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Purified High Molecular Weight Synthetic A β (1-42) and Biological A β Oligomers are Equipotent in Rapidly Inducing MTT Formazan Exocytosis

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Abstract

Synthetic soluble A β oligomers are often used as a surrogate for biologic material in a number of model systems. We compared the activity of A β 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 two hours as opposed to a control toxin (H₂O₂). This effect was comparable for biological and synthetic peptide in both cell types. Monomeric A β attenuated the effect of soluble oligomers. This study suggests that the activities of biological and synthetic oligomers are indistinguishable during early stages of A β oligomer-cell interaction.

Keywords

H4 neuroglioma; C2C12 myoblast; SH-SY5Y neuroblastoma; size exclusion chromatography; ELISA

Introduction

Recent studies have focused on soluble multimeric forms of A β 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 A β (x-42) peptide at the expense of A β (x-40) [1]. The longer 42-amino acid form of A β readily forms soluble oligomeric structures with potent biological activity toward a variety of cell types including neurons [3]. Multiple forms of A β (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

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reveals a profile of apparent oligomer sizes of 100 kDa to 600 kDa and a large peak of monomer with essentially no intermediate size A β -containing species detectable by immunoassay [17]. A similar size distribution of oligomers is produced following incubation of disaggregated A β (1-42) at room temperature for 1–2 hours.

Synthetic A β (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 A β 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 A β 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 A β (1-42) oligomers, many types of cells respond by vacuolation, inhibition of tetrazolium dye reduction activity, and increasing the rate of exocytosis [13]. A β 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 A β (1-42), and myelination inhibition is reminiscent of white matter degeneration in AD [7]. Hence, enhanced exocytosis is an early step in A β oligomer-mediated events, not restricted to neurons, and is disease-relevant.

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 proportions of these forms depend 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 A β assemblies, but not the monomeric peptide, stimulated needle production shortly after exposure of cells to the assemblies. Hence, the initial cellular response to A β assemblies results in an increase in formazan production, not the decrease seen after lengthy incubations (24–48 hours) 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 A β oligomers.

Materials and Methods

Oligomer Generation

Oligomer preparation from synthetic A β (1-42) peptide diluted from DMSO solution was as described [8, 12] and Supplemental Material. Biological A β enriched in oligomers was collected by concentrating (10 $\times$) serum-free culture media supernatant of human H4 neuroglioma cells (H4 –APP) overexpressing human APP695 on a Microcon 10kDa cut-off centrifugal spin filter (Millipore) before chromatography on Sephadex G75 as for synthetic peptide. Synthetic A β (1-42) peptide was purchased from rPeptide (Bogart, GA). Oligomers

were formed by dilution to 2 μ g/ml (443 nM) in 50mM sodium phosphate, 150mM NaCl, pH 7.5, and incubated at room temperature for two hours. 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. A β (1-42) fibrils were produced by incubating the synthetic peptide at 1 mg/ml unstirred in 50mM sodium phosphate, 150mM NaCl, 0.02% sodium azide, pH 7.5 at 37°C for 5 days.

Quantifying A β

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

Cell Culture

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 μ g/ml Hygromycin B.

Partitioning of MTT

Cells were plated in a 96-well tissue culture plate (Greiner bio-one 655180) at 20,000 cells/well in 100 μ l of media. The cells recovered at 37°C under 5% CO₂ overnight. The next day, A β in cell type-specific growth media was pre-warmed to 37°C and added to each well (100 μ l) after aspirating the old media. The plate was returned to the incubator for one hour, then 10 μ l of 5 mg/ml MTT (Sigma-Aldrich) dissolved in growth media was added (final concentration of 0.5mg/ml) and incubated for an additional hour at 37°C.

Tween-soluble formazan was separated from Tween-insoluble MTT by the addition of 10 μ l 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 minutes 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 μ l 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 of wells to which cell media lacking A β were added was set equal to 100.

Effects of Oligomeric A β in the presence of monomer

Purified synthetic A β (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 A β (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.

Results

Oligomer preparations were fractionated on Sephadex G75 (exclusion limit ~70 kDa for globular proteins) in order to measure the amount of A β 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. Figure 1A shows a gel filtration profile of synthetic A β (1-42) oligomers detected by two-site ELISA. Figure 1B shows profiles for biological A β oligomers concentrated from huAPP695-transfected H4 human neuroglioma cell media over three days in serum-free medium. A β concentrations are expressed as monomer equivalents. Only the void volume fractions and the included A β monomer fractions contained significant quantities of A β for both the synthetic peptide and the H4 media. Re-chromatography of purified G75 void volume synthetic or biologic oligomers returned only void volume material with little to no trace of monomer, even if stored at 4 °C for 24 hours, and their size distribution on Sepharose 12 was unaltered, demonstrating their stability in culture media (data not shown).

Figure 2A illustrates the distinction between Tween-insoluble needles and soluble granule structures in SH-SY5Y cells; after Tween-20 extraction only the needles remain. Figure 2B compares the effects of different assembly states of A β (1-42) on the processing of MTT by SH-SY5Y neuroblastoma cells using differential Tween-20 extraction. Neither 0.5 μ M fibrils nor 0.5 μ M monomers changed the proportion of iMTT produced compared to control media. In contrast, a significant increase ($p < 0.001$) in iMTT was induced by the addition of a lower concentration (90 nM) of purified synthetic A β oligomers, and brief treatment with H₂O₂ (0.3%, 3 minutes) reduces iMTT in comparison to controls.

The effect of oligomers relative to monomer or fibrils is dose-dependent and comparable in both SH-SY5Y and C2C12 cells (Figure 3A). Fifty nM of either synthetic or biological monomer had no effect on iMTT for either cell type (circled). There was no statistically significant difference in potency between synthetic and H4-produced (biological) oligomers. Similarly fractionated non-transfected H4 cell-conditioned media had no effect on MTT metabolism. In SH-SY5Y cells, the EC₅₀ for synthetic oligomers was 12.19 nM $\pm$ 0.969 nM, and 13.43 nM $\pm$ 0.725 nM for biologically derived oligomers. Similarly, in C2C12 cells the comparable EC₅₀ values were 15.75 nM $\pm$ 0.838 nM (synthetic) and 19.22 nM $\pm$ 0.932 nM (biological). The dose-responses of synthetic and biological oligomers are positively correlated (Figure 3B). Therefore, biological and synthetic A β oligomers are indistinguishable as monitored by this method.

While oligomeric A β clearly has long term toxic effects on cells, the role of monomer in normal and pathobiological situations is less clear. There are reports of neuroprotective effects of the monomeric peptide in different situations [5]. Since in most studies with synthetic oligomers, as well as physiologically, A β is present as mixtures of oligomers and monomers, we took advantage of the availability of separate pools of purified oligomers and monomeric A β (1-42) to test whether monomer could exert protective effects against oligomer-induced exocytosis of MTT needles. The presence of an equal concentration of monomer partially mitigates the effect of a non-saturating dose of oligomers (Figure 4). While the difference between these two groups did not achieve statistical significance given the limited number of replicates (N=3), the trend suggests oligomer effects may be underestimated in the presence of monomeric peptide.

Discussion

We sought an assay that reflected oligomer effects at early times after exposure to low oligomer concentrations that also gave rise to a positive signal, rather than an inhibition which is a generic terminal response less informative of cellular events. The exocytosis of reduced formazan produced by reduction of the MTT tetrazolium dye by cellular enzymes [14] and the differential effects of A β oligomers on formazan deposits elaborated by Hong *et*

al. [6] provide a rapid and sensitive marker of oligomer activity in a variety of cell types. The ratio of Tween-insoluble formazan to Tween-soluble formazan gives an indication of oligomer activity and a one hour treatment with nM concentrations of chromatographically purified oligomers is sufficient to elicit a maximal response.

The conditions for measurement of the biological activity of A β oligomers were chosen to reflect concentrations of the peptide approaching the nM and lower concentrations believed to be present in the AD brain [4]. At nM concentrations, A β oligomers bind to dendrites of some neurons in primary culture [9]. Binding is reduced by brief mild proteolysis of the cell surface [10], a hallmark of interaction with a receptor(s). In many studies, however, biological effects of A β oligomers are assessed on cultured cells at middle to high micromolar concentrations and following lengthy exposure times, often 24–48 hours, using cell death as an endpoint. A β oligomers are surface active materials and long incubations at high concentrations can lead to non-specific perturbation of the lipid bilayer.

Soluble synthetic or H4 neuroglioma cell culture-derived A β oligomers can be readily separated from monomer by chromatography on Sephadex G75. The lack of a consistent or significant concentration of intermediate size A β -containing structures (15–50 kDa) (also not observed on Sepharose 12 chromatography) suggests either that oligomer shape alters their apparent molecular weight on size exclusion chromatography, or that these structures are not stable on the time scale of the separation (20–30 minutes). The separated oligomers, by contrast, are stable for at least 24 hours at 4 °C.

Monomeric A β (1-42) peptide is believed to be nontoxic, and does not alter MTT metabolism [6, 10, 19], and fibrillar peptide is only weakly toxic. Neither of these forms of A β increased iMTT above controls. Synthetic and H4 neuroglioma-derived oligomers indistinguishably, potently, and dose dependently increase the proportion of iMTT on the surface of SH-SY5Y neuroblastoma cells and C2C12 myoblasts. This equivalence indicates that for this cellular measure of oligomer activity that the synthetic and biological oligomers are comparable. Recent studies with primary rat neuronal cultures report similar effects with synthetic and biological A β oligomers [20]. The potency of purified oligomers and the lack of effect of purified monomer on iMTT production suggested the possibility that the presence of monomeric A β could attenuate the effects of oligomers on cellular systems. Monomer-free A β oligomers are considerably more potent (EC₅₀ 10 – 20 nM, based on A β monomer content) on early stages of cellular activity measured by MTT exocytosis in two cell types than is generally reported with monomer-oligomer mixtures. Addition of monomeric A β (1-42) to fractionated A β oligomers reduced the efficacy of the oligomers by a mechanism that remains to be explored.

The observed similarities between the cellular effects of synthetic A β oligomers and H4-produced oligomers are a significant finding. Whether the similarity extends to oligomers produced in different ways or isolated from different sources should be validated. The mass and size distribution heterogeneity of synthetic A β oligomers depends on the conditions under which they are formed. Their resistance to dissociation by denaturants such as urea and SDS also reveal differences among the preparations in kinetic stability [16], which reflects underlying structural characteristics in protein structure. Increased stability is particularly evident in oligomers from biological sources such as those that accumulate in APP transgenic mouse models of A β pathology [11, 18] and SDS-stable dimeric species postulated to be unique to AD [19]. As the cellular responses elicited by soluble A β oligomers are probed in more detail it is likely that the more subtle structural details of both synthetic and biological oligomers will impact aspects of cellular biology differently. Not all forms of soluble oligomers, either synthetic or biological, may be equivalent

physiologically, which would have consequences for biomarker and therapeutic strategies for Alzheimer's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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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Research Highlights

- Purified soluble A β oligomers rapidly induce cellular MTT formazan exocytosis.
- Synthetic A β (1-42) and cell-derived oligomers equipotently stimulate exocytosis .
- Half-maximal stimulation of exocytosis induced by 10-20 nM oligomers.
- Monomeric A β attenuates the effect of soluble oligomers.

Figure 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).

Figure 2.

(A) Oligomeric Aβ Increases the Proportion of Tween 20-insoluble MTT Formazan in SH-SY5Y Neuroblastoma Cells. Two types of formazan are deposited: intracellular granules and extracellular needles. Cell surface (left) and intracellular (right) focal plane images are shown. Aβ oligomers induce increased formation of the Tween 20-insoluble needles; addition of Tween 20 solubilizes the granules, leaving behind the needle-like formazan. The supernatant is collected and the remaining formazan solubilized in DMSO. Thick lines = needles; dashes = granules. (a) untreated cells metabolizing MTT; (b) cells treated with Aβ oligomers metabolizing MTT; (c) cells treated with Aβ oligomers metabolizing MTT after extraction with Tween 20. **(B)** Effect of Oligomeric, Fibrillar, and Monomeric Synthetic Aβ(1-42) Peptides on MTT Formazan Exocytosis from SH-SY5Y Neuroblastoma Cells. Oligomeric Aβ (90 nM) yields a distinct increase in the proportion of detergent insoluble

MTT (iMTT) over normal baseline production (=100%). No detectable change from control proportions was seen with high concentrations (0.5 μ M) of monomeric or fibrillar A β . Furthermore, the alteration by oligomeric A β is in a direction opposite of acute toxins such as hydrogen peroxide. Each bar represents ten replicates. Asterisk denotes significance from control ($p < 0.001$) and dagger indicates significance from monomer/fibril ($p < 0.001$).

Figure 3.

(A) Dose-dependent increase of iMTT in response to oligomeric A β , but not monomer (50 nM; circled); N=3. SH-SY5Y cells with biological oligomers (closed squares); SH-SY5Y cells with synthetic oligomers (open circles); C2C12 cells with biological oligomers (closed triangle); C1C12 cells with synthetic oligomers (open inverted triangles). (B) Data from the dose-response experiment in (A) plotted as a correlation between the responses elicited from

synthetic A β oligomers versus biological A β oligomers. The diagonal line indicates a linear fit to all of the data on the plot (slope = 1.07; $r^2 = 0.795$). SH-SY5Y (closed circles); C2C12 (open circles). Biologically derived and synthetic oligomeric A β elicit essentially the same effects.

Figure 4. Monomeric A β (1-42) Modulates Oligomer-induced iMTT Exocytosis in SH-SY5Y cells. The addition of 30nM monomeric A β to 30 nM oligomeric A β attenuates the ability of A β oligomers to increase iMTT. * = $p < 0.05$ compared to no A β .