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RESEARCH ARTICLE

Beyond the Amyloid Hypothesis: Proteolytic Dysfunction in Familial Alzheimer's Disease

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ABSTRACT
For over 30 years, the amyloid hypothesis of Alzheimer's disease has dominated this field of biomedical investigation. The hypothesis posits that aggregation of the amyloid β-peptide (Aβ) in the brain triggers a cascade of events that ultimately lead to neurodegeneration and cognitive decline. Early genetic and biochemical evidence supported a critical role of Aβ, particularly the 42-residue form Aβ42: Dominant missense mutations in the substrate (amyloid precursor protein, APP) and enzyme (γ-secretase) that produce Aβ cause early-onset familial Alzheimer's disease (FAD). Nevertheless, serious gaps remain in understanding pathogenic pathways, and despite intense efforts over many years, effective agents for Alzheimer's disease have been elusive. Here I discuss recent efforts to elucidate precisely how FAD mutations alter the complex proteolytic processing of APP substrate by γ-secretase, with results suggesting pathogenic triggers other than Aβ42.

Keywords: amyloid β-peptide, presenilin, γ-secretase, proteolysis

Introduction

Alzheimer's disease (AD) is a devastating neurological disorder in which gradual degeneration of the cerebral cortex leads to progressive loss of cognitive function, particularly learning and memory.[1] In the U.S., over six million are afflicted with the disease, with estimates of over 35 million worldwide. Advanced age is the greatest risk factor for AD: ~99% of cases are in those of age 65 years and older, and the disease afflicts nearly half of those over age 85. Because of this strong correlation with age, AD is expected to dramatically increase in the coming decades due to demographic shifting toward older populations.

AD can be defined pathologically by the presence of specific lesions: amyloid plaques, neurofibrillary tangles, and neuroinflammation.[2] Amyloid plaques and neurofibrillary tangles are proteinaceous deposits, with plaques being extraneuronal and tangles located inside neurons. The plaques are primarily composed of the small (4 kDa) amyloid β-peptide (Aβ), while the tangles are composed of hyperphosphorylated forms of the normally microtubule-associated protein tau. In addition, reactive microglia (immune cells of the brain), and astrocytes (neuronal support cells), which among other things release inflammatory cytokines, are also commonly seen in AD.

Among these pathological features, Aβ aggregation has long been thought to be the trigger of a cascade of events that includes tau assembly into filaments and tangles and activation of microglia and astrocytes resulting in neuroinflammation. Indeed, the Amyloid Hypothesis of AD pathogenesis was first formulated over 30 years ago.[3,4] Cerebral amyloid plaque deposition can occur more than 15 years before the expected age of onset of symptoms.[5] Moreover, while amyloid plaques are unique to AD, pathological tau can be seen in other neurodegenerative diseases.[6] However, over the years the Amyloid Hypothesis has been modified to implicate soluble Aβ oligomeric assemblies, rather than plaques per se, as the neurotoxic trigger in AD.[7]

Familial Alzheimer's disease

The impetus for the original formulation of the Amyloid Hypothesis was the 1991 discovery of the amyloid precursor protein (APP) gene as the site of dominant missense mutations associated with early-onset familial Alzheimer's disease (FAD).[8,9] This gene encodes a single-pass membrane protein that is constitutively and successively cleaved by two proteases to produce the Aβ peptide (Fig. 1). First, the membrane-tethered β-secretase cuts in the lumenal/extracellular region to release the large APP extracellular domain, leaving a 99-residue C-terminal stub (C99) in the membrane.[10] This stub is then proteolyzed within its transmembrane domain by the membrane-embedded γ-secretase complex.[11] Although APP mutations result in FAD, which strikes in midlife, the discovery of these mutations was immediately considered to be likely relevant to the more common sporadic AD of the elderly. This is because, while FAD is a hereditary monogenic disease with an early age of onset, its pathology, presentation, and progression are closely similar to that seen with the more common late-onset sporadic AD.

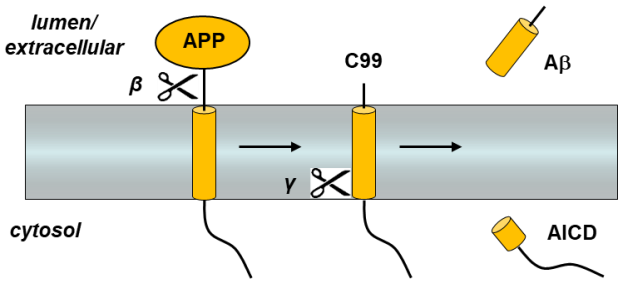

Figure 1. Sequential proteolytic processing of the amyloid precursor protein (APP). Initial proteolysis by β-secretase sheds soluble APP ectodomain (not shown), leaving a membrane-bound 99-residue C-terminal fragment (C99). Further proteolytic processing of C99 by γ-secretase releases Aβ peptides and APP intracellular domain (AICD).

FAD-associated mutations in APP are found in and around the small Aβ region and alter the production or properties of the Aβ peptide.[12] Many of these mutations are found in the transmembrane region, where γ-secretase cleaves, and alter the level and length of the Aβ peptides, skewing production in favor of a highly aggregation-prone 42-residue form (Aβ42). Although Aβ42 is a minor product (most is a shorter, more soluble 40-residue form, Aβ40), this peptide is the principal Aβ variant

found in the pathological cerebral plaques.[13] Other mutations are found near the β-secretase cleavage site and enhance proteolysis by this enzyme to increase C99 and therefore Aβ levels. Some mutations are located within the Aβ sequence and increase the propensity of the peptide to aggregate. Provocatively, mutations that protect against AD onset are also found near the β-secretase cleavage site and lead to decreased proteolysis at this site,[14] findings consistent with the Amyloid Hypothesis.

Several years after the identification of APP as a locus for FAD, two other genes were discovered, encoding homologous multi-pass membrane proteins dubbed presenilin-1 (PSEN1) and presenilin-2 (PSEN1).[15-17] Well over 100 mutations have been identified to date, all missense mutations and predominantly in PSEN1.[12] Very quickly these mutations were found to increase the ratio of Aβ42 to Aβ40 in transfected cells and transgenic mice.[18-22] In subsequent years, presenilin was found to be essential for processing of C99 by γ-secretase[23] and an unprecedented intramembrane aspartyl protease.[24] Activation of presenilin involves assembly with three other membrane protein subunits[25-28] and autoproteolysis of presenilin into an N-terminal fragment (NTF) and C-terminal fragment (CTF)[24,29] Each of these two presenilin subunits contributes one of the conserved catalytic aspartates to the active site of the γ-secretase complex.

Targeting γ-secretase

The search for inhibitors of γ-secretase, to reduce Aβ production and potentially treat AD, began even before the enzyme was identified as a novel intramembrane aspartyl protease complex. Early inhibitors were peptides and peptidomimetics, and these compounds proved to be useful chemical tools and probes to study γ-secretase, identify its substrates, and elucidate its roles in biology and disease.[30] The first γ-secretase substrate discovered after APP was Notch1, a cell-surface receptor essential for development in all metazoans.[31,32] Interaction with a cognate ligand on a neighboring cell triggers Notch1 ectodomain shedding and intramembrane proteolysis to release Notch intracellular domain, translocation to the nucleus, and activation of transcription of genes that regulate cell-fate determinations.[33] The γ-secretase complex is now known to cleave over 100 membrane protein substrates.[34]

The development of inhibitors with more optimal drug-like properties than peptide analogues allowed clinical testing of γ-secretase as a target for the potential treatment of AD. Even before the advent of these compounds, however, the specter of toxicity due to inhibition of Notch1 signaling loomed large. Notch1 is not only essential in development;[35] it also plays signaling roles in cell differentiation in adulthood.[36] Concerns about interfering with Notch function were validated by large clinical trial with two different γ-secretase inhibitors, both of which caused serious adverse events connected with deficient Notch signaling: gastrointestinal effects, immunosuppression, and skin lesions.[37,38] More alarming though was the finding that both clinical drug candidates caused cognitive worsening in AD patients, the opposite of expectations based on the Amyloid Hypothesis.

In the decade since these failed clinical trials, hope for γ-secretase as a target has rested in modulators of the enzyme activity.[39] These compounds lower production of Aβ42 without inhibiting the enzyme. Early compounds were not potent, but over twenty years, many classes of γ-secretase modulators have been discovered. Among these, some have high potencies, good drug-like properties, and excellent safety profiles. Most recently, one such compound was reported with promising pre-clinical results that appear to justify entry into human trials.[40] The promise of these agents, however, entirely depends on the assumption that Aβ42 is the pathogenic entity in AD.

Processive proteolysis

γ-Secretase cleavage of APP substrate C99 does not only result in Aβ40 and Aβ42 production. A series of proteolytic events take place, beginning with endoproteolysis and followed by carboxypeptidase trimming of initially formed long Aβ peptides to shorter secreted forms (Fig. 2).[41] Initial endoproteolysis of C99 occurs at so-called ε cleavage sites to produce Aβ48 or Aβ49 and release the corresponding APP intracellular domains AICD49-99 and AICD50-99, respectively.[42] The long Aβ peptide intermediates are then processively proteolyzed, generally in tripeptide intervals, along two pathways: Aβ49→Aβ46→Aβ43→Aβ40 and Aβ48→Aβ45→Aβ42→Aβ38.[41] Aβ peptides of 43 residues and shorter are secreted once released from the protease complex, while the longer intermediates are retained in the membrane if they are not further processed.[43]

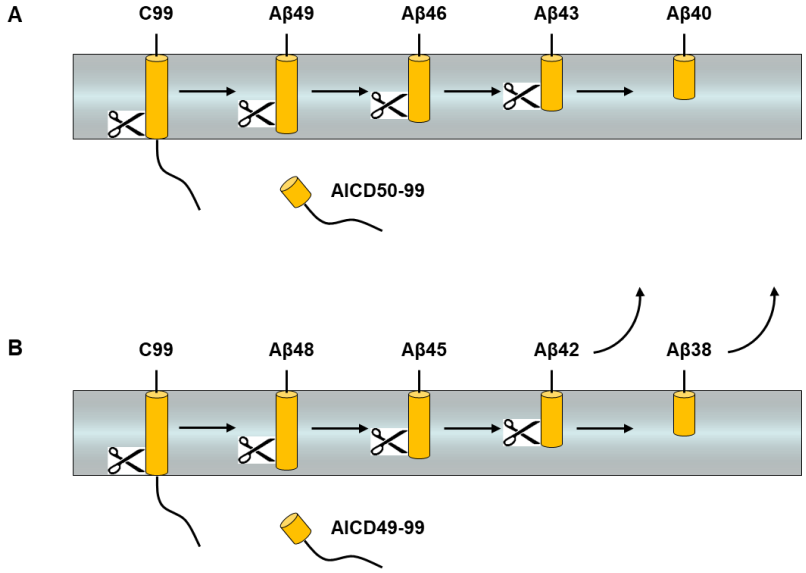

Figure 2. Processive proteolysis of C99 by γ-secretase occurs along two pathways. (A) Initial endoproteolysis of C99 to produce Aβ49 and AICD50-99 is followed by processive carboxypeptidase trimming of Aβ49 to Aβ46, Aβ43 and Aβ40. (B) Alternatively, initial endoproteolysis of C99 can produce Aβ48 and AICD49-99. Subsequent trimming of Aβ48 produces Aβ45, Aβ42 and Aβ38. The last two Aβ peptides along either pathway can be released from the membrane and secreted.

FAD mutations lead to proteolytic dysfunction in the processing of APP substrate C99 by γ-secretase, reducing ε proteolysis and/or one or more specific trimming steps. Early work demonstrated that five FAD PSEN1 mutations in the γ-secretase complex, while generally reducing AICD production (ε cleavage), all skewed the Aβ profile toward peptides of 45 residues and longer compared to the wild-type enzyme.[44] A later report confirmed these findings with other FAD mutations in APP and PSEN1.[45] In another follow-up study, synthetic Aβ48 and Aβ49 were used as substrates, testing the effects on carboxypeptidase trimming to Aβ40 and Aβ42 independently of endoproteolysis.[46] The same five FAD-mutant PSEN1/γ-secretase complexes displayed dramatically reduced trimming function of Aβ49→Aβ40 and Aβ48→Aβ42. Because Aβ49→Aβ40 processing was more severely compromised, all five FAD-mutant enzymes led to increased Aβ42/Aβ40 ratios, widely considered as the critical factor in Aβ aggregations into toxic oligomers and pathological plaques.

More recently, a comprehensive analysis of all proteolytic events by purified wild-type γ-secretase on wild-type and 14 FAD-mutant APP C99 substrates was conducted.[47] Using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), AICD and small-peptide coproducts for each cleavage step were quantified. With this information, the cleavage efficiencies for each step could be deduced and the levels of all Aβ peptide products could be calculated. Validation for the approach was supported in two ways. First, levels of Aβ40 and Aβ42 production calculated from LC-MS/MS results closely matched results obtained by specific ELISAs. Second, total AICD levels closely matched levels of total Aβ production. Moreover, levels of specific AICDs were similar to the sum of the Aβ peptides produced along that pathway (e.g., AICD50-99 ≈ Aβ49+Aβ46+Aβ43+Aβ40). The results showed that, while some of the FAD mutations did not elevate Aβ42/Aβ40, all 14 disease-causing mutations reduced trimming of Aβ peptides of 45 residues and longer, elevating the levels of these peptides. This raises the question of pathogenic roles of these long, membrane-anchored Aβ peptides in FAD.

Implications for pathogenesis and drug discovery

Although the Amyloid Hypothesis has reigned for over 30 years, serious gaps remain in pathogenic mechanisms and pathways. Moreover, despite intense efforts worldwide to target Aβ, particularly aggregation-prone Aβ42, no effective therapies exist that demonstrably slow or halt the neurodegeneration and cognitive decline of AD. How then to reconcile the fact that mutations that

cause early-onset FAD are only found in the substrate (APP) and enzyme (γ-secretase) that produce Aβ? The answer to this conundrum may lie in the multiple proteolytic events carried out by γ-secretase. Conducting full analysis of the effects of FAD mutations on all proteolytic processing of APP substrate by γ-secretase reveals that early, not late, proteolytic steps are compromised, resulting in elevation of longer, membrane-anchored Aβ peptides.

These long Aβ peptides, which we have dubbed "dark amyloid", are difficult to detect but may have profound biological effects. In a recent study,[48] a human neuroblastoma cell line was developed that stably expresses an FAD mutation in APP that dramatically elevates Aβ42/Aβ40 by blocking the Aβ46→Aβ43 trimming step. In parallel, another cell line was developed that expresses this FAD-mutant APP with an additional designed mutation that blocks Aβ45→Aβ42 trimming; these cells produce essentially no Aβ42. Because mitochondrial dysfunction, including decreased oxygen utilization, is seen in AD and other neurodegenerative diseases,[49] oxygen consumption rates in these cell lines were measured. While the FAD-mutant APP cell line showed reduction in basal respiration and respiration associated with ATP production, the addition of an Aβ42-blocking mutation led to even further reductions in oxygen consumption rates. Thus, the observed mitochondrial dysfunction occurred even in the absence of Aβ42 production and instead correlated with blocking trimming of Aβ45 and Aβ46.

Advances in computational biology[50] coupled with structure elucidation of the γ-secretase complex bound to APP substrate[51] has made possible the development of molecular dynamics simulations that provide insight into the structural mechanisms of processive proteolysis by γ-secretase and the effects of FAD mutations.[52,53] These simulations capture the active γ-secretase complex poised for ε proteolysis of APP C99 substrate and predict effects on Aβ48 vs. Aβ49 production by FAD mutations in APP that were verified by biochemical experiments.[52] Most recently, molecular dynamics simulations likewise capture the Aβ49→Aβ46 trimming step, again with predicted deficiencies of certain FAD APP mutations confirmed by LC-MS/MS analysis.[53] In unpublished work, these computational models show that FAD mutations result in less flexible and therefore more stable enzyme-substrate complexes, suggesting how these mutations might reduce proteolytic function.

Taken together these recent findings suggest a path to drug discovery for FAD involving the search for a new class of γ-secretase modulators that stimulate stalled enzyme-substrate complexes and prevent the buildup of long membrane-anchored dark amyloid peptides. Compounds that only stimulate the conversion of Aβ42 to Aβ38, as current γ-secretase modulators do,[39] may not be effective if the pathogenic mechanisms of FAD mutations involve dysfunction of early, not late, proteolytic steps in the γ-secretase processing of APP C99. The new molecular dynamics models of γ-secretase proteolysis of APP substrate may provide a platform for virtual screening to find such novel modulators, with top virtual hits tested in biochemical assays that allow quantification of all proteolytic steps. Optimized compounds could then be tested in cell and animal models of FAD. Such compounds may have potential as therapeutics not only for FAD but also for the much more prevalent late-onset sporadic AD.

Conclusions

The decades-long search for disease-modifying therapeutics for AD that prevent onset or slow progression have not yielded effective medications. The large majority of clinical candidates have targeted secreted Aβ peptides, particularly Aβ42, in light of the prevaling Amyloid Hypothesis of AD pathogenesis. Dominantly inherited early-onset FAD provides an opportunity to elucidate pathogenic mechanisms and identify new therapeutic approaches. Recent studies suggest that inhibition of early proteolytic events in APP processing by γ-secretase and elevation of long membrane-bound "dark amyloid" peptides is a common effect of FAD mutations. Thus, compounds that rescue deficient γ-secretase proteolysis of mutant enzyme or substrate would be expected to prevent or delay onset or slow or halt progression of FAD. Given the close similarities between early-onset inherited FAD and the more common sporadic late-onset AD, advances in understanding and treating FAD are likely to have implications for all AD cases.

Conflicts of Interest Statement

The author declares no conflicts of interest

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