# Physicochemical characteristics of soluble oligomeric A$\beta$ and their pathologic role in Alzheimer's disease

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*Extracellular fibrillar amyloid deposits are prominent and universal Alzheimer's disease (AD) features, but senile plaque abundance does not always correlate directly with the degree of dementia exhibited by AD patients. The mechanism(s) and dynamics of Aβ fibril genesis and deposition remain obscure. Enhanced Aβ synthesis rates coupled with decreased degradative enzyme production and accumulating physical modifications that dampen proteolysis may all enhance amyloid deposit formation. Amyloid accumulation may indirectly exert the greatest pathologic effect on the brain vasculature by destroying smooth muscle cells and creating a cascade of negative impacts on cerebral blood flow. The most visible manifestation of amyloid dis-equilibrium could actually be a defense mechanism employed to avoid serious vascular wall degradation while the major toxic effects to the gray and white matter neurons are mediated by soluble oligomeric Aβ peptides with high β-sheet content. The recognition that dynamic soluble oligomeric Aβ pools exist in AD and are correlated to disease severity led to neurotoxicity and physical conformation studies. It is now recognized that the most basic soluble Aβ peptides are stable dimers with hydrophobic regions sequestered from the aqueous environment and are capable of higher order aggregations. Time course experiments employing a modified ELISA method able to detect Aβ oligomers revealed dynamic intermolecular interactions and additional experiments physically confirmed the presence of stable amyloid multimers. Amyloid peptides that are rich in β-sheet structure are capable of creating toxic membrane ion channels and a capacity to self-assemble as annular structures was confirmed* in vitro *using atomic force microscopy. Biochemical studies have established that soluble Aβ peptides perturb metabolic processes, provoke release of deleterious reactive compounds, reduce blood flow, induce mitochondrial apoptotic toxicity and inhibit angiogenesis. While there is no question that gross amyloid deposition does contribute to AD pathology, the destructive potential now associated with soluble Aβ suggests that treatment strategies that target these molecules may be efficacious in preventing some of the devastating effects of AD.* [Neurol Res 2005; **27**: 000–000]

*Keywords: Oligomer; β-amyloid; Alzheimer's disease; ELISA; neurotoxicity; atomic force microscopy*

## SOLUBLE OLIGOMERIC AND FIBRILLAR A$\beta$: AN OVERVIEW

Alzheimer's disease (AD) neuropathology is characterized by the extracellular deposition of fibrillar amyloid in cortical senile plaques and in the walls of leptomeningeal and parenchymal arteries. These amyloid fibrils form when 40 and 42 amino acid residue A$\beta$ peptides, derived from a larger type I transmembrane molecule amyloid precursor protein (A$\beta$PP), polymerize. When released from A$\beta$PP by the action of the $\beta$- and $\gamma$-secretases into the cytosolic or extracellular milieu, the A$\beta$ peptides generate transient metastable and disordered secondary structures due to the interaction of the hydrophobic domains with the surrounding

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water. Molecular sieving experiments coupled with calibrated column chromatography and atomic force microscopy (AFM) as well as computer modeling reconstructions suggest that the Aβ peptides organize into globular dimeric structures ~3.5 nm in diameter with a hydrophobic core and superficial crevices that shield the
non-polar C-terminal domains[1–4]. Interestingly, apolipoprotein E (Apo E), the best understood Aβ carrier in the brain, has been reported to bind a soluble dimeric form of these peptides[5]. Free soluble dimeric Aβ peptides in the interstitial fluid probably exist only transiently due to their amphipathic nature, which permits interactions with a large number of enzymes and structural proteins via ionic and hydrophobic bonds. A good deal of evidence indicates that these molecules aggregate into large complexes in apparent equilibrium between monomer, dimer, trimer and tetramer masses in the shape of prolate ellipsoids[1,6]. The Aβ peptides may adopt a preponderantly β-sheet conformation conducive to extensive polymerization[7]. The resulting filamentous structures measure ~10 nm in diameter and are of an undefined length. These thermodynamically very stable and water-insoluble structures contain a hydrophobic core surrounded by a hydrophilic shell that interacts with the aqueous environment[4]. The amyloid filaments are observable by light and electron microscopy in the extracellular space of the cerebral cortex and in the walls of leptomeningeal and cortical vessels. An important structural feature is that in AD the amyloid fibrils are composed of ~75% Aβ peptides with the remaining 25% consisting of tightly bound glycoproteins and glycolipids that are responsible for their characteristic insolubility and resistance to proteolysis[8,9].

In spite of having achieved an excellent knowledge concerning the Aβ peptide chemistry[10,11], the exact mechanisms behind their accumulation in the brains of elderly individuals and AD patients still remain mysterious. It is accepted that during the aging process there is a moderate increase in AβPP synthesis that results in an overabundance of this molecule and consequent augmented Aβ deposition[12]. The Aβ overproduction is apparently exacerbated by a sustained and general failure to clear these peptides[13]. It has been suggested that the Aβ/Apo E complexes are removed by endocytosis via the low-density lipoprotein (LDL) receptor and the LDL receptor related protein (LRP). Alternatively, α2-macroglobulin bound Aβ may be cleared through the LRP receptor[14,15]. Interestingly, over-expression of the LRP receptor in PDAPP transgenic mice results in soluble Aβ level increases[16]. In addition, a decreased Aβ intrinsic degradation rate should also be considered as a contributory factor in Aβ accumulation. This may result from down-regulation of specific proteolytic enzymes that are known to have Aβ as a substrate such as neprilysin[17,18], insulin degrading enzyme[19], plasmin[20] and metalloproteinases[21,22]. Two additional structural factors may also impede direct proteolytic degradation of Aβ. First, a large proportion of the fibrillar Aβ is irreversibly denatured into proteolytic resistant oligomeric forms (dimers, trimers and tetramers) that account for up to 45% of the total amyloid plaque core mass[23]. Secondly, Aβ peptides accumulate a vast amount of postranslational modifications such as isomerization of Asp[24], racemization of Asp and Ser[24,25], oxidation of Met[24], cyclization of Glu to pyroglutamyl[26] and tyrosyl cross-linkages[27–30] that strongly hinder proteolytic degradation. Of these postranslational modifications Aβ 1–42 with iso-Asp at positions 1 and 7 showed the fastest rate of oligomerization into dimers, trimers and tetramers on incubation at 37°C followed by Aβ 1–42 and Aβ 3(pyroglutamyl)–42[24]. The association of Aβ molecules with hydrophobic lipid vesicles containing GM1 ganglioside and phosphatidylinositol may also promote Aβ accumulation[31]. Interestingly, soluble dimeric Aβ peptides accumulate in lipid rafts in the AβPP tg2576 transgenic mice[32].

Germane to AD pathology are the deposits of amyloid whose toxicity has been extensively investigated and debated[10,11]. Prior to 50 years of age there is no visible amyloid accumulation in the human brain, but in subsequent years the deposition typically increases substantially[33]. What apparently differentiates AD from normal aging is the degree of amyloid accumulation. AD severity is staged according to neuropathological criteria established by the CERAD[34] and NIA-Reagan[35] and Braak[36] ranking scores but attempts to correlate dementia and amyloid burden have been harshly criticized because senile plaque amyloid load does not always parallel the degree of dementia[37].

Even less clear are clinical correlations of AD with vascular amyloid levels. In the majority of the cases the vascular amyloid load was subjectively measured by observing a limited number of brain histological slices stained with Congo red, thioflavines or silver stains. Histological sections permit neither appreciation of the leptomeningeal and cortical vascular amyloid extent and accordingly, of the consequent hemodynamic impact that pervasive vascular pathology may have upon perfusion of large regions of the brain. We believe that it is at the vascular level where substantial pathological and functional damage of amyloid deposition is exerted resulting in smooth muscle and endothelial cell destruction. It is irrefutable that the demise of vascular myocytes results in a lack of vascular compliance and in the loss of control of regional cerebral blood flow. In hemodynamic terms, when the amyloidosis of the cerebral arteries, arterioles and capillaries is in an advanced condition[38], the diastolic perfusion pressure is severely affected, leaving the brain perfusion largely dependent on the systolic pressure created by the heart. The brain hypoperfusion can be grossly compounded when the vascular amyloidosis coexists with advanced atherosclerosis of the circle of Willis and leptomeningeal arteries[39]. To consider the full pathological impact of the vascular amyloid lesions, it is necessary to prepare whole mounts of the total leptomeningeal membranes. This examination is complemented by the complete detergent-mediated lysis of large blocks of the cerebral cortex followed by the



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staining of the remaining insoluble vascular amyloid by thioflavine-S[40]. In some AD individuals, the amount of vascular amyloid is limited, while in subjects carrying the Apo E $\varepsilon$4 allele, the levels are overwhelming[11,40], an association that still remains to be explained from a pathophysiological point of view. We contend that the amyloid deposited in the cortical senile plaques represents a defense mechanism whereby the soluble and diffusible oligomeric A$\beta$ peptides are sequestered, converted into compact fibrillar amyloid deposits and insulated by glial cells, so as to preclude reaching the vascular walls where considerable damage is elicited[41].

## IS SOLUBLE OLIGOMERIC A$\beta$ NEUROTOXIC AND FIBRILLAR AMYLOID AGGREGATION A PROTECTIVE RESPONSE?

A growing number of *in vitro* and *in vivo* studies support the notion that soluble oligomeric peptides with high $\beta$-sheet content are toxic to neuronal cells and cause their dysfunction and death. In contrast, the aggregation of these peptides in the form of amyloid (defined as a fibrillar, Congo red- and thioflavine-positive polymer) or pre-amyloid (defined as a Congo red- and thioflavine-negative deposits) may represent a protective response that delays neurodegeneration by sequestering soluble A$\beta$ into amyloid fibrils or into amorphous aggregates. In the CNS, this protective response may be reflected in the presence and relative abundance of amyloid plaques (AP) or diffuse plaques (DP) in the neuropil. This hypothesis is presented with the admonition that extracellular AP may also complicate the disease process since they could be perceived as foreign bodies, thus unleashing a microglia-mediated chronic inflammatory reaction with catastrophic consequences, as the one observed in AD.

One prediction of an ''amyloid protection'' hypothesis would be a positive correlation between the number of AP and DP and the duration of the AD from clinical onset to death. Several examples of human diseases characterized by the accumulation of amyloidogenic peptides in the brain support this contention. AD itself, both in its sporadic and familial forms, comprises a group of disorders of relatively long duration defined by the presence of chronic dementia with numerous AP and neurofibrillary tangles (NFT). The autosomal dominant prion disease known as Gerstmann–Straussler–Scheinker (GSS) has a mean duration of 5 years, with some cases reaching 12 years, well beyond the duration of other forms of prion diseases. It is noteworthy that GSS is characterized by a relative abundance of AP and, in some cases, of NFT in the cerebral cortex[42–45]. In striking contrast, other prion diseases with little or no AP or NFT formation, such as sporadic and familial Creutzfeldt–Jakob disease (CJD) or fatal familial insomnia, show a faster clinical course with rampant neurodegeneration and durations ranging from 2 to 35 months[46–48]. An atypical long-course CJD has been associated with the abundance of AP[49]. Moreover, variant CJD caused by bovine spongiform encephalopathy prion infection is characterized by the presence of florid AP and DP and although the plaque count does not correlate with survival, this disease has a mean duration of 14 months, significantly longer than many cases of sporadic CJD[50,51]. Two notable examples, recently described in biochemical and genetic terms, are the familial British and Danish dementias (FBD and FDD) associated with the *de novo* generated peptides ABri and ADan. These 34-mer peptides result from frame-shift mutations in the BRI gene and have C-terminal extensions of 11 residues that render them extremely insoluble at physiological pH and highly neurotoxic to cultured cells[52,53]. Yet, the disease courses are relatively slow, with durations of 10–20 years. Remarkably, both FBD and FDD are characterized by a widespread amyloid and pre-amyloid deposition and numerous *tau*-immunoreactive NFT in the cortex and hippocampus[54,55]. A positive correlation between AP, DP and NFT and duration of disease can be explained by alternative hypotheses such as the necessity of a very long time period for these lesions to develop, or that AP, DP and NFT are coincidental with other unknown factors that determine survival. However, the ''amyloid protection'' theory may be subjected to experimental testing by blocking specifically the steps that result in amyloid formation from soluble oligomers in transgenic (Tg) mice over-expressing amyloidogenic peptides. A sustained increase in the levels of soluble oligomers at the expense of insoluble peptide deposition is predicted to result in more neurodegeneration and shorter survival.

## CHARACTERIZATION OF SOLUBLE A$\beta$ OLIGOMERS

The initial focus on the fibrillar amyloid as the central structure in AD pathology has evolved during the last 10 years. This was due to several outstanding discoveries such as the finding of a soluble fraction of oligomeric A$\beta$ that, at least theoretically, could diffuse along the narrow and convoluted interstitial spaces of the gray matter (GM) and white matter (WM). Being a component of the interstitial fluid, the A$\beta$ could be drained along the periarterial spaces that communicate with the deep lymphatic system of the head and neck[56–62], which empty into the systemic venous circulation. In the early 1990s, small amounts of apparently low molecular weight soluble A$\beta$ peptides were isolated by immunoprecipitation from brain homogenates[63,64] and quantified by ELISA[65]. Likewise, small amounts of SDS-stable soluble oligomeric A$\beta$ were isolated from cell cultures[66], and these oligomers could be stabilized in a monomeric form by Congo red[67]. It was also determined that the presence of soluble A$\beta$ preceded amyloid plaque formation in Down's syndrome[68]. Subsequently, the oligomeric peptides were identified and characterized in normal and AD brain tissue by molecular mass determinations in detergent-free media in GM fractions obtained by differential ultracentrifugation and graded membrane filtration ($<$10, 10–30, 30–100 and $>$100 kDa) of cortical homogenates and quantified by sandwich ELISA. The amount of total soluble A$\beta$ recovered in



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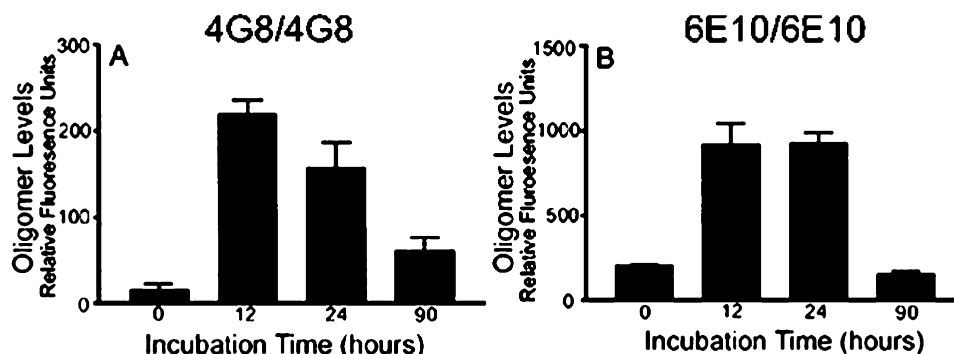


**Figure 1:** The effect of incubation time on A$\beta$ oligomer formation in aqueous solution. A 1 µg/ml solution of A$\beta$ 1–42 in PBS was made from an A$\beta$ 1–42 1 mg/ml DMSO stock solution and incubated at 37°C for 0, 12, 24 or 90 hours. Amyloid-$\beta$ oligomer levels were measured by sandwich ELISA wherein the capture and detection antibodies were the same (**A**) 4G8/4G8 and (**B**) 6E10/6E10. Detection antibodies were labeled with europium, and time resolved relative fluorescence was measured on a Victor plate reader. Fluorescence units are graphed to represent relative oligomer levels

the 220,000 $g$ (2 hours, 4°C) supernatant in the control cases ranged from 5 to 12 ng/gm of cortex, while in the AD cases it ranged from 21 to 90 ng/g of cortex[69]. Isolated soluble oligomeric A$\beta$ forms were obtained from the human brain and characterized by AFM revealing the smallest aggregate to be an A$\beta$ dimer 3–4 nm in diameter[1]. These isolated soluble oligomers were toxic to neurons in culture only in the presence of microglia[1]. Two years later, Klein and colleagues confirmed the presence and toxicity of oligomeric A$\beta$ and proposed the name of ADDLs (A$\beta$-derived diffusible ligands) for these structures[70]. Intriguingly, the deep WM in AD does not generate amyloid deposits despite the fact this tissue contains a considerable level of soluble oligomeric A$\beta$ peptides amounting to an average of 530 ng/g of WM in control non-demented brain and 2200 ng/g of WM in AD ($p$=0.01)[71]. The lower quantities of soluble A$\beta$ present in the GM may be due to its rapid incorporation into the AP or vascular amyloid deposits. It is possible that the significantly elevated quantities of WM-soluble A$\beta$ in AD participate in the gross loss of myelin, oligodendrocytes and axons observed in the areas of WM rarefaction in some AD cases. Alternatively, the soluble A$\beta$ peptides in the WM areas of rarefaction may result from the stagnation of interstitial fluid and edema due to the blockage of the periarterial spaces that drain the brain extracellular fluid[71]. Not unexpectedly, soluble A$\beta$ oligomers were also detected in the CSF and in intracellular medium of neurons[72,73]. The A$\beta$ peptides have been found to be toxic to cultured rodent oligodendrocytes by directly affecting the sphingomyelinase and inducing ceramide accumulation[74]. We propose that the WM oligomeric soluble A$\beta$ originates from the GM since the amounts of these peptides decrease with increasing distance from the GM, being at their lowest concentrations in the periventricular areas[71].

Soluble A$\beta$ oligomers in the physiological fluids and in brain homogenates can be detected and quantified by an ELISA method in which the capture and reporter antibodies are identical. This approach was tested by several *in vitro* techniques with synthetic A$\beta$ 1–42. Amyloid-$\beta$ 1–42 was dissolved in DMSO (1 mg/ml), diluted to a final concentration of 1 µg/ml in phosphate buffered saline (PBS) and incubated (0, 12, 24 and 90 hours) at 37°C. The experiments were conducted using two differentially targeted anti-A$\beta$ monoclonal antibodies, 4G8 and 6E10. At 12 hours, the amount of A$\beta$ oligomerization increased 13 and 4.5 times as detected by the 4G8 and 6E10 antibodies, respectively (*Figure 1*). The intermolecular association rate is solvent dependent. The dilution of A$\beta$ 1–42 from hexafluoroisopropanol (HFIP) into PBS produces a more rapid oligomerization than when the peptide is prepared from dimethyl sulfoxide (DMSO) (*Figure 2*). Subjecting oligomeric A$\beta$ 1–42 to ultrafiltration using graded Centricon filters reveals small amounts of material <10 and <30 kDa, with substantially larger amounts of aggregated A$\beta$ evident in the filtrates obtained employing <100 and <300 kDa (*Figure 3*A). Estimates of the total amount of A$\beta$ 1–42 captured by the various Centricon filters is shown in *Figure 3*B.

As can be appreciated in *Figure 4* severe brain trauma apparently reduces the presence of A$\beta$ oligomers detected in the CSF relative to the control group ($p<0.05$). Oligomerization levels are not always directly proportional to total soluble A$\beta$ 1–42 monomer concentration (*Figure 4*). A comparison of AD, age-matched non-demented controls and trauma patients revealed that comparatively elevated A$\beta$ 1–42 levels do not necessarily provoke commensurate oligomerization. It is important to note that this result is based on a small trauma patient pool ($n$=6) with an average age of 35.5 years. It is tempting to speculate that the oligomerized A$\beta$ pool is dynamic and could increase in an age-dependent manner.

Oligomeric A$\beta$ can generate severe stress in cultured PC12 neuronal cells that can be measured by the inhibition of cellular reduction of 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan, a test widely used to estimate cell viability. Utilizing this technique the degree of neuronal stress was measured in terms of percent of MTT conversion versus A$\beta$ storage time in DMSO. The treatment of PC12



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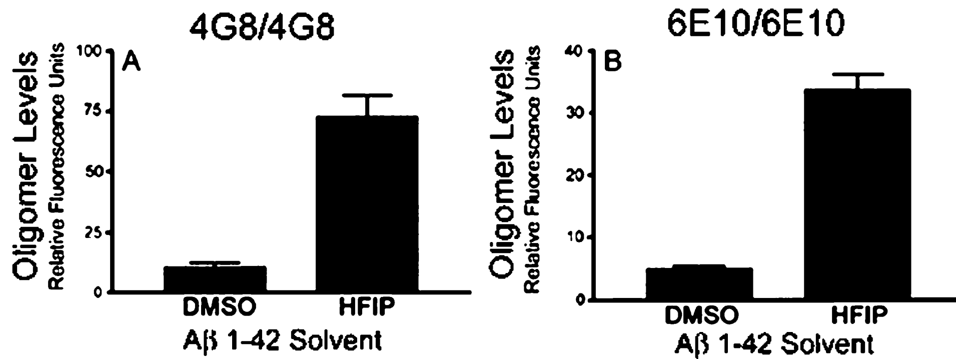


**Figure 2:** The effect of different solvents on oligomer formation. Oligomers are formed instantly upon dilution of A*β* 1–42 from HFIP solution into PBS. A*β* 1–42 was diluted to 1 μg/ml in PBS from a 1 mg/ml stock solution of A*β* 1–42 dissolved in DMSO or HFIP. The relative amount of A*β* 1–42 oligomers were measured by sandwich ELISA using the same antibody as both capture and detection antibodies, either 4G8 (4G8/4G8) or 6E10 (6E10/6E10) shown in (**A**) and (**B**), respectively. Detection antibodies were labeled with europium, and time resolved relative fluorescence was measured on a Victor plate reader. Fluorescence units are graphed to represent relative oligomer levels

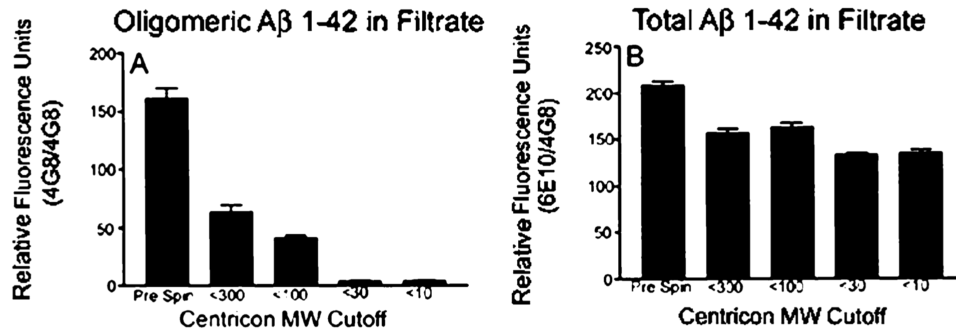


**Figure 3:** Determination of the size of synthetic A*β* 1–42 oligomers by Centricon ultrafiltration. A 1 μg/ml solution of A*β* 1–42 oligomers was prepared by diluting A*β* 1–42 from a 1 mg/ml HFIP stock solution into PBS. Five hundred microliters of A*β* oligomer solution was filtered through a Centricon filtration device of 10, 30, 100 or 300 kDa molecular weight cut-off. The filtrates were collected and total A*β* and oligomeric A*β* levels were measured by ELISA. (**A**) Oligomeric A*β* was measured using 4G8 as capture antibody and europium labeled 4G8 as detection antibody. (**B**) The total A*β* was measured using 6E10 as a capture antibody and europium labeled 4G8 as a detection antibody. Time resolved fluorescence was measured on a Victor plate reader. ELISA fluorescence units are graphed to represent oligomer levels in each fraction collected

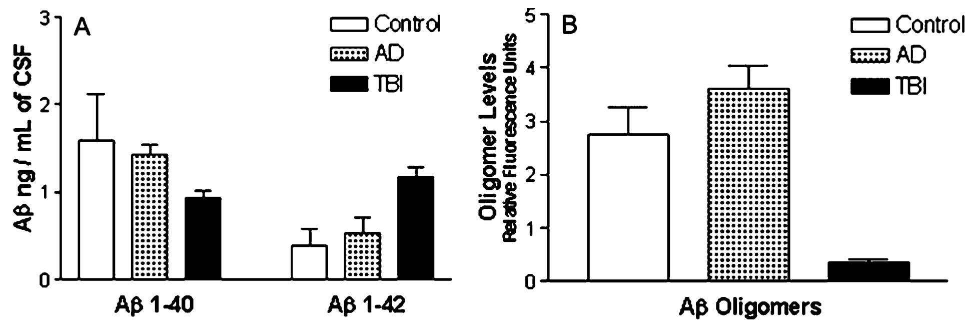


**Figure 4:** Measurement of A*β* 1–40, A*β* 1–42 and oligomeric A*β* levels in control, AD and traumatic brain injury (TBI) patient CSF. CSF from a total of 27 AD patients and 24 age-matched control subjects was obtained from the Sun Health Research Institute (Sun City) rapid autopsy program. CSF was obtained daily from each of six TBI patients for 14–21 consecutive days following injury. (**A**) A*β* 1–40 levels were measured in CSF samples by ELISA wherein biotinylated 6E10 antibody served as detection antibody and R163, an antibody that specifically binds to the C-terminus of 1–40, served as capture antibody. A*β* 1–42 levels were measured in CSF samples by ELISA wherein the biotinylated 6E10 antibody served as a detection antibody and R165, an antibody that specifically binds to the C-terminus of 1–42, served as a capture antibody. The A*β* concentration in each sample was determined by comparison with standards of known A*β* 1–40 and A*β* 1–42 concentrations. The mean ± SEM of A*β* levels measured in CSF samples for each group is graphed. (**B**) Oligomeric A*β* levels were measured using an ELISA wherein biotinylated 6E10 was used as the detection antibody and 6E10 antibody was used as the capture antibody. Relative fluorescence units are graphed to represent mean oligomer levels measured in CSF samples for each group



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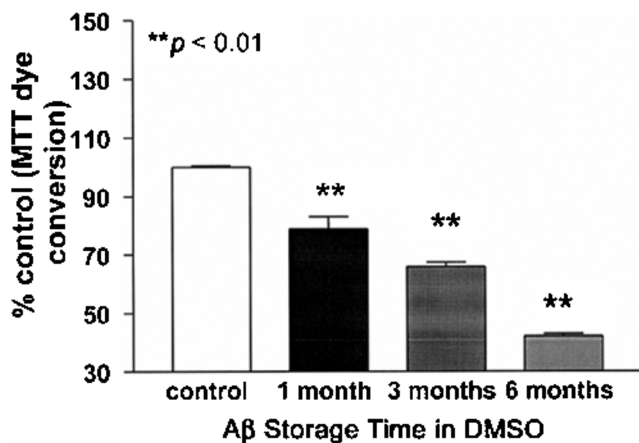


**Figure 5:** Effect of oligomeric A*β* 1–42 on MTT dye conversion by nerve growth factor (NGF) differentiated PC12 cells. PC12 cells were plated onto collagen-coated wells and treated for 6–7 days with RPMI media containing 100 ng/ml NGF to induce a neuronal phenotype. A*β* 1–42 samples were dissolved in DMSO at a concentration of 1 mg/ml and stored at −20°C for 1, 3 or 6 months. NGF differentiated PC12 cells were exposed to 10 μM A*β* 1–42 for 2 hours at 37°C. Following the addition of fresh media to cells, MTT dye was added, and cells were incubated for 2 hours. The cells were then exposed to lysis buffer containing SDS and incubated overnight. The absorbance at 595 nm of each well was measured using a Molecular Devices 96-well plate reader. The results are expressed as percent of control values and represent means±SEM of quadruplicate values. Statistical significance was tested by one-way ANOVA followed by Dunnett's post-test. $^{**}p<0.01$

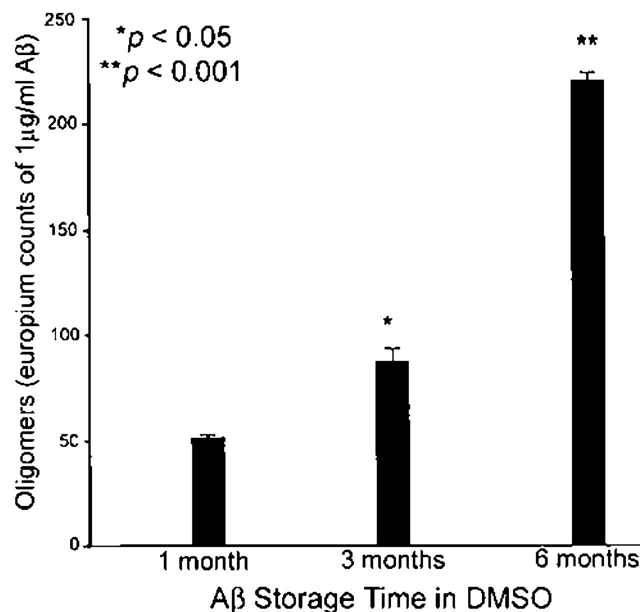


**Figure 6:** A*β* 1–42 oligomer formation in DMSO over time. A*β* 1–42 was dissolved in DMSO at a concentration of 1 mg/ml and stored at −20°C for 1, 3 or 6 months. DMSO stock solutions were diluted to 1 μg/ml in PBS and assayed by sandwich ELISA using 6E10 antibody for both capture and detection. Oligomer levels are expressed as time resolved fluorescence units obtained from 1 μg/ml of A*β* 1–42 by europium immunoassay

cells with older A*β* samples, containing higher amounts of oligomeric A*β*, results in greater inhibition of MTT dye conversion indicating that the oligomers are having a toxic effect on the cells. *Figure 5* shows a significant ($p<0.01$) decrease in MTT dye conversion with an extended storage time of A*β* in DMSO. Oligomerized A*β* levels measured by the 6E10/6E10 antibody combination ELISA increase significantly during this time: 1 versus 3 months: $p<0.05$; 3 versus 6 months: $p<0.001$ (*Figure 6*).

## ATOMIC FORCE MICROSCOPY OF SOLUBLE OLIGOMERIC A*β*

AFM of the monomeric, dimeric and tetrameric fractions purified by FPLC from the AP of AD patients, demonstrated that, on removal of the formic acid by dialysis against water and ammonium bicarbonate, the monomeric A*β* generated long filaments with a diameter of 10 nm. The dimeric and tetrameric forms, in contrast, revealed the presence of discrete prolate ellipsoids measuring 3–4 and 7–8 nm in diameter that corresponded to the volumes of dimeric and tetrameric A*β*, respectively, and were very toxic to hippocampal neurons in culture[1]. Larger globular aggregates of dimeric and tetrameric A*β* were also observed, which could be attributed to octamer, dodecamer and hexadecamer molecules. These observations suggest that the basic soluble form of the A*β* peptides is a stable A*β* dimer[4] in which the hydrophobic regions of the monomers are protected from the aqueous environment[3]. Alternatively, monomers may rapidly associate into very stable filamentous structures with a hydrophobic core made of stacks of antiparallel *β*-sheets perpendicular to the main axis of the filament[75] with lateral hydrophilic domains, transiently effecting a dumbbell-like structure[4]. The lateral association of these protofilaments results in a solid and very stable helical structure in which the hydrophobic regions are entirely shielded by a sleeve of polar amino acids[4,76,77]. The kinetics of *in vitro* aggregation and re-polymerization of A*β* purified from the AD patient AP must be a very complex series of events since a large proportion the A*β* peptides (which vary from individual to individual) are degraded at the N-terminus and contain a high percentage of post-translational modifications[23,24]. Experiments with synthetic A*β* using fluorescence resonance energy transfer and AFM suggest a rapid aggregation of soluble A*β* (∼200 nm), followed by a slow appearance of more organized spheroids (∼30 nm) that eventually convert into filamentous structures[78]. At neutral pH, the A*β* peptides can also generate stable dimers and tetramers with a random secondary structure and can eventually organize into large conglomerates with molecular masses close to 1 million Da[79]. Perfect spheroids of synthetic A*β* 1–40, measuring 10–15 nm, are toxic to cultured neurons[80]. In an independent study, synthetic A*β* 1–42 also generated globular structures with $M_r$ corresponding from trimers to 24-mer as determined by AFM, which were toxic to PC12 cells in culture[81].

An intriguing issue in the biochemistry of AD is the molecular interaction between soluble A*β* and cellular



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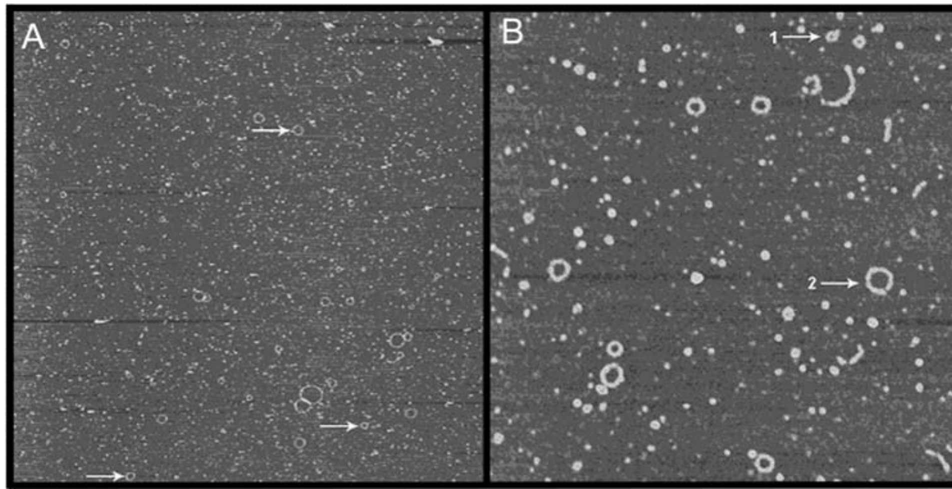


**Figure 7:** Atomic force microscopy images of Aβ. (**A**) A low power caption of Aβ showing numerous rings. Image size is 2.5 × 2.5 μm. (**B**) At higher magnification (field = 1 × 1 μm) small and large rings are indicated by arrows. The rings appear to be formed by a row of several globular molecules measuring between 8 and 12 nm in diameter

membranes[82]. Aβ peptides have the potential to form $Ca^{2+}$ ion-permeable channels in lipid bilayers, which may be very disruptive to cellular homeostasis and cause neuronal death[83–87]. Subsequent studies confirmed that Aβ 1–42 rapidly formed $Ca^{2+}$ channels on endothelial cell surfaces and neurons in culture and elicited cellular degeneration[88–90]. Apparently, the Aβ peptides are capable of forming different types of cation channels (spike mode, burst mode and open mode) with intermediate configurations with positively charged cytotoxic activity[91]. The ability of Aβ peptides, rich in β-sheets with hydrophobic and charged residue domains, to readily create membrane ion channels has stirred speculation regarding the capacity of other homologous amyloids to produce similar ion channels with devastating metabolic consequences for the cell[92]. We used AFM to investigate the capacity of Aβ 1–40 to organize into ring-like structures. Lyophilized Aβ 1–40 was dissolved in DMSO to a concentration of ~1 mg/ml. This stock was diluted in 20 mM PBS pH 7.5 to a final concentration of 50 μg/ml and incubated at room temperature for 24 hours. Five microliter aliquots were deposited on APS-mica after the following time intervals: 0 and 15 minutes, 1, 3, 6 and 24 hours. The samples were imaged using a Nanoscope Multimode IIIa AFM in air. Femtoscan software was then used to measure the height, and inner and outer diameters of the rings present in the images shown in *Figure 7*. A large-scale image (2.5 × 2.5 μm) is shown in *Figure 7*A and a high-resolution image (1 × 1 μm) of one of the sections of the previous figure is shown in *Figure 7*B. Dots and bright globular features on the images correspond to sizes typically observed for Aβ peptide oligomers. In addition, ring-shaped aggregates were visible on both images that formed immediately after dissolving the synthetic peptide stock. Annular features have recently been identified using electron microscopy and AFM and these structures were usually observed to undergo aggregation[93]. These Aβ ring aggregates were relatively stable during the incubation in aqueous solution (PBS buffer) at ambient temperature with no change in the number of rings per micron$^2$ during the first hour (*Figure 8*A). A drop in the total number of ring-shaped morphologies was observed by the end of 24 hours, but the ring size remained constant (*Figure 8*B). It is clear from the images that the size of the rings varies (the smallest and largest rings are indicated with arrows in *Figure 7*). We also measured the diameter of the rings, and the results for the mean number of the outer diameter for several samples taken at different incubation times are shown in *Figure 8*B.

## PATHOLOGICAL ACTION OF SOLUBLE OLIGOMERIC Aβ

The role of soluble oligomeric Aβ in AD pathophysiology is underscored by its direct correlation with the disease severity, an association that is not observed in relation to the insoluble amyloid[94]. Furthermore, we have observed that the soluble Aβ n-40 concentration is a better predictor of synaptic demise in AD than the levels of soluble Aβ n-42 or the cortical amyloid deposits[95]. Soluble Aβ can activate microglia through the His-His-Gln-Lys sequence (Aβ residues 13–16) interaction with cell surface heparan sulfate proteoglycans to induce selective neuron killing[96,97]. There is clear evidence that soluble Aβ induces reactive morphological alterations in astroglial cells and elicits secretion of inflammatory cytokines and nitric oxide (NO) release[98,99]. In the AβPP Tg mice model, presynaptic terminal density decline and synaptic transmission deficits, both influenced by the presence of soluble Aβ, precede fibrillar Aβ deposition[100,101]. Soluble oligomeric Aβ has also been found to be neurotoxic and capable of inhibiting hippocampal long-term potentiation[70,102,103]. A Tg mice model expressing the $A\beta PP_{751}$ isoform in which there is no amyloid plaque accumulation still exhibited severe learning deficits suggesting gross cognitive deficits are associated with



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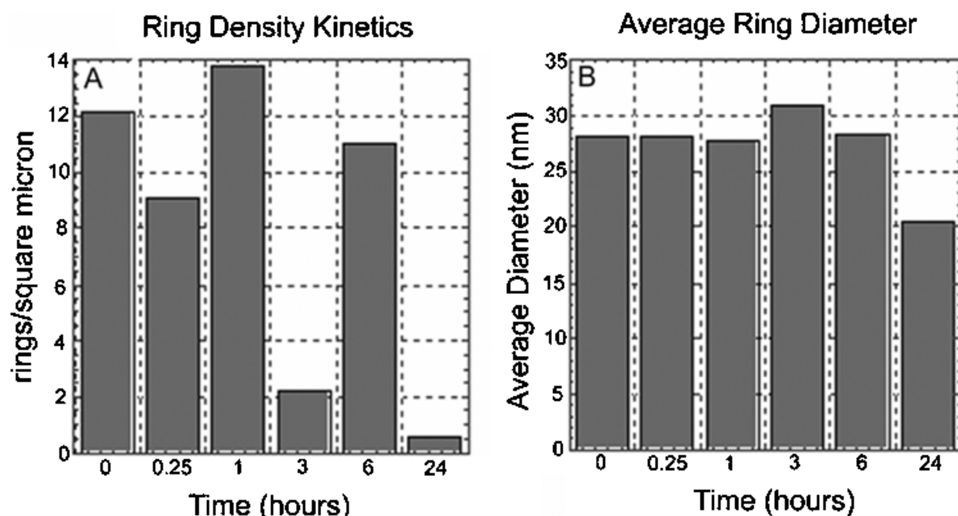


**Figure 8:** (**A**) The dependence of the number of rings per selected area relative to time of incubation in PBS. (**B**) The dependence of the mean diameter of A$\beta$ rings on incubation time

increased levels of soluble A$\beta$[104]. Furthermore, memory loss was reversed by intraperitoneal injections of the anti-A$\beta$ monoclonal antibody BAM-10 without significant reduction in deposited amyloid suggesting decreased soluble A$\beta$ levels correlated most directly with brain function improvement[105]. In addition, early memory impairment in the tg2576 Tg mice was noticed at an age in which deposited A$\beta$ was not yet obvious suggesting soluble A$\beta$ plays a role in memory failure[106]. In the PDAPP Tg mice model, the administration of the monoclonal anti-A$\beta$ m266 reversed memory deficits without changing the brain A$\beta$ load suggesting this functional improvement was due to soluble A$\beta$ peptides clearance rather than extensive amyloid deposit removal[107]. In humans, the soluble A$\beta$ n-40 levels correlated with the degree of cerebrovascular amyloidosis and with Apo E4 allele frequency[95]. Increased levels of the more soluble A$\beta$ n-40 may promote diffusion into the periarterial spaces where amyloid deposition could obstruct interstitial fluid flow and create grave consequences for brain metabolism[40]. In addition, soluble A$\beta$, specifically A$\beta$ 1–40, is a powerful modulator of endothelial cell-mediated vasoconstriction by means of lowering the NO production and decreasing the NO:superoxide ratio[108–111]. Overall, the vasoconstrictive activity of soluble A$\beta$ appears to be mediated by the activation of pro-inflammatory pathway[112–115]. In a similar study, the A$\beta$-mediated vasoconstriction was reversed by free radical scavengers suggesting the direct contribution of soluble A$\beta$ to reactive oxygen species production[116]. The effects of soluble A$\beta$ have also been demonstrated in the APP Tg mice in which alterations in cerebral blood flow and glucose metabolism are observed in the absence of amyloid deposits[117]. Moreover, soluble A$\beta$ can elicit a reduction of blood flow triggered by somatosensory activation[118].

The functional role of soluble A$\beta$ 1–40 was also investigated by our group in a murine model of focal ischemia in which the middle cerebral artery (MCA) was ligated for a period 45 minutes followed by 23 hours of reperfusion of the stroke area. Five control mice were intravenously injected with vehicle alone (200 µl of 2% DMSO in PBS) and five mice were injected with soluble A$\beta$ (4 µg of freshly prepared A$\beta$ 1–40 dissolved in 200 µl of 2% DMSO in PBS). Cerebral blood flow (CBF) measurements were obtained and infarct volumes were calculated as the percent of the ipsilateral hemisphere, which was infarcted. In these experiments the infarct volume was $21.7 \pm 8.4$ and $57.5 \pm 5.1\%$ for the control and A$\beta$ treated mice, respectively ($p=0.007$). Doppler ultrasound was used to measure the relative CBF (CBF=ipsilateral/contralateral CBF $\times$ 100), which were for the control and the A$\beta$ treated mice, $99.2 \pm 1.7$ and $99.4 \pm 1.2$, respectively. Immediately after treatment with vehicle or vehicle plus A$\beta$ (pre-stroke) there was no drop in CBF ($97.4 \pm 1.2$ and $98.0 \pm 1.8$, respectively), suggesting that the A$\beta$ alone did not alter this parameter. After MCA occlusion, the CBF decreased substantially and by a similar amount in both groups ($23.1 \pm 2.1$ and $18.7 \pm 1.9$, $p$=NS) revealing an equivalent degree of ischemia. When the occluding suture is withdrawn (post-reperfusion), blood flow never returns back to baseline due to post-ischemic interference effects. Again there were no differences between control vehicle and A$\beta$-treated mice ($19.2 \pm 3.2$ and $27.6 \pm 12.2$, respectively, $p$=NS). At the final time point of 24 hours, another CBF measurement was taken, and this again showed no difference between control and A$\beta$-treated mice ($23.5 \pm 5.6$ and $33.7 \pm 13.7$, respectively, $p$=NS). In summary, infarct size was ~2.7-fold larger in the A$\beta$-treated animals relative to the control animals not receiving A$\beta$. These differences were not likely due to alterations in CBF, but probably due to alterations in the microvessels caused by the soluble oligomeric A$\beta$ peptides. In a similar study using A$\beta$PP Tg mice, the presence of elevated A$\beta$ peptides also increased the susceptibility of the brain to ischemic injury[119]. Furthermore, the presence of A$\beta$ limits the production of vascular relaxing factors, which alters CBF during hypotensive crisis or induced brain activity[120,121].



**Neurological Research ner05238.3d** 12/9/05 11:52:42
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Soluble Aβ peptides were found recently to elicit a potent anti-angiogenic activity, which may be related to a secondary structure that is rich in β-sheets. The endothelial capacity to form capillaries on Matrigel and *de novo* synthesis of blood vessels in the chorioallantoic membrane and corneal micropocket assays was inhibited by the presence of Aβ. The Aβ inhibition of angiogenesis was also successfully demonstrated *in vivo* on glioblastoma and adenocarcinoma tumors in mice[122].

Soluble oligomeric Aβ may also play a crucial role in mitochondrial toxicity in AD. Apparently, Aβ has a high affinity for the mitochondrial enzyme ABAD (amyloid-β binding alcohol dehydrogenase or hydroxyacyl-Coenzyme A dehydrogenase, type II). Interactions between Aβ and ABAD hinder nicotinamide adenine dinucleotide (NAD) binding, a cofactor indispensable for ABAD–substrate interaction. It has been recently suggested that the ABAD–Aβ complex exists in the mitochondrial matrix[123,124]. A mitochondrial matrix interaction mechanism requires a complex series of biochemical events to allow these molecules to meet in the proper cell compartment. Free soluble Aβ oligomers must be present in the cytosol, perhaps a result of exit from the endoplasmic reticulum via the endoplasmic reticulum associated degradation (ERAD) pathway[125,126]. This multifactorial translocating mechanism is activated when molecules in the ER are misfolded or aggregated, as may be the case for Aβ peptides in AD. Once in the cytosol, the Aβ oligomers must escape the ERAD associated ubiquitin/proteasome machinery degradation to interact with ABAD and proceed either to the inner surface of the plasma membrane, or to the mitochondrion interior[123,127]. It is unclear whether the ABAD–Aβ complex is localized to the mitochondrion interior using the TOM-TIM and associated Hsc70 importing mechanism[128]. Free Aβ peptide oligomers are able to interact with a variety of molecules[129] and may employ another transport system or have the capacity to directly cross the double mitochondrial membranes to interact with ABAD. Confocal microscopy has revealed abundant ABAD–Aβ complexes on neuronal mitochondria surfaces[123]. ABAD plays a critical role in normal neuronal function and patients carrying amino acid substitutions in this molecule showed reduced enzyme activity, manifest psychomotor retardation and a loss of mental and motor skills[130]. Perhaps, the cytosolic interaction of soluble oligomeric Aβ with ABAD does not stimulate translocation, but instead prevents this important enzyme from reaching the mitochondrial matrix influencing neuronal homeostasis.

## ARE SOLUBLE Aβ OLIGOMERS BOTH A FRIEND AND A FOE?

Several studies have suggested that soluble Aβ at low concentrations has a vital biological function. In cell culture, the inhibition of Aβ production by γ- and β-secretase inhibitors or neutralization of Aβ using specific antibodies resulted in a marked reduction in neuron viability, but had no effects on astrocyte and other non-neuronal cell lines[131]. Co-incubation of Aβ1–40 successfully prevented neuronal toxicity elicited by secretase inhibitors or Aβ antibodies. Small amounts of soluble Aβ oligomers have also been shown to promote neurogenesis, which could be important in neuron regeneration[132]. These results suggest that Aβ may have both beneficial and deleterious effects, probably depending on the quantity and quality of the Aβ peptides. Thus, it is possible that Aβ peptides are constitutively synthesized at low levels to sustain neuronal survival and neurogenesis. In Tg mice, newly formed Aβ can be either degraded or transported out of brain parenchyma through the cerebral circulation with a half-life of ~2 hours[133]. In humans, such an equilibrium can be altered by decreasing the expression levels or activity of the Aβ-degrading enzymes or by functional alterations in the cerebral vasculature that would favor the retention and accumulation of Aβ peptides in the brain parenchyma and vascular walls. Insults, such as head trauma, stroke and infections may promote or accelerate this dis-equilibrium. Accumulated soluble Aβ peptides in the brain interstitial fluid gradually aggregate and are converted into rich β-sheet content oligomers. In the AD scenario, the increased Aβ oligomers elicit neurotoxicity, chronic neuroinflammation, vasoconstriction, destruction of the blood vessels and inhibition of angiogenesis. Eventually, toxic Aβ oligomers evolve into fibers which, as we and others suggest, may have a lesser neurotoxic effect[1,69,134,135].

## CONCLUSION

There is no doubt that Aβ accumulation contributes to AD pathology. Apart from the hemodynamic impact of the vascular amyloid deposits, the AP, by their sheer mass and number, also contribute to AD pathology by displacing healthy neuropil and distorting cerebral cortex architecture and function. The fibrillar Aβ, especially the n-42 type, contributes to AD risk. It has been clearly demonstrated that Aβ vaccination improves cognition in Tg mice models of Aβ amyloidosis by reducing the total plaque area in the hippocampus and cerebral cortex[136–140]. In addition, improvements in cognition may also be due to opsonization and Fc receptor-mediated endocytosis of obnoxious soluble oligomeric Aβ.

While extracellular amyloid deposits are an AD hallmark, it is possible that this feature is but a single aspect of the larger pathologic cascade and an indirect consequence of protective responses intended to sequester toxic soluble Aβ molecules. The fact that soluble Aβ levels are better correlated with dementia severity than amyloid plaque burden and the demonstrations that oligomeric molecules are highly toxic species reveal the critical importance of this dynamic Aβ pool.

The tendency of soluble Aβ to aggregate and interact with a wide variety of cell macromolecules, enzymes and organelles has important implications for understanding the full scope of Aβ pathophysiology. Studies



Neurological Research ner05238.3d 12/9/05 11:52:43
The Charlesworth Group, Wakefield +44(0)1924 369598 - Rev 7.51n/W (Jan 20 2003)

of purified Aβ *in vitro* confirm that these molecules assemble spontaneously into oligomeric and perhaps higher order structures over time, a process that clearly occurs *in vivo* as well. Elevated soluble Aβ levels may promote formation of structures that act to physically disrupt cell membranes. Aβ binding to key enzymes such as ABAD may both attenuate activity as well as prevent normal compartmentalization.

The list of processes influenced by soluble Aβ is long and this diversity reflects an extraordinary range of intermolecular interactions. Recent clinical trials in which humans were immunized with Aβ were halted as the result of several cases of encephalitis, probably due to a T-cell response. Neuropathological examination of these brains demonstrated large cortical areas in which there was an apparent clearance of AP, but interestingly the vascular amyloid was seemingly intact suggesting fundamental physicochemical differences between cortical and vascular amyloid[141,142]. In a follow-up study in which the levels of circulating antibodies were measured, those AD patients with higher titers apparently demonstrated some clinical improvement[143]. These observations suggest that amyloid deposits are pathologically as well as physically diverse. New clinical trials are underway utilizing passive vaccination and a variety of shorter Aβ sequences as antigens that may be safer and more efficient in removing the soluble extracellular oligomeric Aβ peptides thus preventing some AD amyloidosis. The evident heterogeneity of amyloid deposits and the potential of vascular amyloid to profoundly affect brain hemodynamics and brain perfusion suggest that amyloid vaccination, if administered at an early stage, will potentially prevent some of the AD pathophysiological deficits.

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