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Perspective

Therapeutic Approaches Related to Amyloid-β Peptide and Alzheimer's Disease

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Introduction and Background

Alzheimer's disease (AD) is a chronic, neurodegenerative disorder which is characterized by a loss of cognitive ability, severe behavioral abnormalities and ultimately death. It is the fourth leading cause of death in industrialized societies, preceded only by heart disease, cancer and stroke. There are currently 2.5–4.0 million AD patients in the U.S. and 17–25 million worldwide. There is no definitive treatment or cure for this devastating disease (Selkoe, 1993).

At autopsy, the AD brain is characterized by a number of important pathological changes. There is a dramatic loss of neurons and synapses in many areas of the central nervous system (CNS), particularly in regions involving higher order cognitive functions such as the hippocampus and the association cortices. In addition, levels of many neurotransmitters are greatly reduced, including but not limited to acetylcholine, serotonin, noradrenaline, dopamine, glutamate, and substance P. This dramatic and global reduction of a number of important CNS neurotransmitters is almost certainly responsible for the broad and profound clinical manifestations of AD, i.e., memory impairment, hallucinations, paranoia, restlessness, and depression (Selkoe, 1993). Currently, most ongoing clinical trials involving compounds to treat AD involve neurotransmitter replacement. In particular, cholinomimetic approaches to elevation of endogenous acetylcholine levels, either through inhibition of acetylcholinesterase (tacrine, E 2020) or by direct stimulation of cholinergic receptors (LY 246708, YM 796, and ABT 418) are being pursued vigorously.

While these approaches have resulted in modest palliative improvement in cognitive function, they do not address the underlying progressive pathology. For these reasons, much effort and enthusiasm has recently been generated toward understanding the unique pathological lesions found in AD brain tissue. Specifically, two microscopic deposits, which were originally defined by Alois Alzheimer in 1907, the neurofibrillary tangle (NFT) and the senile amyloid plaque, remain the pathological hallmarks that define the disease (Figure 1). Plaques and tangles are also highly concentrated in regions of substantial neuronal death in the AD brain, such as the hippocampus and association cortex. A debate continues as to whether plaques and tangles are harmless byproducts or tombstones of the neurodegeneration seen in AD or if they are causal in neuronal death (Selkoe, 1993).

The amyloid plaque as described by Alzheimer was isolated by Glenner and Wong (1984). It is composed of an approximately 4 kDa peptide that aggregates into a fibrillar, β-pleated structure. On the basis of its size and secondary structural characteristics, the peptide has been referred to by a variety of names including A4 peptide, β-peptide, and βA4. According to accepted amyloid nomenclature, the peptide is now referred to as amyloid-β or Aβ. Aggregated Aβ deposits appear green under polarized or fluorescent light after staining with Congo Red, and it is this classic staining characteristic which defines the deposit as being amyloid.

Aβ has been hypothesized to be causally related to AD for a number of reasons: (1) In peripheral amyloidoses (such as primary light chain disease, or secondary AA amyloidosis), large amyloid burdens strongly correlate with tissue and organ dysfunction in those

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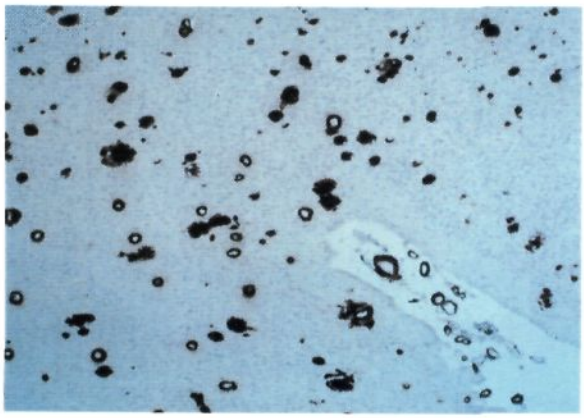

Figure 1. Formalin-fixed section of brain tissue from an Alzheimer's disease patient stained with an antibody 10D5 to the β-peptide. Note that deposits of β-peptide are seen in both the vasculature and in plaques within the parenchyma.

diseases. (2) Aβ deposition is the earliest neuropathological marker in AD and related disorders such as in Down's Syndrome, where it can precede NFT formation by two to three decades (Mann et al., 1992). (3) β-Amyloidosis is relatively specific to AD and related disorders (Selkoe, 1993). (4) Aβ is toxic to cultured neurons (Yankner et al., 1990; Mattson et al., 1992). (5) Rare missense mutations in the structural amyloid precursor protein (APP) gene cause early onset familial AD (Goate et al., 1991; Mullan et al., 1992). Notably, one mutation (Mullan et al., 1992) causes dramatic Aβ overproduction (Citron et al., 1992), while a second mutation at codon 717 of APP (Goate et al., 1991) results in an overproduction of a long form of Aβ that appears to be highly amyloidogenic. Collectively, these findings argue that strategies aimed at blockade of Aβ generation might have therapeutic value for treating AD (see also Williams et al., 1995).

Diagnosis of Alzheimer's Disease. In discussing the role of amyloid-based therapeutic approaches to AD, issues concerning the accurate diagnosis of the disease must be addressed. For example, clinical trial regimens have an inherent potential weakness of including non-AD patients in so called "AD" groups. In addition, no surrogate biological markers of disease currently exist. Such markers would be of enormous value in demonstrating efficacy of a given treatment. Because of these issues, a number of laboratories, including our own, have attempted to identify biological markers that might be helpful in diagnosing and staging AD.

The antemortem diagnosis of AD has been clinically problematic. At postmortem, AD is defined by the presence of a high density of plaques and tangles in specific brain regions in a patient who has experienced progressive dementia during life. The premortem ambiguity in diagnosis arises from the numerous other causes of dementia that are pathologically unrelated to AD. These diseases include as many as 60 disease categories that account for approximately 30−40% of all dementia cases (Schoenberg et al., 1987).

Because of the potential role of APP/Aβ in AD, several investigators have raised the possibility of whether these proteins might be useful diagnostic analytes in demented patients.

The utility of measuring levels of secreted forms of APP in the cerebrospinal fluid (CSF) of AD and control patients has been examined by several laboratories (Wagner et al., 1994; Palmert et al., 1990; Henrikson et al., 1991). These studies have demonstrated that total CSF APP levels in AD patients either do not differ from controls or are only slightly reduced. One explanation for the minor relative discrepancies among these various studies is that AD patients at various stages of disease progression are typically presented as a single group. Thus, if levels of CSF APP change during the course of the disease, this could mitigate the broad diagnostic utility of this analyte.

CSF Aβ levels have also been extensively analyzed for their potential diagnostic utility. Data from several laboratories have shown that levels of total Aβ in CSF do not differ between AD and control patients (Table 2, Shoji et al., 1992; Nakamura et al., 1995). Although these findings initially appeared counterintuitive, recent studies employing an immunoassay specific to Aβ_{1-42} demonstrated a significant reduction of this form of the peptide in AD CSF (Motter, 1995). This finding, if confirmed, suggests that the 42 amino acid form of the peptide, which is known to be more amyloidogenic than the 40 amino acid form, may be poorly cleared from brain parenchyma. In addition, this observation supports the notion that patients with AD may manifest significant plaque burden early in the disease process. Many other questions arise regarding this analyte, including its utility as a surrogate measure of brain amyloid burden and as a measure of the therapeutic efficacy of an amyloid modulating drug. These and other issues will need to be addressed in future diagnostic trials.

Animal Models of Alzheimer's Disease. AD is a uniquely human disease. For this reason, the lack of a reliable animal model of the disease has greatly hampered drug discovery. Recently, a transgenic mouse model was described that overexpresses human APP possessing a codon 717 mutation and which exhibits robust AD-like brain pathology (Games et al., 1995). Various downstream markers of AD neuropathology such as reduced synaptophysin and MAP-2 staining are seen in these transgenic mice. This finding not only substantiates the role of APP and Aβ in contributing to AD pathology but also provides an important model in which to test compounds arising from the multiple therapeutic approaches described below that are aimed at interfering in the pathological cascade seen in the disease. Although this is not the only APP transgenic mouse model (Higgins, 1994; Quon, 1991), it is the first that exhibits sufficient amyloid deposition and attendant neuropathology to enable the meaningful testing of therapeutic agents.

The availability of an animal model that mimics much of the neuropathology of Alzheimer's disease offers several opportunities for drug discovery. For example, since the PDAPP mice described by Games et al. (1995) develop robust numbers of amyloid plaques *and* an apparent loss of synapses, one can address the central hypothesis that decreasing Aβ production will result in reduced neuropathology. In addition, compounds aimed at either the blockade of Aβ aggregation or its associated neurotoxicity can now also be examined in this mouse. Even the efficacy of antiinflammatory agents can be evaluated, since there is evidence that these animals have increased numbers of activated astroglial and

Table 1. Proteases Implicated as Putative β-Secretases[a]

putative enzyme	substrate	reference
multicatalytic protease	Suc-Leu-Met↓MCA	Ishiura, 1991
multicatalytic protease	SEVKM↓DAEFRGHDSGFEVRHQKLVFFAEDVGSNK	Kojima and Omori, 1992
MP-100	DTEEISEVKM↓pNA	Schonlein, 1994
MP-100	Z-Val-LysMet-AMC	
cathepsin D	EVKM↓DAEF	Landror et al., 1994
metalloprotease (EC 3.4.24.15)	Ac-EVKM↓DAEF-NH_2	McDermott et al., 1992 Papastoitsis, 1994
calcium-activated serine protease	HSEVKM↓DAEF	Abraham et al., 1991 Nelson, 1993
rat mast cell protease	EVKM↓pNA	Nelson, 1990, 1993
cathepsin G	SEVNL↓DAEF	Sahasrabudhe, 1993
chymotrypsin	SEVNL↓DAEF	

[a] β-Secretase is defined as an enzyme capable of cleaving at the N-terminus of Aβ (Seubert et al., 1993).

Table 2. Possible Pathology-Related Diagnostic Markers in AD

marker	source	type of marker	comments	references
Aβ	CSF	phenotypic	reduced or unchanged in AD	Nakamura, 1994
$Aβ_{42}$	CSF	phenotypic	reduced in AD	Motter et al., 1995
Tau	CSF	phenotypic	elevated in AD	Vandermeeren et al., 1993 Vigo-Pelfrey et al., 1995
ApoE ε4	Blood	genotypic	increased allele frequency in AD	Strittmatter et al., 1993

microglia cells (Games et al., 1995). Despite the multiple attributes of this model, it should not be assumed that the PDAPP mouse is a perfect model of AD; nevertheless, the anatomic localization of the amyloid plaques, the progressive nature of their deposition, and the obvious neuritic involvement suggest that this model will be a rich source of information that will address key questions about the disease. For example, the temporal relationship of the different types of neuropathology seen in the mice can be readily quantified. Other supportive studies, such as lesioning experiments (e.g., nucleus basalis or entorhinal cortex) can also address the origin of Aβ and how its deposition might be modified. Finally, a precise correlation between neuropathology and behavioral deficits can be assessed. Such studies are ongoing.

Summary of Current Approaches. Therapeutic strategies aimed at the reduction of amyloid-related neuropathology in AD currently fall into three potential areas of intervention: (1) inhibition of proteolytic enzymes involved in the production of Aβ, (2) inhibition of Aβ-induced neurotoxicity, and (3) inhibition of inflammatory processes mediated by Aβ deposition. The purpose of this article is to review the current status of Aβ-related approaches to AD therapeutics.

Pathways Leading to Aβ Formation

Amyloid-β or Aβ as it is isolated from AD brain tissue is between 39 and 43 amino acids long. As sequenced from both cerebrovascular deposits as well as the classic neuritic plaques (Glenner and Wong, 1984), the peptides are heterogeneous at both the amino and the carboxy termini. However, an aspartic acid (Miller et al., 1993; Roher et al., 1993a) is the most prevalent amino-terminal residue detected (this corresponds to position 672 of the 770 amino acid form of APP). Other proteins are also known to exist within the plaque, especially α-1-antichymotrypsin (Abraham et al., 1988), but the cores of these insoluble, extracellular deposits are composed virtually entirely of β-pleated aggregates of the Aβ peptide, primarily $Aβ_{1-42}$ (Roher et al., 1993b).

The Aβ peptide is proteolytically derived from a precursor protein, APP, which is expressed as a family of polypeptides derived from a single gene by alternate splicing (Ponte et al., 1988; Tanzi et al., 1988; and Kitaguchi et al., 1988). The most common forms of this precursor protein are referred to as APP_{695}, APP_{751}, and APP_{770}, the subscripts indicating the number of amino acids in the primary transcripts. The two longer forms are widely expressed in peripheral as well as CNS tissues, whereas the 695 form appears to be largely restricted to the CNS. There are minor forms of APP, including one that lacks the entire carboxy-terminal portion of the molecule, including the Aβ region (de Sauvage et al., 1989). A more recently discovered form, termed L-APP, which lacks exon 15, was first found in lymphocytes, and now has been shown to be expressed in all tissues including brain, except for neurons (Konig et al., 1992). It is presumed that such patterns of regulated expression serve tissue-specific functions, which generally remain unresolved.

Recently, two APP-related gene products (APLP-1 and APLP-2) have been identified in brain tissue (Wasco et al., 1992). Although these proteins share biological activities with APP, they both lack the corresponding Aβ regions and hence are unable to generate Aβ. Nevertheless, understanding their processing and biological functions may shed light on processing of APP.

Studies on the metabolism of APP in tissue culture, using antibodies to various domains of the full-length protein, have revealed several features about its metabolism. These studies (Oltersdorf et al., 1989; Weidemann et al., 1989; Sandbrink et al., 1994) demonstrated that newly synthesized protein, which contains asparagine-linked sugar chains, undergoes posttranslational modifications such as O-linked glycosylation, tyrosine sulfation, and phosphorylation, prior to a secretory cleavage at the α-secretase site (Esch et al., 1990) in a trans-Golgi secretory compartment (Kuentzel et al., 1993). The large extracellular domain is then released into the cell-conditioned medium. The phenomenon of release of secreted APP is widely conserved, across species as well as tissues. In addition, so is the site of cleavage that occurs at the Lys−Leu bond within the Aβ sequence (Esch et al., 1990). Thus, the secretory cleavage of APP is not only a widespread metabolic

event, but necessarily precludes the formation of the Aβ peptide. Although "α-secretase" has not been isolated, the specificity for this enzyme lies in the actual distance of the polypeptide chain from the enzyme rather than the actual amino acid sequence (Maruyama et al., 1991; Gandy et al., 1994).

These discoveries were accompanied by the observations that APP cleavage and secretion often accounted for a relatively small proportion of total APP metabolism (Oltersdorf et al., 1989; Knops et al., 1992), indcating that other metabolic pathways in the cell must play a role in disposing of APP. One of the earlier pathways identified was termed the "endosomal-lysosomal" pathway (Knops et al., 1992; Estus et al., 1992; Golde et al., 1992). Its involvement was based on the finding that acidotropic amines and lysosomal cysteine protease inhibitors, such as leupeptin and E-64, dramatically increased intracellular accumulation of presumptive degradation intermediates of APP. Some of these intermediates, by virtue of their molecular size and antigenic reactivity, contained the entire Aβ sequence and thus were hypothesized to be amyloidogenic. Direct evidence for targeting some cell-surface APP to lysosomes was also obtained (Haass et al., 1992a), which strengthened the hypothesis that "aberrant" processing of APP in the lysosome is responsible for generating Aβ. However, whether Aβ itself was actually made or accumulated under these various lyosomotropic conditions was never established.

A major breakthrough in understanding the metabolic pathways that generate Aβ occurred with the independent discovery by three separate groups that Aβ is normally produced by both primary human cortical cultures (Seubert et al., 1992) and tissue culture cell lines transfected with wild type APP constructs (Haass et al., 1992b; Shoji et al., 1992). Aβ is secreted into the conditioned medium, where it accumulates at nanomolar levels. In addition, Aβ is not detectable in HEK 293 cell extracts, suggesting a generation process tightly coupled to extracellular release. Consistent with extracellular release, circulating soluble Aβ is clearly detectable in the CSF from both normal and AD patients (Seubert et al., 1992; Shoji et al., 1992). Immunologically purified Aβ is heterogeneous; $Aβ_{1-40}$ is the major species produced from fetal human cortical cultures, whereas material purified from pooled human CSF is heterogeneous, predominantly composed of 1−34 and 1−40 (Vigo-Pelfrey et al., 1993). The *in vitro* neurotoxic properties of synthetic $Aβ_{1-40}$ have been well characterized (see below) but the relative toxicity of 1−34 is undefined. The ability of various cell lines transfected with APP to produce Aβ suggests that the enzymatic machinery that generates these peptides from full-length APP is relatively ubiquitously distributed. Nevertheless, it is clear that cells differ in their Aβ production rates, in that cultured neurons, for example, produce at least 5-fold higher levels of Aβ than do fibroblasts (Seubert et al., 1992). It does not appear that the lysosomal degradation pathway contributes significantly to the cellular production of Aβ, since leupeptin an E-64 do not inhibit the secretion of the peptide from cultured cells (Haass and Selkoe, 1993).

Following the publication of the deduced amino acid sequence of APP, (Goldgaber et al., 1987; Kang et al., 1987; Tanzi et al., 1987, 1988; Kitaguchi et al., 1988; and Ponte et al., 1988), a number of laboratories initiated studies to purify and characterize the N-terminal cleaving enzyme of Aβ, termed β-secretase (Figure 2). Cleavage of the Met_{671}−Asp_{672} bond of the full-length protein generates the N-terminal aspartic acid of Aβ (Glenner and Wong, 1984). This cleavage was confirmed by the identification of a novel secreted form of APP, termed sAPPβ, whose C-terminus ends with $methionine_{671}$ (Seubert et al., 1993) (Figure 2). sAPPβ is distinct from the previously characterized sAPP, which extends to position 16 within the Aβ region (referred to as sAPPα). sAPPβ is released into conditioned medium of various cell lines and is also detectable in human CSF (Seubert et al., 1993).

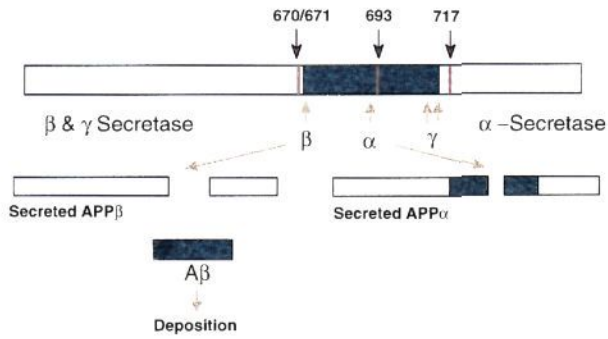

Figure 2. Schematic representation of the possible processing pathways of the amyloid precursor protein (APP). Disease-causing mutations (in red) are indicated by their respective codon numbers.

β-Secretase has not yet been convincingly identified. Several approaches have been used in attempts to identify the crucial protease(s) responsible, since blockade of this activity should lead to reduction in Aβ and the resultant amyloidosis in AD patients. The first strategy has been to follow a traditional biochemical purification approach, using short peptides as substrates (Table 1). Enzymes capable of cleaving at the methionine−aspartic acid junction site or related sites within the synthetic substrates would be implicated as possible β-secretases. Another approach has been to investigate chymotrypsin-like proteases based upon the observation that the P_1 site in APP is a methionine. Cathepsin G or chymotrypsin are examples of enzymes capable of cleaving synthetic substrates at this site (Suhasrabudhe, 1993). The third approach has been to pursue enzymes identified in brain tissue that by nature of their neuronal localization are possible enzymes involved in APP processing and Aβ formation. An example of such an enzyme is calpain 1 (Siman et al., 1989).

In summary, several approaches have been utilized to identify the Aβ N-terminal cleaving enzyme (Table 1). To date, however, the various enzymes implicated as "β-secretases" either do not cleave full-lengh APP (Abraham et al., 1991) singularly at the Met/Asp site, or cleave at multiple sites throughout the polypeptide.

Additional clues to the processing and release of Aβ have been derived from familial AD pedigrees that have point mutations in the APP molecule. The observation that a point mutation at codon 717 (resulting in the substitution of an isoleucine for valine) of APP, which strongly associates with a dominantly inherited form of early onset AD (Goate et al, 1991), strongly suggested that this mutation was pathogenic. Subsequently, 11

different families possessing a valine to an isoleucine, phenylalanine, or glycine mutation were shown to cosegregate with the disease. Separately, a novel double mutation of codons 670/671 of APP was also described in two related families in Sweden (Mullan, 1992). This mutation also segregates with early-onset disease and demonstrates classic neuropathological hallmarks. In this case, the mutation leads to the replacement of Lys_{671}–Met_{671} with Asn_{670}–Leu_{670} in APP.

Curiously, these mutations occur on either side of, but not directly within, the Aβ region itself, suggesting that they may exert their pathogenic effect via an effect on the processing events that release Aβ from the intact precursor (Figure 2). Transfection of tissue culture cells with a cDNA construct coding for the Swedish double mutation led to a 6–8-fold increase in Aβ in the extracellular medium as compared to the wild type APP construct (Citron et al., 1992; Cai et al., 1993). Analysis of the sAPP concurrently released into the medium also demonstrated an increased production of sAPPβ, (Knops et al., 1995; Haass et al., 1995), suggesting that the increased production of Aβ was due to an increased cleavage at the β-secretase site. Subsequent availability of an antibody which specifically recognizes the free carboxy-terminal sequence generated in the secreted APP after β-secretase cleavage of the Swedish APP has confirmed that a substantial portion of total s-APP is cleaved at this site (Knops et al., 1995). Thus, the increased production of Aβ appears to correlate with increased cleavage at the Leu_{671}–Asp_{672} site. Structural analysis of the Aβ purified from the extracellular medium of cells transfected with the Swedish APP mutation demonstrated that although the carboxy termini were heterogeneous, the N-terminus of the major species was Asp_{672} (Dovey et al., 1993). In addition, a significant amount of the 1–42 peptide was produced. The cellular system thus appears to provide a mechanistic explanation of the pathogenicity of the mutation–alteration of the P_1 P_2 residues at the cleavage site for β-secretase leads to a dramatic increase in endopeptidic cleavage by this enzyme, causing increased Aβ production and early-onset disease in affected individuals. An alternative explanation is that the Swedish APP mutation renders the protein susceptible to cleavage by an alternative enzyme distinct from β-secretase. Additionally, it suggests that such cellular systems are relevant models of Aβ production, and can aid in the discovery of compounds that selectively inhibit this process.

The mechanism whereby the mutation at codon 717 leads to disease has been more difficult to unravel. There is no detectable increase in total Aβ production relative to wild–type APP following transfection into human cell lines mutated with APP_{717} cDNAs (P. Seubert et al., unpublished observations). Recently, however, utilizing a sensitive ELISA that selectively measures Aβ species ending at position 42, several investigators have shown that this mutation leads to a significant increase in the proportion of this longer, more insoluble form of Aβ (Suzuki et al., 1994; C. Vigo-Pelfrey et al., unpublished observations). Thus, the APP_{717} mutations may exert their pathogenetic effect not via overproduction of total Aβ, as does the Swedish APP mutation, but rather by increasing the relative proportion of the longer form of the peptide. This likely leads to an increased rate of fibril formation due to

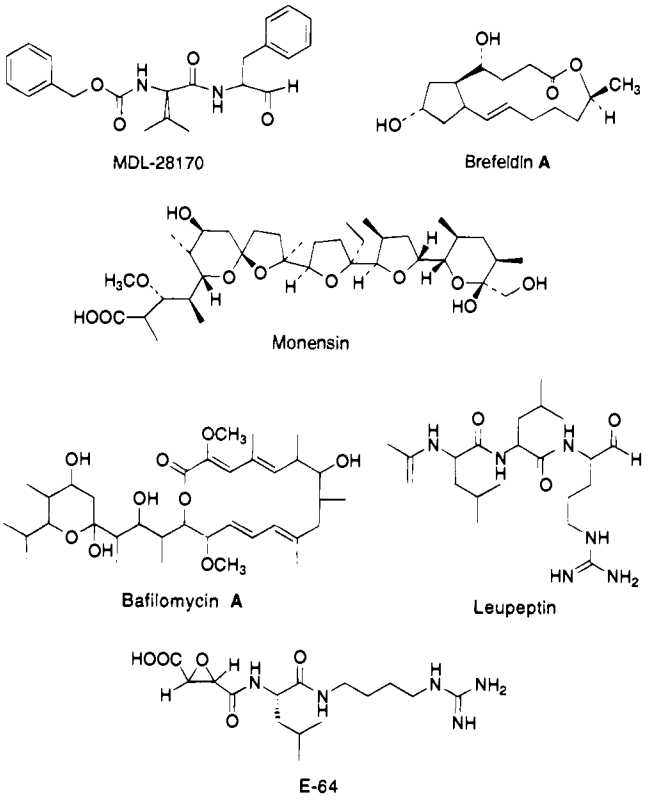

Figure 3. Compounds that have been demonstrated to either reduce soluble Aβ production (MDL 2817, brefeldin A, monensin, and bafilomycin) or stabilize C-terminal APP fragments containing Aβ (E-64 and leupeptin) in cultured cells.

greater quantities of insoluble longer forms of Aβ, thereby accelerating plaque deposition. In this regard, recent studies (Iwatsubo et al., 1994; Roher et al., 1993a) have generated conflicting results regarding the relative ratios of $Aβ_{40}/Aβ_{42}$ in AD brain tissue, with reported ratios ranging from 1:1 to 1:10. Collectively, however, the results support the notion that $Aβ_{1-42}$ is greatly overrepresented in brain relative to CSF (Vigo-Pelfrey et al., 1993) and suggest that it is poorly cleared from brain tissue.

A number of compounds have been demonstrated to reduce Aβ production in cultured cells (Figure 3). Many of these agents, such as ammonium chloride, monensin, and brefeldin probably reduce Aβ through destabilization of pH gradients or disruption of vesicular transport (Haass et al., 1993). It is clear that such agents will not likely result in development compounds, either because of inherent toxicity or nonspecificity of action. Another compound, however, that has recently been shown to potently reduce Aβ production is bafilomycin A (Figure 3). It inhibits Aβ production with an EC_{50} of approximately 50 nM (Knops et al., 1995; Haass and Selkoe, 1995), presumably through its action as an inhibitor of vacuolar H^+-ATPase. Interestingly, no negative effect was detected on cellular viability, nor was a reduction seen in sAPPα production. However, vitually complete inhibition of sAPPβ production was observed, mirroring the inhibition of Aβ production. Thus, inhibition of β-secretase activity in this particular paradigm correlates with reduction of Aβ, whereas α-secretase activity appears to be independently regulated. Although bafilomycin A alters intravesicular pH, it is not clear why this should reduce sAPPβ and Aβ. The simplest explanation is that β-secretase activity

resides in vesicles that are distinct from those involving the α-secretase APP pathway.

Recently, a peptide aldehyde (MDL 28170, Figure 3) at high micromolar levels has also been shown to reduce Aβ production (Higaki et al., 1995). Unlike bafilomycin A, this compound appears to act by stabilizing C-terminal fragments of APP that have already been cleaved by β-secretase but not yet by γ-secretase. Inhibition of γ-secretase would also be therapeutically valuable since the resulting C-terminal APP fragments would likely be shifted into lysosomal compartments and fully destroyed without the release of Aβ. It is unclear whether this compound directly inhibits γ-secretase or works through a yet to be determined mechanism.

The observation that sAPP release is dramatically stimulated (and Aβ release reduced) as a consequence of PKC activation via phorbol esters (Caporaso et al., 1992) or through coupling to muscarinic receptor activation (Nitsch, 1993a,b) has suggested that this may provide another mechanism for inhibition of Aβ production (Buxbaum, 1993; Gabuzda, 1993). The ability of muscarinic agents to stimulate APP secretion has also been shown in hippocampal rat brain slices (Nitsch et al., 1993a,b). What remains to be demonstrated is whether there is a concomitant reduction of Aβ production in this paradigm, and whether such findings can be expanded to *in vivo* studies. If so, this might lead to a relatively straightforward pharmacological route to Aβ reduction. Unrestrained activation of PKC per se has other potential drawbacks that could result in toxicity, although this would need to be thoroughly assessed. In addition, the cellular pathways mediating PKC-activated APP metabolism are still unclear. For example, it is unlikely that this shift in metabolism is due to a simple competition for the same APP pool by either α- or β-secretase activity, since the decrease in Aβ production that accompanies PKC activation can also be detected in the absence of any increase in s-APP release (Gabuzda et al., 1993). Numerous cellular metabolic pathways are activated as a consequence of phorbol ester stimulation, and there does not yet appear to be a clear understanding of which specific biochemical events mediate the effects on Aβ production.

A number of different approaches can and are being pursued to reduce the production of Aβ. From a drug discovery perspective, the most straightforward way of achieving this goal is through direct inhibition of either β- or γ-secretase. As described previously, the true identity of either of these enzymes has not yet been reported, and as a result, no specific inhibitors are currently available. Despite this obvious limitation, a number of compounds do indirectly inhibit either β-secretase (bafilomycin) or γ-secretase (brefeldin or MDL 28170). Although not fully established, these compounds (with the likely exception of MDL 28170) presumably block processing of APP to Aβ through the disruption of vesicular transport. It is anticipated that more specific inhibitors are under development at a number of pharmaceutical companies and that their identities are likely to be revealed in the near future. The ability to broadly screen for inhibitors of Aβ production in tissue culture suggests that the first compounds to enter the clinic in this area may have unknown mechanisms of action. This will eventually be followed by potent, direct inhibitors of β- and γ-secretase.

Aβ Neurotoxicity and the Pathogenesis of AD

The deposition of aggregates of Aβ (Aβ plaques) and neurofibrillary tangles (NFT) are the two brain lesions that define Alzheimer's disease. As such, it is tempting to speculate that Aβ plaques are directly linked to NFT, synaptic loss, and subsequent cognitive dysfunction associated with AD. Although a number of different forms of Aβ plaques have been described in the neuropathology literature, for the purposes of this discussion we will focus on two apparently distinct forms of Aβ plaques: (1) The classic "senile" or "neuritic" plaques consist of fibrillar, congophilic Aβ deposits that are associated with dystrophic and degenerating neurites. Due to their β-pleated nature, these Aβ aggregates stain with dyes such as Congo Red or thioflavin. In addition to Aβ, the prominent presence of activated microglia and reactive astrocytes in and around neuritic plaques in part has given rise to the hypotheses postulating an inflammatory component to AD plaque pathology (see below). (2) "Diffuse" or "preamyloid" plaques consist of amorphous, noncongophilic Aβ deposits that are not associated with significant neuronal pathology. Diffuse plaques contain few, if any, reactive microglia or astrocytes. These plaques were not appreciated until the relatively recent development of sensitive immunocytochemical reagents to visualize Aβ. The regional distribution of diffuse plaques overlaps with that of neuritic plaques in areas of prominent pathology, e.g., hippocampus and association cortices, but interestingly, diffuse plaques also occur in regions typically devoid of neuritic plaques and AD pathology, e.g., cerebellum and corpus striatum (Selkoe et al., 1993). If Aβ does play a role in mediating AD pathology, then studies addressing the biological responses mediated by Aβ should account for the apparent lack of pathology associated with diffuse Aβ plaques, and the association of neuronal pathology, reactive glia, and inflammatory markers, with the β-pleated aggregates found in senile Aβ plaques. In fact, recent *in vitro* studies indicate that the conformation and aggregate states of Aβ are critical determinants of its neurotoxic activity.

Early studies concerned with the neurotoxic properties of Aβ in tissue culture were confounded by the fact that synthetic Aβ peptides which were identical by standard biochemical characterization (reverse phase HPLC chromatography, electrospray mass spectroscopy, and amino acid sequence analysis) displayed dramatically different neurotoxic activities *in vitro* (May et al. 1992). The unwitting use of "inactive" lots of Aβ resulted in some investigators concluding that Aβ was not directly neurotoxic (Koh et al., 1990; Mattson et al., 1992). Interestingly, "inactive" or "fresh" Aβ did potentiate the neurotoxicity of a variety of other insults (e.g., excitotoxins, Ca^{2+} ionophores), a phenomenon described as "indirect toxicity" (Koh et al., 1990; Mattson et al., 1992), and the conformational dependency of this aspect of Aβ activity is relatively unexplored.

Direct *in vitro* neurotoxicity of Aβ is now thought to be at least partially dependent upon achieving a β-sheet conformation in solution (Simmons et al., 1994). Inactive (or less active) lots of Aβ are predominantly random coil in solution, which upon incubation in aqueous solutions for several days ("aging") undergoes a time-

dependent conformational transition from random coil to β-sheet, which correlates with its conversion to a neurotoxic peptide (Simmons et al., 1994). Similar peptide aging paradigms result in aggregation, a process presumably driven by the appearance of β-pleated secondary structure. Several investigators have demonstrated a direct relationship between peptide aggregation and neurotoxic potency of Aβ (Pike et al., 1991, 1993; Busciglio et al., 1992; Mattson et al., 1993). The relationships between conformation/aggregation and toxicity hold for both $A\beta_{1-39/40}$ and $A\beta_{1-42}$. Although the more hydrophobic peptide, $A\beta_{1-42}$, aggregates more readily than $A\beta_{1-40}$, once aggregated, their relative toxicities appear equivalent (Pike et al., 1991). Control Aβ peptides, e.g., reverse peptide $A\beta_{40-1}$, scrambled $A\beta_{1-40}$, and scrambled $A\beta_{25-35}$ peptides, which less readily aggregate, are in general nontoxic (Mattson et al., 1992; May et al., 1993; Busciglio et al., 1992). Thus, it appears that a β-sheet conformation and aggregation are critical determinants of Aβ neurotoxicity. The simple possibility that differential toxicity can be accounted for by differences in purity of different lots of $A\beta_{1-40}$ has been excluded (Simmons et al., 1994; May et al., 1993). Nevertheless, work with $A\beta_{1-42}$ is hampered by potential differences in purification of this extremely hydrophobic peptide (Williams et al., 1995).

Consistent with the hypothesis of conformation-dependent neurotoxicity of Aβ, other amyloid-forming peptides are also neurotoxic to cultured neurons. For example, human amylin, a 37 amino acid peptide, shares several biophysical properties with Aβ, including an ability to aggregate and form congophillic amyloid deposits in the diabetic pancreas (Copper et al., 1987; Westermark et al., 1987). The human amylin peptide is markedly neurotoxic to cultured rat hippocampal neurons (May et al., 1993) and pancreatic islet cell cultures (Lorenzo et al., 1994), while the closely related, but nonamyloidogenic, rat amylin peptide is nontoxic. Similarly, short peptide fragments synthesized from regions of the prion protein predicted to be α-helical (amino acid residues 109–122 and 113–137) instead exhibit predominantly β-pleated sheet secondary structure (Gasset et al., 1992). A prion protein fragment spanning residues 106–126 forms fibrils *in vitro* and is toxic at micromolar concentrations to cultured hippocampal neurons (Forloni et al., 1993a). However, not all prion peptide fragments which form fibrils are neurotoxic (Forloni et al., 1993b). Similarly, $A\beta_{1-28}$ which adopts a β-pleated conformation in solution (Barrow and Zagorski, 1991) and readily forms fibrils in solution (Shen et al., 1993) is uniformly nontoxic toward cultured neurons (Pike et al., 1993; Busciglio et al., 1992). Thus, it appears that a β-sheet conformation and aggregation are critical, but not absolute, determinants of Aβ neurotoxicity.

As $A\beta_{1-40}$ and $A\beta_{1-42}$ constitute the major forms of Aβ found in plaques, most investigators use them interchangeably in neurotoxicity assays. Perhaps more problematic is the widespread use of $A\beta_{25-35}$. This 11mer fragment of Aβ was initially described by Yankner and colleagues as the neurotoxic domain of the full-length peptide (Yankner et al., 1989). Part of the intrigue with this fragment was due to its partial homology to neurokinins such as substance P. Indeed, Yankner and colleagues were able to block Aβ neurotoxicity by cotreatment with substance P; conversely, treatment of immature cultured neurons with substance P antagonists mimicked the toxic effects of Aβ. While the neurotoxicity of $A\beta_{25-35}$ has been replicated by dozens of investigators, rescue of Aβ-treated neurons with substance P has proven elusive (e.g. May et al., 1992; Takadera et al., 1993; Ueda et al., 1994). This, coupled with the lack of evidence for the existence of $A\beta_{25-35}$ *in vivo*, had led us to focus our efforts on the longer forms of Aβ. In conclusion, the likelihood of Aβ interacting with the substance P receptor at present appears remote.

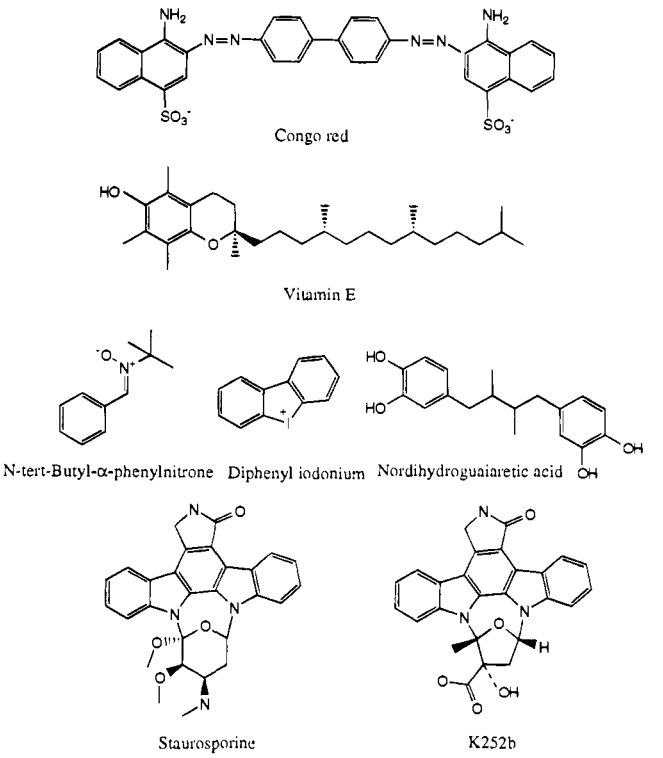

Figure 4. Compounds shown to inhibit Aβ-induced neurotoxicity *in vitro*. Both compounds thought to interact directly with the Aβ peptide and cell-mediated compounds are shown.

Given that neurotoxicity appears to be associated with β-pleated aggregates of Aβ, one therapeutic approach may be to retard or inhibit Aβ aggregation. The advantage of this peptide-based approach is that intracellular mechanisms triggered by Aβ need not be well understood. Various agents that bind to Aβ are capable of inhibiting Aβ neurotoxicity *in vitro*, providing proof of the utility of an Aβ peptide based approach to inhibition of Aβ neurotoxicity. For example, the Aβ-binding dye, Congo Red (Figure 4), completely inhibits Aβ-mediated toxicity in cultured rat cortical and hippocampal neurons and human cortical neurons (Yankner, 1995; R. Rydel and P. C. May, unpublished results). Similarly, the antibiotic rifampicin also prevents Aβ aggregation and subsequent neurotoxicity (Tomiyama et al., 1994). The neuroprotective effects of Congo Red occur at molar ratios of Congo Red: Aβ <1, suggesting that Congo Red is interacting with a subpopulation of the Aβ molecules, possibly the β-pleated aggregates of Aβ. These findings suggest that much lower concentrations of a Congo Red mimetic may effectively block the neurotoxicity associated with nanomolar Aβ *in vivo*. As discussed above, the neurotoxic properties of Aβ are shared by a variety of amyloid-forming peptides, including fragments of the prion protein (Forloni et al., 1993b) and the human amylin peptide (May et al., 1993). In this regard, Congo Red has also been shown to inhibit

the accumulation of protease-resistant prion protein (Caughey and Race, 1992) and scrapie infectivity (Caughey et al., 1993), and to inhibit human amylin neurotoxicity (R. E. Rydel and P. C. May, unpublished results). Recently, polyvinyl sulfonates have been shown to reduce another type of amyloidosis termed inflammation-associated or secondary (AA) amyloidosis (Kiselevsky et al., 1995). It is possible that such compounds might be of utility in reducing Aβ amyloidosis in AD. Unfortunately, it is not clear that such compounds will enter the CNS.

The possible disadvantage of this general therapeutic approach is that compounds of this class might accumulate and become highly toxic in the brain tissue of the AD patient. With the advent of the PDAPP mouse model (described above) that possesses robust Aβ amyloid deposition, these issues of toxicity can finally be assessed before proceeding to the clinic (Games et al., 1995).

A discovery that has added a new dimension of complexity to the understanding of Alzheimer's disease is the recent recognition that an allele of apolipoprotein E, ApoE ϵ4, appears to confer a significantly increased susceptibility to the disease (Strittmatter et al., 1993; Saunders et al., 1993). The mechanism by which this occurs is unknown, but some intriguing histopathological data (Rebeck et al., 1993; Hyman et al., 1995) suggest that this may occur through an increased deposition of amyloid in the brains of the affected ϵ4 carriers. ApoE appears to be able to bind synthetic Aβ peptide and form stable complexes (see below). It has also been suggested that the ApoE ϵ3 isoform, but not the ϵ4, is able to bind to τ, the principal component of paired helical filaments often found in large pyramidal neorons in the AD brain (Strittmatter et al., 1994). Particularly intriguing is the recent observation that the dimeric form of ApoE ϵ3 is very potent at reducing nidus formation of Aβ, whereas the ϵ4 allele might increase it (Hyman et al., 1995; Evans et al., 1995). Whether either of these *in vitro* observations lend any insight into the mechanism of action of the ApoE ϵ4 allele is not obvious at this time. Nonetheless, the genetic and postmortem epidemiological data linking the ApoE ϵ4 allele to an increased risk of Alzheimer's disease appears to be very robust, and the identification of the biochemical pathways which contribute to this linkage might initiate novel therapeutic approaches to inhibiting the pathological deposition of Aβ in Alzheimer's disease.

In this regard, Apo E binds with high avidity to Aβ *in vitro* (Strittmatter et al., 1993), affects fibril formation of Aβ (Ma et al., 1994) and has also been shown to inhibit Aβ-mediated toxicity (Whitson et al., 1994; R. E. Rydel, unpublished data). Interestingly, though the isoform of ApoE appears to influence its avidity for Aβ and its ability to promote fibril formation, both ApoE ϵ3 and ApoE ϵ4 are equally protective against Aβ neurotoxicity (R. E. Rydel, unpublished data). Apolipoprotein J (clusterin), another endogenous lipoprotein, also binds Aβ *in vitro* (Ghiso et al., 1993; Matsubara, 1995) and blocks Aβ neurotoxicity *in vitro* (Fuson et al., 1994). Recently, serum amyloid P has also been shown to bind to Aβ and block proteolysis (Tennent et al., 1995; Hamazaki, 1995).

Thus, direct or indirect manipulation of Aβ peptide aggregation appears to be an attractive therapeutic target unto itself. However, additional studies will be necessary to address the relative beneficial and detrimental effects of these and other Aβ-binding compounds on Aβ deposition, clearance, and neurotoxicity *in vivo*.

An alternative to a peptide-based inhibitor approach is to elucidate the cellular mechanism of Aβ neurotoxicity and develop therapeutics aimed at those biochemical targets. At present, the mechanism by which Aβ affects neuronal viability is not understood. Moreover, the use of disparate *in vitro* model systems, e.g., primary vs cell lines, cortical vs hippocampal cultures, immature vs mature cultures, neuronal-enriched vs mixed neuronal-glial cultures, etc., makes direct comparison of mechanistic studies between various investigators problematic at best. For example, most attempts to verify the early substance P neuroprotection observations (see above) did not replicate the precise culture conditions of Yankner and colleagues. Nonetheless, it now seems unlikely that the effects of Aβ are receptor-mediated given that it requires aggregation, it has a slow time course, and no specific binding sites for Aβ have been detected in neurons. A number of potential mechanisms are currently under investigation.

Calcium dysregulation is a predominant theme in many other neuronal insults. Accordingly, chronic exposure of neurons to aggregated Aβ results in an elevation of rest $[Ca^{2+}]_i$ and increased $[Ca^{2+}]_i$ responses to depolarization and excitatory amino acids (Mattson et al., 1992, 1993). These imaging studies have been complemented by electrophysiological studies describing the induction of irreversible, nonselective currents in cortical neurons treated with Aβ (Furakawa et al., 1994). Electrophysiological effects of Aβ also can be modeled in cell-free systems where Aβ treatment of artifical planar lipid bilayers induces channels that flux cations, particularly calcium (Arispe et al., 1993; Mirzabekov et al., 1994). One cautionary note, Mirzabekov and colleagues were able to measure channl formation in planar bilayers treated with $A\beta_{25-35}$, but observed no effect with $A\beta_{1-40}$ or $A\beta_{1-42}$ of known neurotoxic potency (Mirzabekov et al., 1994). Thus, while attractive as a hypothesis, the relationship between the channel-forming properties of Aβ and its direct neurotoxicity is not well established.

Free radical mediated neuronal damage is another emerging theme as a possible etiology for AD and other age-related neurodegenerative disorders. In this regard, it has been reported that oxidation of Aβ promotes its aggregation (Dyrks et al., 1992), that free radicals are generated by Aβ (Hensley et al., 1994), and that H_2O_2 or related peroxides mediate Aβ neurotoxicity (Behl et al., 1994). Support for the potential role of free radicals as a mechanism of Aβ-mediated damage to neuronal plasma membranes and subsequent disruption of $[Ca^{2+}]_i$ homeostasis are the findings that various antioxidants inhibit Aβ-mediated toxicity in cultured neurons and neuronal cell-lines (Figure 4). for example, vitamin E protects cultured PC12 cells from Aβ toxicity (Behl et al., 1992); catalase, *N-tert*-butyl-α-phenylnitrone, and diphenyleneiodonium inhibit Aβ neurotoxicity in primary rat cortical neurons (Behl et al., 1994); and the lipoxygenase inhibitor/antioxidant, nordihydroguaiaretic acid protects primary rat hippocampal neurons from Aβ-induced accumulation of intracellular calcium and reactive oxygen species (Goodman et al., 1994a). Furthermore, staurosporine and the staurosporine analog, K252b, protect rat hippocampal neurons from both free

radical-mediated injury and Aβ neurotoxicity (Goodman et al., 1994b). These preliminary findings await confirmation, but suggest that antioxidants and agents which attenuate intracellular responses to Aβ may also constitute useful therapeutic strategies for the treatment of AD.

Disruption of Ca^{2+} homeostasis and generation of reactive oxygen species are interrelated, and both can lead to cell death via apoptosis. A number of recent studies have pointed to apoptosis as a mediator of Aβ *in vitro* neurotoxicity (Loo et al., 1993; Forloni et al., 1993a; Gschwind and Huber, 1995). Clearly, many of the morphologic hallmarks of apoptosis are observed in Aβ-treated neuronal cultures, including nuclear condensation and membrane blebbing (Watt et al., 1994). These morphologic changes coupled with biochemical markers of apoptosis such as DNA fragmentation in Aβ-treated neuronal cultures offer compelling evidence for its relevance to some aspects of Aβ-induced *in vitro* neurotoxicity. These *in vitro* studies have prompted an examination of postmortem brain tissue from AD and other neurodegenerative diseases for evidence of apoptosis. Using recently developed sensitive techniques for detecting DNA fragments *in situ*, investigators have obtained evidence that both supports (Su et al., 1994) and refutes (Migheli et al., 1994) a role for apoptosis in AD pathology. One argument against a major role for Aβ-induced apoptosis in AD is the presence of inflammatory markers found in and around amyloid-β plaques (see below). While an inflammatory response is elicited in response to necrotic cell death, apoptosis proceeds without overt cell lysis and, thus, an inflammatory response is avoided (Wyllie, 1993). Given the ability of neurons to undergo both apoptosis and necrosis depending upon the strength of the toxic insult (Hartley et al., 1994), it remains possible that both mechanisms could operate simultaneously in neighboring cells or sequentially within an affected region.

Inflammatory Mechanisms in Alzheimer's Disease

In addition to the classic neuronal pathology associated with AD, there is growing evidence that chronic inflammatory processes contribute to the pathogenesis of AD (reviewed in Eikelenboom and Abraham, 1992; Kalaria, 1993). Numerous immunohistochemical studies have identified proinflammatory cytokines (e.g., IL-1 and IL-6), complement proteins (e.g., C1q, C4, and C3), and additional acute phase proteins (e.g., α1-antichymotrypsin) in senile plaques. Evidence suggests that immobilized Aβ can activate the classical complement cascade by an immunoglobulin-independent pathway through binding to C1q, the first component of the C1 complex (Rogers et al., 1992). It has been suggested that the Aβ:C1q binding interactions require aggregated rather than monomeric Aβ. The degree of complement activation does correlate with increasing aggregation of the peptide and is mediated by the binding of Aβ to a site within the collagen-like domain of C1q (Jiang et al., 1994). Activation of complement will result in a cascade of enzymatic reactions that ultimately leads to the formation of the membrane attack complex, C5b-9. This membrane complex, which has the capacity to lyse cells, has also been identified in dystrophic neurites and neurofibrillary tangles, specifically in the AD brain. Astrocytes and microglia can serve as a local source for these complement proteins, and moreover, Aβ is chemotactic for mononuclear phagocytes (Davis et al., 1992) and has been shown to induce complement C3 production in cultured mouse microglia (Haga et al., 1993). Thus it appears that Aβ accumulation and aggregation may directly contribute to the chronic inflammatory state associated with senile plaques, and to a lesser extent diffuse plaques. Inhibiting the complement system by blocking the Aβ:C1q interaction is one approach that may alter the course of the disease.

Aβ has been reported to promote the release of IL-1 from cultured rat astrocytes and microglia (Araujo and Cotman, 1993) and to promote the release of IL-1, IL-6, IL-8, and TNFα in human cortical mixed brain cultures, the release of IL-8 in human astrocytes, and the release of IL-1, IL-6, and TNF in human microglia (R. E. Rydel, manuscript in preparation). IL-1β and IL-6 have been shown to be elevated in brain and CSF of AD patients (Griffin et al., 1989). Another approach would be the inhibition of the synthesis of these pro-inflammatory cytokines in microglia. Since chronic overexpression of IL-6 induces neurologic disease in transgenic mice (Campbell et al., 1993), it is likely that the chronic inflammatory state associated with senile Aβ plaques contributes to neuronal pathology in AD.

Inflammatory cells such as microglia are also sources of reactive oxygen and nitric oxide (Tanaka et al., 1994; Meda et al., 1995). Reactive oxygen species are important mediators of tissue injury and are central to lipid peroxidation, protein oxidation and to the inflammatory cascade. In addition, iron levels are elevated in AD brain tissue (Gerlach et al., 1994). Iron is intimately involved with free radical reactions such as lipid peroxidation, both in the initiation and propagation phases (Halliwell and Gutteridge, 1992). Interestingly, iron, aluminum, and zinc have been shown to strongly promote Aβ aggregation (Mantyh et al., 1993). In a single unblinded study, intramuscular administration of desferrioxamine (a transition metal chelator) was reported to possibly reduce the progression of AD (Crapper, 1991). This study, however, has not yet been confirmed in a controlled and blinded fashion.

Additional evidence to support a chronic inflammatory state associated with AD is the retrospective finding of the unexpectedly low prevalence of AD in patients with rheumatoid arthritis (McGeer and Rogers, 1992). This is consistent with the fact that rheumatoid arthritis is typically diagnosed at a relatively younger age than AD, and that RA patients would be likely taking antiinflammatory therapy that might afford some protection against the inflammatory component of AD. Additional clinical studies with both indomethacin and prednisone (J. Rogers and L. Thal, respectively, personal communication) are planned to assess their possible utility in delaying progression of AD. Because of the obvious side effects of these antiinflammatory drugs, there is a clear need for better and safer agents.

Future Directions

While a number of compounds aimed at neurotransmitter replacement therapy are currently in clinical trails for treatment of AD, none is likely to impede the demise of the remaining neurons or interfere in the ongoing disease process. In this review, recent therapeutic efforts aimed at the strong association of Aβ with the pathology of the disease, notably neuritic plaque

formation and neuronal death, have been discussed. The most advanced of these approaches involves the inhibition of cleavage and release of the Aβ peptide which leads to plaque formation and eventual neuronal death. Many of the steps involved in this process still remain to be resolved. Nevertheless, prototype compounds that reduce β-peptide release and inhibit its associated neurotoxicity have been identified. In addition, a unique animal model that exhibits much of the pathology seen in AD is finally available. Collectively, there is strong reason to be optimistic that in the near future drugs directed at the neuropathology of AD will be in the clinic and will ultimately reduce or halt the cognitive decline of the AD patient.

Biographies

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