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Purified high molecular weight synthetic Aβ(1–42) and biological Aβ oligomers are equipotent in rapidly inducing MTT formazan exocytosis

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ABSTRACT

Synthetic soluble Aβ oligomers are often used as a surrogate for biologic material in a number of model systems. We compared the activity of Aβ oligomers (synthetic and cell culture media derived) on the human SH-SY5Y neuroblastoma and C2C12 mouse myoblast cell lines in a novel, modified MTT assay. Separating oligomers from monomeric peptide by size exclusion chromatography produced effects at peptide concentrations approaching physiologic levels (10–100 nM). Purified oligomers, but not monomers or fibrils, elicited an increase of a detergent-insoluble form of MTT formazan within 2 h as opposed to a control toxin (H_2O_2). This effect was comparable for biological and synthetic peptide in both cell types. Monomeric Aβ attenuated the effect of soluble oligomers. This study suggests that the activities of biological and synthetic oligomers are indistinguishable during early stages of Aβ oligomer–cell interaction.

Recent studies have focused on soluble multimeric forms of Aβ as potential etiologic factors of Alzheimer's disease (AD) initiation and progression. Familial forms of AD involving mutations in the presenilin component of γ-secretase result in the production of an increased proportion of the longer Aβ(*x*–42) peptide at the expense of Aβ(*x*–40) [1]. The longer 42-amino acid form of Aβ readily forms soluble oligomeric structures with potent biological activity toward a variety of cell types including neurons [3]. Multiple forms of Aβ(1–42) oligomers can be produced from the synthetic peptide depending on the method of solubilization of the peptide and the conditions of incubation in aqueous media [2,3,10]. Size exclusion chromatography of brain tissue from AD cases or APP/PS1 knock-in mice reveals a profile of apparent oligomer sizes of 100–600 kDa and a large peak of monomer with essentially no intermediate size Aβ-containing species detectable by immunoassay [17]. A similar size distribution of oligomers is produced following incubation of disaggregated Aβ(1–42) at room temperature for 1–2 h.

Synthetic Aβ(1–42) is frequently used to prepare oligomers as a surrogate for biologically derived peptide, as a means to reproducibly generate easily characterized material. Biologically derived Aβ oligomers are heterogeneous, and starting concentrations are low in tissue culture media. However, isolation from human or transgenic mouse brain requires a large investment of effort compared to cell culture. Thus, many studies are carried out with more readily available synthetic peptide. Synthetic oligomers are prepared under a variety of conditions that produce different populations of oligomeric and protofibrillar structures that elicit different responses from cells [3]. Despite this, the equivalence of biological and synthetic oligomers in eliciting cellular responses is rarely demonstrated.

A common assay used to detect cellular effects of Aβ assemblies is the metabolism of the dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or a derivative. The reduction of the MTT tetrazole dye to the formazan form is believed to measure mitochondrial activity, which is taken as an indicator of cell viability. When exposed to low concentrations of Aβ(1–42) oligomers, many types of cells respond by vacuolation, inhibition of tetrazolium dye reduction activity, and increasing the rate of exocytosis [13]. Aβ oligomers activate P21-activated protein kinases (PAKs) which regulate actin cytoskeleton organization through cofilin phosphorylation and are translocated from the cytosol to membrane vesicles in cultured hippocampal neurons [10], and in the brains of AD cases and in aged Tg2576 mice [15]. Cultured mature oligodendroglia, but not oligodendroglial precursor cells, also increase exocytosis of formazan when incubated with oligomeric Aβ(1–42), and myelination inhibition is reminiscent of white matter degeneration in AD [7]. Hence, enhanced exocytosis is an early step in Aβ oligomer-mediated events, not restricted to neurons, and is disease-relevant.

Abbreviations: APP, amyloid precursor protein; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Hence, while inhibition of formazan production is generally equated to cell death, this is an oversimplification, as a variety of conditions will interfere with the reduction process though the cells remain alive. Two discrete forms of formazan are visible in cells under the microscope: punctate granules (intracellular) and exocytosed membrane-associated elongated needles or plates (extracellular). The relative proportion of these forms depends on cell type and culture conditions. In 2007, Hong et al. [6] determined that these two forms were differentially soluble in Tween-20, and that soluble Aβ assemblies, but not the monomeric peptide, stimulated needle production shortly after exposure of cells to the assemblies. Hence, the initial cellular response to Aβ assemblies results in an increase in formazan production, not the decrease seen after lengthy incubations (24–48 h) connected with cell death. We have taken advantage of this altered MTT reduction without direct cell death to monitor an early stage in the multistep cellular response to soluble Aβ oligomers.

Oligomer preparation from synthetic Aβ(1–42) peptide diluted from DMSO solution was as described [8,12] and Supplemental material. Biological Aβ enriched in oligomers was collected by concentrating (10×) serum-free culture media supernatant of human H4 neuroglioma cells (H4-APP) overexpressing human APP695 on a Microcon 10 kDa cut-off centrifugal spin filter (Millipore) before chromatography on Sephadex G75 as for synthetic peptide. Synthetic Aβ(1–42) peptide was purchased from rPeptide (Bogart, GA). Oligomers were formed by dilution to 2 μg/ml (443 nM) in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5, and incubated at room temperature for 2 h. After BSA addition (2 mg/ml), oligomers were separated from monomers on a Sephadex G-75 size-exclusion column equilibrated with an appropriate cell culture media containing 2 mg/ml BSA. Aβ(1–42) fibrils were produced by incubating the synthetic peptide at 1 mg/ml unstirred in 50 mM sodium phosphate, 150 mM NaCl, 0.02% sodium azide, pH 7.5 at 37 °C for 5 days.

Aβ content was determined by sandwich ELISA based on [12] using an N-terminal Aβ specific capture antibody (6E10) and a mid-region Aβ-specific biotinylated detection antibody (bio4G8) and a Biotek Synergy HT plate reader. Sample Aβ content was quantified with reference to a concurrently run standard curve of monomeric Aβ(1–40).

SH-SY5Y human neuroblastoma cells, C2C12 mouse myoblast cells, and human H4 neuroglioma cells were grown in standard culture media (MEM, DMEM and GlutaMAX™-I, respectively; InVitrogen/Gibco) containing 10% FBS and 1% Penicillin/Streptomycin. Media for H4 cells stably transfected with human APP695 [17] was supplemented with 200 μg/ml Hygromycin B.

Cells were plated in a 96-well tissue culture plate (Greiner bio-one 655180) at 20,000 cells/well in 100 μl of media. The cells recovered at 37 °C under 5% CO_2 overnight. The next day, Aβ in cell type-specific growth media was pre-warmed to 37 °C and added to each well (100 μl) after aspirating the old media. The plate was returned to the incubator for 1 h, then 10 μl of 5 mg/ml MTT (Sigma–Aldrich) dissolved in growth media was added (final concentration of 0.5 mg/ml) and incubated for an additional hour at 37 °C.

Tween-soluble formazan was separated from Tween-insoluble MTT by the addition of 10 μl of 10% v/v Tween-20 in culture media (final concentration 1% v/v). The plate was shaken at 300 rpm at 37 °C for 10 min to fully solubilize the detergent-soluble fraction of reduced MTT formazan. The media was collected as Tween-soluble MTT (sMTT). The remaining Tween-insoluble MTT (iMTT) was dissolved in 120 μl of DMSO. The absorbance of both MTT pools was read with a Biotek plate reader at 594 nm, subtracting the background absorbance at 690 nm. Using the measured optical densities ($OD_{594\,nm} - OD_{690\,nm}$) of the iMTT and sMTT fractions, the %iMTT was calculated as [iMTT/(iMTT + sMTT)]. For comparison, the %iMTT

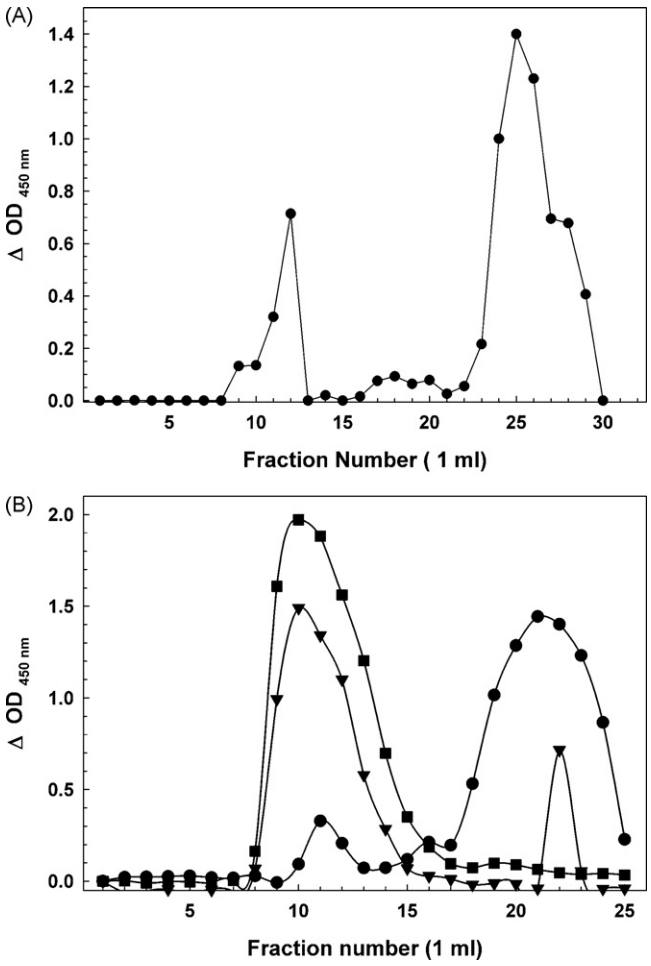

Fig. 1. (A) Sephadex G75 size exclusion chromatography of a synthetic Aβ(1–42) oligomer preparation. The first peak of $OD_{450\,nm}$ (~fractions 9–12) represents the Aβ immunoreactivity of high molecular weight oligomeric Aβ eluting in the void volume, while the second peak (~fraction 23–30) represents primarily monomer and low-m.w. oligomers near the inclusion volume. BSA (66 kDa fr. 13); myoglobin (17 kDa fr. 20). (B) Sephadex G75 size exclusion chromatography of H4 Neuroglioma-derived Aβ Peptides. Similar to the synthetic peptide, the first peak (~fractions 8–15) represents the Aβ immunoreactivity high molecular weight oligomeric Aβ eluting in the void volume, while the second peak (~fraction 18–25) represents primarily monomer and low-m.w. oligomers near the inclusion volume. BSA (66 kDa fr. 13); myoglobin (17 kDa fr. 20). As the H4 cells incubate in serum-free media, the population of Aβ in the media shifts from nearly all monomeric to largely high-m.w. oligomers. Day 1 (circles); Day 2 (inverted triangles); Day 3 (squares).

of wells to which cell media lacking Aβ were added was set equal to 100.

Purified synthetic Aβ(1–42) oligomers and monomers were diluted separately in MEM culture media to 30 nM. Concentrations of oligomers were determined by 2-site immunoassay (6E10/bio4G8) compared to a standard curve generated with Aβ(1–40) monomer. Additionally, they were combined to give 30 nM oligomers and 30 nM monomers. SH-SY5Y cells were treated with these preparations for one hour and processed for MTT partitioning.

Oligomer preparations were fractionated on Sephadex G75 (exclusion limit ~70 kDa for globular proteins) in order to measure the amount of Aβ in oligomers and to separate them from monomeric peptide. The chromatography also permitted the exchange of the peptides into fresh culture media in the presence of BSA to prevent loss of oligomers on the chromatography resin. This choice of fractionation range combined the high molecular weight oligomers into a single pool. Fig. 1A shows a gel filtra-