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Selective Susceptibility of Human Dopaminergic Neural Stem Cells to Dopamine-Induced Apoptosis

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ABSTRACT

Dysfunctions of ubiquitin-proteasome system and toxicity of dopamine have been known as the key mechanisms in the pathogenesis of Parkinson's disease (PD) and proteasome inhibitors are widely used in experimental models of PD to reproduce cell death of dopaminergic neurons. In the present study, immortalized human neural stem cells (HB1.F3, F3) and those transfected with human aromatic acid decarboxylase gene (F3.AADC), were used to investigate the mechanism of selective dopaminergic neuronal cell death mediated by dopamine or proteasome inhibitors. Flow cytometric analysis revealed that F3.AADC was more susceptible to dopamine than parental F3 cell which does not carry dopaminergic phenotype. The dopamine-induced apoptosis was mediated by activation of caspases 3 and 9 and cleavage of PARP. Proteasome inhibitors also induced apoptosis in dose-dependent manner but there was no difference between cell types. Prolonged exposure to subtoxic dose of proteasome inhibitors further enhanced dopamine-induced apoptosis in the F3.AADC, and increased presence of alpha-synuclein and ubiquitin-positive inclusions was noted in the cytoplasm of apoptotic cells by immunocytochemistry. These findings indicate that dopaminergic cells are selectively susceptible to dopamine toxicity and prolonged suppression of proteasome system further enhances selective sensitivity to dopamine toxicity. Chronic subtoxic proteasomal dysfunction of dopaminergic cells might contribute to selective cell death of dopaminergic neurons during the pathogenesis of Parkinson's disease.

Key words: Parkinson's disease, proteasome, dopamine, apoptosis, human neural stem cells

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, which is characterized by motor symptoms, such as bradykinesia, rigidity, tremor, and gait disturbance. PD

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pathology is characterized by selective dopaminergic neuronal death and cytoplasmic Lewy bodies in substantia nigra of midbrain (Forno, 1996). The cause of PD is yet to be clarified but diverse genetic and environmental mechanisms are inferred (Weidong et al., 2009).

Dopamine, a key neurotransmitter in PD, and L-DOPA, a precursor of dopamine cause cell death in cultured neural cells by oxidative and non-oxidative mechanisms (Ahmadi et al., 2008). In addition, dopamine turnover is increased in mesencephalic dopaminergic neurons in PD patients and reactive oxygen species are found to increase following treatment with L-DOPA (Foley at al., 2000). However, there is no convincing evidence of neurotoxic events caused by dopamine or L-DOPA *in vivo* conditions.

The ubiquitin-proteasome system is known to be implicated in pathogenesis of PD. Lewy body, a pathological hallmark of Parkinson's disease, contains a heterogeneous mixture of proteins and lipids, including alpha-synuclein, ubiquitin, synphilin-1, neurofilaments, and oxidized proteins (Olanow and McNaught, 2006). Familial PD cases are caused by abnormalities in genes encoding the proteins alpha-synuclein, parkin, ubiquitin carboxy-terminal hydrolase-L1 and DJ-1, which can cause dysfunctions of proteasome system (Hatano et al., 2009). Proteasome inhibitors are also reported to induce neuronal cells hypersensitive to protein alteration or oxidative stress (Mytilineou et al., 2004; Rideout et al., 2004; Lev et al., 2006). Others in contrast, reported that proteasome inhibitors, such as lactacystin and MG-132, caused the appearance of α-synuclein and ubiquitin inclusions, but prevented dopaminergic degeneration in a 6-hydroxydopamine-induced PD animal model (Inden et al., 2005). To date, the exact role of proteasome inhibition on dopaminergic neurodegeneration and subsequent development of Parkinson's disease is still not well understood.

Human neural stem cells (NSCs) could serve as a good *in vitro* model system representing *in vivo* conditions. In the present study, immortalized human neural stem cells (HB1.F3, F3) and those cells transduced with human aromatic acid decarboxylase gene (F3.AADC), were used to investigate the mechanism of selective dopaminergic neuronal cell death mediated by dopamine or proteasome inhibitors.

MATERIALS AND METHODS

Cell culture

This study used human neural stem cells derived from human embryonic telencephalon (15 week of gestational age), which were immortalized by using a retroviral vector encoding *v-myc* oncogene (Chu et al., 2003). One of the NSC clones, HB1.F3 (F3), expresses phenotypes specific for neural stem cells including ABCG2, nestin and mushashi-1 (Flax et al., 1998). Additionally, F3 expresses Na^+ current when transduced with NeuroD gene and differentiated into neurons (Cho et al., 2002). F3.AADC was also used which was prepared with human aromatic acid decarboxylase gene transfection. Cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Gathersberg, MD) with 10% fetal bovine serum. L-DOPA (5 μM) were added to culture media of F3.AADC. Patterns of growth and morphology were not significantly different from those of non-transfected or supplemented conditions.

Measurement of dopamine

The level of dopamine was measured in cells and cultured media. Medium was collected after 2 days of culture, reacted with alumina (180 mg/ml, Sigma) in 4°C for 24 hours, cleared with sample buffer (0.1 M perchloric acid, 0.1 mM EDTA), and then filtered with 0.22 μm filter. Cells were raked out from culture dish and crushed with sample buffer. The supernatant was collected after centrifuge and filtration. Purified cells and media were transferred through Reverse-Phase HPLC (Waters, Milford, MA) system in 0.5 ml/min mobile phase (0.07 M sodium phosphate monobasic pH 3.4, 1 mM sodium octanesulfonic acid, 0.1 mM EDTA, 8% acetonitrile). We used HR-80 RP-C18 (Esa, Chelmsford, MA) column and analyzed with Coulchem II electrochemical detector (Esa, Chelmsford, MA) in 400 mV.

Measurement of cytotoxicity

Cytotoxicity was evaluated by 3,4,5-dimethyl thiazole-3, 5-diphenyl tetrazolium bromide (MTT) assay test according to standard procedures. The ability of

viable cells to reduce MTT was determined. MTT stock solution (5 mg/ml) was prepared in PBS, pH 7.4, filtered through 0.22 M filter to remove formazan crystals and stored at −20°C in the dark. Cells were plated in a density of 5,000 cells/well on pre-coated 96-well plates and incubated for indicated times. Subsequently, 10-fold diluted MTT with culture medium was added to each well and incubated for 4 hours. Plates were centrifuged at 275×g for 5 min and the supernatant was aspirated. The formazan crystals were completely dissolved in dimethyl sulfoxide by vigorously shaking on microplate shaker. OD was read on microplate ELISA reader (El 312e, Bio-Tek) at wavelength of 550 nm and 630 nm. Survival of each experimental group was compared with that of control cells.

Quantitative assessment of apoptosis

The quantification of apoptotic cells was screened by flow cytometry technique using propidium iodide (PI), identifying cells in late apoptosis of which high permeability allows PI to stain intracellular DNA (Tang et al., 2005). Cells were plated in a 6-well plate and incubated for different time points with L-DOPA and dopamine, alone or in the presence of proteasome inhibitors. Cells were trypsinized and washed with PBS containing 1% bovine serum albumin. Cells were fixed with 70% ethanol containing 0.5% Tween 20 for 30 minutes at 4°C and stained with PBS containing 0.5 mg/ml of PI and 1 μg/ml of RNase A. Fluorescence was measured using a FACScan cytometry system (Beckman Coulter Epics XL, Palo Alto, CA).

The further quantification of apoptotic cells was conducted by dual-color flow cytometry technique using the annexin V conjugated with Alexa 488 and PI. Annexin V detects phosphatidylserine in the outer membrane leaflet, a marker for early apoptosis, whereas PI identifies cells in late apoptosis (Behbahani et al., 2005). Cells were harvested, washed in PBS, resuspended in 100 μl of Annexin binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and treated with Alexa 488-conjugated annexin V (5 μl) and PI (5 μg/ml). Cells were then incubated in room temperature for 15 minutes and analyzed using a FACScan cytometry system.

Measurement of mitochondrial membrane potential (MMP)

Changes in MMP after treatments with L-DOPA and dopamine, alone or in the presence of proteasome inhibitors, were determined by staining cells with JC-1, a mitochondrial potential indicator dye. JC-1 was added directly to the cell culture medium (2 μM) and incubated for 30 minutes at 37°C. After washing with culture medium, cells were trypsinized and resuspended with PBS. Shift from red fluorescent J-aggregates into green JC-1 monomers was immediately measured by flow cytometry.

Western blot analysis

For antibody cleavage blots, 2×10^6 cells treated for the indicated time points with L-DOPA and dopamine, alone or in the presence of proteasome inhibitors, were washed twice with ice-cold PBS, resuspended in 500 μl of ice-cold modified RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, NaCl 150 mm, 25 mM Tris-HEPES pH 7.6). Cells were scraped with a rubber policeman, and transferred into Eppendorf tubes. Cell suspension was rocked on a rocker at 4°C for 15 minutes to lyse cells. The lysates were centrifuged at 14,000 g for 15 min at 4°C. After protein quantification by the method of Bradford, ~20 μg of proteins per well was loaded onto 8~15% SDS-polyacrylamide gel electrophoresis. The gels were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, UK) and reacted with anti-PARP (Oncogene Science, Cambridge, MA), anti-caspase 3, and anti-caspase 7 antibodies (Santa Cruz Biotechnology Inc, CA). Immunological complexes were revealed with the appropriate secondary peroxidase-coupled antibodies (Cell Signaling Technology Inc, Danvers, MA), enhanced chemiluminescence detection system (Amersham Biosciences, UK) and detected by LAS-3000 (Fujifilm, Tokyo, Japan).

Immunocytochemistry

Cells were cultured on plastic chamber slides (Nunc, Naperville, IL), treated with L-DOPA and dopamine, alone or in the presence of proteasome inhibitors, and fixed with 4% paraformaldehyde at room temperature for 15 minutes. After washing, cells were permeabilized with phosphate-buffered

saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4) containing 0.25% Triton X-100 and blocked in 1% bovine serum albumin. Primary antibodies against α-synuclein (1 : 2,500, Sigma, St. Louis, MO) and ubiquitin (1 : 500, Chemicon, Temecula, CA) were incubated overnight at 4°C, followed by secondary antibodies conjugated with FITC or Texas Red (1 : 200, Molecular Probe, Eugene, OR) for 40 minutes at room temperature. Hoechst stain was used to observe apoptosis. The slide was mounted with Vectashield mounting media containing 4,6-diamino-2-phenylindole (Vetcor Lab, Burlingame, CA), and observed under the confocal microscope (LSM510, Carl Zeiss GmbH., Jena, Germany).

Statistical analysis

Data analysis was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL). The significance of intergroup differences was evaluated by one-way analyses of variance (ANOVA) and that of single comparisons was by the Student's *t*-test. Differences were considered significant at $p<0.05$.

RESULTS

Dopamine production

The basal levels of dopamine in cells and culture media were undetectable in human neural stem cells. Addition of L-DOPA to F3.AADC significantly increased the level of dopamine in culture media, while did not induce any noticeable change in cell morphology or growth pattern as compared to those of parental F3 cells. The concentration of dopamine in culture media was 0.9 ng/10^6 cells in F3 and 14.6 ng/10^6 cells in F3.AADC. Therefore, F3.AADC can be regarded as dopamine-secreting dopaminergic cells whereas their prototype cells, F3, as non-secreting control cells.

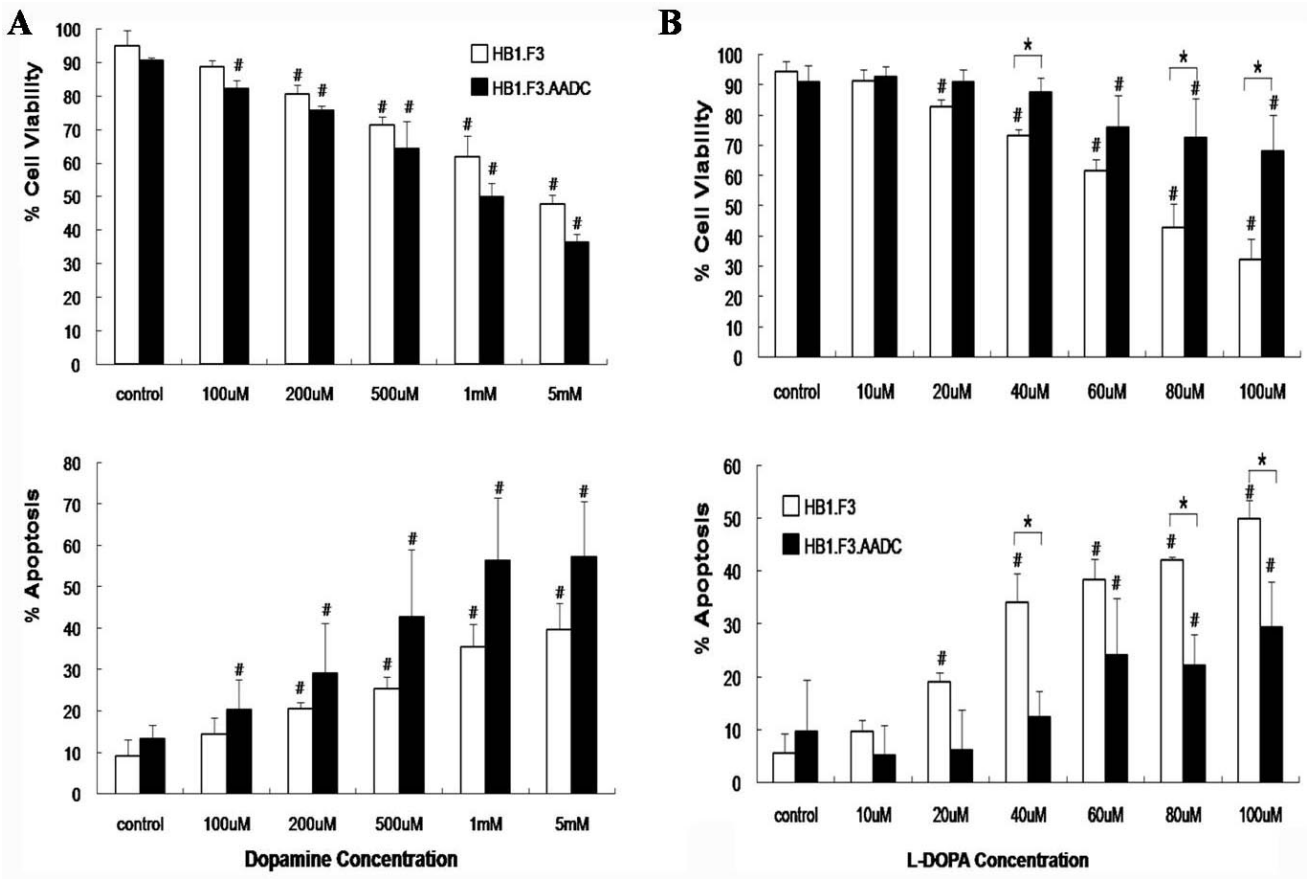

Fig. 1. Dopamine- and L-DOPA-induced cell death. Neural stem cells were incubated with various concentrations of dopamine (A) and L-DOPA (B), and the total cell death after 24 hours was measured using MTT assay. Apoptosis was quantified by PI fluorescence using flow cytometry. #$p<0.05$, vs. control condition, *$p<0.05$, between cell lines.

Cell death by L-DOPA and dopamine

Both L-DOPA and dopamine induced cell death in human neural stem cells was measured by either MTT or flow cytometry using PI assay. Results of cell death assessed by PI assay correlated well with those using MTT assay, indicating that most of cell death induced by L-DOPA or dopamine was apoptosis (Fig. 1). Dopamine-induced cell death was dose-dependent and more prominent in F3.AADC than F3 cells (Fig. 1A). At 500 μM of concentration, dopamine induced 42% of apoptotic cell death after 24 hours in F3.AADC cells compared to 25% in F3 cells (Fig. 1A). However, F3.AADC cells were more resistant to L-DOPA-induced cell death than to dopamine-induced one and these differences were reached statistical significance (Fig. 1B).

Mode of L-DOPA and dopamine-induced apoptosis

To elucidate the pathway of L-DOPA or dopamine-induced apoptotic cell death in dopaminergic cells typical apoptotic markers was examined. Annexin-V assay showed that both L-DOPA or dopamine-induced apoptosis follows typical apoptotic steps involving early apoptosis with translocation of annexin V-positive phosphatidylserine to cell surface and late apoptosis of annexin V-positive and PI-positive fraction (L4 and L2 fraction, respectively in Fig. 2A). Hoechst stain showed increased nuclear condensation and fragmentation in dopaminergic cells by both L-DOPA and dopamine (Fig. 2B). Measurement of mitochondrial membrane potential using JC-1 showed increased shift of red to green fluorescence after 8 hours treatments with L-DOPA or dopamine in dopaminergic cells (shifts from C2 fraction to C4 fraction in Fig. 3A), compared to the typical orange-red fluorescence of JC-1 aggregates in F3 (C2 fraction in Fig. 3A). L-DOPA and dopamine-induced apoptosis caused activation of PARP, caspase 3 and caspase 7, common execution systems in apoptosis by western blot analysis (Fig. 3B). These findings indicate that

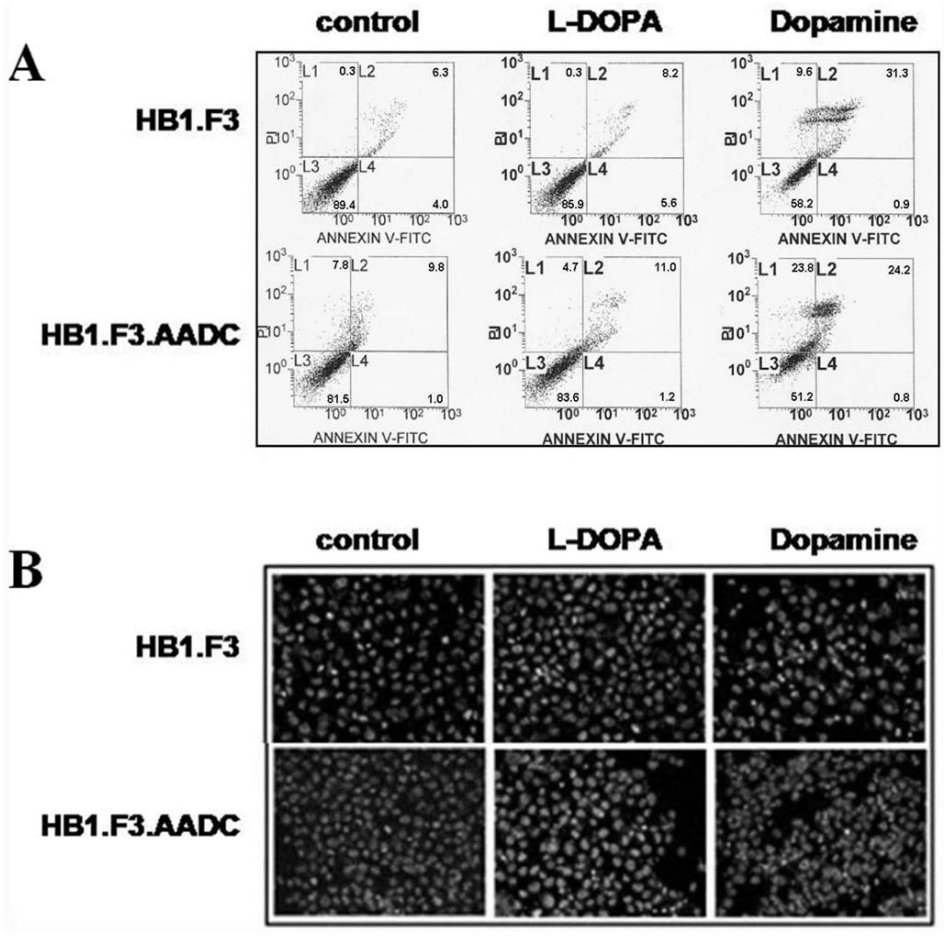

Fig. 2. Dopamine- and L-DOPA-induced apoptosis. Neural stem cells were incubated with dopamine (500 μM) or L-DOPA (100 μM) and apoptotic cell death after 24 hours was quantified by Annexin V and PI fluorescence using flow cytometry (A) and nuclear Hoechst stain (B). Numbers inside the boxes of L4 and L2 represent percentages of cell population in early and late stage of apoptosis, respectively.

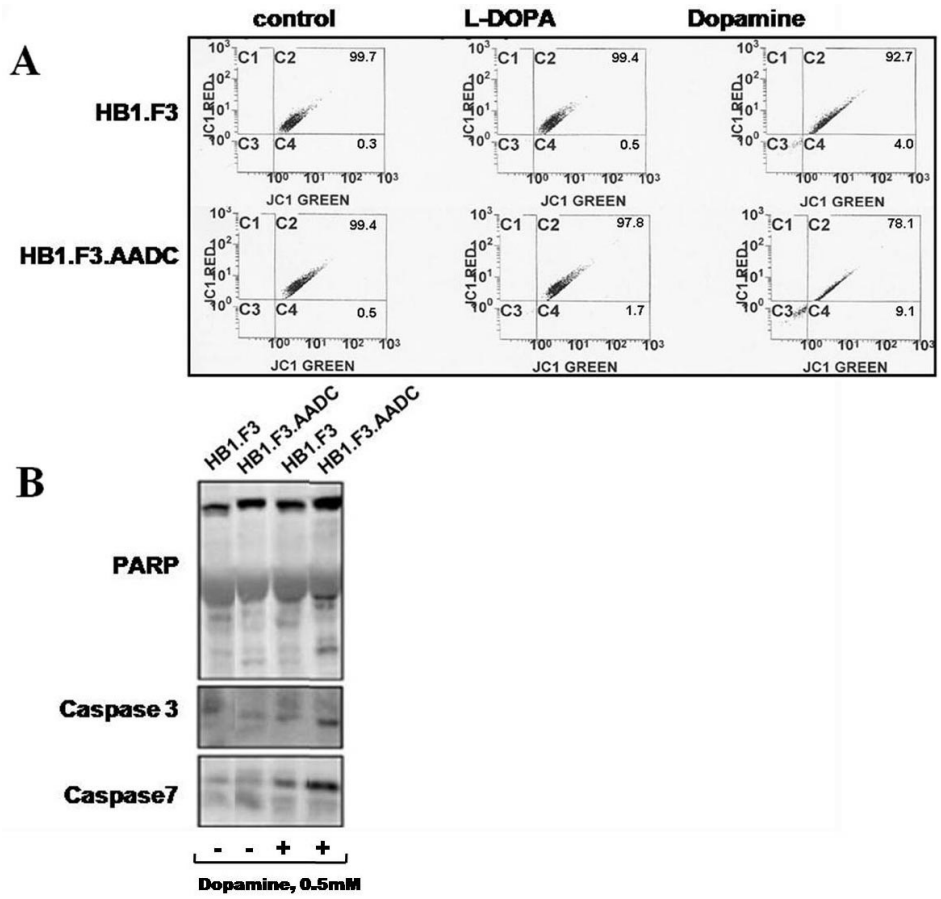

Fig. 3. Mitochondrial, PARP and caspase-dependent apoptosis induced by dopamine and L-DOPA. Neural stem cells were incubated with dopamine (500 μM) or L-DOPA (100 μM) and mitochondrial membrane potential was measured by flow cytometry using JC-1 at 8 hours after treatment (A). Activation of PARP, caspase 3 and caspase 7 was assessed by western blots after 24 hours of treatment (B).

L-DOPA and dopamine-induced apoptosis follows a typical mitochondrial and caspase-dependent pathway.

Apoptosis by proteasome inhibitors

Proteasome inhibitors induced apoptosis in dopaminergic neurons (Fig. 4). PI assay showed PSI was more potent than lactacystin and MG-132, inducing over 40% apoptosis at 10 nM in all the cell types. However, there was no significant difference in sensitivity between F3 and F3.AADC.

Effects of proteasome inhibition on dopamine-induced apoptosis

To examine the combined effect of proteasome inhibition and dopamine-induced apoptosis, F3 and F3.AADC cells were simultaneously treated with dopamine and PSI or lactacystin for a short term at higher concentrations or a long term at lower concentrations. Both proteasome inhibitors and dopamine induced apoptosis when treated alone for 1 day, as shown above (Fig. 1B and 4). However, rate of apoptosis following combined treatments was not increased additively when compared to that of dopamine or proteasome-only condition (Fig. 5A). And when treated together for 1 day at high concentration, a difference in dopamine sensitivity between F3 and F3.AADC was not observed any more. However, long-term culture with combined dopamine and low concentration of proteasome inhibitors (5 days) significantly increased dopamine-induced apoptosis, especially in F3.AADC cells, making dopaminergic cells more sensitive to dopamine (Fig. 5B).

Inclusion body formation in the process of combined dopamine and proteasome inhibitors-induced apoptosis

Immunocytofluorescene study was done after long-term culture with combined dopamine and low concentration of proteasome inhibitors to evaluate the correlation between formation of Lewy body-like cytoplasmic inclusions and apoptosis induced by combined dopamine and proteasome inhibition.

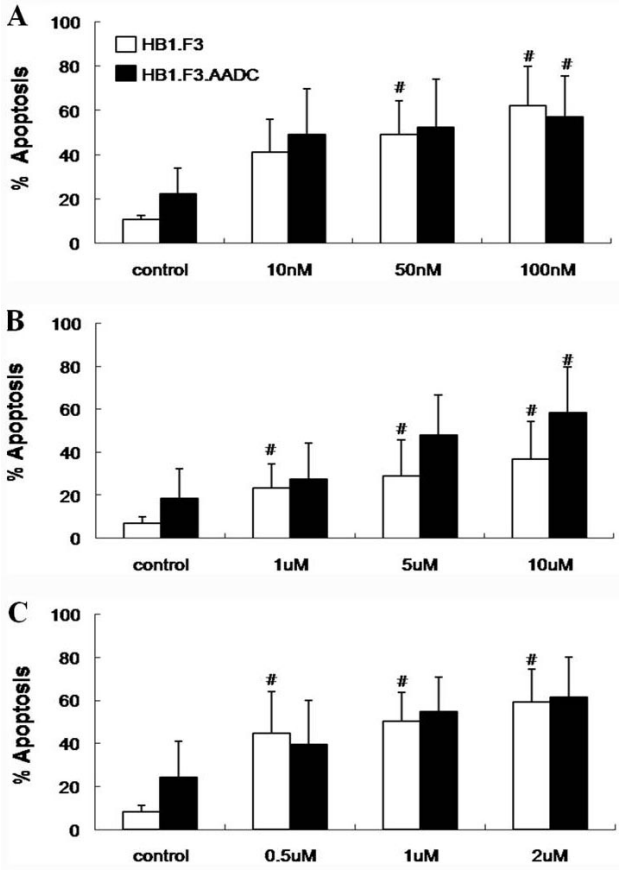

Fig. 4. Apoptosis induced by proteasome inhibition. Neural stem cells were incubated with indicated concentrations of proteasome inhibitor PSI (A), lactacystin (B) and MG-132 (C). Apoptosis was quantified by flow cytometry using PI at 24 hours after treatments. There was no difference between cell lines. $^{\#}p<0.05$, vs. control condition.

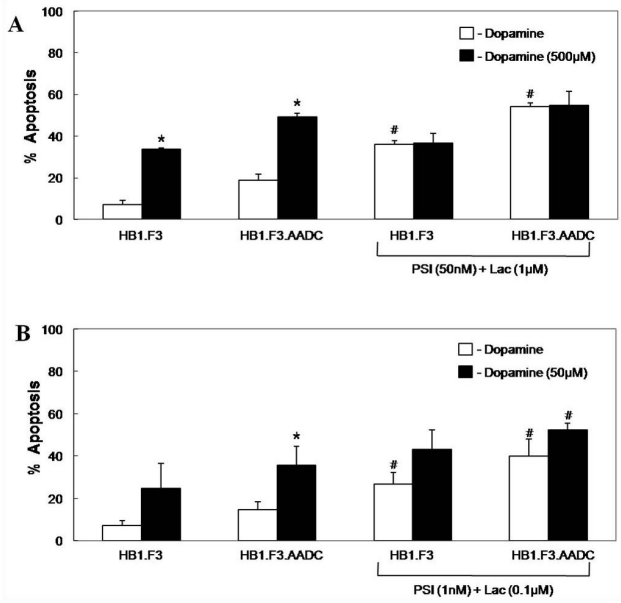

Fig. 5. Effects of proteasome inhibition on dopamine-induced apoptosis. Neural stem cells were incubated with dopamine in the absence or presence of indicated concentrations of proteasome inhibitors, PSI and lactacystin, Apoptosis was quantified by flow cytometry using PI at 24 hours after treatments (A) or 5 days after treatments (B). $^{*}p<0.05$, vs. without dopamine, $^{\#}p<0.05$, vs. without proteasome inhibitors.

Apoptotic cells induced by long-term treatment of dopamine alone showed increased Lewy body-like cytoplasmic inclusions containing both α-synuclein and ubiquitin (Fig. 6). However, α-synuclein and ubiquitin were not completely co-localized in the same Lewy body-like inclusions, so there were some fractions of inclusions stained only with either α-synuclein or ubiquitin. Addition of low concentration of proteasome inhibitors to dopamine further increased α-synuclein and ubiquitin-positive Lewy body like inclusions, especially in the F3.AADC.

DISCUSSION

Human neural stem cells (HB1.F3) immortalized by a retroviral vector encoding *v-myc* were employed in the present study. Dopaminergic transformation of HB1.F3 was carried out with human aromatic acid decarboxylase gene transfection (F3.AADC). Addition of L-DOPA into culture media significantly increased the levels of dopamine in cytoplasm and culture media of F3.AADC, compared to those of F3 cells. Therefore F3.AADC cells were regarded as dopaminergic cell line producing dopamine endogenously, and compared with non-secreting F3 cells to evaluate the difference in dopamine sensitivity.

Dopamine and L-DOPA-induced cell death was found in a dose-dependent manner in all cell lines, and dopaminergic cell lines were more susceptible to dopamine, but not to L-DOPA. F3 was more susceptible to L-DOPA, which might be explained by a preconditioning effect that dopaminergic cells were already exposed to L-DOPA as an additive to culture media or endogenous products. Preconditioning effect of reactive oxygen species (ROS) to protect dopamine-induced apoptosis could contribute the susceptibility of F3 to L-DOPA (Tang et al., 2005).

Most of cell death induced by L-DOPA or dopamine was apoptosis as shown by nuclear staining with PI and flow cytometric analysis using

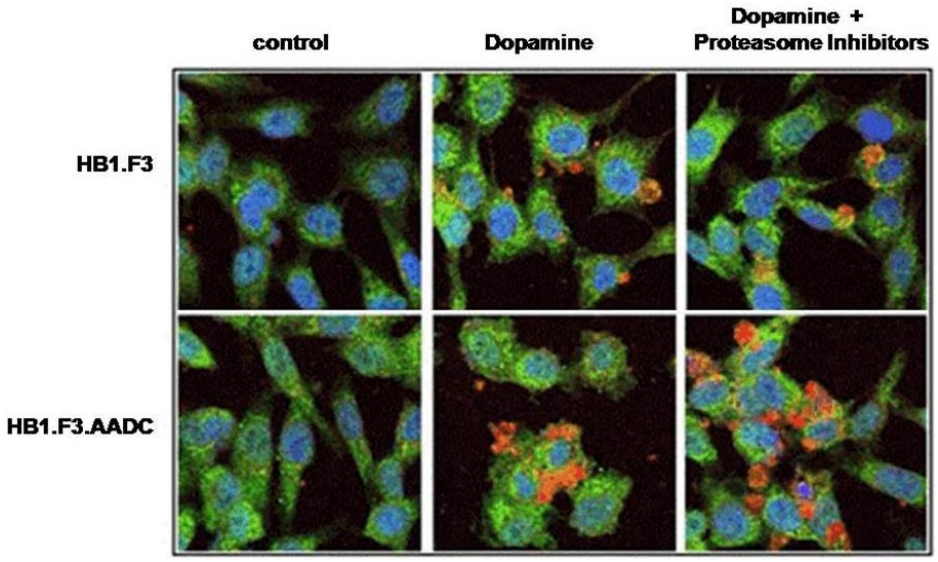

Fig. 6. α-Synuclein positive inclusion body formation in the process of apoptosis induced by dopamine and proteasome inhibitors. Neural stem cells were treated with dopamine (50 μM) in the presence or absence of proteasome inhibitors, PSI (1 nM) and lactacystin (0.1 μM) for 5 days. Inclusion body formation was assessed by immunofluorescence study using α-synuclein (red) and ubiquitin (green).

annexin V/PI. Measurement of mitochondrial membrane potential showed increase of JC-1 monomeric form in dopaminergic cells by dopamine. These results are consistent with previous studies (Lee et al., 2007; Zafar et al., 2007), indicating that disruption of mitochondrial functions had a role in dopamine-induced apoptosis of dopaminergic cells. Caspases play an important role in the apoptotic process both in death receptor pathway and the mitochondrial pathway (Grütter, 2000). Western blot analysis of this experiment revealed dopamine-induced activation of caspase 3 and 7, and PARP in the course of apoptosis in dopaminergic cells.

Proteasomes are the primary enzymes involved in the degradation of proteins within cells. Dysfunction of ubiquitin-proteasome system has taken a great attention in the pathogenesis of Parkinson's disease (Olanow and McNaught, 2006). In the report comparing the effect of dopamine and proteasome inhibitor, dopamine-induced neuronal death was blocked by anti-oxidants but the effect of proteasome inhibitor was not influenced, suggesting that dopamine induced oxidative cell injury but proteasome inhibitor could contribute to cell death by non-oxidative mechanism (Zafar et al., 2007). Several proteasomal inhibitors have been used as key molecules for *in vivo* and/or *in vitro* models of Parkinson's disease. It has been demonstrated that MG132 induced proteasomal inhibition with accumulation of oxidized proteins and subsequent cell death in PC12 cells and N27 cells (Sun et al., 2006). Lactacystin has been documented to have the capability to cause impairment of ubiquitin-proteasome system and sufficient to produce cell death in cultured dopaminergic neuronal cells (Reaney et al., 2006). PSI has drawn a great attention because of the recent report, which asserted development of progressive animal model of PD by systemic exposure of PSI (McNaught et al., 2004). Nonetheless, there has been considerable debate due to the failure of replication of this animal model (Kordower et al., 2006; Mathur et al., 2007).

In this study, proteasomal inhibitors induced dose-dependent apoptosis, and there was no significant difference between F3 and dopaminergic cell (F3.AADC). Proteasomal inhibition further increased dopamine-induced apoptosis in time-dependent manner. Exposure to relatively high concentration of proteasomal inhibitors for short duration (24 hours) did increase apoptosis in dopaminergic cells but did not potentiate the effect of dopamine-induced apoptosis. Chronic exposure (5 days) to low concentration of proteasomal inhibitors significantly increased dopamine-induced apoptosis, especially in dopaminergic cells, resulting in more selective dopamine susceptibility. Immunostaining with α-synuclein and ubiquitin showed formation of α-synuclein and ubiquitin-positive cytoplasmic inclusion bodies in the process of dopamine-induced apoptosis and combination with chronic exposure to proteasomal inhibitors further

increased inclusions, especially in the dopaminergic cell lines. Recent report showed time-dependent increase of cell death by prolonged exposure to proteasome inhibitors with corresponding increase of ROS (Papa et al., 2007). Furthermore, they suggested ROS-dependent collapse of the mitochondrial membrane potential and the nuclear relocalization of apoptosis-inducing factor were decisive events in neuronal cell death, independently on caspase mechanism. Their results indicate that chronic exposure to subtoxic dose of proteasome inhibitors induces neuronal apoptosis by ROS, which is contradictory to those of Zafar et al. (2007). As previously cited, they found that subtoxic dose of proteasome inhibitors even protect 6-hydroxy-dopamine-induced dopaminergic neural loss despite increase of cytoplasmic inclusions suggesting a protective role of α-synuclein-positive inclusions (Inden et al., 2005). In the present study, which was consistent with those of Papa et al. (2007), time-dependent increase of cell death and potentiation of dopamine-induced apoptosis by proteasome inhibitors were observed. And there were increased cytoplasmic inclusions in chronically proteasome inhibitor-exposed dopaminergic cells. These findings could be another *in vitro* evidence to support PD animal model by chronic PSI exposure. However, whether the mechanism of cell death by prolonged exposure to proteasome inhibitors is different from that by acute exposure should be further elucidated.

In conclusion, dopaminergic cells (F3.AADC) were more susceptible to dopamine compared to prototype F3 neural stem cells. Dopamine-induced apoptosis was through activation of caspases and disruption of mitochondrial membrane potential. Chronic exposure to low dose proteasomal inhibitors further increased dopamine-induced apoptosis and Lewy body-like cytoplasmic inclusions selectively in dopaminergic cells. These findings support that dopamine and proteasomal dysfunction play important roles to apoptotic cell death of dopaminergic neurons. These results suggest that chronic proteasomal dysfunction superimposed on dopamine might account for selective dopaminergic neuronal cell death in the pathogenesis of Parkinson's disease.

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