Effect of superoxide dismutase-entrapped liposomes and protein transduction domain-superoxide dismutase on human umbilical vein endothelial cells

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Abstract. Superoxide dismutases (SOD) are able to remove the superoxide anion free radicals produced by environmental stress and thereby protect cells from being injured by reactive oxygen species. However, SOD is unable to transduce automatically across cell membranes. Protein transduction domains (PTDs) are peptides able to mediate protein delivery into cells and were first observed in the HIV-1 Tat protein. In the present study, PTD (RKKRRQRRR) was fused to *Dunaliella salina* (Ds)MnSOD to form PTD-DsMnSOD. This was inserted into pET32a to construct the recombinant plasmid pET32a-PTD-DsMnSOD and transduced into *E. coli* BL21(DE3) to obtain purified PTD-DsMnSOD proteins. Liposome-encapsulated proteins are also able to cross cell membranes. In this study, DsMnSOD proteins were purified and encapsulated by liposomes. The obtained MnSOD, PTD-MnSOD and liposome MnSOD were used to protect human umbilical vein endothelial cells (HUVECs) from injury under oxygen pressure. A cell counting kit 8 was used to test the survival rate of HUVECs and results indicated that the protective effect of MnSOD was limited compared with that of PTD-MnSOD and liposome MnSOD. Thus, PTD and liposomes exhibited improved effects when MnSOD was present in cells.

Introduction

Superoxide dismutases (SODs) are metalloenzymes able to catalyze superoxide free radicals (O^{2-}) and induce dismutation. It is thought that superoxide free radicals may significantly damage a number of large biological molecules and other cell components. Thus, SOD is hypothesized to be the first barrier identified against free radicals *in vivo*. MnSOD is an inducible expression SOD and oxygen free radicals have a significant inductive effect on its expression. Therefore, MnSOD plays an important role in the adaption to environmental change, particularly in coping with environmental stress (1,2).

Dunaliella salina (Ds) is a type of high salt-tolerant single-cell green algae, characterized by its extremely high salt tolerance and ultraviolet (UV) resistance. SODs have been found to exhibit a marked effect on the mechanism of stress resistance in Ds and have advantageous effects, including high activity and strong stress resistance (3). However, exogenous SODs are unable to penetrate cell membranes, therefore the following two methods were used in order to deliver SOD into cells.

Green *et al* (4,5) first reported that the transactivator protein (TAT) of human immunodeficiency virus (HIV)-1 could be transduced into cells using the transmembrane method. Subsequently, Vives *et al* (6) observed that a sequence composed of residue 49-57 of HIV TAT was able to induce complete transduction of proteins. These sequences are known as protein transduction domains (PTDs). I*n vitro* and *in vivo* experiments have demonstrated the newly designed PTD (RKKRRQRRR), whose penetration is stronger and more efficient (7-9). Thus, the use of DNA recombination to fuse the PTD-coded sequence and the SOD genes, prior to transferring PTD-MnSOD into *E. coli* to abundantly express and obtain the PTD fusion proteins, has demonstrated that PTD-SOD can undergo transmembrane transduction (10-14) and function in cells.

Cell entry of liposomes functioning as drug vectors has been widely studied. SODs can be encapsulated in the lipid bilayer of the liposome, facilitating entry of SOD into the cells, reducing enzyme degradation and enabling slow release (15-18).

The liposome-encapsulated DsMnSOD and the purified PTD-DsMnSOD are able to transduce DsMnSOD into cells. In the present study, DsMnSOD, liposome DsMnSOD and PTD-DsMnSOD were transduced into human umbilical vein endothelial cells (HUVECs) to compare the reparative effects on cells subjected to UV radiation and treated with paraquat.

Materials and methods

Chemicals and materials. Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen (Hilden, Germany),

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cellulose nitrate desalting columns were purchased from Pierce Biotechnology Inc. (Rockford, IL, USA) and rabbit anti-histidine polyclonal antibody was obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

For construction of BL21(DE3)-pET30a-DsMnSOD and BL21(DE3)-pET32a-PTD-DsMnSOD, the DsMnSOD gene for constructing BL21(DE3)-pET30a-DsMnSOD had previously been obtained from our laboratory. The expression vector of BL21(DE3)-pETet32a-PTD-DsMnSOD was constructed by designing primers and adding PTD (RKKRRQRRR) to the front end of the sense primers. The primers were as follows: Sense, 5'-GAATTCATGAGGAAGA AGCGGAGACAGCGACGAAGAGGATCCATGGCGTTCG TGCTGCC-3' and anti-sense, 5'-CTCGAGTCACAGCGCTG GCATGCCGCCA-3'. Polymerase chain reaction was used to expand the plasmids of DsMnSOD, obtaining PTD-DsMnSOD with added PTD at the front end. This was ligated into a TA-cloning vector using T4 DNA ligase, and cloned into *E. coli* JM109. PTD-DsMnSOD was excised with *Eco*RI and *Xho*I and subcloned into the *Eco*RI and *Xho*I sites of pET32a to construct pET32a-PTD-DsMnSOD. Following this, the vector was subsequently transferred into BL21(DE3) to obtain BL21(DE3)-pET32a-PTD-DsMnSOD.

Expression and purification of DsMnSOD and the PTD-DsMnSOD fusion protein. BL21(DE3)-PET30a-DsMnSOD was expressed at the optimal inducing temperature of 25˚C and the optimal isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducing concentration of 0.01 mmol/l. DsMnSOD protein was purified by Ni^{2+}-nitrilotriacetic acid sepharose superflow and cellulose nitrate desalting columns.

Constructed BL21(DE3)-PET30a-DsMnSOD was grown at 25˚C in Luria-Bertani broth supplemented with kanamycin and IPTG at concentrations of 100 mg/ml and 0.15 mmol/l, respectively. BL21(DE3)-pet32a-PTD-DsMnSOD was expressed by mass cell culture. The PTD-DsMnSOD fusion protein was obtained by purification through Ni^{2+}-nitrilotriacetic acid sepharose superflow and cellulose nitrate desalting columns. The unit enzyme activity of the two proteins was measured.

DsMnSOD-entrapped liposome preparation. Reverse phase evaporation (19-21) was used. Next, 100 mg lecithin and 50 mg cholesterol were dissolved in 15 ml diethyl ether, DsMnSOD protein was dissolved in phosphate-buffered saline (PBS; pH 7.4) at a final concentration of 4 mmol/l.3 ml, and treated DsMnSOD solution was added to the diethyl ether solution. Next, ultrasonic apparatus was used to emulsify the solution (100 W; 30 sec operation; 30 sec interval in an ice-cold water bath) into a water-in-oil emulsion. A vacuum rotary evaporator was used to remove the organic phase of low boiling point at 25˚C and 53 kPa. The SOD liposome suspension was obtained and the evaporation was repeated at 97 kPa to eliminate the residual organic solvent, until a gel was formed. The phosphate buffer (pH 7.4) was added and the solution was rotated at room temperature until a white suspension was formed. This was centrifuged at 4˚C and 25,070 x g for 40 min. The supernatant was removed to separate the unencapsulated SOD from liposomes. SOD liposome was obtained, followed by deposition, freezing and drying. Next, an SOD assay kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) was used to test the SOD activity. The empty liposomes were prepared using the aforementioned method but without DsMnSOD.

Measurement of the characterization of DsMnSOD and PTD-DsMnSOD. pH resistance of DsMnSOD and PTD-DsMnSOD was measured as follows: 10 μl enzyme [30 U/ml (22)] and 40 μl buffer solutions of various pH values (2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) were added together to a thin-walled tube, mixed well and cultured in a water bath at 37˚C for 1 h. These tubes were subsequently placed on ice and their enzyme activity was tested at room temperature.

The temperature resistance of DsMnSOD and PTD-DsMnSOD was measured as follows: 10 μl enzyme (30 U/ml) and 40 μl PBS (pH 7.4) were added together to a thin-walled tube and mixed well. These tubes were cultured in a water bath at 35, 45, 55, 65, 75 and 85˚C, taken out and placed on ice after 15, 30, 60, 90 and 120 min. Their enzyme activity was measured at room temperature.

The chemical reagent resistance of DsMnSOD and PTD-DsMnSOD was measured as follows: 10 μl enzyme (30 U/ml) and 40 μl PBS (pH 7.4) were added together to a thin-walled tube and mixed well, prior to adding EDTA, SDS and imidazole to final concentrations of 1 and 5 mmol/l, respectively. These tubes were cultured in a water bath at 37˚C for 1 h, placed on ice and their enzyme activities measured at room temperature.

Cell culture. The cell line used was human umbilical vein endothelial cells (HUVECs), which were preserved by the State Key Laboratory of Biotherapy (West China Hospital, Sichuan University, Chengdu, China) and cultured at 37˚C with 5% CO_2. Next, 10% newborn bovine serum was added to RPMI 1640 medium supplemented with penicillin, streptomycin and amphotericin B.

Treatment of HUVECs with the preparations. HUVECs were cultured to 80% confluency in a culture flask, the reaction was terminatd with trypsinisation, pancreatin was extracted, and then new medium was added and the cells were placed into six-well plates with 2.5 ml HUVECs per well. The inoculum concentration was $5x10^5$ cells per well. HUVECs were cultured for 24 h and the cells grew to 80% confluency. The following treatment groups were established: PBS control, DsMnSOD, empty liposome control, liposome-DsMnSOD and PTD-DsMnSOD, whereby 200 μl sample was added to each well (where required) and the activity of each enzyme was controlled to 30 U/ml (22). Following 1 h of treatment (23,24), the medium was removed and cells were washed twice with 1 ml PBS. The cells were digested by trypsin to terminate the reaction and the collected cells were centrifuged at 300 x g for 3 min and stored at -20˚C overnight. Following digestion, the rabbit anti-histidine polyclonal antibody was used for western blotting analysis. The two controls produced no bands, MnSOD had almost no bands and only liposome-DsMnSOD and PTD-DsMnSOD exhibited clear bands (results not shown). MnSOD, liposome-DsMnSOD and PTD-DsMnSOD all had His-tags. When the His-tag entered cells, it produced bands, which indicated that the enzyme had entered the cells.

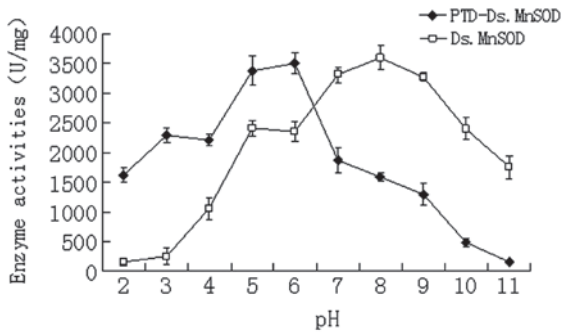

Figure 1. pH tolerance for DsMnSOD and PTD-DsMnSOD. Ds, *Dunaliella salina*; SOD, superoxide dismutase; PTD, protein transduction domain.

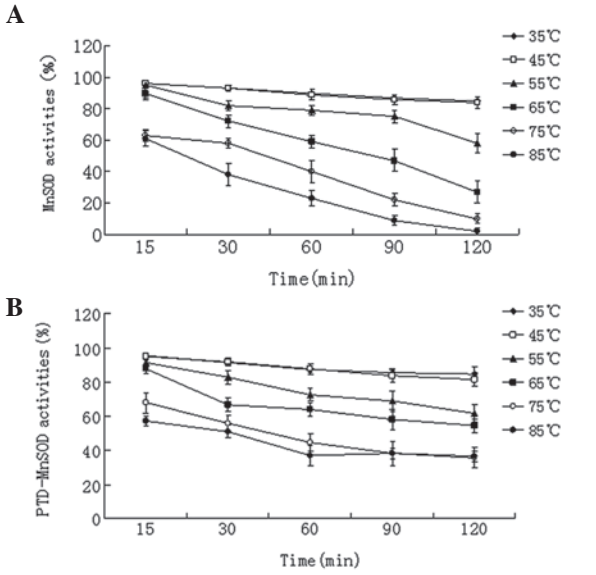

Figure 2. Thermostability of DsMnSOD and PTD-DsMnSOD. PTD-DsMnSOD exhibited greater temperature resistance compared with DsMnSOD. Ds, *Dunaliella salina*; SOD, superoxide dismutase; PTD, protein transduction domain.

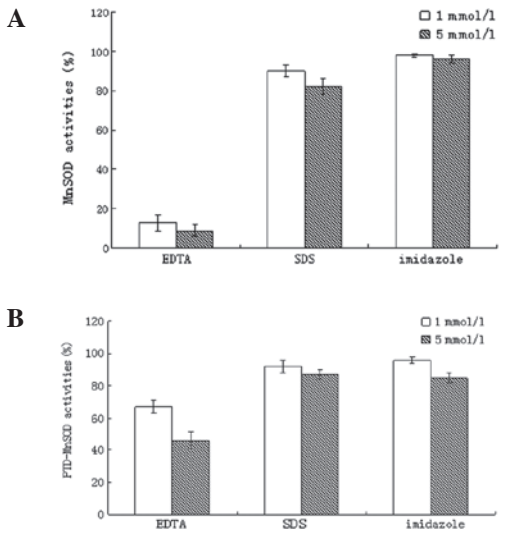

Figure 3. Chemical resistance of DsMnSOD and PTD-DsMnSOD. Ds, *Dunaliella salina*; SOD, superoxide dismutase; PTD, protein transduction domain.

Effect of enzymes on cell viability of paraquat-treated HUVECs. HUVECs were cultured to 80% confluency in a culture flask, the reaction was terminatd with trypsinisation, pancreatin was extracted, and then new medium was added and the cells were placed into 96-well plates with 100 μl HUVECs per well. The inoculum concentration was $1x10^4$ cells per well. HUVECs were cultured for 24 h and the cells grew to 80% confluency. The following treatment groups were established: PBS control, DsMnSOD, empty liposome control, liposome-DsMnSOD and PTD-DsMnSOD. The activity of each enzyme was controlled to 30 U/ml (22). Each treatment group was further divided into three groups and 1, 5 and 10 μl enzyme was added. The various solutions were diluted by PBS to make final concentrations of 0.3, 1.5 and 3 U/ml for the 1, 5 and 10 dμl groups, respectively. Cells were cultured for 1 h (24), and 0Mm paraquat, at a concentration of 5 or 10 mmol/l, was added to each group and cultured for a further 3 h. A cell counting kit-8 (CCK-8; Dojindo, Rockville, MA, USA) was used to measure cell survival.

Effect of enzymes on cell viability of UVB-treated HUVECs. HUVECs were cultured under the conditions and treatment groups described for paraquat treatment. Following enzyme treatment, cells were cultured for 1 h (24), exposed to UVB (wavelength, 253.7 nm; 30 W; 220 V) for 40 min (25,26) and the CCK-8 was used to measure the cell survival following culture for 3 h.

Protein activity assay. Protein concentration was determined using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin as a reference standard. The SOD activity was measured using the SOD assay kit (Nanjing Jiancheng Bioengineering Institute) based on the methods described by Beauchamp and Fridovich (27). The reaction system contained xanthine, and xanthine oxidase which produces superoxide anion free radicals (O^{2-}). Superoxide anion free radicals are able to oxidize hydroxylamine to form nitrite, which reveals an amaranth coloring following addition of a color-developing agent. This color change was assayed by spectrophotometry. When the assayed sample contains SOD, the formation of superoxide anion free radicals is inhibited and the quantity of produced nitrite is reduced. Therefore, as the absorbance of the test tube would be lower than that of the control tube, the activity of SOD in the sample was calculated with the formula: SOD activity (U/mgprot) = [(ODcontrol - O Dassay)/ODcontrol]/50%*[total volume of the reaction solution (ml)/the sampling volume of the samples (ml)]*protein concentration of the homogenate.

Cell viability assay. The CCK-8 (28,29) was purchased from Dojindo and used to measure the cell survival.

Statistical analysis. The statistical difference between means was determined using the Student's t-test and expressed graphically with the standard error of the mean.

Results

Characterization of pH tolerance of DsMnSOD and PTD-DsMnSOD. The enzyme activity of pure DsMnSOD and PTD-DsMnSOD was 5,260.0 and 4,436.7 U/mg, respectively.

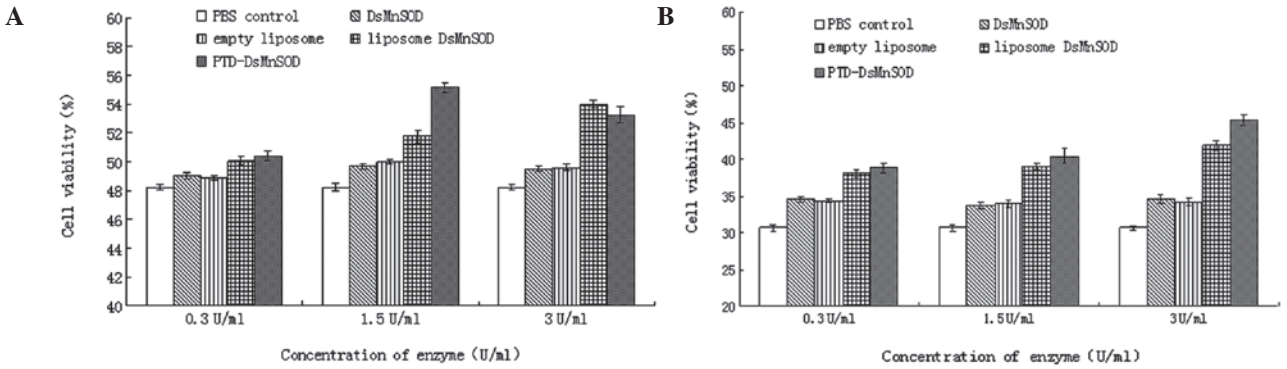

Figure 4. (A) Resistance of DsMnSOD in 5 mmol/l paraquat-treated HUVECs, (B) Resistance of DsMnSOD in 10 mmol/l paraquat-treated HUVECs. The cell survival without adding paraquat or any enzymes was defined as 100% and the survival rate was calculated by comparing others with this. PBS, phosphate-buffered saline; Ds, *Dunaliella salina*; PTD, protein transduction domain; SOD, superoxide dismutase; HUVECs, human umbilical vein endothelial cells.

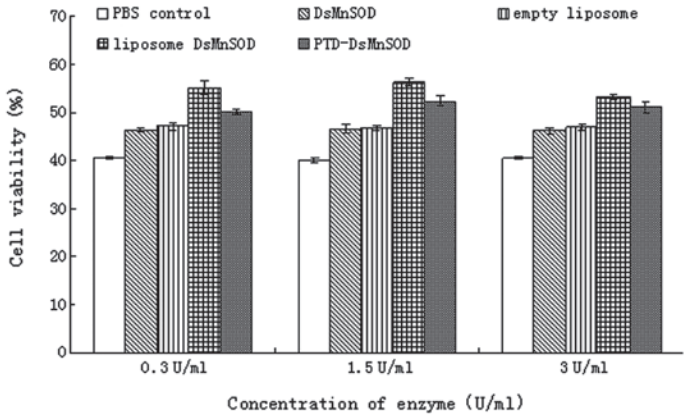

Figure 5. Resistance of DsMnSOD to UVB when HUVECs under various conditions, given as the survival rate of HUVEC cells treated by UVB for 40 min with SOD. UV, ultraviolet; PBS, phosphate-buffered saline; Ds, *Dunaliella salina*; SOD, superoxide dismutase; PTD, protein transduction domain; HUVECs, human umbilical vein endothelial cells.

The pH-resistance test for DsMnSOD and PTD-DsMnSOD (Fig. 1) demonstrated that the optimal pH was 8 and 6, respectively, indicating that PTD-DsMnSOD was able to maintain enzyme activity more effectively under acidic conditions.

Thermostability of DsMnSOD and PTD-DsMnSOD. Analysis of the temperature resistance of DsMnSOD and PTD-DsMnSOD revealed that PTD-DsMnSOD was more temperature resistant than DsMnSOD (Fig. 2). Following treatment at 85˚C for 2 h, DsMnSOD exhibited almost no activity, however, PTD-DsMnSOD activity remained at ~40%.

Chemical resistance of DsMnSOD and PTD-DsMnSOD. The PTD added to DsMnSOD improved the resistance of DsMnSOD to EDTA (Fig. 3), high concentrations of imidazole decreased the activity of DsMnSOD and markedly inhibited PTD-DsMnSOD. SDS had a decreased effect on DsMnSOD and PTD-DsMnSOD activity. PTD-DsMnSOD exhibited higher resistance to EDTA compared with DsMnSOD, whilst resistance to SDS and imidazole was similar. These results indicate that PTD-DsMnSOD is more stable than DsMnSOD.

Resistance of DsMnSOD in paraquat-treated HUVECs (5 and 10 mmol/l). Cell survival in the absence of paraquat or any enzymes was defined as 100% and the survival rate was calculated by comparing others with this. At 5 mmol/l paraquat (Fig. 4A), the cell survival of the PBS control was the lowest, remaining at ~48.2%, which indicated that cells could spontaneously repair the damage caused by paraquat to a certain extent, accourding to our results. In the MnSOD and the empty liposome groups, the cell survival rate increased marginally. This was due to a small amount of MnSOD being able to enter the cells and repair the damage, and also liposomes were able to repair cells to a certain extent. The cell survival was highest in the liposome MnSOD and PTD-MnSOD groups. At a concentration of 0.3 U/ml, the changes in cell survival were not marked but increased quickly when the concentration increased to 1.5 U/ml. The effect of PTD-MnSOD was higher than that of liposome MnSOD. However, when the concentration increased to 3 U/ml, cell survival increased more in the liposome MnSOD group. By contrast, the cell survival decreased in the PTD-MnSOD groups. We hypothesize that liposome MnSOD may have been encapsulated by liposomes, meaning that it functions more slowly but for longer. When the amount of PTD-MnSOD increased to extremely high levels (1.5 U/ml), it exhibited a toxic effect on cells and cell survival decreased.

At a concentration of 10 mmol/l paraquat, the damage to cells caused by paraquat increased and cell survival was lower than with 5 mmol/l paraquat. In the PBS control, the cell survival was ~31%. Under these conditions, MnSOD and the empty liposome

were able to repair cells weakly but repair in liposome MnSOD and PTD-MnSOD was more effective (Fig. 4B). With increasing volumes of paraquat, an increased level of reactive oxygen species (ROS) was produced by cells. Thus, PTD-MnSOD was able to repair cells more rapidly at higher concentrations. When the concentration reached 3 U/ml, the reparative efficiency of PTD-MnSOD was greater than at 1.5 U/ml and greater than that of the liposome MnSOD. Compared with Fig. 4A, in which the cell survival decreased when PTD-MnSOD was 3 U/ml, this indicated that there were a number of toxic effects with excessive levels of SOD entering cells.

Resistance of DsMnSOD in UV-mediated HUVECs. The survival rate of HUVECs treated by UVB (wavelength 253.7 nm; 30 W; 220 V) for 40 min was determined. The cell survival in the absence of UVB irradiation and any enzymes was defined as 100%, with the survival rate calculated by comparing others with this value. Fig. 5 demonstrated that the cell survival in the PBS control was the lowest at ~36%. This increased in the MnSOD and empty liposome groups but the cell survival of these groups was lower than the liposome MnSOD and PTD-MnSOD groups. Compared with the paraquat experiment, the survival of liposome MnSOD was higher than PTD-MnSOD in repairing cells in the UVB experiment. The cell survival was ~55% in the liposome MnSOD group (enzyme activity, 0.3 U/ml) but the cell survival was only ~50% in the PTD-MnSOD group. As the enzyme concentration increased, the cell survival increased in the liposome MnSOD and PTD-MnSOD groups (enzyme activity, ≤1.5 U/ml). The cell survival was lower in the PTD-MnSOD group compared with the liposome MnSOD group. However, when the enzyme concentration was increased and enzyme activity was ≤3 U/ml, the cell survival decreased in the PTD-MnSOD and liposome MnSOD groups.

Compared with results following paraquat treatment, cell survival decreased at higher enzyme concentrations (3 U/ml). These observations indicate cell survival decreased at SOD levels exceeding a certain level.

Discussion

The liposome-encapsulated technique is a highly effective medical preparation method able to transport proteins into cells. However, the stability of liposome encapsulation is not adequate and is therefore not suitable for the preservation of medicines, as part of the proteins may be lost during encapsulation, meaning that it is important to identify a new and effective method.

In this study, the efficacy of the liposome-encapsulated technique for protein transport was compared with the generation of PTD fusion proteins. PTD and SOD were fused together to express PTD-SOD. Next, the ability of the fusion proteins to protect cells from damage caused by UV irradiation and superoxide anions was studied in HUVECs. Western blot analysis was used to measure the ability of the enzymes to enter cells and determine whether they were able to transport proteins into cells. A CCK-8 kit was used to study the tolerance of the cells treated by proteins. The results from the two methods were compared to determine which was more effective.

By comparing the pure protein characteristics of DsMnSOD with those of PTD-DsMnSOD, the unit enzyme activity of DsMnSOD was shown to be higher than that of PTD-DsMnSOD. The promotory enzyme activity of DsMnSOD was 5,260.0 U/mg, whilst that of PTD-DsMnSOD was 4,436.7 U/mg. This difference was due to the high protein molecular mass of PTD-DsMnSOD.

In the experiment measuring pH tolerance, results indicated that PTD-DsMnSOD was more effective than DsMnSOD at resisting acidic conditions. Cells produced more ROS and the cell environment was slightly acidic under environmental stress (30). Therefore, the enzyme activity of PTD-DsMnSOD was greater than that of DsMnSOD and was therefore able to confer greater protection to cells under this environment.

The temperature resistance experiments for DsMnSOD and PTD-DsMnSOD indicated that PTD-DsMnSOD was more effective than DsMnSOD at resisting high temperatures. Following treatment at 85˚C for 2 h, DsMnSOD was almost inactive but PTD-DsMnSOD activity remained at ~40%. This was the advantage of using PTD-DsMnSOD. In the chemical reagent tolerance experiments for the two proteins, PTD-DsMnSOD was more effective than DsMnSOD, and proteins of PTD-DsMnSOD were more stable than those of DsMnSOD.

Although the rate of liposome-encapsulated DsMnSOD production was ~60% in the present study (results not shown), there was still a 40% loss. However, this problem was not observed with PTD-DsMnSOD. It was not necessary to perform the encapsulation experiment when using PTD, which results in reduced time and costs. With liposome-encapsulated DsMnSOD, the existence of liposomes reduces the immunogenicity of MsMnSOD in animals (31-35). However, immunogenicity is not lost with PTD-DsMnSOD.

In the paraquat experiment, the effect of PTD-DsMnSOD on cells was stronger than that of liposome DsMnSOD and the cell survival was higher in PTD-DsMnSOD. In the UVB experiment, cell survival was higher with liposome DsMnSOD than PTD-DsMnSOD and the cell survival of empty liposomes was marginally higher than those treated with PBS. We hypothesize that this was due to the protective effect of liposomes on cells when UVB was applied, as indicated by previous studies (36-38).

It has previously been reported (13) that the ability of SOD to enter cells, and the enzyme activity effect of SOD in cells, increases when PTD is added to the front and rear ends of SOD, instead of only one end. When PTD was added to one end of SOD in the present study, the ability of PTD-SOD to resist ROS in cells was already greater than that of liposome SOD. As a result, resistance increases if PTD is added to both ends. It is clear that adding PTD to SOD is advantageous for resisting ROS, compared with liposome SOD.

In the experiments with paraquat and UVB, SOD enzymes were used at three concentrations (0.3, 1.5 and 3 U/ml). It was concluded that 1.5 U/ml had an increased effect on cells compared with 0.3 U/ml but 3 U/ml SOD exhibited lower levels of cell survival compared with 1.5 U/ml SOD. We hypothesize that there may be a toxic effect on cells at higher concentrations of SOD.

Previous studies on SOD have largely focused on implementing PTD-SOD and liposome SOD experiments separately,

and studies comparing the two techniques have not yet been presented. The present study is the first to compare the two methods and the results showing that PTD-SOD is advantageous for protecting cell compared with liposome SOD may be useful for future studies.

Acknowledgements

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References

1. Haghjou MM, Shariati M and Smirnoff N: The effect of acute high light and low temperature stresses on the ascorbate-glutathione cycle and superoxide dismutase activity in two *Dunaliella salina* strains. Physiol Plantarum 135: 272-280, 2009.
2. Tian J and Yu J: Changes in ultrastructure and responses of anti-oxidant systems of algae (*Dunaliella salina*) during acclimation to enhanced ultraviolet-B radiation. J Photochem Photobiol B 97: 152-160, 2009.
3. Zhang S, Li XR, Xu H, Cao Y, Ma SH, Cao Y and Qiao D: Molecular cloning and functional characterization of *MnSOD* from *Dunaliella salina*. J Basic Microbiol: May 26, 2013 (Epub ahead of print).
4. Green M, Ishino M and Loewenstein PM: Mutational analysis of HIV-1 Tat minimal domain peptides: identification of trans-dominant mutants that suppress HIV-LTR-driven gene expression. Cell 58: 215-223, 1989.
5. Green M and Loewenstein PM: Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell 55: 1179-1188, 1988.
6. Vivès E, Granier C, Prevot P and Lebleu B: Structure activity relationship study of the plasma membrane translocating potential of a short peptide from HIV-1 Tat protein. Lett Pept Sci 4: 429-436, 1997.
7. Beerens AM, Al Hadithy AF, Rots MG and Haisma HJ: Protein transduction domains and their utility in gene therapy. Curr Gene Ther 3: 486-494, 2003.
8. Haenssle HA, Riedl P, Buhl T, Schardt A, Rosenberger A, Schön MP and Schirmbeck R: Intracellular delivery of major histocompatibility complex class I-binding epitopes: dendritic cells loaded and matured with cationic peptide/poly(I:C) complexes efficiently activate T cells. Exp Dermatol 19: 19-28, 2010.
9. Chugh A, Amundsen E and Eudes F: Translocation of cell-penetrating peptides and delivery of their cargoes in triticale microspores. Plant Cell Rep 28: 801-810, 2009.
10. Ding W, Liu S and Rao P: Use of protein transduction domain-superoxide dismutase fusion protein for preparing anti-drunk and anti-alcohol product. China Patent no. C2010H43227. Filed Dec 14, 2009; issued Sep 6, 2010.
11. Liu S and Rao P: Use of fusion protein glutathione-S-transferase-p entanedial-superoxide-dismutase for radio-resistance, comprises 2,4,6-triaziridin-1-yl-1,3,5-triazine protein transduction domain which are connected with super-oxide dismutase. China Patent no. C2008-256140. Filed August 30, 2007; issued March 19, 2008.
12. Liu S, Rao P and Yang Y: Use of protein transduction domain-superoxide dismutase fusion protein in antifatigue product or medicine for protecting or repairing cells with damaged free radical, or improving antifatigue ability of organism. China Patent no. C2009Q75989. Filed November 5, 2008; issued October 14, 2009.
13. Eum WS, Jang SH, Kim DW, Choi HS, Choi SH, Kim SY, An JJ, Lee SH, Han K, Kang JH, Kang TC, Won MH, Cho YJ, Choi JH, Kim TY, Park J and Choi SY: Enhanced transduction of Cu,Zn-superoxide dismutase with HIV-1 Tat protein transduction domains at both termini. Mol Cells 19: 191-197, 2005.
14. Kim DW, Eum WS, Jang SH, Kim SY, Choi HS, Choi SH, An JJ, Lee SH, Lee KS, Han K, Kang TC, Won MH, Kang JH, Kwon OS, Cho SW, Kim TY, Park J and Choi SY: Transduced Tat-SOD fusion protein protects against ischemic brain injury. Mol Cells 19: 88-96, 2005.
15. Kigasawa K, Miyashita M, Kajimoto K, Kanamura K, Harashima H and Kogure K: Efficient intradermal delivery of superoxide dismutase using a combination of liposomes and iontophoresis for protection against UV-induced skin damage. Biol Pharm Bull 35: 781-785, 2012.
16. Umakoshi H, Morimoto K, Yasuda N, Shimanouchi T and Kuboi R: Development of liposome-based mimics of superoxide dismutase and peroxidase based on the "LIPOzyme" concept. J Biotechnol 147: 59-63, 2010.
17. Kanehisa M, Asayama S and Kawakami H: Design of lipo-protein-adsorbed liposomes retaining Mn-porphyrins for SOD mimic delivery to brains. Desalin Water Treat 17: 31-36, 2010.
18. Umakoshi H, Tuan LQ, Shimanocuhi T and Kuboi R: Role of liposome on recognition and folding of oxidized and fragmented superoxide dismutase for its re-activation. Biochem Eng J 46: 313-319, 2009.
19. Szoka F, Olson F, Heath T, Vail W, Mayhew E and Papahadjopoulos D: Preparation of unilamellar liposomes of intermediate size (0.1-0.2 mumol) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes. Biochim Biophys Acta 601: 559-571, 1980.
20. Youan BB: Microencapsulation of superoxide dismutase into poly(epsilon-caprolactone) microparticles by reverse micelle solvent evaporation. Drug Deliv 10: 283-288, 2003.
21. Aoki H, Fujita M, Sun CQ, Fuji K and Miyajima K: High-efficiency entrapment of superoxide dismutase into cationic liposomes containing synthetic aminoglycolipid. Chem Pharm Bull (Tokyo) 45: 1327-1331, 1997.
22. Rengel RG, Filipović-Grcić J, Cepelak I, Zanić-Grubisić T and Barisić K: The effect of liposomes with superoxide dismutase on A2182 cells. Eur J Pharm Biopharm 60: 47-51, 2005.
23. Kim HA, Kim DW, Park J and Choi SY: Transduction of Cu, Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into human chondrocytes. Arthritis Res Ther 8: R96, 2006.
24. Kwon HY, Eum WS, Jang HW, Kang JH, Ryu J, Ryong Lee B, Jin LH, Park J and Choi SY: Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells. FEBS Lett 485: 163-167, 2000.
25. Tsai CF, Lu FJ and Hsu YW: Protective effects of *Dunaliella salina* - a carotenoids-rich alga - against ultraviolet B-induced corneal oxidative damage in mice. Mol Vis 18: 1540-1547, 2012.
26. Pan J, Su Y, Hou X, He H, Liu S, Wu J and Rao P: Protective effect of recombinant protein SOD-TAT on radiation-induced lung injury in mice. Life Sci 91: 89-93, 2012.
27. Beauchamp C and Fridovich I: Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287, 1971.
28. Fung H and Demple B: A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. Mol Cell 17: 463-470, 2005.
29. Hashimoto D, Ohmuraya M, Hirota M, Yamamoto A, Suyama K, Ida S, Okumura Y, Takahashi E, Kido H, Araki K, Baba H, Mizushima N and Yamamura K: Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells. J Cell Biol 181: 1065-1072, 2008.
30. Chwa M, Atilano SR, Reddy V, Jordan N, Kim DW and Kenney MC: Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts. Invest Ophthalmol Vis Sci 47: 1902-1910, 2006.
31. Fabani MM, Gargini R, Taira MC, Iacono R and Alonso-Romanowski S: Study of in vitro stability of liposomes and in vivo antibody response to antigen associated with liposomes containing GM1 after oral and subcutaneous immunization. J Liposome Res 12: 13-27, 2002.
32. Li H, Shu T, Kuma H, Ueda Y, *et al*: Ribonucleoprotein complex originating in Paramyxovirus incapable of expressing some envelope proteins, for insertion into target cell with avoidance of problems with antigenicity and cytotoxicity, for use e.g. in gene therapy. Japan Patent no. C2001-001931. Filed May 18, 1999; issued November 23, 2000.
33. Natsume A, Mizuno M, Ryuke Y and Yoshida J: Cationic liposome conjugation to recombinant adenoviral vector reduces viral antigenicity. Jpn J Cancer Res 91: 363-367, 2000.

34. Steel JC, Cavanagh HM, Burton MA and Kalle WH: Microsphere-liposome complexes protect adenoviral vectors from neutralising antibody without losses in transfection efficiency, in-vitro. J Pharm Pharmacol 56: 1371-1378, 2004.
35. Arora N and Gangal SV: Efficacy of liposome entrapped allergen in down regulation of IgE response in mice. Clin Exp Allergy 22: 35-42, 1992.
36. Costanzo LL, De Guidi G, Giuffrida S, Sortino S and Condorelli G: Antioxidant effect of inorganic ions on UVC and UVB induced lipid peroxidation. J Inorg Biochem 59: 1-13, 1995.
37. Dave N and Liu J: Protection and promotion of UV radiation-induced liposome leakage via DNA-directed assembly with gold nanoparticles. Adv Mater 23: 3182-3186, 2011.
38. Fan R, Gan L, Liu M, Zhu D, Chen L, Xu Z, Hao Z and Chen L: An interaction of helicid with liposome biomembrane. Appl Surf Sci 257: 2102-2106, 2011.
39. Kato S, Kikuchi R, Aoshima H, Saitoh Y and Miwa N: Defensive effects of fullerene-C60/liposome complex against UVA-induced intracellular reactive oxygen species generation and cell death in human skin keratinocytes HaCaT, associated with intracellular uptake and extracellular excretion of fullerene-C60. J Photochem Photobiol B 98: 144-151, 2010.