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NEUROBIOLOGY OF AGING

Neurobiology of Aging 20 (1999) 325–330
Open peer commentary

In vitro and in vivo oxidative stress associated with Alzheimer's amyloid β-peptide (1–42)

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Received 30 October 1998; received in revised form 21 April 1999; accepted 4 June 1999

Abstract

The amyloid β-peptide (Aβ)-associated free radical oxidative stress model for neuronal death in Alzheimer's disease (AD) brain predicts that neuronal protein oxidation is a consequence of Aβ-associated free radicals [8]. In this study we have used both in vitro and in vivo models of β-amyloid (Aβ) toxicity to detect free radical induced oxidative stress by the measure of protein carbonyl levels. These model systems employed cultured hippocampal neurons exposed to exogenous synthetic Aβ(1–42) and transgenic *Caenorhabditis elegans (C. elegans)* animals expressing Aβ(1–42). We also investigated the importance of the Aβ(1–42) Met^{35} residue for free radical formation in peptide solution and for peptide-induced protein oxidation and neuronal toxicity in these model systems. Aβ(1–42) in solution yielded an EPR spectrum, suggesting that free radicals are associated with this peptide; however, neither the reverse [Aβ(42–1)] nor methionine-substituted peptide [Aβ(1–42)Met^{35}Nle] gave significant EPR spectra, suggesting the importance of the methionine residue in free radical formation. Aβ(1–42) addition to cultured hippocampal neurons led to both neurotoxicity (30.1% cell death, $p < 0.001$) and increased protein oxidation (158% of controls, $p < 0.001$), and both of those effects were not observed with reverse or Met^{35}Nle substituted peptides. *C. elegans* transgenic animals expressing human Aβ(1–42) also had significantly increased in vivo protein carbonyls (176% of control animals, $p < 0.001$), consistent with our model. In contrast, transgenic animals with a Met^{35}cys substitution in Aβ(1–42) showed no increased protein carbonyls in vivo, in support of the hypothesis that methionine is important in Aβ-associated free radical oxidative stress. These results are discussed with reference to the Aβ-associated free radical oxidative stress model of neurotoxicity in AD brain.

Keywords: Amyloid β-peptide; Cell culture; *C. elegans*; Free radical; Protein oxidation; Alzheimer's disease; Methionine

1. Introduction

Amyloid β-peptide (Aβ), the major constituent of the senile plaques (SP) that characterize Alzheimer's disease (AD) [38], is a 39–43 amino acid peptide derived from proteolytic processing of amyloid precursor protein (APP), a transmembrane glycoprotein that is expressed during normal cellular metabolism [17]. Synthetic Aβ is toxic to neuronal cultures [18,31,34,45], red blood cells [32] or clonal cell lines [4], and its neurotoxic properties may be involved in the neuronal degeneration seen in AD [39,44].

An increasing amount of experimental and genetic evidence points to an essential role of Aβ in AD. Mutations in the APP encoding gene are found in early-onset AD patients [15,33], and mutations in presenilin 1 and presenilin 2, which lead to early-onset AD, are associated with excess Aβ deposition and oxidative stress [16,39]. Transgenic animals overexpressing APP also show increased Aβ content in the brain and exhibit oxidative stress [13,39,41].

Evidence for involvement of free radicals in AD include the presence of elevated levels of protein oxidation [20], lipid peroxidation products [27,30,36], and oxidative damage to mitochondria [2,3,40] in AD brain. Our laboratory reported enhanced protein oxidation in Aβ-containing SP-rich regions of AD brain but not in SP-poor cerebellum [20]. The ability of toxic Aβs to induce protein oxidation and inhibit the activity of oxidation-sensitive enzymes is consistent with the hypothesis that Aβ can act like a pro-oxidant [1,8,18,19,37,42,45,46]. In addition to numerous reports supporting the free radical hypothesis in Aβ toxic-

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0197-4580/99/$ – see front matter
PII: S0197-4580(99)00056-1

ity, a sizable body of evidence supports the involvement of free radicals indirectly. For example, antioxidants and free radical scavengers including propylgallate, N-tert-butyl-α-phenylnitrone (PBN), vitamin E, and EUK-8 protect Aβ-treated cells or synaptosomes from cytotoxicity and oxidative stress [4,5,7,8,10,11,18,22,29,42,43,45,47]. Localized to the C-terminal region of Aβ, Met^{35} may be critical for aggregation [35] and toxicity [8,35].

In this study we report formation of free radicals in Aβ(1–42) solution which are not detected in the reverse, non-toxic peptide Aβ(42–1) nor in Aβ(1–42) Met^{35}Nle. We also report Aβ(1–42)-associated free radical oxidative stress by the measure of protein oxidation in in vitro rat embryonic hippocampal neuronal culture, and in vivo in transgenic *Caenorhabditis elegans (C. elegans)* nematodes expressing human Aβ(1–42) [12,26]. Finally, we demonstrate the importance of Met^{35} in the toxicity of Aβ in these in vitro and novel in vivo models.

2. Materials and methods

2.1. Peptides, enzymes, and other chemicals

Synthetic Aβ(1–42) and Aβ(1–42)Met^{35}Nle were purchased from AnaSpec (San Jose, CA) Aβ(1–42), Lot # 5811 and Lot # 7266; Aβ(1–42)Met^{35}Nle, Lot # 8388. Aβ(42–1) was purchased from Bachem (King of Prussia, PA) Lot # 517383. Peptides were stored in the dry state at 4°C. The protein carbonyl Oxyblot kit was purchased from Oncor, Cat # S7150 (Gaithersburg, MD). PBN was synthesized in our laboratory and purified by repeated recrystallization and sublimation. HPLC, EPR, MS and NMR verified purity of the PBN. All other chemicals used were at highest purity.

2.2. Neuronal cultures

Neuronal cultures were prepared from 18-day-old Sprague-Dawley rat fetuses. Rat hippocampi were dissected and incubated for 15 min in a solution of 2 mg/mL trypsin in Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (HBSS) buffered with 10 mM HEPES (Gibco, Paisley, Scotland). The tissue was then exposed for 2 min to soybean trypsin inhibitor (1 mg/mL in HBSS) and rinsed 3 times in HBSS. Cells were dissociated by trituration and distributed to 60 mm^2 polyethlyleneimine-coated plastic culture dishes (Costar, Cambridge, MA). Initial plating densities were 75–100 cells/mm^2. At the time of plating, the culture dishes contained 2 mL of Eagle's minimum essential medium (MEM; Gibco) supplemented with 100 mL/L fetal bovine serum (Sigma, St. Louis, MO), 1 mM L-glutamine, 20 mM KCl, 1 mM pyruvate, and 40 mM glucose. After a 5 h period to allow cell attachment, the original medium was removed and replaced with 1.6 mL of fresh medium of the same composition. After a 24 h period, 1.2 mL MEM was replaced with 1.0 mL of Neurobasal medium (Gibco) supplemented with 2% v/v B-27 (Gibco), 2 mM L-glutamine (Gibco), and 0.5% w/v D-(+) glucose. On the fifth day two-thirds of the Neurobasal medium was replaced with freshly prepared Neurobasal medium of the same composition. Cultures were maintained at 37°C in a 5% CO_2/95% room air-humidified incubator at all times. Cultures were used for the experiments between 9–11 days. In B27/Neurobasal medium, glial growth is reported to be less than 0.5% of the nearly pure neuronal population [6].

2.3. Experimental treatment of cultures

Synthetic Aβ(1–42) and variants were dissolved in double-distilled deionized water at a concentration of 1 mg/mL, and incubated at 37°C for 24 h for the aggregation. This stock solution was added to cultures to produce a final concentration of 10 μM. The same volume of sterile deionized water was added to control cultures. Neuronal cultures were treated with peptide for 2 days.

2.4. Sample preparation from transgenic Caenorhabditis elegans

The construction and characterization of the transgenic strains used in this study (CL2120, CL3115, and CL2122) have been previously described [12]. These strains use the *unc-54* body wall muscle myosin promoter to drive high level expression of either Aβ(1–42) (CL2120) or Aβ(1–42)Met^{35}Cys (CL3115); these lines express comparable levels of Aβ. Control transgenic strain CL2122 contains only the *unc-54* expression vector and the *mtl-2*/GFP marker gene, and lacks immunoreactive Aβ deposits. Staged 4-day old gravid adults were washed free of bacteria and resuspended in a minimal volume of water before being quick-frozen in liquid N_2. Aβ production in these preparations was confirmed by immunoblots of sub-aliquots.

2.5. EPR spectroscopy and spin trapping

Aβ peptides were solubilized to a concentration of 1 mg/mL by addition of a solution of 50 mM PBN in PBS solution [150 mM NaCl, 5 mM sodium phosphate buffer, pH 7.4] stored over chelex-100 beads and containing the iron chelator deferoxamine mesylate (2.5 mM). The peptide solutions and controls were incubated at 37°C for up to 60 h. EPR spectra were obtained at appropriate time points by placing 300 μL of the solutions into a quartz flat cell using a Bruker (Billerica, MA) EMX EPR spectrometer equipped with computerized data acquisition software. EPR parameters used were: microwave power = 20 mW, modulation amplitude = 0.30 G, gain = 1×10^5, and conversion time = 10.28 ms.

2.6. Neuronal survival

Cell death was assayed by counting the number of neurons that internalized the Trypan blue dye. Cells were rinsed 3 times with 1 mL PBS (pH = 7.4). Trypan blue (0.4%-Sigma) was added to cells along with 300 μL PBS and incubated for 10 min. Sixteen different microscopic areas were counted for uptake of Trypan blue, an index of cell death. Data are given as percentages of corresponding vehicle-treated values.

2.7. Protein carbonyl assay (oxyblot method)

To determine the level of protein oxidation an Oxidized Protein Detection Kit (Oxyblot, ONCOR Cat # S7150-Kit) was used. This kit is based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH) [24,25].

The samples were treated with 20 mM DNPH in 10% trifluoroacetic acid and Derivatization-Control solution, and incubated for 15–30 min. After derivatization and neutralization with 2 M Tris/30% glycerol (Neutralization Solution, Oxyblot Kit), and 19% 2-mercaptoethanol, cell proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (SDS-PAGE). Oxidized bovine serum albumin (BSA) with known concentration of carbonyls (20 nmol of carbonyls/mg of protein) were treated with DNPH and loaded as a standard (1 pmol of protein carbonyl per lane) with each set of the samples. For the standard preparation, the BSA (Standard for Gel Filtration Chromatography, Cat # A3581, Sigma) was dissolved in double-deionized water at 2 mg/mL and oxidized by Fe^{2+}/H_2O_2 (100 μM/1 mM) for 2 h at 37°C. The reaction was stopped with deferoxamine mesylate, and the small molecular weight substances were removed from the protein by passage through a Sephadex G-25 desalting column. The concentration of carbonyl groups per mg of protein was determined by colorimetric carbonyl assay [25]. SDS-PAGE was performed in mini-slabs (0.75 × 60 × 70 mm, 12% total acrylamide) according to method of Laemmli [23] using identical protein amounts per lane determined by the BCA assay and verified by image analysis of Coomassie blue stained gels. Following electrophoresis, proteins were transferred to nitrocellulose paper for further immunoblotting analysis. Western blotting was performed according to the procedure adapted from Glenney [14]. The transfer of proteins to nitrocellulose paper after SDS-PAGE was completed in two hours. The transfer buffer used was Tris-Glycine pH 8.5 containing 20% methanol. After the transfer, membranes were blocked in 3% BSA (in PBS with 0.01% sodium azide and 0.2% Tween-20) for 1 h at room temperature. Rabbit anti-DNP antibody from ONCOR Oxyblot Kit (1:150 working dilution) was used as a primary antibody. Secondary antibodies (anti-Rabbit IgG conjugated with alkaline phosphatase, Sigma) were diluted in the blocking solution 1:15,000 and incubated with the membrane for 1 h. Membranes were washed after every step in washing buffer (PBS with 0.01% sodium azide, and 0.2% Tween 20) for 10 min at room temperature. Washed membranes were developed using BCIP-NBT solution (SigmaFast tablets, Sigma). Western blots were analyzed using computer-assisted imaging [MCID/M4 software supplied by Imaging Research (St. Catharines, Ontario, Canada)]. Data are given as percentages of corresponding vehicle-treated values.

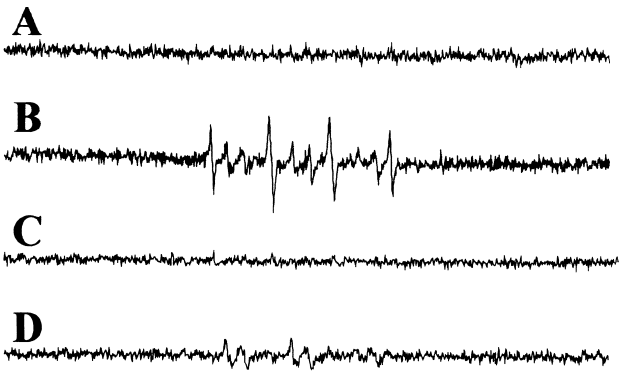

Fig. 1. EPR spectra of Aβ(1–42) and variants after 60 h of incubation at 37°C in PBN/PBS solution containing metal ion chelators. (**a**) PBN solution lacking peptide gives no signal. (**b**) Aβ(1–42) yields a prominent 4-line EPR spectrum. (**c**) Aβ(1–42) Met^{35}Nle gives no EPR spectrum suggesting the importance of methionine in Aβ(1–42) free radical formation. (**d**) The non-toxic Aβ(42–1) reverse peptide yields no 4-line EPR signal, and only an extremely weak 6-line spectrum was observed.

3. Results and discussion

Direct evidence of the formation of free radicals in Aβ(1–42) solution was obtained using EPR spin trapping methods [8]. Similar to Aβ(25–35) but for the first time with Aβ(1–42), a 4-line EPR spectrum of Aβ(1–42) in PBN/PBS solution was observed (Fig. 1b). No EPR spectrum was observed for the spin trap PBN alone (Fig. 1a), i.e., control solutions give no signal, indicating that the radical originates from the peptide Aβ(1–42). The reverse peptide Aβ(42–1) did not result in a 4-line spectrum; rather, an extremely weak 6-line spectrum was observed. Given the suggestion from previous studies that methionine residue 35 may be important to the toxicity properties of Aβ [8,35], we substituted norleucine for methionine in Aβ(1–42). Norleucine has the same side chain length and hydrophobicity as methionine, but does not contain a thio ether functionality. The Met^{35}Nle substituted peptide did not yield an EPR spectrum, suggesting that methionine near the C-terminus is important in the free radical formation (Fig. 1c,d). Recently, Bush and co-workers also reported the presence of peptide associated free radicals in Aβ(1–42) with a different method [21]. Those researchers showed that Aβ(1–42) can reduce Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+}, i.e., an electron to reduce the metal ion is derived from the peptide, thereby forming a peptide radical.

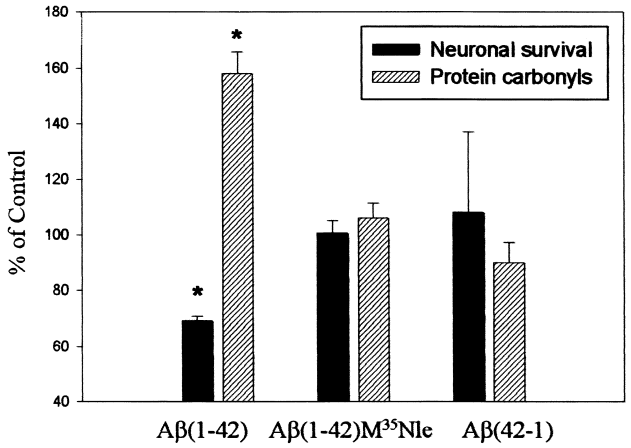

Fig. 2. The relative changes of neuronal survival and protein carbonyl content in rat embryonic hippocampal neuronal culture treated with 10μM of Aβ(1–42) or variants for 2 days. Both neuronal survival and protein carbonyl content are expressed as mean % of control. Statistical comparisons were made using ANOVA followed by Dunnett's test or Student's *t*-test. Error bars represent SEM values. Significance at $^*p < 0.001$ (n = 3–6).

The amount of protein carbonyls is a measure of protein oxidation [9], and it is well established now that synthetic Aβ(25–35) and Aβ(1–40) can give rise to an increase in protein carbonyl levels in cell culture [18,42,45,46]. However, to our knowledge, Aβ(1–42)-mediated increase in protein carbonyl levels has not been studied before in cell culture systems. Here we report that after 2 days of incubation of 10 μM Aβ(1–42) with hippocampal neuronal culture, protein carbonyl levels were increased 158% relative to controls ($p < 0.001$, Fig. 2), suggesting a peptide-induced oxidative insult. Treatment of cultures for this same period with Aβ(1–42) decreased the neuronal survival to 69.1% ($p < 0.001$) of the control (Fig. 2). Neither the biologically inactive reverse peptide, Aβ(42–1) nor the Met^{35}Nle substituted peptide led to increased protein oxidation or decreased neuronal survival in cell culture. Taken together with the EPR results, these observations suggest that it is the Aβ peptide (1–42)-induced free radicals that lead to protein oxidation and death of neurons, and that methionine plays a role in these effects. Further, that the reverse peptide was not neurotoxic, did not lead to neuronal protein oxidation, and did not yield a 4-line EPR spectrum with PBN, all in contrast to Aβ(1–42), suggest that the specific sequence, and therefore the structure, of Aβ is critical for its oxidative stress and neurotoxicological properties.

Analogous to our finding that AD brain regions containing SP show increased protein oxidation [20], we reasoned that *C. elegans* transgenic animals expressing full-length human Aβ(1–42) also would show evidence of protein oxidation. It was previously demonstrated that transgenic *C. elegans* strains can be engineered to express an *unc*-54/β 1–42 minigene [26]. In the present study three transgenic strains were used: an *unc*-54/β 1–42 transgenic line expressing Aβ(1–42) (CL2120); a control *unc*-54 vector-only transgenic line (CL2122); and an *unc*-54-based transgenic line expressing Aβ(1–42) Met^{35}Cys variant (CL3115). A typical Oxyblot image representing carbonyl content in *C.*

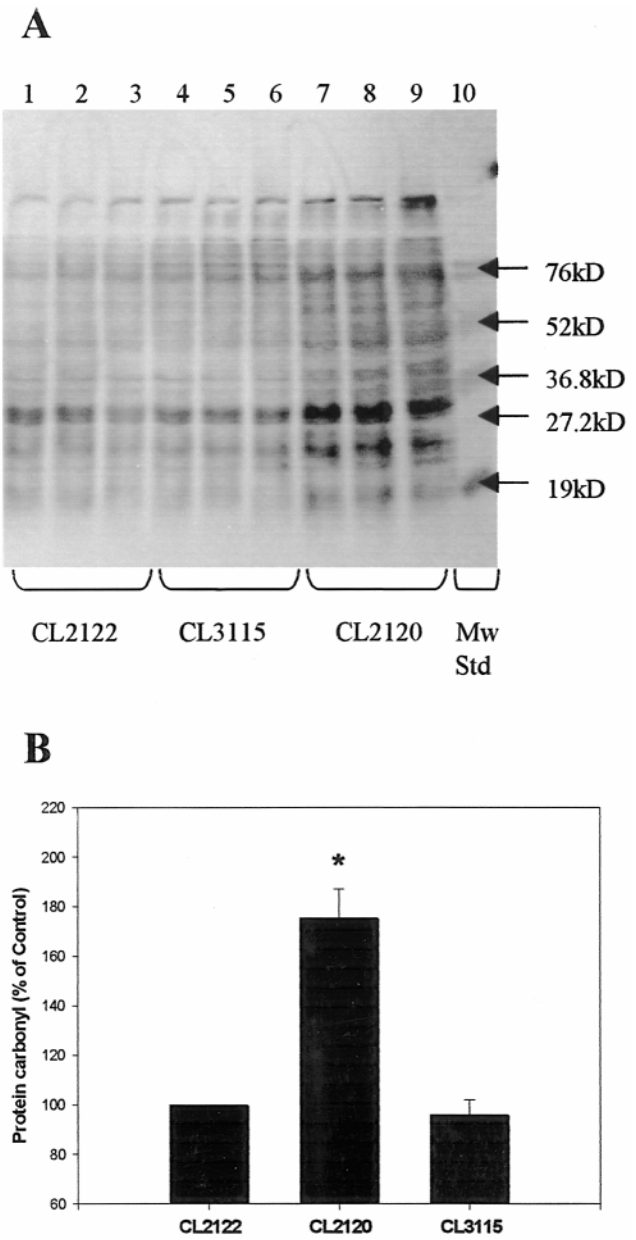

Fig. 3. Protein carbonyl content in *C. elegans* transgenic animals expressing full-length human Aβ(1–42). (**a**) Typical Oxyblot image representing the protein carbonyl content of *C. elegans*. Lanes 1,2, and 3 show protein carbonyl content of *unc-54* vector-only transgenic line (CL2122); lanes 4, 5, and 6 show the protein carbonyl content of *unc-54*-based transgenic line expressing Aβ(1–42)Met^{35}Cys variant (CL3115); lanes 7, 8, and 9 show the protein carbonyl content of *unc-54*-based transgenic line expressing human Aβ(1–42) (CL2120). Molecular weight standards are shown in lane 10. (**b**) The relative changes of protein carbonyl content in transgenic *C. elegans* expressing Aβ. Statistical comparisons were performed using ANOVA followed by Dunnett's test. Mean ± SEM values are shown. Significance at $^*p < 0.001$ [Five different samples (in triplicate) of each transgenic animal line were analyzed].

elegans expressing human Aβ peptide is shown in Fig. 3a. Lanes 1, 2, and 3 show the results with the vector only transgenic line (CL2122), lanes 4, 5, and 6 show the results using Met^{35}Cys substituted Aβ(1–42) expressing transgenic line (CL3115), and the results with Aβ(1–42)-expressing transgenic line (CL2120) are shown in lanes 7, 8, and 9. Molecular weight standards are shown in lane 10. The lanes from all the experiments were digitized, and Fig. 3b demonstrates the relative carbonyl amounts in the above mentioned strains. CL2120, which expresses Aβ(1–42), showed carbonyl amounts of 176% relative to the vector-only control, ($p < 0.001$); however, CL3115, which has a cysteine substitution instead of methionine at the 35th position of Aβ(1–42), showed no increase in protein carbonyl, pointing toward a critical role of Met^{35} in Aβ-induced oxidative stress, consistent with the in vitro results above. Interestingly, although the Aβ levels in the CL3115 strain are similar to those observed in CL2120 [12], the CL3115 strain fails to make detectable thioflavin S-reactive amyloid deposits, which are readily observed in strains expressing wild-type Aβ(1–42) (i.e., CL2120) [12].

These free radical oxidative stress results employing Aβ(1–42), the predominant form of Aβ found in AD brain, are consistent with the Aβ-associated free radical oxidative stress model of neuronal death in AD [8]. Previous studies of the 11-mer, Aβ(25–35), showed that the C-terminus, containing Met^{35}, was critical for toxicity, [35] and for EPR signal formation [8]. The results reported here using Cys-substituted Aβ(1–42) in an in vivo model are consistent with these findings. In agreement with our results, others showed that Aβ(1–42) with a Met^{35}norleucine substitution added to hippocampal neurons led to a significant, nearly 50% increased cell survival over that of unsubstituted Aβ(1–42) [28]. Although the precise molecular mechanisms by which Aβ(1–42) lead to oxidative stress and neurotoxicity in AD remain unknown, the results presented here support the notion that Aβ-associated free radicals may play a key role and that Aβ(1–42) methionine residue 35 may be important in this role. Additional in vivo and in vitro studies of Aβ(1–42) designed to gain further insight into free radical mechanisms and neurotoxicity are in progress.

Acknowledgments

This work was supported in part by grants from NIH to D.A.B. (AG-10836; AG-05119) and C.D.L (AG-12423).

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