Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells

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Abstract A human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) gene was fused with a gene fragment encoding the nine amino acid transactivator of transcription (Tat) protein transduction domain (RKKRRQRRR) of HIV-1 in a bacterial expression vector to produce a genetic in-frame Tat–SOD fusion protein. The expressed and purified Tat–SOD fusion protein in *Escherichia coli* can enter HeLa cells in a time- and dose-dependent manner when added exogenously in a culture media. Denatured Tat–SOD protein was transduced much more efficiently into cells than were native proteins. Once inside the cells, transduced Tat–SOD protein was enzymatically active and stable for 24 h. The cell viability of HeLa cells treated with paraquat, an intracellular superoxide anion generator, was increased by transduced Tat–SOD. These lines of results suggest that the transduction of Tat–SOD fusion protein may be one of the ways to replenish the Cu,Zn-SOD in the various disorders related to this antioxidant enzyme.

Key words: Transduction; Copper,zinc-superoxide dismutase; Human immunodeficiency virus type 1 transactivator of transcription

1. Introduction

The reactive-oxygen species are inevitably formed as by-products of various, normal cellular processes involving interactions with oxygen. These reactive-oxygen species damage macromolecules in the cells, and therefore, sometimes make significant contributions to the several pathological processes of human diseases, including carcinogenesis, ischemia, radiation injury, and inflammation/immune injury [1,2]. Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is among the key cellular enzymes by which cells detoxify free radicals and protect themselves from oxidative damage [3].

Since every biological macromolecule can serve as a target for the damaging action of the abundant oxygen radicals, interest has grown over the therapeutic potential of SOD. There has been considerable interest in the use of SOD because of its role in protection against human diseases. A wide variety of Cu,Zn-SOD, conjugated or encapsulated, are available, including SOD conjugated with polyethylene glycol, Ficoll, lecithin, albumin, and SOD encapsulated with liposome [2,4–6]. All of the above have longer circulating half-lives than the untreated SOD molecules. In addition, gene therapy and transgenesis have been suggested as a clinical delivery system of SOD [7–9]. Currently, gene therapy has been widely exploited, and it is considered a promising method to introduce therapeutic proteins into the cells. However, this technique may have some constraints, including gene delivery, prolonged gene expression, efficacy, and toxicity [10].

It has been recently reported that the basic domain of human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) protein possesses the ability to traverse biological membranes efficiently in a process termed 'protein transduction' [11–14]. Although the mechanism is unknown, transduction occurs in receptor- and transporter-independent fashion that appears to target the lipid bilayer directly [14,15]. Furthermore, HIV-1 Tat proteins have recently been shown to serve as carriers to direct uptake of heterologous proteins, including ovalbumin, β-galactosidase, and horseradish peroxidase, into the cells in vitro and in vivo [16–18]. Thus, HIV-1 Tat proteins have tremendous potential to deliver large-sized compounds into the cells.

In the present study, we describe the transduction of full-length Tat–SOD fusion protein into HeLa cells and whether this transduced Tat–SOD has a protective effect against oxidative stress in the cells.

2. Materials and methods

2.1. Materials

Restriction endonucleases and T4 DNA ligase were purchased from Promega Co. *Pfu* polymerase was obtained from Stratagene. Oligonucleotides were synthesized from Gibco BRL custom primers. Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. Plasmid pET15b and *Escherichia coli* strain BL21 (DE3) were from Novagen. Ni^{2+}-nitrilotriacetic acid Sepharose superflow was purchased from Qiagen. A human Cu,Zn-SOD cDNA fragment was isolated using the polymerase chain reaction (PCR) technique from the λ_{Zap} human placenta cDNA library [19] and a monoclonal antibody raised against human Cu,Zn-SOD was produced in our laboratory.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; Tat, transactivator of transcription; Cu,Zn-SOD, Cu,Zn-superoxide dismutase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

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2.2. Expression and purification of Tat–SOD

The pTat–SOD expression vector was constructed to express the basic domain (amino acids 49–57) of HIV-1 Tat as a fusion with Cu,Zn-SOD as follows: First, two oligonucleotides were synthesized and annealed to generate a double-stranded oligonucleotide encoding nine amino acids from the basic domain of HIV-1 Tat. The sequences are (top strand) 5′-TAGGAAGAAGCGGAGACAGCGACGAAGAC-3′ and (bottom strand) 5′-TCGAGTCTTCGTCGCTGTCTCCGCTTCTTCC-3′. The double-stranded oligonucleotide was directly ligated into the *Nde*I–*Xho*I-digested pET15b in frame with the six histidine open-reading frame to generate the HisTat expression plasmid, pHisTat. The sequences of the polylinkers cloned into the plasmid were confirmed with a fluorescence-based automated sequencer (model 373A; Applied Biosystems, Inc.).

Next, on the basis of the cDNA sequence of human Cu,Zn-SOD, two oligonucleotides were synthesized. The top strand, 5′-CTCGAGGCGACGAAGGCCGTGTGCGTG-3′, contains an *Xho*I restriction site, and the bottom strand, 5′-GGATCCTTATTGGGCGATCCCAATTAC-3′, contains a *Bam*HI restriction site. The reaction mixture was made up in a 50 μl siliconized reaction tube and heated at 94°C for 5 min. The program for PCR consisted of 30 cycles of extension at 94°C for 40 s, denaturation at 54°C for 1 min, annealing at 70°C for 3 min, and the final extension at 72°C for 10 min, 20°C for 5 min.

The PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into a TA-cloning vector (Invitrogen) and then transformed into a competent cell. The plasmids of selected colonies were purified by using an alkaline lysis method [20]. The purified TA vector containing human *SOD1* cDNA was digested with *Xho*I and *Bam*HI, and then subcloned into a pET15b and pTat expression vector which had been digested with the same restriction enzymes (Fig. 1). The host *E. coli* BL21(DE3) was transformed with pSOD and pTat–SOD, and then the transformants were selected on a LB plate containing ampicillin. The selected colonies were cultured in a LB medium containing ampicillin at 37°C with shaking at 200 rpm. After the cells had grown until OD_{600} = 0.5∼1.0, IPTG was added to a concentration of 0.5 mM, and the incubation was continued for 3 h. The cells were harvested, and a 5 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) was added and sonicated. The recombinant Tat–SOD was purified under denaturing and native conditions, respectively. To denature Tat–SOD, harvested cells were disrupted by sonication in a binding buffer containing 6 M urea. After centrifugation, supernatants containing Tat–SOD were immediately loaded onto a 2.5 ml Ni^{2+}-nitrilotriacetic acid Sepharose column. After the column was washed with 10 volumes of a binding buffer and six volumes of a washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9), the fusion protein was eluted with an elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The fusion protein containing fractions were combined and the salts were removed using Sephadex G-15 column chromatography. The protein concentration in fractions was estimated by the Bradford procedure using bovine serum albumin as a standard [21].

2.3. Transduction of Tat–SOD into cultured HeLa cells

The HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum, and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under a humidified condition of 95% air and 5% CO_2. For the transduction of Tat–SOD, HeLa cells were grown to confluence on a 6 well plate for 4–6 h. And then, the culture medium was replaced with 1 ml of fresh solution without a fetal bovine serum. After HeLa cells were treated with various concentrations of Tat–SOD for 1 h, the cells 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 SOD enzyme assay and Western blot analysis. The dismutation activity of SOD in cell extracts was measured by monitoring the inhibition of ferricytochrome *c* reduction by the xanthine/xanthine oxidase reaction described previously [22]. For Western blotting, proteins in cell extracts were separated on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were transferred onto a nitrocellulose membrane. The membranes were incubated with 5% BSA in PBS, and then with a monoclonal anti-human Cu,Zn-SOD antibody (hSOD mAb-1.4) for 1 h at room temperature. After they were washed, the membranes were then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma, diluted 1:5000) for 1 h at room temperature. Immunoreactive bands were visualized with the alkaline phosphatase conjugate substrate kit (Bio-Rad) according to the manufacturer's protocol. The intracellular stability of transduced Tat–SOD fusion protein was estimated as follows: After HeLa cells were treated with 2 μM denatured Tat–SOD for 1 h, the cells were washed and changed with a fresh culture medium to remove Tat–SOD that was not transduced. And then, cells were further incubated for 3, 6, 12, 24 and 48 h, followed by preparations of cell extracts for a SOD enzyme assay and Western blot analysis.

2.4. Effect of transduced Tat–SOD on cell viability of paraquat-treated HeLa cells

The biological activity of transduced Tat–SOD was assessed by the cell viability of HeLa cells treated with paraquat (methyl viologen), which is well known as an intracellular superoxide anion generator [2]. Cells were plated into 6 well trays at 70% confluence, and they were allowed to attach cells per well. After the cells were treated with 0.1∼2 μM denatured Tat–SOD and control SOD for 1 h, respectively, then the 5 mM paraquat was added to the culture medium for 12 h. Cell viability was estimated by with a colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

3. Results and discussion

Cu,Zn-SOD is one of the cell's primary defenses against oxygen-derived free radicals, and it is vital for maintaining a healthy balance between oxidants and antioxidants. Recently, it has been reported that the point mutations of Cu,Zn-SOD have been linked to familial amyotrophic lateral sclerosis

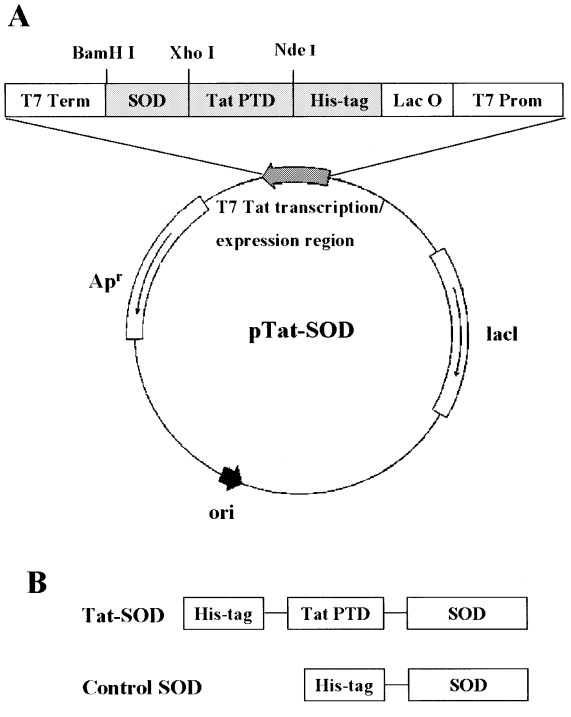

Fig. 1. Construction of Tat–SOD expression vector system (pTat–SOD) based on the vector pET15b (A). The synthetic Tat oligomer was cloned into the *Nde*I, *Xho*I sites, and 486 bp of human Cu,Zn-SOD cDNA was cloned into *Xho*I, *Bam*HI sites of pET15b. The expression vector is under the control of the T7 promoter and lacO-operator. The expression is induced by the addition of IPTG. Diagram of expressed Tat–SOD and control SOD fusion proteins (B).

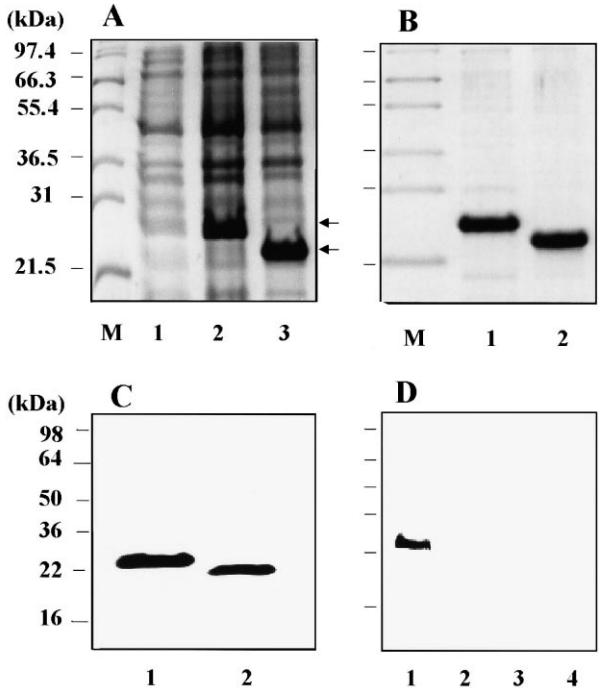

Fig. 2. Expression and purification of Tat–SOD in *E. coli*. Protein extracts of cells (A) and purified fusion proteins (B) were analyzed by 15% SDS–PAGE gel and subjected to Western blot analysis with a monoclonal antibody to human Cu,Zn-SOD (C). Lanes in A are as follows: extracts of bacteria containing expression vector pET (lane 1), pTat–SOD (lane 2), and pSOD (lane 3). Lanes in B and C are as follows: lane 1, Tat–SOD; lane 2, SOD. Transduction of Tat–SOD fusion proteins into cultured HeLa cells (D). 1 μM of Tat–SOD and control SOD were added to the culture media for 1 h and analyzed by Western blot analysis. Lanes are as follows: lane 1, denatured Tat–SOD; lane 2, denatured SOD; lane 3, native Tat–SOD; lane 4, native SOD.

(FALS) [23]. Transgenic mice that express a mutant Cu,Zn-SOD have been shown to develop amyotrophic lateral sclerosis (ALS) symptoms [24]. In an effort to replenish the Cu,Zn-SOD activity in the patients like FALS, we are describing a genetic approach of the Tat–SOD fusion protein into the cells.

To develop an expression system to overexpress and simply purify the cell-permeable SOD protein, we constructed the Tat–SOD expression vector (pTat–SOD), which contains consecutive cDNA sequences encoding the human Cu,Zn-SOD, Tat-protein transduction domain (Tat_{49-57}) and six histidine residues at the amino-terminus (Fig. 1A). We also constructed the SOD-expression vector (pSOD) to produce control SOD protein without an HIV-1 Tat-protein transduction domain (Fig. 1B).

Transfected bacterial cells induced with IPTG were lysed at 4°C in a PBS buffer. The crude cell extracts obtained from *E. coli* were used for electrophoresis in 15% SDS–PAGE. Fig. 2A shows the protein bands visualized by staining with Coomassie brilliant blue. The bands marked by arrows in lane 2 (Tat–SOD) and lane 3 (SOD), in comparison with that of the lane 1 (pET15b vector) alone, indicate that the protein was expressed at a very high level with a major component of the total soluble proteins in the cells. Recombinant proteins were purified homogeneously from cell lysates by Ni^{2+}-nitrilotriacetic acid Sepharose affinity chromatography under denaturing and native conditions, respectively (Fig. 2B). The yields of fusion proteins purified were approximately 20 mg/l culture. The recombinant Tat–SOD and SOD proteins have an estimated molecular mass of approximately 18 and 17 kDa, respectively. However, it was detected that recombinant fusion proteins migrate to bands with a higher molecular weight than those of the expected sizes on the SDS–PAGE (as shown in Fig. 2B), which is consistent with the previous reports [25,26]. The purified products were further confirmed by Western blot analysis using a monoclonal antibody to a human Cu,Zn-SOD (Fig. 2C). Tat–SOD and SOD were detected at the corresponding bands in Fig. 2B.

To evaluate the transduction ability of Tat–SOD, 1 μM of Tat–SOD proteins purified under denaturing and native conditions were added to the culture media of HeLa cells for 1 h, and they were analyzed by the Western blot technique. As shown in Fig. 2D, denatured Tat–SOD was successfully delivered into the cells, whereas native Tat–SOD and the control SOD were not delivered into the cells. These results indicate that protein unfolding is required for efficient transduction of Tat–SOD into HeLa cells. Although the exact mechanisms for protein transduction are still entirely unclear, Derossi et al. demonstrated that the Antennapedia homeodomain (Antp), another protein transduction domain from *Drosophila*, was internalized by cells in a receptor- and transporter-independent mechanism [15].

As shown in Fig. 3, we then analyzed the transduction of denatured Tat–SOD fusion protein into the cells. Denatured Tat–SOD proteins were added to the culture media of HeLa

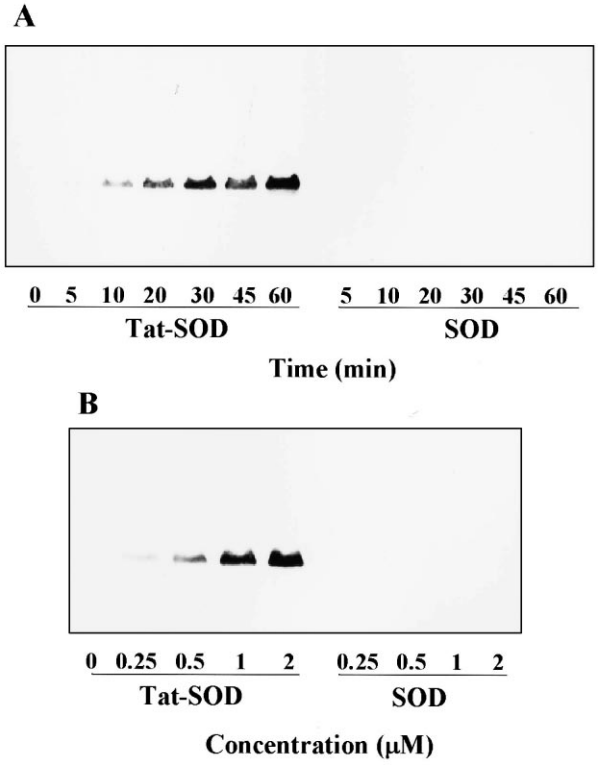

Fig. 3. Time- and dose-dependent transduction of Tat–SOD into cultured HeLa cells. 1 μM of denatured Tat–SOD and control SOD were added to the culture media for 5 min–1 h (A), 0.25–2 μM of denatured Tat–SOD and control SOD were added to the culture media for 1 h (B). Transduced Tat–SOD into the cells was analyzed by Western blotting.

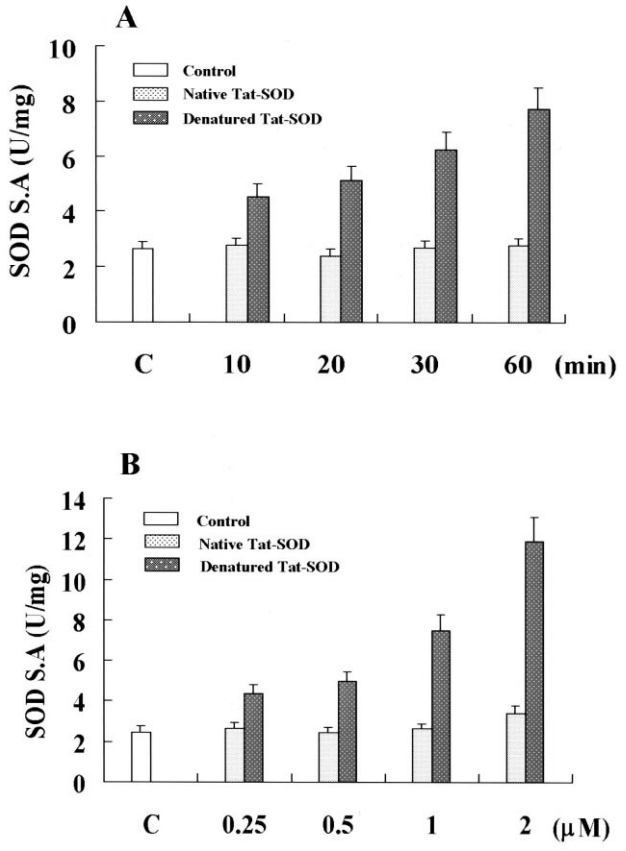

Fig. 4. The specific activities of SOD in cultured HeLa cells treated with Tat–SOD. 1 μM of denatured Tat–SOD was added to the culture media for 5 min–1 h (A), 0.25–2 μM of denatured Tat–SOD was added to the culture media for 1 h (B). Each bar represents the mean ± S.E.M. obtained from four experiments.

cells at 1 μM concentration for various times, and the level of transduced proteins was analyzed by Western blotting. The intracellular concentration of transduced Tat–SOD into cultured cells was detected after 5 min and gradually increased by 1 h (Fig. 3A). This time-dependent manner of transduction indicated that Tat–SOD was 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 [17]. This little difference in time course may derive from the properties of transduced Tat fusion protein, such as the unfolding degree, polarity, and the molecular shape of the protein.

The dose-dependency of the transduction of denatured Tat–SOD fusion proteins was further analyzed. Various concentrations of denatured Tat–SOD proteins were added to the culture media of HeLa cells for 1 h, and the levels of transduced proteins were measured by Western blotting. As shown in Fig. 3B, the levels of transduced proteins in the cultured HeLa cells were increased concomitantly with the amounts of fusion proteins treated in media.

The restoration of authentic properties of transduced protein in the cells is a key point in application of the protein-transduction technology to the therapeutic use. Therefore, we determined the dismutation activities of SOD in the cells treated with denatured Tat–SOD. As shown in Fig. 4, the enzyme activities of Cu,Zn-SOD in the cultured HeLa cells were increased in a time- and dose-dependent manner. The

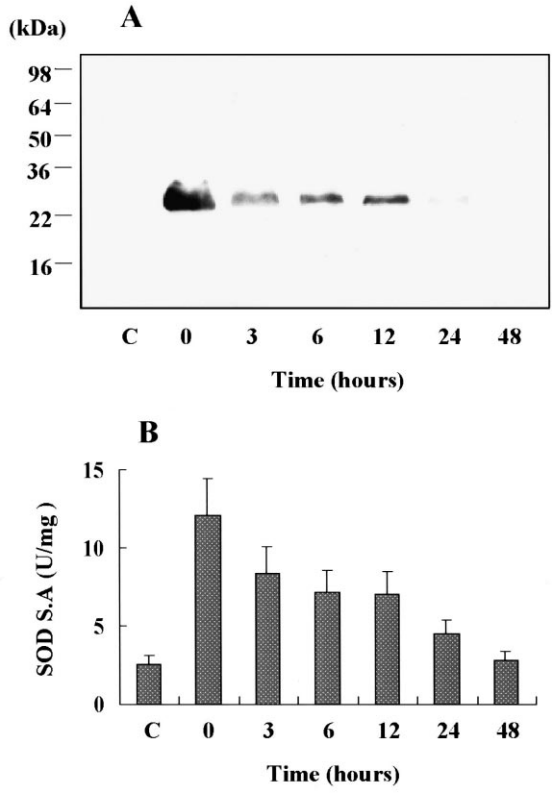

Fig. 5. The stability of transduced Tat–SOD into cultured HeLa cells. Cells pretreated with 2 μM Tat–SOD were incubated for 3–48 h. Transduced Tat–SOD was identified by Western blot analysis (A) and by an enzyme assay (B), respectively. Each bar in B represents the mean ± S.E.M. obtained from four experiments.

enzyme-specific activities were increased about 2–5-fold by treatment with various concentrations of denatured Tat–SOD, but they were not in cells treated with native Tat–SOD. These results implicate that denatured Tat–SOD in HeLa cells may be correctly refolded by the mechanism of

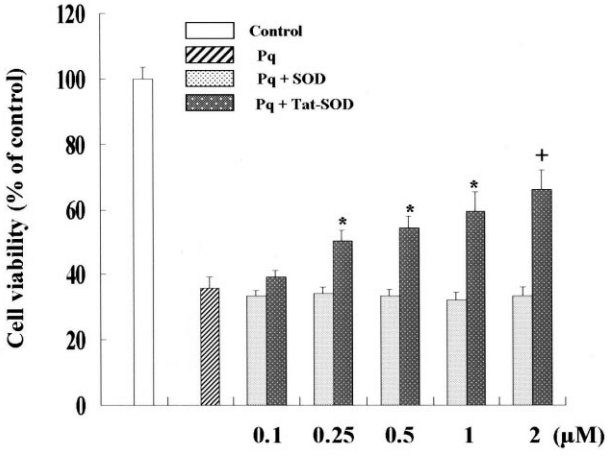

Fig. 6. Effect of transduced Tat–SOD on cell viability of HeLa cells treated with paraquat (Pq, 5 mM) was added to the HeLa cells pretreated with 0.1–2 μM Tat–SOD, and control SOD for 1 h, respectively. Cell-viability was estimated by with a colorimetric assay using MTT. Each bar represents the mean ± S.E.M. obtained from five experiments. Asterisks and crosses denote statistical significance at $P < 0.05$ and $P < 0.01$, respectively. The statistical analysis was evaluated by Student's t-test.

molecular chaperone or by a spontaneous process. However, intracellular refolding mechanisms for transduced proteins wait to be elucidated in the future.

The intracellular stability and enzymatic activity of transduced Tat–SOD into HeLa cells was shown in Fig. 5. The apparent degradation of transduced Tat–SOD was observed as a function of incubation time. However, significant levels of transduced protein and enzyme activity persisted in HeLa cells until 24 h. To determine whether transduced Tat–SOD can play its biological role in the cells, we have tested the effect of transduced Tat–SOD on cell-viability under oxidative stress. After the cells were exposed to 5 mM paraquat without Tat–SOD, only 36% of the HeLa cells were viable; the viability was significantly increased when Tat–SOD was pretreated in a dose-dependent manner (Fig. 6). The cell-viability of HeLa cells pretreated with 0.25∼2 μM Tat–SOD was increased by approximately 40–80% as compared with that of the control. These results indicate that transduction of Tat–SOD was definitely effective against superoxide anion induced by paraquat in HeLa cells.

Taken together, the present experimental 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 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.

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