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Review

Molecular mechanisms initiating amyloid β-fibril formation in Alzheimer's disease

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The deposition of aggregated amyloid β-protein (Aβ) in the human brain is a major lesion in Alzheimer's disease (AD). The process of Aβ fibril formation is associated with a cascade of neuropathogenic events that induces brain neurodegeneration leading to the cognitive and behavioral decline characteristic of AD. Although a detailed knowledge of Aβ assembly is crucial for the development of new therapeutic approaches, our understanding of the molecular mechanisms underlying the initiation of Aβ fibril formation remains very incomplete. The genetic defects responsible for familial AD influence fibrillogenesis. In a majority of familial cases determined by amyloid precursor protein (APP) and presenilin (PS) mutations, a significant overproduction of Aβ and an increase in the Aβ42/Aβ40 ratio are observed. Recently, it was shown that the two main alloforms of Aβ have distinct biological activity and behaviour at the earliest stage of assembly. *In vitro* studies demonstrated that Aβ42 monomers, but not Aβ40, form initial and minimal structures (pentamer/hexamer units called paranuclei) that can oligomerize to larger forms. It is now apparent that Aβ oligomers and protofibrils are more neurotoxic than mature Aβ fibrils or amyloid plaques. The neurotoxicity of the prefibrillar aggregates appears to result from their ability to impair fundamental cellular processes by interacting with the cellular membrane, causing oxidative stress and increasing free Ca^{2+} that eventually lead to apoptotic cell death.

Keywords: Alzheimer's disease, amyloid-β, APP mutations, fibrillogenesis, neurotoxicity, protein aggregation

AMYLOID CASCADE HYPOTHESIS OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss and personality changes (Alzheimer, 1906). The pathological hallmarks of AD are senile plaques and neurofibrillary tangles (NFT) in the brain, accompanied by neuronal and synaptic loss. NFT are composed of hyperphosphorylated microtubule associated protein tau which assembles in paired helical filaments (PHF) and accumulates in the cytoplasmic compartments in neurons. The senile plaques are extracellular deposits in the brain parenchyma, mainly consisting of a 40–43 amino-acid peptide named amyloid β (Aβ). In 1964, Aβ was identified in a fibrillar form within the senile plaques by electron microscopy (Terry *et al.*, 1964). Aβ fibril formation is associated with a cascade of neuropathogenic events that induces brain neurodegeneration leading to the cognitive and behavioral decline characteristic of AD. Therefore, a detailed knowledge of Aβ assembly is crucial for the development of new therapeutic approaches designed to prevent fibril formation and/or to dissociate existing fibrils. Yet, our understanding of the molecular mechanisms underlying the initiation of Aβ fibrillogenesis remains very incomplete.

Amyloid β-peptide is produced by specific endoproteolytic cleavages of the amyloid precursor protein (APP) which is a type I cell surface glycoprotein of up to 770 amino acids involved in nuclear signaling. The protein may also have a growth-stimulating function and is important in wound repair. The *APP* gene, localized on chromosome 21q21.2, is a house-keeping gene since it is expressed abundantly in a variety of tissues. After synthesis on ribosomes, a small fraction of APP molecules reaches the plasma membrane where they undergo specific endoproteolysis by three proteases: α-, β-, and γ-se-

Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; ADDLs, Aβ-derived diffusible ligands; APP, amyloid precursor protein; ARC, Arctic variant of Aβ; BACE, β-site APP-cleaving enzyme; NFT, neurofibrillary tangles; PHF, paired helical filaments; PS presenilin; TACE, tumor necrosis factor-alfa converting enzyme.

cretases. The present candidates for α-secretase activity are three members of the ADAM (a disintegrin and metalloprotease) family: ADAM-9, ADAM-10, and TACE (tumor necrosis factor-α converting enzyme)/ADAM-17 (Buxbaum *et al*., 1998). The protein responsible for β-secretase activity was identified as an aspartyl protease and named BACE (β-site APP-cleaving enzyme) (Hussain *et al*., 1999). A large complex of different proteins, including neprilysin, insulin-degrading enzyme, and presenilins as the part of catalytic centre, is suggested to be responsible for the γ-secretase cleaving activity (Esler & Wolfe, 2002). Due to the action of the secretases, 40- to 43-residue-long Aβ peptides are produced. α-Secretase cleaves APP inside the Aβ sequence generating non-amyloidogenic peptide fragments: a soluble N-terminal part of APP (αAPPs) and a C-terminal fragment C83 anchored in the membrane (Fig. 1). Further γ-secretase cleavage of the C-terminal fragment releases a 3-kDa peptide, p3. β-Secretase cleaves APP at the N-terminal of the Aβ sequence leading to the formation of an N-terminal part of APP (βAPPs) and the C-terminal fragment C99. Subsequent cleavage of the C99 protein intermediate at the C-terminal side of the Aβ sequence by γ-secretase generates the amyloidogenic form of the peptide. The γ-secretase processing is a heterogenous event forming Aβ with different C termini. Aβ40 and Aβ42 are the most common forms. γ-Secretase usually cuts at Val at position 40 or/and at Ala at position 42. By contrast to Aβ40, Aβ42 has a greater tendency to form fibrillary Aβ. The deposition of "seeding" Aβ42 accelerates Aβ40 accumulation and stimulates a cascade of processes leading to the formation of plaques and neurofibrillary tangles followed by neuron death (Jerret & Lansbury, 1993). According to the "amyloid cascade hypothesis" (Hardy & Higgins, 1992; Hardy & Selkoe, 2002) abnormalities in APP metabolism with subsequent accumulation of Aβ peptides in the brain are a primary event in the pathogenesis of AD. Other pathological features (neurofibrillary tangles, neuronal damage and cell death) are regarded as secondary.

AMYLOID β-PROTEIN OLIGOMERIZATION AND FIBRIL FORMATION

The fibrillization of Aβ into senile plaques is a complex process involving several discrete steps (Teplow, 1998; Tseng *et al*., 1999; Walsh *et al*., 2000). After the release of Aβ from cells, it can bind to several proteins, for example α1-antichymotrypsin, albumin, apolipoprotein E, and complement proteins (Abraham *et al*., 1988; Ma *et al*., 1994). Aβ is also present as stable soluble dimers which are detected in both brain homogenates and cell culture media (Enya *et al*., 1999). The total Aβ concentration may be the critical determinant of fibril formation. In young and healthy brains, Aβ is fully catabolised immediately after its secretion from the cell before it can be deposited. In the aging brain, enhanced production of Aβ and its reduced clearance may lead to Aβ deposition. Recent *in vitro* studies have identified three types of Aβ oligomers: 1) very short oligomers ranging from dimer to hexamer size (Le Vine, 1995; Bitan *et al*., 2003a); 2) Aβ-derived diffusible ligands (ADDLs), small oligomers ranging from 17 to 42 kDa (Lambert *et al*., 1998); and 3) protofibrills, that can be seen in electron microscopy, short fibril intermediates of < 8 nm in diameter and < 150 nm in length (Harper *et al*., 1997; Walsh *et al*., 1997; Yong *et al*., 2002). Protofibrils are transient structures observed during *in vitro* formation of mature amyloid fibrils (Harper *et al*., 1997; Walsh *et al.* 1997; 1999; Yong *et al*., 2002). Detailed relationships between these different oligomers are not clear. All oligomeric Aβ assembly intermediates: oligomers, ADDLs, protofibrils and also mature Aβ fibrils are neurotoxic and may be the key effectors of neurotoxicity in AD. It has been suggested that oligomers inhibit neuronal viability up to ten times more intensely compared to fibrils, further emphasizing the importance of the regulation of oligomer/protofibril formation in Alzheimer's disease (Dahlgren *et al*.,

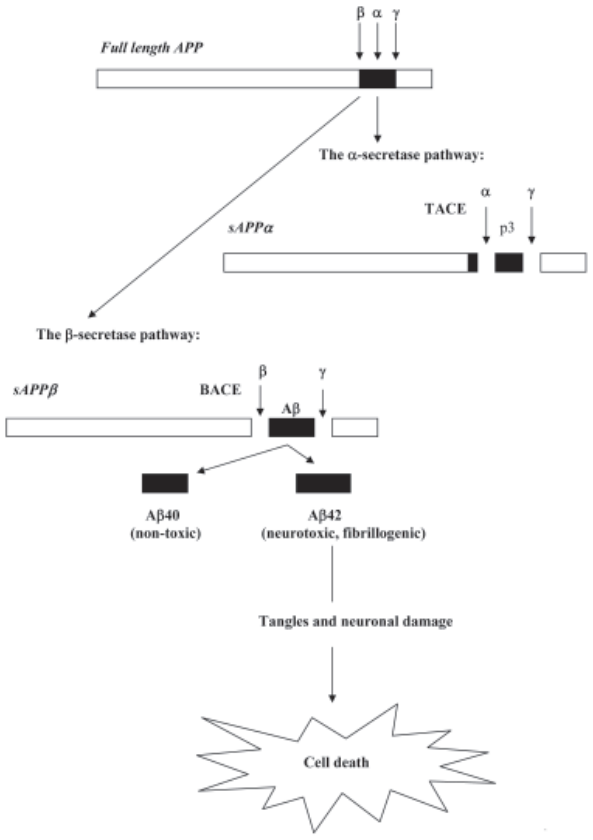

Figure 1. Endoproteolytic cleavages of the amyloid precursor protein (APP) leading to the formation of non-amyloidogenic (the α-secretase pathway) or amyloidogenic (the β-secretase pathway) products which are essential for the pathogenesis of Alzheimer's disease.

2002). Agents preventing the assembly of toxic Aβ oligomers could be potential therapeutic means to treat AD (Klein, 2001).

Recently it was shown that two predominant *in vivo* Aβ alloforms, Aβ40 and Aβ42, have distinct oligomerization pathways (Kirkitadze *et al.,* 2002; Bitan *et al*., 2003a). The peptides showed different behaviour at the earliest stage of assembly, monomer oligomerization. Studies of the kinetics of Aβ fibril formation have shown that Aβ42 forms fibrils much faster than Aβ40 (Walsh *et al*., 1997; Kirkitadze *et al*., 2001). Aβ42 is more fibrillogenic and more neurotoxic than Aβ40. It was demonstrated (Bitan *et al*., 2003a) that the initial phase of oligomerization of Aβ42 monomers involves formation of pentamer/hexamer units, so-called paranuclei (Fig. 2). Paranuclei are initial and minimal structures that can oligomerize to larger forms: large oligomers, protofibrils, fibrils. Monomers, paranuclei and large oligomers are predominately unstructured with only short β-sheet/β-turn and helical elements. During protofibril formation essential conformational changes occur when the unstructured, α-helix, and β-strand elements transform into β-sheet/β-turn structures. Paranuclei could not be observed for Aβ40 at similar concentrations of the peptide. Carefully prepared aggregate-free Aβ40 existed as monomers, dimers, trimers, and tetramers, in rapid equilibrium (Bitan *et al*., 2003a). The critical residue promoting the formation of pentamer/hexamer units is Ile-41. Addition of Ile-41 to Aβ40 is sufficient to induce formation of paranuclei (Bitan *et al*., 2003a). A natural tendency to form paranuclei seems to be only a feature of Aβ42. This finding may explain the particularly strong association of Aβ42 with AD. Oxidation of Met35 in Aβ42 blocked paranucleus formation and produced oligomers indistinguishable in size and morphology from those produced by Aβ40 (Bitan *et al*., 2003b). Systematic structural alterations of the $C^{35}\gamma$-substituent group revealed that its electronic nature, rather than its size (van der Waals volume), was the factor controlling oligomerization pathway choice. Preventing the assembly of toxic Aβ42 paranuclei through selective oxidation of Met35 thus represents a potential therapeutic approach for AD. A comparison of 34 physiologically relevant Aβ alloforms revealed that the most important feature controlling early oligomerization was the length of the C terminus (Bitan *et al*., 2003c). The side-chain of residue 41 in Aβ42 was important both for effective formation of paranuclei and for self-association of paranuclei into larger oligomers. Also the side-chain of residue 42 and the C-terminal carboxyl group affected paranucleus self-association. Aβ40 oligomerization was particularly sensitive to substitutions of Glu22 or Asp23 and to truncation of the N terminus (Bitan *et al*., 2003a). Aβ42 oligomerization, in contrast, was largely unaffected by substitutions at positions 22 or 23 or by N-terminal truncations, but was affected significantly by substitutions of Phe19 or Ala21. These results revealed that the specific regions and residues that control Aβ oligomerization differ between Aβ40 and Aβ42.

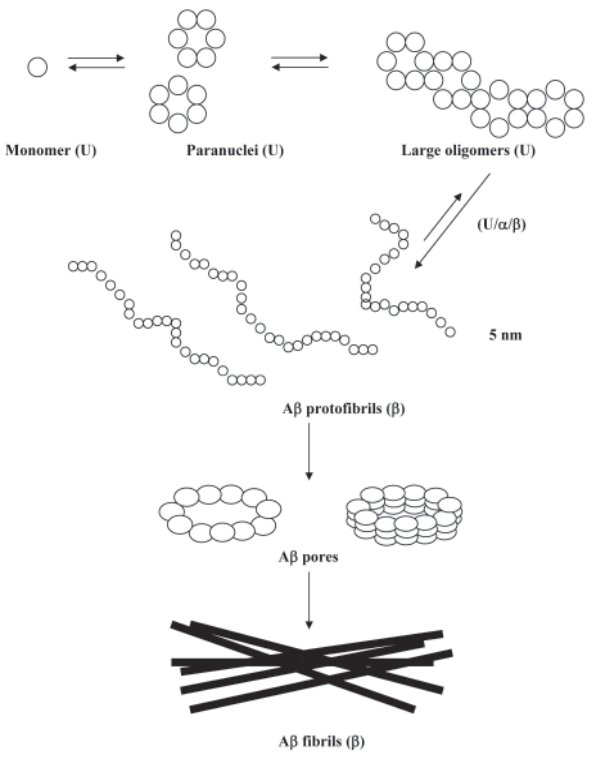

Figure 2. A model of Aβ42 oligomerization and fibril formation.

The equilibrium between monomer and paranuclei is rapid and reversible. The conversion to protofibrils is slower but also reversible. The further oligomerization of protofibrils into fibrils appears irreversible. Monomers, paranuclei and large oligomers are predominately unstructured (U) with only some β-sheet/β-turn (β) and helical (α) elements. During protofibril formation essential conformational changes occur when the unstructured, α-helix, and β-strand elements transform into β-sheet/β-turn structures (U/α/β). The diameteres of the protofibrils and fibrils are indicated in nm.

EFFECTS OF FAMILIAL ALZHEIMER'S DISEASE (*FAD*) GENE MUTATIONS ON Aβ OLIGOMERIZATION

The majority of AD cases have a multifactoral etiology. Only a few percent of familial cases show a clear autosomal dominant pattern of inheritance. To date, three genes responsible for familial autosomal dominant AD have been identified in the human genome: *amyloid precursor protein* gene (*APP;* chromosome 21q21.1), *presenilin 1* gene (*PS1*; chromosome 14q24.3), and *presenilin 2* gene (*PS2*; chromosome 1q42.1). So far, 20 pathogenic mutations in the *APP*

gene, 124 mutations in the *PS1* gene and 8 mutations in the *PS2* gene have been described worldwide in families with AD (Kowalska, 2004). The mutations have a direct effect on Aβ fibril formation. Both APP and PS mutations affect the activity of α-, β-, and γ-secretases during APP processing leading to overproduction of amyloidogenic Aβ42 (Borchelt *et al*., 1996). A significant increase in the Aβ42/Aβ40 (concentration) ratio is observed in the majority of familial cases determined by the mutations. Presenilin mutations probably disturb the whole protein network of the large complex which is responsible for the γ-secretase processing of the APP protein through subtle conformational alterations. They may also downregulate unfolded protein response (UPR), an intracellular signaling pathway that controls transcription of genes for chaperones, which prevent improper folding of proteins (Steiner & Haass, 2000). Mutations within codons 692–694 of the APP protein (Fig. 3) cause amino acid substitutions that alter the biophysical properties of Aβ (Kowalska, 2003). The Aβ peptides with the Flemish mutation (A692G) assemble into protofibrils and fibrils at a slower rate compared to the wild type peptide (Walsh *et al*., 2001). In contrast, the Dutch mutant Aβ (E693Q) forms protofibrils and fibrils markedly faster than the wild type (Kirkitadze *et al*., 2001). While patients with the Dutch APP mutation have predominantly hemorrhagic strokes, Flemish APP patients develop both strokes and AD. Recent studies on Dutch mutation have shown (Bornebroek, *et al*., 2003) that the concentration of Aβ42 is significantly decreased whereas the concentration of Aβ40 remains the same as of the wild type peptide. This finding contrasted with the elevated concentrations found in Alzheimer's disease. Therefore it is suggested that the Dutch AβPP693 mutation located within the Aβ encoding region of the APP gene has a different effect not only on the clinical and pathological expression but also on Aβ processing than other mutations. To determine whether these diverse clinical and pathological presentations are due to mutant Aβ or APP, Kumar-Singh and colleagues studied the effect of Flemish, Dutch, and wild-type Aβ/APP on phosphorylation of specific microtubule-associated tau protein epitopes observed in AD (Kumar-Singh *et al*., 2002). No effect was observed in differentiated SH-SY5Y cells either stably expressing APP or treated with synthetic Aβ (12–42). However, a paradoxical temporal difference was observed in the neurotoxic potential of mutant and wild-type Aβ. While long 24-h incubation at a physiological level of Aβ (2 μM) showed a higher incidence of apoptosis for Dutch Aβ, a short 2-h incubation showed elevated apoptosis for Flemish and wild-type Aβ. The altered aggregation properties of Aβ, with Dutch Aβ aggregating faster and Flemish Aβ aggregating slower than the wild type, elucidated a discrete two-phase Aβ neurotoxicity. The results imply that, at least *in vitro*, Aβ might be neurotoxic in an initial phase due to its soluble oligomeric or other early toxic Aβ intermediate(s), which is perhaps distinct from the late neurotoxicity incurred by aggregated larger assemblies of Aβ. The Arctic mutation (E693G) also forms protofibrils at a higher rate and in greater quantities than the wild-type peptide (Nilsberth *et al*., 2001). In contrast to the effects of other APP mutations, carriers of Arctic mutation show decreased plasma levels of Aβ40 and Aβ42 (Kirkitadze *et al*., 2001). Chromatographically isolated populations of different sized profibrils (Kheterpal *et al*., 2003) of Aβ40, both the wild type and the Arctic variant, showed about 40% protection of the backbone amide hydrogens. These Aβ protofibrils were exposed to hydrogen-deuterium exchange analysis and monitored by mass spectrometry (HX-MS). The results revealed that Aβ protofibrils appeared to be highly resistant to exchange with deuterium even after 2 days of incubation in aqueous deuterated buffer, implying a very stable, presumably H-bonded, core structure. This is in contrast to mature amyloid fibrils, whose equally stable structure protects about 60% of the backbone amide

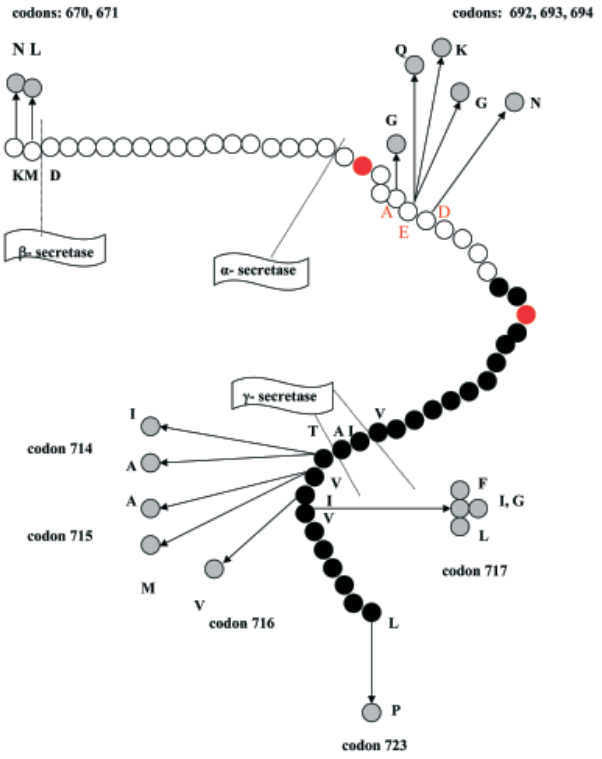

Figure 3. A fragment of APP containing Aβ sequence (from the β-secretase cleavage site before residue D1 of Aβ to the γ-secretase cleavage site after residue Val40 or Ala42 of Aβ).

The major pathogenic mutations responsible for familial Alzheimer's disease are indicated by arrows (one is a double mutation: K/M670/671N/L). The sequence located in the membrane is indicated by filled circles.

hydrogens over the same time frame (Kheterpal *et al*., 2003). The authors also found that wild type Aβ was preferentially excluded from both protofibrils and fibrils grown from an equimolar mixture of wild type and Arctic mutant peptides. The analysis of the structural properties of protofibrils formed by the Arctic variant of Aβ, Aβ40(ARC), and the effect of wild type Aβ40 on the distribution of the protofibrillar species formed by Aβ40(ARC) in the conditions that mimic the situation in heterozygous patients (Lashuel & Hartley, 2003) revealed that the Arctic mutation accelerates both Aβ oligomerization and fibrillogenesis *in vitro*. In addition, Aβ40(ARC) was observed to affect both the morphology and the size distribution of Aβ protofibrils. Electron microscopy examination of the protofibrils formed by Aβ40(ARC) revealed several morphologies, including relatively compact spherical particles roughly 4–5 nm in diameter, annular pore-like protofibrils, large spherical particles 18–25 nm in diameter, and short filaments with chain-like morphology (Lashuel & Hartley, 2003; Caughey & Lansbury, 2003). Therefore, protofibrils may act as pathogenic amyloid pores (Fig. 2). Conversion of protofibrils to fibrils occurred more rapidly for Aβ40(ARC) than for Aβ40/Aβ40(ARC). These results imply that co-incubation of Aβ40(ARC) with wild type Aβ40 leads to kinetic stabilization of Aβ40(ARC) protofibrils. An increase in the ratio of Aβ40/Aβ40(ARC) may result in the accumulation of potential neurotoxic protofibrils and acceleration of disease progression in familial Alzheimer's disease mutation carriers. As regards the Iowa APP mutation (D694N), it increases the fibril formation rate of Aβ, but the kinetics and stability of protofibrils remain unknown (Van Nostrand *et al*., 2001).

Overall, both *in vitro* and *in vivo* studies have indicated that soluble, oligomeric forms of Aβ have potent neurotoxic activities, and in fact, may be the proximate effectors of the neuronal injury and death occuring in Alzheimer's disease. It is now apparent that Aβ oligomers and protofibrils are more neurotoxic than mature Aβ fibrils or amyloid plaques (Haass & Steiner, 2001). The accumulation of Aβ peptides mediates neuronal cytotoxicity which induces a cascade of neuropathogenic events leading to brain neurodegeneration (Glenner & Wong, 1984; Eikelenboom *et al*., 1998; Hardy & Selkoe, 2002; Mousseau *et al*., 2003). Several mechanisms have been proposed to be involved in this process including direct cytotoxicity, production of reactive oxygen species, increased intracellular response to excitatory amino acids, and disruption of calcium homeostasis (Smith *et al*., 1996; Hardy & Selkoe, 2002). The strong cytotoxicity of the pre-fibrillar amyloid aggregates may be a direct consequence of their interactions with cell membranes causing membrane damage *via* the formation of non-specific ion channels. These channels have already been described for Aβ peptides (Lin *et al*., 2001). They could disrupt cellular homeostasis impairing fundamental cellular processes by oxidative stress and by increasing free Ca^{2+} that eventually cause apoptotic cell death (Stefani & Dobson, 2003). Future progress in our understanding of the molecular mechanisms of the neurotoxicity of Aβ aggregates will be crucial for the development of new therapeutic strategies in the treatment of both Alzheimer's disease and many other neurodegenerative disorders, including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, spinocerebellar ataxias, and prion diseases, caused by abnormal protein folding (misfolding) and aggregation.

Aβ AGGREGATION AND CHOLESTEROL METABOLISM

So far, no evidence of an altered Aβ generation in sporadic late-onset AD, the major form of the disease, has been provided. Thus, it is reasonable to assume that Aβ aggregation in sporadic AD may be induced by an as yet unknown post-translational modifications of Aβ and/or by an altered mechanism of Aβ clearance. Biochemical and cell biological studies confirm that changes in cholesterol metabolism in neurons may underlie the pathological processes in AD. The APOE*4 allele of *apolipoprotein E* gene encoding a protein directly involved in the regulation of lipid metabolism, is a major risk factor in sporadic late-onset AD. Several lines of evidence suggest that apolipoprotein E modulates the distribution and metabolism of cholesterol in neuronal membranes in an allele-dependent manner. Moreover, it was suggested that Aβ begins to be aggregated in the brain *via* its binding to a glycolipid molecule, GM1 ganglioside (Yanagisawa *et al*., 1995). The GM1 ganglioside may be considered as a molecular chaperone for conversion of Aβ. Based on the unique molecular characteristics of GM1 ganglioside-bound Aβ, including its extremely high aggregation potential and altered immunoreactivity, it was hypothesized that Aβ adopts an altered conformation *via* binding to GM1 and accelerates aggregation of soluble Aβ by acting as a seed (Yanagisawa & Ihara, 1998). Recently, it has been found that the binding of Aβ to GM1 is significantly accelerated in cholesterol-rich domains. There are at least several reports indicating that Aβ initially accumulates in the fractions with the lipid composition similar to that of lipid rafts (Sawamura *et al*., 2000; Tun *et al*., 2002; Ehehalt *et al*., 2003). Therefore, it is very likely that membrane lipids, including cholesterol and gangliosides, are highly involved in the aggregation of soluble Aβ in AD brains.

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