In Vivo Protein Transduction: Penetration of the Biologically Active HIV-1 Tat Mediated Cu,Zn-Superoxide Dismutase Rescues Murine Skin from Oxidative Stress

Tsang-Pai Liu[3,4], Yi-Ping Chen[1], Chih-Ming Chou[1,2], Yuan-Ching Chang[3,4], Ting-Ting Chiu[2] and Chien-Tsu Chen[1,2*]

[1]Department of Biochemistry, School of Medicine, Taipei Medical University, Taipei, Taiwan
[2]Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan
[3]Department of Surgery, Mackay Memorial Hospital, Taipei, Taiwan
[4]Mackay Medicine, Nursing and Management College, Taipei, Taiwan

ABSTRACT

Reactive oxygen species (ROS), derived from the metabolism of oxygen and exist inherently in all aerobic organisms, lead to oxidative damage in a cell, tissue, or organ. This damage can affect a specific molecule or the entire organism. The level of oxidative stress is determined by the balance between the rate at which oxidative damage is induced and the rate at which it is efficiently repaired and removed. Antioxidant enzymes, such as superoxide dismutase (SOD) have been encouraging to have a beneficial effect against various diseases mediated by the ROS. We have reported that the denatured Tat-SOD1 was successfully transduced into PC12 cells in vitro and retained its activity which rescued the cells from paraquat-induced oxidative stress. We assess the ability of in vivo delivery of Tat-SOD1 fusion protein into the mammalian. The sodium nitroferricyanide (SNP)-induced expressions of COX-2 and nitroxide were reduced by the presence of Tat-SOD1 protein. Our results demonstrated that Tat-SOD1 proteins efficiently penetrated into the epidermis and the dermis of murine skin, moreover, successfully inactivated the induced oxidative damage. In vivo transduction of functioning protein may open the door to protein therapy for human diseases related to this antioxidant enzyme and to ROS.

Keywords: oxidative stress, Tat-SOD1, murine, in vivo, COX-2, NO

INTRODUCTION

. Amyotrophic lateral sclerosis (ALS) is a neurological disorder characterized by progressive and irreversible degeneration of motor neuron cells in the spinal cord and brain, which results in paralysis and death. There is still no cure for this disastrous disease. Mutations in the SOD gene lead to impaired cellular defense against oxidative stress, which takes blame for one fourth of the familial ALS[1, 2]. These findings suggest free radicals may have a key role in the pathogenesis of ALS. There are studies demonstrated that the basic domain of human immunodeficiency virus[i] type 1 (HIV-1) transactivator of transcription (Tat) protein can directly and efficiently penetrate cell membrane in a process called protein transduction[3, 4]. Moreover, Tat-proteins have encouraging results to serve as efficient carriers to direct uptake of different proteins, including ovalbumin, L-galactosidase, and horseradish peroxidase, Cu, Zn-superoxidase dismutase into the cells in vitro and in vivo[5-7]. Recently, we reported wild-type and mutant Tat-SOD1 fusion protein effectively transduced into rat pheochromocytoma PC12 cells, and the protein functioned well in protecting these cells from paraquat-induced cell death. In the present study, we investigated the in vivo delivery of Tat-SOD into mammalian skin. Our results revealed not only an efficiently in vivo transduction of Tat-SOD protein but also an effective in vivo biological activity of this protein. We suggest in vivo delivery of Tat-SOD proteins into different tissue or organ opens the possibilities of "protein therapy" for patients suffering from disorders related to ROS

MATERIALS AND METHODS

Cell Culture and Animal Treatment

The rat pheochromocytoma cell line PC12 was obtained from the American Type Culture Collection (Manassas, VA). 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. Sodium nitroferricyanide (SNP), and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celecoxib (CELEBREX^{TM}) was from PHARMACIA [(Packer) Phamacia Ltd., Northumberland, England].

PC 12 cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Gibico/BRL, Grand Island, NY) supplemented with 5% 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. Other culture reagents were purchased from HyClone.

Female ICR mice (6–7 weeks of age) were supplied from the Laboratory Animal Center (College of Medicine,

National Taiwan University, Taipei, Taiwan).The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light/12 h dark cycle. The dorsal side of the skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used. Mice were topically treated on their shaven backs with vehicle (acetone, 200 μl/site), TPA (10 nmol/200 μl/site), or sodium nitroferricyanide (20 μmol/200 μl/site) for 4 h. For the Tat-SOD1 protein treatments, mice were treated with Tat-SOD1 protein at the indicated doses for 30 min, then further treated with TPA for 4 h.

Superoxide dismutase (SOD) activity was measured according to the method of Marklund[8]. The optical density of each sample was measured at 420 nm before the addition of pyrogallol. The absorbance was measured at 10-s intervals and lasted for 3 min. SOD specific activity was expressed as units per milligram (U/mg) of protein.

Immunohistochemical Staining for Tat-SOD, COX-2 and Nitrotyrosine

PC12 cells were fixed with 4% paraformaldehyde and permeralized with 0.1% Triton X-100. After washed in PBS, cells were blocked with 3% skim milk for 1 hour and then incubated with a polyclonal rabbit anti-human Cu,Zn-SOD1 antibody (hSOD1) for 16 hours at 4°C. The cells visualized with anti-rabbit Cy3-labeled secondary antibody at a 1/1000 dilution for 2 hours, and counterstained for nuclei with DAPI (DNA marker) for 5 minutes. The cells were analyzed with an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT system (Diagnostic Instruments, Sterling Heights, MI) and assembled by PhotoShop program (Adobe System, CA).

The immunohistochemical staining was performed as described by Chun et al. [9] with minor modifications. The mice were sarcrified by cervical dislocation. Sections (4 μm) were formalin-fixed, paraffin-embedded. For the antigen retrieval, the deparaffinized sections were heated and boiled in 10 mM citrate buffer (pH 6.0) and then rinsed with PBS containing 0.05% Tween-20 for 5 min. Each section was treated with 3% hydrogen peroxide in methanol for 15 min; then, washed with PBS mixed with 1% bovine serum albumin for 30 min and PBST twice for 5 min. The slides were incubated with 2% normal goat serum in PBA for 30 min and then incubated with polyclonal COX-2 antibody (Cayman Chemical, Ann Arbor, MI) or polyclonal anti-nitrotyrosine antibody (Upstate, Lake Placid, NY) at room temperature for 1 to 2 h. The slides were developed using the HPR EnVisionTM system (Dako, Glostrup, Denmark) to stain with 3,3'-diaminobenzidine tetrahydrochloride. Mayer's hematoxyline was finally used as counterstaining.

RESULTS AND DISCUSSION

ALS is a very good example that imbalance between oxidants and antioxidants resulting from mutant Cu,Zn-SOD protein impairs cell's self-defense systems against oxygen-derived free radicals. Gene therapy has been considered a promising method for resolving problems coming from imbalance functioning protein production[10]. We have yet developed adequate efficiency targeting and stable regulation of long term protein functioning. Alternative approach of protein therapy may address this issue.

Previously, we have developed a simple and efficient system to express and purify the cell-permeable SOD1 protein. We constructed the Tat-SOD expression vector (pQE-Tat-SOD), which contains SOD1 cDNA sequences encoding the human Cu,Zn-SOD1 and Tat protein transduction domain (Tat 49-57) at the amino terminus[11]. This constructed gene was transformed into E. coli and a recombinant pQE-SOD1 protein with a specific activity of 1335 SOD1 unit/mg could be produced. To further evaluate the transduction ability of Tat-SOD1, 1.5 uM of Tat-SOD1 protein purified under denaturing conditions was added to the culture media of PC12 cells for 1, 2, and 3 hours. Analysis of protein penetration ability assessed by immunofluorescence staining in the PC12 cell revealed that Tat-SOD1 was successfully transduced into cells in a time-dependent manner (Figure 1).

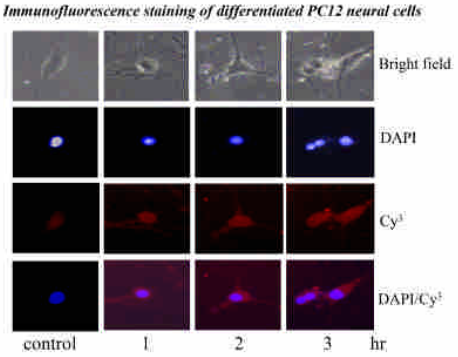

Figure 1. Transduction of the deantured Tat-SOD1 protein into the differentiated PC12 cells. Differentiated PC12 cells in a six-well plate were treated with 1.5 μM of denatured Tat–SOD protein and incubated for various time intervals. The immunocytochemical staining by DAPI, nuclear staining, and Cy3, SOD1 staining was analyzed by microscopy.

The activity of denatured Tat-SOD1 protein in PC12 cells was further analyzed by western blotting and enzyme activity (data not shown). The protein has restoration of basal activity and even higher enzyme activity with co-treatment of metal ions. We assessed the cell viability under paraquat (70 mM) induced oxidative stress to test the biologically active effect of transduced Tat-SOD1 protein. DNA fragmentation demonstrated that transduced Tat-SOD1 protein restored authentic properties of antioxidants to protect PC12 cells from the attack of superoxide anion.

Not only restoration of biologically activity of this transduced protein, but in vivo transduction of "therapeutic protein" into mammalian is key point in application of this methodology to future targeted protein therapy.

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061836 Vol. 2, 2007

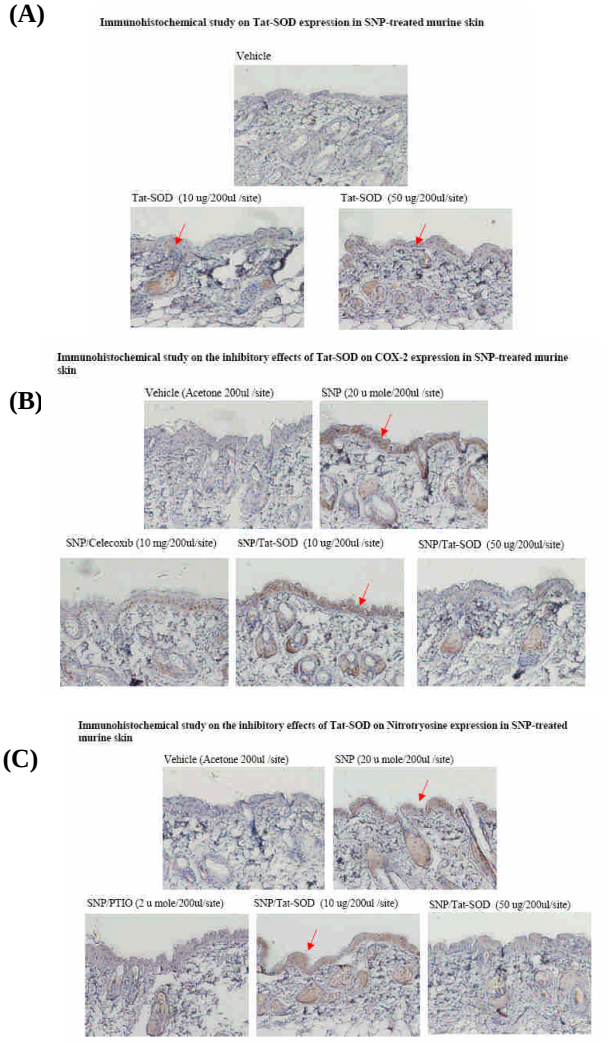

Figure 2. Transduction of the deantured Tat-SOD1 protein into the the murine skin. Mice were topically treated on their shaven backs with vehicle (acetone, 200 µl/) and Tat-SOD1 (10ug and 50ug) (A); treated with SNP (20 µmol) for 4 h and celecoxib, Tat-SOD1 (10ug and 50ug) (B); treated with SNP (20 µmol) for 4 h and PITO, Tat-SOD1 (10ug and 50ug) (C).

To determine whether Tat-SOD1 fusion protein could be successfully transduced and restoring the function in vivo, murine skin model was used. Tat-SOD1 was sprayed onto murine skin for 2 hours and the degree of transduction of the fusion protein was assessed by immunohistochemical staining. Penetration signals were significantly detectable in the epidermis and dermis treated with Tat-SOD1 fusion protein (Figure 2A). Results observed in Figure 2A showed that Tat-SOD1 fusion protein could penetrate the living animal skin as well as PC 12 cells. To evaluate whether penetrated Tat-SOD1 still has antioxidants activity in vivo, we examined the protective biological activity on murine skin after an oxidative stress insult generated by the treatment of SNP. Results from Immunohistochemical staining after two hours of treatment demonstrated that Tat-SOD1 efficiently reduces thethe oxidative stress caused inflammatory effects generated by the introduction of SNP. In comparison with PTIO (SNP antagonist) and celecoxib (Cox-2 inhibitor) groups, Tat-SOD1 shows a similar detoxication effect in a dose-dependent manner (Figure 2B and C). This result related with the NF-kB signals to mediate the inflammation response was found (data not shown).

Recommended oxygen radical scavenging therapy is continuing to grow, based primarily on inferential evidence suggesting a potential role for oxygen-derived free radicals in various types of pathophysiology. Some distinct advances in pharmacologic manipulation of protein scavengers have been made which could ultimately greatly enhance the use of these reagents as drugs, as well as some innovative techniques for drug delivery. Unfortunately, most of the therapeutic reports in the literature, almost all of which are based on usage of standard (native) SOD and/or catalase, are still anecdotal and/or uncontrolled[12]. Intracellular delivery of macromolecules remains problematic because of the bioavailability restriction imposed by the cell membrane. Recent studies on protein transduction domains have circumvented this barrier, however, and have resulted in the delivery of peptides, full-length proteins, iron beads, liposomes, and radioactive isotopes into cells in culture and animal models in vivo[13]. Therefore, in order to replenish the antioxidant activity in cells and tissues, we have focused the possibility of a protein transduction, an alternative approach, based on SOD enzyme to address this problem. Full length fusion proteins are generated that contained an NH2-terminal 11 amino acid protein transduction domain (PTD) from human immunodeficiency (HIV) Tat protein[4]. These denatured proteins could be transduced into cells in a rapid, concentration-dependent manner that seemed to be independent of receptors and transporters[14]. Over 50 proteins with different size were then successfully transduced into human and murine cell types in vitro[15-17].

To ensure this method could be useful for delivering peptides in vivo, we developed an expression system for over-expressing the Tat-SOD1 protein. We confirmed the Tat-SOD1 fusion protein was well expressed by Western blotting experiment. For the transduction into the cells, our results observed from the transduction of the denatured Tat-SOD1 protein suggested that it can successfully be delivered into cells and restored its bioactivity to protect PC12 cells from oxidative stress attacked by superoxide generator, paraquat. It is widely accepted that the Tat protein transduction domain of HIV can be efficiently transduced through the plasma membrane as well as PEP-1; in spite of unclear mechanism of penetration. Some reports questioned the Tat fusion protein transduction about the artificial redistribution caused from cell fixation[19, 20], Most data supported the involvement of endocytosis via a cellular internalization by the HIV basic domain peptides.

Our results consistently demonstrated that the exogenous human Cu,Zn-SOD1 fused with Tat peptide can directly transduced into the PC 12 cells and murine skin cells, and it delivered as an active Tat-SOD1 exhibiting the anti-inflammation against oxidative stress.

Our murine skin model demonstrated that a significant IHC signal in skin tissue confirms the efficient in vivo uptake of the Tat-SOD1. Oxidative stress was generated by SNP via the formation of toxic ROS in the skin tissue was confirmed by IHC. In vivo transduced Tat-SOD1 showed the protective effect against ROS as PTIO and Celecoxib. Moreover, it indicated a dose dependent fashion. Therefore, transduction of the fused antioxidant enzyme Tat-SOD1 offered more promising potential for in vivo protein therapy.

In conclusion, we demonstrated that human Cu, Zn-SOD1, an important antioxidant enzyme, fused with Tat peptide can be efficiently delivered in vivo with restoration of biological activity in mammalian tissue. This success in protein transduction lays the groundwork for future complementation experiments and for eventual transduction of therapeutic proteins into patients in the form of protein treatment of ROS-mediated diseases. Potential immune responses and toxicity associated with transduction of proteins in vivo are important issues that need to be elucidated.

REFERENCES

1. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX *et al*: Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993, 362(6415):59-62.
2. Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, Getzoff ED, Hu P, Herzfeldt B, Roos RP *et al*: Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* 1993, 261(5124):1047-1051.
3. Frankel AD, Pabo CO: Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 1988, 55(6):1189-1193.
4. Green M, Loewenstein PM: Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* 1988, 55(6):1179-1188.
5. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J: Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci U S A* 1994, 91(2):664-668.
6. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF: In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 1999, 285(5433):1569-1572.
7. Eum WS, Choung IS, Li MZ, Kang JH, Kim DW, Park J, Kwon HY, Choi SY: HIV-1 Tat-mediated protein transduction of Cu,Zn-superoxide dismutase into pancreatic beta cells in vitro and in vivo. *Free Radic Biol Med* 2004, 37(3):339-349.
8. Marklund S., Marklund G.. 1974. Involvement of the superoxide anion radical in the autoxidation of pyragallol and a convenient assay for superoxide dismutase. *Eur.J. Biochem.* **47:** 469–474.
9. Chun, K. S., Cha, H. H., Shin, J. W., NA, H. K., Park, K. K., Chung, W. Y., and Surh, Y. J. (2004) Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-kappaB. *Carcinogenesis*, **25**, 445-454.
10. Anderson WF: Human gene therapy. *Nature* 1998, 392(6679 Suppl):25-30.
11. Chou CM, Huang CJ, Shih CM, Chen YP, Liu TP, Chen CT: 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. *Ann N Y Acad Sci* 2005, 1042:303-313.
12. Greenwald RA: Superoxide dismutase and catalase as therapeutic agents for human diseases. A critical review. *Free Radic Biol Med* 1990, 8(2):201-209.
13. Wadia JS, Dowdy SF: Protein transduction technology. *Curr Opin Biotechnol* 2002, 13(1):52-56.
14. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A: Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* 1996, 271(30):18188-18193.
15. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF: Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat Med* 1998, 4(12):1449-1452.
16. Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC, Dowdy SF: Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc Natl Acad Sci U S A* 1997, 94(20):10699-10704..
17. Vocero-Akbani AM, Heyden NV, Lissy NA, Ratner L, Dowdy SF: Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein. *Nat Med* 1999, 5(1):29-33.
18. Lundberg M, Wikstrom S, Johansson M: Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* 2003, 8(1):143-150.
19. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B: Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 2003, 278(1):585-590.

*corresponding author: Professor Chien-Tsu Chen, chenctsu@tmu.edu.tw