Oxidative stress precedes fibrillar deposition of Alzheimer’s disease amyloid β-peptide (1–42) in a transgenic Caenorhabditis elegans model

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Abstract

Alzheimer’s disease is a progressive, neurodegenerative disorder characterized by senile plaques and neurofibrillary tangles, and synapse loss [22]. The major constituent of senile plaques is the amyloid beta peptide (Aβ), a 39–43 amino acid length peptide. Aβ1–42 is the most abundant in senile plaques and self-associates faster than Aβ1–40 [19,42]. Aβ is thought by many to be central to the pathogenesis of the disease. The Alzheimer’s disease brain is under significant oxidative stress, and the Aβ1–42 peptide is known to cause oxidative stress in vitro. One controversy in the amyloid hypothesis is whether or not Aβ plaques are required for toxicity. We have employed a temperature-inducible Aβ expression system in Caenorhabditis elegans to create a strain of worms, CL4176, in which Aβ1–42 is expressed with a non-permissive temperature of 23 °C. The CL4176 strain allows examination of the temporal relationship between Aβ expression, oxidative stress, and Aβ fibril formation. CL4176 were under increased oxidative stress, evidenced by increased protein oxidation indexed by increased carbonyl levels, 24 and 32h after temperature upshift as compared to the control strain, CL1175. The increased oxidative stress in CL4176 occurred in the absence of Aβ fibril formation, consistent with the notion that the toxic species in Aβ toxicity is pre-fibrillar Aβ and not the Aβ fibril. These results are discussed with reference to Alzheimer’s disease.

Keywords: Alzheimer’s disease; Senile plaque; Caenorhabditis elegans; Protein oxidation; Amyloid beta-peptide; Oxidative stress

1. Introduction

Alzheimer’s disease is a neurodegenerative disorder characterized pathologically by senile plaques, neurofibrillary tangles, and synapse loss [22]. The major constituent of senile plaques is the amyloid beta peptide (Aβ), a 39-43 amino acid length peptide. Aβ1–42 is the most abundant in senile plaques and self-associates faster than Aβ1–40 [19,42]. Aβ is thought by many to be central to the pathogenesis of Alzheimer’s disease [44]; however, the hypothesis is controversial because of differing views on the relationship between aggregation of Aβ to neurotoxicity. One view is that the neurotoxicity of Aβ is directly linked to its state of aggregation in that only fibrillar Aβ is neurotoxic [30,36]. Another hypothesis has emerged stating that fibrils are not necessary for neurotoxicity and that aggregates or oligomers of Aβ are the neurotoxic species [24,46,48,49].

Transgenic Caenorhabditis elegans (C. elegans) that express human Aβ1–42 through a body-wall muscle myosin promoter and an Aβ minigene [27] have been used as in vivo models to study Aβ toxicity and deposition [28,51]. Previously, we have shown that transgenic C. elegans expressing Aβ1–42 (C. elegans strain CL2120) have increased protein carbonyl formation, suggesting that Aβ toxicity is associated with oxidative stress [51]. Further, consistent with in vitro studies suggesting that methionine residue 35 is important in the oxidative stress and neurotoxic properties of Aβ1–42 [8,20,21,51], replacement of the codon for methionine in these C. elegans with a codon for a different amino acid abrogated the protein oxidation associated with the peptide in vitro [51]. In order to examine the role of Aβ aggregation in neurotoxicity, we have employed an in vivo model of inducible Aβ production in transgenic C. elegans. The temperature-inducible Aβ expression system in the C. elegans created a model in which the relationship between Aβ toxicity, fibril formation, and oxidative stress could be examined temporally. If aggregated Aβ1–42 is the
neurotoxic species involved in oxidative stress, oxidative stress and phenotypic paralysis of the nematode *C. elegans* is predicted to precede fibrillar deposition of Aβ.

2. Materials and methods

The Oxyblot kit was purchased from Intergen (Purchase, NY). All other chemicals were purchased from Sigma-Aldrich in their highest purity.

2.1. *C. elegans*

The construction and characterization of temperature-inducible transgenic strains CL4176 [{*smg-1*}({*cc546ts*}; *dvIs27*({*pAF29*}+{*pRF4*}))] and CL1175 [{*smg-1*}({*cc546ts*}; *dvIs175*{*pRF4*})] have been described elsewhere [29]. Briefly, the mRNA-surveillance system of the *C. elegans* (the *smg* system [31]) was used to create the temperature-inducible system. At non-permissive temperatures, the *smg-1* system is inactive, allowing translation of the stabilized transgene mRNA for human Aβ(1–42). The inducible Aβ-expression transgene present in strain CL4176 was constructed by inserting a signal peptide/Aβ minigene fragment (recovered from pCL12 [27]) into expression vector pPD118.60. This expression vector contains the *myo-3* body-wall muscle-specific myosin promoter and an abnormally long 3' untranslated region, which makes efficient transgene expression dependent on *smg-1* function (see Fire lab 1997 expression vector kit, http://ftp.ciwemb.edu/PNF:byName:/FireLabWeb/FireLabInfo/FireLabVectors/). Both CL4176 and matched transgenic control CL1175 also contain pRF4, which encodes the rol-6(su1006) morphological marker used in the initial identification of these transgenic strains.

Transgenics were grown at a permissive temperature at 16 °C and then upshifted to the non-permissive temperature of 23 °C for a designated period of time. At the upshifted temperature of 23 °C, CL4176 is strongly induced for expression of human Aβ(1–42), while the control inducible strain CL1175 does not express Aβ at any temperature. Transgenic *C. elegans* strain CL2006 has been described previously [27]. These transgenic nematodes contain pCL12, a chimeric construct that employs the *unc-54* body-wall muscle-specific myosin promoter to produce constitutive Aβ(1–42).

For X-34 staining of transgenic *C. elegans* animals [28], synchronized populations of CL4176 and CL2006 animals were grown at 16 °C until reaching the third larval stage, then upshifted to 23 °C. After 24 h of upshift, live animals were incubated for 2 h in a 25 ml drop of 1 mm X-34, then returned to the growth plate for 6 h for destaining before imaging (staining and destaining also performed at 23 °C).

2.2. Protein carbonyl measurement

*C. elegans* were washed free of bacteria and resuspended in water before being frozen in liquid N2. Samples were

![Graph](image-url)

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**Fig. 1.** Transgenic *C. elegans*, CL4176 and CL1175, grown at 16 °C were upshifted to the non-permissive temperature of 23 °C, inactivating the *smg-1* system. CL4176 thereby expressed human Aβ(1–42) for 0, 4, 8, 12, 24, or 32 h, while the control animal, CL1175, expressed no Aβ(1–42). Samples were derivatized with DNPH and protein carbonyl levels determined. CL4176 at 24 h has a significantly increased levels of protein carbonyls compared to the control animal, CL1175, at 24 h after temperature upshift (**P < 0.002**). Protein carbonyl levels of the CL4176 animal at 32 h are significantly increased as compared to CL1175 at 0 h (**P < 0.008**), and 32 h (*****P < 0.0002). CL4176 animals at 32 h have significantly more protein carbonyls as compared CL4176 at 0 h (****P < 0.009) and 24 h (*****P < 0.005). Samples are expressed as percent control ± S.E.M., normalized to CL1175 at 0 h of upshifted temperature. *N* = 3–9.
then sonicated, and protein concentration was determined by the BCA method, using BSA as a standard. The immunoblot technique for determining protein carbonyl levels is based on DNPH derivatized detection of carbonyls [26]. Two hundred mM 2,4-dinitrophenylhydrazine (DNPH) stock solution was diluted 10 times with water, 5 μl of sample was incubated at room temperature with 5 μl of 12% SDS and 10 μl of the diluted DNPH for 20 min with vortexing. The samples were neutralized with 7.5 μl of the neutralization solution (2 M Tris in 30% glycerol). Two hundred fifty nanograms of the sample solution was loaded into the wells of the slot blot apparatus. Proteins were transferred directly to the nitrocellulose paper under vacuum pressure and standard immunochemical techniques were performed. A rabbit-anti DNPH polyclonal primary antibody (Oxyblot kit (S-7150-kit), Intergen) and a goat anti-rabbit IgG alkaline phosphatase (Sigma) secondary antibody were used. Samples were developed using SigmaFast Tablets (BCIP/NBT substrate), and blots were scanned into Adobe PhotoShop and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

Fig. 2. Amyloid deposits in transgenic C. elegans animals with induced (CL4176) or constitutive (CL2006) Aβ expression. Shown are the anterior portions of young adult animals grown in parallel and vitally stained with the amyloid-specific dye X-34 [28]. Left panel, DIC image; right panel, epifluorescence (false color) image. Note the appearance of numerous X-34-reactive deposits in the CL2006 animal (arrows), and their absence in CL4176. When these animals were imaged, the CL4176 animal, which had been induced to express Aβ by a 32 h temperature upshift, was paralyzed. These results indicate pathology precedes detectable amyloid formation in this transgenic model. Size bar = 50 mm.

2.3. Statistics

One-way ANOVA was used to determine statistical significance. P values less than 0.05 were considered significant.

3. Results

C. elegans were engineered to express human Aβ(1–42) in a temperature-inducible manner, allowing temporal examination of oxidative stress and formation of fibrils. Transgenic animals inducibly expressing Aβ(1–42), CL4176, have a significantly increased protein carbonyl content at 24 (P < 0.002) (139%) and 32 h (P < 0.0002) (196%) as compared to the inducible control strain, CL1175, at 24 and 32 h, respectively, indicating that the CL4176 worm is under significant oxidative stress (Fig. 1). Additionally, the protein carbonyl content in the CL4176 animal at 32 h is significantly increased from the CL4176 protein carbonyl content at 24 h (P < 0.005) (Fig. 1).

Although induced CL4176 animals have significantly increased protein carbonyl levels relative to controls,
detectable intracellular amyloid deposits are very rare in these animals, in contrast to previously described transgenic lines with constitutive expression of Aβ(1–42) [28]. Although all CL4176 animals examined 32 h after upshift are paralyzed, amyloid deposits could only be detected in 3% of these animals (average of 0.06 deposits/animal, n = 100). In comparison, when CL2006 animals, which have constitutive Aβ(1–42) expression, were propagated in parallel, all examined animals had obvious amyloid deposits (average of 12.6 deposits/animal, ±0.6 S.E.M., n = 80), but did not yet show paralysis. This difference in detectable amyloid deposits is illustrated in Fig. 2. CL4176 animals maintained longer than 32 h at the non-permissive temperature exhibit developmental arrest and eventual death, but never attain the degree of amyloid deposits seen in CL2006 animals. These results demonstrate that in this transgenic C. elegans model, gross pathology and protein oxidation are clearly not correlated with fibrillar β amyloid deposition.

4. Discussion

The Alzheimer’s disease brain is under significant oxidative stress, evidenced by various markers of protein, lipid, DNA oxidation, and others [7,9,33]. Aβ is thought to be central to the pathogenesis of Alzheimer’s disease [41]. People with mutations in the genes for APP, presenilin 1, or presenilin 2 gene expression, oxidative stress and neurotoxicity have significantly at 24 and 32 h after temperature upshift.

Interestingly, oxidative stress in Aβ(1–42) expressing animals, in the absence of monomers and fibrils, disturb synaptic plasticity in vivo at physiological levels [49]. We have demonstrated that fibrils, per se, are not toxic by studying Aβ (1–42)Met35Nle peptide and Aβ(1–42)MetSox, in which the sulfur atom of the methionine 35 residue is replaced with a methylene group or methionine sulfoxide, respectively [45,51]. Aβ(1–2)Met35Nle did not cause cell death or induce oxidative stress in cultured hippocampal neurons [51]. However, this non-toxic peptide formed fibrils, indistinguishable from Aβ(1–42) [46,47]. Similarly, if the sulfur atom in methionine of Aβ(1–42) is already oxidized, no oxidative stress nor neurotoxicity occur, yet fibrils form [45]. These findings emphasize the key role of methionine in Aβ-induced oxidative stress and neurotoxicity and suggest that fibrils do not necessarily correlate with toxicity. Additionally, Vitamin E can prevent cell death and decrease oxidative stress induced by Aβ(1–42) in cell culture [50]; however, Vitamin E does not affect the peptide’s fibril formation [47], suggesting that inhibition of fibril formation does not necessarily lead to abrogation of toxicity.

Much debate has centered on whether fibrils are necessary for toxicity of Aβ [11,17]. There are many observations that are inconsistent with the hypothesis that fibrils are necessary for Aβ toxicity, i.e. amyloid deposits do not correlate with dementia [2,5,37,38,44]; non-demented individuals may exhibit plaques [10]. The data presented in this paper, assuming similar behavior of the peptide in brain, may answer some of this criticism. In CL4176, significant oxidative stress preceded fibrillogenesis of Aβ, causing phenotypic paralysis of the C. elegans individual. Similarly, the oxidative stress observed in AD brain may, in fact, be caused by non-fibrillar forms of Aβ. There has been speculation that fibrils might be protective agents [34] that rid the cell of more deleterious toxic intermediates, pre-fibrillar Aβ [25]. If correct, the lack of correlation between plaques and dementia may be explained by the toxicity of non-fibrillar Aβ.

We note that previous studies with transgenic C. elegans animals expressing variant Aβ (1–42) peptides suggested a correlation between Aβ fibrilization and toxicity: transgenic animals expressing an Aβ(1–2) variant containing a Met35Cys substitution have dramatically reduced amyloid formation [12] and reduced protein oxidation [51], while animals expressing a single chain Aβ dimer have no detectable amyloid formation [28] and reduced toxicity [14]. However, these previous results are consistent with this current study if it is postulated that Aβ toxicity involves a multimer or conformer that serves as an intermediate in fibril formation. In this model, the Met35Cys and single chain dimer Aβ variants have reduced toxicity because of a reduced ability to form this toxic intermediate, with a downstream outcome of no or little amyloid formation. The more severe phenotype exhibited by the inducible CL4176 strain relative to the constitutive CL2006 strain may result because induction of
AJ-I in CL4176 leads to a rapid build-up of this hypothetical toxic intermediate. CL2006 animals, which actually hatch with amyloid deposits [28], may actually be protected by the sequestration of the toxic intermediate into amyloid fibrils.

If non-fibrillar Aβ is the more toxic species, some proposed therapeutic strategies for AD may be questionable. Many drugs are being designed to inhibit fibril formation of Aβ with the idea that fibrillar Aβ is the cause of cell death in AD [13]. Additionally, the now-halted immunization experiments against Aβ plaques [39] were designed to stop the formation of fibrils. However, both of these therapies drive the equilibrium of Aβ aggregation to the non-fibrillar form which may in turn cause more damage to the cell than Aβ plaques. The idea of non-fibrillar intermediates being toxic instead of fibrils is not exclusive to AD. There is growing evidence that oligomers may be toxic species in other neurodegenerative diseases such as Parkinson’s disease [15], Huntington’s disease [6], etc. As such, the role of non-fibrillar peptides in the disease process will need to be considered when developing future therapeutics.

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References


