# Identification of Three Mutations in the Cu,Zn-Superoxide Dismutase (Cu,Zn-SOD) Gene with Familial Amyotrophic Lateral Sclerosis

## Transduction of Human Cu,Zn-SOD into PC12 Cells by HIV-1 TAT Protein Basic Domain

CHIH-MING CHOU,[a] CHANG-JEN HUANG,[b] CHWEN-MING SHIH,[a] YI-PING CHEN,[a] TSANG-PAI LIU,[c] AND CHIEN-TSU CHEN[a]

[a]*Department of Biochemistry, Taipei Medical University, Taipei, Taiwan*

[b]*Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*

[c]*Division of General Surgery, Mackay Memorial Hospital, Taipei, Taiwan*

**ABSTRACT: The most frequent genetic causes of amyotrophic lateral sclerosis (ALS) determined so far are mutations occurring in the gene coding for copper/zinc superoxide dismutase (Cu,Zn-SOD). The mechanism may involve the formation of hydroxyl radicals or malfunctioning of the SOD protein. Wild-type SOD1 was constructed into a transcription–translation expression vector to examine the SOD1 production *in vitro*. Wild-type SOD1 was highly expressed in *Escherichia coli*. Active SOD1 was expressed in a metal-dependent manner. To investigate the possible roles of genetic causes of ALS, a human Cu,Zn-SOD gene was fused with a gene fragment encoding the nine amino acid transactivator of transcription (Tat) protein transduction domain (RKKRRQRRR) of human immunodeficiency virus type 1 in a bacterial expression vector to produce a genetic in-frame Tat–SOD1 fusion protein. The expressed and purified Tat–SOD1 fusion proteins in *E. coli* can enter PC12 neural cells to observe the cellular consequences. Denatured Tat–SOD1 was successfully transduced into PC12 cells and retained its activity via protein refolding. Three point mutations, E21K, D90V, and D101G, were cloned by site-directed mutagenesis and showed lower SOD1 activity. In undifferentiated PC12 cells, wild-type Tat–SOD1 could prevent DNA fragmentation due to superoxide anion attacks generated by 35 mM paraquat, whereas mutant Tat–D101G enhanced cell death. Our results demonstrate that exogenous human Cu,Zn-SOD fused with Tat protein can be directly transduced into cells, and the delivered enzymatically active Tat–SOD exhibits a cellular protective function against oxidative stress.**

**KEYWORDS: amyotrophic lateral sclerosis (ALS); copper/zinc superoxide dismutase (Cu, Zn-SOD); transactivator of transcription (Tat) protein**

Address correspondence to: Chien-Tsu Chen, Department of Biochemistry, Taipei Medical University, No. 250, Wu-Hsing St., Taipei 110, Taiwan. Voice: +886-2-2736-1661 ext. 2500; fax: +886-2-2738-6527.

chenctsu@tmu.edu.tw

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## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive and irreversible disease characterized by degeneration of the motor neurons. This disease leads to weakness of the muscles, causing difficulties with walking, speech, and swallowing. The precise cause of the disease remains unclear. Previously, mutations of the Cu,Zn-superoxide dismutase gene (*SOD1*) were identified by Rosen *et al.* in a rare subgroup of patients, and most of the patients were found to have a family history of ALS.[1,2] Most ALS cases are sporadic, although more than 10% are familial cases. Most of the familial ALS (FALS) cases have autosomal-dominant traits, and about 25% of the FALS cases are reported to have a mutation in the *SOD1* gene.[1,3] SOD1 catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen to provide cellular defense against oxidative damage.[4] More than 90 mutations, involving 59 of 153 amino acid residues, have been described previously.[5] Identification of these mutations revealed that free radicals may play a critical role in the pathogenesis of the disease.[3] The mechanism of pathogenesis—whether SOD mutation produced motor neuron death because of the loss of SOD enzymatic activity or gain of an adverse function—needs to be further elucidated. In other studies involving the transduction of mutant SOD genes into nonneuronal cells and transgenic mice, the data demonstrated that there is no correlation between SOD activities and the frequency or severity of the disease.[7,8] These data suggest that mutant SOD does not cause FALS due to the lack or decrease of SOD activity.[6–8] In addition, in studies of SOD-knockout mice, the observed viability of motor neurons also supports this contention.[9] Wong *et al.* recently proposed that the FALS-linked mutant SOD failed to bind or shield copper ($Cu^{2+}$) as effectively as wild-type enzyme, and this change led to the enhancement of $Cu^{2+}$-catalyzed oxidative reactions.[10]

Currently, gene therapy has been widely exploited and is considered a promising method to introduce therapeutic proteins into cells.[11] There are also reports indicating that the basic domain of human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) protein can traverse biological membranes efficiently in a process termed protein transduction.[12–15] Although the mechanism is still unclear, it is widely accepted that the transduction occurs in a receptor- and transporter-independent fashion that appears to target the lipid bilayer directly.[15,16] Furthermore, Tat proteins have recently been shown to serve as carriers to direct uptake of heterologous proteins, including ovalbumin, L-galactosidase, and horseradish peroxidase, into the cells *in vitro* and *in vivo*. These data suggest that HIV-1 Tat proteins have tremendous potential to deliver large compounds into the cells.[17,18]

In this study, we report an effective approach for studying the mechanisms underlying neuronal death induced by the mutant SOD. Our results suggested that the wild-type and mutant Tat–SOD1 fusion protein effectively transduced into differentiated rat pheochromocytoma PC12 cells, and the function of the transduced Tat–SOD1 was found to possess a protective effect against oxidative stress in these cells.

## MATERIALS AND METHODS

### *Materials*

Restriction endonucleases and $T_4$ DNA ligase were purchased from Promega (Madison, WI). Oligonucleotides were synthesized from Gibco BRL (Gaithersburg, MD) custom primers. Isopropyl-L-D-thiogalactopyranoside (IPTG) was obtained from Promega. Plasmid pQE30 and $Ni^{2+}$–nitrilotriacetic acid (NTA) Sepharose superflow was purchased from Qiagen (Valencia, CA). *Escherichia coli* strain JM109 (DE3) was obtained from Stratagene (La Jolla, CA). Human Cu,Zn-SOD1 cDNA fragment was isolated using the polymerase chain reaction (PCR) technique using the human liver cDNA library. Polyclonal antibodies raised against human Cu,Zn-SOD were produced in our laboratory.

### *Isolation of the Full-Length Human Cu,Zn-SOD1*

Total RNA was isolated from human liver using the RNAzol reagent (Tel-Test, Friendswood, TX) according to the instructions of the manufacturer. To isolate the cDNA covering the complete open reading frame (ORF) of human Cu,Zn-SOD1 according to the sequences of one human expressed sequence tag accession number K00065, Genbank), the full-length human Cu,Zn-SOD1 was amplified with a Cu,Zn-SOD sense primer, CuZn-SOD-F: 5′-ATG GCG ACG AAG GCC GTG TGC GTG CTG-3′, and a Cu,Zn-SOD antisense primer, CuZn-SOD-R: 5′- TTA TTG GGC GAT CCC AAT TAC ACC ACA-3′. PCR amplification was performed in a 50-mL reaction mixture containing 2 mL of first-strand cDNA, 0.5 mg of primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U of HiFi-DNA polymerase (Yeastern Biotech, Taipei, Taiwan). Samples were incubated in a thermal cycler (Hybaid MultiBlock System; Hybaid Limited, Franklin, MA). The cDNA from brain as template was used for PCR amplification using the program of 94°C for 3 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension step at 72°C for 15 min. All RACE products were ligated into pGEM-T easy vector (Promega) and subjected to sequence analysis.

### *Construction of Expression Clones*

The expression plasmid pQE–SOD1 was constructed by inserting the full-length human Cu,Zn-*SOD1* cDNA into pQE30 at the *Sph*I and *Pst*I sites, which allows generation of the Cu,Zn-SOD1 protein with in-framed His-tag at the N-terminal end.

The pQE–Tat-SOD1 expression vector was constructed to express the basic domain (amino acids 49–57) of HIV-1 Tat as a fusion with Cu,Zn-SOD1 as follows. In brief, two oligonucleotides were synthesized and annealed to generate a double-stranded oligonucleotide encoding nine amino acids from the basic domain of HIV-1 Tat. Their sequences are (*Bam*HI-Tat-F) 5′-GAT CCG GAA GAA GCG GAG ACA GCG ACG AAG ACG-3′ and (*Bam*HI-Tat-R) 5′-GAT CCG TCT TCG TCG CTG TCT CCG CTT CTT CCG-3′. The double-stranded oligonucleotide was directly ligated into the *Bam*HI-digested pQE–SOD1 to generate the His–Tat-SOD1 expression plasmid pQE–Tat-SOD1. Similarly, other expression constructs with three point mutations, such as pQE–Tat-SOD1-E21K, pQE–Tat-SOD1-D90V, and pQE–Tat-

SOD1-D101G, respectively, were also generated by using site-directed mutagenesis. The mutated sequences were confirmed by sequence analysis.

### *Expression and Purification of Recombinant Proteins*

Five expression constructs mentioned above, for example, pQE–SOD1, pQE–Tat-SOD1, pQE–Tat-SOD1-E21K, pQE–Tat-SOD1-D90V, and pQE–Tat-SOD1-D101G, were expressed in *E. coli* JM109. A colony of *E. coli* cells was separately inoculated into Luria-Bertani broth in the presence of 100 mg/ml ampicillin, and the culture was grown overnight at 37°C until its optical density at 600 nm reached 1.2. To induce expression of these recombinant proteins, IPTG was added to a final concentration of 1.0 mM, and the incubation was continued for 4 h. All recombinant proteins were collected and purified by $Ni^{2+}$–NTA Sepharose metal affinity resins according to the manufacturer's instructions (Qiagen). The purity of these proteins was examined by 10% SDS-PAGE.

### *Transduction of Wild-Type and a Variety of Mutant Forms of Tat–SOD Proteins into PC12 Cells*

Rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT), 10% horse serum (HS), penicillin G (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) in a humidified atmosphere of 5% $CO_2$ at 37°C. Various culture reagents used were purchased from HyClone.

For the transduction of Tat–SOD1 and mutant SODs, PC12 cells were grown to confluence on a six-well plate. The culture medium was then replaced with 1 mL of fresh DMEM without FBS. After PC12 cells were treated with various concentrations of Tat–SOD for 1 h, the cells were harvested with trypsin–EDTA and washed with phosphate-buffered saline (PBS). The cells were then lysed, and cell extracts were subjected to SOD enzyme assay and Western blot analysis. The dismutation activity of SOD in cell extracts was measured with the pyrogallol reaction.[19]

For Western blotting, cell lysates were separated on a 10% SDS-PAGE , and the proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane according to the method of Towbin.[20] The membranes were incubated with 5% skim milk in PBS, and then with a polyclonal anti–human Cu,Zn-SOD antibody (hSOD) for 16 h at 4°C. The PVDF membranes were extensively washed and incubated with a horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin G antibody (diluted 1:3000; Jackson ImmunoResearch Labs, West Grove, PA) for 1 h at room temperature. Immunoreactive bands were visualized with the enhanced chemiluminescence substrate kit (NEN, Boston, MA) according to the manufacturer's protocol. The intracellular stability of transduced Tat–SOD1 and mutant forms of SOD1 was estimated as follows: PC12 cells were treated with 0.5 µg/mL denatured Tat–SOD1 for 0, 1, 2, 4 and 6 h; the cells were washed; and cell extracts for a SOD enzyme assay and Western blot analysis were prepared. The biological activity of transduced Tat–SOD1 and mutant forms of SOD1 was assessed by the cell viability of PC12 cells treated with paraquat (methyl viologen), which is well known as an intracellular superoxide anion generator. Cells ($2 \times 10^5$) were incubated in six-well plate at 70% confluence and were allowed to attach. The cells

were first treated with 1.5 μM denatured Tat–SOD1 and mutant forms of SOD1 for 4 h, respectively, followed by the addition of 35 mM paraquat for 2 h. Cell viability was estimated using the dye exclusion method.[26]

### *Determination of Superoxide Dismutase Activity*

Superoxide dismutase (SOD) activity was measured according to the method of Marklund.[19] Superoxide anion radical is involved in the auto oxidation of pyrogallol. At alkaline pH, SOD dismutates superoxide, thereby inhibiting the auto-oxidation of pyrogallol. The stock pyrogallol solution contained 1.0 mL of 0.5 M HCl and was stored at 30°C. The assay mixture, containing 500 μL of buffer (50 mM Tris–cacodylic acid buffer, pH 8.4, 1 mM diethylenetriamine pentaactic acid, 1 mM potassium phosphate buffer, pH7.0), 50 μL of sample, and 400 μl of deionized water, was put into a cuvette. The control sample contained 500 μL of assay buffer and 450 μL of water. The optical density of each sample was measured at 420 nm before the addition of pyrogallol. The increase in absorbance was measured at 10-s intervals and lasted for 3 min. SOD specific activity was expressed as units per milligram.

## RESULTS AND DISCUSSION

The Cu,Zn-SOD protein plays an important role to maintain a healthy balance between oxidants and antioxidants and is one of the cell's self-defense systems against oxygen-derived free radicals. Recently, studies revealed that the point mutations of Cu,Zn-SOD have been linked to FALS.[25] Using the transgenic mouse model that expresses a mutant Cu,Zn-SOD, previous work has shown the mice to develop symptoms of ALS.[18,21,22]

To develop a simple but efficient system to express and purify the cell-permeable SOD protein, we constructed the Tat–SOD1 expression vector (pQE–Tat-SOD1), which contains *SOD1* cDNA sequences encoding the human Cu,Zn-SOD, Tat protein transduction domain (Tat 49–57), and His-tagged sequence at the amino terminus (FIG. 1A). We prepared recombinant pQE–Tat with wild-type and mutant SODs as vectors for gene delivery into PC12 cells (FIG. 1B). The three point mutations of E21K, D90V, and D101G were identified from FALS-related patients in Taiwan. The presence of the mutation in the SOD cDNA was confirmed by PCR and DNA sequencing (data not shown). The Tat–wt, Tat–E21K, Tat–D90V, and Tat–D101G Tat fusion proteins were constructed. We also constructed the SOD expression vector (pQE–SOD1) to produce control SOD protein without an HIV-1 Tat protein transduction domain (FIG. 1B).

Transfected bacterial cells induced with IPTG were lysed in PBS buffer. The recombinant proteins were purified by $Ni^{2+}$–NTA Sepharose affinity chromatography and then subjected to SDS–10% PAGE. FIGURE 2 shows the protein bands visualized by Western blot analysis. The recombinant SOD1 and Tat–SOD1 proteins have an apparent molecular mass of 17 and 18 kDa, respectively (FIG. 2). However, the recombinant fusion proteins migrated to higher-molecular-weight positions than those of the expected sizes on the SDS–PAGE (as shown in FIG. 2), which is consistent with the previous reports.[23,24]

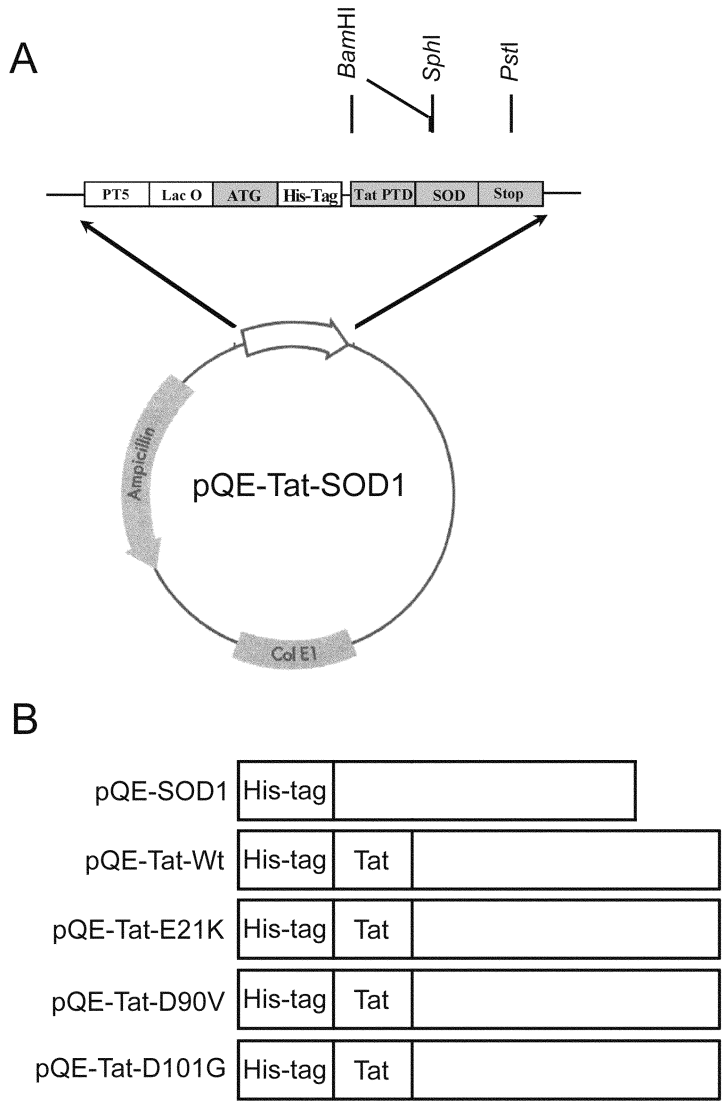


**FIGURE 1.** Contrustion of SOD1 and Tat–SOD1 expression system (pQE–Tat-SOD1) based in the vector pQE30. The synthetic Tat oligomer was cloned into the *Bam*HI site, and human Cu,Zn *SOD* cDNA was cloned into *Sph*I and *Pst*I sites of pQE (**A**). Expressed Tat–wt, Tat–E21K, Tat–D90V, Tat–D101G, and control SOD fusion proteins (**B**).

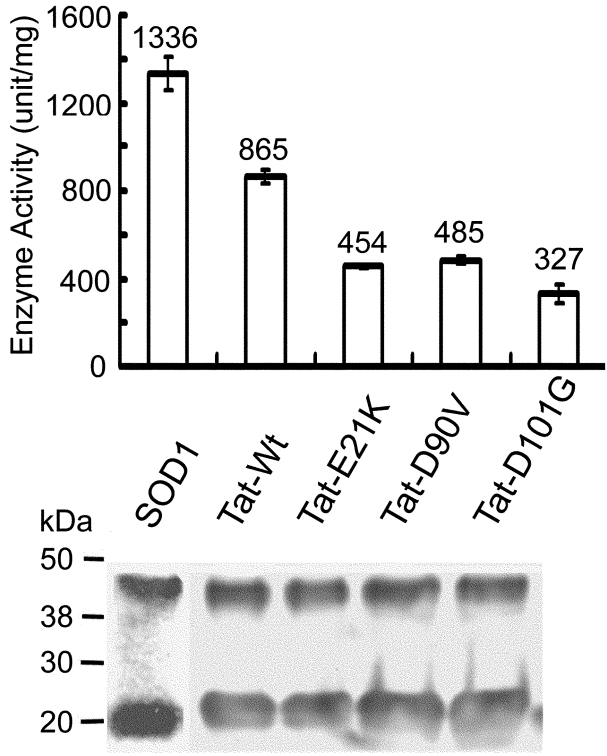


**FIGURE 2.** Active human wild-type SOD1 and Tat–SOD fusion protein expressed in *E. coli* indicates two forms, 23 kDa (monomer) and 46 kDa (dimer) in Western blot. Rather than expression in a mammalian cell, we constructed the *SOD1* gene into pQE30 vector and transformed it into *E. coli*. Active protein was produced with an activity of 1335 SOD U/mg; the other Tat fusion proteins, Tat–wt, Tat–E21K, Tat–D90V, and Tat–D101G, were produced with an activity of 865, 485, 454, and 327 SOD U/mg, respectively. The purification protein were subjected to 10%SDS–PAGE, transferred to PVDF, and detected with anti–human Cu,Zn-SOD antiserum. The protein molecular mass markers (kDa) are indicated on the left. The result illustrates that two protein forms, 23 and 46 kDa, representing the monomer and dimer, respectively, of SOD1.

The recombinant protein pQE–SOD1 has been produced with a specific activity of 1335 SOD U/mg (FIG. 2), whereas the other Tat fusion proteins, Tat–wt, Tat–E21K, Tat–D90V, and Tat–D101G, produced less specific activity of 865, 485, 454, and 327 SOD U/mg , respectively (FIG. 2).

To further evaluate the transduction ability of Tat–SOD1, 1.5 μM of Tat–SOD1 proteins purified under denaturing conditions was added to the culture media of PC12 cells for 1, 2, 4, and 6 h and then were analyzed by the immunofluorescence staining technique. As shown in FIGURE 3A, the time-dependent manner of transduction indicated that Tat–SOD1 was rapidly transduced into cells and that the levels of transduced proteins in the cultured PC12 cells were increased during the treatments.

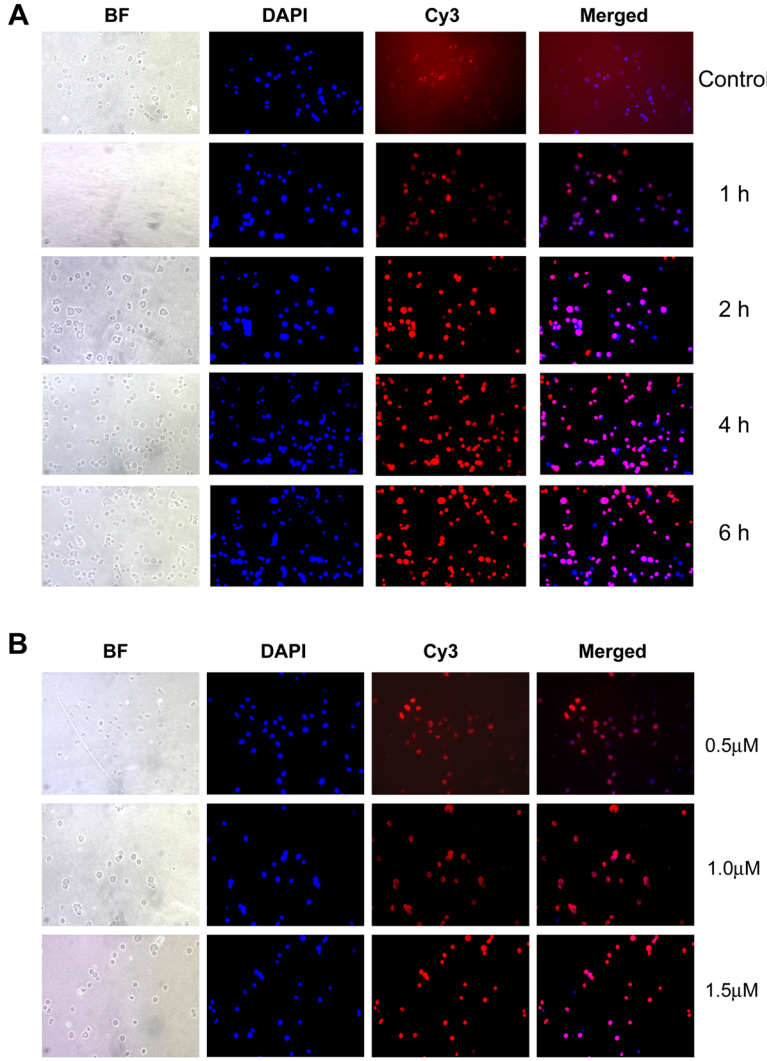


**FIGURE 3.** Visualization of Tat–SOD transduced into undifferentiated PC12 cells by immunofluorescence assay. PC12 cells plated in a six-well plate were treated with 1.5 μM of Tat–SOD to culture media and incubated for various time intervals (**A**). PC12 cells were treated with Tat–SOD at concentrations of 0.5–1.5 μM for 1 h (**B**). Then, the transduced fusion proteins into the cells were analyzed by immunofluorescence microscopy. DAPI, nuclear staining; Cy3, SOD1 staining.

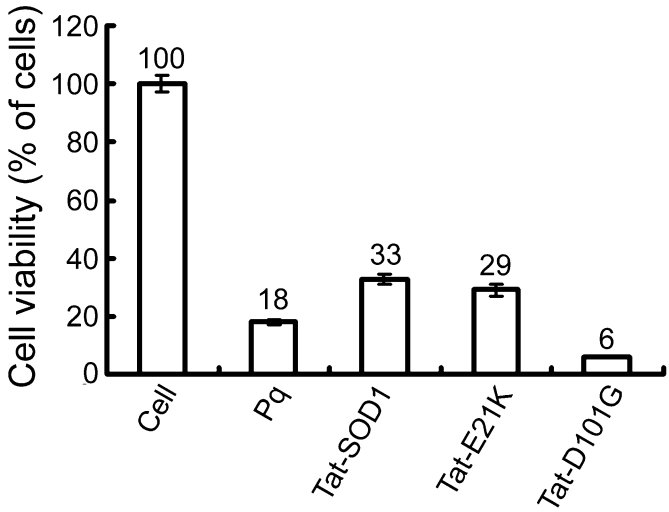


**FIGURE 4.** Tat–SOD protects PC12 cells from attack of the superoxide anion generated by 35 mM paraquat, but Tat–D101G enhanced cell death. Tat-tagged wild-type and mutant SOD were transduced into PC12 undifferentiated cells as shown. Cell viability experiment was performed in presence of paraquat (35 mM) and revealed that Tat–SOD protects PC12 cells from superoxide anion attack. After the cells were exposed to 35 mM paraquat without Tat–SOD, only 18% of the PC12 cells were viable; the viability was significantly decreased when pretreated with Tat–D101G. The cell viability of PC12 cells pretreated with 1.5 μM Tat–SOD, Tat–E21K, and Tat–D101G, was 33%, 29%, and 6%, respectively.

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.[18] The difference in the transduction period may be due to the properties of transduced Tat fusion protein, such as the degree of unfolding, polarity, and the molecular shape of the protein. The dose dependency of the transduction of denatured Tat–SOD1 fusion proteins was further analyzed. Various concentrations (0.5, 1.0, and 1.5 μM) of denatured Tat–SOD1 proteins were added to the culture media of PC12 cells for 1 h, and the levels of transduced proteins were measured by immunofluorescence staining. As shown in FIGURE 3B, the PC12 cells were treated with 1.5 μM Tat–SOD protein; more than 90% of the cells were transduced in 1 h.

To determine whether transduced Tat–SOD protein could play its biological role in the cells, we tested the Tat–SOD and mutant SODs on cell viability under oxidative stress. After the cells were exposed to 35 mM paraquat without Tat–SOD for 2 h, approximately 18% of the PC12 cells were viable, and the viability was significantly decreased when pretreated with Tat–D101G (FIG. 4). When the cells were pretreated with 1.5 μM Tat–SOD, Tat–E21K, and Tat–D101G, the viability of PC12 cells was 33%, 29%, and 6%, respectively (FIG. 4).

We further analyzed SOD1 activity of three point mutations, E21K, D90V, and D101G, which were cloned via site-directed mutagenesis in our laboratory. All three

point mutations were found to exhibit lower SOD1 activity. In undifferentiated PC12 cells, wild-type Tat–SOD1 escaped DNA fragmentation due to superoxide anion attacks generated by 35 mM paraquat; however, mutant Tat–D101G enhanced cell death.

In conclusion, these results demonstrate that exogenous human Cu,Zn-SOD fused with Tat protein can be directly transduced into the cells, and the delivered enzymatically active Tat–SOD fusion exhibits a cellular protective function against oxidative stress. Therefore, this transduction may allow the therapeutic delivery of Cu,Zn-SOD for the various disorders related to this antioxidant enzyme. However, whether mutant Tat-D101G–enhanced cell death may be due to increased production of hydroxyl radicals needs to be elucidated.

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