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Effects of Newly Synthesized Recombinant Human Amyloid-β Complexes and Poly-Amyloid-β Fibers on Cell Apoptosis and Cognitive Decline

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The main pathological hallmark of Alzheimer's disease is the deposition of amyloid-beta (Aβ) peptides in the brain. Aβ has been widely used to mimic several aspects of Alzheimer's disease. However, several characteristics of amyloid-induced Alzheimer's disease pathology are not well established, especially in mice. The present study aimed to develop a new Alzheimer's disease model by investigating how Aβ can be effectively aggregated using prokaryotes and eukaryotes. To express the Aβ42 complex in HEK293 cells, we cloned the Aβ42 region in a tandem repeat and incorporated the resulting construct into a eukaryotic expression vector. Following transfection into HEK293 cells via lipofection, cell viability assay and western blotting analysis revealed that exogenous Aβ42 can induce cell death and apoptosis. In addition, recombinant His-tagged Aβ42 was successfully expressed in *Escherichia* coli BL21 (DE3) and not only readily formed Aβ complexes, but also inhibited the proliferation of SH-SY5Y cells and *E. coli*. For in vivo testing, recombinant His-tagged Aβ42 solution (3 μg/μl in 1× PBS containing 1 mM Ni^{2+}) was injected stereotaxically into the left and right lateral ventricles of the brains of C57BL/6J mice (n = 8). Control mice were injected with 1× PBS containing 1 mM Ni^{2+} following the same procedure. Ten days after the sample injection, the Morris water maze test confirmed that exogenous Aβ caused an increase in memory loss. These findings demonstrated that Ni^{2+} is capable of complexing the 50-kDa amyloid and that intracerebroventricular injection of Aβ42 can lead to cognitive impairment, thereby providing improved Alzheimer's disease models.

Keywords: Amyloid-beta, recombinant protein, apoptosis, exogenous Aβ42 complex

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is associated with impairment in cognitive function and memory and is the most common cause of dementia. The most pronounced histopathological hallmark of AD is the deposition of amyloid-beta (Aβ) peptides in the brain, which is accompanied by calcium metabolism disorder, mitochondrial disorder, and oxidative stress [1–3]. However, the mechanisms underlying AD pathogenesis remain largely unknown. Amyloid refers to fibrillar aggregates consisting of common β-pleated structures; neuritic plaques of this type appear to be strongly associated with reactive and degenerative processes; this is in contrast to diffuse plaques,

which are thought to be relatively harmless [4]. One of the major components of neuritic plaques is a 42-amino-acid Aβ peptide derived from amyloid precursor protein (APP), an integral membrane protein expressed in many tissues, including the synapses of neurons. APP has been highlighted as a key regulator of synapse formation, neural plasticity, and iron homeostasis [5]. Previous studies have reported that 1-42 Aβ (Aβ42) is produced in lower amounts than the 1-40 Aβ (Aβ40) peptide. However, preferential deposition of Aβ42 is attributed to the COOH-extended form of the peptide, which is more insoluble than Aβ40 and more prone to aggregation and formation of anti-parallel β-pleated sheets. Aβ42 can also act as seeds to initiate Aβ40 aggregation. Several studies have delineated two additional pathways of APP processing that involve both amyloid polypeptides. First, an endosomal/lysosomal pathway generates a complex set of APP-related membrane-bound fragments, some of which contain the entire Aβ sequence [6]. Second, through mechanisms that are not fully understood, Aβ1-40 is secreted into the conditioned medium and is present in cerebrospinal fluid in vivo. The proteolytic enzymes responsible for cleaving the NH_2 and COOH termini of Aβ are termed β and γ secretases, respectively [7]. However, the presence of Aβ in conditioned medium in a wide variety of cultured cells and in human cerebrospinal fluid suggests that it is produced as a normal function of cells.

Various animal disease models can be efficiently used to examine the effectiveness and problems in the regulatory mechanisms of aging and age-related chronic diseases, as well as for the development of therapeutic drugs. Animal models used for testing preclinical efficacy can be divided into two types: models induced by treatment with exogenous material that cause artificial brain damage, and models generated via gene editing. The model induced by exogenous material is generated by injecting CNS-toxic substances into the animal brain and has been widely used for preclinical research on drugs to reduce AD symptoms. The latter model is produced via gene editing and results in overexpression of Aβ or tau hyperphosphorylation, leading to pathological changes in the brain.

Models of brain disease for testing preclinical efficacy have mainly been produced using rodents. However, rodent models have several limitations, considering that amyloid plaques are not deposited in rodent brains, unlike in humans, although memory loss symptoms still develop. Thus, the present study focused on the development of a new model of amyloid deposition using recombinant Aβ polypeptide plaques and repeated cloning of the Aβ gene.

Materials and Methods

Animals

All mice used in the study were maintained at the Catholic University of Daegu (CU) animal facility under specific pathogen-free conditions in a temperature-controlled environment of 22 ± 2°C with a 12-h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of the CU (IACUC-2015-038) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, 2011). All animals were provided free access to food and water.

Amyloid-Beta Aggregation Assay and Treatment of SH-SY5Y Cells

To express His-tagged Aβ42, DNA fragments encoding Aβ42 were amplified by PCR, ligated into the pCOLD vector (Takara Bio., Japan) and then transformed into *Escherichia coli* BL21 (DE3). *E. coli* cells expressing the His-tagged proteins were lysed by sonication in phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 1% protein inhibitor cocktail. After centrifugation at 16,000 ×*g* for 10 min, the recombinant protein was affinity-purified on a His-binding agarose resin column (ELPIS BIOTECH, Korea) as previously described [8]. Freshly purified 100 μM Aβ42 was then incubated with 1 mM Ni^{2+} in 1× PBS at 4°C for 48 h. The sample was then subjected to SDS-PAGE to analyze the complex formation of recombinant Aβ42 through interaction between poly-histidine and Ni^{2+}. For the cell viability assay, SH-SY5Y cells were seeded at 2×10^3 per well in 96-well plates in DMEM supplemented with 10% fetal calf serum (ThermoFisher Scientific, USA), penicillin (100 U/ml), and glutamine (0.3 mg/ml) and subsequently incubated in a humidified tissue culture incubator containing 5% CO_2 at 37°C. Afterwards, recombinant human Aβ42 (rAβ42) complexes were added to the cultures at varying concentrations (0.02, 0.2, 2, and 4 μM). After 24 h, the medium containing rAβ42 complexes was replaced with fresh normal cell medium for continued culture [9]. Water-soluble tetrazolium salt reagent (Dogen, Korea) was added to the maintenance cell medium at different time points and incubated at 37°C for an additional 1 h. Absorbance values were determined using the enzyme-linked immunosorbent assay reader (Omega; BMG LABTECH, Germany) at 490 nm [10].

DNA Cloning

For amplification of DNA fragments encoding the poly-Aβ42 region, six oligonucleotide primers were designed (Table 1). PCR was performed for 35 cycles at 94°C for 60 sec, 60°C for 60 sec, and 72°C for 120 sec, using a PCR thermal cycler (Takara Bio). The PCR products were cloned, sequenced, and subjected to 1.2% agarose gel electrophoresis. The desired PCR products were then purified using a gel extraction kit in accordance with the manufacturer's guidelines. Purified DNA fragments were cloned in DH5α cells using the pEGFP-N1 vector kit (Takara Bio). After

Table 1. Primer sequences for construction of multi-human amyloid-beta complex.

Sense primer (5′-3′)	Antisense primer (5′-3′)
CTCGAGGATGCAGAGTTCCGACATGAC	AAGCTTCGCTATGACAACACCGCCCAC
GAATTCGATGCAGAGTTCCGACATGAC	GGATCCCGCTATGACAACACCGCCCAC
AAGCTTGATGCAGAGTTCCGACATGAC	GTCGACCGCTATGACAACACCGCCCAC

plasmid DNA isolation, inserts were sequenced using the vector-specific sequencing primer on an ABI 3100 DNA sequencer (Applied Biosystems, USA).

HEK293 Cells and Preparation of Protein Extracts

DNA fragments encoding the multi-Aβ42 regions were introduced into a pEGFP-N1 vector. HEK293 cells were transfected with the plasmid constructs (5 μg) using FuGENE (Promega, USA) and subsequently cultured in Dulbecco's modified Eagle's medium/10% heat-inactivated fetal bovine serum in the presence of 0.5 mg/ml G418 at 37°C in 5% CO_2 and 95% air [11]. Cultured cells were collected via centrifugation at 800 ×*g* for 10 min and then washed with PBS. Proteins were extracted using 20 mM Tris/HCl (pH 7.4) supplemented with 1% Triton X-100, 0.15 M NaCl, and 1% protease inhibitor cocktail (Sigma-Aldrich, USA) on ice for 2 h. The cell suspension was then centrifuged at 13,000 ×*g* for 10 min at 4°C. The protein concentration was determined using a Coomassie protein assay kit (Pierce, USA) [12, 13].

Western Blot Analysis

Proteins were separated using SDS-PAGE and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 2% skim milk in 50 mM Tris/HCl (pH 7.5) supplemented with 0.3 M NaCl and 0.1% Tween-20. The blots were probed with the abovementioned primary antibodies and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies [14]. Protein bands were visualized using an enhanced chemiluminescence western blotting detection kit (ELPIS BIOTECH, Korea) in accordance with the manufacturer's instructions. Next, the blots were probed with various antibodies, including anti-VPS35 [15], anti-caspase-3 (Abcam, ab44976, UK) [16], anti-caspase-8 (Abcam, ab25901) [17], and anti-caspase-9 [18], as previously described.

Measurement of Antibacterial Activity

For measurement of antimicrobial activity of rAβ42 against *E. coli* (KCTC 2571), bacterial cultures were incubated at 37°C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.15 M NaCl, 1.5% agar) and assessed using a broth microdilution assay. Briefly, a single bacterial colony was inoculated and cultured overnight at 37°C. The bacterial concentration was controlled to approximately 1×10^6 CFU/ml. To determine the effects of rAβ42, an aliquot of the culture was transferred to 10 ml of fresh LB medium containing rAβ42 and incubated for an additional 1 to 12 h at 35°C. Bacterial growth was measured on the basis of optical density at 600 nm (OD_{600}) using a spectrophotometer.

Behavioral Analysis Using the Morris Water Maze Test

C57BL/6J mice were used for all behavioral studies, and all experimental animals were allowed to adapt to the environment prior to the Morris water maze (MWM) test. Before this test, mice were randomly assigned to one of two groups (n = 8/group) [19]. The solution containing recombinant His-tagged Aβ was dissolved in 1× PBS containing 1 mM Ni^{2+} at a final concentration of 3 μg/μl and subsequently incubated at 37°C for 4 days before use. Then, stereotaxic surgery was performed under xylazine (10 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.) anesthesia. Holes were drilled in the skull over the lateral ventricle using the following coordinates: AP −0.2 mm, ML 1.0 mm, and DV −2.4 mm from the bregma. The needle of the Hamilton microsyringe (26-gauge) (Hamilton Company) was inserted 3.5 mm beneath the surface of the skull. Three microliters of the recombinant His-tagged Aβ solution was injected to the right lateral ventricle, after which the same volume of the solution was injected into the left cerebral ventricle. Control mice were injected with 1× PBS containing 1 mM Ni^{2+} following the same procedure. At 10 days after sample injection, the animals were placed in a circular pool (diameter, 150 cm; height, 35 cm) filled with water; the container was made opaque using non-allergenic, water-soluble white paint. The water was maintained at a temperature of 22 ± 2°C. A platform (diameter: 12 cm) was hidden at a permanent location (the quadrant center) within the pool, with its top surface submerged 1.5 cm below the water surface. A video camera was mounted on the ceiling above the pool and connected to a video recorder and a tracking device, allowing on- and off-line automated tracking of the path traveled by each mouse. The mice were trained to move to the location of the platform for four consecutive days; three trials lasting for a maximum of 1 min each were conducted per day. The latency time required to find the platform was recorded as a measure of spatial memory. On day 5, the mice were released in the quadrant opposite to the target and forced to swim for 1 min in the pool without the platform. The total time spent searching for the platform in the target zone (*i.e.*, the previous location of the platform) was recorded as a measure of memory retention. All behavioral studies were carried out by well-trained experimenters blinded to the mouse genotypes.

Statistical Analysis

Variations in the performance of the mice in both groups were analyzed using repeated measures ANOVA and Tukey's test.

Results of the cell viability assay examination were statistically analyzed using independent *t*-tests in the Statistical Package for the Social Sciences for Windows, ver. 18.0 (SPSS Inc., USA). $p < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error of the means (SEM).

Results

Exogenous Poly-His-Tag-Fused rAβ42 Polypeptide Suppressed Proliferation of SH-SY5Y Cells and *E. coli* and Induced Upregulation of Apoptosis-Related Proteins

In the present study, we attempted to generate an approximately 8-mer complex of Aβ42 using Ni^{2+}. We induced the binding of a string of histidine (His) residues to several types of immobilized Ni^{2+} under specific buffer conditions, given that the expressed His-tagged rAβ42 can bind easily. To examine whether rAβ42 aggregates successfully formed oligomers, Aβ aggregation assay was performed using immobilized Ni^{2+}. Results revealed the expression of a recombinant protein with a 6× His fused to the Aβ42 N-terminus. As described in the experimental procedures, rAβ42 was incorporated into the oligomeric assembly using Ni^{2+}. The molecular size of the rAβ42 complex was estimated to be 50 kDa based on SDS-PAGE

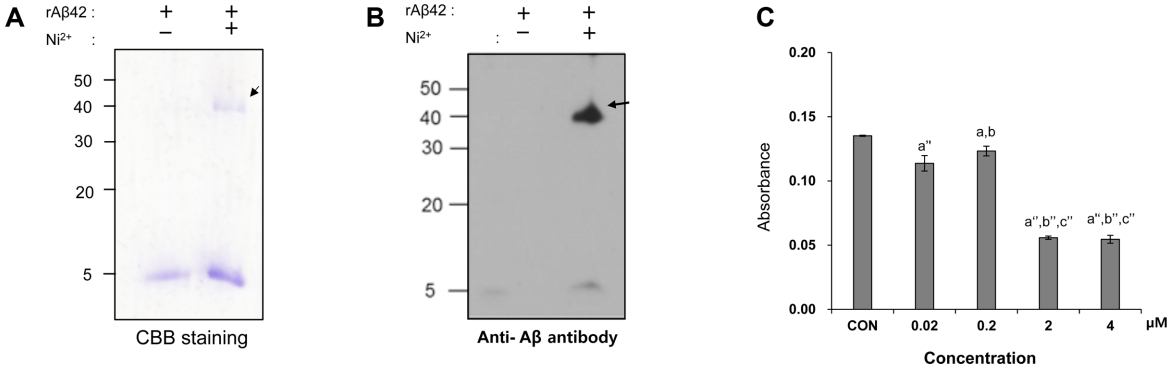

Fig. 1. Induction of Ni^{2+}-dependent poly-histidine-coupled Aβ42 (rAβ42) complexes.

(**A**) rAβ42 was incubated with Ni^{2+} for 12 h and then separated by SDS-PAGE. Blots were stained with Coomassie Brilliant Blue (CBB) and anti-Aβ 42 antibody. (**B**) The rAβ42 complexes prevented SH-SY5Y cell proliferation. (**C**) Repeated Aβ42 DNA-mediated SH-SY5Y cell death (a″, b″, and c″ indicate statistically significant differences compared with SH-SY5Y cells).

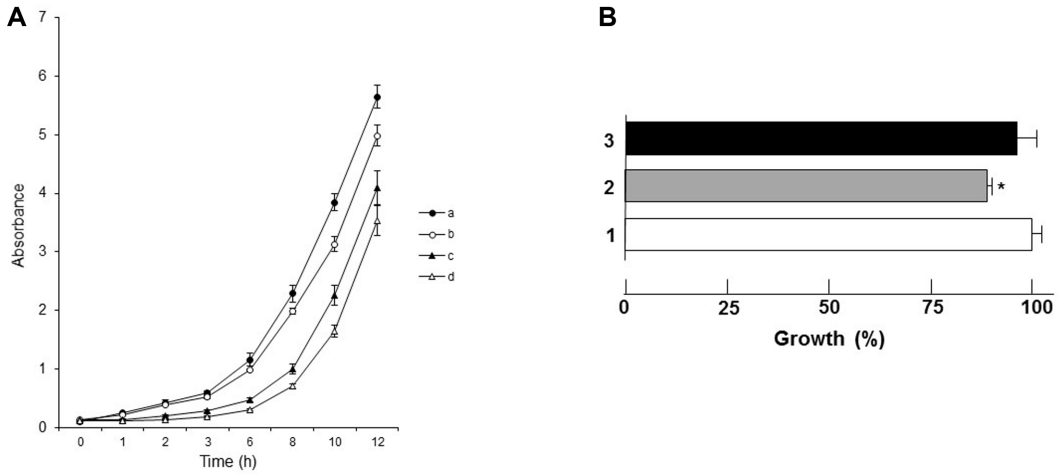

Fig. 2. Recombinant human Aβ42 (rAβ42) inhibition of *E. coli* growth.

(**A**) *E. coli* were cultured alone (circle), with Ni^{2+} only (diamond), with 50 μg/ml rAβ42 (triangle), or with Ni^{2+}-conjugated rAβ42 complexes (square). Bacterial growth was monitored by inoculation of agar diluted with cells at different time points and counting the number of CFUs. (**B**) rAβ42 was incubated with polyclonal anti-Aβ42 antibody conjugated with protein-A beads. After removal of the beads, samples were used for assessing *E. coli* growth. 1: Ni^{2+}-contained media; 2: Ni^{2+}-contained media with rAβ42; 3: rAβ42-removed media. The asterisk indicates statistically significant differences compared with 1 and 3.

(Figs. 1A and 1B). Additionally, results of the cell bioassay showed that the rAβ42 complex suppressed cell proliferation in all treatment groups (Fig. 1C). To investigate the inhibitory effect of rAβ42 on *E. coli* growth, we measured changes in bacterial proliferation, as shown in Fig. 2. *E. coli* exposed to rAβ42 and Ni^{2+}-conjugated rAβ42 complexes showed complete growth inhibition for the first 6 h (Fig. 2A). On the other hand, immunodepletion using anti-Aβ42 antibody of rAβ42 restored the antimicrobial activity (Fig. 2B).

Repeated Amyloid-Beta 42 Polypeptides Produced Using pEGFP N1 Vector Induced Apoptosis in HEK Cells

Although cognitive deficits or hippocampal formation reduction were observed in young (3–4 months old) animals treated with Aβ deposits, not all Tg2576 mice were cognitively impaired. Hence, transgenic mouse models do not cover the full spectrum of AD pathologies, such as neuronal loss. Thus, whether repeated Aβ42 amino acid sequences can promote neuronal cell death was investigated. To analyze the effects of Aβ on apoptosis, we designed three Aβ42 complexes at the DNA level, as shown in Fig. 3A. We cloned three variants of repeated Aβ42 (Fig. 3B) and performed western blot analysis. The three types of Aβ42 complexes were successfully expressed in HEK293 cells (Fig. 3C). Cell death rates and expression levels of apoptosis-related proteins transfected with the Aβ42 vector were significantly higher in treated mice than in the control at 48 h after transfection (Figs. 3D and 3E). No significant differences were observed among the two-, four-, and eight-repeat Aβ variants (Fig. 3E). We then examined caspase 9 activity in HEK293 cells. Treatment with the repeated Aβ42 complexes increased caspase 9 activity in HEK293 cells compared with that in control HEK293 cells. Consistent with the results described above, western blot analysis also detected the activated form of caspase 3 after 48 h in HEK293 cells (Fig. 3D), thereby

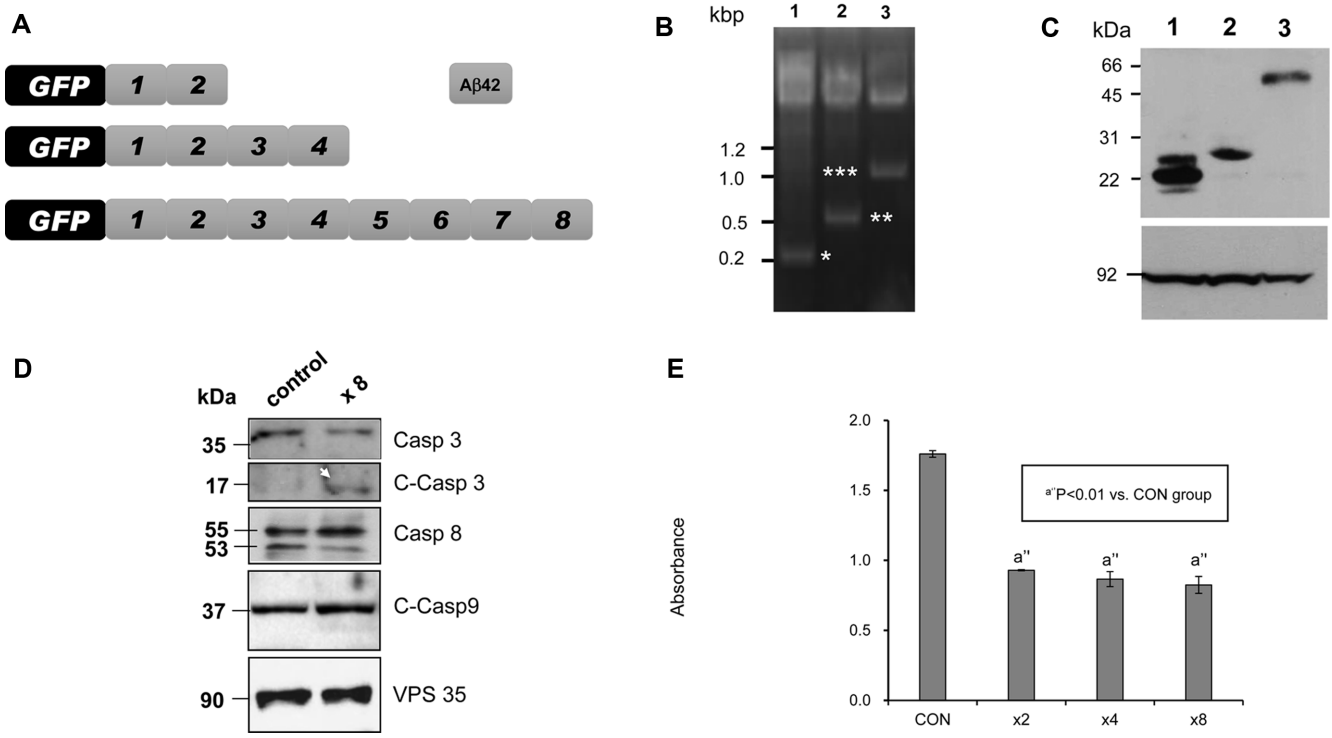

Fig. 3. Various forms of repeated Aβ42 inhibit HEK293 cell proliferation by upregulating the expression of apoptotic factors.
(**A**) Construction of GFP-coupled poly-Aβ42. The human Aβ42 region was amplified by PCR and inserted into the pEGFPN1 vector. Cloning was repeated two, four, and eight times to generate vectors encoding 2-, 4-, and 8-mer Aβ42, respectively, followed by insertion of the PCR products amplified with primers containing enzyme cut sites at the 3' end. (**B**) Agarose gel electrophoresis of DNA after restriction enzyme digestion. Asterisks indicate the poly-Aβ42 DNA fragments. (**C**) Transfection of pEGFPN1-poly-Aβ42 into HEK293 cells. HEK293 cells were lysed with 1% Triton X-100 and separated by SDS-PAGE. Blots were stained with anti-GFP antibody; anti-VPS35 antibody was used as an internal control. (**D**) Western blot analysis. The expression of the representative apoptosis molecules was significantly upregulated in the transfected HEK293 cells. The arrowhead indicates cleaved caspase-3. C-Caspase 9: cleaved caspase-9. VPS35 was used as an internal standard. (**E**) Poly-Aβ42-mediated HEK293 cell death. a" indicates statistically significant differences ($^{a"}$, $p < 0.001$ vs. control group) compared with HEK293 cells.

demonstrating that Aβ42 promoted cell apoptosis in the Aβ-induced cells.

His-Tagged Amyloid-Beta 42 Plaques and Ni^{2+} Assembly Induced Cognitive Dysfunction

Results revealed that His-tagged rAβ42 formed rAβ42 complexes owing to the presence of Ni^{2+} (Figs. 1A and 1B). The mice were subjected to the MWM test to analyze the effects of intracellular rAβ42 accumulation-induced neurodegeneration. On day 1 (hidden platform trials), no differences were observed between the recombinant Aβ42 complex-treated and control groups in terms of latency and path length, indicating similar motor and visual capabilities. Thus, we assumed that the mice could see the flagged platform, respond to cues in the surrounding environment, and swim acceptably. From days 2 to 5 (days 1–4 of the hidden platform trials), a difference in the escape latency was observed between the groups, suggesting that the treated mice had significantly lower cognitive performance than the controls over time. Probe trial results on the last day (day 5) showed that the number of times the mice traveled to the third quadrant, in which a hidden platform was previously placed, was significantly lower in the treatment group than in the control (Fig. 4B). These data indicate a significant association between the Ni^{2+}-linked poly-His-tagged recombinant Aβ42 and the observed memory deficits (Fig. 4). Control mice were quick in finding the hidden platform, whereas rAβ42 ICV-injected mice exhibited severe memory impairment. During the probe test, animals in the Aβ-treated group spent less time in the retention platform than did the mice in the control group.

Discussion

Previous studies have suggested the direct involvement of the Aβ42 peptide in neuronal diseases, indicating that this peptide is necessary for the development of diseases such as AD [7]. A representative Alzheimer's mouse model, the human mutant APP transgenic mouse model overexpressing the Swedish double mutant form of APP695, was introduced as the Tg2576 mouse [6]. Aggregates of Aβ polypeptides have long been implicated as triggers of AD. Among the different types of Aβ aggregates, soluble oligomers, namely, low-molecular-weight species ranging from approximately 2 to 10 mers, have emerged as the species responsible for early synaptic dysfunction and neuronal loss [9]. The present study focused on the development of an Alzheimer's animal model by implementing two different approaches. In the first approach, the mouse model was produced following the existing amyloid injection method, which was modified for the Aβ42 complex. The second approach in the experiment for inducing cell death was conducted by producing a fragment encoding multiple Aβ42 units by repeatedly combining the gene Aβ42 region, followed by transformation into cells to induce apoptosis. AD models have generally been produced in rodents through the accumulation of Aβ42. However, many researchers have pointed out that the rodent AD model is fairly dissimilar

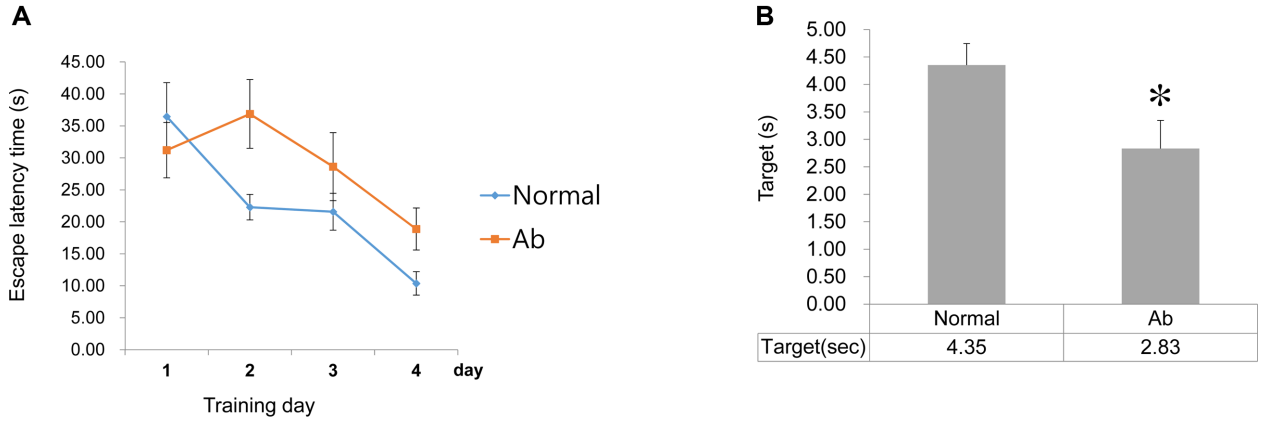

Fig. 4. Comparison of swim latency periods in the Morris water maze test.

Mice were injected with amyloid-beta (Aβ) complexes conjugated with Ni^{2+} ($n = 8$) or Ni^{2+} solution only (control group) ($n = 8$). (**A**) On day 1 of the hidden platform tests, Aβ42 complex-treated and control mice showed a shorter latency time to escape onto the hidden platform than on days 2, 3, and 4; $p < 0.001$ by repeated measures ANOVA. (**B**) In the probe trial on day 5, Aβ42 complex-treated mice traveled to the third quadrant, where the hidden platform had previously been placed, significantly more frequently than the controls. * $p < 0.005$ by Student's *t*-test.

from the AD in humans. As a result, efforts have been made to use miniature pigs and non-human primates, which are anatomically similar to humans. However, amyloid plaque formation in the brain, which is dominant in AD in humans, is not observed in rodents. Hence, the purpose of this study was to provide preliminary findings to develop an AD model using mice. We developed a method of accumulating Aβ42 complexes using Ni^{2+}, which has been commonly used to purify recombinant proteins for biochemical and structural studies [20]. rAβ42 DNA was engineered to allow the addition of a poly-histidine tag (His-tag) to the N-terminus of the Aβ42 polypeptide. The His-tag then binds to the Aβ42 complexes to enable purification of the tagged Aβ protein via immobilized metal affinity chromatography. Whether Aβ42 aggregates are the main cause of AD remains a controversy. However, the new rAβ42 produced in this study was associated with apoptosis at low concentrations following injection into cultured SH-SY5Y cells. Therefore, the complexes can be used to generate an effective AD model. A recent study showed that Aβ42 was toxic not only to eukaryotic cells, but also to prokaryotic cells and yeast [21]. As shown in Fig. 2, Ni^{2+}-conjugated rAβ42 complexes were more resistant to bacteria-mediated degradation than rAβ42. These results indicate that oligomerization of rAβ42, at least in part, exerts antimicrobial activity against *E. coli* by disrupting cells. The advantage of the rAβ42 used in the present study is its hydrophilicity, which makes it easier to combine with Ni^{2+}. Moreover, as shown in Fig. 4, injection of the amyloid complexes into the mouse brain impaired cognitive ability more than injection of amyloid alone. Thus, rAβ42 injection may be necessary for the development of induced Alzheimer's animal models for miniature pig or non-human primates. As mentioned above, the rAβ42 proteins developed in the present study have the advantage of producing plaques that can be easily injected and can thus be used as a basic material for developing various Alzheimer's models. At present, the standard transgenic animal model of AD is produced via genetic modification of the APP gene. Moreover, AD does not develop from a single gene abnormality, but from double gene modification due to co-factors such as BACE, apolipoprotein E, and presenilin [22–26]. However, in the existing AD models, there are many cases in which Aβ plaque formation does not occur or is delayed. To fill this gap, we produced a complex at the gene level using Aβ, which consists of 42 amino acids. Additionally, we successfully constructed a new gene comprising 2- to 8-mer Aβ42 (Fig. 3A), which can form β42 complexes and also induce apoptosis. In addition, results demonstrated that apoptosis was primarily responsible for the observed cell apoptosis (Fig. 3C). Caspase-3, which interacts with caspase-8 and caspase-9, has been shown to play a central role in the execution phase of apoptosis, thereby providing evidence that apoptosis was induced by the Aβ42 complexes or by overexpression of the Aβ42 gene (Fig. 3D). These findings reflect that it is possible to develop a gene modification model using the repeated Aβ42 units. Hyper-deposition of Aβ due to aberrant production of APP has been implicated in the etiology of AD in the human brain. Thus, there is an obvious need to generate animal models that are more similar to human AD for research, as well as for the development of new drugs for effective AD treatment.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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