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ORIGINAL ARTICLE

Generation of reactive oxygen species by beta amyloid fibrils and oligomers involves different intra/extracellular pathways

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Abstract A neuropathological characteristic of Alzheimer's disease is the extracellular accumulation of amyloid beta peptide (Aβ) in neuritic plaques. Recent evidences suggested that soluble Aβ oligomers are the predominant neurotoxic species for neurons. Thus, considerable attention has been paid to discriminate the cytotoxic pathways of Aβ pre-fibrillar aggregates and mature fibrils. We showed that the mechanisms by which Aβ oligomers and fibrils generated reactive oxygen species differ in terms of site of production and kinetics, suggesting the involvement of different intra/extracellular pathways.

Keywords Bet amyloid · Neurotoxicity · Free radicals · Oligomers

Introduction

Among the numerous mechanisms hypothesized to cause Alzheimer's Disease (AD), much evidence supports the view that beta amyloid (Aβ) is neurotoxic and that it initiates a cascade of events eventually leading to synaptic failure and neuronal death. The strongest evidence for amyloid being a cause, and not just a consequence of the disease comes from genetics (Selkoe 2001). Familial or early onset form of AD mutations in the amyloid precursor protein (APP) and the enzymes that clip it to form Aβ, lead to a massive overproduction of Aβ and to a swift descent into disease.

The amyloid cascade hypothesis has undergone some revision (Selkoe 2002). Initially it was believed that the plaques themselves were causing the disease. Based on that idea, it has been proposed that removing the plaques might retard or even reverse the memory loss and cognitive decline seen in AD. Recent studies on the neurotoxic properties of Aβ have opened a new vision on this process. Aβ toxicity, at least in cell culture, was found to be dependent on the fibrillar state of the peptide. Recent evidence suggests that soluble Aβ oligomers are the predominant neurotoxic species for neurons (Klein 2002; Glabe 2005; Tamagno et al. 2006). In this regard Aβ oligomers were found, even at nanomolar concentration to kill mature neurons in hippocampal slices (Lambert et al. 1998). Moreover, Aβ oligomers appeared to interfere with many critical neuronal activities, including inhibition of long-term potentiation (LTP) in organotypic hippocampal slices (Lambert et al. 1998; Wang et al. 2002). Aβ oligomers can also cause calcium dysfunction and membrane disruption, thus interfering with overall cell functioning (Demuro et al. 2005; Kayed et al. 2004). Aβ oligomer toxicity has also been shown in vivo (Walsh et al. 2002). In particular, i.c.v. injection of Aβ oligomers inhibits LTP and disrupts cognitive function (Walsh et al. 2002; Cleary et al. 2005). Importantly, the concomitant injection of the anti-Aβ antibody 6E10 with oligomers, neutralized the oligomer-induced LTP dysfunction (Klyubin et al. 2005). These data support the idea that oligomers represent a fundamental species responsible for mediating Aβ toxicity in AD (Selkoe 2001).

On the other hand, it is well established that the aggregated Aβ, referred to as fibrils, is toxic for cultured neurons. The standard protocol to obtain fibrillar Aβ

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foresees the incubation of the peptide at 37°C for hours or even days, to produce neurotoxicity (May et al. 1992). Depending upon the time expected for Aβ40/Aβ42 in vitro incubations, such a preparation contains a plethora of Aβ forms (oligomers, pre-fibrils and fibrils); some or all of which may have biological activity. Thus, using this protocol it is difficult to discriminate the effects of the individual aggregate state of Aβ, since the effects observed should be attributed to all of them. Recently, a new protocol has been developed to obtain different aggregated species of Aβ (Dahlgren et al. 2002; Stine et al. 2003) separately. This new approach allows better investigation of the biological properties of the individual Aβ aggregated species.

The present study aimed to clarify the specific effects of the individual aggregated isoforms of Aβ. This is relevant to deriving an appropriate therapeutic approach to the disease.

Materials and methods

Cell culture

The neuroblastoma cell line SH-SY5Y was routinely cultured in 1:1 Ham's F12:Dulbecco modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 100 μg/ml streptomycin and was kept at 37°C in humidified 5% CO_2/95% air. For differentiation, cultures were seeded at approximately 10^5 cells/dish and retinoic acid was added to a final concentration of 10 μM. The cultures were allowed to differentiate for 1 week. All the experiments were then done with differentiated cells.

Peptide preparation

Synthetic wild-type Aβ1-42 (Biosorce) was initially dissolved to 1 mM in hexafluoroisopropanol (Sigma) and separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was removed under vacuum and the peptide film was stored desiccated at −20°C. The aggregation protocol was done as described by Dahlgren et al. (2002). Briefly, the peptide was first resuspended in dry dimethyl sulfoxide (Me_2SO, Sigma) to a final concentration of 5 mM. For oligomeric conditions, Ham's F-12 (phenol red-free, BioSource, Camarillo, CA) was added to bring the peptide to a final concentration of 100 μM, and incubated at 4°C for 24 h. For fibrillar conditions, 10 mM HCl was added to bring the peptide to a final concentration of 100 μM and incubated for 24 h at 37°C. For unaggregated conditions, the 5 mM Aβ in Me_2SO was diluted directly into cell culture media.

Time-course of amyloid aggregate binding to cell surface membrane and their internalization

Aggregate adsorption to cell surface was analyzed as previously described (Cecchi et al. 2006). Briefly, 1.5×10^4 SH-SY5Y cells were seeded on glass coverslips, differentiated with 10 μM retinoic acid for 7 days and exposed to unaggregated, oligomeric and fibrillar species of Aβ 1-42 peptide for 2, 4 and 6 h. Then the cells were counterstained with fluorescein-conjugated wheat germ agglutinin (50 μg/ml) for 10 min to detect plasma membrane profiles and fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature. The plasma membrane was permeabilized by treating cells with a 3.0% glycerol solution in PBS containing 0.5% BSA. After washing, the coverslips were incubated with mouse monoclonal anti-Aβ antibodies 6E10 (Signet, DBA, Italy) diluted 1:1,000 in PBS containing 1.0% FBS for 60 min. The immunoreaction was revealed by incubating the samples for 90 min with Texas Red-conjugated anti-mouse secondary antibodies (Vector Laboratories, DBA, Italy) diluted 1:1,000 in the same solution. Negative controls were obtained by substituting the washing solution for the primary antibody (not shown). The fluorescence was analyzed using a confocal (Bio-Rad MCR 1024 ES) scanning microscope (Hercules, CA) equipped with a krypton/argon laser source (15 mW) for fluorescence measurements using two excitation wavelengths at 568 and 488 nm for Texas Red and fluorescein, respectively. The observations were performed using a Nikon Plan Apo 60× oil immersion objective. A series of optical sections (512 × 512 pixels) 1.0 μm in thickness was taken through the cell depth at intervals of 0.8 μm. A number of optical sections ranging from 10 to 20 for each examined sample were then projected as a single composite image by superimposition. Quantification of surface bound or internalized aggregates was achieved by the freely available Java image processing program (ImageJ). Texas Red fluorescence was expressed as fractional change above the resting value, $\Delta F/F$, where F is the average baseline fluorescence before the application of the peptide aggregates and ΔF represents the fluorescence changes over the baseline.

Evaluation of cell viability

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), showing the mitochondrial activity of living cells. Differentiated SH-SY5Y neuronal cells in 96-well plates were challenged with different aggregated species of Aβ at different concentrations for 72 h, then 500 μg/ml MTT (final concentration) was added in each well and cells were incubated at 37°C for additional 3 h. MTT was removed, and cells were lysed with dimethyl

sulfoxide. The absorbance at 595 nm was measured using a Bio-Rad 3350 microplate reader. Control cells were treated in the same way without the peptides, and the values of different absorbances were expressed as a percentage of control.

Reactive oxygen species detection

Detection of intracellular ROS was performed as previously described (Uberti et al. 2007), neuronal cell cultures grown on 35-mm dishes were incubated with 10 μM 2′,7′-dichlorofluorescin (DCF) diacetate (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C after a time-course with different Aβ species. The cells were then rinsed with Kreb's ringer solution. Intracellular esterases convert DCF diacetate to anionic DCFH which is trapped in the neuron. The fluorescence of DCF, formed by the reaction of DCFH with ROS was examined with Fusion TM Master (Packard Bioscience Company).

Statistical evaluation

Results were given as mean ± standard error mean values. Statistical significance of differences was determined by mean values of the ANOVA, followed by Student's t test. Significance was accepted for a p value < 0.05.

Results

Both fibrils and oligomers induce ROS generation

Differentiated SH-SY5Y neuronal cells were exposed to monomeric, oligomeric and fibrillar species of Aβ 1-42 at different concentrations and 48 h later cell viability was measured using the MTT assay. At 10 nM concentration, both monomeric and fibrillar Aβ 1-42 peptides were not toxic, while oligomers reduced cell viability by about 25%. Oligomeric and fibril isoforms reduced cell viability in a dose-dependent manner, with oligomers being more toxic than fibrils at each concentration examined. Monomeric Aβ 1-42 peptide displayed neurotoxic properties only at concentrations higher than 1 μM and the degree of cell loss was consistently lower in comparison with similar concentrations of oligomers and fibrils (Fig. 1a). One of the more accredited mechanism involved in Aβ toxicity is the production of free radicals. Here we investigated whether monomeric, oligomeric and fibrillar species of Aβ differently activated ROS generation (Fig. 1b). Fibrillar and oligomeric Aβ 1-42 peptide induced ROS generation already after 3 h and this effect lasted for at least 24 h (Fig. 1b). At 3 h, fibrils and oligomers increased ROS by about 25% and 20%, respectively. As shown in Fig. 1b, monomeric Aβ species induced a lower ROS increase (15%) as measured at 6 and 24 h.

Aβ1-42 fibrils bind to cell surface membrane while oligomers enter the cells

To understand whether oligomers- and fibrils-induced ROS generation was mediated by an extracellular or intracellular signal, we investigated the adsorption of the amyloid assemblies on the cellular surfaces and the internalization within neuronal cells. To that purpose, differentiated SY5Y cells were exposed to 1 μM monomeric, oligomeric and fibrillar species of Aβ 1-42 peptide and at different time points immunofluorescence confocal microscopy analysis was performed using 6E10 monoclonal anti-Aβ antibodies.

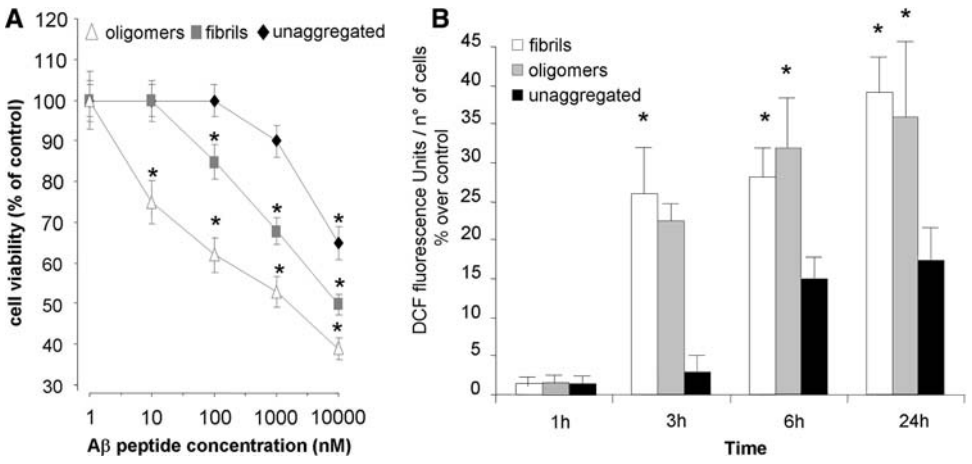

Fig. 1 **a** Cells were treated with oligomers, fibrils and unaggregated peptide at different concentrations and then cell viability was measured using MTT assay. **b** Cells were treated with oligomers, fibrils and unaggregated peptides at the concentration of 1 μM and 24 h later ROS generation inside the cells was measured using DCF fluorescence. Data represents mean ± SEM of at least three different experiments from three separate cell preparations. *$p < 0.01$ versus the corresponding control values

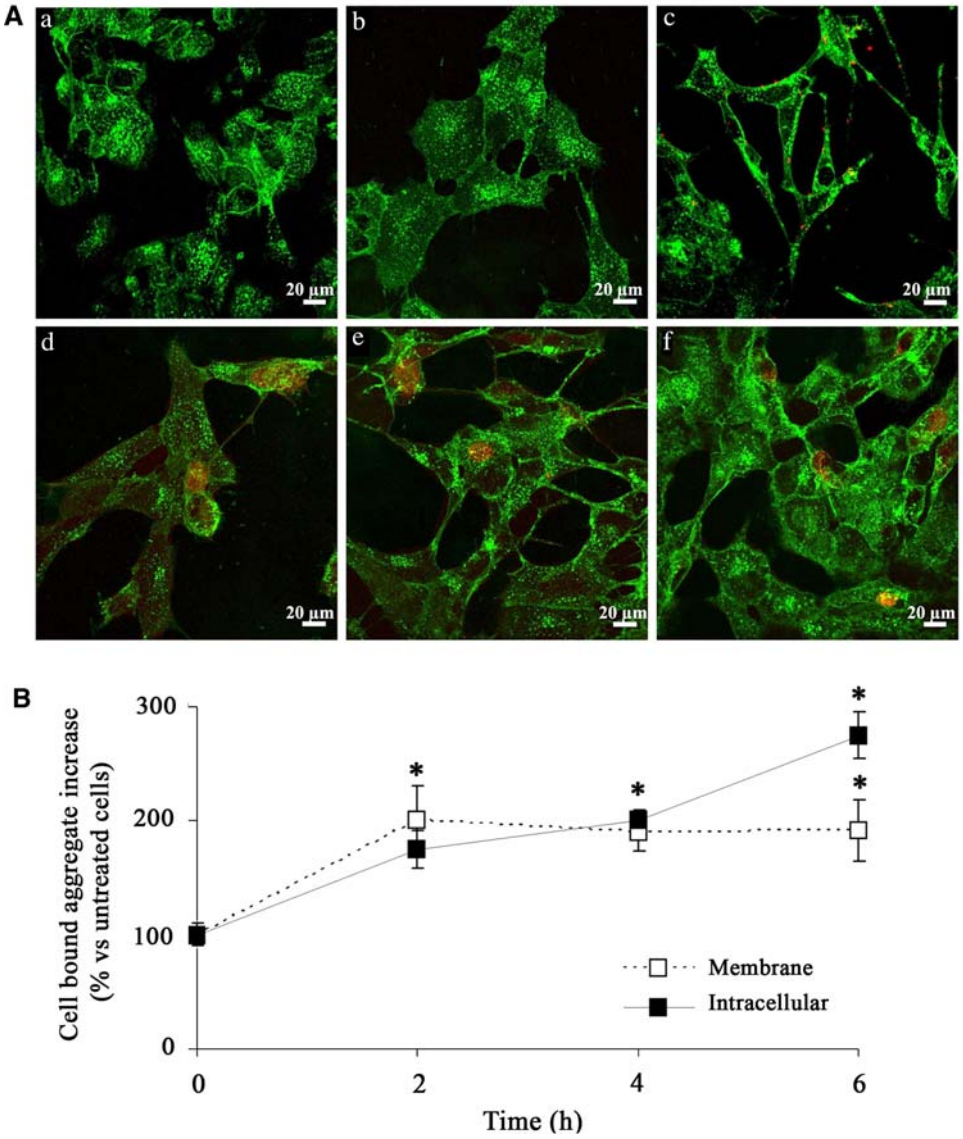

Fig. 2 **a** Representative confocal microscopy images showing the effect of various species of Aβ1-42 peptide on aggregate interaction with cellular surface, and internalization within the cytoplasm, of SH-SY5Y cells. (*a*) untreated cells; (*b*) cells treated with 1 μM unaggregated Aβ1-42 for 6 h; (*c*) cells treated with 1 μM fibrillar Aβ1-42 for 6 h; (*d–f*) cells treated with 1 μM oligomeric Aβ1-42 for 2 (*d*), 4 (*e*) and 6 (*f*) hours. Counterstaining was performed with fluorescein-conjugated wheat germ agglutinin to detect plasma membrane profile (*green*). The aggregates were labeled with monoclonal mouse 6E10 anti-Aβ antibodies and Texas Red-conjugated anti-mouse secondary antibodies with plasma membrane permeabilization with glycerol as specified under "Materials and methods". **b** Time-course of the oligomers bounded to cell surface membrane of SH-SY5Y cells or aggregate internalization within the cells. Quantification analysis of Texas Red fluorescence signals are expressed as % changes (mean ± SEM) versus untreated cells (assumed as 100%) of three independent experiments. *Significant difference ($p \leq 0.01$) versus untreated cells

As shown in Fig. 2a, no significant difference in red fluorescence signal between untreated (a) and monomeric Aβ1-42 treated (b) cells can be observed, even after 6 h of exposure, whereas Aβ1-42 fibrils showed a moderate binding to cell surface membranes (c). On the other hand, Aβ1-42 oligomers, added to the cell culture medium, were able to accumulate quickly near the plasma membrane and to be internalized into the cytoplasm of neuroblastoma cells after 2, 4 and 6 h treatment (d–f). Quantification analysis of the amount of oligomeric aggregates bounded to cell membranes and inside the cells, supported the evidence on the internalization process of Aβ oligomeric species (Fig. 2b). When the cells were pre-treated with colchicine, which causes disruption of microfilaments and microtubules and inhibits phagocytosis and endocytosis, before adding oligomers in the medium, ROS generation was prevented. At variance colchicine did not affect fibrils-induced ROS production (Fig. 3).

Discussion

This study demonstrated that fibrils and oligomers, that are two different aggregated states of the same peptide, endow different properties. One of the substantial differences was the neurotoxic potency. Aβ1-42 oligomers were found to be the most potent species, being active at nanomolar concentrations. At the concentration of 10 nM oligomers affected cell viability, while fibrils at the same concentration were not toxic. At higher concentration fibrils became toxic even if their effects were less potent in comparison with oligomers. These data are in accordance with many

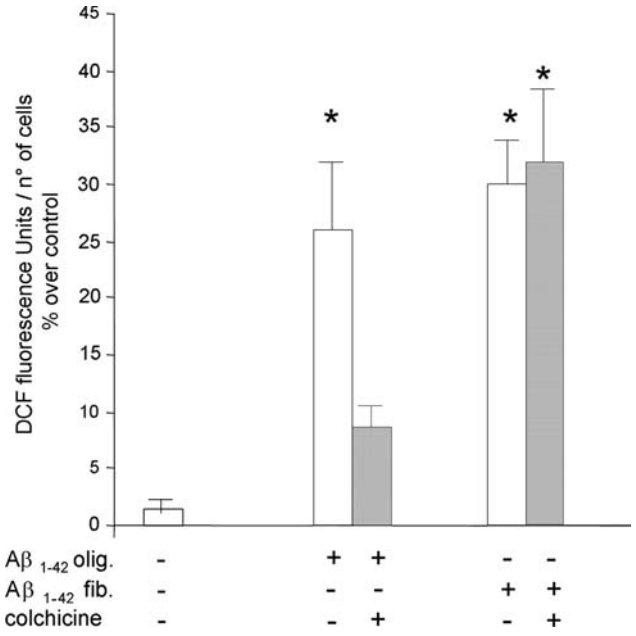

Fig. 3 Cells were treated with oligomers, fibrils in the presence or absence of colchicine then 24 h later ROS generation inside the cells was measured using DCF fluorescence. Data represent mean ± SEM of at least three different experiments from three separate cell preparations. *$p < 0.01$ versus the corresponding control values

other studies (Lambert et al. 1998; Hartley et al. 1999; Walsh et al. 2002; Wang et al. 2002) and among them Dahlgren et al. (2002) demonstrated that oligomers inhibited neuronal viability 10-fold more than fibrils and 40-fold more than unaggregated peptides.

A growing body of evidence suggests the contribution of oxidative stress in the neurodegenerative processes associated with AD. Oxidative stress seems to have a dual role in the pathogenesis of the disease. From one side, oxidative stress has been reported to induce Aβ production (Cleary et al. 2005; Pratico et al. 2001; Paola et al. 2000). On the other hand, numerous reports support the hypothesis that Aβ itself acts as a pro-oxidant agent. In fact, the toxic properties of Aβ-derived free radicals have been well documented (Misonou et al. 2000; Butterfield et al. 1994; Harris et al. 1995, 1996; Mark et al. 1995). Here we demonstrated that both oligomers and fibrils induced ROS production inside the cells. The ROS production caused by monomers was 2.5-fold lower than that derived from oligomers and fibrils. Generation of oxidative stress seems to be independent of Aβ aggregation states; in fact, all individual Aβ species are able to reduce copper II to copper I leading to free radical generation (Opazo et al. 2000). The time-course study and the confocal analysis, however, suggested that oligomers and fibrils can activate ROS in a different way. Fibrils, for the entire period of examination (6 h), did not enter the cells and showed a moderate binding to cell surface membranes. On the other hand, fibrils activated ROS generation after 3 h of exposure suggesting that ROS can be produced by lipid peroxidation of the plasma membrane. At variance, ROS generation induced by oligomers was presumably an intracellular event. In fact, Aβ1-42 oligomers, added to the cell culture medium, quickly accumulated near the plasma membrane and internalized in the cytoplasm. These observations are further supported by the finding that the endocytosis inhibitor Colchicine prevented oligomer-induced ROS enhancement.

Accumulating data point to the relevance of intraneuronal Aβ as a trigger of the pathological cascade of events leading to neurodegeneration (Wirths et al. 2004; Tseng et al. 2004; Billings et al. 2005; Oddo et al. 2006). Herein an additional concept is emerging: besides intraneuronal Aβ production, cellular uptake of Aβ from the extracellular space is considered as an additional mechanism that contributes to intracellular Aβ accumulation. In line with our data, Knauer et al. (1992) demonstrated that selective intracellular accumulation of Aβ synthetic peptides can be prevented under experimental conditions that do not allow endocytosis. Furthermore it is a matter of common knowledge that the extracellular Aβ aggregates are of neuronal origin and are secreted as soluble peptide (Wirths et al. 2004). Thus, it can be hypothesized that, in AD pathology, Aβ overproduced by few cells and released in the extracellular space may enter bystander cells propagating, along a specific neuronal circuit, the deleterious Aβ-induced effects.

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