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Small heat shock proteins differentially affect Aβ aggregation and toxicity

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Abstract

β-Amyloid (Aβ) is the primary protein component of senile plaques in Alzheimer's disease (AD) and has been implicated in neurotoxicity associated with the disease. Aβ aggregates readily *in vitro* and *in vivo*, and its toxicity has been linked to its aggregation state. Prevention of Aβ aggregation has been investigated as a means to prevent Aβ toxicity associated with AD. Recently we found that Hsp20 from *Babesia bovis* prevented both Aβ aggregation and toxicity [S. Lee, K. Carson, A. Rice-Ficht, T. Good, Hsp20, a novel alpha-crystallin, prevents Abeta fibril formation and toxicity, Protein Sci. 14 (2005) 593–601.]. In this work, we examined the mechanism of Hsp20 interaction with Aβ1–40 and compared its activity to that of other small heat shock proteins, carrot Hsp17.7 and human Hsp27. While all three small heat shock proteins were able to prevent Aβ aggregation, only Hsp20 was able to attenuate Aβ toxicity in cultured SH-SY5Y cells. Understanding the mechanism of the Hsp20-Aβ interaction may provide insights into the design of the next generation of Aβ aggregation and toxicity inhibitors.

Keywords: Alzheimer's disease; Amyloid; Aggregation; Toxicity; Cell culture; Hsp27; Hsp17.7; Hsp20; Chaperone; α-Crystallin

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is the most commonly occurring cause of dementia. Upon autopsy, the two primary histopathological features observed in AD are senile or amyloid plaques and neurofibrillary tangles. β-Amyloid peptide (Aβ) is the major protein component of senile plaques. Many hypothesize that the accumulation of Aβ during disease is linked to the neurodegeneration observed during AD.

Aβ aggregates readily both *in vitro* and *in vivo* into fibrils, protofibrils, and smaller diffusable species (derived diffusible ligands or ADDLs), and its toxicity is linked to its aggregation state [2–10]. Based on the Aβ aggregation hypothesis, many research groups have focused on finding agents that prevent or reduce Aβ aggregation as a potential means to attenuate Aβ toxicity and treat AD [11–22]. Aggregation prevention strategies include the use of chemical chaperones such as polyphenol [15,16], apomorphine [17], and hexadecyl-*N*-methylpiperidinium (HMP) bromide [18], and short peptides, KLVFF [14,19,20], LPFFD [21], GVVIN, and RVVIA [22], which are believed to bind to a key sequence of Aβ associated with aggregation. At near equimolar concentrations of these agents and Aβ, the chemical chaperones and peptides have proved useful in preventing both aggregation and toxicity.

Small heat shock proteins (sHsps) which have chaperone-like activity have also been examined for their ability to prevent Aβ aggregation or toxicity. While there have been several reports that sHsps and/or α-crystallins, including human Hsp27, rat Hsp20, αA-crystallin, and αB crystallin, attenuate Aβ fibril formation [23–28], there are fewer (and conflicting reports) on the affects of sHsps or α-crystallins on Aβ toxicity [24,28,29].

Recently we found that a novel heat shock protein, Hsp20 from *B. Bovis*, can prevent Aβ aggregation and toxicity at very low concentrations of small heat shock protein

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relative to Aβ (1:1000) [1]. In the work presented here, we describe a series of experiments in which we examine the mechanism of *B. bovis* Hsp20 activity and compare it to activity of other small heat shock proteins, Hsp17.7 from carrot, and human recombinant Hsp27. While all three small heat shock proteins (sHsps) attenuated Aβ1–40 aggregation, only Hsp20 attenuated Aβ toxicity. Electron microscopy, turbidity measurements, and Congo red binding results suggest that Hsp20 formed large transient multimeric complexes with Aβ under conditions that led to productive aggregation and toxicity attenuation, while such large multimeric complexes were not detected in the other sHsp-Aβ mixtures. From these results, we postulated a mechanism for Hsp20–Aβ interaction that leads to both toxicity and aggregation prevention. Understanding how proteins prevent aggregation and toxicity of Aβ may provide insight into how best to design the next generation of Aβ aggregation inhibitors to be used in AD.

Materials and methods

Materials. Aβ1–40, the peptide comprised of the first 40 amino acids of the human Aβ sequence, was purchased from AnaSpec (San Jose, CA) and Biosource International (Camarillo, CA). Recombinant human heat shock protein 27 (Hsp27) was purchased from MBL International Corporation (Woburn, MA). Human neuroblastoma SH-SY5Y cells (ATCC No.: CRL-2266) were purchased from ATCC (Manassas, VA). Cell culture reagents and cell dissociation buffer were purchased from Invitrogen Life Technologies (Carlsbad, CA). Congo red was purchased from Fisher Chemicals. (Pittsburgh, PA). Annexin-PE, 7AAD, and Annexin V binding buffer were purchased from Becton–Dickinson Biosciences Pharminogen (San Diego, CA). All other chemicals, unless otherwise specified, were obtained from Sigma–Aldrich Co. (St. Louis, MO).

Heat shock protein 20 (Hsp20) preparation. Hsp20 was originally isolated from *B. bovis* [30]. Hsp20 with an N-terminal polyhistidine tag was produced in recombinant *Escherichia coli* as described previously [1]. Protein purity and molecular weight were confirmed by SDS–PAGE.

Heat shock protein 17.7 (Hsp17.7) preparation. The gene encoding Hsp17.7 from carrot was cloned into *E. coli* [31]. The recombinant *E. coli* strain (a gift from Professor Lynn Zimmerman, Department of Biology, UMBC) was grown in LB medium (tryptone (10 g/L), yeast extract (5 g/L), and NaCl (10 g/L)) with 50 μg/mL kanamycin in an aerobic environment at 37 °C overnight. IPTG (100 mM) was added to cells in order to induce Hsp17.7 when *E. coli* reached an OD_{600} of 0.6. The cells were harvested by centrifugation at 6000*g* for 10 min and frozen at −20 °C overnight. The cells were lysed with 20 mM imidazole and 10 min sonication. Hsp17.7 was purified using metal affinity chromatography on a copper binding resin (POROS® 20MC, Framingham, MA). The protein was eluted by pH gradient from pH 7.4 (phosphate buffer, 50 mM NaH_2PO_4, and 300 mM NaCl) to pH 4.5. Protein purity and molecular weight were confirmed by size-exclusion chromatography.

Protein sample preparation. Aβ1–40 was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) at the concentration of 10 mg/mL. This solution was incubated at room temperature for 20–30 min in order to completely dissolve the Aβ. Filtered phosphate-buffered saline (PBS, 4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, and 1.4 mM KH_2PO_4, pH 7.4) was added to the Aβ solution to make the final concentrations used in experiments. For cell viability assays, MEM was used instead of PBS buffer. Aβ samples were mixed on a rotator at 18 rpm and 37 °C, and samples were taken out as a function of time. sHsps were always added to the Aβ samples before the samples were incubated (prior to aggregation).

Congo red binding. Congo red was dissolved in PBS at the concentration of 120 μM and syringe filtered. The Congo red solution was mixed with protein samples at 1:9 (v/v) ratios to make the final concentration of Congo red 12 μM. After a short vortex, the mixtures were incubated at room temperature for 30–40 min. Absorbance measurements from 400 nm to 700 nm were taken (UV–vis spectrometer model UV2101, Shimadzu Corp.; Kyoto, Japan). Alternatively, Congo red absorbance was read at 405 nm and 540 nm using an Emax Microplate Reader (Molecular Devices, Sunnyvale, CA). In both cases, PBS buffer solution was used as a blank. The concentration of Aβ fibrils was estimated from Congo red binding via Eq. (1):

$$[A\beta_{FIB}] = ({}^{541}A_t/4780) - ({}^{403}A_t/6830) - ({}^{403}A_{CR}/8620) \quad (1)$$

where ${}^{541}A_t$ and ${}^{403}A_t$ are the absorbances of the Congo red-Aβ mixtures at 541 nm and 403 nm, respectively, and ${}^{403}A_{CR}$ is the absorbance of Congo red alone in phosphate buffer [32]. When using the microplate reader, absorbances at 405 nm and 540 nm were assumed to be same as those at 403 nm and 541 nm.

Turbidity assay. Protein samples (100 μM Aβ and 100 μM Aβ + sHsps) were prepared as described above. At every 30 min until 8 h, the turbidity of protein samples was monitored at 405 nm using UV–vis spectrometer. PBS buffer solution was used as a blank.

Electron micrograph (EM). Two-hundred microliters of Aβ peptide solution or Aβ-sHsp mixtures, prepared as described above, was mixed, placed on glow discharged grids, and then negatively stained with 1% aqueous ammonium molybdate (pH 7.0). Grids were examined in a Zeiss 10C transmission electron microscope at an accelerating voltage of 80 kV. Calibration of magnification was done with a 2160 lines/mm crossed line grating replica (Electron Microscopy Sciences, Fort Washington, PA).

Cell culture. SH-SY5Y cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 25 mM sodium bicarbonate, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in a humidified, 5% (v/v) CO_2, 37 °C incubator. Low passage number cells were used (less than passage 20) in all experiments to reduce the instability of the cell line.

Viability assay. SH-SY5Y cells at a density of 1×10^6 cells/mL were grown in 96-well plates. Cells were fully differentiated by addition of 20 ng/mL NGF for 8 days. Aβ samples in MEM were added to the differentiated SH-SY5Y cells and the cells were incubated with Aβ samples at 37 °C for 2 h. Negative controls (cells in medium with no Aβ) and positive controls (cells treated with 800 μM H_2O_2 in 50% (v/v) medium for 2 h) were also prepared. At least three wells were prepared for each Aβ treatment, and each positive and negative control.

To stain cells with 7-AAD (a membrane impermeant nucleic acid dye) and annexin-PE (which binds to phosphatidylserine on the outside of apoptotic cells), culture medium was replaced with 100 μL of 1× binding buffer (0.01 M Hepes/NaOH, pH 7.4, 0.14 M NaCl, 2.5 mM and $CaCl_2$). Thereafter, 5 μL of both annexin-PE (5 ng/well) and 7AAD (0.25 μg/well) was added, followed by 10-min incubation in the dark at 25 °C. One hundred and fifty microliters of 2× binding buffer was then added. Finally, cells were removed from plates by mechanical scraping, then analyzed using the BD FACSArray Bioanalyzer flow cytometer (BD Bioscience; San Jose, CA). Annexin-PE was excited with the 532 nm laser and detected using the yellow (564–606 nm) filter, while 7AAD was detected using the red (653–669 nm) filter. Spillover for the two detectors was calculated using three samples one of which was unstained, another stained with only annexin-PE, and the third stained only with 7AAD [33].

Positive and negative control populations were used to set up quadrant gates of dot plots of annexin-PE staining versus 7-AAD staining. Live cells were taken as those that were neither stained with annexin-PE nor with 7-AAD. Relative cell viabilities were calculated using Eq. (2)

$$\text{Relative cell viability } (\%) = \frac{(\text{L.C.}_{\text{sample}} - \text{L.C.}_{\cdot H_2O_2})}{(\text{L.C.}_{\text{NControl}} - \text{L.C.}_{\cdot H_2O_2})} \times 100 \quad (2)$$

where $\text{L.C.}_{\text{sample}}$ is live cells (%) of cells treated with Aβ and sHsps, $\text{L.C.}_{\text{Ncontrol}}$ is live cells (%) of negative control, and $\text{L.C.}_{\text{A}\beta}$ is live cells (%) of cells treated with only Aβ.

Data analysis. For each experiment, at least three independent determinations were made. Data are plotted as means plus or minus the standard error of the measurement. Significance of results was determined via the Student's *t* test with $p < 0.05$ for individual comparisons and