이학석사학위논문

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화학부 생화학 전공

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안준성의 석사학위논문을 인준함

2012 년 12 월

위 원 장 _____ (인)

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Heat shock protein 27 reduces phosphorylated tau and rescues cell death in human neuroblastoma cell line SH-SY5Y

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A Thesis for the M.S. Degree in Biochemistry

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Abstract

The two pathological hallmarks characterizing Alzheimer's disease are the formation of extracellular deposit of amyloid-β in the form of senile plaques and intracellular neurofibrillary tangles (NFTs), which consist of pathological hyperphosphorylated tau protein aggregated into insoluble paired helical filaments (PHFs). Tau protein, a microtubule-associated protein, is abundant in neurons of the central nervous system. To keep nerves functioning properly, it stabilizes microtubules, which are important for supporting cell structure and cellular processes. When tau protein is modified, it stops functioning and a cell can't maintain cell structure. Therefore hyperphosphorylated tau is believed to be a pathological hallmark of Alzheimer's disease. We use the okadaic acid, phosphatase inhibitor, to preserve hyperphosphorylated tau and develope a cellular model where apparently authentic PHF-like tau hyperphosphorylation is induced.

Heat shock protein 27(Hsp27), one of the sHsp (small heat shock protein) group, is known as a chaperone, which prevents protein aggregation and stabilizes denatured protein. This protein was obtained by expressing in Escherichia coli and separating Hsp27 from other proteins. Here we show that Hsp27 reduces hyperphosphorylated tau by helping its degradation. Moreover, Hsp27 rescues from hyperphosphorylated tau mediated cell death in neuroblastoma cell line SH-SY5Y.

Keywords: Hyperphosphorylated tau, Alzheimer's disease, Hsp27, SH-SY5Y.

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1. Introduction

Alzheimer's disease is a cause of dementia, which means a loss of brain function. The cause and process of Alzheimer's disease has not been discovered. However Alzheimer's disease is known to be relevant to plaques and tangles in the brain [1]. Extracellular amyloid β deposits in the shape of amyloid plaques and intracellular neurofibrillary tangles are present in the brain 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 by alternative splicing of the tau gene. Each plays different physiological role during the different developmental steps [4]. Tau protein has two domains, projection domain and assembly domain. Moreover, there are specific serine or threonine phosphorylation sites, related to several neurodegenerative disorders, on the tau protein [5]. The ability of tau protein to stabilize microtubules by binding them is dependent on degree of site specific phosphorylation on tau protein [6]. Since tau protein in Alzheimer' disease brain aggregates into paired helical filament (PHFs) and neurofibrillary tangles (NFTs), they are believed to hardly bind on microtubules and function [7]. Tau phosphorylation is attenuated by several

phosphatases, PP1A, PP2A, PP2B and PP5 to the relation of tau phosphorylation [8]. The small heat shock protein from human (Hsp27) reduces hyperphosphorylated tau-induced cell death of human cortical neuronal cell line HCN2A by binding of pathological tau [9]. We also examined the effect of Hsp27 on the pathological tau of the human neuroblastoma SH-SY5Y cell line.

2. Materials and Methods

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). Dulbecco'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)-2H-tetrazolium, monosodium salt] was purchased from Dojindo (Korea).

Expression and purification of HSP27 protein

The pET28a vector containing hexahistidine (His6) 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℃. Single colony was inoculated into 20 ml of LB medium containing 1 mM kanamycin and incubated at 37℃ with shaking at 200 rpm. 10 mL overnight culture was inoculated in 500 ml medium within a 1 L flask. Cultures were incubated at 37 ℃, to OD_{600} of 0.5. Proteins were expressed by adding isopropyl-b-D-thiogalactopyranoside at final concentration of 1 mM for 4 h. The cells were centrifuged at 6,000 rpm for 15 min at 4 ℃, resuspended in 20 mM Tris–HCl (pH 7.0), 500 mM NaCl, 35 mM imidazole buffer (Binding buffer) and sonicated on ice with 1 s pulses between 8 s pauses by using a sonicator (Sonics Vibra-Cell VCX 750, Sonic & Materials Inc., USA) for 30 min. After sonication, the lysates were centrifuged at 10,000rpm for 15 min and the soluble fractions were collected. The clarified supernatant fraction was applied to a preequilibrated HisTrap 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, 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), 100 mM NaCl.

Cell Culture

SH-SY5Y human neuroblastoma cells were grown in in Dulbecco's Modification of Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic in an atmosphere containing 5% CO2 and 95% air. Cell below passage number of 20 were used for experiments.

Western blotting

For immunoblotting analysis, cells were harvested, washed in DPBS, resuspended, and homogenized using RIPA buffer. Lysates were then centrifused at 15,000 g for 15 min at 4℃. Lysates cleared by centrifugation were adjusted to 50 μg protein concentration by micro BCA Protein Assay Kit, boiled with 2X sample buffer for 5 min, separated by SDS-PAGE and then transferred to AmershamHybond ECL Nitrocellulose Membrane, which were blocked for 30 min 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 each 15 min and incubated with primary antibodies overnight at 4 ℃. They were washed and applied with appropriate second antibodies (peroxidase-linked anti-rabbit or anti-mouse IgG) and AmershamECL Western Blotting Detection Reagents.

Cytotoxicity assay

The cytotoxicity assay was performed by using Cell Counting Kit-8. 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 incubation for 48 h, cells were treated with 10 mL of polyplex solution, Hsp27 solution, and 50 nM okadaic acid for 24 h in each step. Then, 10 μL of CCK-8 solution was added to each well. After 4 h of incubation at 37 ℃, the absorbance was measured at 450 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA).

3. 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 in which the Hsp27 is fused to a hexahistidine (His6), can be cleaved off. 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 with expected size (25kDa) (Figure 1(c) and (f)). To confirm expression of Hsp27, western blotting assay was performed. Hsp27 was detected on a 25 kDa 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 western blotting assay (Figure 2). SH-SY5Y cell lysates were immunoblotted with HT7 (Pierce), a monoclonal antibody that recognizes normal tau from human and bovine brain and PHF-tau; ptau 199/202 (Millipore), a polyclonal antibody that recognizes tau pSerine 199/202 in samples of recombinant human; Tau-1 (Millipore), a monoclonal antibody that recognizes dephosphorylated serine site of tau at 195, 198, 199, 202 Serine; HSP27 (Pierce), a polyclonal antibody that recognizes human, mouse Hsp27. When plasmid inserted with tau cDNA is transfected, the amount of tau increased in those cell lysates (Figure 2(a)). We delivered Hsp27 into SH-SY5Y and confirm successful delivery by western blotting analysis (Figure 2(e)). In the presence of okadaic acid, small amount of tau phosphorylated at 199/202 Serine increased. However, they decreased in those cell lysates to which the Hsp27 was delivered (Figure 2(b)). We found that the pathological features of tau protein depend on phosphorylation of residues 199/202 serine. Moreover, we confirmed that Hsp27 recognizes pathological tau protein and facilitates degradation of them.

Hsp27 decreases hyperphosphorylated tau-induced cytotoxicity

To investigate the effect of Hsp27 on hyperphosphorylated tau induced-cell death, the cytotoxicity assay was performed by Cell Counting Kit-8. As shown in Figure 3, 50 nM okadaic acid displayed significant cytotoxicity and the relative cell viability (RCV) of okadaic acid were less than 50%. When cells were delivered with Hsp27, cell viability was recovered about 40% (Figure 3(c) and (d)). However there was no therapeutic effect in cells to which okadaic acid was not treated. Overexpression of tau protein did not affect cell viability (Figure 3(b)). Even when cells were exposed with okadaic acid, overexpression of tau protein has no significant influence on cell viability (Figure 3(c) and (d)). These results indicate that the cause of tau mediated cell death is the post translational hyperphosphorylation of tau protein, which leads to release from the microtubules and destabilize them, but not overexpression of tau.

Morphology and cell structure

As untreated SH-SY5Y cells, these have several neurites to the surrounding area (Figure 4(A)). Moreover, transfection with pJDK-Tau makes no difference to cell morphology (Figure 4(C)). When SH-SY5Y cells were treated with 50 nM okadaic acid, the cell rounding and detachment of SH-SY5Y cells were observed presumably due to dissolution of the microtubule network (Figure 4(E) and (G)). In addition, cells adhered together and formed large clusters. This result explained that okadaic acid-induced tau hyperphosphorylation makes tau separated from microtubule, destabilizes

microtubule, and leads to the collapsed cell structure.

To prevent the collapse of the cell structure and the detachment from the bottom, we delivered Hsp27 into the cells. We found that hsp27 can't prevent the collapse of the cell structure (Figure 4(F) and (H)). This is probably because Hsp27 degrades pathological hyperphosphorylated tau using the proteosome [10], but not dephosphorylation of pathological hyperphosphorylated tau. However, when Hsp27 were delivered into the cells, the amount of detachment and condensation is decreased. These round cells treated with Hsp27 were believed to be alive. When we tested cell viability using MTT assay, insoluble formazans were appeared in the medium. So, it is hard to remove medium without preserving insoluble formazans. The existence of formazans in the medium indicated that Hsp27 degrades pathological hyperphosphorylated tau and enhances cell survival.

5. Conclusions

We confirmed that pathological hyperphosphorylated tau is one of the main causes of Alzheimer disease rather than overexpression of tau protein in human neuroblastoma cell line SH-SY5Y. It is thought that hyperphosphorylated tau can hardly bind to microtubules and function. Therefore microtubules become dissociated and disabled in the cells containing hyperphosphorylated tau. Hsp27 (heat shock protein), one of the

sHsp (small heat shock protein) group, recognize pathological tau protein, stabilizes denatured protein and prevents its aggregation. However Hsp27 can’t rescues SH-SY5Y cells from collapse of cell structure. This result indicates that Hsp27 enhances cell viability by decreasing pathological proteins, not refolding denatured proteins. To examine how to reduce hyperphosphorylated tau and recover cell viability, further studies are needed. Also, additional studies in animal models need to be conducted to clarify the role of Hsp27 on hyperphoshporylated tau aggregation.

6. References

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7. Figures

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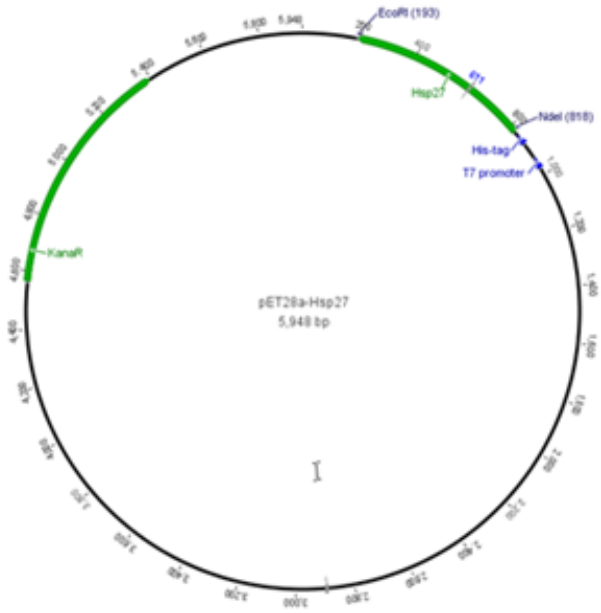

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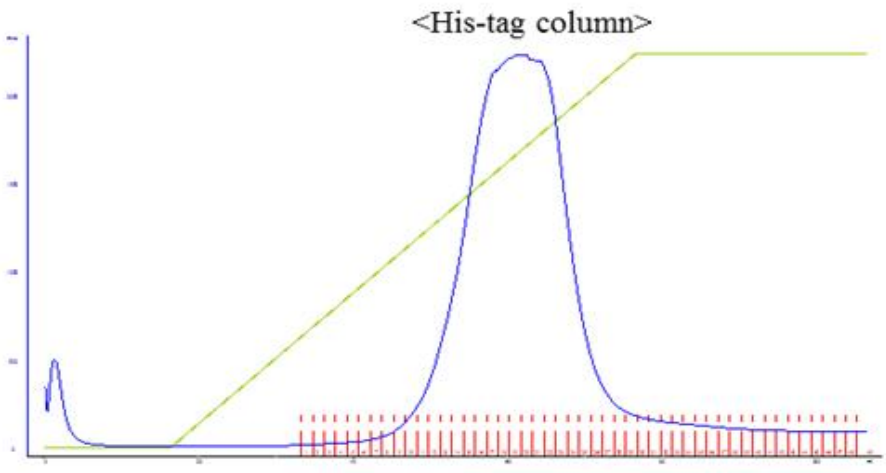

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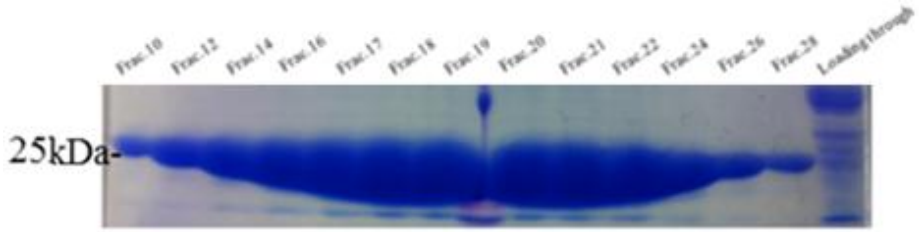
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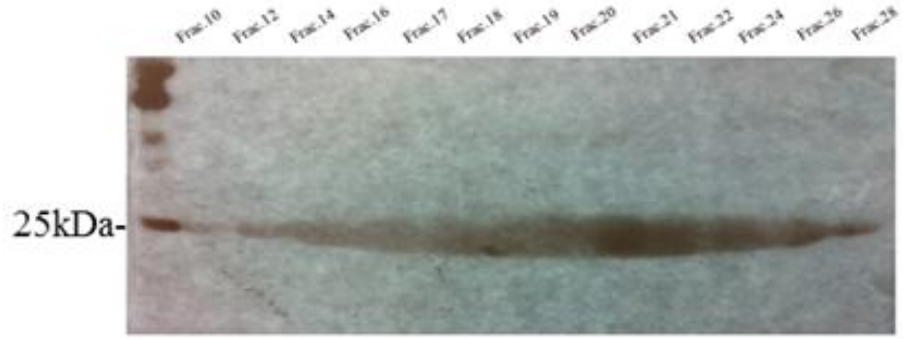
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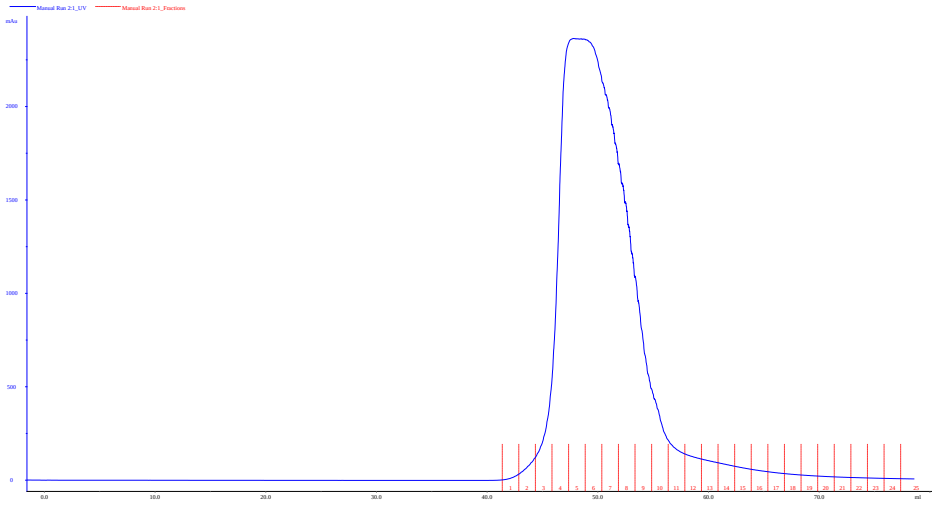

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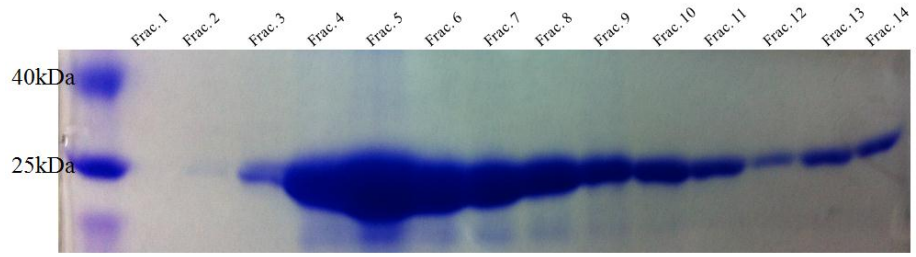

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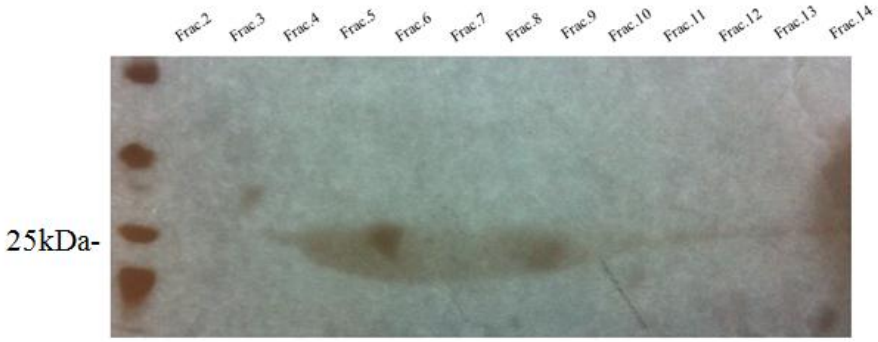

Figure 1. purification of HSP27 protein. (A)pET28a vector containing hexahistidine(His6) and Hsp27 (B) The HiTrap Chelating HP column bound protein was eluted with a linear gradient of 20mM Tris–HCl (pH 7.5), 500mM NaCl, 1M imidazole (elution buffer)(C)(F)SDS-PAGE was performed on samples eluted using the HiTrap and HiLoad 16/600 Superdex 200 prep grade column(D)(G) western blotting analysis with Hsp27 antibody (E) Superdex200 column was eluted with20 mMTris–HCl (pH 7.5), 100 mMNaCl.

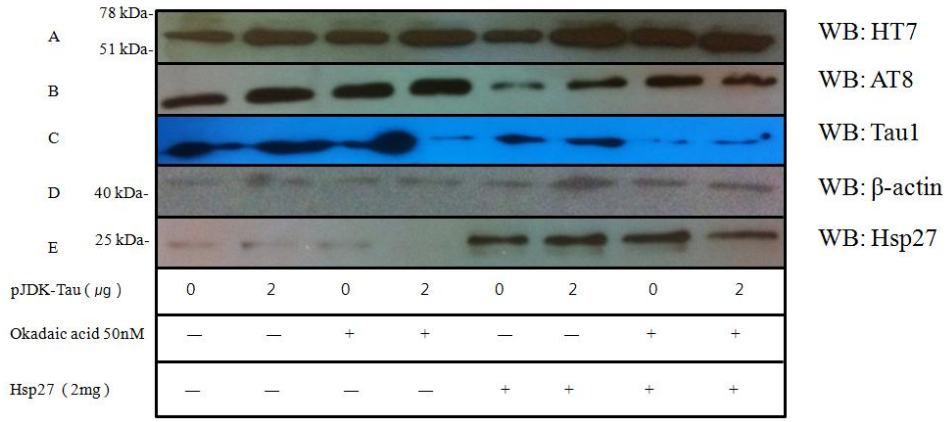

Figure 2. Western blotting analysis with (A) HT7, a monoclonal antibody that recognizes normal tau from human and bovine brain and PHF-tau (B) pTau 199/202, a polyclonal antibody that recognizes tau pSerine 199/202 in samples of recombinant human (C) Tau-1, a monoclonal antibody that recognizes dephosphorylated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

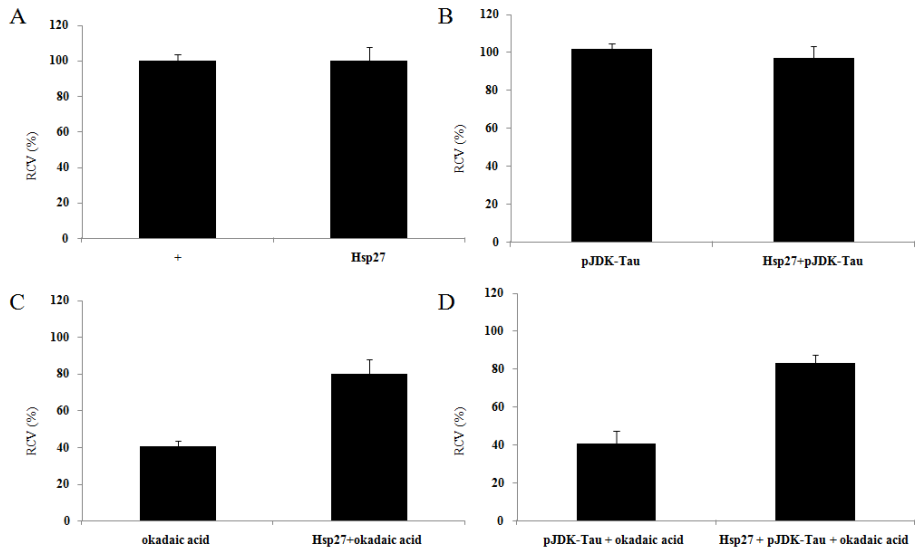

Figure 3. CCK assay results of SH-SY5Y treated with (A) Hsp27. (B) tau cDNA and Hsp27. (C) okadaic acid, and Hsp27. (D) tau cDNA, okadaic acid, and Hsp27. RCV (relative cell viability) was represented as relative absorbance (%) to cell only. Cells were seeded at 3.0 x 10^4 cells/well in 96 well plates. 0.1mg of Hsp27, 0.1 μg of tau cDNA, and 50nM okadaic acid were added onto the cells. Data are presented as mean ± SD (n=4).

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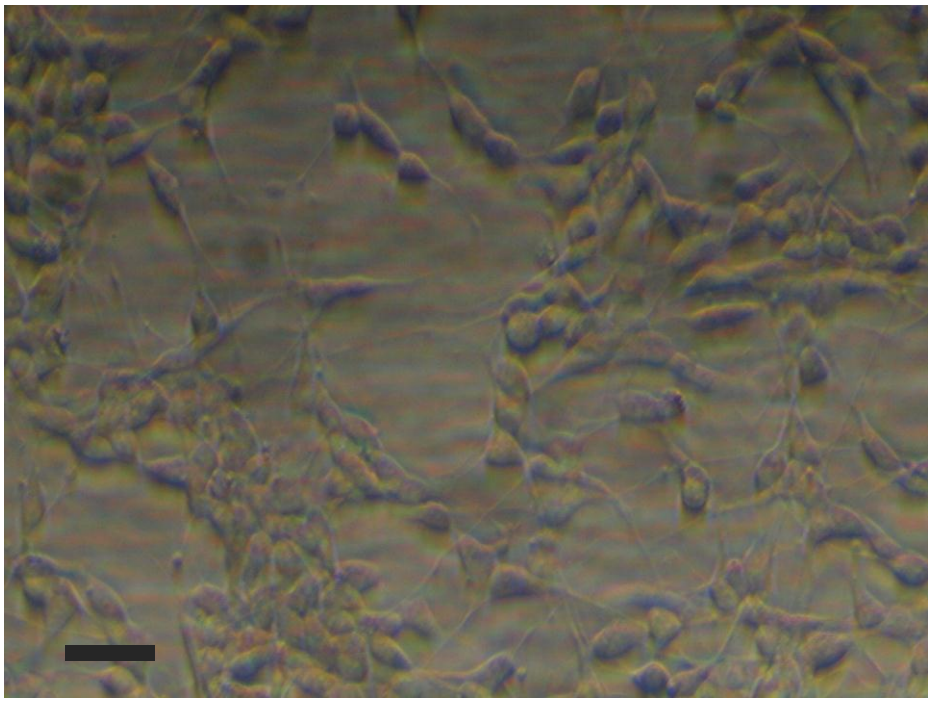

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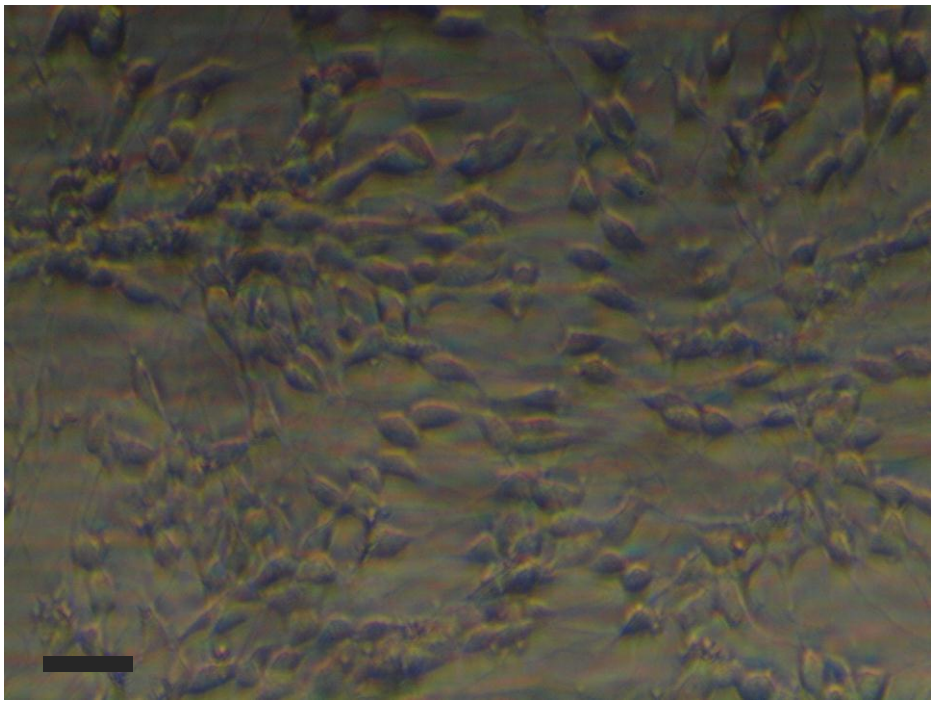

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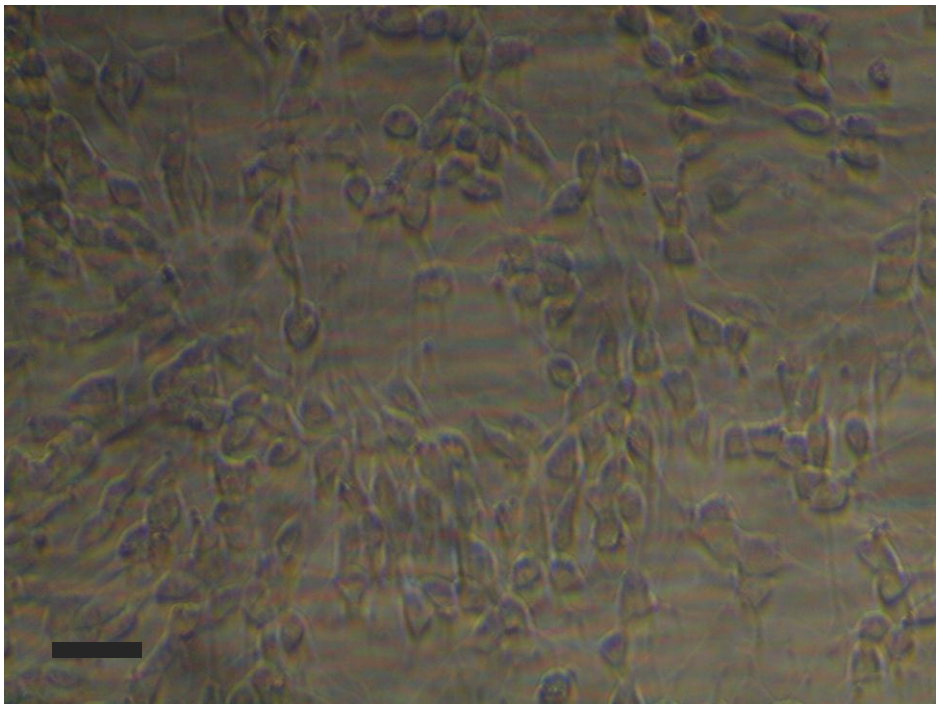

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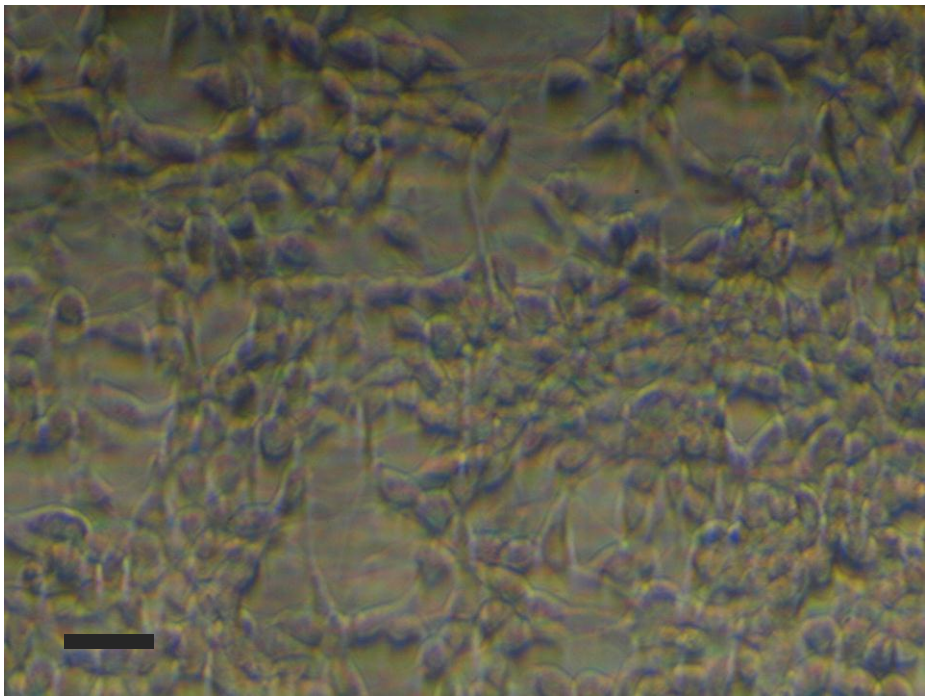

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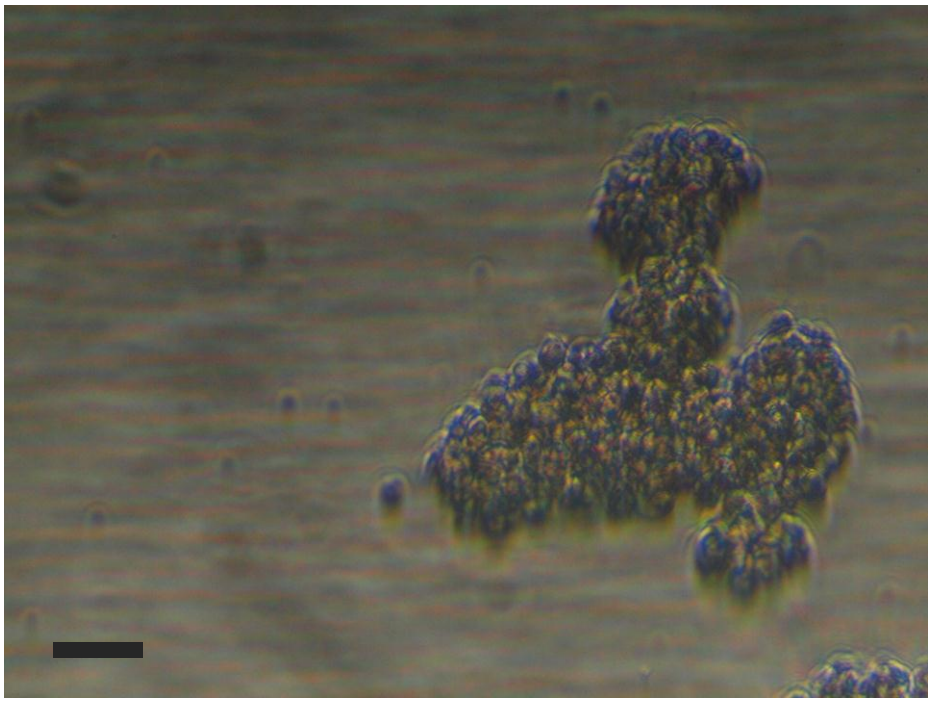

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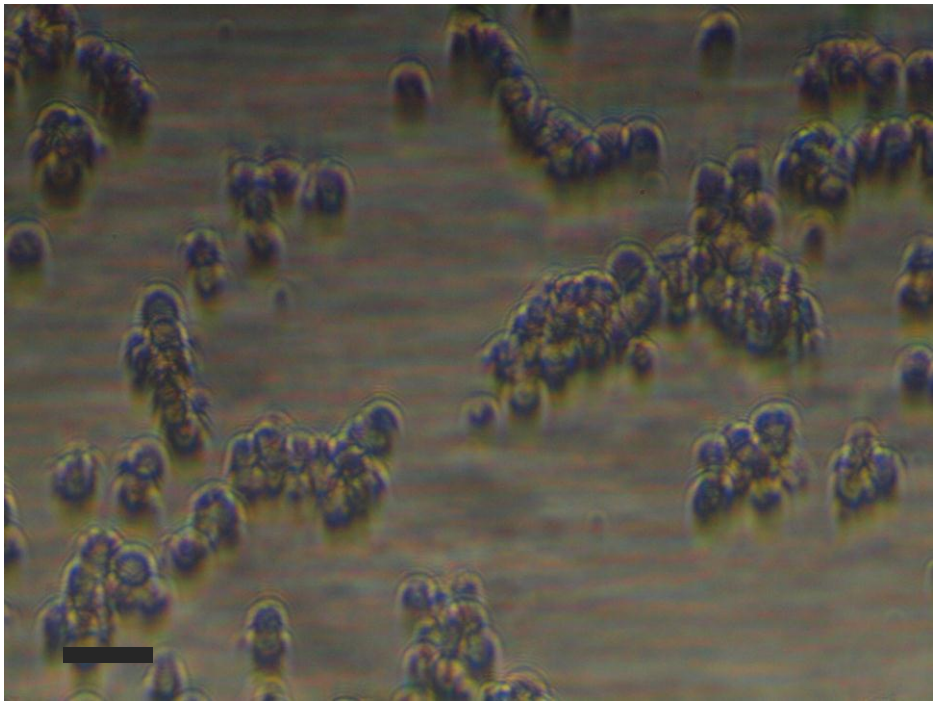

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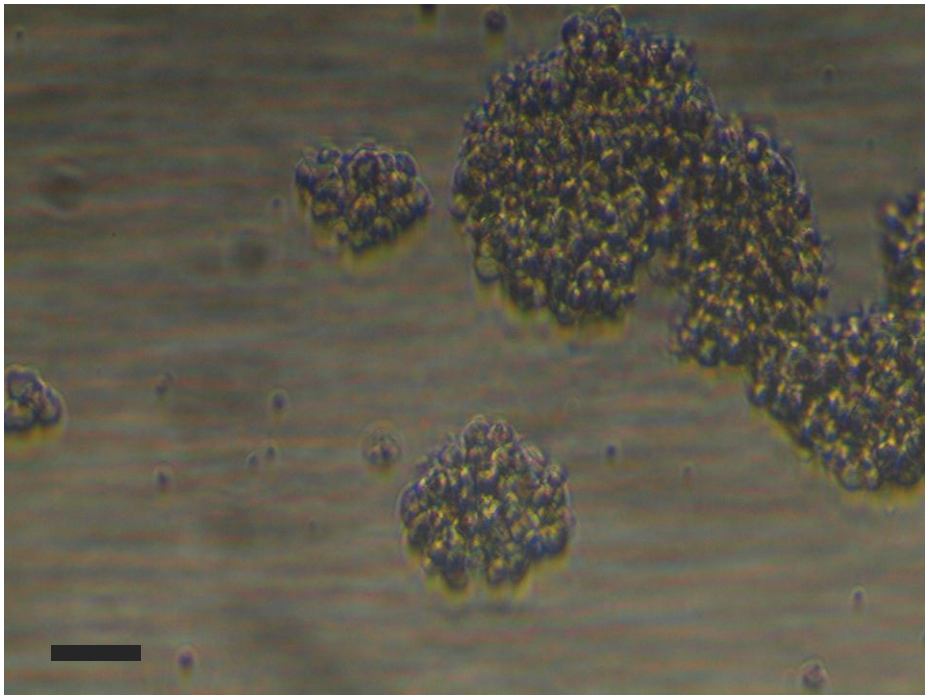

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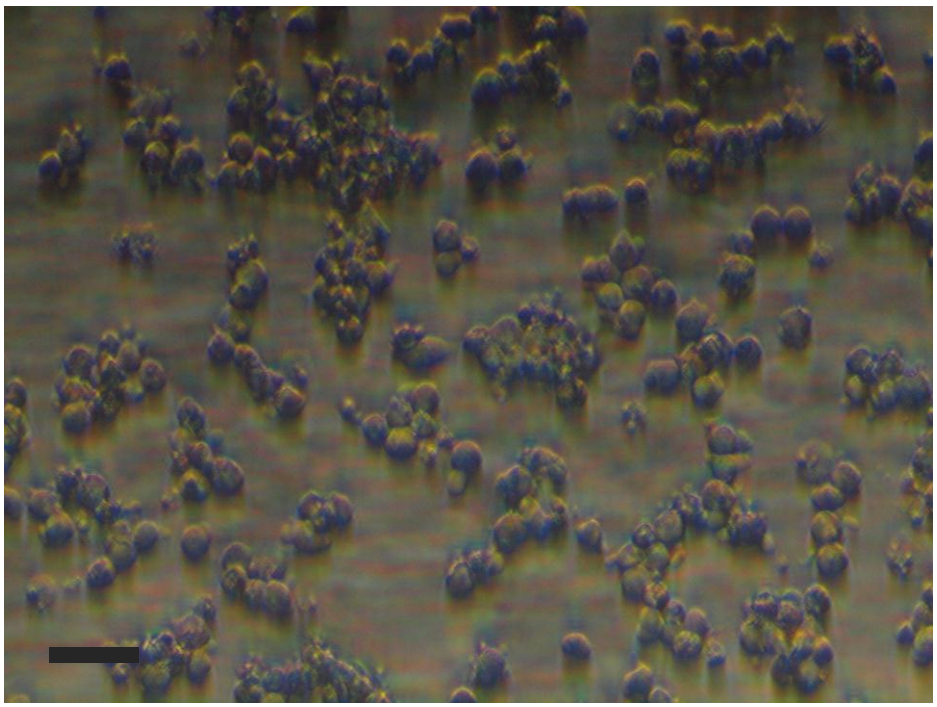

Figure 4. Morphology of SH-SY5Y. Cells were seeded at 1.0 x 10^6 per well in 6-well plate. (A) control group. Cells were treatet with (B) 2 mg of Hsp27 (C) 2 μg of pJDK-Tau, (D) 2 mg of Hsp27 and 2 μg of pJDK-Tau (E) 50nM okadaic acid for 24 h, (F) 2 mg of Hsp27 and 50nM okadaic acid, (G) 2 μg of pJDK-Tau and 50nM okadaic acid, and (H) 2 mg of Hsp27, 2 μg of pJDK-Tau and 50nM 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 polyamidoamine dendrimer). Scale bar = 25 μm

Abstract in Korean (국문초록)

알츠하이머를 특징짓는 두 가지 병리학적 증상은 세포 밖에서 amyloid-β 침전의 형성과 세포 안에서 신경원섬유엉킴 neurofibrillary tangles (NFTs) 의 형성이다. 신경원섬유엉킴 neurofibrillary tangles (NFTs) 은 과량으로 인산화가 된 불용해성 섬유로 뭉쳐진 tau 단백질들로 구성되어있다. Tau 단백질은 microtubule-associated protein 라고도 불리며, 중추신경계의 신경세포에 집중되어 있다. 신경세포들의 기능을 정상상태로 유지하기 위해서는 tau 단백질들이 세포 골격과 세포 생존에 필요한 세포소기관들을 수송하는 기능을 하는 미세소관을 안정화 시켜야 한다. Tau 단백질이 변형되면 기능을 하지 못하게 되고, 그 세포는 더 이상 세포 구조를 유지할 수 없게 된다. 과량의 인산화가 된 tau는 알츠하이머의 병리학적 지표로 생각된다. 우리는 okadaic acid라는 phosphatase 억제제를 사용하여 tau 단백질의 인산화를 유지하고 알츠하이머 세포 모델을 재현 하였다.

열 충격 단백질 27은 sHsp (small heat shock protein) 계열 중 하나로 단백질의 비정상적인 집합을 막고 단백질의 기능을 제대로 6하도록 접힘을 돕는 역할을 한다. 이 단백질을 대장균에서 발현시켰고, 여러 정제과정을 거쳐서 다른 단백질로부터 분리하였다. 우리는 열 충격 단백질 27을 세포 내로 전달하였고, 세포 내

독성을 띄는 인산화된 tau protein이 줄어드는 것을 관측하였다. 또한 신경아세포종 SH-SY5Y에서 열 충격 단백질 27은 과량으로 인산화된 tau protein으로 인한 세포사를 감소하는 것을 알 수 있었다.

주요어: 과인산화된 tau, 알츠하이머 병 , 열 충격 단백질 27, SH-SY5Y

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