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Fibrillar seeds alleviate amyloid-β cytotoxicity by omitting formation of higher-molecular-weight oligomers

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ABSTRACT

Amyloid-β (Aβ) peptides can exist in distinct forms including monomers, oligomers and fibrils, consisting of increased numbers of monomeric units. Among these, Aβ oligomers are implicated as the primary toxic species as pointed by multiple lines of evidence. It has been suggested that toxicity could be rendered by the soluble higher-molecular-weight (high-n) Aβ oligomers. Yet, the most culpable form in the pathogenesis of Alzheimer's disease (AD) remains elusive. Moreover, the potential interaction among the insoluble fibrils that have been excluded from the responsible aggregates in AD development, Aβ monomers and high-n oligomers is undetermined. Here, we report that insoluble Aβ fibrillar seeds can interact with Aβ monomers at the stoichiometry of 1:2 (namely, each Aβ molecule of seed can bind to two Aβ monomers at a time) facilitating the fibrillization by omitting the otherwise mandatory formation of the toxic high-n oligomers during the fibril maturation. As a result, the addition of exogenous Aβ fibrillar seeds is seen to rescue neuronal cells from Aβ cytotoxicity presumably exerted by high-n oligomers, suggesting an unexpected protective role of Aβ fibrillar seeds.

1. Introduction

Amyloid-β (Aβ) peptide, which is believed to play a causative role in the pathogenesis of Alzheimer's disease (AD), can exist in multiple forms including monomers, oligomers or protofibrils, fibrils, and amyloid plaques that contain densely packed Aβ fibrils and other molecules [1]. Amyloid fibrils or plaques are considered to be a pathological hallmark of AD. It has been suggested that the two extremes of Aβ forms, monomers and insoluble fibrils are unlikely the candidates for synaptic plasticity and memory impairment in AD [2]. As such, non-fibrillar Aβ aggregates are more critical in the pathogenesis of AD [3–6].

The identification of the toxic oligomeric variants responsible for long term potential (LTP) impairment and synaptic dysfunction *in vivo* has been a focus of current research [6–9]. On one hand, Soluble low-molecular-weight (low-n) oligomers including Aβ dimers [5,8,10,11], trimers [12], tetramers [13], 12-mers [6,13] are individually proven to be neurotoxic and to be able to impair synaptic functions. On the other hand, high-molecular-weight (high-n) oligomers which are spherical Aβ assemblies [14–16], have also been isolated from AD brain tissues and suggested to be the main toxic Aβ oligomeric species. It seems plausible that both soluble low-n oligomers and high-n oligomers could be responsible for the pathogenesis of AD.

However, whether the low-n oligomer species are the main responsible neurotoxic Aβ forms is much disputed. In most of the relevant literatures, Aβ oligomers were analyzed by SDS–PAGE [7]. As the essential material in this method, the detergent SDS, is known to induce the artificial oligomerization of Aβ [17] – a inherent caveat to prevent the precise determination of Aβ stoichiometry. Take the Aβ dimer as an example, this low-n oligomer was investigated as the minimal sized toxic species *in vivo*[5,8]. Due to the intrinsic thermodynamic trend to assemble into high-n metastable oligomers, whether the synaptotoxicity observed was indeed directly caused by the dimers present in their purified fractions remains unproven [7]. Recently, the synaptic toxic Aβ dimers

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; ITC, isothermal titration calorimetry; SEC, size exclusion chromatography; PBS, phosphate buffered saline; ThT, Thioflavin T; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazoliumbromide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential media; FBS, fetal bovine serum; TEM, transmission electron microscope; FTIR, Fourier transform infrared spectroscopy.

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were captured by cross-linking, recapitulating the transient nature of these low-n oligomers [11].

Since the metastable high-n assemblies could be the potential culprit for AD development [7,11], it is of great importance to re-evaluate the roles of monomers and fibrils because they might act as sources and reservoirs of the those soluble synaptotoxic and neurotoxic oligomers [2]. That mature Aβ fibrils can seed accelerated aggregation of both monomer and oligomer Aβ hints at the possible interactions between fibrils and other forms of Aβ [18–20]. Despite the great progress made in this field, the following mechanistic questions remain unanswered: Why can fibrillar seeds regulate the Aβ aggregation process and how do fibrillar seeds affect the levels of the different Aβ aggregated forms, especially toxic soluble high-n oligomer species? To our best knowledge, little literature is available on the detailed interaction – on a molecular level – between fibrils or amyloid plaques and other forms of Aβ [21–23]. In this study, we found that the interaction between the monomeric and fibrillar Aβ is a pivotal event for fibrillar seeds to modulate Aβ toxicity. Once monomer Aβ binds to fibrillar seed, its conversion to fibril is greatly accelerated, as evidenced by significantly reduced levels and shortened lifespan of the oligomeric forms, especially the high-n species. The thermodynamic favorable interaction of monomeric Aβ with fibrillar seeds results in less cytotoxicity of Aβ in neuroblastoma N2a cell line and rescues cells by omitting the formation of high-n oligomer. Since the high-n oligomer Aβ species, not other Aβ species, have been implicated as the primary pathological species in the pathogenesis of AD [2,7,11], our results imply that amyloid plaques may play a positive protective role by interacting with monomers, subsequently accelerating their fibrillation and thus preventing them from forming the neurotoxic oligomers.

2. Materials and methods

2.1. Preparation of monomer Aβ

Aβ42 was from R-peptide Company, USA. Monomeric forms of Aβ42 were prepared by dissolving to 5 mg/ml in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Acros, USA), incubated overnight at room temperature and stored at −20 °C in HFIP. The HFIP was then evaporated off by a speed vac before using. After that, dimethyl sulfoxide (DMSO) was added and the solutions (5 mg/ml) were sonicated for 15 min before using.

2.2. Preparation of fibrillar Aβ seeds

Aβ42 fibrils were prepared by incubating 50 μM monomer Aβ42 for 84 h. The fibrils were centrifuged at 10,000*g* at 4 °C for 30 min (Biofuge, Heraeus Instruments, Germany) and 100 μl of supernatant was injected into a size exclusion chromatography column (TSK-GEL G3000PW$_{XL}$, TOSOH corporation) to quantify the relative amount of soluble monomer Aβ and oligomer Aβ in PBS solution. By comparing with the amount of the fresh monomer Aβ that was initiated to form fibrils, the amount of formed fibrils was estimated from the area of relative peaks (Fig. S1). The pellet was reconstituted into PBS (1×, pH 7.4) with v/v 1% DMSO and the sample contained fibrillar Aβ42 at the estimated concentration of 75 μM. The solution was mechanically fragmented into smaller fibrillar structures by sonication on ice for 45 min. The resultant stock solution was used as fibrillar Aβ seeds in following experiments.

2.3. Isothermal titration calorimetry (ITC) analysis

All calorimetric experiments were performed on a fully computer-operated and thermostated VP-ITC calorimeter (Microcal Inc., USA) at 30 °C. Each titration to determine the enthalpy changes of the adsorption of monomer Aβ on the fibrillar seeds consisted of 14 successive injections of 20 μl seed solution (75 μM) into the reaction cell (1.4616 ml) containing 20 μM Aβ42 in the same buffer.

2.4. Quenched tyrosine fluorescence titration

20 μM Aβ42 or PBS blank solution with v/v 1% DMSO was placed in a four-sided quartz fluorescence cuvette with total volume of 700 μl. Fibrillar seed stock solution, 9.58 μl each time, was added into the cuvette. Such volume was designed to simulate the ITC condition. Fluorescence spectra were collected using a Hitachi FP-4500 fluorescence spectrophotometer (Japan). An excitation wavelength of 280 nm (slit width = 5 nm) was used and data were collected over 290–350 nm (slit width = 5 nm).

2.5. Fibrillation experiments

Aβ42 fibrillation was studied at 37 °C in the absence or presence of fibrillar seeds. The monomer Aβ42 concentration was 20 μM in PBS. Fibrillar seeds at different concentrations (1, 2, and 4 μM, respectively) were incubated with Aβ42. Each sample of 1.2 ml was shaken in a 1.5 ml Eppendorf tube at 200 rpm. To monitor the growth of fibrils, 20 μl aliquots from the tubes were taken at different time points and mixed with Thioflavin T (ThT, 10 μM) in a four-sided quartz fluorescence cuvette. The ThT fluorescence was measured at 489 nm with excitation at 440 nm in a Hitachi FP-4500 fluorescence spectrophotometer (Japan).

2.6. Analytical size exclusion chromatography (SEC)

Analytical SEC was utilized to quantify the relative amounts of soluble (monomer and oligomer) Aβ42 in solution at selected time points during Aβ aggregation process. An SEC column TSK-GEL G3000PW$_{XL}$ (TOSOH Corporation) was connected to a Waters 600 (Waters, USA). Aliquots (200 μl) of the samples were injected into the column. The running buffer was PBS (1×, pH 7.4) and the elution was monitored by UV 215 and 280 nm at a flow rate of 0.5 ml/min. Integration was operated on Origin 7.0 ®Software.

2.7. Cell viability experiments

N2a WT cells were cultured in the medium containing 42.5% (v/v) DMEM, 42.5% (v/v) Optical MEM, 5% (v/v) FBS, 100 units/ml penicillin, 0.1 mg/ml of streptomycinin a density of 5000 per well in 96 wells plate for first 24 h. After the medium was replaced by 50% (v/v) DMEM and 50% (v/v) Optical MEM, 20 μM Aβ with or without 1, 2 , and 4 μM of seeds was adding into wells for further incubation for 10 or 20 h. 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium-bromide (MTT) was added to each well for 3.5 h. MTT reduction was assessed by measuring the absorption at 570 nm by a BioTek Synergy 4 microplate reader.

3. Results

3.1. Fibrillar seeds interact with monomer Aβ at the ratio around 1:2

We first characterized the interaction between Aβ fibrillar seeds and monomers by measuring the changes of tyrosine intrinsic fluorescence. Tyrosine intrinsic fluorescence has been applied to assess interactions of Aβ with other molecules [24,25]. Tyrosine intrinsic fluorescence emission at 309 nm (excited at 280 nm) is usually quenched on the occasion of molecular interactions. A marked quenched tyrosine fluorescence signal at 309 nm was observed

when Aβ fibrillar seeds were titrated into Aβ (20 μM) (Fig 1A). This quenching of fluorescence signal was increasing when the ratio of seed to monomer was less than 0.5. With the further addition of Aβ fibrillar seeds, the quenched intensity reached at a plateau. The interaction of monomer Aβ with the fibril occurred very rapidly, and we observed an immediate quenching of the tyrosine fluorescence during the titration. The time was short enough (∼min) to exclude the possibility that the quenched fluorescence is due to the fibril formation of monomeric Aβ (∼h).

To further investigate the interaction between Aβ fibrillar seeds and monomers, we measured the enthalpy change when fibrillar seeds interact with monomers by isothermal titration calorimetry (ITC) analysis. First, ITC experiment was performed to measure the self-association of 20 μM Aβ monomers alone as the control, in order to exclude the possibility that the self-assembly of Aβ monomers in PBS may cause interfering ITC background signal during the experimental time course (~ 3hr) (Figure S2). In addition, the prepared fibrillar seeds also remained thermodynamic stable during the titration process (data not shown). ITC experiments were then carried out by titrating fibrillar seed solution (75 μM) into the sample cell containing Aβ monomer (20 μM). As seen from the titration curve (Fig. 1B), the binding of Aβ monomers to fibril seed is exothermic during experimental time course. Seeds exhibit the binding stoichiometry of 1:2 with monomers, namely each Aβ molecule of seed binding to two Aβ monomers. Structurally, Aβ fibrillar seed is a nano-structure with β-sheet motif within the full length of the fibril [22,26]. This nano-structure ideally can provide a reasonable template for the spontaneous adsorption of monomeric Aβ. From the ITC result, each Aβ molecule of seed can provide two different binding sites for two monomer molecules at a time, which is consistent with the previously reported data from cryoEM [27].

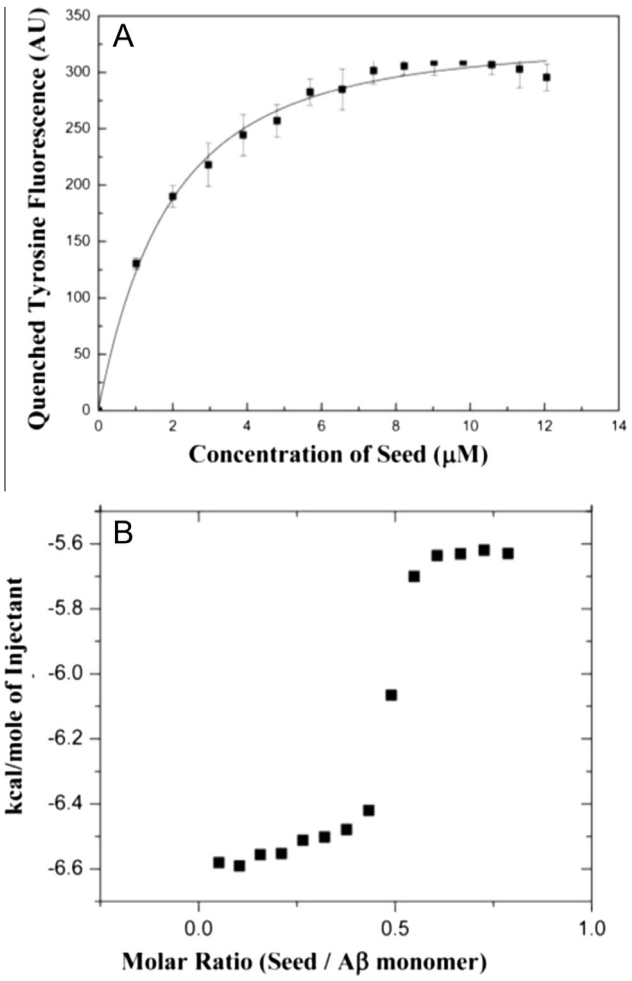

Fig. 1. Fibrillar Aβ seeds interact with monomer Aβ. Fibrillar seed stock solution was 75 μM and monomer was 20 μM. (A) Intrinsic tyrosine fluorescence of monomer Aβ quenched by adding fibril seeds. The values are means ± SD, $n = 3$. (B) ITC results for the interaction of monomer Aβ with fibrillar Aβ seeds. Results are representative of two independent measurements.

The fast interaction of monomeric Aβ with seed could be described as two following steps:

$$\text{Seed}\, x\mathrm{H_2O} + 2(A\beta\, y\mathrm{H_2O}) \leftrightarrow [(A\beta - \text{seed} - A\beta)(x+2y)\mathrm{H_2O}]^{\#} \quad \text{(Step 1)}$$

$$[(A\beta - \text{seed} - A\beta)\,(x+2y)\mathrm{H_2O}]^{\#} \leftrightarrow (A\beta - \text{seed} - A\beta)\, z\mathrm{H_2O} + (x+2y-z)\mathrm{H_2O} \quad \text{(Step 2)}$$

According to hypothetical Step 1 and 2, the thermodynamics of interaction depends on two continuous processes featuring the possible non-covalent bond formation and solvent reorganization. From an enthalpic viewpoint, the formation of non-covalent bonds is exothermic while the disruption of structurally well-defined solvent shells is endothermic. The unfavorable desolvation enthalpy, in this case, was overcome by the more favorable interaction between Aβ monomer and fibrillar seed [28].

During protein–ligand interactions, solvent reorganization may contribute greatly to changes in entropy [29,30]. In this study, the interaction of Aβ monomers with the seeds is believed to reduce the solvent-accessible surface area, leading to the release of highly ordered solvent molecules into the bulk solution. It is possible that the entropic gain from the desolvation is large enough to compensate for the entropy lost due to the restricted of conformational space of monomeric Aβ, another contributing factor to further favor the surface adsorption.

3.2. Fibrillar Aβ seeds accelerate monomer Aβ fibrillation and inhibit its oligomerization

Upon the addition of the fibrillar seeds into the solution of Aβ monomers, the aggregation progress of the Aβ monomer would be altered compared to un-seeded scenario due to a high Aβ monomer local concentration around the nano-structure surface of seed caused by the fast adsorption. We adopted 20 μM as initial peptide concentration in order to monitor Aβ fibrillation by thioflavin T assay. Consistent with previous reports [19,31], the presence of fibril seeds shortened the lag time of the key nucleation in the Aβ aggregation (Fig. 2). The fibrillar seeds clearly accelerated the Aβ aggregation even at the low concentration of 1 μM and this behavior was in a dose-dependent manner with the seed concentration.

The results provided by negatively stained TEM analysis (Fig. S3) and Fourier Transform Infrared Spectroscopy (FTIR) analysis (Fig. S4) also confirmed that the fibrillar seeds accelerated fibril formation of monomer Aβ while omitting the oligomer formation (See supporting information).

3.3. Fibrillar Aβ seeds reduce the level of high-n oligomer species and shorten its lifespan

To kinetically explore how fibrillar seeds affect the levels of the different Aβ aggregated forms, especially toxic soluble high-n oligomers, size exclusion chromatography (SEC) analysis was performed to monitor the progression of Aβ forms. Unlike SDS–PAGE, SEC can provide a precise definition of Aβ species under non-denatured conditions. According to the standard protein calibrated SEC curve and the retention time, the molecular weight of those high-n oligomers was estimated (See support information). Their molecular weights mainly varied from 80 to 150 kDa, which approximates 18–35mer (Fig. 3A).

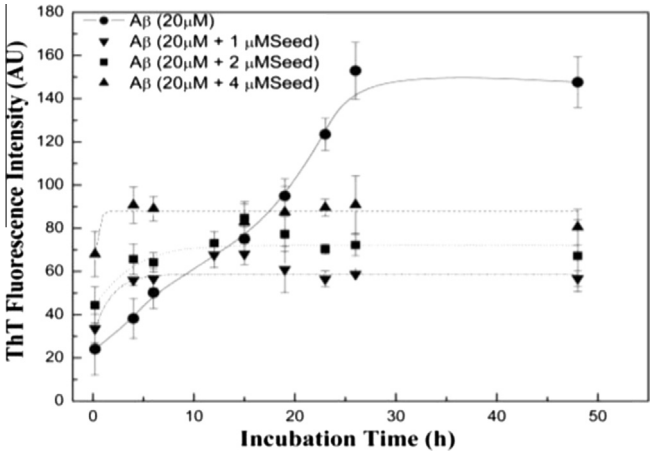

Fig. 2. Fibrillar Aβ seeds accelerate monomer Aβ fibrillation as assessed by ThT assay. Fibrillar seed stock solution is 75 μM and monomer is 20 μM. Monomeric Aβ is added with seeds at different concentrations (1, 2 and 4 μM). The values are means ± SD, $n = 3$.

The peak of low-n Aβ species included monomers, dimers and trimers from the curve. We found that fibrillar seeds reduced high-n oligomer species levels during the experimental process of Aβ aggregation (Fig. 3A). The level of high-n oligomers was gradually accumulated within the incubation time of 26 h in the Aβ alone group (Fig. 3A and D). About 65% monomer Aβ converted into high-n oligomers by 26 h according to the integral areas under SEC peaks (the protein concentrations of the various Aβ fractions are linearly correlated with UV absorbance at 215 nm or 280 nm) [32,33]. 35% low-n Aβ species including monomers still existed in the sample. Compared to Aβ incubated alone, Aβ incubated with fibrillar seeds clearly had fewer amounts of high-n and low-n oligomers. The highest levels of high-n oligomers corresponding to Aβ was incubated with different concentrations of fibril seeds (2 and 4 μM) (Fig. 3B and C) were about 14% and 7% of total Aβ, much lower than the high-n oligomer level in the sample of Aβ alone (Fig. 3A and D). Thus high-n oligomer level is reduced by introducing fibrillar seeds.

More importantly, the addition of fibrillar seed can regulate the lifespans of high-n oligomers. Fig. 3A and D showed that for the sample of Aβ incubated alone, the high-n oligomers can exist for 30 h, which was long enough to generate neurotoxicity (see Fig. 4 for 10 and 20 h incubation). But in the presence of fibrillar seeds, high-n oligomers at detectable levels can be observed only within first 10 h. Furthermore, we showed that monomer Aβ incubated with seeds formed insoluble fibrils much faster than the monomer incubated alone (Fig. 3D), which is consistent with the data from the ThT assay (Fig. 2).

3.4. Fibrillar seeds alleviate Aβ cytotoxicty

All *in vitro* results so far supported the view that the seed-accelerated fibrillation can reduce the formation and lifespan of the toxic high-n oligomers. Considering the low levels and the short lifespan of Aβ high-n oligomers regulated by the fibril seeds, the toxicity of Aβ could be evidently counteracted when mixed with fibrillar seeds. If true, it may support the idea that the fibrils or plaques play a protective role in the pathogenesis of AD by bypassing highly toxic oligomers. Given much emphasis on the neuronal toxicity of the soluble high-n oligomers, we used MTT assay to assess the cell survival in 20 μM with or without fibrillar seeds. When Aβ was incubated with the neuroblastoma N2a cell for the first 10 h, the cell viability was reduced to 55% comparing with the control group, while a further 10 h incubation exacerbated the cell damage

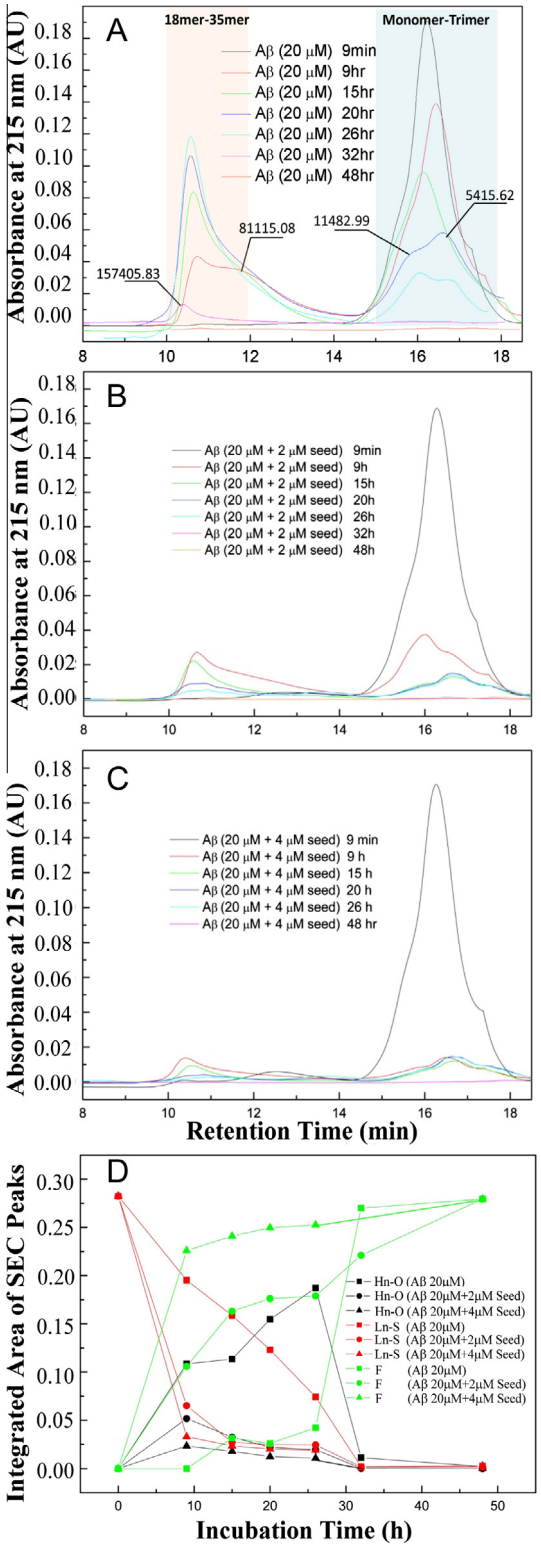

Fig. 3. Fibrillar Aβ seeds at (A) 0 μM, (B) 2 μM and (C) 4 μM regulate level of high-n oligomer by SEC analysis. Fibrillar seed stock solution is 75 μM and is 20 μM for monomeric Aβ. Results are representative of two experiments. The molecular weight of Aβ42 oligomers is calculated according to the SEC traces calibrated by standard proteins curve (See Supporting information). (D) Kinetic curves of low-n species including monomers, high-n oligomers, and fibril for the samples in which Aβ is incubated alone or with 2 μM seeds or 4 μM seeds. "Hn-O" stands for high-n oligomer, "Ln-S" low-n Aβ species and "F" fibril.

to 40% viability (Fig. 4). This result indicated the correlation of Aβ cytotoxity with both formation and lifespan of the high-n

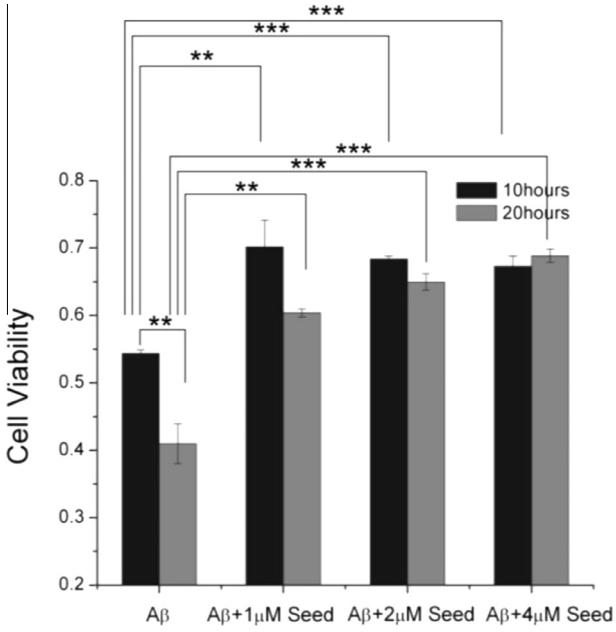

Fig. 4. MTT assay shows that fibrillar Aβ seeds can rescue N2a WT cell from the cytotoxic Aβ at both 10 and 20 h incubations. Fibrillar seed stock solution is 75 μM and monomer is 20 μM. The values are means ± SD, $n = 5$. $^{**}P < 0.01$ and $^{***}P < 0.001$.

oligomers species. In contrast, in the presence of fibrillar seeds, all assayed concentrations of seeds displayed the ability to rescue cell from the Aβ toxicity. Combined with the fact that fibrillar seeds can promote the transformation of Aβ monomer to fibrils and thus prevent formation of the more toxic high-n oligomers, we hypothesize that the behavior of fibrillar seeds for reducing Aβ Cellular toxicity may play certain protective role in AD pathogenesis.

4. Discussion

Our results presented herein show that seed-mediated amyloid fibrillation is actually nanostructure-modulated aggregation process [25,34,35]. The fibrillar seed can bind to Aβ monomer at the stoichiometry of 1:2, which dramatically reduces the critical concentration of the Aβ solution required for the fibrillation process. Thus seed can promote Aβ fibrillation, which is much different from the pathway of Aβ self-aggregation.

In our study, Aβ forms fibrils form by nucleated conformational conversion of high-n oligomers in the absence of seeds, which suggests that toxic soluble high-n oligomers are the essential intermediates when monomer Aβ is incubated alone. In the presence of seeds, Aβ monomers interact with seeds and bind to the surfaces of the seeds to propagate fibrils spontaneously. The high-n oligomer formation is thus omitted. Since the high-n oligomer species but not the low-n species are proposed as the potent mediators of Aβ toxicity, fibrillar seeds could be protective in AD pathogenesis.

A question arises though: why do people with plaques develop full-blown AD if the plaques play a protective role? Two explanations may exist. One possibility is the existence of other factors that reverse fibrils or plaques to oligomers *in vivo*. If true, fibrils or plaques serve as a pool of the toxic oligomers. Alternatively, the extracellular fibrils or plaques may have little regulatory effect on the intracellular Aβ concentration, which may be more critical than extracellular Aβ in the development of AD. Therefore, considering the possible protective mechanism of fibrils or amyloid plaques formation *in vivo*, it is urgent to explore the true causes like the possible reversing factors that convert fibrils to oligomers or the role of intracellular Aβ, which could be helpful to develop therapeutic drugs to cure AD eventually.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.088.

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