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Transduced HSP27 protein protects neuronal cell death by enhancing FALS-associated SOD1 mutant activity

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Familial Amyotrophic lateral sclerosis (FALS) is a progressive neurodegenetative disorder induced by mutations of the SOD1 gene. Heat shock protein 27 (HSP27) is well-defined as a stress-inducible protein, however the its role in ALS protection has not yet been established. To investigate the role HSP27 may have in SOD1 mutant-mediated apoptosis, human SOD1 or HSP27 genes were fused with a PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame fusion protein, which was then transduced into cells. We found the purified PEP-1-HSP27 fusion proteins can be transduced efficiently into neuronal cells and protect against cell death by enhancing mutant SOD1 activity. Moreover, transduced PEP-1-HSP27 efficiently prevents protein aggregation produced by oxidative stress. These results suggest that transduced HSP27 fusion protein may be explored as a potential therapeutic agent for FALS patients. [BMB reports 2009; 42(3): 136-141]

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal progressive degenerative disorder characterized by gradual decline of muscle strength, respiratory failure and selective degeneration of motor neurons mainly in the cortex, brainstem and spinal cord. Approximately 10% of ALS patients are familial cases exhibiting an autosomal dominant inheritance (1, 2) with more than 100 mutations having been identified and associated with ALS. ALS is fatal within 3-5 years of the onset of symptoms in most patients. There is more strong evidence for a gain of function mutation of superoxide dismutase (SOD), such as protein misfolding, aggregation, pro-oxidant activity and impaired proteasomal turnover (3-5).

Heat shock proteins (HSPs) are major stress proteins induced in response to a variety of stresses including oxidative stress (6) and are implicated as modulators of disease pathology in many neurological conditions including ALS, Alzheimer's disease, Parkinson's disease and Huntington's disease (7-10). Several studies have shown that HSP70 and HSP40 protect against the pro-apoptotic activity of mutant SOD1, which interacts with HSP70, HSP40, alpha B-crystallin, and HSP27 in NIH3T3 and N2a cells (11-14). A study by Vleminckx *et al*. (2002) showed HSP27 levels increased in mice with ALS while others found HSP27 expression levels decreased (15-17). Therefore, the precise role of HSP27 in ALS remains unclear.

Presently, many researchers have demonstrated the development of protein transduction technology in which several small protein regions called protein transduction domains (PTDs) allow the delivery of exogenous protein into living cells (18-21). This has been utilized in the successful delivery of full-length Tat fusion proteins. By using this protein transduction technology, we have reported how various fusion proteins can be efficiently transduced into mammalian cells and animal tissues and markedly protect against reactive oxygen species (22-26).

In the present study, we examine how HSP27 protects against neuronal cell damage induced by FALS-related SOD1 mutant and oxidative stress. Our results demonstrate that the PEP-1-HSP27 fusion protein is efficiently transduced and protects against neuronal cell death. Therefore, we suggest that the PEP-1-HSP27 fusion protein could be useful as a potential therapeutic agent for FALS.

RESULTS AND DISCUSSION

Purification of PEP-1-HSP27 and PEP-1-SOD1 fusion proteins

Construction, expression and purification of the PEP-1-HSP27 and PEP-1-SOD1 fusion proteins are all previously described (27, 28). Briefly, PEP-1-HSP27 and PEP-1-SOD1 fusion proteins were purified following the induction of expression using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10

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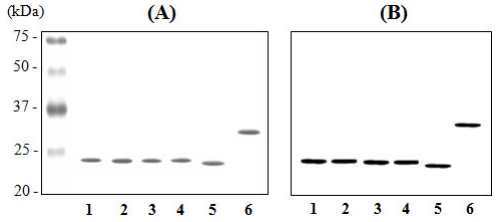

Fig. 1. Purification of PEP-1-HSP27 and PEP-1-SOD1 fusion proteins. Purified PEP-1-HSP27 and PEP-1-SOD1 fusion proteins were analyzed by 12% SDS-PAGE (A) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (B). Lanes in panels A and B are as follows: lane 1, wild type; lane 2, A4V; lane 3, G93A; lane 4, G85R; lane 5, D90A; lane 6, HSP27.

column chromatography. SDS-PAGE and Western blot analysis of the purified PEP-1-HSP27 and PEP-1-SOD1 fusion proteins shows both were purified and the fusion proteins were confirmed by an anti-rabbit polyhistidine antibody (Fig. 1).

Transduction of PEP-1-HSP27 and PEP-1-SOD1 fusion proteins into astrocyte cells

We previously reported that transduction of PEP-1-SOD1 fusion proteins into astrocyte cells was dose- and time-dependent, and that direct fluorescence analysis confirmed its intracellular delivery. The intracellular distribution of the wild type PEP-1-SOD1 fluorescence signal in non-fixed cells was similar to that of mutants PEP-1-SOD1 (27).

The intracellular delivery of PEP-1-HSP27 fusion proteins into astrocyte cells was therefore confirmed by direct fluorescence analysis. As shown in Fig. 2, almost all cultured cells were found to be transduced with PEP-1-HSP27 fusion proteins (Fig. 2). However, fluorescence was not detected in the negative control cells and in cells treated with control HSP27 (Fig. 2A and B, respectively).

Peptides and fusion proteins, including HIV-1 Tat and $(Arg)_9$ PTD, do not transduce across the cell membrane of living cells, instead being internalized by endocytosis. Therefore, any apparent entry is an artifact caused by fixation, which disrupts the cell membrane and therefore cannot be reliably used to study membrane-translocating proteins. For that reason, fixation should be avoided in studies of protein transduction into living cells (29). To exclude the possibility that cell fixation with paraformaldehyde may have affected PEP-1-HSP27 fusion proteins transduction, we transduced FITC conjugated PEP-1-HSP27 fusion proteins into fixed and non-fixed astrocyte cells. The intracellular distribution of the PEP-1-HSP27 fluorescence signal in non-fixed cells was similar to that of fixed cells (Fig. 2C and Fig. 2D). These results indicate not only are artifacts of protein transduction not induced, but that cell fixation with paraformaldehyde is actually not required for PEP-1-HSP27 fusion protein transduction. In addition, previous observations similarly indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation (18, 30).

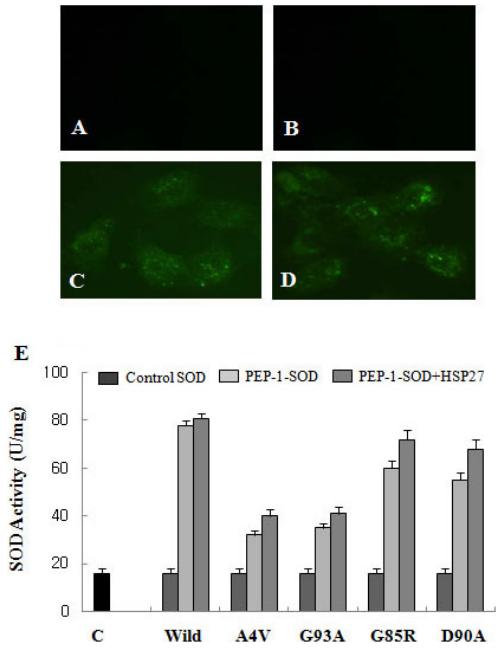

Fig. 2. Transduction of PEP-1-HSP27 (A-D) and changes in SOD activity (E). After FITC-labeled PEP-1-HSP27 (3 μM) was transduced into astrocyte cells, the cells were washed twice with trypsin-EDTA, PBS and immediately observed by fluorescence microscopy. Negative control cells alone (A), positive control cells treated with HSP27 (B), non-fixed cells treated with PEP-1-HSP27 (C), and fixed cells treated with PEP-1-HSP27 (D). PEP-1-SOD1 and PEP-1-HSP27 (3 μM) were co-transduced for 1 h and the SOD activities were analyzed (E).

Next, the SOD1 activity in astrocyte cells co-transduced with PEP-1-SOD1 and PEP-1-HSP27 fusion proteins was assessed. As shown in Fig. 2E, the enzyme activity of mutant SOD1 were significantly increased by HSP27 proteins when compared to PEP-1-SOD1 alone. Wild type SOD did not change the SOD activity either. In addition, an assessment of SOD1 in astrocyte cells treated with PEP-1-GFP fusion protein as a control showed PEP-1-GFP did not change the SOD activity at all (data not shown). These results demonstrate that PEP-1-HSP27 fusion protein plays a critical role in astrocyte cells as a stimulator of the mutant SOD1 enzyme.

Effect of transduced PEP-1-HSP27 fusion proteins on the viability of cells under oxidative stress

Heat shock proteins (HPSs) have very important functions, such as acting as molecular chaperons in physiological conditions or in response to stress (31, 32). To determine whether the HSP27 fusion protein has a functional role in cells under oxidative stress, we examined the viability of cells upon co-transduction of the PEP-1-SOD1 and PEP-1-HSP27 fusion proteins after the administration of hydrogen peroxide. When the cells were exposed to 1.2 mM hydrogen peroxide, only 35% of cells were viable. However, the viability of cells significantly increased af-

ter pre-treatment with the PEP-1-HSP27 (3 μM) fusion protein (Fig. 3). This result suggests the PEP-1-HSP27 fusion protein protects against cell death induced by oxidative stress. In particular, over-expressed HSP27 belongs to the family of small heat shock proteins, which protect against apoptotic cell death triggered by various stimuli such as oxidative stress by decreasing reactive oxygen species (ROS), thereby improving overall anti-oxidant defense (33). Recently, HSPs have been implicated as modulators of disease pathology in many neurological conditions such as ALS, Parkinson's disease (PD), and Alzheimer's disease (AD) and several hereditary diseases (7-9).

Effects of PEP-1-HSP27 on the aggregation of SOD1

It has been reported that FALS models exhibit the formation of SOD1 aggregations in astrocytes and motor neurons as one of its earliest pathological features (34, 35). The functional role of HSP27 in the aggregation of SOD1 by hydrogen peroxide under oxidative stress was determined by measuring the change of SOD1 aggregation in the presence and absence of HSP27. As shown in Fig. 4, a band shift was observed when wild type PEP-1-SOD was incubated with hydrogen peroxide but then decreased in the presence of HSP27, which indicates that

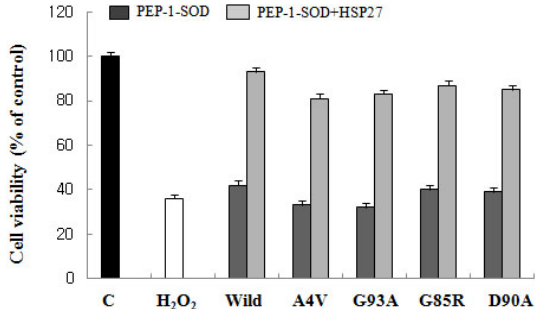

Fig. 3. Effect of PEP-1-HSP27 transduction on cell viability under oxidative stress. Hydrogen peroxide (1.2 mM) was added to astrocyte cells pre-treated with 3 μM PEP-1-SOD and 3 μM PEP-1-HSP27 for 1 h. Cell viability was estimated by MTT colorimetric assay.

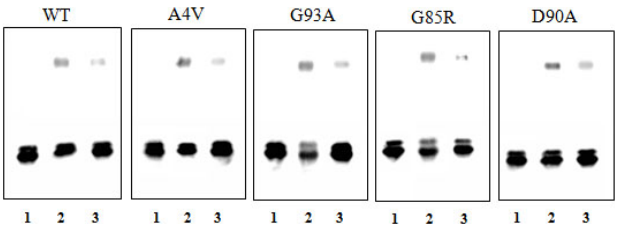

Fig. 4. Effect of PEP-1-HSP27 on aggregation of SOD1 under oxidative stress. Aggregation of SOD1 caused by oxidative stress was analyzed by Western blotting. PEP-1-SOD fusion proteins (0.2 μg) were incubated at 37°C for 1 h under hydrogen peroxide (1 mM) in both the absence and presence of PEP-1-HPS27. Lanes are as follows: lane 1, control; lane 2, SOD1 + Hydrogen peroxide; lane 3, SOD1 + Hydrogen peroxide + HSP27.

HSP27 efficiently prevents the aggregation of SOD1. It is worthwhile to mention that mutant SOD becomes more aggregated under oxidative stress.

It has been reported that the hydroxyl radicals produced by the free radical generating activity of FALS mutants with hydrogen peroxide is consistently enhanced in comparison with that of wild type SOD1 and thereby may directly contribute to the oxidative damage of macromolecules. It is this oxidative damage of macromolecules is associated with the aggressiveness diseases progression (36). These results show that transduced PEP-1-HSP27 fusion protein significantly inhibits the formation of protein aggregation induced by the hydrogen peroxide.

In summary, we have demonstrated for the first time that human HSP27 fused with PEP-1 peptide (PEP-1-HSP27) can be efficiently co-transduced into neuronal cells. Furthermore, PEP-1-HSP27 fusion protein was shown to markedly protect against neuronal cell death induced by hydrogen peroxide. Although the detailed mechanism remains to be further elucidated, our success in the protein transduction of PEP-1-HSP27 may ultimately be beneficial for patients with FALS related to mutant SOD1 and neurodegenerative disorders.

MATERIALS AND METHODS

Materials

Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen (GmbH, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co (Haarlen, Netherlands). Fetal bovine serum (FBS), DMEM and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). The rabbit anti-histidine polyclonal antibody was purchased from the Santa Cruz Biotechnology company (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest analytical grade available.

Expression and purification of PEP-1-HSP27 fusion proteins

The PEP-1-SOD1 (wild & mutant) and PEP-1-HSP27 expression vectors were constructed as previously described (27, 28). Two oligonucleotide primers were synthesized based on the cDNA sequence of human HSP27 and were ligated into a *Nde*I-*Xho*I-digested pET-15b vector for PCR amplification. The sense primer, 5′-CTCGAGATGACCGAGCGCCGCGTCCCCTTC-3′, contains an *Xho*I site, and the antisense primer, 5′-GGATCCTTACTTGGCGGCAGTCTCATCGGA-3′, contains a *Bam*HI restriction site. A PCR reaction was performed and the PCR product was excised with *Xho*I and *Bam*HI (Invitek, Berlin, Germany), eluted and then ligated into a TA-cloning vector (Promega, Madison, WI, USA) and a pPEP-1 vector using T4 DNA ligase (Takaka, Otsu, Shiga, Japan). The PEP-1-HSP27 was then cloned in *E. coli* DH5α cells and sequences were confirmed by sequence analysis.

To produce the PEP-1-HSP27 fusion proteins, plasmids were transformed into *E. coli* BL21 cells, which were grown in 100 ml of LB media at 37°C to a D_{600} value of 0.5∼1.0 then in-

duced with 0.5 mM IPTG at 37°C for 4 h. Harvested cells were lysed by sonication at 4°C in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), and the recombinant PEP-1-HSP27 was purified. Cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA) under native conditions for column purification. After the column was washed with 10 volumes of binding buffer and six volumes of wash buffer (25 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9), the fusion proteins were eluted using eluting buffer (0.25 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fractions containing the PEP-1-HSP27 fusion proteins were combined and the salts were removed using PD-10 column chromatography (Amersham, Braunschweig, Germany). The protein concentration was estimated by Bradford assay using bovine serum albumin as a standard (37).

Transduction of PEP-1-HSP27 into astrocyte cells

Astrocyte cells were grown to confluence on a 6-well plate, upon which the culture medium was replaced with 1 ml of fresh solution. Astrocyte cells were transduced by treatment of PEP-1-HSP27 at various concentrations for 1 h. The cells were harvested prior to the preparation of cell extracts for Western blot analysis by treatment with trypsin-EDTA (Gibco, Grand Island, NY, USA) and washing with phosphate-buffered saline (PBS).

MTT assay

The biological activity of the transduced PEP-1-HSP27 fusion proteins was assessed by a colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide) in which the cell viability of astrocyte cells treated with hydrogen peroxide was measured. The cells were seeded onto 6 well plates at 70% confluence then pretreated for 1 h with 3 μM PEP-1-HSP27, either in the presence or absence of mutant PEP-1-SODs. 1.2 mM of hydrogen peroxide was next added to the culture medium for 4 h. Cell viability was estimated using MTT colorimetry.

Enzymatic assay of SOD and Western blot analysis

The enzymatic assay measuring the dismutation activity of SOD was performed by monitoring the inhibition of the ferricytochrome c reduction reaction by xanthine/xanthine oxidase described by McCord and Fridovich (38). The reaction mixture contained 10 μM cytochrome c, 50 μM xanthine and sufficient xanthine oxidase to produce a reduction rate of cytochrome c of 0.025 absorbance units per min at 550 nm. The assay was performed in 3 ml of 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA in a cuvette at 25°C. Under these defined conditions, the amount of superoxide dismutase required to inhibit the reduction rate of cytochrome c by 50% (to a rate of 0.0125 absorbance unit per min) is defined as 1 unit of activity.

For Western blot analysis, the transduced PEP-1-HSP27 and PEP-1-SOD1 fusion proteins were electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane. The membrane was blocked for 2 h in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% tween-20 (TBST) and was then incubated for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted to 1:10000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ, USA) for detection.

Analysis of SOD1 aggregation

PEP-1-SOD1 fusion proteins (0.2 μg) were incubated at 37°C for 1 h with 1 mM hydrogen peroxide, either in the presence or absence of PEP-1-HSP27 (0.2 μg), for a total volume of 20 μl. The reaction was stopped by the addition of 1 μl catalase (1 mg/ml). The samples were treated with a sample buffer and then boiled at 100°C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE and Western blotting as previously described.

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