

A β toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets

William L. Klein*

Department of Neurobiology and Physiology, Cognitive Neurology and Alzheimer's Disease Center, Northwestern University Institute for Neuroscience, 2153 North Campus Drive, Evanston, IL 60208, USA

Received 26 November 2001; accepted 27 January 2002

Abstract

Over the past several years, experiments with synthetic amyloid-beta peptide (A β) and animal models have strongly suggested that pathogenesis of Alzheimer's disease (AD) involves soluble assemblies of A β peptides (Trends Neurosci. 24 (2001) 219). These soluble neurotoxins (known as *ADDLs* and *protofibrils*) seem likely to account for the imperfect correlation between insoluble fibrillar amyloid deposits and AD progression. Recent experiments have detected the presence of ADDLs in AD-afflicted brain tissue and in transgenic-mice models of AD. The presence of high affinity ADDL binding proteins in hippocampus and frontal cortex but not cerebellum parallels the regional specificity of AD pathology and suggests involvement of a toxin receptor-mediated mechanism. The properties of ADDLs and their presence in AD-afflicted brain are consistent with their putative role even in the earliest stages of AD, including forms of mild cognitive impairment. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Amyloid; Protein misfolding; Degenerative disease

1. Introduction

No one knows for certain what causes Alzheimer's disease (AD). Many factors are involved, including inflammation, oxidative damage, and cytoskeletal abnormalities. The dominant hypothesis of the past 10 years, however, has been the "amyloid cascade" (Klein, 2000). In this hypothesis, dementia in AD depends on neuron death caused by amyloid fibrils; these fibrils, which are found in senile plaques, are large insoluble polymers generated from the 42 amino-acid, self-aggregating amyloid-beta peptide (A β).

The amyloid cascade hypothesis, despite its many strengths, has significant flaws, and it has not been fully accepted. This article reviews recent evidence that fibrillar amyloid is not the only toxic form of A β , perhaps not even the most relevant form. Evidence now points to a pathogenic role for small toxins that comprise globular A β oligomers (Klein et al., 2001). These soluble oligomers, which have been called "ADDLs," present novel opportunities to develop AD vaccines and therapeutic drugs.

This article will discuss five issues: (i) the ADDL hypothesis, and how it solves the major problem with the amyloid cascade hypothesis; (ii) the experimental basis for

the ADDL hypothesis, from toxin structure to experimental nerve cell biology; (iii) preliminary attempts at validation, comprising recent efforts to measure and characterize ADDLs in AD patients and in transgenic-mice models of AD; (iv) the mechanism of toxicity, with links to particular signal transduction pathways and toxin receptors; (v) use of ADDLs for development of therapeutic drugs and AD vaccines.

2. The ADDL hypothesis: missing links in A β toxicity

Over the past 10 years, there has been a profound interest in the possibility that toxins made from the small A β cause neuronal dysfunction and death in AD. In the early 1990s, two landmark findings from experimental nerve cell biology provided a direct link between A β and neurodegeneration. First, Yankner and Cotman and their groups at Harvard and UC-Irvine showed that solutions containing synthetic A β peptide can be toxic to CNS neurons (Busciglio et al., 1992; Pike et al., 1993). Second, these groups found that toxicity requires peptide reorganization, as fresh solutions containing only A β monomer were innocuous, but stored solutions developed neurotoxicity (Loo et al., 1993; Lorenzo and Yankner, 1994). The most obvious constituents to emerge in typical stored solutions were large amyloid fibrils, analogous in structure to the fibrils found in senile plaques of AD.

* Tel.: +1-847-491-5510; fax: +1-847-491-5211.

E-mail address: wklein@northwestern.edu (W.L. Klein).

These findings provided a strong rationale for the hypothesis that build-up of amyloid fibrils in the brain is the primary step in AD pathogenesis. Illustrating the enthusiasm of the AD research community for this concept, a search of PubMed on the subject “A β ” will generate over 8000 citations, with studies ranging from biophysics and cellular biology to transgenic-mice models and human pathology.

Given a decade of strong genetic, molecular and cellular support for the amyloid cascade hypothesis, why is it necessary to suggest a new hypothesis for A β toxicity? In fact, despite the strong evidence, there is no consensus in support of the amyloid hypothesis, largely because brain cell damage and dementia do not correlate well with plaque location and quantity (Terry, 1999). It is not uncommon for pathologists to find abundant insoluble amyloid deposits in individuals with normal cognition. Reciprocally, many APP-transgenic-mice that have no insoluble amyloid deposits show significant brain dysfunction and damage (Table 1) (Klein et al., 2001).

The central question can be put as follows: if dementia correlates so poorly with amyloid plaque deposits, why is A β the apparent key to AD pathogenesis?

A clue can be found in recent studies of multiple strains of APP-transgenic-mice that show synapse loss, an important aspect of AD pathology. Mucke et al. at the Gladstone Institute and the University of California, San Francisco, have observed that synapse loss, while independent of amyloid deposits, nonetheless correlates with levels of A β immunoreactivity in *soluble* extracts (Mucke et al., 2000). Similar results have been obtained for humans by Lue et al. at Sun City (Lue et al., 1999).

The significance of the correlation between synapse loss and soluble A β can be explained by the ADDL hypothesis (Lambert et al., 1998). ADDLs are potent A β 1–42 neurotoxins that are non-fibrillar. Instead of fibrils, their structure comprises relatively small globular oligomers.

Table 1
Amyloid plaque-independent brain damage in APP-transgenic-mice

Description	References
Loss of synaptic terminals	Mucke et al. (2000)
Recognition memory deficits	Dodart et al. (2000)
Apoptotic neurons, behavioral deficiencies	Kumar-Singh et al. (2000)
Loss of synaptic terminals, neurons	Hsia et al. (1999)
Faster decay of LTP	Larson et al. (1999)
Deficits in Y-maze performance	Holcomb et al. (1999)
Dysregulation of hypothalamic-pituitary-adrenal function	Pedersen et al. (1999)
Accelerated neurodegeneration	Chui et al. (1999)
Aggression, neophobia, deficits in LTP maintenance	Moechars (1999)
Reduced alternation performance in Y-maze	Holcomb et al. (1998)
Hypoactivity, spatial learning deficits	D’Hooge et al. (1996)
Neophobia, impaired spatial alternation, diminished glucose use	Hsia (1995)
Spatial navigation deficits	Czech et al. (1994)
Difficulties in water maze learning	Yamaguchi et al. (1991)

Synthetic ADDLs are soluble, *in vitro*, and as discussed later, these nonfibrillar assemblies of A β 1–42 recently have been found in soluble brain extracts from APP-transgenic-mice and AD patients.

The ADDL hypothesis says that a major factor in Alzheimer’s dementia is the neurological impact of soluble, globular oligomers of A β 1–42 (“ADDLs”). By causing specific aberrations in synaptic signaling, ADDLs first cause early-stage memory loss, with broader-scale effects of ADDLs subsequently leading to synapse degeneration and nerve cell death. The ADDL hypothesis includes three distinguishing features: (1) involvement of small globular neurotoxins, which would be missing links that account for the imperfect correlation between dementia and the large insoluble fibrils of amyloid plaques; (2) a role for ADDLs at very early stages in the disease, given that ADDLs form at low concentrations and would precede plaque formation; and (3) a mechanism in which early stage memory impairment stems not from neuron death but rather from malfunctions in memory-specific signal transduction.

3. Experimental origin of the ADDL hypothesis

The first indication that A β neurotoxicity might not require large fibrillar aggregates came from studies by Caleb Finch’s group at University of Southern California (Oda et al., 1994, 1995). They found that a glia-secreted protein called ApoJ blocks formation of large A β aggregates. This appears to be a chaperone-like activity, as inhibition requires only 5% molar amounts of ApoJ relative to A β . Most importantly, blocking formation of sedimentable aggregates does not block toxicity; in fact, ApoJ-treated solutions actually show enhanced toxicity.

These and related A β preparations formed in the absence of ApoJ subsequently have undergone extensive structural and functional analysis. Atomic force microscopy (AFM; see Stine et al., 1996) has been a particularly valuable tool in verifying the absence of fibrils or rod-like subfibrillar molecules in these preparations (Fig. 1, right panel). The toxic preparations contain exclusively small globular proteins, which we have named ADDLs, for “A β -derived diffusible ligands.”

Toxicity of ADDL preparations is readily detected using the commercially available MTT assay, which monitors vesicle trafficking and oxidative metabolism (Liu and Schubert, 1997). The widely-used PC12 cell line shows sensitivity to micromolar ADDLs within 4 h. Not all cell lines, however, respond to ADDLs. The most sensitive cells found so far have been differentiated cortical neurons, which, after 24 h exposure, have maximum responses to oligomerized A β at doses under 50 nM (total A β peptide).

In terms of biological responses, it is the neurological impact of ADDLs that is of most significant interest. In experiments with rat and mouse CNS tissue, ADDLs have been found to impair synaptic plasticity in the short-term

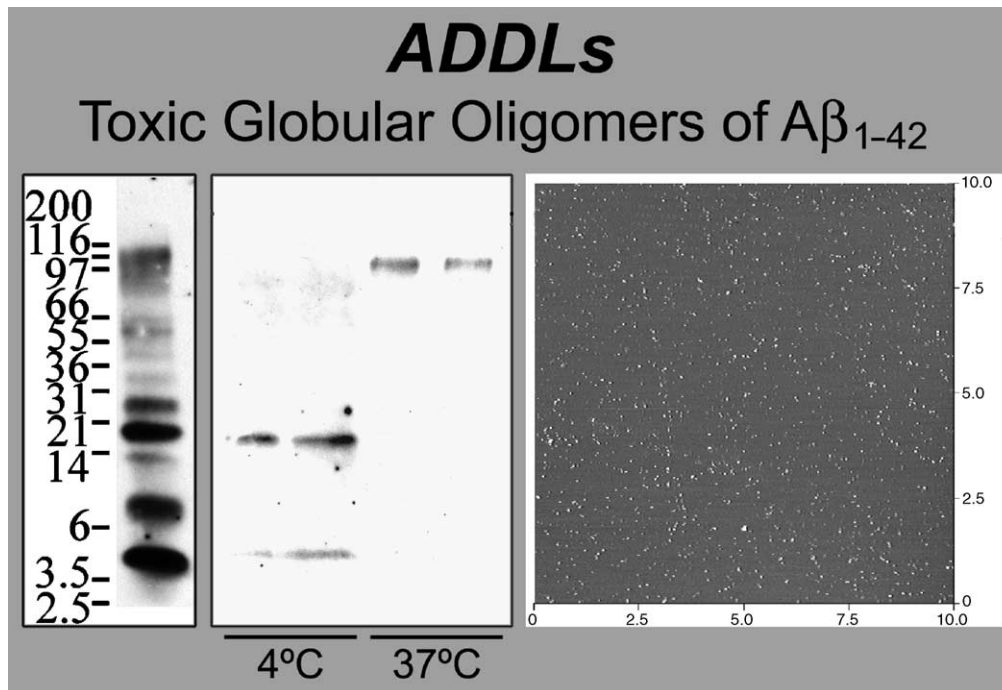


Fig. 1.

and cause selective neuron death in the longer-term. In addition, indirect evidence suggests ADDLs may cause loss of synaptic terminals.

In the short-term, ADDLs inhibit hippocampal LTP, a form of synaptic plasticity that is widely studied as a paradigm for learning and memory. Inhibition occurs within 45 min, the shortest time tested, and it is complete at sub-micromolar doses. Inhibition by ADDLs has been observed in brain slices and in vivo and can be elicited by stereotaxic delivery to synapses (Klein, 2000).

The impact on LTP is highly specific. Short-term plasticity is not lost, although somewhat reduced, and no impact is evident on evoked action potentials (EPSPs), or induction of long-term depression (LTD), another form of synaptic plasticity (Wang et al., 2001). Interestingly, ADDLs do inhibit recovery from LTD, which like LTP is brought about by a short rapid burst of spikes. ADDLs thus shift synaptic plasticity in a negative direction, blocking LTP while reinforcing LTD. This negative shift ultimately may be related to a destabilization of synapses.

Overall, inhibition of synaptic plasticity by ADDLs is consistent with a fast, localized effect at synapses. It is not due to broad disruption of neuronal function or wide-spread neuronal degeneration. Instead, the mechanism appears to be highly selective for particular aspects of synaptic plasticity.

Degenerative effects, however, are evident over longer time-frames. The second neurological consequence of ADDLs appears to be synapse loss, suggested by the indirect evidence from transgenic-mice discussed above. Hypothetical alternatives for synapse loss are ADDL-induced

synaptosis (whereby synapses are destabilized and removed) or ADDL-inhibited synapse replacement (whereby synaptogenesis required to replace synapses during normal turnover is blocked). Such effects, like the impact of ADDLs on LTP, could be caused by localized action of ADDLs at synaptic terminals. Because of known links between functional and structural synaptic plasticity (Mattson and Duan, 1999; McEachern and Shaw, 1999; Steward and Schuman, 2001; Yuste and Bonhoeffer, 2001), it is plausible that ADDL-disrupted signaling mechanisms that inhibit LTP also could cause synapse loss.

The third neurological consequence of ADDLs is selective neuron death. Death occurs within several days and can occur at low ADDLs doses. Unlike the synaptically-localized impact of ADDLs on LTP, however, it may be necessary for ADDLs to cover larger areas of neuronal cell surfaces for death to occur. Death due to ADDLs is routinely observed in differentiated organotypic cultures prepared from mature hippocampi, but younger tissue may be less vulnerable, suggested by preliminary results with cell lines and acute brain slice cultures.

An especially significant aspect of ADDL-induced neuron death is its regional specificity. In parallel to Alzheimer's pathology, ADDLs kill hippocampal but not cerebellar neurons (Klein et al., 2001). Regional specificity has not been observed for fibril preparations, suggesting differences in their toxic properties. ADDLs but not fibrils appear to be cell-specific.

Structurally, ADDLs have been found to comprise a heterogeneous population of oligomers. Characterization has

Table 2
Preparation of ADDLs

Monomerization by HFIP and storage of A β peptide

- (1) Solid A β peptide (1–42) is stored as a solid at -80°C . Remove and place on ice when ready to prepare stock peptide films.
- (2) Place 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma H-8508) on ice in the hood and allow to cool. HFIP is highly corrosive and very volatile. Add enough HFIP to solid A β peptide such that the final peptide concentration is 1 mM (e.g. 2.217 ml cold HFIP to 10 mg A β 1–42). Rinse vial thoroughly.
- (3) Incubate at room temperature for 60 min, keeping vial closed. Solution should be clear and colorless. Any traces of yellow color or cloudy suspension indicates poor peptide quality and should not be used.
- (4) Place peptide—HFIP solution back on ice for 5–10 min.
- (5) Aliquot solution into non-siliconized microcentrifuge tubes (e.g. 100 μl = 0.45 mg for A β 1–42). Do not close tubes.
- (6) Allow HFIP to evaporate overnight in the hood at room temperature.
- (7) Transfer tubes to a speedvac and dry down for 10 min. All traces of HFIP must be removed. The resulting peptide should be a thin clear film at the bottom of the tubes. The peptide should not be white or chunky.
- (8) Store dried peptide films over desiccant at -80°C . These stocks should be stable for several months.

ADDL preparation

- (9) Remove peptide film from -80°C freezer and put on ice.
 - (10) Make 5 mM A β stock in 100% DMSO by adding 20 μl fresh anhydrous DMSO (Sigma Hybri-Max D-2650) to 0.45 mg peptide. Pipette thoroughly, washing down the sides of the tube to ensure complete resuspension of peptide film. DMSO stock should be clear and colorless. Note: DMSO is very hygroscopic and fresh ampoules should be used for each preparation.
 - (11) Dilute 5 mM peptide stock into F12 medium without phenol red (BioSource Inc., custom preparation). For the best yield, the ADDL solution should be no greater than 100 μM (e.g. 20 μl DMSO stock to 980 μl F12). A volume of 5 mM peptide stocks should be prepared fresh. Do not store peptide as a DMSO stock as protofibrils will form!
 - (12) Incubate at 5°C for 24 h.
 - (13) Following incubation, centrifuge at $14,000 \times g$ for 10 min in the cold.
 - (14) Transfer supernatant to a new tube. This supernatant is the ADDL preparation.
-

relied on atomic force microscopy and immunoblot analyses (Chromy et al., 2001; Lambert et al., 1998). Other methods such as light scattering, NMR, or analytical centrifugation present multiple difficulties, including the fact that they require concentrations of A β that are too high and cause formation of larger structures.

By AFM, ADDLs are globular molecules, and they are heterodisperse. Size and heterogeneity of molecules seen in AFM images are consistent with the analysis obtained SDS-PAGE immunoblots. By gel analysis, ADDL preparations contain oligomers that range from trimer to 24 mer (approximately 108 kDa) (Fig. 1, left panel).

The A β -derived neurotoxins in ApoJ-chaperoned solutions have proven *not* to be ApoJ–A β complexes. Confirming this, it has been found that fibril-free populations of toxic, globular oligomers will form under certain circumstances without addition of ApoJ (Lambert et al., 1998). A simple and reliable procedure to form ADDLs without ApoJ is included in Table 2.

Consistent with their putative role in pathogenesis, ADDLs do not form from A β 1–40 but require A β 1–42, the form of A β linked to AD (Younkin, 1998). While A β 1–40 apparently can form oligomers (Bitan et al., 2001), these are unstable, and their formation appears to require high peptide concentrations.

A critical question is whether ADDLs are transient or relatively stable structures. The answer is important because of the existence of a second type of sub-fibrillar A β neurotoxin known as the protofibril (PF). PFs initially were regarded as inert intermediates en route to assembly of toxic fibrils (Lansbury, 1997; Walsh et al., 1997), but more recently they

have been found to be neuroactive (Hartley et al., 1999; Walsh et al., 1999).

Protofibrils are large rod-shaped molecules different in structure from ADDLs. Theoretically, the smaller ADDLs, which are globular molecules, might serve as precursors to the rod-shaped PFs. Electron microscopy experiments have suggested, moreover, that a typical PF preparation might also contain ADDLs (see, e.g. Fig. 1c in the publication by Walsh et al., 1999).

Typical ADDL solutions, however, held at 1 μM and 37°C for 24 h do not show any accumulation of protofibrils by AFM, nor is there any build-up of fibrillar material incapable of entering the separating gels of SDS-PAGE. (Fig. 1, middle and right panels, kindly provided by R. Novak and B.A. Chromy.) ADDLs thus are relatively stable entities, and they can exist free of PFs. The proportion of particular oligomers found within an ADDL population can shift, however, and it is common for the abundant tetramer fraction typically present at the outset to be replaced by 12–24 mers.

The extent to which ADDLs exist in dynamic equilibrium with PFs and whether conditions exist that favor ADDL–PF interconversions remain unknown.

Another distinguishing feature of ADDLs is that they form readily at low concentrations that could be attainable in vivo. The lower limit has not been determined, but even in low nM solutions of A β held at 4°C ADDLs form within min (Chang et al., 2001). Significantly higher levels of A β monomer are required to foster PF formation, suggesting that of the various neurotoxic forms of A β , the ADDLs would be first to appear.

4. Validation: are ADDLs the missing links in AD pathogenesis?

An important question now under study is whether ADDLs, which form readily *in vitro*, actually accumulate in brains of APP-transgenic-mice and AD patients. Detection of endogenous ADDLs would provide strong *prima facie* evidence for the ADDL hypothesis.

As mentioned, analyses of aqueous extracts from brains of TG mice models and AD autopsies suggest a correlation between synapse loss and the levels of soluble amyloid peptides. Correlations in these ELISA studies were imprecise, however, probably due in part to use of antibodies that detect physiological monomer as well as higher order pathogenic species.

To overcome poor signal-to-noise ratios, we have developed a dot-blot assay using antibodies that discriminate oligomers from monomers (Lambert et al., 2001). This assay reveals major differences in soluble oligomer levels between strains of mice even when no differences are detected by the commonly used monoclonal antibody 4G8.

ADDLs now have been detected in soluble extracts of human brain tissue (Lambert et al., 2001). Preliminary molecular analyses indicate that endogenous forms of human ADDLs are equivalent to the synthetic species used for toxicity experiments *in vitro*. Immunoblots after two-dimensional gel separations show isoelectric points near 5.6 for synthetic ADDLs and for the endogenous ADDLs found in soluble extracts of AD brain tissue. A common oligomer in AD brain is a 12-mer, although smaller and bigger species have been found. The size of endogenous ADDLs is consistent with the spectrum of oligomers found with synthetic ADDLs. Conformations of synthetic and endogenous ADDLs also are equivalent since they cross-react with the same quaternary structure-sensitive antibodies. Regional analysis suggests that oligomers are more abundant in AD cerebral cortex than in cerebellum.

Evidence for endogenous PFs also has been found, as A β -immunoreactive material can be observed that enters stacking but not separating gels in one-dimensional analysis, consistent with the larger PF mass.

In a limited comparison of tissue from human subjects (five AD brains versus five aged normals), we have found robust oligomer-immunoreactivity in dot-blots from all AD-afflicted brains, but essentially background immunoreactivity in all controls. The molar levels of oligomers are difficult to quantify at this time because of uncertainty regarding the size of the species present, but the ratio of AD versus control brains is greater than 20–1. Replicates for a given brain sample are uniform. Regional analysis suggests that oligomers are more abundant in AD cerebral cortex than in cerebellum. Despite the small sample size, the conclusion is compelling—unlike normal elderly patients, AD patients have significant levels of soluble oligomers, as predicted by the ADDL hypothesis.

5. Mechanism: toxin receptors and signal transduction

ADDLs are neurotoxic molecules that accumulate in AD brains. It clearly is important, therefore, to establish the molecular basis for their toxicity. Our current model is that ADDLs cause neuronal dysfunction and degeneration by specific disruption of neuronal signal transduction (Klein, 2000). We propose that these specific signaling effects depend on interaction between ADDLs and differentially-expressed toxin receptors.

The specificity implied by the model is consistent with the differential vulnerability of particular CNS neurons to AD. Hippocampal and cortical neurons, e.g. are at risk in AD but cerebellar neurons are not. In parallel with AD pathology, cultures of differentiated hippocampal or cortical neurons exposed to low doses of ADDL exhibit impaired MTT reduction and eventually undergo cell death, but cerebellar neurons do not. Within the hippocampus, neurons from CA1 appear particularly susceptible to ADDLs. Although preliminary results indicate low endogenous levels of cerebellar ADDLs also may be a factor, insensitivity to ADDLs may be a major reason why cerebellar neurons are largely unscathed in AD.

A biochemical basis for differential sensitivity to ADDLs lies in differential expression of putative ADDL toxin receptors (Gong et al., 2001). The size and solubility of ADDLs, along with the specificity imparted by their hydrophilic surface domains, indicate a capacity to act as highly specific ligands. To search for binding proteins, a ligand blot assay has been developed that uses oligomer-selective antibodies to detect bound ADDLs. In this assay, membranes from rat cortex and hippocampus show two binding proteins, p140 and p260, that readily bind low doses of ADDLs ($\sim 10^{-9}$ M). These two high-affinity ADDL binding proteins are absent from cerebellum.

Differential expression of ADDL binding proteins has been confirmed by immunofluorescence microscopy. As with the ligand blot assay, microscopy shows ADDL binding sites on neurons from hippocampus and cortex but not cerebellum. Binding sites are small puncta (Lambert et al., 2001) reminiscent of receptor clusters. Such clusters are known to occur at synaptic receptor hot-spots, focal contacts, and membrane rafts or caveolae. The hot spots show greatest density on neurites, and they can be detected on unpermeabilized cells, verifying that binding is to cell surface sites.

Besides occurring in lower mammals (rat, mouse, pig), the ADDL binding proteins p140 and p260 also are present in humans (Gong et al., 2001). Comparisons of ligand blots with silver-stained gels shows these proteins are not abundant. Both p140 and p260 are sensitive to low amounts of trypsin, and p260 disassembles in beta mercaptoethanol. Both proteins are difficult to extract with nonionic detergents suggesting a possible association with rafts or the cytoskeleton.

We currently are investigating further the hypothesis that the p140 and p260 binding proteins actually are toxin

receptors. Our expectation is that such receptors should decrease in abundance in proportion to neuron loss in AD. Very preliminary results indicate a trend consistent with this prediction.

Efforts also are underway to discover down-stream signaling events that may be triggered by ADDL toxin receptor. Data indicate ADDLs have an impact on signaling pathways linked to Fyn, a Src-family protein tyrosine kinase (Lambert et al., 1998). Fyn not only is important physiologically to synaptic sculpting and memory mechanisms, but previous evidence strongly suggests it is relevant to AD. In AD brains, Fyn is over-expressed 400% in tangle-containing neurons (Shirazi and Wood, 1993). Consistent with this observation, tau phosphorylation in cultured cells, via GSK-3 β , can be stimulated by Fyn activation (Lesort et al., 1999). A connection between GSK-3 β and the important *wnt* signaling pathway, which has been linked to amyloid toxicity (De Ferrari and Inestrosa, 2000), is well-established, but no direct evidence has yet coupled Fyn to *wnt* pathways. Fyn also may be upstream in signaling pathways leading to reactive oxygen species (ROS), and ADDLs have been found to elicit ROS formation in neuronal cell lines (Longo et al., 2000). Finally, amyloid preparations, which stimulate tau phosphorylation (Lambert et al., 1994), also activate fyn, which can be detected by its increased association with focal adhesion kinase (Zhang et al., 1996).

In healthy neurons, Fyn is tethered to postsynaptic densities via association with PSD-95 and is found in the signaling scaffolds of focal contacts, rafts and caveolae. Functionally, Fyn has been linked to synaptic sculpting, tetanus-induced LTP, and spatial memory (reviewed in Klein, 2000). When organotypic brain slice cultures from fyn knockout mice are tested for their response to ADDLs, the answer is very clear: no Fyn, no death (Lambert et al., 1998).

6. Use of ADDLs in vaccines and drug development

Great interest is now focused on vaccine development as a therapeutic approach to AD thanks to the pioneering work of Dale Schenk and his colleagues at Elan Pharmaceuticals (Schenk et al., 1999). Schenk's group found that vaccinating transgenic APP-mice with typical fibrillar preparations of A β (which are mixtures of large fibrillar aggregates, protofibrils, ADDLs, and monomers) can reduce plaque-burden and help maintain healthy neurite structure.

We have begun to investigate the possibility that significant improvements in therapeutic efficacy might be achieved by using ADDLs for vaccines. Initial results have shown that vaccinating rabbits with very low doses of ADDLs can generate a robust immune response (Lambert et al., 2001). A key feature of the vaccination protocol has been to verify the structure and bioactivity of the preparations used to vaccinate. Preparations in these experiments are fibril and

protofibril-free and have maximal impact on PC12 cells at under 5 μ M (total A β).

Although the specificity of the Elan antisera has not yet been reported, we have found that ADDL vaccines generate antisera that are extremely selective for assembled forms of A β (roughly 1000-fold higher affinity for oligomers compared to monomers). Specificity for assembled A β , i.e. for the toxic species, presumably is generated because ADDLs are "foreign" to the rabbits, whereas monomers are recognized as "self." Rabbits and humans have the same amino-acid sequence for A β 1–42.

The significance of this specificity is two-fold (1) autoimmune responses may be low, and, (2) the antisera can target traces of the toxic species within a milieu of abundant, physiological monomer.

The specificity of ADDL-generated antisera has practical use on several fronts, including receptor and ADDL-blocker assays (Lambert et al., 2001). The dot-blot assay used detect ADDLs in AD brains, e.g. has been used to screen combinatorial libraries for their ability to block ADDL formation in vitro. Rapid structural screening can be used in tandem with neuroprotection assays to discover lead compounds for ADDL-directed therapeutics. In early screening trials, we have found that certain cyclodextrin-based libraries identified for the presence of structural blockers also give neuroprotection in the MTT assay (Chang et al., 2001). We also have used the dot-blot assay to verify the recent report that neuroprotective Ginkgo biloba extracts block ADDL formation (Yao et al., 2001).

The most significant property of the antisera generated by the ADDL vaccine is its ability to neutralize ADDL toxicity (Lambert et al., 2001). In dose-response experiments, the toxic impact of ADDL preparations is completely neutralized by pretreatment with antisera. It is noteworthy that the ADDL preparations contain excess monomer, but that within this milieu the antisera target the toxic species. Results have been obtained so far using cell biological assays. We predict that antibodies generated in vivo after vaccination with ADDLs would analogously target endogenous toxic species with high specificity and sensitivity.

The predicted immuno-targeting of soluble toxic species is in harmony with results reported by Morgan et al. (Morgan et al., 2000). In a remarkable finding, Morgan's group observed that vaccination with an amyloidogenic A β preparation strongly protects against cognitive decline in APP-transgenic-mice. Most significantly, the beneficial effects of vaccination do not correlate with removal of amyloid deposits, or "plaque-burden." The implication is that the cognitive deficit in these mice models is due to soluble A β -derived neurotoxins, and that vaccination has neutralized these soluble neurotoxins.

Experiments are underway between our group and that of Lennart Mucke at the Gladstone Institute and UCSF to determine if ADDL vaccines, enriched in the putative pathogenic target, might provide the most effective means to eliminate cognitive impairment in transgenic-mice models.

In summary, work over the past several years has given us a new view of A β toxicity in AD in which soluble, subfibrillar neurotoxins have an important role in pathogenesis, perhaps even more so than the familiar insoluble amyloid deposits of plaques. The relative impact of soluble toxins may be particularly significant at the earliest stages of AD, including the transition to AD from mild cognitive impairment. Early-stage involvement of ADDLs is consistent with their potent bioactivity and their ability to form at extremely low peptide concentrations. Only small amounts of A β would be needed to generate endogenous toxic ADDLs, which we anticipate would precede protofibrils and the insoluble amyloid deposits of plaques. ADDLs now have been detected in AD-afflicted brains and in transgenic APP-mice models of AD.

Given the presence and properties of ADDLs, exciting new outcomes for early-stage AD therapeutics can be envisioned. Cognitive impairment once was believed to be caused solely by the physical degeneration of neurons and their death. Putative involvement of ADDLs, however, suggests that early-stages of the disease might be due to a specific impact on the signaling biochemistry required for synaptic plasticity, not neuron death. If so, new treatments such as ADDL vaccines or ADDL-targeting drugs may not only stop AD's inexorable course, but may actually reverse the dysfunction in mild cognitive impairment and early-stage AD.

Acknowledgements

This article is based on a lecture presented at the ISN satellite conference "Cell communication in the nervous system: function and dysfunction." The author would like to thank the organizers of the meeting, Drs. Marco Prado and Fernando de Mello for their efforts in creating a most successful and stimulating symposium. He also is grateful for support from the National Institutes of Health, from the Boothroyd, Feiger and French Foundations, from benefactors of Northwestern University, and from the Institute for the Study of Aging.

References

- Bitan, G., Lomakin, A., Teplow, D.B., 2001. Amyloid-beta-protein oligomerization: prenucleation interactions revealed by photo-induced cross-linking of unmodified proteins. *J. Biol. Chem.* 276, 35176–35184.
- Busciglio, J., Lorenzo, A., Yankner, B.A., 1992. Methodological variables in the assessment of beta amyloid neurotoxicity. *Neurobiol. Aging* 13, 609–612.
- Chang, L., Lambert, M.P., Viola, K.L., Gong, Y., Yu, J., Venton, D.L., Krafft, G.A., Finch, C.E., Klein, W.L., 2001. Nonfibrillar A β toxins in AD: an immunoassay to characterize ADDL formation and identify ADDL-blocker compounds. *Soc. Neurosci. Abs.* 27 (11), 322.
- Chromy, B.A., Nowak, R., Lambert, M.P., Viola, K.L., Chang, L., Stine, W.B., Priebe, G., Krafft, G.A., Klein, W.L., 2001. Self-assembly of A β 1–42 into globular neurotoxins (ADDLs). *Biochemistry*.
- Chui, D.H., Tanahashi, H., Ozawa, K., Ikeda, S., Checler, F., Ueda, O., Suzuki, H., Araki, W., Inoue, H., Shirotani, K., Takahashi, K., Gallyas, F., Tabira, T., 1999. Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. *Nat. Med.* 5, 560–564.
- Czech, C., Masters, C., Beyreuther, K., 1994. Alzheimer's disease and transgenic mice. *J. Neural Transm. Suppl.* 44, 219–230.
- De Ferrari, G.V., Inestrosa, N.C., 2000. Wnt signaling function in Alzheimer's disease. *Brain Res. Brain Res. Rev.* 33, 1–12.
- D'Hooge, R., Nagels, G., Westland, C.E., Mucke, L., De Deyn, P.P., 1996. Spatial learning deficit in mice expressing human 751-amino acid beta-amyloid precursor protein. *Neuroreport* 7, 2807–2811.
- Dodart, J.C., Mathis, C., Bales, K.R., Paul, S.M., Ungerer, A., 2000. Behavioral deficits in APP(V717F) transgenic mice deficient for the apolipoprotein E gene. *Neuroreport* 11, 603–607.
- Gong, Y., Chang, L., Lambert, M.P., Viola, K.L., Krafft, G.A., Finch, C.E., Klein, W.L., 2001. Nonfibrillar A β toxins in Alzheimer's pathogenesis: presence of ADDLs and ADDL-binding proteins in Alzheimer's disease brains. *Soc. for Neurosci. Abs.* 27 (10), 322.
- Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vasquez, S., Vassilev, P.M., Teplow, D.B., Selkoe, D.J., 1999. Protofibrillar intermediates of amyloid-beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J. Neurosci.* 19, 8876–8884.
- Holcomb, L., Gordon, M.N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., Sanders, S., Zehr, C., O'Campo, K., Hardy, J., Prada, C.M., Eckman, C., Younkin, S., Hsiao, K., Duff, K., 1998. Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat. Med.* 4, 97–100.
- Holcomb, L.A., Gordon, M.N., Jantzen, P., Hsiao, K., Duff, K., Morgan, D., 1999. Behavioral changes in transgenic mice expressing both amyloid precursor protein and presenilin-1 mutations: lack of association with amyloid deposits. *Behav. Genet.* 29, 177–185.
- Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., Mucke, L., 1999. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3228–3233.
- Klein, W.L., 2000. A β toxicity in Alzheimer's Disease. In: Chesselet, M.F., (Ed.), *Contemporary Clinical Neuroscience: Molecular Mechanisms of Neurodegenerative Diseases*. Humana Press, Totowa, pp. 1–47.
- Klein, W.L., Krafft, G.A., Finch, C.E., 2001. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci.* 24, 219–224.
- Kumar-Singh, S., Dewachter, I., Moechars, D., Lubke, U., De Jonghe, C., Ceuterick, C., Checler, F., Naidu, A., Cordell, B., Cras, P., Van Broeckhoven, C., Van Leuven, F., 2000. Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. *Neurobiol. Dis.* 7, 9–22.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., Klein, W.L., 1998. Diffusible, nonfibrillar ligands derived from Abeta 1–42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6448–6453.
- Lambert, M.P., Stevens, G., Sabo, S., Barber, K., Wang, G., Wade, W., Krafft, G., Snyder, S., Holzman, T.F., Klein, W.L., 1994. Beta/A4-evoked degeneration of differentiated SH-SY5Y human neuroblastoma cells. *J. Neurosci. Res.* 39, 377–385.
- Lambert, M.P., Viola, K.L., Chromy, B.A., Chang, L., Morgan, T.E., Yu, J., Venton, D.L., Krafft, G.A., Finch, C.E., Klein, W.L., 2001. Vaccination with soluble A β oligomers generates toxicity-neutralizing antibodies. *J. Neurochem.* 79, 595–605.
- Lansbury Jr., P.T., 1997. Structural neurology: are seeds at the root of neuronal degeneration? *Neuron* 19, 1151–1154.

- Larson, J., Lynch, G., Games, D., Seubert, P., 1999. Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP mice. *Brain Res.* 840, 23–35.
- Lesort, M., Jope, R.S., Johnson, G.V., 1999. Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3 β and fyn tyrosine kinase. *J. Neurochem.* 72, 576–584.
- Liu, Y., Schubert, D., 1997. Cytotoxic amyloid peptides inhibit cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by enhancing MTT formazan exocytosis. *J. Neurochem.* 69, 2285–2293.
- Longo, V.D., Viola, K.L., Klein, W.L., Finch, C.E., 2000. Reversible inactivation of superoxide-sensitive aconitase in A β 1–42-treated neuronal cell lines. *J. Neurochem.* 75, 1977–1985.
- Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J., Cotman, C.W., 1993. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7951–7955.
- Lorenzo, A., Yankner, B.A., 1994. Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. U.S.A.* 91, 12243–12247.
- Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E., Rogers, J., 1999. Soluble amyloid-beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am. J. Pathol.* 155, 853–862.
- Mattson, M.P., Duan, W., 1999. Apoptotic biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders. *J. Neurosci.* 19, 152–166.
- McEachern, J.C., Shaw, C.A., 1999. The plasticity-pathology continuum: defining a role for the LTP phenomenon. *J. Neurosci.* 19, 42–61.
- Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M., Arendash, G.W., 2000. Abeta peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 408, 982–985.
- Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., McConlogue, L., 2000. High-level neuronal expression of Abeta 1–42 in wild-type human amyloid protein precursor transgenic-mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058.
- Oda, T., Pasinetti, G.M., Osterburg, H.H., Anderson, C., Johnson, S.A., Finch, C.E., 1994. Purification and characterization of brain clusterin. *Biochem. Biophys. Res. Commun.* 204, 1131–1136.
- Oda, T., Wals, P., Osterburg, H.H., Johnson, S.A., Pasinetti, G.M., Morgan, T.E., Rozovsky, I., Stine, W.B., Snyder, S.W., Holzman, T.F., Krafft, G.A., Finch, C.E., 1995. Clusterin (ApoJ) alters the aggregation of amyloid-beta-peptide (Abeta 1–42) and forms slowly sedimenting Abeta complexes that cause oxidative stress. *Exp. Neurol.* 136, 22–31.
- Pedersen, W.A., Culmsee, C., Ziegler, D., Herman, J.P., Mattson, M.P., 1999. Aberrant stress response associated with severe hypoglycemia in a transgenic mouse model of Alzheimer's disease. *J. Mol. Neurosci.* 13, 159–165.
- Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W., 1993. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* 13, 1676–1687.
- Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandeventer, C., Walker, S., Wogulis, M., Yednock, T., Games, D., Seubert, P., 1999. Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400, 173–177.
- Shirazi, S.K., Wood, J.G., 1993. The protein tyrosine kinase, fyn, in Alzheimer's disease pathology. *Neuroreport* 4, 435–437.
- Steward, O., Schuman, E.M., 2001. Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* 24, 299–325.
- Stine Jr., W.B., Snyder, S.W., Lador, U.S., Wade, W.S., Miller, M.F., Perun, T.J., Holzman, T.F., Krafft, G.A., 1996. The nanometer-scale structure of amyloid-beta visualized by atomic force microscopy. *J. Protein Chem.* 15, 193–203.
- Terry, R.D., 1999. The neuropathology of Alzheimer disease and the structural basis of its cognitive alterations. In: Terry, R.D., Katzman, R., Bick, K.L., Sisodia, S.S. (Eds.), *Alzheimer Disease*. Lippincott Williams, and Wilkins, Philadelphia, PA, pp. 187–206.
- Walsh, D.M., Hartley, D.M., Kusumoto, Y., Fezoui, Y., Condron, M.M., Lomakin, A., Benedek, G.B., Selkoe, D.J., Teplow, D.B., 1999. Amyloid-beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. *J. Biol. Chem.* 274, 25945–25952.
- Walsh, D.M., Lomakin, A., Benedek, G.B., Condron, M.M., Teplow, D.B., 1997. Amyloid-beta-protein fibrillogenesis: detection of a protofibrillar intermediate. *J. Biol. Chem.* 272, 22364–22372.
- Wang, H.-W., Pasternak, J.F., Kuo, H., Ristic, H., Lambert, M.P., Chromy, B., Viola, K.L., Klein, W.L., Stine, B., Krafft, G.A., Trommer, B.L., 2001. Soluble oligomers of β amyloid (1–42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. *Brain Res.*
- Yamaguchi, F., Richards, S.J., Beyreuther, K., Salbaum, M., Carlson, G.A., Dunnett, S.B., 1991. Transgenic mice for the amyloid precursor protein 695 isoform have impaired spatial memory. *Neuroreport* 2, 781–784.
- Yao, Z., Drieu, K., Papadopoulos, V., 2001. The Ginkgo biloba extract EGb 761 rescues the PC12 neuronal cells from beta-amyloid-induced cell death by inhibiting the formation of beta-amyloid-derived diffusible neurotoxic ligands. *Brain Res.* 889, 181–190.
- Younkin, S.G., 1998. The role of Abeta 42 in Alzheimer's disease. *J. Physiol Paris* 92, 289–292.
- Yuste, R., Bonhoeffer, T., 2001. Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annu. Rev. Neurosci.* 24, 1071–1089.
- Zhang, C., Qiu, H.E., Krafft, G.A., Klein, W.L., 1996. Abeta peptide enhances focal adhesion kinase/fyn association in a rat CNS nerve cell line. *Neurosci. Lett.* 211, 187–190.