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Transduced Tat-α-Synuclein Protects against Oxidative Stress *In vitro* and *In vivo*

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Parkinson's disease (PD) is a common neurodegenerative disorder and is characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Although many studies showed that the aggregation of α-synuclein might be involved in the pathogenesis of PD, its protective properties against oxidative stress remain to be elucidated. In this study, human wild type and mutant α-synuclein genes were fused with a gene fragment encoding the nine amino acid transactivator of transcription (Tat) protein transduction domain of HIV-1 in a bacterial expression vector to produce a genetic in-frame WT Tat-α-synuclein (wild type) and mutant Tat-α-synucleins (mutants; A30P and A53T), respectively, and we investigated the protective effects of wild type and mutant Tat-α-synucleins *in vitro* and *in vivo*. WT Tat-α-synuclein rapidly transduced into an astrocyte cells and protected the cells against paraquat induced cell death. However, mutant Tat-α-synucleins did not protect at all. In the mice models exposed to the herbicide paraquat, the WT Tat-α-synuclein completely protected against dopaminergic neuronal cell death, whereas mutants failed in protecting against oxidative stress. We found that these protective effects were characterized by increasing the expression level of heat shock protein 70 (HSP70) in the neuronal cells and this expression level was dependent on the concentration of transduced WT Tat-α-synuclein. These results suggest that transduced Tat-α-synuclein might protect cell death from oxidative stress by increasing the expression level of HSP70 *in vitro* and *in vivo* and this may be of potential therapeutic benefit in the pathogenesis of PD.

Keywords: Dopaminergic neurons, HSP70, Paraquat, Parkinson's disease, Tat-α-synuclein

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that results in the degeneration of dopaminergic neurons in the substantia nigra (SN) and dopamine depletion in the striatum. It is characterized by disabling motor abnormalities that include tremor, muscle stiffness, bradykinesia and postural instability (Forno, 1996; Janko and Tolosa, 1998). Although the mechanism of cell death is not fully understood, many researchers have suggested that the pathogenesis of PD is related with many factors including aging, inflammation, chemicals and genetic factors (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Hunot and Hirsch, 2003; Warner and Schapira, 2003).

α-synuclein is a major constituent of Lewy bodies (LBs) in PD and is an acidic neuronal protein that is composed of 140 amino acids (Goedert, 1997). Although the etiology of PD remains unknown, several kinds of evidence suggest that the protein α-synuclein has a pathogenetic role in PD. In particular, two missense mutations (A30P and A53T) in α-

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synuclein gene that are responsible for familial PD have been identified and the mutant proteins are more aggregated than the wild type protein (Narhi *et al.*, 1999; Li *et al.*, 2001). Indeed, LBs found in PD patients are primarily composed of aggregated α-synuclein, and the overexpression of α-synuclein in transgenic mice resulted in LBs formation associated with the degeneration of dopaminergic neurons (Conway *et al.*, 1998; Masliah *et al.*, 2000; Warner and Schapira, 2003).

Oxidative stress has been suggested to play a central role in the pathogenesis of PD, the increase of aggregated α-synuclein and mitochondrial alterations that could lead to dopaminergic neuronal cell death (Cohen, 2000; Hsu *et al.*, 2000). Recently, we reported that α-synuclein was significantly aggregated by the copper-catalyzed oxidative reaction in the Cu,Zn-SOD/H_2O_2 system (Kim *et al.*, 2002). These findings support the contention that free radicals may play a critical role in the aggregation of α-synuclein. Several epidemiological studies have suggested that exposure to agricultural chemicals is associated with an increased risk of developing PD. The herbicide paraquat (PQ; 1,1'-dimethyl-4,4'-bipyridium dichloride) may contribute to the pathogenesis of PD based on both epidemiological studies of parkinsonism corrected with exposure to the agent and its structural similarity to the active metabolite of the parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridium ion (MPP^+) (Hertzman *et al.*, 1990; Semchuk *et al.*, 1993; Forno, 1996; Liou *et al.*, 1997; Gorell *et al.*, 1998; Peng *et al.*, 2005).

Recently, several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells. These include, carrier peptides derived from the HIV-1 Tat protein, *Drosophila* Antennapedia (Antp) protein, and herpes simplex virus VP22 protein (Fawell *et al.*, 1991; Vives *et al.*, 1997; Prochiantz, 2000). Up to the present, many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology (Wadia and Dowdy, 2002). We also successfully transduced Tat-SOD directly into various cell lines including pancreatic islet cells and transduced Tat-SOD increased radical scavenger activity (Eum *et al.*, 2004a). More recently, we transduced PEP-1-SOD into neuronal cells across the blood-brain barrier and efficiently protected against ischemic insults (Eum *et al.*, 2004b).

In the present study, we constructed and expressed the Tat-α-synuclein fusion proteins (WT, A30P and A53T) to determine the protective effects of α-synuclein (WT) against oxidative stress. We found that Tat-α-synuclein (WT) fusion protein protects neuronal cells against paraquat-induced cell death *in vitro* and *in vivo* via the induction of heat shock protein 70 (HSP70).

Materials and Methods

Cloning, expression, and purification of Tat- α-synuclein. HIV-1 Tat expression vector and α-synuclein were prepared as described previously (Kwon *et al.*, 2000; Kim *et al.*, 2002). Briefly, synthesized oligonucleotide was ligated into a *NdeI-XhoI* digested pET-15b vector. Next, on the basis of the cDNA sequence of human α-synuclein, two primers were synthesized. The sense primer, 5'-CTCGAAATGGATGTATTCATGAAAGGACTT-3' contains an *XhoI* site, and the antisense primer, 5'-GGATCCTTAGGCTTCA GGTTCGTAGTCTTG-3', contains a *BamHI* restriction site. The resulting PCR products were digested with *XhoI* and *BamHI*, eluted, ligated into a TA-cloning vector and pTat vector using T4 DNA ligase and cloned in *E. coli* BL21 (DE3) cells.

To produce the Tat-α-synuclein fusion proteins (WT, A30P and A53T), the plasmid was transformed into *E. coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of LB media at 37°C to a D_{600} value of 0.5~1.0 and induced with 0.5 mM IPTG at 37°C for 3-4 h. Harvested cells were lysed by sonication at 4°C in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) that contained 6 M urea. After centrifugation, the supernatant was immediately loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. After the column was washed with 10 volumes of a binding buffer and 6 volumes of a washing buffer (60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9), the fusion protein was eluted with an eluting buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fusion proteins were combined and the salts were removed using a PD-10 column chromatography. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

Transduction of Tat-α-synuclein fusion proteins into astrocyte cells. For the transduction of Tat-α-synuclein (WT, A30P and A53T), cells were grown to confluence on a 6-well plate. Then the culture medium was replaced with 1 ml of fresh solution. After the cells were treated with various concentrations of Tat-α-synuclein for 1 h, they were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform a Western blot analysis. The intracellular stability of transduced Tat-α-synuclein fusion proteins were estimated as follow: After the cells were treated with 3 μM Tat-α-synuclein for 1 h, the cells were washed and changed with a fresh culture medium to remove Tat-α-synuclein that was not transduced. And then cells were further incubated for 72 h, followed by preparations of cell extracts for Western blot analysis.

Western blot analysis. The transduced Tat-α-synuclein proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% tween-20 (TBST) for 2 h and was then incubated for 1 h at room temperature with anti-α-synuclein antibody and anti-HSP70 antibody (dilution 1 : 400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1 : 10000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL.

Fluorescence analysis. For direct detection of fluorescein-labeled protein, purified Tat-α-synucleins were labeled using EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit. The labeling

of FITC was carried out according to the manufacturer's instructions. Cultured cells were grown on glass coverslips and treated with 3 μM Tat-α-synuclein fusion proteins. Following incubation for 1 h at 37°C, the cells were washed twice with PBS and trypsin-EDTA. For the fixed cells, they were fixed with 4% paraformaldehyde for 10 min at room temperature. The distribution of fluorescence was analyzed on a fluorescence microscopy.

Analysis of α-synuclein aggregation. Tat-α-synuclein fusion proteins (2 μg) were incubated at 37°C for 4 h with 10 μM copper (II) and 1 mM H_2O_2 in a total volume of 20 μl. The reaction was stopped by the addition of 1 μl catalase (1 mg/ml). The samples were treated with sample buffer and were boiled at 100°C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE and Western blotting.

MTT assay. The biological function of the transduced Tat-α-synuclein fusion proteins was assessed by measuring the cell viability of astrocyte cells treated with paraquat dichloride hydrorate, which is well known as an intracellular superoxide dismutase anion generator (Choi *et al.*, 2004; Moosavi *et al.*, 2005). The cells seeded into 6-well plates at 70% confluence. The cells were pretreated with 3 μM Tat-α-synucleins for 1 h, then the 30 mM paraquat was added to the culture medium for 12 h. Cell viability was estimated with a colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetra zolium bromide).

Animals and treatment. Male 8 weeks old (22-25 g) C57BL/6 mice were used. The animals were provided with a commercial diet and water ad libitum under controlled temperature, humidity, and lighting conditions (22 ± 2, 55 ± 5% and a 12 : 12 light/dark cycle with lights). The animals used in this experiment were treated according to the 'Principles of Laboratory Animal Care' (NIH publication No. 86-23).

Mice were divided into five groups with 10 animals in each group. Mice in groups 2, 3, 4, and 5 were given a single intraperitoneal (i.p.) injection of 10 mg/kg paraquat dichloride hydrorate freshly dissolved in sterile saline to induce Parkinson's disease, whereas normal mice in group 1 were injected with an equivalent volume of saline once a week for 3 consecutive weeks. Mice in groups 3, 4, and 5 were injected two times (pre- and post-day after paraquat injection) with 300 μg of Tat-α-synuclein (wild type [WT], A30P and A53T). The mice were killed by cerevical dislocation at 1 week after the last injection.

Immunohistochemistry and statistical analysis. The frozen and sectioned midbrain were prepared and fixed with 4% paraformaldehyde for 10 min. For removal of nonspecific immunoreactivity, free-floating sections were first incubated with 0.3% Triton X-100 and 10% normal goat serum in PBS for 1 h. They were then incubated with a rabbit anti-tyrosine hydroxylase (TH) monoclonal antibody (dilution 1 : 400) for 24 h room temperature. After being washed with PBS, the sections were incubated for 1 h with biotinylated goat anti-rabbit antibody (Vector Laboratory, Burlingame, CA, USA; dilution 1 : 200) and then visualized with 3,3-diaminobenzidine (40 mg DAB, 0.045% H_2O_2 in 100 ml PBS) mounted on gelatin-coated slides.

For calculating the number of survival neurons, sections representing the same level of the substantia nigra (SN) were selected for measurement. Each studied field in each tissue was selected within the midpoint of the SN region including all layers. Tissue images were obtained through an Axiophot light microscope connected via CCD camera to a PC monitor. Images of cresyl violet positive neurons were captured with an Applescanner. Measurement of the number of neuronal somata was performed using an image analyzing system equipped with a computer-based CCD camera. Cell counts were obtained by averaging the counts from 70 sections taken from each animal. The number of cresyl violet positive neurons was compared to the control normal group. Finally, a Student's *t*-test was performed to investigate the protective effect of Tat-α-synuclein (Kang *et al.*, 2002).

Results

Expression and purification of Tat-α-synucleins. To develop an expression system to overexpress and simply purify the cell-permeable α-synuclein protein, we constructed the Tat-α-synuclein (wild type; WT, mutants; A30P and A53T) expression vector (pTat-α-synuclein), which contains consecutive cDNA sequences encoding the human α-synuclein, Tat-protein transduction domain (Tat_{49-57}) and six histidine residues at the amino-terminus. We also constructed the α-synuclein (WT, A30P and A53T) expression vector to produce control α-synuclein protein without an HIV-1 Tat protein transduction domain (data not shown).

Following the induction of expression, Tat-α-synuclein and α-synuclein fusion proteins were purified. Briefly, the fusion proteins were expressed in *E. coli* and clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. Fusion protein containing fractions were combined and salts were removed using a PD-10 column. The crude cell

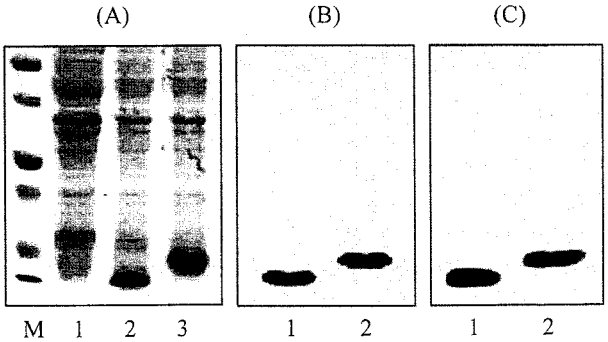

Fig. 1. Expression and purification of Tat-α-synuclein fusion proteins in *E. coli*. Protein extracts of cells (A) and purified fusion proteins (B) were analyzed by SDS-PAGE on 15% gel and subjected to Western blot analysis with antibodies to anti-histidine (C). Lanes in A are as follows: lane 1, non-induced; lane 2, induced control α-synuclein; lane 3, induced Tat-α-synuclein. Lanes in B and C are as follows: lane 1, purified control α-synuclein; lane 2, purified Tat-α-synuclein.

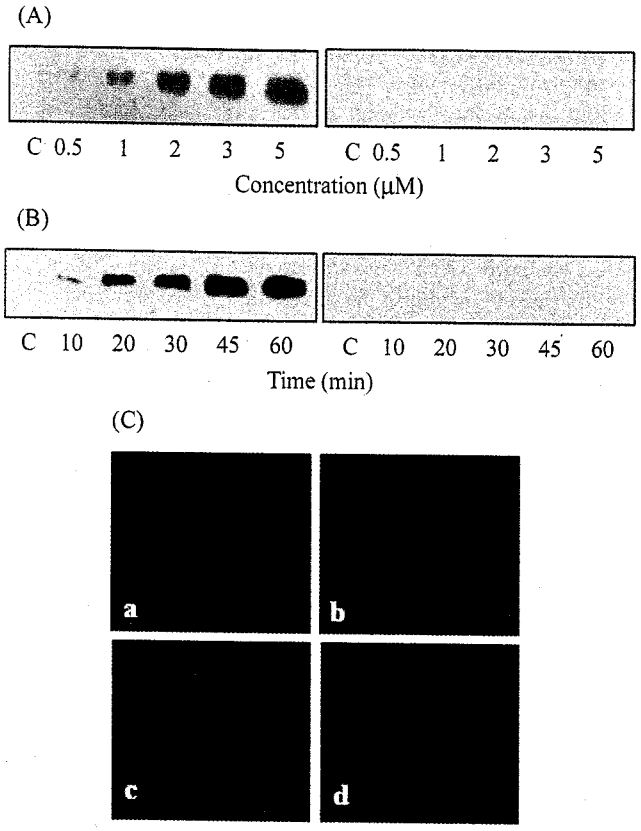

Fig. 2. Transduction of Tat-α-synuclein fusion proteins into astrocyte cells. 3 μM Tat-α-synuclein fusion proteins were added to the cultured media for 10-60 min, respectively (A). 0.5-5 μM Tat-α-synuclein fusion proteins were added to the cultured media for 60 min, respectively (B). Transduced Tat-α-synuclein fusion proteins into cells were analyzed by Western blotting. Fluorescence analysis of transduced Tat-α-synuclein proteins when the cells were fixed or nonfixed with paraformaldehyde, respectively (C). After FITC-labeled Tat-α-synuclein proteins (3 μM) were transduced into astrocyte cells, the cells were washed twice with trypsin-EDTA, PBS and immediately observed by fluorescence microscopy. Negative control cells themselves (a), Positive control cells treated with WT α-synuclein (b), non-fixed cells treated with WT Tat-α-synuclein (c), fixed cells treated with WT Tat-α-synuclein (d).

extracts obtained from *E. coli* and purified Tat-α-synuclein fusion proteins were electrophoresed in 15% SDS-PAGE. The expression and purification results are shown in Figs. 1A and 1B. The recombinant Tat-α-synuclein and α-synuclein fusion proteins have an estimated molecular mass of approximately 17 and 15 kDa, respectively. The purified fusion proteins were further confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. Tat-α-synuclein was detected at the corresponding bands in Fig. 1C. In the same experiment conditions, we purified α-synuclein and mutant Tat-α-synuclein fusion proteins (A30P and A53T) and they were confirmed by SDS-PAGE and Western blotting (data not shown).

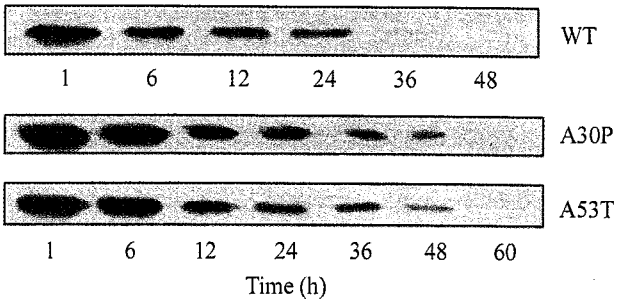

Fig. 3. Stability of Tat-α-synuclein fusion proteins tranduced into cells. Cells pretreated with 3 μM Tat-α-synuclein fusion proteins were incubated for various time periods. The Tat-α-synuclein fusion proteins transduced into cells were analyzed by Western blotting.

Transduction of Tat-α-synuclein into astrocyte cells. To determine whether WT Tat-α-synuclein fusion protein could transduce into cells, we added various concentrations (0.5-5 μM) of fusion proteins to the culture medium for 1 h and then determined the levels of protein transduction into the cells by Western blotting. As shown in Fig. 2A, WT Tat-α-synuclein fusion protein was successfully delivered into astrocyte cells, whereas control α-synucleins without Tat was not transduced into cell. The transduction of Tat-α-synuclein into cultured cells was increased in a dose-dependent manner. In addition, the intracellular concentration of transduced Tat-α-synuclein into cultured cells was detected after 10 min and gradually increased by 1 h (Fig. 2B). This time-dependent manner of transduction indicated that Tat-α-synuclein was rapidly transduced into the cells.

The intracellular delivery of Tat-α-synuclein into astrocyte cells was further confirmed by direct fluorescence analysis. As shown in Fig. 2C, almost all cultured cells were found to be transduced with Tat-α-synucleins. To exclude the possibility that cell fixation with paraformaldehyde may affect Tat-α-synuclein transduction by direct fluorescence, we used FITC conjugated Tat-α-synuclein fusion proteins to transduce into non-fixed or fixed astrocyte cells. The intracellular distribution of the WT Tat-α-synuclein fluorescence signal of non-fixed cells was similar to that of fixed cells. In the non-fixed condition, mutant Tat-α-synuclein fusion proteins fluorescence signals were similar to those of WT Tat-α-synuclein. These results indicate that cell fixation with paraformaldehyde is not required for Tat-α-synuclein fusion protein transduction.

The intracellular stability of transduced Tat-α-synuclein into astrocyte cells was shown in Fig. 3. The Tat-α-synuclein protein was added to the culture media of astrocyte cells at a concentration 3 μM for various time periods and the resulting levels of transduced protein were analyzed by western blotting. As shown in Fig. 3, the intracellular levels of transduced Tat-α-synuclein into cells were initially detected after 1 h, and then declined gradually over the period of observation. Significant levels of transduced Tat-α-synuclein (WT) persisted

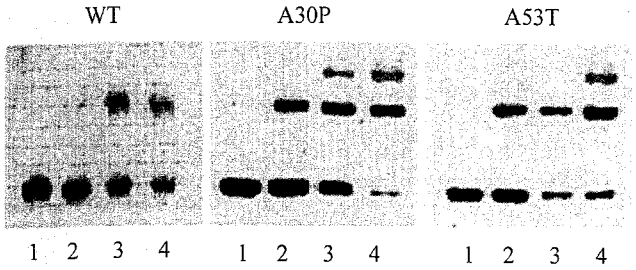

Fig. 4. Aggregation of α-synuclein by the copper (II) and H_2O_2 system analyzed by Western blotting. Tat-α-synuclein fusion proteins (2 μg) were incubated at 37°C for 4 h under various conditions. Lanes in A and C are as follows: lane 1, α-synuclein control; lane 2, α-synuclein with 10 μM copper (II); lane 3, α-synuclein with 1 mM H_2O_2; lane 4, α-synuclein with 10 μM copper (II) and 1 mM H_2O_2.

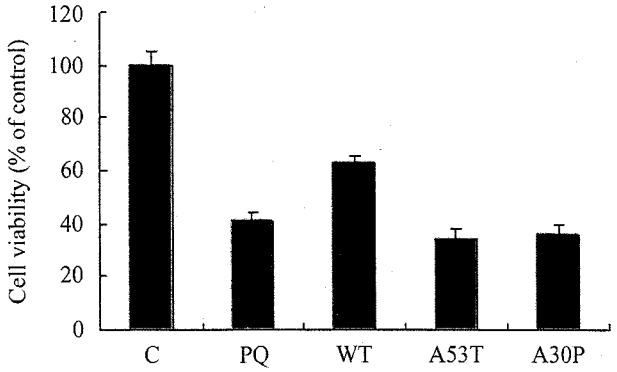

Fig. 5. Effect of transduced Tat-α-synuclein on cell viability. The paraquat (PQ, 30 mM) was added to the astrocyte cells pretreated with 3 μM Tat-α-synuclein fusion proteins for 1 h, respectively. Cell viabilities were estimated with a colorimetric assay using MTT. Each bar represents the mean ± S.E.M. obtained from five experiments.

in microglia cells for 24 h. However, Tat-α-synuclein (A30P and A53T) stabilities were markedly increased compared with WT and persisted in cells for 48 h.

Aggregation of α-synuclein. Recently, it has been reported that α-synuclein was significantly aggregated by the paraquat and copper-catalyzed oxidative reaction (Kim *et al.*, 2002; Manning-Bog *et al.*, 2002). To clarify aggregation of α-synuclein by oxidative stress, the aggregation of α-synuclein was induced by copper (II) and H_2O_2 systems. As shown in Fig. 4, the band shift was observed when WT Tat-α-synuclein was incubated with copper (II), or H_2O_2 and copper (II) and H_2O_2. In contrast, the mutant Tat-α-synuclein band was shifted more than that of WT Tat-α-synuclein. These results suggest that the aggregation of mutant α-synuclein is more sensitive to oxidative stress.

Effect of transduced Tat-α-synuclein fusion protein on the viability of neuronal cells. To determine whether the transduced fusion proteins have a functional role in cells, we tested the effects of transduced Tat-α-synuclein (WT, A30P and A53T; 3 μM) fusion proteins on cell viability. As shown in Fig. 5, when the cells were exposed to 30 mM paraquat without Tat-α-synuclein, only 40-45% of cells were viable. However, the viability of the cells pretreated with WT Tat-α-synuclein markedly increased, whereas the cell viabilities were slightly decreased when the A30P and A53T mutant proteins were pretreated. The increased viability of cells transduced with Tat-α-synuclein (WT) suggests that this fusion protein has a protective effect on cells against oxidative stress.

To verify whether the toxicity of Tat-α-synuclein (WT, A30P and A53T) depended on the protein intracellular concentration, we incubated microglia cells with increasing concentrations (0.5-7 μM) of fusion proteins. At low concentrations of Tat-α-synuclein (0.5-3 μM), no toxicity was observed in either the WT or mutant Tat-α-synuclein. However, at a higher concentration of Tat-α-synuclein (5-7 μM), more toxicity occurred with mutant Tat-α-synucleins in comparison to the WT Tat-α-synuclein (data not shown).

Transduced Tat-α-synuclein (WT), not mutants (A30P and A53T) fusion protein, induced HSP70 protein in neuronal cells. We assessed the effects of Tat-α-synuclein transduction on the expression of heat shock protein 70 (HSP70), which is known as a potent antiapoptotic protein against several cytotoxic stimuli. In particular, HSP70 has been reported to play a protective role against paraquat toxicity (Manning-Bog *et al.*, 2003). After transduction of WT Tat-α-synuclein (3 μM) into astrocyte cells for 1 h, the expression level of HSP70 markedly increased (Fig. 6A). However, after transduction of mutant Tat-α-synuclein (A30P and A53T), the HSP70 expression was not detected. As the control experiment, we examined whether Tat alone has some effect on HSP70 expression. When we used Tat-GFP fusion protein as a negative control, the HSP70 expression level was not affected in both astrocyte and HeLa cells (Fig. 6B).

We then examined the stability of HSP70 protein after transduction of Tat-α-synuclein into astrocyte cells. The Tat-α-synuclein protein was added to the culture media of astrocyte cells and the expression levels of HSP70 protein were analyzed by Western blotting. As shown in Fig. 6C, the expression levels of HSP70 into cells were initially detected after 1 h, and then declined gradually over the period of observation. HSP70 expression markedly increased at 1 h, slowly returned to the basal level at 12 h, and decreased again at 24 h by transduction of WT Tat-α-synuclein fusion proteins. However, when mutant Tat-α-synuclein (A30P and A53T) fusion proteins transduced into cells, the HSP70 expression transiently increased up to 6 h, and then it rapidly declined. In the WT Tat-α-synuclein fusion proteins transduced cells, the HSP70 expression remained high and independent of paraquat treatment. However, in the Tat-α-synuclein (A30P and A53T) fusion proteins transduced cells, the level of HSP70 expression

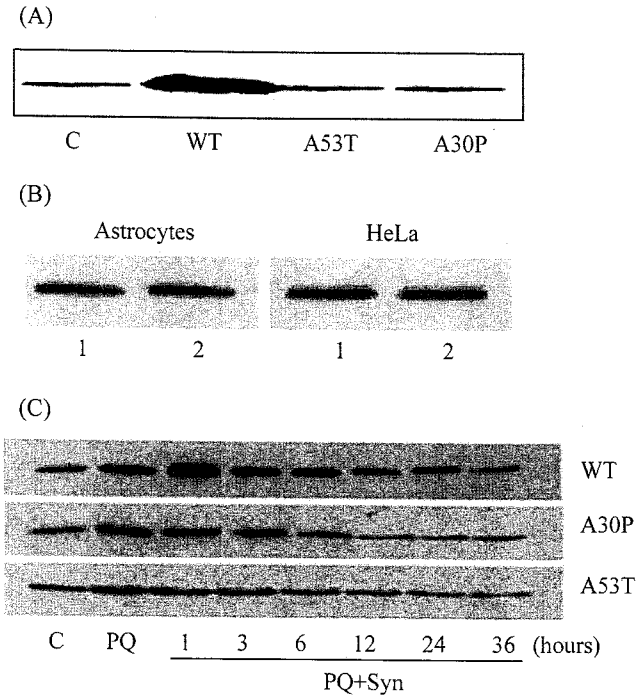

Fig. 6. Effects of Tat-α-synuclein (A) and Tat-GFP (B) fusion protein transduction on the expression of HSP70 in astrocyte cells and exposed to paraquat (C). After the cells were treated with 3 μM Tat-α-synuclein and 3 μM Tat-GFP fusion proteins for 1 h, 30 mM paraquat was added to the culture medium for 36 h. The expression of HSP70 was assessed from cell lysates by Western blotting. Lanes in B are as follows: lane 1, control cells; lane 2, Tat-GFP tranduced cells.

markedly decreased compared with WT Tat-α-synuclein fusion protein.

Paraquat-induced neurodegeneration in mice. In C57BL/6 mice, paraquat has been reported to cause dopaminergic cell degeneration (Manning-Bog *et al.*, 2002; McCormack *et al.*, 2002; Manning-Bog *et al.*, 2003). To determine whether the transduced Tat-α-synuclein protects against neurodegenration in the paraquat-treated animal model, we compared dopaminergic neuronal cell death after transduction of WT and mutant α-synuclein by immunohistochemistry using the tyrosine hydroxylase (TH) antibody. Animals were treated with a weekly intraperitoneal (i.p.) injection of 10 mg/kg paraquat for 3 consecutive weeks, and the dopaminegic neuronal cell levels were estimated by TH immunostaining. The protective effects of Tat-α-synuclein fusion protein under the same experimental conditions were evaluated by cresyl violet histochemistry (data not shown). As shown in Fig. 7, the neuronal cells in paraquat-treated group mice were significantly reduced, however no difference in the extent of the cell death was observed between WT Tat-α-synuclein treated mice and control group mice. In the WT Tat-α-

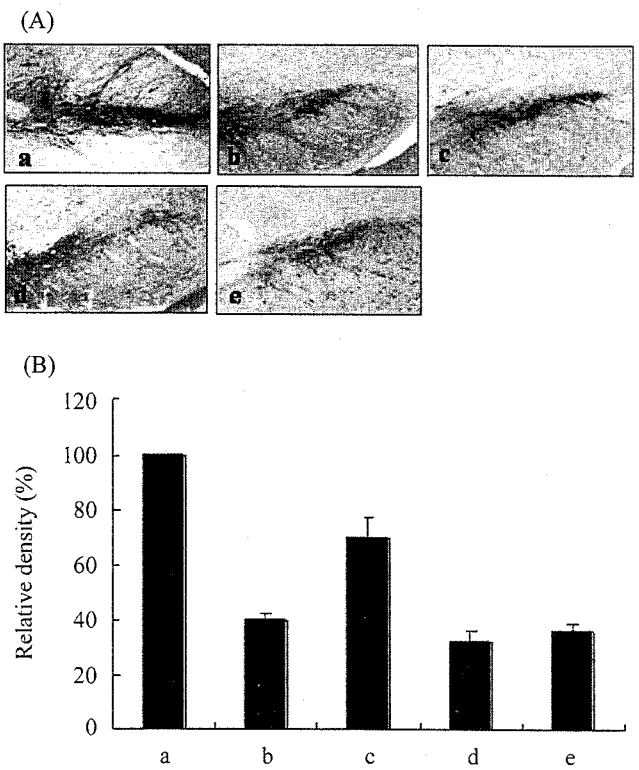

Fig. 7. Effect of transduced Tat-α-synuclein fusion proteins on neuronal cell viability. (A) Representative photomicrographs of the TH-immunostained substantia nigra (SN) of the control and Tat-α-synuclein transduced mice. (B) Neuronal cell density in the SN after paraquat and transduced Tat-α-synuclein fusion proteins. Animals were treated once a week for 3 consecutive weeks and killed at 7 d after the last injection of saline, paraquat and Tat-α-synuclein and paraquat. Negative control (a), positive control (b), and Tat-α-synuclein injected mice (c, WT; d, A30P; e, A53T).

synuclein-treated group, 70-75% of the neuronal cell remained compared to the paraquat-treated group. Therefore, these lines of results indicate that WT Tat-α-synuclein fusion protein protected against dopaminergic neuronal cell damage caused by paraquat-induced Parkinson's disease model mice. Furthermore, we confirmed the protective effect of transduced Tat-α-synuclein fusion proteins *in vivo* by comparison with HSP70 levels in both mice pretreated with WT and mutant α-synuclein, respectively. Dopaminergic neurons in each group of mice were immunostained with antibodies against HSP70. As shown in Fig. 8, the mice treated with WT Tat-α-synuclein fusion protein showed an enhancement of HSP70 immunoreactivity, whereas no changes of HSP70 levels were detected in the mice treated with mutant proteins. These results indicate that WT Tat-α-synuclein fusion proteins are transduced *in vitro* and *in vivo* and effectively protect against neuronal cells by induction of HSP70 protein.

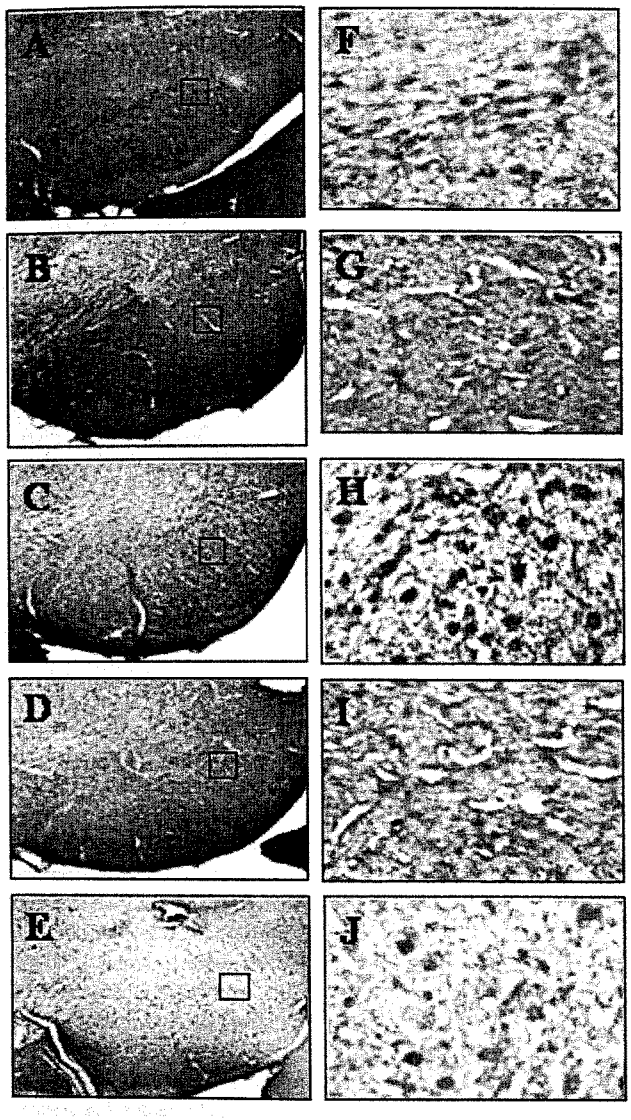

Fig. 8. Enhanced HSP70 levels in transduced Tat-α-synuclein fusion protein in mice. Representative photomicrographs of the HSP70-immunostained substantia nigra (SN) of the control and Tat-α-synuclein transduced mice. Negative control (A), positive control (B), and Tat-α-synuclein injected mice (C, WT; D, A30P; E, A53T). Insets show the expression of HSP70 (right; F, G, H, I, and J), respectively.

Discussion

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is associated with progressive degeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta. In the pathogenesis of PD, α-synuclein is implicated as a pivotal genetic factor. Moreover, two missense mutations (A53T and A30P) in the gene encoding α-synuclein are linked to dominantly inherited PD, thereby directly implicating the role of α-synuclein in PD is widely studied (Kruger *et al.*, 1998; Clayton and George, 1999; Feany and Bender, 2000). Although the physiological and pathological roles of α-synuclein are unclear yet, they might play an important role in neuroplasticity and in response to neuronal cell damage (Iwai *et al.*, 1995; Clayton and George, 1998). Up to date, various studies have demonstrated that overexpression of α-synuclein proteins in neuronal cells enhanced cell death or protective effect against oxidative stress (Conway *et al.*, 1998; Hashimoto *et al.*, 2002; Manning-Bog *et al.*, 2003; Zourlidou *et al.*, 2004).

In the present study, we provide the evidence in an *in vitro* and *in vivo* study that transduced WT Tat-α-synuclein can directly prevent paraquat-induced dopaminergic neuronal cell death by induction of HSP70.

To clarify the relevance of WT and mutants α-synuclein neuroprotective function against oxidative stress, we used Tat-α-synuclein (WT, A30P and A53T) fusion proteins in neuronal cell and animal model mice for PD.

As shown in Fig. 1, the purified WT Tat-α-synuclein fusion proteins were confirmed by SDS-PAGE and Western blot analysis using an anti-rabbit polyhisitidine and anti-α-synuclein antibody. The expressed Tat-α-synuclein fusion proteins were purified homogeneously from cell lysates by Ni^{2+}-nitrilotriacetic acid Sepharose affinity chromatography. The Tat-α-synuclein proteins have an estimated molecular mass of approximately 17 kDa.

We found that WT Tat-α-synuclein fusion proteins could be transduced into astrocyte cells in a time- and concentration-dependent manner (Fig. 2). This time- and concentration-dependent transduction indicates that Tat-α-synuclein fusion proteins were rapidly transduced into cells. It was reported that Tat-β-galactosidase fusion protein was transduced rapidly into HepG2 cells, reaching near maximum intracellular concentrations in less than 15 min. Also, we reported that Tat-SOD and Tat-catalase fusion protein was transduced into mammalian cells and tissues within 1 h (Schwartze *et al.*, 1999; Kwon *et al.*, 2000; Jin *et al.*, 2001). This little difference in time and course may derive from the properties of transduced Tat fusion proteins, such as the unfolding degree, polarity, and the molecular shape of the protein. The intracellular delivery of Tat-α-synuclein into the cells was further confirmed by direct fluorescence analysis. In this study, we used FITC-conjugated Tat-α-synuclein fusion protein to transduce into non-fixed or fixed cells. As shown in Fig. 2C, we could not detect any differences in the fluorescence distribution of transduced Tat-α-synuclein between fixed and non-fixed cells. These results demonstrate that cell fixation with paraformaldehyde was not required for Tat-α-synuclein transduction. Recently, we reported that the transduction of Tat-SOD or PEP-1-SOD fusion proteins into cells was not affected by paraformaldehyde fixation (Eum *et al.*, 2004a; 2004b).

The intracellular stability of transduced Tat-α-synuclein (WT, A30P and A53T) fusion proteins into astrocyte cells were shown in Fig. 3. The apparent degradation of transduced Tat-α-synuclein was observed as an incubation time. Transduced WT Tat-α-synuclein protein persisted in astrocyte cells until

24 h, whereas the stability of mutant (A30P and A53T) Tat-α-synuclein protein was markedly increased compared with WT. These results indicate that mutant α-synuclein may be present as aggregated form in the cells. It is known that the synaptic protein α-synuclein was found to abnormally accumulate in Lewy bodies (LBs) in PD. Similar observations which indicate that WT α-synuclein is normally degraded but mutant α-synuclein (A30P and A53T) degradation has a long half-life have been reported (Garrido *et al.*, 2001).

We then examined the relationship between cell viability and heat shock protein 70 (HSP70) of the WT and mutant α-synuclein proteins in transduced astrocyte cells (Fig. 6). It is well known that HSP70, a potent antiapoptotic intracellular chaperone, is readily induced by several stresses, such as heat, oxidative stress, or anticancer drugs, and up-regulation of HSP70 has been shown to increase resistance against ROS and NO (Bellmann *et al.*, 1995; Garrido *et al.*, 2001). Although the protective mechanism mediated by HSP70 remains unclear, transduction of WT Tat-α-synuclein resulted in a sustained increase of HSP70 expression in paraquat treated astrocyte cells, which might be responsible for the protective effect of WT Tat-α-synuclein. However, the mutant Tat-α-synuclein did not show any of the protective effect. We previously reported that the transduced Tat-SOD enhanced the expression of potent antiapoptotic proteins, Bcl-2 and HSP70 in streptozotocin treated MIN6N cells (Choung *et al.*, 2003). Those lines of results indicate that higher levels of HSP70 could conceivably contribute to neuroprotection after paraquat exposure to transduced cells treated with proteins which have anti-oxidant effects.

To examine the protective effect of transduced WT Tat-α-synuclein fusion protein in the PD mice model, we produced an animal PD mice model by exposure to paraquat. It is well known that exposure of C57BL/6 mice to either paraquat or the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides one of the most valuable approaches to analyze critical aspects of PD in the animal model. Recently, several investigators have reported on two animal models of selective dopaminergic cell death induced by exposure of rats to rotenone and by injections of mice with paraquat (Manning-Bog *et al.*, 2002; McCormack *et al.*, 2002; Kuhn *et al.*, 2003). In addition, the features of dopaminergic cell injury that characterize mice exposure to paraquat make this experimental model particularly suitable for investigation into the role of α-synuclein in neurotoxic processes (Manning-Bog *et al.*, 2002; McCormack *et al.*, 2002).

As shown in Figs. 7-8, WT Tat-α-synuclein fusion protein protected cells from the toxicity induced by paraquat exposure, whereas mutant Tat-α-synuclein fusion proteins did not. Several studies have shown that the overexpression of WT α-synuclein protected cells from toxicity induced by paraquat exposure and this effect was associated to a specific HSP70 protein induction in the PC12 cells. In addition, it is suggested that HSP70 may directly interact with α-synuclein itself in Lewy bodies (Manning-Bog *et al.*, 2002; Dou *et al.*, 2003; Albani *et al.*, 2004). However, further studies are necessary in order to clarify the mechanism of neuroprotection by α-synuclein overexpression.

In summary, we report for the first time that transduced Tat-α-synuclein fusion protein protected against oxidative stress-induced neuronal cell death by induction of HSP70 *in vitro* and *in vivo*. Therefore, our success in the transduction of Tat-α-synuclein may provide a new strategy for the identification of relationships between α-synuclein and PD and this transduction technology may be of potential therapeutic use for the pathogenesis of PD.

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