Intracellular APP Processing and Aβ Production in Alzheimer Disease

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Abstract. Senile plaques composed of Aβ peptides are a histopathological hallmark of Alzheimer disease (AD). A role for Aβ in the etiology of AD has been argued from analysis of mutations associated with a subset of early-onset familial AD (FAD). Expression of autosomal dominant mutations in the genes for the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) in affected patients, cultured cells, or transgenic mice leads to increased production of total Aβ or increased production of Aβ ending at residue 42(Aβ42). Since Aβ42 is the more amyloidogenic and toxic species in vitro and is the major component of amyloid senile plaques in vivo, overproduction of this peptide may play a crucial role in the pathogenesis of AD. Thus, an understanding of the production of Aβ within the cell in normal and pathological conditions is critical to understanding early events in AD. Studies in cell culture have established that processing of APP to form Aβ can occur at multiple locations within the cell and leads to the production of 2 pools of Aβ: a secreted pool composed predominantly of Aβ40 and a nonsecreted, intracellular pool composed preferentially of more amyloidogenic Aβ42. The purpose of this review is to provide a summary of our current understanding of APP processing in the generation of the secreted and intracellular pools of Aβ and to propose a model linking the intracellular pool to the formation of extracellular plaques and neuronal pathology in AD.

Key Words: Intracellular Aβ; Insoluble Aβ42; Endoplasmic reticulum; Senile plaques.

INTRODUCTION

Alzheimer disease (AD) is a progressive neurodegenerative dementia afflicting 1% of the population over age 65. Characteristic features of the disease include neurofibrillary tangles composed of abnormal tau paired helical filaments, neuronal loss, and alterations in multiple neurotransmitter systems. A most significant pathological feature, however, is an overabundance of diffuse and compact senile plaques in association and limbic areas of the brain. Although these plaques contain multiple proteins, their cores are composed primarily of β-amyloid, a 39–42 amino acid proteolytic fragment derived from the amyloid precursor protein (APP; for review, see 1).

APP is a single-transmembrane protein with a 590–680 aa long extracellular amino terminal domain and an approximately 55aa cytoplasmic tail that contains intracellular trafficking signals (Fig. 1). mRNA from the APP gene on chromosome 21 undergoes alternative splicing to yield 8 possible isoforms, 3 of which (the 695, 751 and 770 amino acid isoforms) predominate in the brain (2, 3). APP695 is the shortest of the 3 isoforms and is produced mainly in neurons. Alternatively, APP751, which contains a Kunitz-protease inhibitor (KPI) domain, and APP770, which contains both the KPI domain and an MRC-OX2 antigen domain, are found mostly in non-neuronal glial cells. All 3 isoforms share the same Aβ, transmembrane, and intracellular domains and are thus all potentially amyloidogenic. The normal function of APP is currently unknown, although in neurons it has been demonstrated to be localized in synapses where it may play a role in neurite extension or memory (4).

APP is trafficked through the constitutive secretory pathway, where it undergoes post-translational processing including a variety of proteolytic cleavage events. APP can be cleaved by 3 enzymatic activities termed α-, β-, and γ-secretase (Fig. 1). α-secretase cleaves APP at amino acid 17 of the Aβ domain, thus releasing the large amino-terminal fragment APPα for secretion. Since α-secretase cleaves within the Aβ domain, this cleavage precludes Aβ formation. Rather, the intracellular carboxy-terminal domain of APP generated by α-secretase cleavage is subsequently cleaved by γ-secretase within the predicted transmembrane domain to generate a 22–24 residue (~3kD) fragment termed p3 which is non-amyloidogenic (5). Alternatively, APP can be cleaved by β-secretase to define the amino terminus of Aβ and to generate the soluble amino-terminal fragment APPβ. Subsequent cleavage of the intracellular carboxy-terminal domain of APP by γ-secretase yields full-length Aβ. Carboxy-terminal cleavage of Aβ by γ-secretase results in the generation of multiple peptides, the 2 most common being 40-amino acid Aβ (Aβ40) and 42-amino acid Aβ (Aβ42). Aβ40 comprises 90%–95% of secreted Aβ and is the predominant species recovered from cerebrospinal fluid (6). In contrast, less than 10% of secreted Aβ is Aβ42. Despite the relative paucity of Aβ42 production, Aβ42 is the predominant species found in plaques and is deposited initially (7), perhaps due to its ability to form insoluble amyloid aggregates more rapidly than Aβ40 (8, 9).
ALTERATIONS OF Aβ PRODUCTION IN AD

Aβ has been postulated to be a causal factor in the pathogenesis of AD. The presence of Aβ-containing amyloid plaques is necessary for the neuropathological diagnosis of AD, suggesting that these entities may be involved in the etiology of the disease. Supportive evidence for the causal role of Aβ in AD can be found in patients with Down syndrome, who often develop AD-like symptoms and pathology after age 40 (10). Down syndrome patients produce elevated APP presumably due to an additional copy of chromosome 21 and exhibit florid AD-like amyloid plaques prior to the onset of other AD symptoms, suggesting that amyloid deposition is an initial event (11). Furthermore, alterations in APP processing have been linked to a subset of familial AD patients (FAD) with autosomal dominant mutations in APP (12, 13), presenilin 1 (PS1; 14), and presenilin 2 (PS2; 15). FAD individuals comprise 10% of all AD cases and generally exhibit symptoms of the disease much earlier than sporadic AD patients. For example, a double mutation of amino acids 670 and 671 in APP from Lys-Met to Asn-Lys immediately upstream of the β-cleavage site of Aβ ("Swedish" mutation or APP<sub>AnL</sub>) results in a 5–8-fold increase in the formation of Aβ by cells (13, 16, 17, 18). Furthermore, mutations adjacent to the carboxy-terminal γ-cleavage site of Aβ, such as the Val177 mutations, specifically increase the production of the more amyloidogenic Aβ42 relative to Aβ40 (19). The fact that these alterations are sufficient to cause AD-like pathology is supported by studies which show that transgenic mice overexpressing either APP<sub>AnL</sub> (20) or APP<sub>V717F</sub> (21) produce higher levels of Aβ prior to the exhibition of other AD pathological features such as abnormal phosphorylation of cytoskeletal tau, microgliosis, reactive astrocystosis, reduced levels of synaptic marker proteins, and memory deficits (22-24).

FAD-associated mutations in genes other than APP also affect Aβ production. The presenilins, multi-transmembrane proteins localized predominantly to the ER and Golgi, play a crucial role in APP processing. APP and PS1 may form a complex in vivo (25) and PS1 is necessary for the production of Aβ, as PS1 knockout mice produce less Aβ due to impaired γ-secretase cleavage of APP (26). Over 40 dominant point mutations in PS1 (chromosome 14) and PS2 (chromosome 1) as well as 1 splice site mutation in PS1 have been associated with FAD phenotypes to date (27). FAD-linked mutations in the presenilins also provide a link between Aβ and AD in that expression of these mutants in cells favors the production of Aβ42 (28). Thus, presenilins are involved in the carboxy-terminal cleavage of APP in both normal and pathological states. Since there may be different γ-secretases for the generation of Aβ40 and Aβ42 (29, 30), it is interesting to speculate that presenilin mutations may increase the ratio of Aβ42/Aβ40 by preferentially encouraging the activity of the 42-specific γ-secretase.
PRODUCTION OF SECRETED AND INTRACELLULAR Aβ

The study of Aβ has historically focused on extracellular (i.e. secreted) Aβ, as these Aβ molecules are assumed to be the building blocks of the extracellular plaques in AD. Extracellular Aβ may have an intracellular origin prior to secretion, as Aβ can be detected endogenously within neuronal cells such as NT2N (31–33) as well as within other cell types (34, 35). Both Aβ40 and Aβ42 can be produced intracellularly (31). Furthermore, although much of the intracellularly generated Aβ is en route to secretion, there is a significant pool of Aβ that is not secreted (33, 36, 37). Thus, there appear to be 2 distinct pools of intracellularly-generated Aβ: a pool that is eventually secreted, and a pool that is destined to remain within the cell.

Given the evidence that altered production of Aβ may be an initial event in the development of AD, much research has focused on understanding the mechanisms by which APP is processed to generate Aβ. The main cleavage pathways appear to be conserved in both neuronal and non-neuronal cells, but the predominant intracellular sites of production and the particular products formed are cell-type dependent. Non-neuronal cells preferentially process APP via α- and γ-secretase cleavage to generate APPα and the non-amyloidogenic fragment p3. Thus, non-neuronal cells are not a significant source of Aβ under normal conditions. However, although non-neuronal cells predominantly utilize α-secretase, neurons do not rely heavily on this pathway and produce very low levels of p3 (38). Regardless of the cell type, α-secretase cleaves APP constitutively (5) and is thought to occur mainly at the cell surface since APPα cannot be detected intracellularly (38, 39) and cell-surface labeled APP can be recovered as APPα in the medium (40). In addition to constitutive α-secretase activity, α-secretase cleavage can also be regulated in both neurons and non-neuronal cells via activation of glutamate, muscarinic receptors, and protein kinase C (PKC) (see 41 for review). PKC-stimulated α-secretase activity requires the activity of tumor necrosis factor-α converting enzyme (TACE), which can cleave APP at the α-cleavage site in vitro. TACE has broad sequence specificity and appears to cleave a wide range of proteins at extracellular residues near their transmembrane domains (42).

Cleavage by β- and γ-secretases yields Aβ and is a constitutive event, as Aβ can be detected in normal brains in picomolar to nanomolar concentrations (43, 44). APP is trafficked intracellularly through the default secretory pathway and generation of Aβ can occur at several distinct locations along this route. APP produced in the endoplasmic reticulum (ER) transits to the Golgi, where it is post-translationally modified via N- and O-linked glycosylation and tyrosine sulfation before vesicular transport to the cell surface (45). Cell surface APP is then reinternalized into the endosomal/lysosomal system where it may be degraded (46). Aβ can be produced in at least 3 sites along this pathway: the endosomal-lysosomal system, the Golgi apparatus, and the ER (Fig. 2).

APP enters the endosomal-lysosomal system following reinternalization from the cell surface mediated by the APP carboxy-terminal NPTY motif (46, 47). Amyloidogenic carboxy-terminal fragments (CTFs) of APP have been demonstrated to exist in the endosomes/lysosomes, where they may serve as a substrate for γ-secretase cleavage to generate Aβ (47). Indeed, treatment of cells with agents that interfere with the pH of lysosomes such as chloroquine or NH4Cl reduce the secretion of Aβ (48, 49). However, Aβ itself cannot be purified from lysosomes of radiolabeled cells (49), suggesting that Aβ produced in this organelle is rapidly secreted, and more recent studies have shown that blocking the endosomal/lysosomal system has no effect on the production of intracellular Aβ in neurons (50). Thus, the endosomal/lysosomal system contributes a small amount of Aβ exclusively to the secreted pool. This pathway may be mostly involved in non-neuronal Aβ production, which has been shown by immunoelectron microscopy to occur near the surface of cells (51).

A second intracellular site of Aβ production is within the Golgi apparatus. This pathway was originally identified in non-neuronal cells expressing APP with the Swedish APPs717 mutation. For example, undifferentiated neuro2a (N2a) cells that overexpress wild-type APP cleave it mainly by α-secretase cleavage at the surface of the cell. However, N2a cells that overexpress APP with the Swedish mutation tend to process APP via β-secretase cleavage to form Aβ (18, 52). The intracellular localization of this β-cleavage was investigated by blocking APP exit from the ER with the fungal antibiotic brefeldin A (BFA). This abolishes secreted Aβ (53) as well as intracellular Aβ40, demonstrating that these species are produced downstream of the ER. The specific location was determined by treatment of N2a cells with the ionophore monensin or incubation at 20°C, both of which block protein trafficking past the trans-Golgi network. These treatments reveal that the Golgi is the main site of intracellular Aβ40 production. Secreted Aβ40 and Aβ42 may be produced here as well (52), although this is controversial (53). By correlating the formation of APPβ with APP post-translational modifications known to occur at specific points in the Golgi apparatus, it has been demonstrated that β-cleavage can occur as early as the medial Golgi (18). Endogenous β-cleavage in the Golgi was also demonstrated in non-neuronal H4 cells and PC12 cells, although subsequent γ-cleavage was not detected (54, 35).

Although this pathway contributes to the pool of Aβ in non-neuronal cells expressing the FAD Swedish APP.
Fig. 2. Three intracellular pathways of Aβ production. APP is synthesized in the endoplasmic reticulum (ER) and is trafficked through the Golgi network to the cell surface. From the cell surface it is internalized via endocytosis into the endosomal/lysosomal system. Cleavage of APP to form Aβ can occur at 3 sites along this pathway. The endosomal/lysosomal system may contribute minor amounts of secreted Aβ, particularly in non-neuronal cells. The trans-Golgi network (TGN) is the major site of intracellular Aβ40 production in neurons and in non-neuronal cells engineered to express the FAD APP<sub>sw</sub> "Swedish" mutation. In addition, either the TGN or post-Golgi vesicles are responsible for the production of secreted Aβ in neurons. Finally, the ER is a site for the production of Aβ42 that is destined to remain within the cell. (Figure courtesy of D. Skovronsky)

mutation, Golgi processing also appears to be constitutively active in neurons expressing wild-type APP (39). Expression of APP<sub>sw</sub> in non-neuronal cells results in an 8-fold increase in Aβ production, whereas expression in neurons results in a very modest, less than 2-fold increase in Aβ levels, suggesting that this pathway is already active. As further evidence of the importance of this pathway in neurons, immunoelectron microscopy using an Aβ40 end-specific antibody has demonstrated Aβ40 to be localized predominantly in the trans-Golgi network (51, 56). As Aβ40 is the main secreted and intracellular Aβ species produced, the Golgi appears to be the main site of Aβ production in neurons.

A third pathway for the production of Aβ was recently identified in the endoplasmic reticulum/intermediate compartment (ER/IC) (36, 37, 51). Although treatment of cells with BFA (35) or incubation at 15°C to retain proteins in the ER abolishes Aβ secretion, some β-cleavage can still occur in the ER compartment, as amyloid-containing APP CTFs are produced (55) and APPβ can be detected intracellularly in neuronal NT2N cells (38). Furthermore, γ-cleavage occurs as well, as intracellular Aβ can be detected in transfected kidney 293 cells (37), NT2N neurons (36), or a cell-free reconstitution system (56) following treatment with BFA. Moreover, formation of Aβ when protein exit from the ER is blocked is not simply due to nonphysiological retention of β- and γ-secretases in the ER, as identical results were obtained when APP alone was retained in the ER with a carboxy-terminal dilsine retention motif (36). Interestingly, the intracellular Aβ produced in the ER is almost exclusively Aβ42 (36, 37, 51) and is not destined for secretion. Immunoelectron microscopy studies using an end-specific antibody for Aβ42 offer further confirmation that Aβ42 can be localized to the ER in neurons (51, 56).

Taken together, these results point to the production of 2 pools of Aβ. The first is a secretable pool that consists preferentially of Aβ40 and is generated mainly in the Golgi and/or post-Golgi vesicles in neurons and in the endoplasmic/lysosomal system in non-neuronal cells. The second is a nonsecreted pool consisting of Aβ40 generated in the TGN as well as Aβ42 generated in the ER/IC. Significantly, relative levels of Aβ40 and Aβ42 are different between the secreted and intracellular pools. Although absolute levels of secreted Aβ are higher than levels of intracellular Aβ, secreted Aβ has a much lower ratio of Aβ42/Aβ40 (1:10) than intracellular Aβ (1:3, 32, 33, 36, 37, 50). Given that Aβ42 is the more amyloidogenic species and may serve as the nidus for amyloid plaques, the higher intracellular ratio of Aβ42/40 may be important for AD. Indeed, the FAD V717F mutation has differential effects on secreted and intracellular Aβ and elevates intracellular Aβ42 following retention of APP in the ER with BFA (37).
PROPOSED ROLES OF INTRACELLULAR Aβ IN AD

Intracellularly-generated Aβ may be important in understanding the role of amyloid in senile plaque formation. While senile plaques are large extracellular accumulations of Aβ, they likely result from Aβ produced intracellularly and secreted by neurons. However, secretion of Aβ alone is insufficient to explain the pathogenesis of senile plaques, as CSF levels of Aβ in normal or AD patients are too low to initiate fibril formation. Rather, there must be some mechanism for concentrating Aβ to form a nidus for a plaque. One possible mechanism may arise if Aβ, particularly the more amyloidogenic Aβ42, is concentrated intracellularly. The ER-generated Aβ42 that is not secreted may increase slowly over time within the cell until it reaches concentrations necessary for fibril formation. This idea is supported by the recent discovery of a large, detergent-insoluble pool of Aβ in NT2N cells that can be liberated by extraction with formic acid (33). This pool has a much higher Aβ42/A40 ratio than the detergent-soluble pool, with Aβ42 surpassing Aβ40 as the predominant Aβ species. The detergent-insoluble Aβ42 is generated within the ER/IC and increases with time in culture due to slower overall turnover of Aβ42 than Aβ40. Moreover, since this Aβ accumulates intracellularly in the absence of any FAD mutations it is possible that pathologicial mutations may accelerate this phenomenon, as mutations in APP and PS1 increase the relative production of Aβ42. This may involve intracellular Aβ42, since presenilins are localized within the ER where intracellular Aβ42 is generated (27) and the APP V717 mutations can increase the relative production of intracellular Aβ42 (37).

Since the detergent-insoluble intracellular pool of Aβ42 is degraded slowly, it might form an overwhelming amyloid burden within the cell that may lead to the formation of extracellular amyloid plaques. In transgenic mice harboring the V717F FAD APP mutation, Aβ fibrils were detected intracellularly, particularly in the vicinity of the rough endoplasmic reticulum (57), and Aβ can also be found inside neurons prior to plaque formation in aged macaque brains (58). Neuronal cell death is a prominent feature of AD (59), and dying neurons might rupture and release the accumulated intracellular Aβ42 into the surrounding extracellular milieu. Once released, Aβ42 could have multiple effects. First, Aβ42 might stimulate further production of amyloidogenic APP fragments in neighboring neurons. Bahr et al. (60) demonstrated that exogenous Aβ42 added to hippocampal slice cultures was selectively internalized within AD-vulnerable CA1 neurons and induced a buildup of C99, the amyloidogenic precursor to Aβ. Thus, a local release of Aβ42 could amplify the levels of intracellular Aβ in surrounding cells. Secondly, the release of insoluble intracellular Aβ42 from dying neurons might form a nidus for the accumulation of secreted Aβ into diffuse or senile extracellular plaques. It has been observed that extracellular deposition of Aβ plaques is localized within the vicinity of neuronal cell death and often is seen surrounding dead neurons (61). More striking evidence for intracellular Aβ serving as a seed for subsequent plaque formation is the identification and amplification of mRNAs from senile plaques. Ginsberg et al. (62) recently demonstrated that the majority of mRNAs which can be amplified from immunohistochemically-defined senile plaques in AD hippocampus are of neuronal origin, suggesting the contribution of neuronal cell components in the development of senile plaques.

Although neuronal cell death in AD might occur for multiple reasons unrelated to the buildup of intracellular Aβ, the possibility exists that the intracellular Aβ itself is causally involved in neuronal death and other features of AD pathology. Aβ42 has been shown to have greater toxicity in vitro than Aβ40 (63). Thus, accumulation of high levels of intracellular Aβ42 as neurons age may eventually prove toxic to the cell. Correlational evidence for the toxicity of intracellular Aβ is derived from the observation that neurons with TUNEL-positive DNA damage in AD brains contain intracellular Aβ (61). Detectable intracellular Aβ is also found in association with characteristic features of AD pathology. In AD brain tissue, intracellular Aβ is found in the same neurons which contain neurofibrillary tangles (64) and has been shown to overlap with intracellular neurofibrillary tangles when the tissue is treated with 10% formic acid (65). Additionally, consistent with the relative levels of secreted and intracellular Aβ described in previous sections, intracellular neurofibrillary tangles are often associated with the more amyloidogenic Aβ42 (66), whereas extracellular neurofibrillary tangles are more likely to be associated with Aβ40 (67). It has also been demonstrated that an intracellular increase in potentially amyloidogenic fragments is correlated with a decrease in synaptic marker protein staining (60), suggesting that intracellular Aβ may be involved in AD-associated synaptic deficits. Furthermore, these effects can be exerted in the absence of or prior to the formation of senile plaques, suggesting the potential involvement of intracellular Aβ. For example, Hsia and colleagues recently demonstrated that transgenic mice harboring the FAD APP V717F mutation display morphological and electrophysiological deficits in the CA1 and CA3 regions of the hippocampus several months prior to the development of amyloid plaques (23). These deficits include diminished presynaptic terminals, decreased neuronal number, and impairments in synaptic transmission that do not correlate with the level of plaque burden. Although the presence of extracellular plaques is apparently not necessary, the authors demonstrated that increased Aβ levels do play a role. Transgenic mice with both the Swedish and V717F FAD mutations produce
very high levels of Aβ and have correspondingly more severe deficits in synaptic transmission.

Thus, we propose a theoretical model for the generation of senile plaques as well as other features of AD pathology (Fig. 3). Aβ42 is generated in the neuronal ER under normal circumstances, and this production may be increased by the presence of FAD mutations in APP or the presenilins. Due to reduced clearance, Aβ42 preferentially accumulates inside the cell and becomes insoluble. The insoluble, intracellular Aβ42 may eventually achieve a local concentration within neurons sufficient for the formation of fibrils. This process may require decades, thus accounting for the late onset of AD symptoms. Eventually, the neuron may die due to causes related or unrelated to the accumulation of this Aβ and thereby release the aggregated Aβ42. The now extracellular Aβ42 fibrils are free to complex with secreted Aβ from other neurons (or from non-neuronal cells in some rare cases), forming extracellular amyloid plaques. Thus, in this model the extracellular plaque is seeded from intracellular sources. Furthermore, intracellular Aβ may have other effects inside the cell, contributing directly or indirectly to the etiology of other features of AD such as synaptic degeneration and hyperphosphorylated PHF tau. Currently, this theory of intracellular Aβ42 in the pathogenesis of AD remains hypothetical and clearly requires more experimentation to substantiate or refute it. However, by shifting the focus of early events in AD from outside of the neuron to inside, this model may be useful in illuminating new directions of research as well as identifying new targets to combat the progression of AD.

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