic cells

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Abstract

Parkin, a product of the gene responsible for autosomal recessive juvenile parkinsonism (AR-JP), is an important player in the pathogenic process of Parkinson's disease (PD). Despite numerous studies including search for the substrate of parkin as an E3 ubiquitin–protein ligase, the mechanism by which loss-of-function of parkin induces selective dopaminergic neuronal death remains unclear. Related to this issue, here we show that antisense knockdown of parkin causes apoptotic cell death of human dopaminergic SH-SY5Y cells associated with caspase activation and accompanied by accumulation of oxidative dopamine (DA) metabolites due to auto-oxidation of DOPA and DA. Forced expression of α-synuclein (α-SN), another familial PD gene product, prevented accumulation of oxidative DOPA/DA metabolites and cell death caused by parkin loss. Our findings indicate that both parkin and α-SN share a common pathway in DA metabolism whose abnormality leads to accumulation of oxidative DA metabolites and subsequent cell death.

Keywords: Parkin; Apoptosis; Antisense; Knockdown; Neuroblastoma; Synuclein dopamine metabolism; Quinone

Parkinson's disease (PD) is the second most common neurodegenerative disorder primarily caused by selective loss of dopaminergic neurons in the midbrain substantia nigra pars compacta. Familial PD has been highlighted to study the mechanisms underlying neurodegeneration in PD, although only 5–10% of patients with PD are of the familial form of PD [1,2]. To date, 10 causative genes have been mapped and cloned in familial PD by linkage studies, which have significantly enhanced our understanding of the genetic mechanisms of PD [3]. Of these genes, *parkin*, the causative gene (*PARK2*) of AR-JP, representing the most prevalent form of familial PD [4], is of special interest, because it encodes an E3 ubiquitin–protein ligase [5], which

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covalently attaches ubiquitin to target proteins, designating them for destruction by the proteasome [6,7]. These findings suggest that impediment of *parkin* leads to deterioration of dopaminergic neurons and that PD, at least AR-JP, is caused by the failure of proteolysis mediated by the ubiquitin–proteasome system [8]. Since then, our knowledge about parkin has expanded, and indeed at present various putative substrates, e.g., CDCrel-1, synphilin-1, α-SN22 (O-glycosylated form of α-synuclein [α-SN]), Pael-R, and cyclin E have been identified [9–14]. Moreover, negative regulation of parkin E3 activity by parkin modification, such as nitration and phosphorylation, has been reported [15–17], but the pathophysiological role of parkin is still poorly understood.

One crucial issue that needs to be investigated is why AR-JP brains show severe neuronal loss with gliosis in the substantia nigra and mild neuronal loss in the locus coeruleus and why dopaminergic neurons in the substantia nigra are particularly vulnerable to the loss-of-function effect of parkin, though parkin is expressed ubiquitously throughout the brain. To define how the loss-of-function of parkin induces selective dopaminergic neuronal death in the midbrain, we interfered with endogenous parkin mRNA, a potentially suitable in vitro model of AR-JP for investigating the mechanism of selective dopaminergic neuronal death. To knock down the level of parkin in cells, we designed a full-length human parkin antisense (abbreviated as-parkin) using an adenovirus vector that has a high multiplicity of infection (moi) toward post-mitotic cells or cell lines which has neuronal characteristics and is an excellent tool to search for the effect of as-parkin on differentiated SH-SY5Y cells that exhibit features characteristic of dopaminergic neurons.

Here, we report that as-parkin selectively induced apoptosis of SH-SY5Y cells in a caspase-dependent manner. We also found that loss of parkin resulted in accumulation of endogenous L-3,4-dihydroxyphenylalanine (DOPA)- and dopamine (DA)-chromes derived from auto-oxidation of DOPA/DA-quinones, which mediates the toxic effect by covalently binding to the thiol group of proteins and consequently disintegrates cellular integrity and eventually causes cell death [18–20]. α-SN is a putative protein associated with membrane transport or signal transduction but of unknown function. α-SN gene mutations such as missense or multiplication cause familial autosomal dominant PD [21–27]. We found that forced expression of α-SN suppressed the loss of cell viability and accumulation of oxidative DOPA/DA metabolites caused by loss of parkin. Based on these findings, we propose that parkin and α-SN contribute to a common DA metabolic pathway; the impairment of which may lead to selective degeneration of dopaminergic neurons and consequently to PD.

Materials and methods

Adenoviruses. We used the adenoviral plasmid (pAdEasy-1) and the shuttle vector (p-shuttle-CMV) (Q.Bio gene). Various cDNAs used were inserted into the shuttle vector. The shuttle vector plasmid was linearized with *Pme*I. Electrocompetent *Escherichia coli* BJ5183 cells were added and electroporation was performed in 2-mm cuvettes in a Gene Pulser electroporator. Cells were inoculated onto 10-cm Petri dish containing LB-agar and 50 μg/ml kanamycin. Smaller colonies were picked and grown in 2 ml LB-broth (Sigma Chemical St. Louis, MO) containing 50 μg/ml kanamycin. Recombination was confirmed with *Pac*I. Approximately 5×10^6 cells were plated onto 10-cm culture dish. Ten micrograms of plasmid DNA linearized by *Pme*I, 12 μl FuGENE6 (Roche Molecular Systems, NJ), and 500 μl OptiMEM (Gibco-BRL) were mixed and transfected, according to the protocol provided by the manufacturer. After 7–10 days, the cells were collected by scraping off the 10-cm dish together with floating cells in the culture. The supernatant was removed after low-speed centrifugation. After sonicating the pellet, the cells were resuspended into 1 ml Dulbecco's modified Eagle's medium (DMEM) and frozen to −80 °C. In the next step, 500 μl of viral lysate was used to infect 7×10^7 cells in 15-cm dish. This process was repeated 1–3 times. Viruses were purified by CsCl banding; the final yield was 10^{10} plaque forming units.

Cells and cell culture. Human neuroblastoma cells (SH-SY5Y) and HeLa cells were obtained from American Type Culture Collection. The cells were maintained in growth medium (DMEM, Sigma, supplemented with 10% fetal bovine serum [Gibco-BRL, Gaithersburg, MD], 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C under 5% CO_2. SH-SY5Y cells were cultured with 100 μM of all *trans*-retinoic acid in dimethyl sulfoxide (DMSO) (Sigma R-2625) for 3 to 4 days for differentiation. The cells were infected with the antisense adenovirus at 150 moi; LacZ at 150 and 5 moi, wild and mutant α-SN adenovirus at 5 moi. Cells were collected 36 h after infection, centrifuged, and analyzed.

Western blotting. Infected or control cells were lysed in Laemmli SDS sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (NuPAGE, Invitrogen, San Diego, CA) and transferred onto polyvinylidene difluoride (PVDF) membrane. Western blotting was performed according to the ECL protocol provided by the supplier (Invitrogen, San Diego, CA) using specific antibodies of parkin and cleaved caspases (Cell Signaling Technology, Beverly, MA), α-SN (BD Transduction Laboratories, Lexington, KY), and β-Gal (Promega, Madison, WI).

Cell survival assay. Cells were infected with as-parkin or LacZ adenovirus and incubated for 48 h in 96-well plate. Cell viability was evaluated using the WST8, MTT reduction assay. Briefly, the solution of 0.1 mg/ml MTT in DMEM was added to each well and incubated for 2 h. The transmission was evaluated at 450 and 655 nm by 96-well microplate reader (Bio-Rad, Richmond, CA).

TUNEL assay. Terminal-deoxynucleotidyl transferase mediated d-UTP nick end labeling (TUNEL) assay was performed using formalin-fixed, ApopTag In Situ Apoptosis Detection Kits (Intergen, Purchase, NY). Fragmented DNA was labeled by fluorescein isothiocyanate (FITC) and observed under a fluorescence microscope.

Measurements of DOPA/DA-chromes. Thirty-six hours after infection, cells were solubilized in 500 μl of 1% Triton X-100 solution for 2 h and then centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was used as cell extract and was incubated for 3 min at room temperature. After 10% TCA protein precipitation, the generation of DOPA/DA-quinones was estimated by measuring the absorbance of the incubation supernatant at 475 nm based on the formation of DOPA/DA-chromes. The amount of DOPA/DA-chromes was calculated from a standard curve constructed using known amounts of DA and 0.01 mg/ml tyrosinase. The protein concentration in the cell extracts was determined by using the BCA Protein Assay Reagent Kit

(Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Statistical analysis. All data were expressed as means ± SEM. Differences between groups were examined for statistical significance using Dunnett's *t* test or Turkey's multiple *t* test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results and discussion

Antisense parkin causes loss of viability of SH-SY5Y cells

We first examined the effect of knockdown of parkin on the viability of human neuroblastoma cells (SH-SY5Y). These cells contain dopaminergic machinery and can differentiate into neuronal-like phenotypes when treated for 3–5 days with retinoic acid (RA), accompanied by arrest of cell proliferation and increased dopamine metabolism [28,29]. Infection of SH-SY5Y with full-length human as-parkin adenovirus caused deterioration of cell viability, as monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The differential SH-SY5Y cell death effect was observed in the range between 50 and 250 moi titer of as-parkin adenovirus (data not shown) and thereafter we routinely used 150 moi titer (Fig. 1A, left panel). Control β-galactosidase (β-Gal) adenovirus had no effect on cell viability, although β-Gal was highly expressed in SH-SY5Y cells (Fig. 1A, right panel). Infection of cells with as-parkin caused a marked decrease of endogenous parkin protein level in differentiated SH-SY5Y cells, without altering actin level in the cells (Fig. 1A, right panel). The effect of as-parkin was abrogated, upon co-infection with sense-parkin (data not shown).

Effect of antisense parkin is cell-type specific

Intriguingly, we found that the effect of as-parkin on cell viability was much less in undifferentiated growing SH-SY5Y cells compared with differentiated cells (Fig. 1B, left panel). In addition, as-parkin did not influence cell viability of HeLa cells derived from human adenocarcinoma of the uterine cervix, which do not express parkin protein and lack the dopamine metabolic pathway (Fig. 1B, right panel). Thus, antisense knockdown of parkin exerts its effect based on the cell type, and the effects are observed in a dopaminergic neuron-specific manner, and depend on the differentiation state of dopaminergic neurons.

Antisense parkin induces apoptotic cell death

As shown in Fig. 1C, the cells appeared clear when their morphology was compared with uninfected (control) and β-Gal expressing cells. SH-SY5Y cells infected with as-parkin adenovirus showed morphological

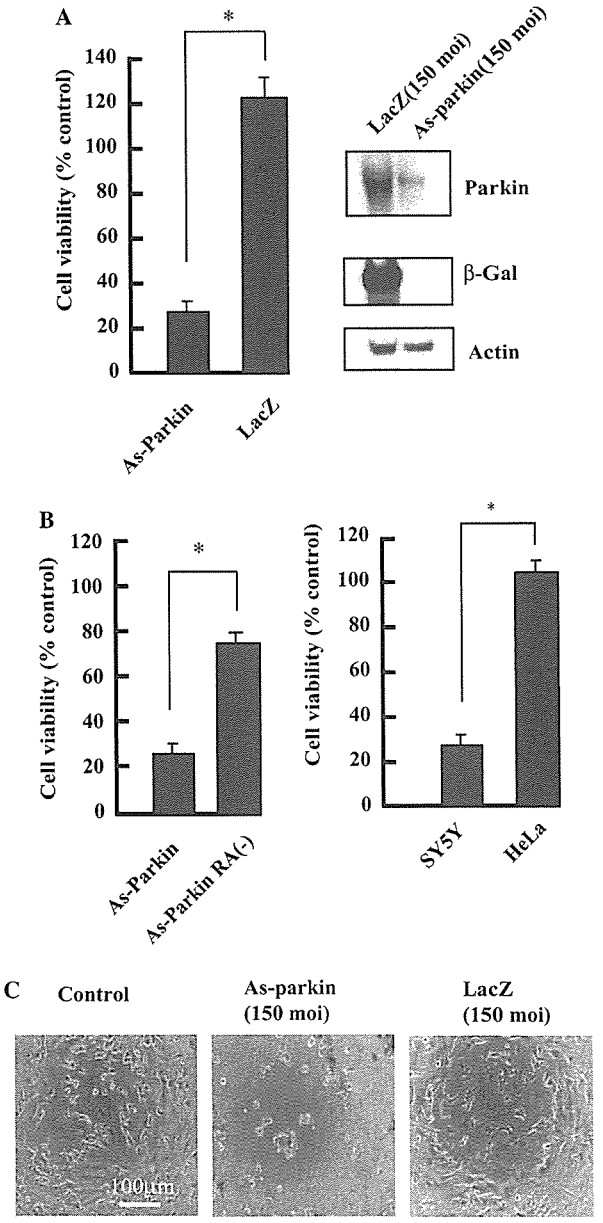

Fig. 1. Parkin knockdown is associated with loss of SH-SY5Y cell viability. (A) Effects of antisense parkin (as-parkin) and LacZ. Adenoviruses were infected for 48 h with 150 moi titers as indicated on the differentiated SH-SY5Y cells that had been pre-cultured with RA for 4 days. Cell viability was determined by the MTT assay (left panel). The results are expressed as percentage of MTT activity of uninfected cells (control). Data represent means ± SEM of 8 determinations. $*P < 0.01$ versus control group (Dunnett's *t* test). Cells that had been treated for 48 h with 150 moi titers of as-parkin and LacZ adenoviruses were lysed in Laemmli SDS sample buffer, and the proteins were separated by SDS–PAGE, followed by Western blotting with antibodies against parkin, β-galactosidase (β-Gal), and actin (right panel). (B) Undifferentiated SH-SY5Y cells without treatment with RA and HeLa cells were treated for 48 h with as-parkin adenovirus. The cell viability was measured and represented as indicated. (C) Morphological changes in differentiated SH-SY5Y cells upon knockdown of parkin. The cells were infected for 48 h with as-parkin and LacZ adenovirus vectors or left uninfected (control). Note the presence of apoptotic cells. Bar, 100 μm.

changes typical of apoptosis. To determine the nature of cell death induced by as-parkin, we performed TUNEL assay. As shown in Fig. 2A, as-parkin-treated SH-SY5Y cells showed nuclear condensation and fragmentation. In contrast, these changes were rarely observed in β-Gal-expressing SH-SY5Y cells. In support

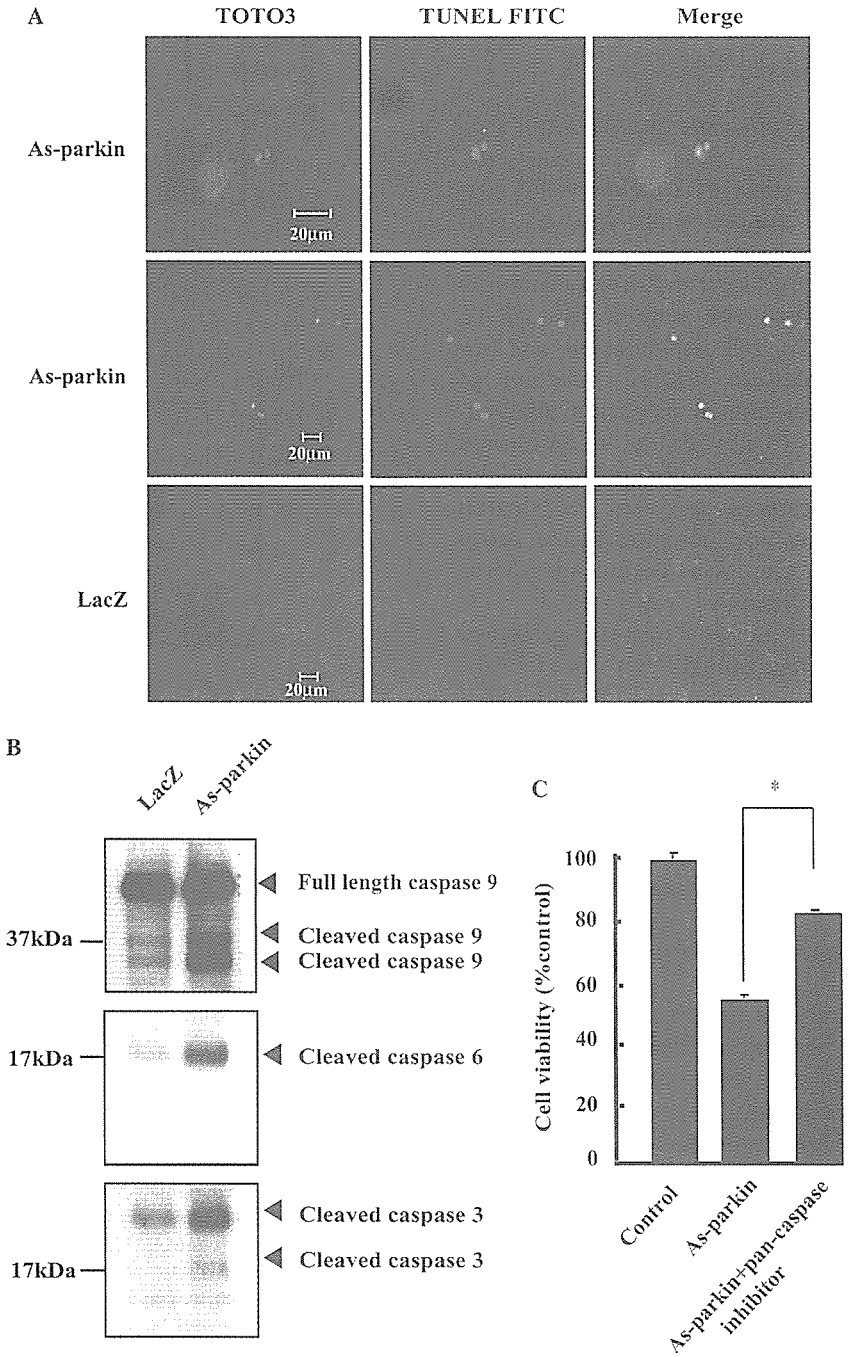

Fig. 2. Parkin knockdown induces apoptosis. (A) Detection of cells with nuclear DNA fragmentation due to parkin knockdown by TUNEL assay. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin and LacZ adenoviruses (150 moi). TUNEL assay was performed to detect apoptotic cells. TUNEL-positive cells (green) were detected (TdT enzyme is labeled with FITC green fluorescence). Nuclei were counterstained with TOTO3 (red). Bar, 20 μm. (B) Activation of caspase-3, -6, and -9 by as-parkin. After infection with as-parkin and LacZ adenoviruses as for a, the cell extracts were used for Western blot analysis using antibodies against cleaved caspase-3, -6, and -9. Arrowheads on the right indicated corresponding caspases. Note that anti-cleaved caspase-3 and -6 antibodies did not react with their native forms. (C) Effects of a 'pan' caspase inhibitor on apoptosis induced by the loss of parkin. The inhibitor was added at 100 μM when cells were treated by as-parkin adenovirus as for (A). Note that the caspase inhibitor significantly blocked parkin knockdown-induced deterioration of cell viability. Data represent means ± SEM of 8 determinations. $^*P < 0.05$ versus control (uninfected) group (Dunnett's t test).

of the TUNEL findings, we also detected activation of caspase-3, -6, and -9 in SH-SY5Y cells under parkin knockdown (Fig. 2B). In addition, Western blot analysis showed cleaved poly(ADP)-ribose polymerase (PARP) in the course of as-parkin infection and a time-dependent increase of the 25-kDa cleaved fragment, confirming the activation of caspase(s) (data not shown). Further experiments showed that application of a pan-caspase inhibitor for 6 h before infection significantly prevented apoptotic cell death as determined by the MTT reduction assay (Fig. 2C). Taken together, these results suggest that as-parkin-induced SH-SY5Y cell death is likely to be mediated by activation of the caspase cascade.

Antisense parkin increases DOPA/DA metabolites

Next we examined the effect of α-SN on the viability and DOPA/DA-chrome level in differentiated SH-SY5Y cells with a reference to parkin loss. These experiments were based on previous studies describing abnormal DA metabolism in α-SN-deficient mice [30] and α-SN binding to DA-quinones [31]. For this purpose, we constructed adenovirus vectors expressing α-SN, and first tested its effect on the expression of parkin. α-SN and its PD-linked mutants (Ala30Pro and Ala53Thr) had no effect on the levels of parkin, irrespective of the treatment of as-parkin (Figs. 4A and B). It is of note that α-SN did not express at significant levels in SH-SY5Y cells under present conditions. Then, we investigated the effect of α-SN on the as-parkin-induced loss of cell viability. As shown in Fig. 4A, infection of SH-SY5Y cells by both adenovirus vectors expressing α-SN and as-parkin caused marked reduction of their cellular chrome levels and resulted in amelioration of as-parkin-induced deterioration of cell viability (Fig. 4B). Intriguingly, coinfection of cells with wild-type α-SN and as-parkin adenoviruses abrogated as-parkin-induced accumulation of DOPA/DA-chrome. However, α-SN mutants (Ala30Pro and Ala53Thr) and β-Gal expression did not reduce the generation of DOPA/DA-chrome by as-parkin. Thus, it seems that the α-SN-induced suppression of apoptosis was associated with a reduction in the DOPA/DA-chrome level in α-SN expressing SH-SY5Y cells. These results suggest that α-SN inhibits apoptosis induced by parkin knockdown by blocking the generation of DOPA/DA-chromes; i.e., DOPA/DA-quinones.

Antisense parkin-induced extensive apoptosis of differentiated dopaminergic SH-SY5Y cells but limited apoptosis of undifferentiated SH-SY5Y cells and no apoptosis of HeLa cells, indicating cell-type specificity. With regard to the cell-specific vulnerability, an important factor seems to be dopamine (DA) metabolism, which is a peculiar feature of dopaminergic neurons. Indeed, the differentiated SH-SY5Y cells retain a high DA metabolic pathway [28,29]. DA is a molecule prone to oxidation and it contributes to the generation of reactive oxygen species, which when in abundance can cause oxidative injury of various cellular components[18,20]. Indeed, abnormally high levels of these free radicals in dopaminergic neurons have been implicated as environmental factors causing not only sporadic PD but also AR-JP [32,33]. We tested the effects of as-parkin infection on the level of endogenous DOPA- and DA-chromes (DOPA/DA-chromes), which are derived from DOPA- and DA-quinones, respectively, whose metabolites could originate from cytosolic DOPA or DA oxidation [18,20]. Thus, the amounts of DOPA/DA-chromes reflect those of endogenous DOPA/DA-quinones. DOPA/DA-chrome levels were significantly high in parkin knockdown cells whereas there was no change in β-Gal expressing ones (Fig. 4A). These findings suggest that parkin knockdown-induced apoptosis is mediated by an increase in DOPA/DA-chromes.

Recently, four groups independently reported the generation of a mouse model that lacks the *parkin* gene, which display certain abnormalities of dopamine metabolism [34–37]. However, these parkin knockout mice had only subtle phenotypes exhibiting a largely normal gross brain morphology. Based on the pathologic findings, all the parkin null mice showed no neuronal loss in the SN. This is in marked contrast to our in vitro system described in this study, in which parkin knockdown induced activation of the caspase cascade and apoptosis of dopaminergic SH-SY5Y cells. Why do parkin knockout mice lack the abnormalities seen in AR-JP patients? One plausible explanation is the presence of a putative molecule(s) that suppresses the defect induced by loss-of-function of parkin, and the abundant presence of such molecule(s) in the brain should be linked to the pathogenesis of PD. Here, we propose that α-SN is the molecule that compensates for the loss of parkin, since α-SN prevented apoptotic cell death induced by as-parkin. In this regard, Western blot analysis showed that the dopaminergic SH-SY5Y cells did not express α-SN at significant levels (Fig. 3A, lanes 2 and 4), which is in marked contrast to the high abundance of dopaminergic neurons in vivo [38]. Regardless of the compensatory role of α-SN for the loss-of-function of parkin in the AR-JP, α-SN probably cannot cope with the accumulation of toxic molecules in the absence of parkin and thus apoptotic neuronal death perhaps occurs gradually, leading to degeneration of dopaminergic neurons and consequently the development of early-onset PD. We provide the first evidence for the anti-apoptotic role of α-SN and its involvement in the common pathway of parkin.

To date, several studies have demonstrated that α-SN exerts protective effects against various cellular stresses such as oxidative damage and related apoptosis of neurons [39,40]. Considering the reason why muta-

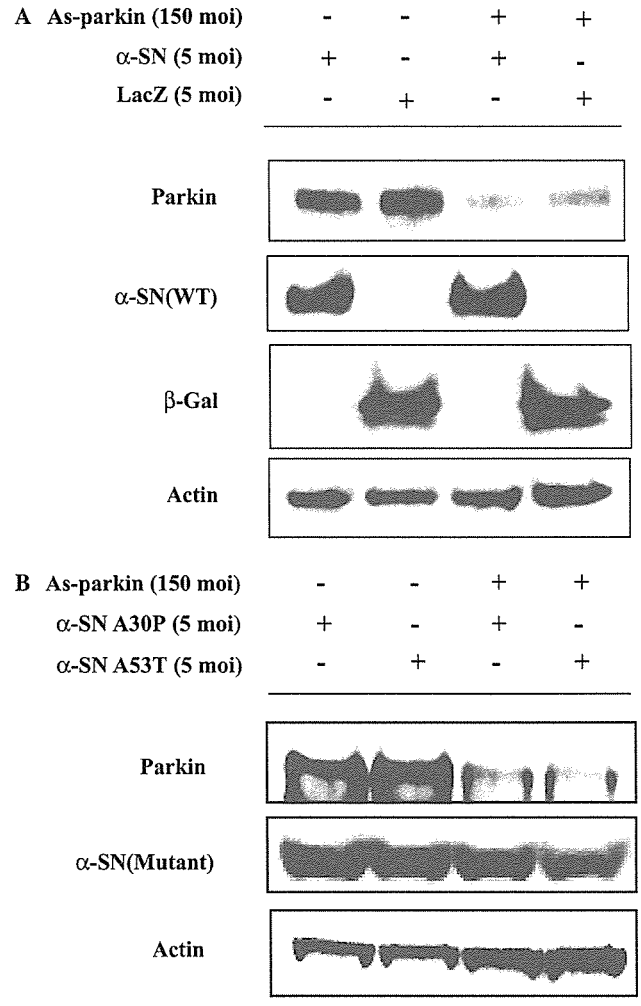

Fig. 3. Expression levels of parkin, α-SN, and LacZ. The expression levels of parkin, wild, and mutant α-SN, and LacZ in SH-SY5Y cells treated for 48 h with adenoviruses were monitored by Western blot analysis.

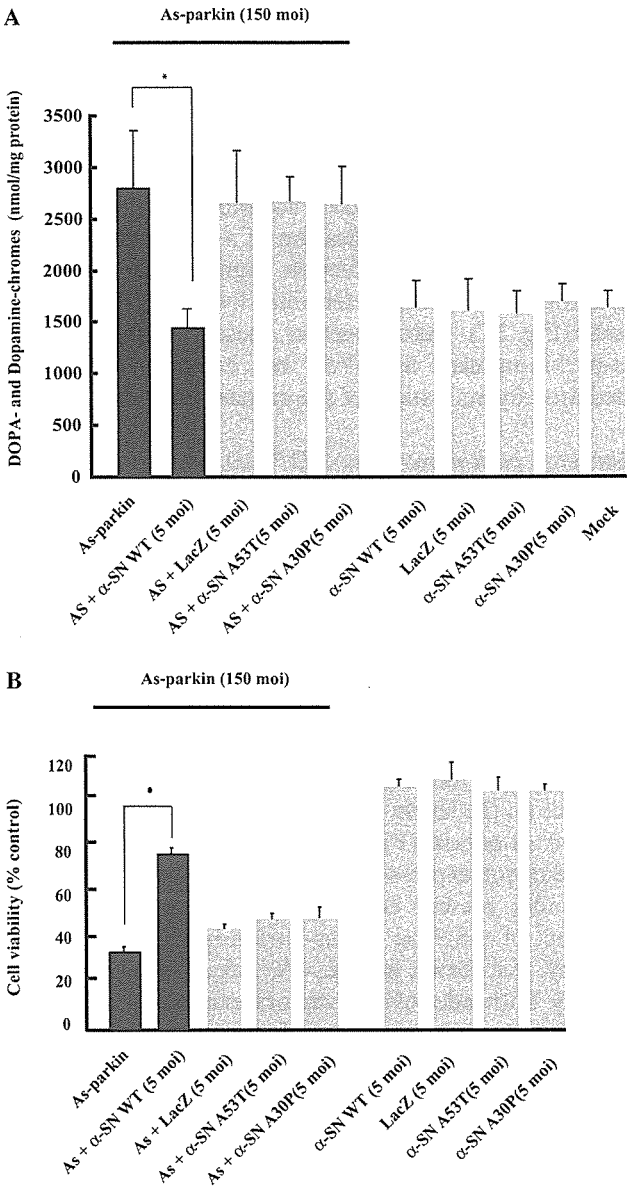

Fig. 4. α-Synuclein inhibits parkin knockdown-induced apoptosis and accumulation of DOPA- and DA-quinones. (A) Cellular level of DOPA/DA-chromes. After the differentiated SH-SY5Y cells were treated for 36 h with as-parkin, wild, and mutant α-SN, LacZ, and adenoviruses, cellular DOPA/DA-chromes were measured. Note the profound decrease of DOPA/DA-chromes in α-SN-expressing SH-SY5Y cells. Data are means ± SEM of 10 determinations. $^*P < 0.05$ versus control group (Turkey's multiple t test). (B) Effects of overexpression of wild and mutant α-SN on as-parkin-induced deterioration of cell viability. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin adenovirus. Cells were coinfected with LacZ and α-SN adenovirus (5 moi) and at 150 moi titers of as-parkin adenovirus. The cell viability was measured and represented as in Fig. 1A (left panel).

tion of α-SN causes familial PD, it is clear that this type of disease is due to the gain-of-toxic function of the α-SN mutants with missense mutations, differing from the neuroprotective roles of the wild-type α-SN. In addition, α-SN proteins with disease-causing missense mutations tend to generate protofibrils [31,41], suggesting that protein misfolding including α-SN plays a key role in the pathogenesis of PD. In contrast, at high concentrations, it oligomerizes to β-pleated sheets known as protofibrils (i.e., fibrillar polymers with amyloid-like characteristics). Indeed, multiplication of α-SN has been reported in the autosomal dominant form of PD, indicating that overproduction of this protein affects the cellular damages. In this regard, there is a discrepancy between the protective role of α-SN in the present study and combination of PD and α-SN multiplication. This could be explained by appropriate physiological level of synuclein [40]. Thus, in patients with α-SN multiplication, the copy numbers of this gene may be related to the clinical severity of PD; patients with triplicate α-SN show dementia with Lewy bodies [24]; while those with duplicate levels do not show dementia [26,27].

It remains unclear why dopaminergic neurons of the substantia nigra are selectively vulnerable to the loss of parkin in AR-JP patients. In the present study, we provided a clue for this enigmatic puzzle. Considering the specificity of the lesions in PD, it is possible that the high oxidative state associated with DA metabolism may cause deterioration of dopaminergic neurons. The mechanism underlying increased oxidative stress may involve DA itself, because oxidation of cytosolic DOPA/DA may be deleterious to neurons. Indeed, DA causes apoptotic cell death as evident by morphological nuclear changes and DNA fragmentation [42–44]. In this regard, we showed here that as-parkin directed loss of parkin leads to abnormality of DOPA/DA metabolism, which resulted in the generation of DOPA/DA-quinones in SH-SY5Y cells. Thus, DA and its metabolites seem to exert cytotoxicity mainly by generating highly reactive quinones through auto-oxidation. On the other hand, the toxicity of DOPA and DA is due to the generation of reactive oxygen species that could disrupt cellular integrity, causing cell death. However, the reason for the production of oxidative DOPA/DA-metabolites following loss of parkin is not clear at present.

Our results showed for the first time that loss of parkin leads to death of differentiated dopaminergic cells in vitro. This cell-based experiment enhances our understanding of the pathophysiology of PD and could be potentially useful for drug screening. Our results also showed that α-SN and parkin are involved in DA metabolism and that aberrant regulation of DA is accompanied by accumulation of oxidative DOPA/DA metabolites.

Acknowledgments

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REGULAR PAPER

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Expression of 8-oxoguanine DNA glycosylase (OGG1) in Parkinson's disease and related neurodegenerative disorders

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Abstract Oxidative stress including DNA oxidation is implicated in Parkinson's disease (PD). We postulated that DNA repair enzymes such as 8-oxoguanosine DNA glycosylase (OGG1) are involved in the PD process. We performed immunohistochemical and biochemical studies on brains of patients with PD and those of patients with progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) as disease controls, and control subjects. We found higher expression levels of mitochondrial isoforms of OGG1 enzymes in the substantia nigra (SN) in cases of PD. Furthermore, Western blot analysis revealed high OGG1 levels in the SN of the patients with PD. Our results indicate the importance of oxidative stress within the susceptible lesions in the pathogenesis of PD.

Keywords 8-Oxoguanosine DNA glycosylase · Mitochondrial DNA · Parkinson's disease · Progressive supranuclear palsy · Corticobasal degeneration

Introduction

The primary cause of Parkinson's disease (PD) is still unknown; however, oxidative stress and mitochondrial dysfunction have been implicated as major contributors to neuronal death in the substantia nigra (SN) [1, 11, 21, 27, 28, 29, 32, 33]. Reactive oxygen species (ROS) are highly reactive and oxidize nucleic acids, increasing the frequency of mutations in DNA. 8-Oxoguanosine (8-oxoG) is one of the oxidized forms of guanine. Because 8-oxoG mispairs with adenine and cytosine, 8-oxoG induces the occurrence GC:CG to T:A transversion mutation. There are various error-avoiding mechanisms in organisms against oxidative DNA damage [20, 31]. MutM, one of the DNA repair enzymes found in *Escherichia coli*, removes 8-oxoG paired with cytosine in DNA [19]. The human OGG1 gene encodes an 8-oxoG DNA glycosylase (hOGG1), which is a functional homolog of MutM [4, 23]. In human tissues, there are two major isoforms of hOGG1; hOGG1-1a and hOGG1-2a [23]. Whereas hOGG1-1a is located in the nucleus, hOGG1-2a is located on inner mitochondrial membrane, on which mitochondrial DNA (mtDNA) is situated [23]. The hOGG1-2a plays important roles in repairing errors caused by oxidative stress in mtDNA [23]. In the present study, we examined immunohistochemically and biochemically the expression of hOGG1-2a in human brains of normal subjects, patients with parkinsonism including PD, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD).

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Materials and methods

Patients and brain tissues (paraffin-embedded sections)

We examined autopsied brains of seven patients with PD, four with PSP, four with CBD, and seven aged-matched control subjects. The pathological diagnosis was confirmed in all patients and aged-matched control subjects by the Department of Neurology, Juntendo University School of Medicine. The clinical profiles of the patients are summarized in Table 1. To determine whether the disease duration of PD influenced the expression of hOGG1-2a, we divided PD patients into two groups by disease duration. We defined disease duration less than

Table1 clinical summary of all subjects (*ALS* amyotrophic lateral sclerosis,*CBD* corticobasal degeneration, *CML* chronic myelocytic leukaemia,*Fr* fresh frozen brain, *P* paraffin-embedded section,*PD* Parkinson's disease, *PN* pontine nucleus,*PSP* progressive supranuclear palsy, *SN* substantia nigra)

Case	Diagnosis	Age	Sex	Duration (years)	Postmortem delay (h)	Cause of death	Sample
1	Control	65	F	-	1.5	ALS	P
2	Control	38	M	-	10	Malignant lymphoma	P
3	Control	82	F	-	1.5	Cerebral infarction	P
4	Control	60	M	-	5.5	Liver cirrhosis	P
5	Control	67	M	-	3.5	CML	P
6	Control	55	M	-	11	Gastric cancer	P
7	Control	81	M	-	2.5	Cerebral infarction	P
8	Control	89	M	-	2.5	Lung cancer	Fr (SN1, PN1)
9	Control	66	M	-	5	Gallbladder cancer	Fr (SN2, PN2)
10	Control	57	M	-	5	Chronic renal failure	Fr (SN3, PN3)
11	Control	48	F	-	5.5	SLE	Fr (SN4)
12	Control	70	F	-	2.5	Laryngeal cancer	Fr (SN5)
13	PD	72	F	5	< 24	Lung haemorrhage	P
14	PD	51	M	11	3.5	Pneumonia	P
15	PD	65	F	4.5	2	Asthma	P
16	PD	74	M	17	1	Liver cirrhosis	P
17	PD	37	F	7	< 24	Unknown	P
18	PD	71	F	13	8	Pneumonia	P
19	PD	63	M	12	20	Unknown	P
20	PD	56	F	17	6	Pneumonia	Fr (SN6)
21	PD	71	M	Unknown	< 24	Unknown	Fr (SN7)
22	PD	75	M	11	5	Pneumonia	Fr (SN8)
23	PD	85	F	13	5	Pneumonia	Fr (SN9)
24	PD	66	M	10		Pneumonia	Fr (SN10)
25	PSP	71	F	13	13	Lung cancer	P
26	PSP	85	F	3	3.5	Pneumonia	P
27	PSP	77	M	6	12	Pulmonary embolism	P
28	PSP	69	M	4	6.5	Pneumonia	P
29	PSP	86	F	6	< 12	Pneumonia	Fr (PN4)
30	PSP	74	M	6	3	Pneumonia	Fr (PN5)
31	CBD	61	M	8	8	Pneumonia	P, Fr (PN6)
32	CBD	81	F	8	9	Pneumonia	P
33	CBD	59	F	3	4.5	Ileus	P
34	CBD	65	M	6	9	Pneumonia	P, Fr (PN7)

10 years as short-duration group and over 10 years as long-duration group. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

Antibodies

A rabbit polyclonal anti-hOGG1-2a antibody was prepared as described previously [23]. The antibody recognizes the C terminus of hOGG1-2a and its specificity has been confirmed [23]. Anti-TOM40, cytochrome oxidase subunit I, tyrosine hydroxylase (TH), and GAPDH antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA), Molecular Probes (Eugene, OR), Calbiochem (La Jolla, CA) and Chemicon (Temecula, CA), respectively.

Immunohistochemistry and immunofluorescence for hOGG1-2a

The deparaffinized sections were microwaved in phosphate buffer (Antigen Retrieval Citra, BioGenex, San Ramon, CA) for antigen retrieval. Endogenous peroxidases were quenched by incubation with 3% hydrogen peroxide. After incubating the slides with blocking solution (10% normal goat serum), sections were treated overnight at 4°C with an anti-hOGG1-2a antibody. These sections were incubated with biotinylated anti-rabbit IgG from goat (DAKO, Carpinteria, CA; 1:100). After incubation with streptavidin conjugated to horseradish peroxidase (HRP) (DAKO; 1:200), we treated sections with biotinyl tyramide and hydrogen peroxide. Sections were then incubated with streptavidin conjugated to HRP and visualized with 3',3'-diaminobenidine (DAB). Double immunofluorescence was performed with anti-hOGG1-2a and anti-cytochrome oxidase subunit I antibodies, or with anti-hOGG1-2a and TH antibodies. These sections were treated with Alexa fluor goat anti-rabbit IgG (Molecular Probes; 1:200) and Alexa fluor goat anti-mouse IgG (Molecular Probes; 1:300). Signal was observed under Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

Semiquantitative analysis

For semiquantitative analysis, microscopic photographs of the whole SN and pontine nuclei (PN) were prepared.