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Hsp27 Reduces Phosphorylated Tau and Prevents Cell Death in the Human Neuroblastoma Cell Line SH-SY5Y

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The two major symptoms characterizing Alzheimer's disease are the formation of amyloid-β extracellular deposits in the form of senile plaques and intracellular neurofibrillary tangles (NFTs) that consist of pathological hyperphosphorylated tau protein aggregated into insoluble paired helical filaments (PHFs). Neurons of the central nervous system have appreciable amounts of tau protein, a microtubule-associated protein. To maintain an optimal operation of nerves, the microtubules are stabilized, which is necessary to support cell structure and cellular processes. When the modified tau protein becomes dysfunctional, the cells containing misfolded tau cannot maintain cell structure. One of the pathological hallmarks of Alzheimer's disease is hyperphosphorylated tau protein. This paper shows that the small heat shock protein from humans (Hsp27) reduces hyperphosphorylated tau and prevents hyperphosphorylated tau-induced cell death of the human neuroblastoma cell line SH-SY5Y.

Key Words : Hyperphosphorylated tau, Alzheimer's disease, Hsp27, SH-SY5Y

Introduction

Alzheimer's disease is one of the causes of dementia, which is an indication of brain function loss. The exact cause and process of Alzheimer's disease has not been identified as well as other neurodegenerative disorders. However, Alzheimer's disease is thought to be caused by plaques and tangles in the brain.[1] Extracellular amyloid β deposits in the form of amyloid plaques and intracellular neurofibrillary tangles are found in the brains of patients with Alzheimer's disease.[2]

Tau, a microtubule-associated protein, stabilizes microtubules in neuronal cells and modulates microtubule dynamics.[3] There are six tau protein isoforms in human CNS from the alternative splicing of the tau gene. Each plays a different physiological role during the distinct developmental steps.[4] Moreover, the tau protein has two domains: projection and assembly. This protein has specific serine or threonine phosphorylation sites on these two domains, which are related to several neurodegenerative disorders.[5] The ability of the tau protein to stabilize microtubules by binding them is dependent on the degree of site specific phosphorylation on the tau protein.[6] Since hyperphosphorylated tau protein in the Alzheimer's disease brain aggregates into paired helical filament (PHFs) and neurofibrillary tangles (NFTs), they are believed to scarcely bind on microtubules and function.[7] Tau phosphorylation is attenuated by several phosphatase, PP1A, PP2A, PP2B and PP5, to the relation of tau phosphorylation.[8] Okadaic acid, a phosphatase inhibitor, is used to preserve hyperphosphorylated tau and develop a cellular model where seemingly authentic PHF-like tau hyperphosphorylation is induced.[9]

Heat shock protein 27(Hsp27), one of the members of the sHsp (small heat shock protein) group, is known as a chaperone. This means that it reduces protein aggregation and stabilizes misfolded protein. This protein was obtained by expressing in Escherichia coli and separating Hsp27 from other proteins. The small heat shock protein from humans (Hsp27) reduces hyperphosphorylated tau-induced cell death of the human cortical neuronal cell line HCN2A by the binding of pathological tau.[10] We also examined the effect of Hsp27 on the pathological tau of the human neuroblastoma SH-SY5Y cell line.

Experimental

Materials. *Escherichia coli* BL21 (DE3) was purchased from Enzynomics (Korea). Kanamycin and okadaic acid were purchased from Sigma Aldrich (USA). Luria-Bertani broth agar was purchased from Merck (USA). Isopropyl β-D-1-thiogalactopyranoside was purchased from MB cell (Korea). Dulcecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered Saline (DPBS) and fetal bovine serum (FBS) were purchased from WelGENE (USA). Trypsin-EDTA was purchased from Invitrogen (USA). Antibiotic-antimycotic was purchased from Gibco (USA). Pro-Ject Protein Transfection Reagent kit, Ripa buffer, and BCA Protein Assay kit were purchased from Pierce (USA). Peroxidase-linked *anti*-rabbit and *anti*-mouse IgG were purchased from Santa Cruz Biotechnology (USA). Rabbit polyclonal *anti*-tau phosphoserine 199/202 antibody and mouse monoclonal *anti*-tau-1 were purchased from Millipore (USA). *Anti*-human tau monoclonal antibody and rabbit *anti*-hsp27 polyclonal antibody were purchased from Pierce Biotechnology (USA). WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium

salt] was purchased from Dojindo (Korea).

Expression and Purification of HSP27 Protein. The pET28a vector containing hexahistidine(His_6) and Hsp27 was transformed into the Escherichia coli strain BL21 (DE3) for protein expression. Luria–Bertani broth agar plate containing kanamycin was spread with *E. coli* and incubated overnight at 37 °C. A single colony was inoculated into 20 mL of LB medium containing 1 mM kanamycin and incubated shaking at 200 rpm at 37 °C. 10 mL of overnight culture was inoculated in 500 mL medium in a 2 L flask, where the cultures were incubated at 37 °C to OD_{600} of 0.5. Proteins were expressed by adding isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1 mM for 4 h. The cells were centrifuged at 6,000 rpm for 15 minutes at 4 °C and resuspended in 20 mM Tris–HCl (pH 7.0), 500 mM NaCl, and 35 mM imidazole buffer (binding buffer). This was sonicated on ice with 1 second pulses every 8 seconds by using a sonicator (Sonics Vibra-Cell VCX 750, Sonic & Materials Inc., USA) for 30 minutes. After sonication, the lysates were centrifuged at 10,000 rpm for 15 minutes and the soluble fraction was collected. The clarified supernatant fraction was applied to a preequilibrated HiTrap Chelating HP column (GE Healthcare) with 5 mL bed volume. Bound protein was eluted with 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 1 M imidazole (elution buffer). Further purification was carried out with HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare) using 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl.

Cell Culture. SH-SY5Y human neuroblastoma cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM; Welgene) supplemented with 10% fetal bovine serum (FBS; Welgene) and 1% antibiotic-antimycotic (Gibco) in an atmosphere containing 5% CO_2 and 95% air. A Cell below passage number of 20 was used for the experiments.

Treatment of SH-SY5Y Cells. The cells were seeded at 3.0×10^4 and 1.0×10^5 for each 96 and 6 well. They were grown in the medium for 48 h at 37 °C before the transfection of pJDK-Tau. The polyplexes were formed with 2 μg of pJDK-Tau and 8 μg of PAMAM-Arg in FBS-free medium, and the mixtures were incubated for 30 minutes at room temperature. The cells were further incubated for 24 h without exchanging medium. After transfection, 2 mg of Hsp27 were placed in the cells using the Pro-Ject Protein Transfection Reagent Kit (Pierce) according to manufacturer's guidelines. The cells were then treated with 50 nM okadaic acid for 24 h.

Western Blotting. For immunoblotting analysis, cells were harvested, washed in DPBS, resuspended, and homogenized using RIPA buffer (Pierce). Lysates were then centrifused at 15,000 rpm for 15 minutes at 4 °C. Lysates separated by centrifugation were adjusted to 50 μg using the micro BCA Protein Assay Kit (Pierce), boiled with 2x sample buffer for 5 minutes, separated by SDS-PAGE, and transferred to Amersham Hybond ECL Nitrocellulose Membrane (GE Healthcare). This was blocked for 30 minutes at room temperature with 5% skim milk/TBST (Tris-Buffered Saline and 0.1% Tween 20). The membranes were washed three times in TBST for 15 minutes each and incubated with primary antibodies overnight at 4 °C. They were washed and applied with appropriate second antibodies (peroxidase-linked *anti*-rabbit or *anti*-mouse IgG) and Amersham ECL Western Blotting Detection Reagents (GE Healthcare).

Cytotoxicity Assay. The cytotoxicity assay was performed by using the Cell Counting Kit-8 (Dojindo). The SH-SY5Y cells were seeded in a 96-well plate at 3.0×10^4 in 80 μL DMEM medium containing 10% FBS. After incubating for 48 h, cells were treated in each step with 10 μL of polyplex solution, Hsp27 solution, and 50 nM of okadaic acid for 24 h before 10 μL of CCK-8 solution was added to each well. After 4 h of incubation at 37 °C, the absorbance was measured at 450 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA).

Results and Discussion

Purification of Recombinant Hsp27 in *Escherichia coli.* Human Hsp27 cDNA was inserted into the pET28a expression vector (Figure 1(a)). This vector produces a fusion protein where the Hsp27 is fused to a hexahistidine (His_6). Hsp27 was purified by using a HiTrap Chelating HP column (Figure 1(b)) and HiLoad 16/600 Superdex 200 prep-grade column (Figure 1(e)). Analysis using SDS-PAGE (12%) revealed the presence of Hsp27 at the expected size (25 kDa) (Figure 1(c) and (f)). To confirm the expression of Hsp27, a western blotting assay was performed. That detected Hsp27 on the 25kDa band (Figure 1(d) and (g)).

Okadaic Acid-Induced Hyperphosphorylated tau is Inhibited by Hsp27. To determine the effect of Hsp27 on hyperphosphorylated tau, SH-SY5Y cells were transfected with pJDK-tau, treated with 50 nM okadaic acid, delivered with Hsp27, and then analyzed by the western blotting assay (Figure 2). SH-SY5Y cell lysates were immunoblotted with HT7 (Pierce), ptau 199/202 (Millipore), Tau-1 (Millipore), and HSP27. HT7 (Pierce) is a monoclonal antibody that recognizes normal tau from human and bovine brain and PHF-tau. Ptau 199/202 (Millipore) is a polyclonal antibody that recognizes tau pSerine 199/202 in samples of recombinant human. Tau-1 is a monoclonal antibody that recognizes dephosphorylated serine sites of tau at 195, 198, 199, and 202 Serine. Finally, HSP27 is a polyclonal antibody that recognizes human and mouse Hsp27. When plasmid inserted tau cDNA was transfected, the amount of tau increased in those cell lysates (Figure 2(a)). We delivered Hsp27 into SH-SY5Y and confirmed successful delivery by checking through the western blotting analysis (Figure 2(e)). In the presence of okadaic acid, phosphorylated tau at 199/202 Serine increased a small amount. However, they decreased in the cell lysates where the Hsp27 was delivered (Figure 2(b)).

Hsp27 Decreases Hyperphosphorylated Tau-induced Cytotoxicity. To investigate the effect of Hsp27 on SH-SY5Y's cytotoxicity, the cytotoxicity assay was performed by using the Cell Counting Kit-8 (Dojindo). As shown in Figure 3, 50 nM of okadaic acid displayed significant cyto-

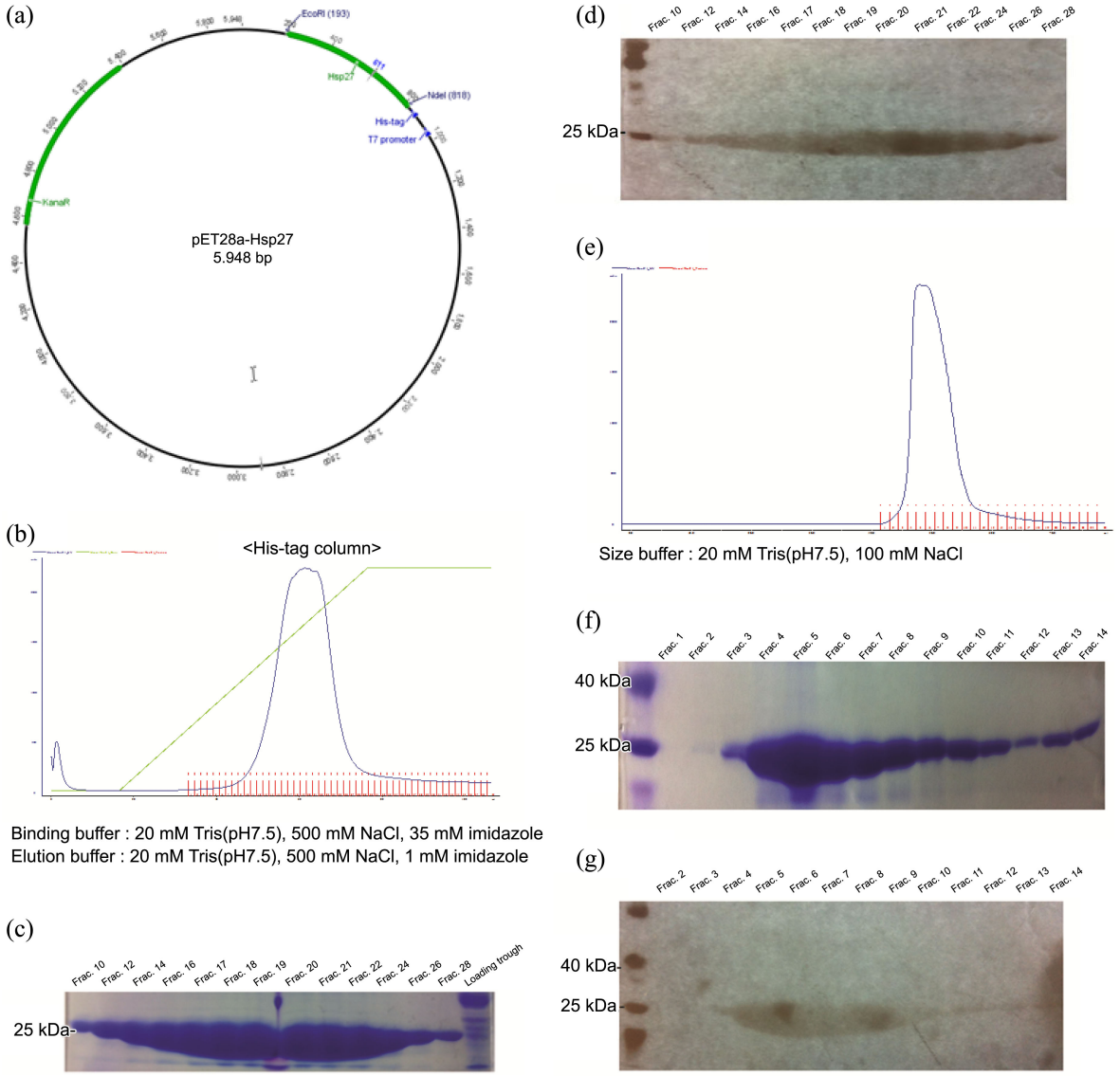

Figure 1. Purification of HSP27 protein. (a) pET28a vector containing hexahistidine (His6) and Hsp27 (b) The HiTrap Chelating HP column bound protein was eluted with a linear gradient of 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 M imidazole (elution buffer) (c), (f) SDS-PAGE was performed on samples eluted using the HiTrap and HiLoad 16/600 Superdex 200 prep grade column (d), (g) western blotting analysis with Hsp27 antibody (e) Superdex 200 column was eluted with 20 mM Tris–HCl (pH 7.5) and, 100 mM NaCl.

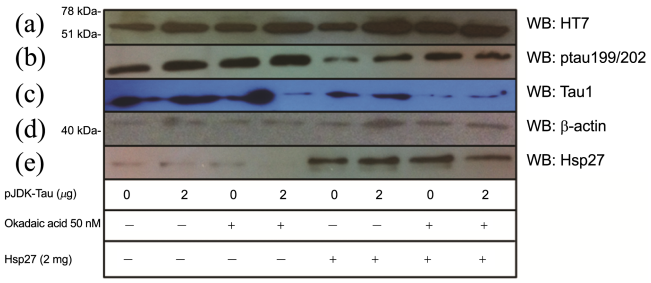

Figure 2. Western blotting analysis with (a) HT7, a monoclonal antibody that recognizes normal tau from human and bovine brain, and PHF-tau (b) ptau 199/202, a polyclonal antibody that recognizes tau pSerine 199/202 in samples of recombinant human (c) Tau-1, a monoclonal antibody that recognizes dephosphorylated serine site of tau at 195, 198, 199, 202 Serine (e) HSP27, a polyclonal antibody that recognizes human, mouse Hsp27.

toxicity and had less than 50% relative cell viability (RCV). When cells were delivered with Hsp27, cell viability recovered about 40% (Figure 3(c) and (d)). However, there were no therapeutic effects in cells where okadaic acid was not treated. An overexpression of tau protein did not affect cell viability (Figure 3(b)) and even when cells were exposed to okadaic acid, an overexpression of the tau protein had no significant influence on cell viability (Figure 3(c) and (d)).

Morphology and Cell Structure. Untreated SH-SY5Y cells have several neuritis in the surrounding area (Figure 4(a)) and transfection with pJDK-Tau makes no difference on cell morphology (Figure 4(c)). When SH-SY5Y cells were treated with 50 nM okadaic acid, we observed cell rounding and detachment of SH-SY5Y cells presumably due to the dissolution of the microtubule network (Figures 4(e)

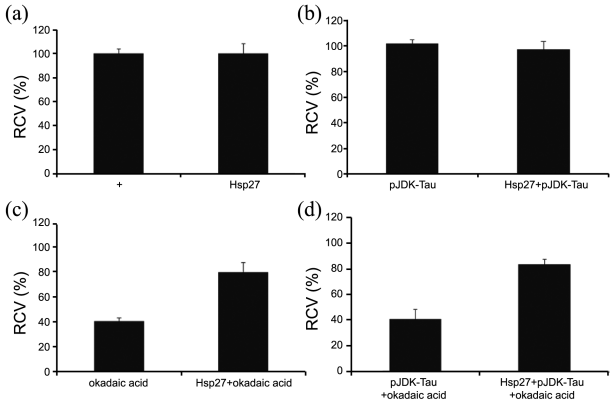

Figure 3. CCK assay results of okadaic acid, tau cDNA, and Hsp27. (a) Hsp27. (b) tau cDNA and Hsp27. (c) okadaic acid, and Hsp27. (d) tau cDNA, okadaic acid, and Hsp27. RCV (relative cell viability) was represented as relative absorbance (%) to the cell only. Cells were seeded at 3.0×10^4 cells/well in 96 well plates. 0.1 mg of Hsp27, 0.1 μg of tau cDNA, and 50 nM okadaic acid were added to the cells. Data are presented as mean ± SD (n = 4).

and (g)). In addition, cells adhered together and formed large clusters, which explained how the okadaic acid-induced tau hyperphosphorylation made tau separate from the microtubule, destabilized the microtubule, and led to the collapsed cell structure.

To prevent the collapse of the cell structure and the detachment from the bottom, Hsp27 was delivered into the cells, but found that hsp27 cannot prevent the collapse of the cell structure (Figures 4(f) and (h)). This is probably because Hsp27 degrades pathological hyperphosphorylated tau using the proteosome but not the dephosphorylation of pathological hyperphosphorylated tau.[11] However, when Hsp27 was delivered into the cells, the amount of detachment and condensation decreased. These round cells treated with Hsp27 were believed to be alive. When we tested cell viability using the MTT assay, insoluble formazans appeared in the medium. Because it is hard to remove the medium without preserving insoluble formazans, we had to test the cell viability test by using the CCK analysis. The existence of formazans in the medium indicated that Hsp27 degrades pathological hyperphosphorylated tau and enhances cell survival.

Conclusion

We confirmed that pathological hyperphosphorylated tau is one of the main causes of Alzheimer's disease rather than just an overexpression of tau protein in the human neuroblastoma cell line SH-SY5Y. The hyperphosphorylated tau can barely bind to microtubules and function. Hsp27 (heat shock protein), one of members of the sHsp (small heat shock protein) group, prevents tau protein aggregation and stabilizes the denatured protein. However, Hsp27 cannot rescue SH-SY5Y cells from cell structure collapse, which indicates that Hsp27 enhances cell viability by decreasing pathological proteins. Further studies are needed to examine

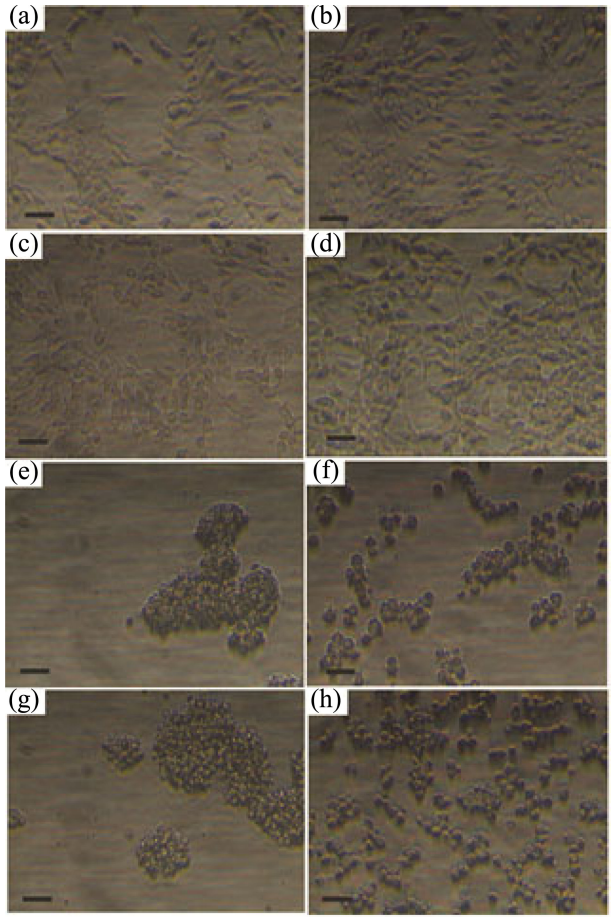

Figure 4. Morphology of SH-SY5Y. Cells were seeded at 1.0×10^6 per well in a 6-well plate. (a) control group. Cells were treated with (b) 2 mg of Hsp27 (c) 2 μg of pJDK-Tau (d) 2 mg of Hsp27 and 2 μg of pJDK-Tau (e) 50 nM okadaic acid for 24 h, (f) 2 mg of Hsp27 and 50 nM okadaic acid, (g) 2 μg of pJDK-Tau and 50 nM okadaic acid (h) 2 mg of Hsp27, 2 μg of pJDK-Tau and 50 nM okadaic acid. Hsp27 was delivered by using Pro-Ject Protein Transfection Reagent. pJDK-Tau was transfected into SH-SY5Y cells by using PAMAM-R G4 (arginine conjugated polyamidoaminedendrimer). Scale bar = 25 μm.

how to reduce hyperphosphorylated tau and recover cell viability. Also, additional studies in animal models need to be conducted to clarify the role of Hsp27 on hyperphoshporylated tau aggregation.

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