이학석사학위논문

마우스 해마 신경세포에서 신경돌기 퇴화 및 신경독성을 일으키는 육합체 Aβ 단백질의 효과

Amyloid-β protein hexamers induced neuritic degeneration and neurotoxicity in the mouse hippocampal neurons

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Abstract

Amyloid-β protein hexamers induced neuritic degeneration and neurotoxicity in the mouse hippocampal neurons

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Alzheimer's disease (AD) has become one of the greatest threat to global well-being and economy. Despite the fact that about 46.8 million people worldwide are currently affected by the disease and this number is expected to be tripled by 2050, there is currently no treatment available to halt the progression of disease despite many years of extensive research.

Early reports stated amyloid fibrils were the cause of AD, but the recent research have found that soluble beta-amyloid (Aβ) oligomers are key pathological agents which are responsible for initiating a complex cascade that ultimately results in AD. However, the precise mechanism underlying AD is still unclear, and this is partly due to the unresolved nature of Aβ conformations that exert neurotoxicity and the difficulty of preparing the oligomers due to their thermodynamic

instability.

Here, we developed a novel method to prepare Aβ oligomers that stably maintain each oligomeric state from dimer to hexamer, respectively. We constructed the expression vectors to express Aβ and fused proteins that have been reported to form oligomers in a stable manner (leading proteins) at its N-terminus. We expect, in this way, this would help inducing a stable formation of each form of Aβ oligomers. With this method, we prepared leading proteins, Aβ_{1-40} and Aβ_{1-42} present in each of five different oligomeric state.

The mouse hippocampal neurons treated with hexameric Aβ_{1-42} exhibited a severely disrupted morphology, suggesting the Aβ_{1-42} hexamers, but not other types of oligomers, play a key role in AD pathogenesis. We concluded that Aβ_{1-42} hexamers are neurotoxic species, stressing the importance of the hexamer as a potential target in development of AD immunotherapy.

Keywords: Alzheimer's diesase, beta-amyloid, oligomers, neurotoxicity, immunotherapy
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Introduction

A. Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia. Currently, about 46.8 million people worwide are being affected by AD, and this number is believed to be close to 131.5 million by 2050 when all of surviving babyboomers turn at least 85 years old (World Alzheimer Report, 2015). The estimated prevalence is about 10%-30% among those over 65 years of age, and the average life expectancy for those diagnosed with AD is around 8-12years (Masters et al., 2015).

AD is a progressive neurodegenerative disease, characterized clinically by cognitive and behavioural impairment, and neuropathologically by extraneuronal accumulation of Aβ protein in senile plaques and intraneuronal deposition of hyperphosphorlylated tau. At early stages of AD, the disease affects hippocampus, which is located in the medial temporal lobe and responsible for learning and memory. As the disease advances, damage spreads to other areas of the neocortex and the resultant widespread cell death causes brain shrinkage at a different rate between individuals.

The current clinical treatment strategies against AD is to relieve symptoms of AD, but do not prevent or slow the progression of AD (Prince et al., 2011). One of the reasons is uncertainty over genetic and environmental risk factors despite many efforts to understand the mechanism responsible for AD pathology.

B. Familial/Sporadic forms of Alzheimer's Disease

There are two major forms of AD exitst; familial and sporadic AD. The sporadic form accounts for more than 95% of all cases, is commonly developed among individuals over 65 years of age , and is characterized by a late onset (mean age of 80－90 years). The main cause is the failure to clear the beta-amyloid (Aβ) peptide from the interstices of the brain. However, the etiology of the sporadic form is not fully understood. A large number of genetic risk factors have been implicated in a AD such as *APOE, PICALM, CD33, TREM2, ABA7, CLU, CR1,* although their exact roles in the disease remain to be elucidated (Masters et al., 2015).

Unlike the sporadic form, the familial form is exceedingly rare, making up less than 1% of reported AD cases. It is also called "autosomal dominant inherited Alzheimer's disease (DIAD)". The patients with this form of AD are reported to have pathogenic mutations in three genes that are responsible for processing of Aβ; amyloid precursor protein (*APP*), presenilin 1 (*PS1*), and presenilin 2 (*PS2*). These mutations leads to overproduction of an aberrant form of Aβ, and as a result, the disease occurs at a significantly younger age (mean age of 45 years) compared to the patients with sporadic AD. The sporadic and familial forms are comparable in terms of symptoms, disease progression and biomarker profiles (Masters et al., 2015).

Since Aβ is involved in both forms of the disease, many research has been aimed at explore highly dynamic nature of Aβ aggregation.

C. Processing of amyloid precursor proteins (APP)

Amyloid precursor protein (APP) is a type I, transmembrane protein that resembles a signal transduction receptor, and is encoded on chromosome 21. APP is highly expressed in neuronal cells but also in many other cell types (Forloni et al., 1992), but its precise function remains unknown.

Aβ is a 40-43 amino acid peptide derived by proteolytic cleavage of APP. There are two pathways that have been described for APP processing; the non-amyloidogenic and the amyloidogenic pathway (Ehehalt et al., 2003).

Most APPs have been known to undergo non-amyloidogenic pathway, in which APP is cleaved within the Aβ domain by α-secretase resulting in secretory amyloid precursor protein α (sAPPα) and a C-terminal fragment (CTF)-83. CTF-83 can then be cleaved by γ-secretase producing P3 (Aβ17-40/42) peptide and APP intracellular domain (ACID), and thus preclude the formation of pathogenic Aβ (Chow et al., 2009).

In amyloidogenic pathway, alternatively, APP is initially cleaved by β-secretase at the N-terminus of the Aβ domain, generating β-secretase-cleaved soluble APP (sAPPβ) and CTF-99. Subsequential processing of CTF-99 by γ-secretase results in ACID and a mixture of 38-43 amino acid Aβ peptides with carboxyl-terminal heterogeneity, and these peptides are the main constituents of the plaques (Chow et al., 2009).

The two most common isoforms implicated in AD are $A\beta_{1-40}$ and $A\beta_{1-42}$, and they are very comparable in terms of molecular structures, except that $A\beta_{1-42}$ contains two additional hydrophobic amino acids, Ile-41 and Ala-42, at its carboxyl terminal. Since the carboxyl terminal region of $A\beta$ is highly hydrophobic, whereas the amino terminal is relatively hydrophilic, two hydrophobic residues make $A\beta_{1-42}$ far more hydrophobic than $A\beta_{1-40}$, and therefore, due to its hydrophobicity, they are less soluble and aggregate much more quickly (Liu et al., 2005).

$A\beta_{1-40}$ is the most prevalent soluble species which accounts for more than 90% of total secreted $A\beta$ in cerebrospinal fluid(CSF) at low nanomolar concentrations (Vigo-Pelfrey et al. 1993). Also, $A\beta_{1-40}$ presents at about 10-fold higher levels than $A\beta_{1-42}$ in plasma (Zou et al., 2003). Unlike $A\beta_{1-42}$, this species exhibits a slow rates of fibil formation due to its hydrophilic nature (Liu et al., 2005).

Although $A\beta_{1-42}$ is a minor species and less abundant than $A\beta_{1-40}$, it is more fibilogenic and cytotoxic and have a strong tendency to aggregate to form the plaques (Selkoe, 2001). Numerous evidences have also demonstrated that $A\beta_{1-42}$ is more heavily enriched in AD brains than in age-matched control brains, indicating $A\beta_{1-42}$ is a major species that correlates with AD pathogenesis (Kuperstein et al., 2010).

D. Amyloid cascade hypothesis

Early versions of amyloid cascade hypothesis, which posited Aβ fibrillar aggregates found in extracellular plaques drive neurodegeneration in AD, has dominated the field of AD research for the past decades.

Accumulating evidences, however, suggest that plaques prefibrillar soluble Aβ oligomers are the pathological initiator of a cascade leading to neurodegeneration, and this modified version is often called "amyloid beta cascade hypothesis".

E. Current treatment of AD

Currently, there are no treatment available to slow or stop the progression of the disease. Since soluble Aβ_{1-42} oligomers are known to play crucial roles in AD pathogenesis, immunotherapy targeting soluble Aβ_{1-42} oligomers has been regarded as a potential treatment for AD. However, recently-developed anti-Aβ immunotherapies has revealed unsatisfactory results because they failed to slow or improve cognitive decline and to extent life span (Kohyama et al., 2015).

One of the most challenging issues in AD research is that little is known about the nature of the Aβ mediating the pathology. Also, these oligomers are thermodynamically unstable, thereby making even more difficult to prepare soluble Aβ oligomers at different oligomeric state.

Therefore, we employed a novel method to overcome this issues. We have chosen the five diffrent proteins (leading proteins) that have been previously shown to stably form oligomers from dimers to hexamers, respectively. Also, they haven't been reported to induce neurotoxicity. Fusion of each of these leading proteins at N-terminus of Aβ would result in formation of stable Aβ oligomers at each oligomeric state, as their leading proteins induce. We also inserted thrombin cleavage site between each of these proteins and Aβ, and this is to obtain Aβ oligomers without leading proteins after cleavage reaction, and these oligomers are expected to maintain their oligomeric states stably.

We expected this would enable to reveal which form(s) of Aβ_{1-42} oligomers are neurotoxic, thereby providing potentials to develop a novel immunotherapy that specifically removes neurotoxic Aβ oligomers.

MATERIALS AND METHODS

Construction of expression vectors. The hexahistidine tag coding sequence, each of leading proteins (Hrs, Foldon of bacteriophage T4 fibritin, Cytidine Deaminase (CDA), Nucleoplasmin (NPM), Heat shock protein 90 (HSP90)), thrombin cleavage site and Aβ-$_{42}$ or Aβ-40 were synthesized and amplified by PCR. The resulting PCR products were cloned into multiple cloning sites (MCS) of Plasmid pET28a that carries the T7 promoter and kanamycin resistance gene.

Protein expression and purification. All recombinant proteins were produced in E. coli strain BL21(DE3) transformed with the appropriate plasmid. Bacteria were grown at 37° C in LB-agar plates, supplemented with appropriated antibiotics [kanamycin (Km), 50 μg/ml; chloramphenicol (Cam), 25 μg/ml]. Exponential 500-mL cultures grown with shaking at 37° C in LB medium supplemented with 50 μg/ml kanamycin and 25 μg/ml chloramphenicol were induced at OD_{600} = 0.5 with 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for an additional 4 hours. Then the cells were harvested by centrifugation, washed twice with PBS, resuspended in lysis buffer, and disrupted by sonication at 4° C. The homogenates were cleared at 12,000g for 10min. The obtained clarified extracts were used for purification by fast liquid chromatography (FPLC), while proteins present mainly in inclusion bodies were solublized by 4M UREA and refolded in favorable conditions.

Western blotting and Coomassie blue staining. Purified recombinant proteins were separated by SDS-PAGE. For Western blotting, the gel was transferred onto PVDF membrane, and probed with antibodies against hexahistidine tag. Proteins were detected with HRP-conjugated anti-mouse IgG antibody. For coomassie blue staining, the gel was stained with Coomassie blue for 40 min, and de-stainied with the destaining buffer containing 5% Methanol and 7% acetic acid every 1.5 hr for multiple times.

Establishment of primary mouse neuron cultures. Hippocampi were isolated from E17.5 fetuses in Hepes-buffered Hank's balanced salt solution, trypsinized (0.25%) for 20 min at 37 ° C, and plated in MEM growth medium containing 10% FBS (heat-inactivated), 2.25% glucose, 2mM Glutamine and 1% antibiotic, on poly-L-lysine-coated culture dishes. After 4hours, the medium was replaced with Neurobasal medium containing B27 supplement, 2mM Glutamine and 1% antibiotic. Subsequent half-media changes were performed every 3-4 days. All cell culture supplies were obtained from GIBCO Life Sciences (Gaithersburg, MD, USA).

Result

A. Generation of expression vectors that induce formation of soluble Aβ_{1-40} and Aβ_{1-42} oligomers from dimer to hexamer.

First, to induce oligomerization of Aβ_{1-42} or Aβ_{1-40}, we have chosen five different proteins (that is, Hrs, Foldon of bacteriophage T4 fibritin, Cytidine Deaminase (CDA), Nucleoplasmin (NPM), Heat shock protein 90 (HSP90)) that have been reported to have capacity to form oligomers from dimers, trimers, tetramers, pentamers and hexamers, respectively. Therefore, we expected that we could obtain the stable Aβ oligomers at each oligomeric states by fusing leading proteins at their N-terminus (Table 1).

Plasmid pET28a was used for construction and expression of recombinant proteins in *E.Coli.* Each of leading proteins was inserted into the vector in order to stimulate oligomerization. A hexa-histidine tag was added to the its N-terminus to facilitate the purification of the proteins by Ni^{2+}-NTA affinity chromatography. Also, thrombin cleavage site was incorporated between each of leading protein and Aβ_{1-42} or Aβ_{1-40}, and this is to gain Aβ_{1-42} or Aβ_{1-40} oligomers with no leading protein attached. With this strategy, we assumed that these constructs would yield soluble Aβ_{1-42} and Aβ_{1-40} oligomers that are aligned towards C-terminus (Fig 1).

Figure 1. Schematic representation of expression vector (pET28). All expression vectors for bacterial expression were modified variants of a pET28a vector containing an N-terminal hexahistidine tag, gene of oligomerization-inducing domain, thrombin cleavage site and genes of proteins we are interested in (that is, $A\beta_{1-40}$, $A\beta_{1-42}$), respectively.

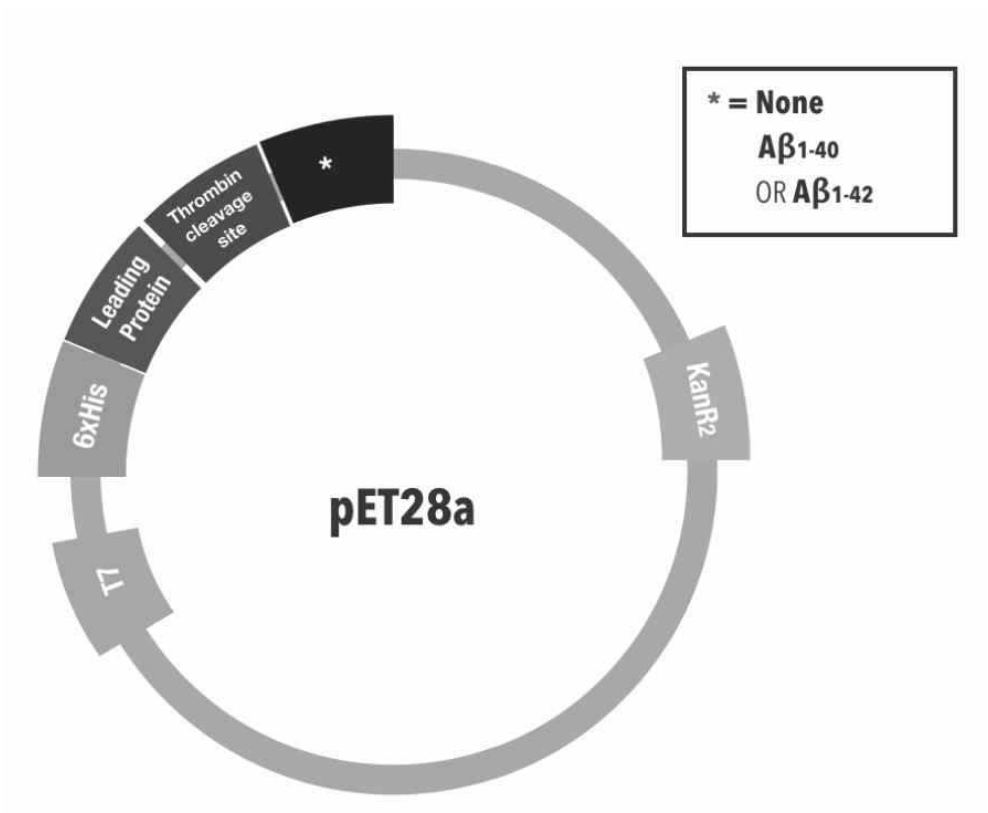
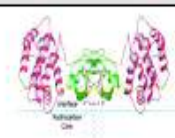

* = None
Aβ1-40
OR Aβ1-42
*
Thrombin cleavage site
Leading Protein
6xHis
T7
KanR2
pET28a

Table 1. Summary of amino acid regions, predicted structures and crystal structures of leading proteins.

Inducer molecule	Amino acid region	Expected structure	Crystal structure
Hrs tandem domains (Hrs)	1-221	Dimer	
Foldon domain of bacteriophage T4 fibritin (Foldon)	457-483	Trimer	
Cytidine deaminase (CDA)	1-146	Tetramer	
Nucleoplasmin (NPM)	1-122	Pentamer	
Heat shock protein 90 (HSP90)	293-732	Hexamer	

B. Formation of oligomers at five different oligomeric states

To determine whether Aβ exerts neurotoxicity on the mouse primary hippocapal neurons, the recombinant proteins were purified from *E.coli.* The proteins that were soluble at high levels of expression were purified by Ni^{2+}-NTA affinity chromatography. On the other hand, the proteins that were soluble at low levels of expression and found mostly in inclusion bodies (mostly, Aβ_{1-42} and Aβ_{1-40} fused with leading proteins rather than leading proteins only) were denatured with chaotropic agent, 8M UREA, and refolded in appropriate conditions.

Both affinity-purified and refolded proteins were prepared and analyzed on SDS-PAGE. Most of the proteins migrated as the predicted size, while some proteins present majorly as monomers, and this was revelead by Coomassie blue staining and Werstern blot analysis (Fig 2).

C. Validated use of leading proteins as control proteins

Before we determine which form of soluble Aβ oligomers at each oligomeric state from dimer to hexamer, respectively, is the neurotoxic species responsible for AD pathogenesis, it is imeprative to check whether leading proteins only have no neurotoxic effects. Since leading proteins with no Aβ fused would be used as control proteins for later experiments, this validation step is compulsory to exclude this possibility.

Figure 2. Monomeric and oligomeric recombinant proteins purified from *E.Coli.* The overexpressed proteins were purified by distinct methods depending on their solubility. The purified proteins were separated by SDS-PAGE, and Coomassie blue staining and Western blot with hexa histidine tag antibody was performed, and we confirmed that proteins are at the expected oligomeric states being induced by the oligomer-forming ability of their leading proteins. **(a) Hrs (dimer)** size of monomeric leading protein (lane1,2); ~24kDa, of $A\beta_{1-40}$, $A\beta_{1-42}$ (lane3,4, respectively); ~29kDa. **(b) Foldon (trimer)** size of monomeric leading protein (lane1,2); ~3kDa, of $A\beta_{1-40}$, $A\beta_{1-42}$ (lane3,4, respectively); ~8kDa. **(c) CDA (tetramer)** size of monomeric leading protein (lane1,2); ~16kDa, of $A\beta_{1-40}$, $A\beta_{1-42}$ (lane3,4, respectively); ~22kDa. **(d) NPM (pentamer)** size of monomeric leading protein (lane1,2); ~14kDa, of $A\beta_{1-40}$, $A\beta_{1-42}$ (lane3,4, respectively); ~19kDa. **(e) HSP90 (hexamer)** size of monomeric leading protein (lane1,2); ~48kDa, of $A\beta_{1-40}$, $A\beta_{1-42}$ (lane3,4, respectively); ~53kDa. (+); boiled, (-); unboiled.

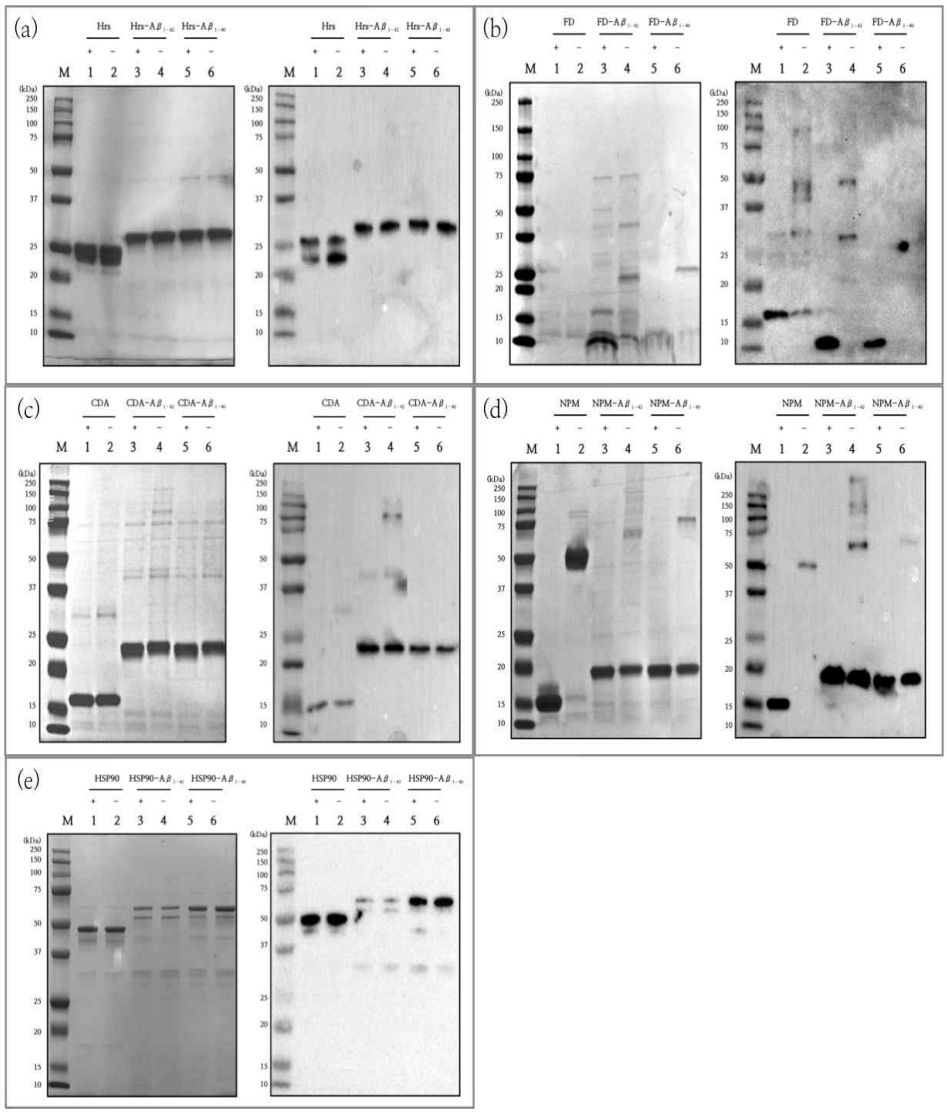
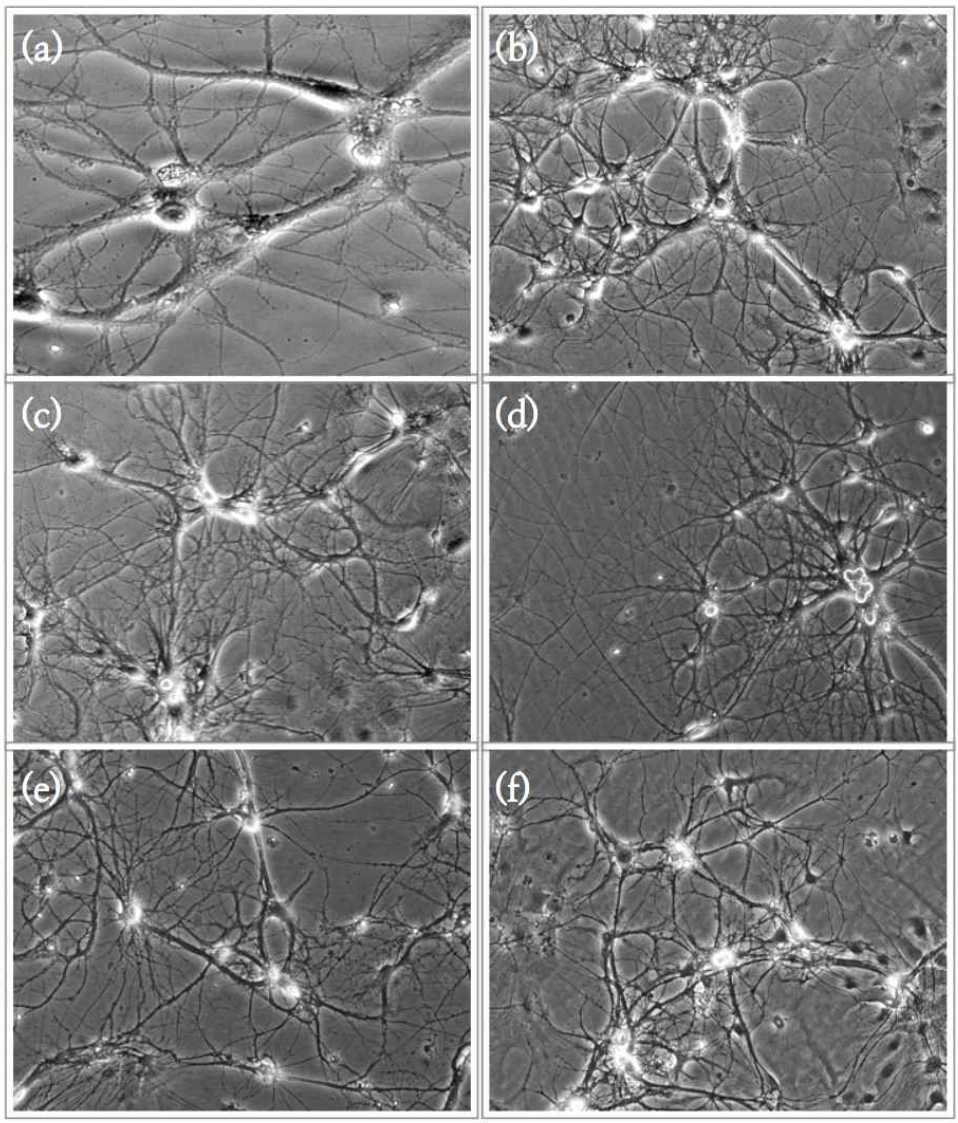

(a)
Hrs
Hrs-Aβ1-42
Hrs-Aβ1-40
M 1 2 3 4 5 6
(kDa)
250
150
100
75
50
37
25
20
15
10
(b)
FD
FD-Aβ1-42
FD-Aβ1-40
(c)
CDA
CDA-Aβ1-42
CDA-Aβ1-40
(d)
NPM
NPM-Aβ1-42
NPM-Aβ1-40
(e)
HSP90
HSP90-Aβ1-42
HSP90-Aβ1-40

Figure 3. Validated use of leading proteins as control proteins. Each leading proteins (10nM) were treated to the mouse hippocampal neurons after cultured for 6 days, and the cells were examined after 12 days of treatment. Microscopic observation revealed treatment of each leading protein did not alter neuronal cell viability compared with non-treated control, indicating all of leading proteins doesn't have toxic effect at 10nM. (a); Non-treated, (b); Hrs (dimer), (c); Foldon (trimer), (d); CDA (tetramer), (d); NPM (pentamer), (e); HSP90 (hexamer).


(a)
(b)
(c)
(d)
(e)
(f)

To this purpose, we cultured the mouse primary neuronal cells from hippocampus where is known to be particulary affected by Alzheimer's disease. Each of five leading proteins were treated once at concentration of 10nM after 6 days of culture when the neuronal cells were thought to be fully differentiated. On day 12, cell viability was determined by microscopic observation and compared with that of non-treated cultures. Leading proteins did not show any notale effect on cell viability marked by normal dendritic growth and branching (Fig 3), indicating that the leading proteins does not have neurotoxic effects on the cells and can be used as control proteins.

D. Non-neurotoxic effects of $A\beta_{1-40}$ hexamers

$A\beta_{1-42}$ is one of the two major isoforms that constitues the senile plaque which is one of the neuropathological characteristics of Alzheimer disease (AD), and is generated through the sequential cleavage of APP by β- and γ-secretase (Liu et al., 2005).

Although $A\beta_{1-42}$ is much less abundant than $A\beta_{1-40}$, making up only about 10% of total secreted $A\beta$ (Selkoe, 2002), many reports have previously demonstrated that $A\beta_{1-42}$ is more pathogenic than $A\beta_{1-40}$ and prone to oligomerization and more readily forms the fibrilar deposits due to its hydrophobic nature. (Liu et al., 2005). Therefore, $A\beta_{1-42}$ is regarded to be more strongly implicated in pathogenesis of Alzheimer's disease than $A\beta_{1-40}$ although the reason for this is not completely understood. While this concept is generally accepted at

present, relatively little is known about at which oligomeric state(s), $A\beta_{1-42}$ exerts neurotoxic effects.
Soluble $A\beta_{1-42}$ oligomers have been demonstrated to be responsible for the initiation of a complex cascade which leads to neurodegeneration in Alzheimer's disease. On the basis of previous studies, we hypothesized that there might be one or more types of soluble $A\beta_{1-42}$ oligomers that induce neurodegeneration.

To test this hypothesis, cultures were prepared from mouse hippocampus on embryonic day 17.5, and the cells were treated with $A\beta_{1-42}$ at each oligomeric state after 6 days of culture at concentrations of 10nM. On day 12, cell viability was determined by microscopic observation and compared with that of cultures treated with leading proteins only. Exposure to $A\beta_{1-42}$ hexamer at a concentration of 10nM resulted in the complete neuronal loss (Figure 4). Under the same condition, on the other hands, other oligomers did not significantly affect cell survivals.

E. Non-neurotoxic effects of $A\beta_{1-40}$ oligomers

$A\beta_{1-40}$ is a major soluble $A\beta$ species produced from the sequential APP processing by β- and γ-secretase just as $A\beta_{1-42}$, and is the most abundant isoform, making up about 90% of the total $A\beta$ present in CSF (Selkoe, 2002). Unlike $A\beta_{1-42}$, two hydrophobic amino acids at its C-terminal are absent in $A\beta_{1-40}$, which makes it more soluble and slowly converts into the plaque (Liu et al., 2005). It is still unclear

Figure 4. $A\beta_{1-42}$ hexamers exhibited neurotoxicity.

$A\beta_{1-42}$ fused with five different leading proteins (10nM), thereby present in five different oligomeric states from dimer to hexamer, respectively, were treated to the mouse hippocampal neurons after cultured for 6 days as described, and the cells were examined after 12 days of treatment. In the culture treated with hexameric $A\beta_{1-42}$, significant neurodegeneration was observed, whereas the cell viability of cultures treated with at other $A\beta_{1-42}$ oligomer forms (that is, Hrs(dimer), Foldon(trimer), tetramer(CDA) and NPM(pentamer)) was comparable to non-treated control.

(a); Non-treated, (b); Hrs (dimer), (c); Foldon (trimer), (d); CDA (tetramer), (d); NPM (pentamer), (e); HSP90 (hexamer).

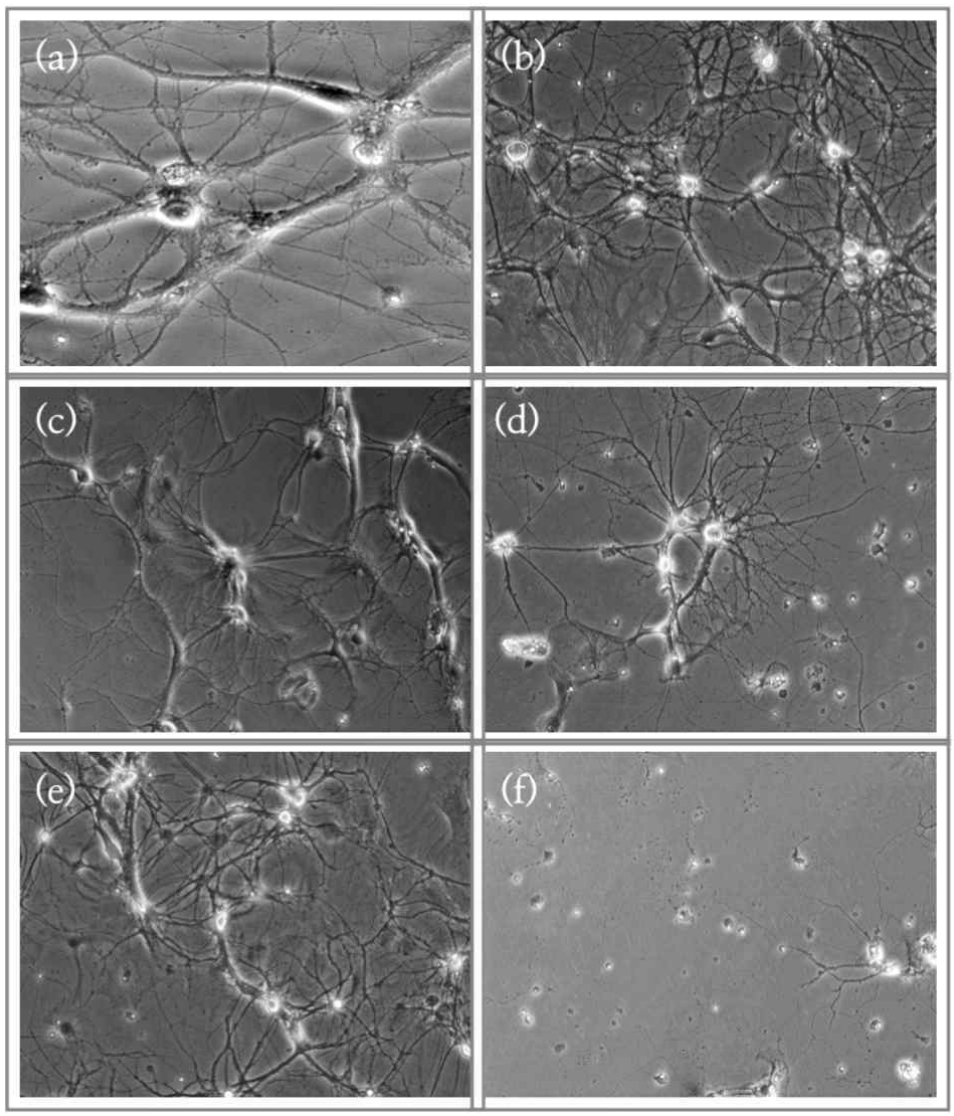

(a)
(b)
(c)
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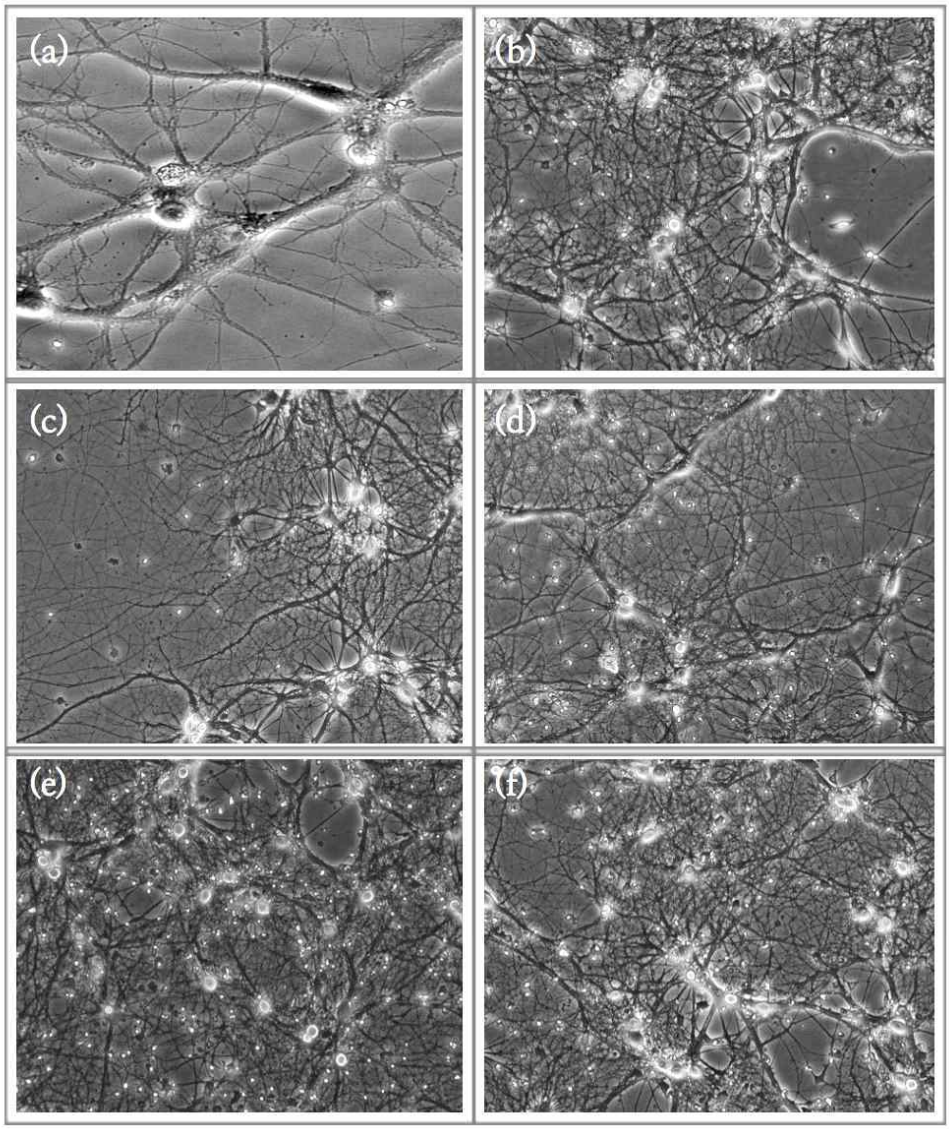
Figure 5. None of $A\beta_{1-40}$ oligomers did not affect the cell vialbility

$A\beta_{1-40}$ fused with five different leading proteins (10nM), thereby present in five different oligomeric states from dimer to hexamer, respectively, were treated to the mouse hippocampal neurons after cultured for 6 days as described and the cells were examined after 12 days of treatment. $A\beta_{1-40}$ oligomers in any oligomeric state did not affect neuronal survival at any concentrations, indicating $A\beta_{1-40}$ oligomers less pathogenic compared to $A\beta_{1-42}$ oligomers consistent with many of previously reported studies.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whether $A\beta_{1-40}$ is associated with AD pathology. Thus, we decided to test whether the $A\beta_{1-40}$ oligomers have no toxic effects on primary neuronal cells.

Cultures of dissociated mouse hippocampal neurons were prepared from E17.5 mouse embryos. After 6 days *in vitro*, each form of $A\beta_{1-40}$ oligomers was treated at concentrations of 10nM. On day 12, cell viability was determined by microscopic observation and compared with that of cultures treated either with leading proteins only or $A\beta_{1-42}$. Unlike $A\beta_{1-42}$, none of the five different types of oligomers displayed the neurotoxic activity on the hippocampal neuronal cells. We determined that, consistent with many previous observations, soluble $A\beta_{1-40}$ are non-neurotoxic.

Taken together, consistent with many previous studeis, our results indicate that treatment of $A\beta_{1-42}$ significantly reduced the neuronal viability, but strikingly this phenomenon was only observed for the cells treated with hexameric $A\beta_{1-42}$ at a concentration of 10nM. To our knowledge, none of research groups prepared different types of soluble $A\beta$ at once, even though there are papers showing one specific type of oligomers is neurotoxic or non-neurotoxic. Therefore, this study is important in that five different forms of stably-maintained $A\beta$ oligmers were tested at once and one specific form only showed neurodegeneration marked by neuritic degeneration.

Discussion

Currently, there are drug and non-drug treatment options available for AD, but these can only temporarily alleviate symptoms, but cannot cure the disease. Many scientists continue to believe that $A\beta_{1-42}$ is the main pathogenic factor responsible for the degenerative changes that occur in the brain during AD. Therefore, immunotherapy that uses antibodies against $A\beta$ has been believed to prevent the thier further aggregation in the form of the plaque, and so far this has been regarded one of the most promising strategies for the treatment of AD. Consequently, large pharmaceutical companies in the world such as Eli Lilly, Merk and Biogen have invested the significant amount of time and capital in devoping a drug to tackle the underlying causes of the disease, the recent AD trials have been unsuccessful. Some companies have been frustrated by the repeated failures and high costs, and abandoned to develop AD treatment drugs.

One of the causes of failures comes from the complexity of the disease. Currently, most of the AD research is based on the belief that soluble $A\beta$ is the main pathogenic factor that trigger a complex degenerative cascade that ultimately leads to AD. However, it is still largely unknown which form of $A\beta$ oligomers induce neurotoxicity due to the unresolved nature of the $A\beta$ conformations. The fact that there are no standard methods to prepare $A\beta$ at each oligomereic state and every research group differentially prepare $A\beta$ oligomers impedes the precise understanding of the nature of $A\beta$ oligomers.

To overcome this issue, we designed the expression vectors to express Aβ fused with five proteins, and each protein stimulate the formation of Aβ oligomers at each oligomeric state from dimer to hexamer at its N-terminus. This way, we expected that we would be able to obtain the Aβ oligomers which maintain their original oligomeric states unlike Aβ oligomers which is unstable thermodynamically in physiological conditions whose conformations are constantly changed. Notably, None of these protein have been reported to have neurotoxicity.

To obtain Aβ oligomers purely in the absence of the leading proteins, we incorporated the thrombine cleavage sequence between the leading proteins and Aβ. The recombinant proteins were purified either by FPLC when the majority of proteins are soluble or a series process of denaturing and refolding when proteins mainly present in the inclusion bodies, and we confirmed whether they appropriately formed oligomers by coomassie blue staining and western blot.

The present study found that Among Aβ_{1-42} at five other oligomeric states from dimer to hexamer, only hexameric Aβ_{1-42} exhibited a severe neurotoxic effect on the cells, marked by severe fragmentation of neurites and cell death. As the neurotoxicity of leading proteins themselves have not been reported so far, neurodegeneration was not observed for the cells treated with each leading protein. Also, Aβ_{1-40} that has taken any form of oligomers did not affect the viability of neurons at any treated concentrations.

This study is significant in that it has revealed the replationship between a specific form of Aβ_{1-42} and AD pathogenesis for the first time by

utilizing the novel method of purifying five different forms of oligomers that has been shown to maintain their oligomeric state in a stable manner.

However, the present study leaves a few questions unanswered. One is as to whether the induced-oligomeric states would be preserved following the removal of leading proteins by thrombin. The ultimate purpose of this study is to develop antibodies that specifically binds to pathological form of $A\beta_{1-42}$, thereby prevent the formation of the aggregates implicated in AD. Therefore, distruption of conformations would preclude the further development. To test this, we have performed the thrombin cleavage assay on hexameric $A\beta_{1-42}$. However, the cleavage efficiency was very low, and only about 20% of the total $A\beta_{1-42}$ seemed to have when confirmed by both Western blotting and Coomassie blue staining. Therefore, we decided to replace the thrombin cleavage tag with four other cleavage tags, that can be cleaved by Human Rhinovirus 3C Protease (3C), Enterokinase (EKT), Factor Xa (FXa) and Tobacco Etch Virus Protease (TEV), respectively. We are now expecting that this replacement achieves full cleavage efficiency.

Another concern is that the neurotoxic effect of hexamer $A\beta_{1-42}$ is virtually the consequences of the hexameric $A\beta_{1-42}$ without any effect of leading compound involved. If this effect is entirely due to the hexameric conformation of $A\beta_{1-42}$, the identical consequences have to be observed on neurons when treated when the leading protein is substituted with other proteins that have capacity to form hexamer.

Therefore, finding another proteins that can replace HSP90 will be needed to reassure the neurotoxic effect that hexameric conformation uniquely have.

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국문초록

마우스 해마 신경세포에서 신경돌기 퇴화 및 신경독성을 일으키는 육합체 Aβ 단백질의 효과

알츠하이머병은 전 세계 사람들의 건강과 경제를 위협하고 있다. 현재 전 세계적으로 약 4천 6백 8십만 명이 이 질환을 앓고 있다고 보고되고 있으며, 2050년에는 그 수가 세배에 다다를 것이라고 예상되고 있다. 알츠하이머병에 대한 연구는 오랜 기간 이어져 왔음에도 불구하고, 현재 이 병의 진행을 막을 수 있는 치료법은 존재 하지 않는다.

초기의 보고들은 아밀로이드 섬유가 병의 원인일 것이라고 생각했으나, 근래 연구 결과를 통해 가용성 베타 아밀로이드(beta-amyloid, Aβ) 올리고머가 신경독성을 가진 물질로서, 일련의 복잡한 과정을 시작하여 최종적으로 병에 이르게 한다는 것을 밝혔다. 하지만 여전히 Aβ가 정확히 어떤 기전으로 신경퇴화를 일으키는지는 정확히 밝혀지지 않았으며, 그 원인 중 하나는 여러 형태의 올리고머를 취하는 가용성 Aβ 올리고머 중 정확히 어떤 형태가 신경 독성을 일으키는지 알려지지 않은 것이라고 보고 있다.

따라서, 이 연구를 통해 이합체부터 육합체 Aβ를 안정적으로 유지할 수있는 새로운 방법을 개발하고자 하였다. 먼저, 신경독성이 보고되지 않은 단백질들 중 이합체부터 육합체를 안정적으로 형성한다고 알려진 단백질들(leading proteins, 리딩 단백질)을 선정하였고, 이들 각각을 Aβ의 N-말단에 결합시킴으로서 각 형태의 Aβ 올리고머 형성을 유도하고자 하였다. 이 방법을 통해, 이합체부터 육합체까지 각각의 올리고머 형태를

유지하는 Aβ$_{1-40}$과 Aβ $_{1-42}$를 얻을 수 있었다.

이 후, 마우스 해마 신경세포에 이들을 처리했을 때, 육합체 베타 Aβ$_{1-42}$를 처리한 세포에서만 뉴런의 신경돌기가 퇴화되는 것을 확인하였다. 이 연구를 통해 신경 독성을 갖는 Aβ $_{1-42}$를 찾음으로서 향후 면역치료체 개발 가능성이 높아졌다.

주요어: 알츠하이머병, 베타 아밀로이드, 올리고머, 신경독성, 면역치료제
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