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Review

Beta-amyloid oligomers: recent developments

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Abstract

Recent studies point to a critical role of soluble β-amyloid oligomers in the pathogenesis of one of the most common neurodegenerative diseases, Alzheimer's disease (AD). Beta-amyloid peptides are cleavage products of a ubiquitously expressed protein, the amyloid precursor protein. Early studies suggested that accumulation of extracellular β-amyloid aggregates are the most toxic species causing synaptic dysfunction and neuronal loss in particular regions of the brain (neurobiological features underlying cognitive decline of the AD patients). In recent years, a shift of pardigm occurred, and now there is accumulating evidence that soluble oligomeric forms of the peptide are the most toxic to neuronal cells. In this review, we discuss recent findings on the toxic effects of amyloid-β oligomers, their physico-chemical properties and the possible pathways of their formation *in vitro* and *in vivo*.

Keywords: Alzheimer's disease; β-amyloid; neuronal cell death.

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and is the leading cause of late-life dementia accounting for more than 50% of all dementias. Clinically, AD is manifested as progressive cognitive decline, loss of memory and changes in personality. Histopathologically, these symptoms are accompanied by the loss of hippocampal and cortical neurons (particularly those projecting from the basal forebrain to the cerebral cortex) and accumulation of neuritic plaques that contain dying neurons, inflammatory-activated astroglial cells and β-amyloid peptides (Aβ). These peptides tend to aggregate and form complexes of varying size and different morphology – from small soluble oligomers, bigger protofibrils and large insoluble fibrils. The dominant hypothesis on the pathogenesis of AD suggests that Aβ aggregates into toxic species which disrupt synaptic functions and cause neuronal loss [for reviews see (1, 2)]. Initially it was believed that formation of fibrilar Aβ deposits outside the cells is the crucial event in the development of AD. However, in recent years more and more experimental evidence suggests that soluble oligomers represent the primary cytotoxic forms of Aβ.

In this review we discuss recent advances in the understanding of the chemistry of Aβ peptides and their oligomeric assemblies and the mechanisms of Aβ oligomer-induced neurotoxicity, particularly the mechanisms of Aβ oligomer-induced neuronal death.

Biochemistry of Aβ peptides

Aβ peptides are cleavage products of amyloid precursor protein (APP) by complexes of proteases known as α-, β- and γ-secretases. APP is a single-pass transmembrane protein located in plasma membranes of various cells within a body. There are three isoforms of APP and the isoform consisting of 695 amino acid residues (APP695) is predominantly expressed in neuronal cells (3). Cleavage of APP by α-secretase produces non-amyloidogenic soluble Aβ peptides, whereas sequentional action of β- and γ-secretases produces Aβ peptides of various length – from 37 to 43 amino acid residues, recently reviewed in (4). These Aβ peptides are less soluble and more prone to oligomerization/fibrilization, particularly $Aβ_{1-42}$ which spontaneously aggregates in aqueous solutions (5–7). $Aβ_{1-40}$ is the most abundant species produced by proteolytic cleavage of APP in the brain, and $Aβ_{1-42}$ normally makes up 1%–10% of the total Aβ peptides pool (4, 8). However, in certain forms of AD, the levels of $Aβ_{1-42}$ in brains were found to be significantly increased (9). In brains of normal elderly and AD patients, various forms of N-terminally truncated variants of Aβ have been detected, though at reletively lower amounts, and the most abundant forms among them were found to be $Aβ_{11-42}$ and pyroglutamate-$Aβ_{11-42}$ (9). Based on data obtained on human mixed neuronal cultures, it has been suggested that $Aβ_{11-42}$ may be a secretory product from the cells (10).

APP has also been found in the membranes of Golgi complex (11), endoplasmic reticulum, endosomal, lysosomal (12) and mitochondrial membranes (13). Intracellular APP can be proteolytically cleaved in a similar way to that in plasma membrane, producing Aβ peptides which, in contrast to extracellular species, were found to be predominantly $Aβ_{1-42}$ (14). Another source for intracellular Aβ is thought to be an internalized extracellular Aβ. For example, it has been reported that Aβ can be taken up by neurons and microglial cells through the scavenger receptor for advanced glycation

end products (RAGE) (15, 16). Internalization was found to be rapid and resulted in the appearance of cytoplasmic Congo Red stained structures in macrophages (17), suggesting that intracellular Aβ can rapidly undergo oligomerization/fibrilization. Aβ can be also internalized associated with other receptors such as NMDAR, low-density lipoprotein receptor, nicotinic acetylcholine receptor (18).

In addition to secretases, cytosolic activated caspases can cleave APP at aspartate in the position 664. A peptide of 31 amino acid residues (C31) is formed which can be additionally cleaved by γ-secretase generating so-called Jcasp fragment (19). Both peptides, C31 and Jcasp, are thought to be involved in Aβ-induced neurotoxicity (20, 21).

The physiological functions of APP and Aβ peptides remain largely undetermined. It has been suggested that APP may be involved in synaptogenesis, transmembrane signal transduction, cell adhesion, regulation of calcium homeostasis, etc. (4, 22). Concerning Aβ peptides, it was generally considered that these species are abnormal, toxic and found only in aged and diseased brains. However, Aβ peptides were found to be present in cerebrospinal fluid and blood of healthy humans or in the media from cultured brain cells (10, 23) suggesting that Aβ peptides may have a physiological function. Moreover, recent findings revealed that $A\beta_{1\text{-}40}$ as well as $A\beta_{1\text{-}42}$, at physiologically relevant concentrations exerted a protection in certain models of neuronal death (24, 25). $A\beta_{1\text{-}42}$ monomers and oligomers at picomolar concentrations were shown to enhance hippocampal long-term potentiation, whereas high nanomolar concentrations led to the well established reduction of potentiation (26). Thorough investigations of the roles of APP and Aβ in the functioning of normal, non-diseased cells are necessary, as they may provide important clues to understanding what goes wrong during development of Alzheimer's and other neurodegenerative diseases.

Chemical composition and properties of Aβ peptides

The amino acid sequence of $A\beta_{1\text{-}42}$ monomer associated with the sporadic and familial forms of AD is Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala. The polypeptide chain of $A\beta_{1\text{-}40}$ is shorter than $A\beta_{1\text{-}42}$ by two residues Ile and Ala at the carboxy end of the chain. $A\beta_{1\text{-}40}$ is the most abundant form of amyloid, while C-terminally extended polypeptide $A\beta_{1\text{-}42}$ exhibits significantly higher propensity for fibrilization (27). Both polypeptides are composed of 16 different amino acids without any Thr, Trp, Pro and Cys residues. An additional Thr residue at the C-terminus extends the amyloid peptide chain to $A\beta_{1\text{-}43}$, which according to recent findings (28) comprises a significant portion of the proteinaceous plaques in the brain of AD patients. The polypeptide molecules exhibit a rich hydrophilic amino acid region at the N-terminus, while the mid-chain (central hydrophobic cluster) and C-terminus are heavily populated with hydrophobic aromatic and aliphatic amino acid residues. The net charge of the molecules is -3, though it obviously depends on the pH and dielectric properties of the medium in which the charged residues are located. At low pH, the charge of the polypeptide reduces to 0. It is suggested (29) that a nucleus for intra-molecular folding exists within the decapeptide region Ala21–Ala30. A number of mutations associated with the familial forms of AD are located in this region. In particular, the mutations (named after the ethnic regions where they were identified) due to intra-Aβ mutations in the APP sequence have the following amino acid substitutions: Arctic Glu22→Gly (30), Dutch Glu22→Gln (31), Italian Glu22→Lys (32), Flemish Ala21→Gly (33) and Iowa Asp23→Asn (34). Less common APP mutations causing intra-Aβ substitutions at the N-terminal include: Ala2→Val (35), 'English' His6→Arg (36), 'Tottori-Japanese' Asp7→Asn (37) and at the C-terminal: Ala42→Thr (38).

The monomer form of the full-length Aβ polypeptide is unstable in aqueous environment, and comprises a mixture of diverse ordered and disordered conformational species (39). For this reason, most of the information about possible structures of the monomers is derived from molecular simulations supplemented by liquid- and solid-state NMR data. The latter is typically obtained using either truncated or chemically stabilized forms of monomers or low molecular weight oligomers. Most of the monomer structures in the literature refer to the so-called 'monomer in fibril' structure, which reflects a structural motif of polypetide or its fragment embedded into a beta sheet forming amyloid fibrils. Such structural studies were recently meticulously reviewed (40, 41). Because of the lack of well-resolved spectral features for different comformers such techniques as NMR have limited power to identify individual members and their distribution in ensembles. Recent molecular dynamics simulations combined with simulations of two-dimensional chirality-induced IR spectra demonstrated high potential of this novel instrumental technique for differentiating various β-amyloid monomer structures in water solutions (42). Fluoroorganic compounds such as hexafluoroisopropanol (HFIP) and trifluoroacetic acid (TFA) tend to stabilize monomeric forms of Aβ by driving conformational transformation into α-helical rich structures; for example $A\beta_{1\text{-}42}$ in HFIP (43) (see PDB entry 1Z0Q) and $A\beta_{1\text{-}40}$ in TFA (44) (see PDB entry 1AML). Circular dichroism spectroscopy indicates predominantly α-helical (~80%) stucture of $A\beta_{1\text{-}40}$ in trifluoroethanol (TFE) (>20% vol/vol)/water (45), and ~70% α-helical conformation in pure HFIP (46). Comparative molecular dynamics studies confirm the ability of HFIP and TFE to stabilize monomer forms of $A\beta_{1\text{-}42}$ containing two α-helical fragments in the mid- and C-terminal regions of the polypeptide (47, 48). The tendency for adoption of a helical structure in HFIP/water mixtures was predicted for the $A\beta_{25\text{-}35}$ fragment by replica exchange molecular dynamics simulation (49). The same trend was observed for $A\beta_{12\text{-}36}$ in TFE/water mixtures (50).

There is an abundant evidence of the capability of the Aβ peptides to bind metals, in particular Cu, Zn, Fe (51) and

Al (52). The polar $A\beta_{1-16}$ region is widely considered as a metal binding domain (41, 53). Aβ peptides form multiple metal binding sites of various affinities (54). At pH 7.4 the highest affinity (~7 aM) was reported for $A\beta_{1-42}$ binding to Cu^{2+}; $A\beta_{1-40}$ binds copper with ~50 pM affinity (54). Aβ peptides bind Zn^{2+} with significantly lower affinities at or above ~100 nM (53). It was pointed out that buffer components may affect measured affinities; authors report 'buffer-independent' dissociation constants of $A\beta_{1-40}$ complexes (1:1) 35 nM and 2 μM for Cu^{2+} and Zn^{2+}, respectively (55). Stronger binding of Cu^{2+} is consistent with recent NMR data indicating more efficient binding of Cu^{2+} in comparisson to Zn^{2+} (56). Earlier experiments with $A\beta_{1-16}$ (57) demonstrated Zn^{2+} binding to the histidine imidazole ring forming intermolecular His-Zn-His bridges, which leads to oligomerization and precipitation of Aβ, while the chelation of Cu^{2+} by histidine and main-chain amide groups resulted in soluble Cu-$A\beta_{1-16}$ complexes at physiological pH. Recently, using the isothermal titration calorimetry, the $A\beta_{6-14}$ region has been identified as the minimal Zn^{2+} binding site wherein the ion is coordinated by His6, Glu11, His13 and His14 (58). Quantum/molecular mechanics simulation allowed authors to conclude that in monomer $A\beta_{1-16}$, Zn^{2+} is recognized and captured by the Glu-Val-His-His region and temporarily coordinated by water as the fourth chelator. This state exists until His6 replaces the water molecule and becomes fourth chelator of Zn^{2+} (58). Recent molecular dynamics simulations (41) suggest that Zn^{2+} can simultaneously coordinate intra- and intermolecularly bridging two peptides, thus, promoting aggregation. In the most stable modeled conformers, Zn^{2+} is coordinated intramolecularly by His6, Glu11, His13 and His14, while in the intermolecular coordination Zn^{+} is surrounded by His6, His13 and His14 with Asp7 contributed by neighboring peptide.

Earlier studies of binding of Cu^{2+} and Cu^{+} ions was reviewed by Bush (51) and Rauk (40). There is less consensus on how copper ions are coordinated by Aβ. While there is general agreement on participation of His13 and His14 residues in the coordination sphere around Cu^{2+}, the nature of the third N and O is less clear. Recent site-specific 13C and 15N labeling, in conjunction with hyperfine sublevel correlation spectroscopy, points to the carbonyl of Ala2 as an oxygen ligand. X-ray absorbtion spectroscopy data indicate two possible Cu^{2+} intramolecular coordinations: 3N1O and 2N2O. One involves residues Hys6, Tyr10, His13, His14, another involves residues Arg7, Tyr10, His13 and His14 (59). The former is supported by electron spin resonance measurements indicating the involment of all three His residues in coordination (60). Electrospray ionization mass spectroscopy confirmed that both His13 and His14 are involved in coordination of Cu^{2+} and Cu^{+} (61). Interesting data were recently obtained using electrochemical methodology. The direct heterogeneous or mediated homogeneous electron transfer to Cu^{2+} and from Cu^{+} occurs via a small fraction of the Cu-Aβ complex molecules in which the electron transfer occurs involving a remarkably small reorganization energy (0.3 eV) (62).

Less is known about Aβ monomer binding to Fe^{3+} and Al^{3+}. Some authors argue that aluminum and iron ions are a key to precipitation in senile plaques cores (52), however, the mechanism of incorporation of these metals is still unclear. These ions in the uncomplexed form are present at very low concentrations at physiological pH, however, their binding to Aβ may occur via direct interaction of the peptide and biological complexes containing these metals. Recently it has been demonstrated (63) that Aβ binds through His13 and His14 to the iron-containing heme complex with Arg5 forming a second sphere coordinating residue. The formation of such complexes results in the peroxidase activity which, in addition to abnormal iron homeostasis and decay of iron regulatory proteins, may lead to dysfunction in mitochondrial complex IV and oxidative stress. A detailed picture of the biological function of metals in AD has been recently described in several reviews (64–66).

Aggregation of Aβ peptides

Aggregation of Aβ is considered to be a key process in AD, in which neurotoxic soluble low molecular weight species are produced. Aggregation occurs spontaneously and is dependent on the conditions such as peptide concentration, pH, ionic strength, temperature, presence of metal ions and organic components in the media in which the oligomerization takes place. Recent reviews summarize current knowledge on morphology (67) and structure of toxic Aβ oligomer species (40). The morphology of Aβ oligomer species spans from linear protofibrils to spherical, disc-like and annular shaped nanoscale objects. The interrelationship between those structures and their contributions to AD pathogenesis are not fully understood. Nevertheless, there is mounting evidence that different morphologies may determine different functions and toxicity of soluble amyloid species (68). The molecular weight of the soluble oligomers implicated in neurotoxicity ranges from 8 to 9 kDa (dimers) to >100 kDa soluble entities. Under interaction with phospholipid membranes they may further reorganize into multimeric channel-like structures (69). Structural classification of toxic Aβ oligomers was recently reviewed in (39).

Mounting experimental evidence suggests that the low molecular weight species are the main malignant actors in AD (46, 70–73). For this reason, dimers, being the smallest entities that may be involved in pathogenesis, attracted much attention during the last few years. They may be isolated by purification of naturally or synthetically made Aβ mixtures (see below), or they may be prepared by chemical cross-linking of Aβ monomers. Synthetic, chemically linked dimer was produced by the substitution Ser26→Cys, and then by oxidation producing cross-linked dimer (73). Such dimers exhibited no secondary structure, however, aggregated more rapidly than either Aβ S26C or wild-type monomers and formed para-stable beta-sheet rich, thioflavin T-positive, protofibril-like assemblies (73). Similar substitution Ala2→Cys in $A\beta_{1-40}$ allowed production of disulfide linked dimers, which were more inclined to form β-sheet rich entities than wild type $A\beta_{1-40}$ monomers. Even though their morphology

exhibits a curved linear shape dimer, the aggregates formed differ in morphology from Aβ fibers.

Mutations in Aβ affect the rate of oligomer formation. In the case of the Arctic mutation (30), which results in only one amino acid substitution Glu22→Gly22, monomers exhibit significantly higher peak concentrations and production rates of small size $A\beta_{1\text{-}40}$ oligomers, and the fibrilization rate remains unaffected in mutated $A\beta_{1\text{-}40}$. In contrast, another group reported significant acceleration of both oligomer and fibril formation processes (74). Interestingly, an equimolar mixture of $A\beta_{1\text{-}40}$ (wt) and $A\beta_{1\text{-}40}$ (Arc) (a mimic of the situation in heterozygous carriers) resulted in the accumulation of oligomers stable for tens of hours at 25°C. This experimental fact points to an interesting parallel between the structure and function of the polypeptide. Both the experimental NMR (29) and computational data (75) point to the direct involvement of Glu22 (and Asp23) in the initial steps of the intramolecular nucleation through the stabilization of a turn conformation formed by Val24-Lys28 residues. The stabilization occurs via long-range Coulombic interaction between Glu22 and Asp23 with Lys28. This is in accord with the molecular dynamics simulation data (75) indicating that the region is directly involved in the initial phase of Aβ oligomer formation.

Aggregation of Aβ is drastically affected by the trace amounts of Cu^{2+} and Zn^{2+} which interact with the amino acid residues located in the polar region of the polypeptide chain. The atomic force microscopy study indicates that instead of fibrils, small globular and ill-structured microaggregates are obtained in the presence of Cu^{2+} and Zn^{2+}, respectively (76).

Typically, the aggregation process proceeds toward the generation of the mixtures of different soluble species. This makes the precise physical characterization of the Aβ oligomer preparations extremely difficult. However, surfactants such as sodium dodecyl sulfate (SDS) may effectively stabilize certain oligomer forms thus producing 'clean' populations of species suitable for structural characterization by advanced instrumental techniques (77). Further investigations of the structure and function relationships will possibly be dependent on the availability of the well-characterized preparations of Aβ oligomers.

Aβ oligomers used in cytotoxicity and neuronal dysfunction studies

As the nature of the molecular species causing dementia is one of the central unresolved problems in AD etiology, we review here in detail methods and physical properties of Aβ used for cytotoxicity, neural dysfunction and cognitive studies. Both natural and synthetic Aβ peptides are used in these studies. Natural Aβ oligomers are obtained by two preparative techniques: from cultured mammalian cells (70, 78), or extraction from post-mortem brain tissues (79, 80). The amyloid material isolated from microsomes of the Chinese hamster ovary cell line referred to as 7PA2 contained SDS-stable Aβ dimers and trimers, as detected by the electrophoresis. They were resistant to 8 M urea and 50% formic acid treatment, while the immune-electron microscopy and ultracentrifugation revealed no protofibrils and fibril Aβ assemblies in the preparations. Together these observations suggest that most of the Aβ material, the concentration of which in conditioned media was 5–10 nM, was distributed between monomer, dimer and trimer forms of Aβ (70, 81). While in most studies using natural preparations, no attempts were made to isolate individual Aβ members, in some cases (80), cell-derived Aβ species were fractionated by the size exclusion chromatography (SEC) to obtain monomer-, dimer- and trimer-enriched fractions. In other recent work, neuroblastoma (Neuro-N2a) cell-derived preparations from conditioned media indicated significantly higher (~485 nM) concentration of total Aβ, and matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) mass spectroscopy detected various N-terminal degraded and oxidized Aβ segments, in addition to the full length $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides (78).

Brain-derived soluble Aβ preparations can be obtained from human post-mortem or transgenic mice sources. Protocols include homogenization, lysis and ultra-centrifugation. The preparations purified by immunoaffinity chromatography (80) contained monomers, trimers and a 12-mer dubbed Aβ*56 species (according to SEC and electrophoresis data). The aqueous soluble Aβ species obtained by sequential ultracentrifugation of human brain homogenates revealed elevated amounts of Aβ monomers, dimers and trimers (79) in samples from AD patients. In addition, the insoluble amyloid cores isolated from AD cortex may release Aβ monomers and dimers by formic acid treatment (79). Untreated human CSF containing Aβ (82) was recently used in cell function tests. Hitherto, no precise structural or morphological characterization was attempted in studies with naturally derived Aβ species. One of possible reasons impeding direct application of the advanced instrumental methods to characterize naturally produced Aβ oligomers is a very low concentration of the Aβ species in such preparations. The Aβ monomer concentration of the total Aβ rarely exceeds 5–20 nM. Concentrating protocols so far have had limited applicability because elevating concentrations into the micromolar range may result in noticeable changes in Aβ aggregation resulting in decreased total protein in solution.

Most of the *in vitro* and *in vivo* studies were carried out using artificial oligomer species made of synthetic or recombinant Aβ peptide material. Due to the propensity of Aβ peptide to populate polymorphous structural states, it is likely that most if not all of the *in vitro* and *in vivo* preparations used in the toxicity tests bear certain 'memory' about the procedures used for their preparation (83).

The artificial methods of Aβ oligomer preparation may be divided into two groups: (1) methods that do not employ inhibitors of oligomerization, and (2) methods that require special inhibitors such as TFA, HFIP, DMSO and others. Agregate free preparations of low molecular weight Aβ species may be prepared by centrifugation and subsequent size exclusion chromatography or ultrafiltration through a 10 kDa molecular weight cutoff centrifugal filter device (84).

Fluoroorganic compounds are frequently used for both initial disintegration and subsequent oligomerization of Aβ. For example, in the TFA method (85), a TFA Aβ polypeptide salt is used as a starting material, while the oligomerization is carried out at neutral pH in PBS. Fibrils may be prepared by incubation of Aβ in PBS for 3 days at 37°C, while the low molecular weight Aβ oligomers are obtained by incubation at room temperature for 2–3 days followed by ultracentrifugation at 16 000 *g* (10 min) and SEC separation. Obviously, traces of TFA may be present in the final preparation. In another method, HFIP is used for both initial disintegration and control of oligomerization (86). The effect of different small molecular inhibitors of aggregation has been reviewed in (87).

The question of whether or not application of fluoroorganic compounds yields biologically relevant Aβ oligomeric particles remains unclear. Different morphology and stability properties were reported for $A\beta_{1\text{-}40}$ obtained with and without HFIP (88). It was demonstrated (83) that HFIP-derived Aβ oligomer preparations contain residual HFIP, which may be responsible for membrane damage, and, consequently, for cytotoxicity observed in studies *in vitro*.

Even though Zn, Cu, Fe and Al strongly interact with the Aβ polypeptides, most of the protocols for artificial Aβ oligomer preparations do not use these metals. There are just a few works in which introduction of metals into the oligomerization media was attempted. The oligomerization of $A\beta_{1\text{-}42}$ in the presence of Cu^{2+} (complexed with glycine) yields histidine-bridged dimers as detected by EPR spectroscopy at Cu^{2+}/peptide ratios >0.6 (89). At the same time, further fibrilization that proceeds quickly in metal-free media, is inhibited by Cu^{2+}-glycine complex. The form in which the metal is introduced into the media is obviously important. As recently reported (90), introduction into the medium of Cu^{2+} or Fe^{2+} in the form of simple inorganic salts did not result in changes of morphology of $A\beta_{1\text{-}40}$ preparations.

Even though the artificial preparations reproduce many biological functions of naturally occurring Aβ species, the question whether such oligomers are adequate models of the species responsible for triggering the pathological sequence of events in AD remains open.

Neurotoxicity of Aβ oligomers

Aβ oligomer neurotoxicity may be manifested through different mechanisms involving disruption of synaptic activity, direct induction of neuronal death, induction of inflammatory responses in brain leading to secondary neuronal death or primary phagocytosis of neurons [see (91)].

Parameters determining Aβ toxicity

It has been established that toxicity of Aβ oligomers depends on the length of the monomeric peptide so that the most toxic are $A\beta_{1\text{-}42}$ and the least toxic $A\beta_{1\text{-}40}$. Using cultured Neuro-2A cells, Dahlgren et al. (92) have shown that oligomers of $A\beta_{1\text{-}42}$ decreased cell viability about 10-fold more than fibrillar $A\beta_{1\text{-}42}$: 50% decrease in cell viability was detected for oligomeric $A\beta_{1\text{-}42}$ at about 0.4 μM vs. 4.0 μM for fibrillar $A\beta_{1\text{-}42}$. In contrast, $A\beta_{1\text{-}40}$ oligomers and fibrils were non-toxic to neuronal cells up to 10–20 μM concentrations (92). This may be mainly due to the aggregation capacity of the peptides as mentioned above. So, one may conclude that toxicity may be a parameter of aggregation that differs between $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$.

The second obvious parameter is the concentration of Aβ oligomers. In many studies on Aβ toxicity in cell cultures, the oligomers were reported to cause significant cell death at 5–20 μM concentrations (93–96). Compared to physiological levels of Aβ, such concentrations seem to be excessive as concentrations of Aβ *in vivo* are estimated to be in the nanomolar range. In contrast, synaptic dysfunction was found to be affected by much lower, pathophysiologically relevant 5–100 nM concentrations (79, 97). Low molecular weight oligomers (dimers-pentamers) have been reported to induce significant cell death already at 250–500 nM concentrations (77), and one report demonstrated significant $A\beta_{1\text{-}42}$ oligomer toxicity at as low as 10 nM concentrations (92).

This points to the feasible dependence of Aβ oligomer toxicity on the size of oligomeric particles. Increasing evidence suggests that the most toxic aggregates of Aβ range between dimers and trimers up to dodecamers. Studies on the electrophysiological effects of Aβ demonstrated that a trimeric form of $A\beta_{1\text{-}42}$ most powerfully inhibits long-term potentiation in rodents (97). Aβ dimers were found to disrupt learning and memory, synaptic function and long-term potentiation (70, 98), and soluble 56-kDa dodecamers (termed as Abeta*56) affected cognition and memory in transgenic mouse models (99). Dimeric and tetrameric forms of $A\beta_{1\text{-}42}$ have been shown to be particularly toxic to hyppocampal neurons due to their high binding capacity to lipid membranes (93). In accord, we have recently demonstrated a sharp correlation between the size of $A\beta_{1\text{-}42}$ oligomers and their toxicity to primary neuronal cells in culture. Small oligomeric forms of $A\beta_{1\text{-}42}$, with a particle z-height of 1–2 nm (possibly dimers-pentamers) were found to be the most toxic species that induced rapid necrotic neuronal death at low submicromolar concentrations, whereas aggregates above 4–5 nm (oligomers with n>14) did not cause significant cell death (46).

Aβ oligomers may have different conformations and shapes which may have an impact on their cytotoxicity. Small globular Aβ oligomers with 5 nm diameter have been shown to form in media from cultured cells and induce neuronal cell death (100). Other studies have reported formation of annular Aβ oligomers with 8–12 nm outer diameter and pores of about 2 nm (7). Such structures were formed preferentially from mutant Aβ peptides and were suggested to be responsible for Aβ oligomer toxicity by a mechanism similar to bacterial pore-forming toxins [see (101) and references there]. In contrast, other toxic Aβ species have different conformations: Aβ dimers and trimers most likely are relatively compact spheroidal particles, a planar hexagon form is assumed for $A\beta_{1\text{-}42}$ tetramers and stacked hexamer structures were suggested for $A\beta_{1\text{-}42}$ dodecamers [see (101)].

At present there is no clear answer as to whether size, structure or conformation determine the toxicity of oligomeric Aβ particles.

Aβ oligomers and synaptic dysfunction

There are many studies demonstrating that Aβ oligomers can induce synaptic dysfunction. For example, several studies have shown that synthetic and natural Aβ oligomers inhibit hippocampal long-term potentiation, enhance long-term depression, decrease density of dendritic spines and numbers of electrophysiologically active synapses, and that the levels of Aβ oligomers required for such effects correlate well with the observed amount of these species *in vivo* (70, 79, 82, 97). The effects seem to be related to the Aβ oligomer-induced disruption of synaptic vesicle release at the presynaptic terminals (102), disruption of trafficking of glutamate receptors, and activation of cofilin and calcineurin (71), though the precise mechanisms of the impairment remain largely unclear.

How do Aβ oligomers induce cell death?

Aβ oligomers have been reported to induce cell death in virtually all cell types, but neurons seem to be particularly sensitive. Higher sensitivity of neurons to Aβ oligomer-induced cell death was demonstrated in primary neuronal and astroglial cultures comparing toxic concentrations of Aβ oligomers (46, 103).

Numerous studies have shown that natural and synthetic Aβ oligomers can induce cell death when applied extracellularly to cultures suggesting that Aβ exerts toxic action from the outside of the cells (95, 104). It seems that physical interaction of Aβ oligomers with cell membranes is required as it has been reported that the human lymphoma U937 cell line, which does not bind $A\beta_{1\text{-}42}$, is resistant to Aβ cytotoxicity (105).

Analysis of literature reveals two main mechanisms by which Aβ oligomers initiate cell death directly acting on target cells. One mechanism involves direct interactions of Aβ oligomers with plasma membrane phospholipids causing disruption of membrane integrity (86). As a result, changes in cellular ion homeostasis may occur. In concert, it has been shown that Aβ oligomer toxicity and membrane permeabilization can be reversed using the membrane sealant copolymer poloxamer 188 (106). Another mechanism explaining Aβ neurotoxicity suggests interaction of oligomeric particles with cell surface receptors such as NMDAR (71), α7nAChR (107) or interaction with prion protein (108). In agreement, it was found that oligomeric Aβ suppresses NMDA-induced currents in neurons that lead to alteration in signaling and endocytosis of NMDA receptors (109). Aβ oligomers were also shown to modulate α7nAChR-mediated Ca^{2+} influx in brain synaptosomes (110) and neuronal cells (111) leading to activation of α7nAChR-associated kinases and rapid Tau phosphorylation (112). Activation of NMDA and α7nAChR receptors may lead to an increase in cellular Ca^{2+} which may disrupt neuronal transmission and induce excitotoxic death of neurons. Indeed, dysregulation of cellular Ca^{2+} homeostasis is increasingly considered as an important aspect of Aβ toxicity (113). Glabe's group has shown that application of oligomeric forms of Aβ rapidly (approx. in 5 s) elevated intracellular Ca^{2+}, whereas equivalent amounts of monomers and fibrils did not (114). Elevation of intracellular Ca^{2+} may lead to endoplasmic reticulum stress. Such a mechanism has been reported recently by Resende's group (94), who showed that freshly prepared oligomeric assemblies of $A\beta_{1\text{-}42}$ rapidly induce endoplasmic reticulum stress resulting in activation of caspases and apoptosis of cortical neurons in culture. Alternatively, an increase in intracellular Ca^{2+} may lead to activation of calpains or opening of mitochondrial permeability transition pore and neuronal death (115).

Aβ oligomers were reported to interact with other receptors such as nerve growth factor receptor (116), insulin receptor (117), and Frizzled receptor (118). Binding of Aβ oligomers to the receptors may trigger downstream signaling cascades involving various protein kinases (c-Jun, Wnt, glycogen synthase kinase-3β) which may lead to cell dysfunction and apoptotic cell death (119, 120). Binding of Aβ to RAGE in neurons has been shown to result in oxidative stress and NF-κB activation (16). Despite intensive investigations, we have to admit that at present the sequence of signaling events triggered by Aβ oligomers and the downstream targets remain poorly understood. It is possible that multiple pathways may be activated by Aβ oligomers bound to various receptors, which makes the matter even more complicated.

Aβ oligomer toxicity has also been suggested to be associated with the internalization of the oligomers. In this regard, it has been shown that $A\beta_{1\text{-}42}$ oligomers, but not fibrils, are readily taken up by neuronal and HeLa cells by endocytosis and are transported into lysosomes (95). $A\beta_{1\text{-}42}$ oligomers are poorly degraded by lysosomes and their accumulation in this compartment may cause leakage of lysosomal enzymes leading to cell death (95). The inhibition of the uptake of Aβ oligomers usually reduces their cytotoxicity. It was also suggested that Aβ oligomer-induced proteasomal dysfunction may lead to cell death based on recent findings that Aβ oligomers inhibited the ubiquitin-proteasome system (121, 122).

Another hypothesis relates intracellular Aβ oligomer toxicity with the mitochondrial pathway of apoptosis and is based on findings that intracellular deposits of $A\beta_{1\text{-}42}$ in AD brains correlate with apoptotic cell death mediated by p53 and Bax (123). In line with this, accumulation of Aβ in mitochondria, and its binding to cyclophilin D, a component of the mitochondrial permeability transition pore, have been reported (124). Opening of the permeability transition pore is known to be involved in trigering of the intrinsic apoptotic pathway by releasing cytochrome c and other apoptogenic proteins. It was established that Aβ oligomeric forms have a high affinity of binding to cyclophilin D: K_d values for the interaction of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ oligomers with cyclophilin D were found to be 4 nM and 227 nM, respectively, whereas the K_d for monomeric forms were 164 nM and 1.7 μM (124). It has been suggested that binding of Aβ oligomers with cyclophilin D may enhance the translocation of cyclophilin

D from the matrix to the inner mitochondrial membrane and to facilitate the opening of mitochondrial permeability transition pore leading to cell death. An other study indicated that Aβ oligomers applied extracellularly caused rapid alterations in mitochondrial functions. It was shown that $A\beta_{1\text{-}42}$ oligomers, at low nanomolar concentrations that do not cause significant neuronal death during short time incubations, caused fast decline in mitochondrial membrane potential and ATP production (96). This may lead to synaptic dysfunction or slower necrotic neuronal death due to energy depletion over longer time.

Another intramitochondrial protein that binds Aβ is amyloid β-peptide-binding alcohol dehydrogenase (ABAD) (125). Aβ-ABAD complexes were detected in the mitochondria of AD patients and transgenic mice (126). It has been shown that inhibition of the Aβ-ABAD interaction suppressed Aβ-induced mitochondrial dysfunction, ROS generation and cell death in cultured neurons and AD mouse models (126, 127). Cytotoxicity of freshly prepared Aβ was found to correlate with decreased ABAD enzymatic activity in neurons (128). Furthermore, micromolar concentrations of synthetic Aβ, likely of oligomeric form, were shown to inhibit the activity of purified ABAD (126). Nevertheless, the link between Aβ-induced ABAD inhibition and neuronal dysfunction is still unclear. It has been suggested that Aβ-induced inhibition of ABAD may result in accumulation of toxic metabolites such as 4-hydroxy-2-nonenal (4-HNE), a non-enzymatic end-product derived from lipid peroxides (129), accumulation of which has been reported in AD (130). These data indicate that ABAD-Aβ interaction may have an impact on mitochondrial and neuronal dysfunction. However, it is still unclear which aggregation forms of Aβ inhibit ABAD and how the Aβ-induced ABAD inhibition causes neuronal death.

What type of cell death is induced by extra- or intracellular Aβ oligomers is also an open question. Most of the studies on Aβ toxicity use the MTT assay to detect changes in cell viability. Among other disadvantages, this method does not allow determination of a death pathway as it measures cellular redox activity rather than cell death itself. However, there are several studies suggesting apoptosis to be induced by Aβ oligomers (94, 96, 119, 120, 131). On the other hand, there are studies, including our own, demonstrating that Aβ oligomers, even at submicromolar concentrations, cause primarily necrotic cell death, particularly in primary neuronal cell cultures (46, 132, 133). The question relating to the Aβ-oligomer-induced cell death pathway is important as there are more possible pharmacological means to control apoptosis than necrosis, and if the mode of cell death is defined, this would be helpful in defining strategy for development of new pharmacological treatments of AD.

Expert opinion and outlook

Despite a large volume of the experimental data obtained with synthetic Aβ oligomer particles, the ambiguity regarding the molecular structure of naturally formed toxic oligomers is still present. This is primarily related to the fact that most of the modern instrumental techniques cannot be applied because of very low concentration levels of naturally occurring Aβ species. One of the possible solutions would involve the selective concentration and alignment of the oligomeric species at the interphases, such that surface specific structural techniques such as neutron reflectometry and vibrational sum-frequency spectroscopy may be used to probe molecular structure and the 'molecular-fingerprint' of Aβ oligomers. First attempts to characterize synthetic preparations of Aβ have been already made (134, 135). These techniques in combination with the immunoaffinity methodologies will provide new avenues for the precise molecular characterization of naturally occurring toxic Aβ entities.

There is now strong evidence coming from many laboratories that small oligomeric particles of Aβ are the most neurotoxic species causing synaptic dysfunction, neuronal loss and inflammatory responses in the brain. Nevertheless, many questions remain to be answered in this field as well. These include elucidation of pathways of formation of various forms of Aβ and identification of factors affecting oligomerization/fibrilization of Aβ. In particular, the nature of the aggregation state of intraneuronal Aβ stores and factors controlling this process remain to be determined. It will be important to identify targets on cellular membranes that interact with various forms of Aβ and trigger downstream responses. It is highly imperative to determine molecular mechanisms of neurotoxicity of small oligomers of Aβ that lead to apoptotic or necrotic neuronal death. Do oligomers trigger particular signaling events or prevent binding of some other potential ligands to neurons? Why are neurons particularly sensitive to Aβ oligomers? Elucidation of the precise molecular mechanisms of Aβ oligomer-induced cell death will be crucial for the development of the selective drugs to prevent neuronal loss during Alzheimer's disease. But first and most of all, we need to get a clear understanding of physiological functions of APP and Aβ, as this knowledge may be essential for elucidation of molecular mechanisms of pathogenesis, as well as for the development of new strategies for the treatment and prevention of Alzheimer's and other neurodegenerative diseases.

Highlights

- Soluble Aβ oligomers are produced when APP cleavage products (Aβ peptides of various length) or synthetic Aβ peptides undergo specific pathways of oligomerization *in vivo* or *in vitro*. Among various Aβ peptides, $A\beta_{1\text{-}42}$ are most prone to oligomerization/fibrilization in aqueous solutions. These species have been found to be the most toxic to neurons.
- Metal binding to Aβ plays a pivotal role in structure formation and function of Aβ oligomers. However, there are few *in vitro* studies in which the levels of metals and their chemical state are maintained in a controllable fashion.
- Most of the methods by which artificial oligomer preparations are made for biological studies employ special

organic compounds which strongly interact with both monomer and oligomer forms of Aβ. To what extent the biological activity data and conclusions obtained using artificial Aβ species may be extrapolated to the processes in the brain remains unanswered.

- Cell or tissue-derived Aβ oligomers are the closest entities to those that may be directly involved in AD pathology. The precise structural characterization of such species is currently inaccessible for most advanced instrumental techniques due to very low concentrations and the structural and morphological instability of the Aβ species.
- Toxicity of Aβ oligomers depends on various factors that are not yet fully determined yet. These include: primary structure and length of Aβ peptides, conformation, size and shape of oligomeric particles, concentration of Aβ oligomers.
- Aβ oligomers seem to affect synaptic activity of neurons at low nanomolar or picomolar concentrations whereas cell death is induced at higher nanomolar and micromolar concentrations of Aβ oligomers.
- Aβ oligomers have been reported to induce apoptotic as well as necrotic cell death.
- In the literature, at least two main mechanisms of extracellular Aβ-oligomers-induced cell death are discussed. The first mechanism suggests binding of extracellular Aβ oligomers to plasma membrane causing disruption of membrane integrity leading to disturbances of intracellular ion homeostasis. The second mechanism involves interaction of Aβ oligomers with various receptors that may trigger signaling events leading to cell death. Extracellular Aβ oligomers may also be internalized and accumulate in lyzosomes. Downstream events may include endoplasmic reticulum stress, inhibition of ubiquitin-proteasome system, and mitochondrial permeability transition. At the current stage of research, we do not have a full picture of the sequence of events triggered by Aβ oligomers and leading to neuronal death.

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