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Clearing the Brain's Amyloid Cobwebs

Minireview

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Elevated cerebral levels of amyloid β-protein occur universally in Alzheimer's disease, yet only a few patients show evidence of increased Aβ production. Therefore, defects in proteases that degrade Aβ could underlie some or many cases of familial and sporadic AD. This previously neglected topic has begun receiving serious attention. Understanding how proteolysis regulates Aβ levels in the cerebral cortex has implications for both the pathogenesis and the treatment of this protean disorder.

By now, many in the scientific community have warmed to the hypothesis that the accumulation of thin filaments of the amyloid β-protein (Aβ) in limbic and association cortices constitutes a major threat to neuronal function. Although the amyloid hypothesis (more correctly, the Aβ hypothesis) for the causation of Alzheimer's disease (AD) still stirs strong passions, the number of genetic, biochemical, and animal modeling studies supporting its validity is impressive and growing apace. But proof can only come from successful trials of Aβ-lowering therapies in humans. Happily, this process has recently begun.

Although a concept that seemed simplistic and farfetched just 15 years ago has now entered the clinic, we are left with many unresolved and fascinating biological questions about how a small hydrophobic peptide accumulates in the brain and then apparently initiates cytopathology. Perhaps foremost among these is exactly why cerebral Aβ levels are elevated in patients with the disease. While the field has focused enormous attention on the complex mechanisms of Aβ production, including the role of the β- and γ-secretases which generate Aβ from its precursor protein (APP), increases in production currently explain a small minority of cases, specifically those bearing inherited mutations in APP or in presenilins 1 or 2. This realization leaves open the likelihood that many, perhaps most, cases of AD are caused by faulty clearance of a peptide that is produced at normal levels throughout life. The mechanisms of Aβ degradation and clearance have received remarkably little attention, but that has begun to change, prompting this review.

Support for the concept that decreased clearance of Aβ can actually precipitate the AD phenotype came first from studies of how the inheritance of one or two alleles of apolipoprotein E4 predisposes one to the disease. Humans bearing ApoE4 alleles show no evidence of a general increase in Aβ production (for example, plasma levels seem not to be elevated), and coexpression of APP with each of the human ApoE isoforms causes no detectable change in cellular Aβ generation. Instead, human neuropathological analyses and mouse modeling suggest that inheritance of ApoE4 leads to a rise in the steady state levels of Aβ in the brain, presumably by enhancing its fibrillogenic potential and/or decreasing its clearance from the brain's extracellular space. While the precise mechanism is unclear, it is likely that ApoE4-mediated rises in brain Aβ levels represent a clearance failure. This review will now focus on another potential basis for Aβ accumulation—defects in proteases that normally degrade the peptide shortly after its production.

Neprilysin and Insulin-Degrading Enzyme: Two Candidates for Regulators of Aβ Levels in Human Brain

Metabolic labeling studies in living mice show that newly generated Aβ is very rapidly turned over in the brain (Savage et al., 1998), suggesting that Aβ-degrading proteases help regulate its levels. There are numerous proteases in the brain that could potentially participate in Aβ turnover, and the goal of finding a single, dominant protease that is responsible for the majority of Aβ degradation may be elusive. Indeed, there is already evidence that several enzymes may contribute to the degradation of Aβ peptides in brain tissue (Table 1). At the outset, one must distinguish between proteases that appear to degrade Aβ only in its monomeric state and those that can degrade oligomeric and/or highly aggregated (fibrillar) forms of the peptide. Very few examples of the latter class have been documented, perhaps because the Aβ peptide becomes resistant to an array of proteases as a result of structural changes associated with its polymerization into amyloid fibrils. Among the former class, two proteases, neprilysin and insulin-degrading enzyme (IDE), have received the most attention to date.

Both of these candidate proteases were first chosen to be tested on synthetic Aβ by laboratories already interested in their ability to degrade other peptides (Howell et al., 1995; Kurochkin and Goto, 1994). But in the case of IDE, evidence that it could degrade naturally secreted Aβ also arose independently from an unbiased screen of cultured cell lines for Aβ-degrading proteases (Qiu et al., 1998). The principal such activity turned out, upon partial purification and inhibitor characterization, to be an ~110 kDa thiol metalloendopeptidase indistinguishable from IDE. IDE had previously been shown to degrade insulin, glucagon, atrial naturetic peptide (ANF), and TGF-α, among other small peptides of diverse sequence. The recent addition of amylin to this list (Bennett et al., 2000) has furthered the hypothesis that IDE has little sequence specificity but recognizes a conformation that is prone to conversion to a β-pleated sheet structure. Such a property could explain its propensity to degrade several peptides that undergo concentration-dependent formation of amyloid fibrils (e.g., insulin, ANF, amylin, calcitonin, and Aβ). Importantly, IDE has been found to degrade rat and human amylin peptides similarly, despite the fact that only the latter can form amy-

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Table 1. Some Proteases Capable of Degrading Aβ

Protease	Class	Some Substrates (besides Aβ)	Principal Subcellular Loci	Recent Findings Relevant to a Role in Aβ Degradation
Insulin-degrading enzyme (insulysin)	metallo	insulin, glucagon, ANF, TGF-α, β-endorphin, amylin	cytosol, peroxisomes, extracellular fluid, plasma membrane, internal membranes	• secreted by microglia • present on internal membranes at sites of Aβ generation • gene located near putative FAD locus
Neprilysin (NEP)	metallo	endorphins, enkephalins	plasma membrane, internal membranes	• capable of degrading membrane-associated Aβ • deletion causes rise in cerebral Aβ levels in vivo
Plasmin	serine	fibrin, other matrix proteins	extracellular fluid	• can degrade both monomeric Aβ and fibrils in vitro
uPA/tPA	serine	plasminogin	extracellular fluid	• can be activated by Aβ aggregates to generate plasmin • uPA gene located near putative FAD locus
Endothelin converting enzyme-1	metallo	big endothelins, bradykinin, substance P, oxidized insulin B chain	plasma membrane, internal membranes	
Matrix metalloproteinase-9	metallo	Collagen, elastin, laminin, pro-TNFα	extracellular fluid	• latent form of MMP-9 accumulates in AD brain

loid fibrils. It appears, therefore, that the motif recognized by IDE is not the β-pleated sheet region per se but a conformation of the monomer in a pre-amyloid state (Bennett et al., 2000).

One concern about the physiological relevance of IDE's ability to degrade secreted peptides such as insulin and Aβ has been that the enzyme occurs principally in a soluble form in the cytoplasm. However, a form of IDE can be labeled on the cell surface, including in neurons, and is also present on intracellular membranes (Vekrellis et al., 2000). Its mode of entry into membranes and the nature of its membrane anchor need to be resolved, as IDE does not have a known signal peptide or transmembrane domain. Nevertheless, the existence of a membrane-anchored form of the protease suggests that it could help regulate insulin signaling at the plasma membrane and could also participate in the degradation of both soluble and membrane-associated forms of Aβ. The cleavage products of Aβ produced by IDE are not neurotoxic and not prone to depositing on amyloid plaques, and therefore recombinant IDE reduces Aβ toxicity in cortical neuronal cultures (Mukherjee et al., 2000). While endogenous IDE has been specifically shown to degrade synthetic Aβ monomers in homogenates and membrane fractions of human brain (Perez et al., 2000; K. Vekrellis and D.J.S., unpublished data), confirmation of the effects of this protease in vivo, e.g., in mice lacking the IDE gene, is now required.

The intracerebral injection of synthetic Aβ peptides in the presence or absence of various protease inhibitors provided evidence that neprilysin is a major $A\beta_{42}$-degrading protease in rat brain, although the enzyme did not mediate $A\beta_{40}$ degradation in this paradigm (Iwata et al., 2000). The approach involved microinjecting a 250 μM solution of radiolabeled $A\beta_{1-42}$ into the hippocampi of 2-month-old rats, waiting 0–30 min, extracting the isolated hippocampi in 0.1% SDS buffer, and visualizing the catabolic products by reverse-phase HPLC. The injected peptide underwent degradation with a half-life of 15–20 min, and a single major peak representing a catabolic intermediate of residues 10–37 was obtained at 30 min. Importantly, SDS was required to extract Aβ immediately after its in vivo injection and at all time points thereafter; no peptide was recovered without this detergent. The authors surmised that the peptide rapidly entered an insoluble phase, probably representing membrane association, before proteolysis occurred. This interpretation is consistent with the conclusion, based on preinjecting different protease inhibitors, that neprilysin produced the $A\beta_{10-37}$ intermediate, since neprilysin is a member of the neutral endopeptidase family of membrane-anchored proteases found on the cell surface. Subsequent work provided important evidence that the steady state levels of endogenous Aβ are elevated in the brains of young neprilysin-deficient mice (Iwata et al., 2001). The rise, while highly significant, was not large, and plaque formation was not observed. Given the rapid turnover of Aβ in the brain (Savage et al., 1998), if neprilysin were the major degrader of Aβ, its deletion should produce an even greater accumulation. Therefore, other proteases, including additional members of the neutral endopeptidase family, may compensate in part for the loss of neprilysin. Indeed, chronic thiorphan infusion, which should inhibit several proteases, led to actual plaque formation in rats (Iwata et al., 2000), a more robust effect than that of deleting just neprilysin.

An interpretation that could explain the existing data on both IDE and neprilysin is that IDE mediates much of the degradation of soluble, monomeric Aβ but has less ability to degrade Aβ once it becomes insoluble and/or oligomeric, whereas neprilysin has little role in degrading soluble Aβ but can degrade buffer-insoluble, SDS-extractable Aβ associated with membranes. Consistent with this hypothesis are three additional findings. First, naturally occurring oligomers (dimers and trimers) of secreted Aβ in culture medium are resistant to IDE, while Aβ monomers in the same sample are avidly degraded by the enzyme (Qiu et al., 1998; Vekrellis et al., 2000). Second, IDE occurs abundantly in a soluble, extracellular form in the nervous system (as documented in human CSF and neuronal and microglial culture media [Qiu et al., 1998; Vekrellis et al., 2000]) in addition to a

membrane-anchored form, whereas neprilysin occurs almost exclusively in a membrane-anchored form. And third, degradation of Aβ in the soluble fraction of brain appears not to be decreased by inhibition or deletion of neprilysin (Iwata et al., 2000), whereas degradation of Aβ in the membrane fraction of brain is decreased ~25%–35% by neprilysin inhibitors and ~70% by IDE inhibitors (K. Vekrellis and D.J.S., unpublished data). In summary, both neprilysin and IDE may contribute to overall Aβ degradation in the brain, but each may play a role in distinct subcellular loci and with different forms of Aβ. The possibility that different proteases principally degrade different pools of Aβ in the brain will need to be borne in mind as additional enzymes are evaluated.

Other Candidate Aβ-Degrading Proteases

The plasmin proteolytic cascade, known to be crucial for fibrinolysis and cell migration, has recently been implicated in Aβ clearance as well. In this cascade, either of two activators of plasmin, tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), can be posttranslationally activated by binding to fibrin aggregates and then cleave plasminogen to yield the active serine protease, plasmin, which proteolyzes fibrin and other substrates. In vitro studies have suggested that Aβ aggregates can substitute for fibrin aggregates in activating tPA. In the nervous system, plasminogen, tPA and uPA are expressed in neurons, and tPA is also synthesized by microglia. Experiments in primary neuronal cultures show that pure plasmin (at 10–30 nM) can significantly decrease the amount of neuronal injury induced by aggregated Aβ (16–30 uM) (Tucker et al., 2000). In vitro biochemical assays indicate that pure plasmin can proteolyze fibrillar Aβ, although at an efficiency about 100-fold less than that for freshly dissolved (i.e., largely monomeric) Aβ. While these results make plasmin the first physiologically relevant protease shown to degrade aggregated Aβ in vitro, the reaction was about 20-fold less efficient than that involving aggregated fibrin (Tucker et al., 2000). Moreover, this in vitro effect may not predict similar activity on amyloid fibrils in vivo, because serum amyloid P component, which is associated with amyloid deposits in AD brain, is able to bind to and prevent the proteolysis of isolated cerebral amyloid (Tennent et al., 1995). Evidence for a role of plasmin in regulating Aβ monomer or polymer levels in vivo has not yet appeared.

Another protease expressed in brain that has been evaluated for its ability to degrade Aβ is endothelin converting enzyme-1 (ECE-1) (Eckman et al., 2001). This integral membrane zinc metalloprotease, with its active site located in the lumen and extracellularly, can cleave the endothelin precursors and several other biologically active peptides, including bradykinin, substance P, and the oxidized insulin B chain. Cellular overexpression of ECE-1 leads to a marked reduction in the levels of naturally secreted Aβ40 and Aβ42 peptides in Chinese hamster ovary cells. The purified enzyme directly proteolyzes both synthetic peptides in vitro (Eckman et al., 2001). Whether this protease can alter Aβ levels in vivo remains to be determined. Other purified proteases that have been reported to digest synthetic Aβ peptides under in vitro conditions include matrix metalloproteinase-9 (Backstrom et al., 1996) and cathepsin D (McDermott and Gibson, 1996).

As candidate enzymes that degrade Aβ in the brain are confirmed, it will become important to identify other proteases that activate them and proteins that normally inhibit them, as these regulatory molecules represent attractive therapeutic targets. In this regard, the metalloendopeptidase 24.15 has been reported to indirectly regulate Aβ degradation. Whereas this enzyme does not proteolyze synthetic Aβ, decreasing its activity (e.g., via antisense treatment) leads to increased Aβ levels in cell culture (Yamin et al., 1999). It is thus possible that MP24.15 processes a zymogen of an Aβ protease or degrades its endogenous inhibitor. Evidence that enhancing the cerebral levels of a protease inhibitor can increase Aβ deposition in vivo comes from recent studies of the effects of the serine protease inhibitor, α1-antichymotrypsin, in APP transgenic mice (Mucke et al., 2000; Nilsson et al., 2001).

A potential clearance mechanism for Aβ aggregates is the phagocytosis of amyloid fibrils by cerebral microglia and their subsequent degradation by acid hydrolases in late endosomes and lysosomes. While a contribution by this route is possible, microaggregates of synthetic Aβ42 shown to be internalized by cultured microglia and transported to lysosomes were remarkably stable and were degraded very slowly, leading to their severe accumulation inside cells (Paresce et al., 1997). Therefore, it is unclear whether attempted Aβ removal by this route is beneficial or rather induces a microglial-associated inflammatory response. Of related interest is the current debate as to whether the dramatic Aβ-clearing effects of active and passive Aβ immunization are due to the clearance of Aβ-antibody complexes by local microglial (Bard et al., 2000) and/or the transport of such complexes out of the brain and into CSF and blood (DeMattos et al., 2001).

Searching for Genetic Evidence of a Primary Defect in Aβ Degradation in Alzheimer's Disease

Taken together, the biochemical studies in neuronal cultures, mice, and human brain tissue reviewed above suggest that specific neural proteases actively degrade Aβ monomers under physiological conditions. Therefore, inherited or acquired defects in Aβ-degrading proteases could chronically elevate Aβ levels in a manner mechanistically distinct from but quantitatively similar to presenilin mutations. In attempts to date to implicate Aβ proteases in familial AD, early work is pointing to IDE and uPA as candidate genes. Both genes are located on chromosome 10q, and different sets of late-onset AD pedigrees have shown linkage to DNA markers in the vicinity of these genes. In the NIMH registry of 435 families with late-onset AD, genetic linkage to markers near 10q23–q25 as well as allelic association of one of these markers with AD have been documented. (See accompanying minireview by Tanzi and Bertram for more details and references therein [this issue of *Neuron*].) IDE is located within the linkage region. However, any association of the AD phenotype with polymorphisms in the IDE gene itself remains to be examined. It is of related interest that missense mutations in IDE which decrease its ability to degrade insulin in muscle have been discovered in the inbred GK diabetic rat, a compelling model of type 2 diabetes mellitus (Fakhrai-Rad et al., 2000).

In separate experiments, an apparently distinct linkage region has been localized to chromosome10q,

roughly 40 cM centromeric to the IDE region. In this work, linkage was shown not only to AD per se, but also to elevation of plasma Aβ levels, which are transmitted as a heritable quantitative trait in some families. Of particular interest is the fact that the uPA gene maps to a position near the center of this linkage region. (See accompanying minireview by Tanzi and Bertram for more details and references therein [this issue of *Neuron*].) Work is also underway to search for evidence of linkage between AD and markers on chromosome 3, where the neprilysin gene is found.

New Directions in Elucidating Aβ Degradation

As the mechanisms of Aβ degradation receive increasing attention, it is important that we design experiments which will yield physiologically and pathologically relevant information. Biochemical experiments in which purified proteases are tested on synthetic Aβ peptides are becoming of limited interest. The ability of a particular protease to degrade naturally produced Aβ species at physiological concentrations of enzyme and substrate is paramount. While important clues can be obtained from cell culture studies, particularly with primary neurons, this work should be extended increasingly to in vivo systems. Each candidate protease will need to be tested in gene-deleted mice to determine its effects on normal Aβ economy, as has already been successfully done for neprilysin (Iwata et al., 2001). Such knockout lines as well as mice transgenic for a protease of interest should be bred to APP-overexpressing mice to discern any quantitative effects on cerebral Aβ deposits and their associated glial and neuronal cytopathology. Human brain tissue should also be studied, taking into account in which subcellular locus and under which conditions (pH, etc) a protease is expected to encounter and cleave Aβ.

Given the large percentage of familial AD cases that are not currently explained by abnormalities of Aβ production, efforts to rule in or out candidate Aβ-degrading proteases in genetic studies of AD pedigrees are crucial. Depending on the outcome of such work, searches for mutations, AD-promoting polymorphisms, or other gene defects should also be conducted in so-called "sporadic" forms of the disease. And as soon as a candidate protease or its activator(s) or inhibitor(s) are implicated genetically, new animal models which reproduce the genotype and potentially the phenotype of the defect should be developed. Such studies may reveal in relatively short order whether inherited abnormalities of Aβ degradation contribute to the genesis of AD. But even if none are found, pharmacologically upregulating certain Aβ-degrading proteases or, more plausibly, interfering with the production or processing of their natural inhibitors could have great therapeutic potential. Time will tell whether AD represents, at least in part, a clearance disease.

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