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Molecules and Cells

Transduced Tat-DJ-1 Protein Protects against Oxidative Stress-Induced SH-SY5Y Cell Death and Parkinson Disease in a Mouse Model

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Parkinson's disease (PD) is a well known neurodegenerative disorder characterized by selective loss of dopaminergic neurons in the substantia nigra pars compact (SN). Although the exact mechanism remains unclear, oxidative stress plays a critical role in the pathogenesis of PD. DJ-1 is a multifunctional protein, a potent antioxidant and chaperone, the loss of function of which is linked to the autosomal recessive early onset of PD. Therefore, we investigated the protective effects of DJ-1 protein against SH-SY5Y cells and in a PD mouse model using a cell permeable Tat-DJ-1 protein. Tat-DJ-1 protein rapidly transduced into the cells and showed a protective effect on 6-hydroxydopamine (6-OHDA)-induced neuronal cell death by reducing the reactive oxygen species (ROS). In addition, we found that Tat-DJ-1 protein protects against dopaminergic neuronal cell death in 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP)-induced PD mouse models. These results suggest that Tat-DJ-1 protein provides a potential therapeutic strategy for against ROS related human diseases including PD.

INTRODUCTION

Parkinson's disease (PD) is a common age-related neurodegenerative disease well known for the selective loss of dopaminergic neurons in the substantia nigra (SN) and dopamine depletion in the striatum (Dawson and Dawson, 2003; Eriksen et al., 2005; Moore et al., 2005). It is characterized by disabling motor abnormalities such as tremors, muscle stiffness, bradykinesia, and postural instability (Forno, 1996; Janko and Tolosa, 1998). It has been well established that the pathogenesis of PD is related to many factors including aging, inflammation, chemicals, and genetic factors (Dawson and Dawson, 2003; Dauer and Przedborski, 2003; Honot and Hirsch, 2003; Warner and Schapira, 2003). Although the exact mechanism of cell death is not fully understood, many studies have focused on understanding the PD mechanism using the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in animals (Burns et al., 1983; Heikkila et al., 1984).

Reactive oxygen species (ROS) are natural and inevitable by-products of various cellular processes components and damage macromolecules in cells by altering their structures and functions. Prolonged exposure to ROS contributes significantly to the pathological processes of a number of human diseases and is a major factor in the cause of various neurodegenerative disorders including PD (Hald and Lotharius, 2005; Pong et al., 2001; Rastogi et al., 2006; Tamagno et al., 2003). In a previous study, we showed that the administration of a herbicide paraquat (1,1′-dimethy-4,4′-bipyridinium) triggers selective dopaminergic neuronal cell death and the exposure of mice to which is an effective model for studying the pathological aspects of PD (Choi et al., 2006a). Also, another study has suggested that paraquat is associated with an increased risk of PD (Synder and D'Amato, 1985).

DJ-1 is a homodimeric protein belonging to the Thi/Pfp1 superfamily consisting of 189 amino acids. It is highly conserved from human to *Escherichia coli* and is localized in the cytoplasm, nucleus, and mitochondria (Bonifati et al., 2003; Gupta et al., 2008; Lee et al., 2003; Wilson et al., 2004). This protein has multiple functions including antioxidant activity, chaperone-like properties, and transcriptional regulation (Menzies et al., 2005; Xu et al., 2005; Zhou et al., 2006). In addition, it is well known that the loss of the DJ-1 function by oxidative stress and mutation leads to human PD pathogenesis (Bonifati et al., 2003).

Since cell-permeable peptides, called protein transduction domains (PTDs) or cell penetrating peptides (CPPs), were identified two decades ago, these peptides have numerous therapeutic applications and are used to successfully deliver a range of molecules into cells. Among the cell-permeable peptides, Tat peptide is well known for its ability to deliver exoge-

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nous proteins into cells (Wadia and Dowdy, 2002; 2003). Although Tat fusion proteins have been used to deliver therapeutic proteins *in vitro* and *in vivo*, the exact mechanism remains unclear. In previous studies, we have shown *in vitro* and *in vivo* that various transduced fusion proteins efficiently protected against cell death (An et al., 2008; Choi et al., 2006a; 2006b; Eum et al., 2004; Kim et al., 2009; Kwon et al., 2000).

In this study, we designed a Tat-DJ-1 protein for direct transduction *in vitro* and *in vivo*. The results demonstrated that Tat-DJ-1 protein efficiently transduced into cells and protected against cell death *in vitro* and *in vivo* leading us to suggest that Tat-DJ-1 protein may be a potential therapeutic agent for various diseases related to oxidative stress, including PD.

MATERIALS AND METHODS

Materials

Human neuroblastoma SH-SY5Y cells were obtained with from Korean Cell Line Research Foundation, Seoul, Korea. Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen. The FBS and antibiotics were purchased from Gibco BRL. Ni^{2+}-nitrilotri-acetic acid sepharose superflow was purchased from Qiagen.

2′,7′-dichlorofluorescein diacetate (DCF-DA) was purchased from Sigma (USA). Primary antibodies against cleaved caspase-3 (Cell Signaling Technology, USA) and actin (USA) were obtained commercially. All other chemicals and reagents were of the highest analytical grade available.

Expression and purification of Tat-DJ-1 proteins

A cell-permeable HIV-1 Tat expression vector was prepared in our laboratory as described previously (Kwon et al., 2000). The cDNA sequence for human DJ-1 was amplified by PCR using the sense primer 5′-CTCGAGGCTTCCAAAAGAGC-3′ and the antisense primer, 5′-GGATCCCTAGTCTTTAAGAA-3′. The resulting PCR product was sub-cloned in a TA cloning vector and ligated into the pTat expression vector, with six histidine open-reading frames to generate the expression vector, and cloned into *E. coli* DH5α cells.

The recombinant Tat-DJ-1 plasmid was transformed into *E. coli* BL21 cells and induced with 0.5 mM IPTG at 37°C for 3-4 h. Harvested cells were lysed by sonication and the recombinant Tat-DJ-1 was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

Cell culture and transduction of Tat-DJ-1 into SH-SY5Y cells

The SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under humidified conditions of 95% air and 5% CO_2. For the transduction of Tat-DJ-1, SH-SY5Y cells were treated with various concentrations of Tat-DJ-1 fusion protein (0.5-3 μM) for various durations (10-120 min). The cells were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS) and harvested for the preparation of cell extracts to perform Western blot analysis.

Western blot analysis

The proteins in cell lysates were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were electrotransferred to a nitrocellulose membrane, which was then blocked with 5% non-fat dry milk in PBS. The membrane was probed with a rabbit antihistidine polyclonal antibody (1:1000; Santa Cruz Biotechnology, USA), followed by incubation with goat antirabbit immunoglobulins (dilution 1:10,000; Sigma-Aldrich, USA). The bound antibodies were then visulalized by enhanced chemilluminescence according to the manufacturer's instructions (Amersham, USA).

Fluorescence microscopy analysis

SH-SY5Y cells were grown on coverslips treated with 3 μM of Tat-DJ-1. Following incubation for 1 h at 37°C, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. The cells were permeabilized and blocked for 30 min with 3% bovine serum albumin, 0.1% Triton X-100 in PBS (PBS-BT) and washed with PBS-BT. The primary antibody (His-probe, Santa Cruz Biotechnology) was diluted 1:2000, and incubated for 3 h at room temperature. The secondary antibody (Alexa fluor 488, Invitrogen) was diluted 1:15000, and incubated for 45 min at room temperature in the dark. Nuclei were stained for 30 min with 1 μg/ml DAPI (Roche). The distribution of fluorescence was analyzed by fluorescence microscopy (Nikon eclipse 80i, Japan).

3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay

An MTT assay was used to determine the viability of SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA). The cells were pretreated with Tat-DJ-1 (0.5-3 μM) for 1 h, after which 6-OHDA (50 μM) was added to the culture medium for 24 h. The absorbance was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340) and the cell viability was defined as the % of untreated control cells.

Measurement of reactive oxygen species (ROS)

ROS levels were determined using the ROS sensitive dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), which is converted by ROS into the highly fluorescent 2′,7′-dichlorofluorescein (DCF). SH-SY5Y cells were incubated in the absence or presence of Tat-DJ-1 (3 μM) for 1 h, and then treated with 6-OHDA (50 μM) for 30 min. Cells were treated with DCF-DA (15 μM) for 15 min and then washed twice with PBS. The level of DCF fluorescence, reflecting the concentration of ROS, was measured by fluorescent microscopy using a fluoroskan enzyme-linked immunosorbent assay (ELISA) plate reader calibrated for excitation at 485 nm and emission at 538 nm (Labsystems Oy, Finland).

TUNEL assay

SH-SY5Y cells were incubated in the absence or presence of Tat-DJ-1 (3 μM) for 1 h, and then treated with 6-OHDA (80 μM) for 10 h. Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using the Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. Images were taken using a fluorescence microscope (Nikon eclipse 80i, Japan).

Caspase-3 activation measurement

SH-SY5Y cells were incubated in the absence or presence of Tat-DJ-1 (3 μM) for 1 h, and then treated with 6-OHDA (80 μM) for 10 h. The expression of active caspase-3 in whole cell lysates was analyzed by Western blotting using cleaved caspase-3 antibodies. Band intensity was measured by densitometer (Image J software; NIH, USA).

Experimental animals and immunohistochemistry

Male 8-week C57BL/6 mice (22-25 g) were used in this study.

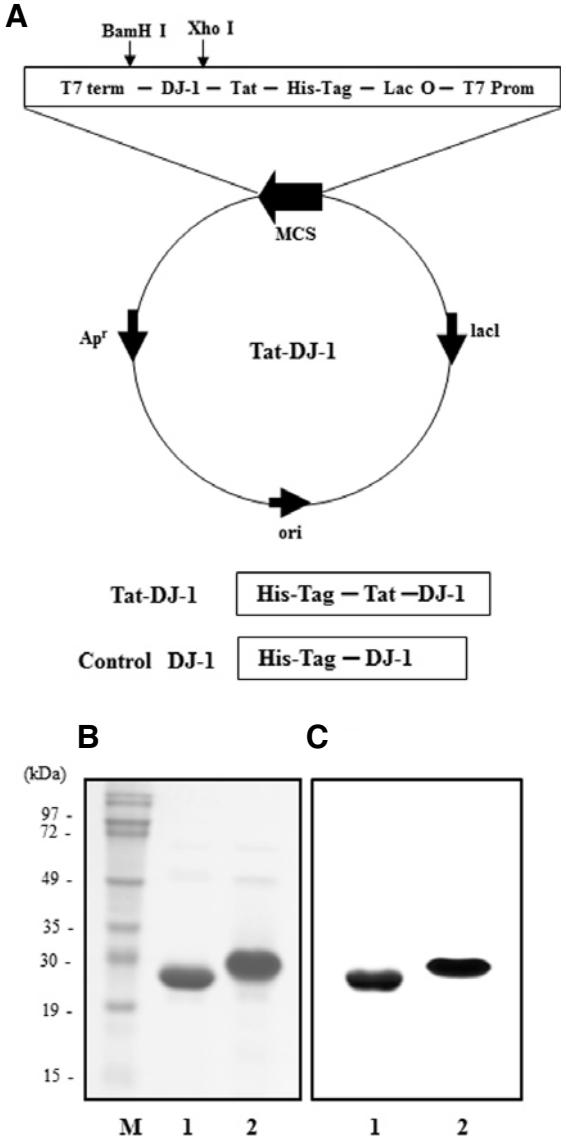

Fig. 1. Construction and purification of Tat-DJ-1 protein. Tat-DJ-1 expression vector system was constructed using vector pET-15b. Schematic sequences of Tat-DJ-1 and control DJ-1 proteins (A). Purified Tat-DJ-1 proteins were analyzed by 12% SDS-PAGE (B) and subjected to Western blot (C) analysis with an anti-rabbit poly-histidine antibody. Lanes in B and C are as follows: lane 1, purified control DJ-1 protein; lane 2, purified Tat-DJ-1 protein.

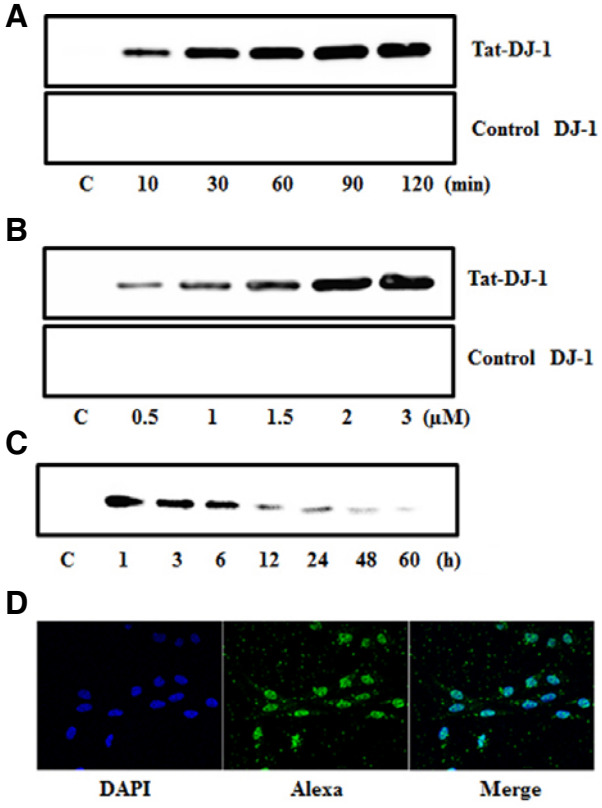

Fig. 2. Transduction of Tat-DJ-1 protein into SH-SY5Y cells. (A) Tat-DJ-1 (3 μM) and control DJ-1 were added to the culture media for 10-120 min, (B) Tat-DJ-1 (0.5-3 μM) and control DJ-1 were added to the culture media for 60 min and (C) cells pretreated with 3 μM Tat-DJ-1 were incubated for 1-60 h and analyzed by Western blot analysis. (D) The distributions of transduced Tat-DJ-1 proteins were observed by fluorescence microscopy.

The animals were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light: 12 h dark cycle and free access to food and water. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee. The mice received four injections of MPTP (20 mg/kg) at 2 h intervals. To determine whether transduced Tat-DJ-1 protects against PD, mice were i.p. injected with Tat-DJ-1 (2 mg/kg) 12 h before the MPTP treatment. Mice (n = 5 for each group) were divided into the following groups: 1) non-treated controls, 2) MPTP-treated, 3) MPTP + Tat-DJ-1 treated, 4) MPTP + control DJ-1 treated, and 5) MPTP + Tat peptide treated. The mice were killed 1 week after the last injection. The frozen and sectioned midbrains 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 (Vector Laboratories, USA; dilution 1:200) and then visualized with 3,3-diaminobenzidine (DAB) (40 mg DAB, 0.045% H_2O_2 in 100 ml PBS) mounted on gelatin-coated slides.

Behavioural test

Motor function of mice was assessed by the Rotarod test (Iwamoto et al., 2003). In brief, mice were positioned on a Rotarod (IITC Life Science, USA). The rod rotated gradually increasing from 5 to 30 rpm over the course of 15 min. The total length of time and distance moved while staying on the accelerating Rotarod was automatically measured.

Quantitative analysis

The data were analyzed using one-way ANOVA to determine statistical significance.

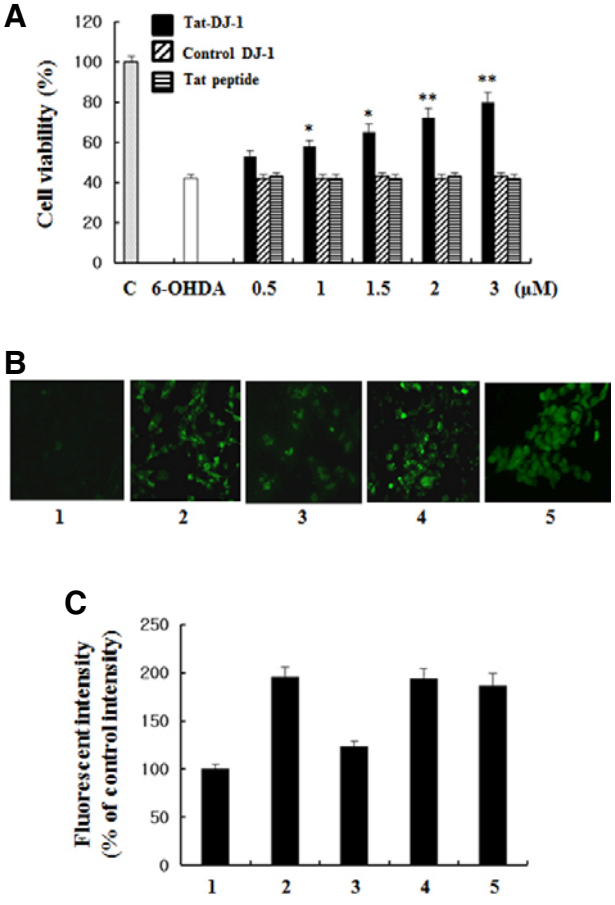

Fig. 3. Effects of transduced Tat-DJ-1 on cell viability and ROS generation in the SH-SY5Y cells. 6-hydroxydopamine (6-OHDA) (50 μM) was added to the cells pretreated with Tat-DJ-1 (0.5-3 μM) for 1 h. (A) Cell viabilities were estimated by with a colorimetric assay using MTT. **P* < 0.05 and ***P* < 0.01, compared with 6-OHDA treated cells. (B) The cells were treated with Tat-DJ-1 (3 μM) for 1 h, and then exposed to 6-OHDA (80 μM) for 30 min. Intracellular ROS levels were measured after staining with a fluorescent dye, DCF-DA. (C) The fluorescent intensity was measured by an ELISA plate reader. Lanes in (B) and (C) are as follows: Lane 1, control cells; lane 2, 6-OHDA treated cells; lane 3, Tat-DJ-1 treated cells; lane 4, control DJ-1 treated cells; lane 5, Tat peptide treated cells.

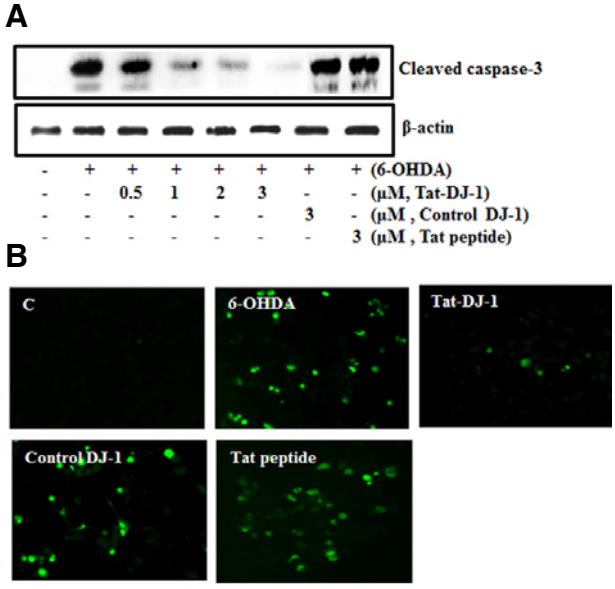

Fig. 4. Tat-DJ-1 protein protects against 6-OHDA-induced caspase-3 activation and DNA fragmentation in SH-SY5Y cells. The cells were treated with Tat-DJ-1 (3 μM) for 1 h, and then exposed to 6-OHDA (80 μM) for 10 h. (A) The caspase-3 activation was measured by Western blotting. (B) DNA fragmentation was detected by TUNEL staining.

RESULTS

Expression and purification of Tat-DJ-1 protein

A human DJ-1 gene was amplified by RT-PCR using a set of primers designed from a database of DJ-1 genes and a cDNA template prepared from the human brain cDNA library. To develop an expression and purification system for the cell-permeable DJ-1 protein, we constructed a Tat-DJ-1 expression vector (pTat-DJ-1), which contained a consecutive cDNA sequence encoding the human DJ-1, a Tat protein transduction domain (Tat49-57), and six histidine residues at the amino-termius. We also constructed a DJ-1 expression vector to produce control DJ-1 protein without an HIV-1 Tat protein transduction domain (Fig. 1A).

After induction of expression, Tat-DJ-1 proteins were purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. Purified Tat-DJ-1 proteins were electrophoresed in 12% SDS-PAGE. The purification results are shown in Fig. 1B. The purified recombinant Tat-DJ-1 proteins had estimated molecular masses of approximately 30 kDa. The proteins were further confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. Tat-DJ-1 proteins were detected at the corresponding bands in Fig. 1C.

Transduction of Tat-DJ-1 into SH-SY5Y cells

To determine whether Tat-DJ-1 protein is able to transduce into SH-SY5Y cells, we added 3 μM Tat-DJ-1 protein to the culture medium for various periods of time (10-120 min), and then analyzed the transduced protein levels by Western blotting.

The intracellular concentration of transduced Tat-DJ-1 proteins in cells was detected within 10 min and gradually increased until 60 min. The dose-dependency of the transduction of Tat-DJ-1 proteins was also analyzed. Various concentrations (0.5-3 μM) of Tat-DJ-1 proteins were added to the cells in culture for 60 min, and the levels of transduced proteins were measured by Western blotting. The proteins transduced into the cells in a concentration dependent manner. As shown in Figs. 2A and 2B, Tat-DJ-1 protein efficiently transduced into SH-SY5Y cells in a time- and dose-dependent manner. However, the control DJ-1 did not transduce into the cells.

The intracellular stability of transduced Tat-DJ-1 proteins in SH-SY5Y cells is shown in Fig. 2C. The Tat-DJ-1 proteins were added to the culture media of the cells at a concentration of 3 μM for various time periods and the resulting transduced protein levels were analyzed by Western blotting. The intracellular level of transduced Tat-DJ-1 in cells was initially detected after 1 h. Though the level declined gradually over the period of observation, significant levels of transduced Tat-DJ-1 protein persisted in the cells for 48 h.

To further clarify the cellular localization of transduced proteins in the cells, transduced cells were double stained with the nucleus specific marker DAPI. As shown in Fig. 2D, Tat-DJ-1

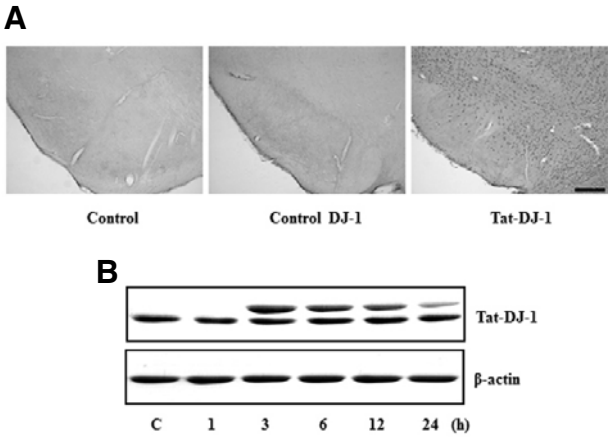

Fig. 5. Transduction of Tat-DJ-1 protein across the blood-brain barrier. (A) Transduction of Tat-DJ-1 protein in mouse brain was analyzed by immunohistochemistry using anti-histidine antibody. Animals were treated with a single injection of Tat-DJ-1 and killed after 8 h. Scale bar = 100 μm. (B) The intracellular stability of transduced Tat-DJ-1 proteins in mouse brain. The transduced Tat-DJ-1 protein levels were analyzed by Western blotting using DJ-1 antibody.

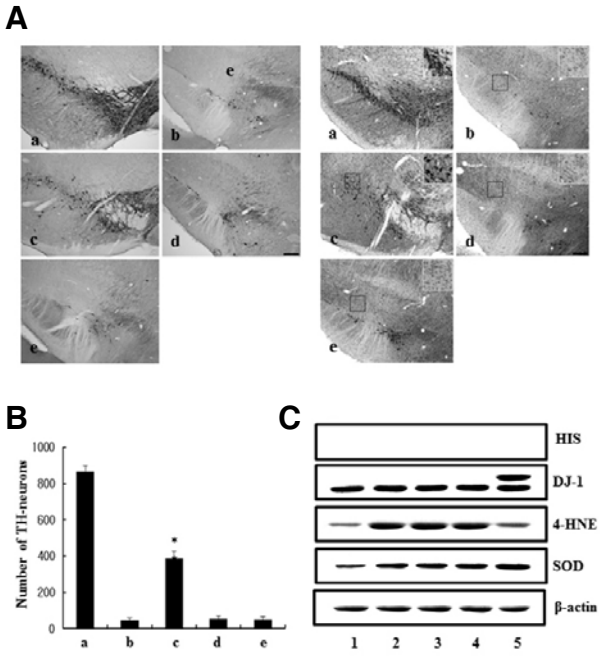

Fig. 6. Effect of transduced Tat-DJ-1 on dopaminergic neuronal cell viability. (A) Representative photomicropraphs of the cresyl-violet and TH-immunostained SN of Tat-DJ-1 treated mice. (B) The number of TH-immunoreactivity neurons was counted in the SN of mice. $^{**}P < 0.01$ compared to MPTP treated mouse. Negative control (a; saline treated), positive control (b; MPTP-treated), Tat-DJ-1 treated (c), control DJ-1 treated (d) and Tat peptide treated (d). (C) Protective effect of transduced Tat-DJ-1 protein in the mice brain analyzed by Western blotting using histidine, DJ-1, 4-HNE and SOD antibodies. Lane 1, control; lane 2, MPTP-treated; lane 3, Tat peptide treated; lane 4, control DJ-1 treated; lane 5, Tat-DJ-1 treated.

protein was detected in the cytoplasm and in the nucleus of transduced cells. These results indicate that Tat-DJ-1 proteins efficiently transduced into the cells and were stable for at least 48 h.

Transduced Tat-DJ-1 protein affect the viability of cells under oxidative stress

To determine whether transduced Tat-DJ-1 protein protects cells under oxidative stress, cell viability was measured after the administration of 6-hydroxydopamine (6-OHDA) using the MTT assay. When the cells were exposed to 50 μM 6-OHDA for 24 h, only 43% of cells were viable. As shown in Fig. 3A, the viability of cells exposed to 6-OHDA increased up to 81% in those pretreated with Tat-DJ-1 proteins. However, control DJ-1 protein did not show a protective effect under the same conditions. These results indicate that transduced Tat-DJ-1 protein plays a defensive role against cell death induced by oxidative stress in the cells.

Reactive oxygen species (ROS) mediated many of the pathophysiological events that cause several diseases including PD (Hald and Lotharius, 2005; Pong et al., 2001; Rastogi et al., 2006; Tamagno et al., 2003) and has been suggested to be involved in apoptotic cell death (Ahn et al., 2009). 6-OHDA administration results in the formation of ROS (Glinka et al., 1997). Therefore, we investigated whether Tat-DJ-1 protein inhibits the intracellular ROS generation by 6-OHDA. To determine the generation of ROS, we used the intracellular oxidation of DCF-DA fluorescent dye. When the cells were exposed to 80 μM 6-OHDA for 30 min, 6-OHDA markedly increased the DCF signal. However, ROS generation by 6-OHDA was decreased by the presence of Tat-DJ-1 protein (Fig. 3B). ROS generation was quantified using a Fluoroskan ELISA plate reader. As shown in Fig. 3C, transduced Tat-DJ-1 protein significantly inhibited the ROS production by 6-OHDA compared to control DJ-1 protein in SH-SY5Y cells. These results indicate that Tat-DJ-1 protein efficiently inhibits 6-OHDA-induced ROS generation.

Since caspase-3 plays a central role in apoptosis in mammalian cells (Park et al., 2010), we examined whether Tat-DJ-1 protein inhibits caspase-3 activation and DNA fragmentation in SH-SY5Y cells. To detect caspase-3 activation in 6-OHDA treated cells, caspase-3 activation was determined by western blotting using antibodies against cleaved caspase-3. 6-OHDA increased the caspase-3 activation when compared with the control. However, transduced Tat-DJ-1 protein significantly inhibited caspase-3 activation in a dose-dependent manner (Fig. 4A). Next, the protective effect against DNA fragmentation of transduced Tat-DJ-1 protein was determined by TUNEL staining. As shown in Fig. 4B, the negative control cells were not colored. However, 6-OHDA markedly increased the number of cells stained compared with the control, whereas cells treated with transduced Tat-DJ-1 protein were only slightly stained. These results indicate that caspase-3 activation and DNA fragmentation resulted from 6-OHDA-induced ROS and these are efficiently inhibited by transduced Tat-DJ-1 protein.

Transduction of Tat-DJ-1 protein into substantia nigra

To determine whether the Tat-DJ-1 protein crossed the blood-brain barrier, we performed immunohistochemistry on brain sections of Tat-DJ-1-treated mice for substantia nigra (SN). As shown in Fig. 5A, transduced Tat-DJ-1 protein levels were significantly increased throughout the SN of Tat-DJ-1-treated animals. However, control Tat-DJ-1 protein was not transduced into the SN. These results indicate that Tat-DJ-1 protein efficiently transduced into SN beyond the mice blood-brain bar-

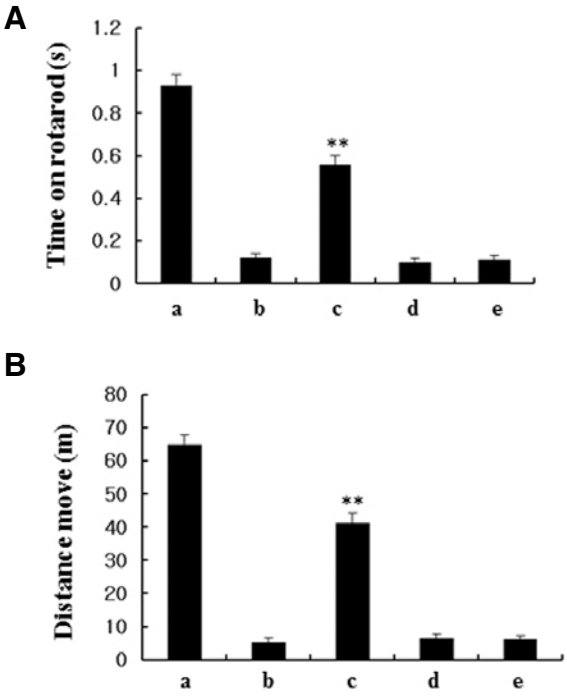

Fig. 7. Effects of transduced Tat-DJ-1 on behavior test. Mice were positioned on a Rotarod and the time (A) and total distance (B) that the mice stayed on the rod without falling was recorded. Lanes in A and B are as follows: 1, control; 2, MPTP treated; 3, Tat-DJ-1 treated; 4, control DJ-1 treated; 5, Tat peptide treated. $^{**}P < 0.01$, compared with MPTP-treated mice.

rier.The intracellular stability of transduced Tat-DJ-1 proteins in mouse brain under the same conditions is shown in Fig. 5B. The transduced Tat-DJ-1 protein levels were analyzed by Western blotting using a DJ-1 antibody. The intracellular level of transduced Tat-DJ-1 in brain was initially detected after 1 h and significant levels of transduced Tat-DJ-1 protein persisted in the brain for 24 h.

Transduction of Tat-DJ-1 protein protects against Parkinson disease in an animal model

To determine whether Tat-DJ-1 protein plays biological roles *in vivo*, we examined the effects of transduced Tat-DJ-1 protein on dopaminergic neuronal cell viabilities in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model. The mice were intraperitoneal (i.p.) injected with Tat-DJ-1 (2 mg/kg) 12 h before the MPTP treatment. To evaluate the protective effects of transduced Tat-DJ-1 protein against dopaminergic neuronal injury, dopaminergic neuronal cell levels were estimated by tyrosine hydroxylase (TH) immunostaining. As shown in Fig. 6, Tat-DJ-1 protein efficiently protected against dopaminergic neuronal injury caused by MPTP treatment. However, control DJ-1 did not protect compared to MPTP treated mice.

We also directly evaluated by stereological counts of TH-immunoreactive neurons. In the MPTP-treated mice, the number of TH-positive cells in the SN was markedly reduced compared to the control mice. However, the neurons were significantly increased by transduced Tat-DJ-1 protein compared to those in the mice treated with MPTP and control DJ-1. These results indicate that Tat-DJ-1 protein efficiently transduces into the brain and effectively protects against neuronal cell injury by detoxifying MPTP-induced oxidative stress *in vivo*. Furthermore, we examined the mechanism by which transduced Tat-DJ-1 protein protects the mice brain. As shown in Fig. 6C, Tat-DJ-1 protein transduced into the mice brain analyzed by Western blotting using histidine, DJ-1, 4-HNE and SOD antibodies. In the MPTP treated mice brain, the levels of 4-HNE were significantly increased. However, increased 4-HNE is reduced to similar extent compared with normal brain by transduced Tat-DJ-1 protein. Interestingly, the levels of SOD were markedly increased by transduced Tat-DJ-1 proteins in the mice brain. These results indicate that the exogenous transduction of Tat-DJ-1 protein induces the expression of SOD protein and may produce a synergistic protective effect by Tat-DJ-1 and this anti-oxidant enzyme.

To assess the influence of transduced Tat-DJ-1 protein on clinical improvement, the Rotarod test was performed. The time spent on the accelerating Rotarod was significantly decreased for MPTP-treated mice compared to control mice, whereas the times of Tat-DJ-1 protein-treated mice were recovered compared to those of MPTP-treated mice (Fig. 7). Tat-DJ-1 protein-treated mice demonstrated significantly attenuated movement compared to MPTP-treated mice.

DISCUSSION

Parkinson's disease (PD) is a neurodegenerative disorder affecting approximately 1-2% of the population aged 65 years or older and is associated with progressive degeneration of dopaminergic neurons in the substantia nigra (SN) pars compactra (Fahn, 2003). DJ-1 is conserved in bacteria, plants, animals, and humans (Lucas and Marin, 2007). It is well known that DJ-1 has multiple functions including anti-oxidant and chaperone functions associated with PD pathogenesis (Menzies et al., 2005; Xu et al., 2005; Zhou et al., 2006). However, DJ-1 deficiency or loss-of-function leads to oxidative stress-induced cell death *in vitro* and *in vivo* (Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005). Although anti-oxidant enzymes have been considered as potential therapeutic agents against oxidative stress-mediated diseases, the enzymes inability to enter cells hinders this despite its apparent potential. So far, protein transduction using various exogenous proteins and protein transduction domains (PTDs) into cells for therapeutic application (Wadia and Dowdy, 2002; 2003). Thus, we prepared a cell-permeable Tat-DJ-1 fusion protein to protect against oxidative stress-induced neuronal cell death *in vitro* and *in vivo*.

Cell-permeable Tat-DJ-1 protein was highly expressed in cells and was nearly homogeneous at more than 95% pure, as determined by an SDS-PAGE analysis. The purified Tat-DJ-1 proteins were confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. Purified Tat-DJ-1 protein was efficiently transduced into SH-SY5Y cells in a time-/dose-dependent manner and transduced Tat-DJ-1 protein persisted in the cells for 48 h. In addition, we further examined Tat-DJ-1 protein localization into the cells using DAPI and Alexa fluorescence. These results indicate that Tat-DJ-1 protein efficiently transduced into SH-SY5Y cells without becoming attached to the outside membrane of the cells.

Reactive oxygen species (ROS) are inevitably formed as by-products of various normal cellular processes involving interactions with oxygen. ROS is an important mediator of neuronal cell death in PD. In addition, over-expression of DJ-1 leads to increased resistance to dopamine toxicity and reduced intracellular ROS (Inden et al., 2006; Junn et al., 2009; Kim et al., 2005). Thus, we examined the viability of cells with transduced

Tat-DJ-1 proteins after the administration of 6-hydroxydopmine (6-OHDA), which is a toxin derived from dopamine. When the cells were exposed to 50 μM 6-OHDA for 24 h, only 43% of cells were viable. The viability of cells exposed to 6-OHDA increased up to 81% when the cells were pretreated with Tat-DJ-1 proteins. We also examined whether transduced Tat-DJ-1 protein can inhibit the oxidative stress induced by 6-OHDA using ROS sensitive dye 2′,7′-dichlorofluorescein diacetate (DCF-DA). As shown in Fig. 3, transduced Tat-DJ-1 protein significantly inhibited 6-OHDA induced ROS production in SH-SY5Y cells. Furthermore, we found that transduced Tat-DJ-1 protein protects against 6-OHDA-induced apoptotic cell death in SH-SY5Y cells by the inhibition of caspase-3 activation.

Caspase-3 activation is well known as a maker of apoptotic cell death (Park et al., 2010). These results indicate that transduced Tat-DJ-1 protein plays a defensive role against oxidative stress-induced neuronal cell death by efficiently inhibiting 6-OHDA-induced ROS generation and csapase-3 activation. Recent reports have demonstrated that DJ-1 protein protects against neuronal cell death resulting from 6-OHDA-induced ROS. After exposure to dopaminergic neurotoxins including rotenone and 6-OHDA, intracellular ROS levels were inversely correlated with DJ-1 expression levels, suggesting that DJ-1 may mitigate 6-OHDA-induced oxidative stress and help protect cells against oxidative stress (Menzies et al., 2005; Xu et al., 2005; Zhou et al., 2006).

To examine the protective effect of transduced Tat-DJ-1 protein in animal models, we used a PD mouse model where mice were exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT). It is well known that exposure of C57BL/6 mice to the neurotoxin MPTP is one of the most valuable approaches to analyze critical aspects of PD in animal models. MPTP, a selective neurotoxicant, is known to deplete striatal dopamine initiated by the 1-methyl-4-phenylpyridinium ion (MPP^+) (Araki et al., 2001; Heikkila et al., 1984). As shown in Fig. 6, Tat-DJ-1 protein efficiently protected cells from the toxicity induced by MPTP exposure. Also the number of neurons significantly increased when the Tat-DJ-1 protein was transduced. However, the increased cell number was not observed when the control DJ-1 protein was used. These results indicate that Tat-DJ-1 protein protects against dopmainergic neuronal cell death in the SN region after PD, and attenuates neuronal damage after PD insults. However, the precise mechanisms remain to be studied further. Paterna et al. (2007) has shown that overexpression of DJ-1 significantly reduces MPTP-induced TH neuron loss in mice. Although the mechanism is unclear, overexpression of DJ-1 may reduce DAT functions which contribute to the increased resistance of DJ-1 expression dopamine neurons against MPTP. Also, overexpression of DJ-1 reduced postsynaptic dopamine receptor responses in normal mice. These results suggest that further exploration of the molecular functions of DJ-1 for *in vitro* and *in vivo* is warranted before DJ-1 therapy for PD can commence (Paterna et al., 2007). In agreement with our results, transduced Tat-DJ-1 protein markedly decreased MPTP-induced TH neuron loss in mice. However, a better understanding of DJ-1 on behavior and dopamine neurotransmission is required.

In summary, we demonstrated that human DJ-1 fused with Tat peptide (Tat-DJ-1) can be efficiently transduced *in vitro* and *in vivo* and it markedly protects against oxidative stress-induced neuronal cell death. Although the detailed mechanism remains to be explored, Tat-DJ-1 protein provides a strategy for therapeutic delivery in various ROS related human diseases including PD.

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