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Aβ Amyloidogenesis: Unique, or Variation on a Systemic Theme?

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ABSTRACT: For more than a century amyloid was considered to be an interesting, unique, but inconsequential pathologic entity that rarely caused significant clinical problems. We now recognize that amyloid is not one entity. *In vivo* it is a uniform organization of a disease, or process, specific protein co-deposited with a set of common structural components. Amyloid has been implicated in the pathogenesis of diseases affecting millions of patients. These range from Alzheimer's disease, adult-onset diabetes, consequences of prolonged renal dialysis, to the historically recognized systemic forms associated with inflammation and plasma cell disturbances. Strong evidence is emerging that even when deposited in local organ sites significant physiologic effects may ensue.

With emphasis on Aβ amyloid, we review the present definition, classification, and general *in vivo* pathogenetic events believed to be involved in the deposition of amyloids. This encompasses the need for an adequate amyloid precursor protein pool, whether precursor proteolysis is required prior to deposition, amyloidogenic amino acid sequences, fibrillogenic nucleating particles, and an *in vivo* microenvironment conducive to fibrillogenesis. The latter includes several components that seem to be part of all amyloids. The role these common components may play in amyloid accumulation, why amyloids tend to be associated with basement membranes, and how one may use these findings for anti-amyloid therapeutic strategies is also examined.

KEY WORDS: Aβ, βPP, amyloid, glycosaminoglycans, perlecan, laminin, apolipoprotein E, Apo E, fibrillogenesis, basement membranes, presenilins.

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I. INTRODUCTION

Beta-amyloid (Aβ), found in association with Alzheimer's disease (AD), and a familial form of cerebral amyloid angiopathy first identified in Holland, has been the subject of numerous reviews (Checler, 1995; Coria and Rubio, 1996; Hendriks and van Broeckhoven, 1996; Haass, 1996; Selkoe, 1996; Beyreuther et al., 1996). These previous reviews have focussed almost exclusively on Aβ as it relates to AD omitting a wealth of information obtained from research examining amyloid as a generic entity. This broader literature offers some unifying perspectives that are applicable to the process of Aβ deposition. The present review examines Aβ amyloidogenesis as a specific case of amyloid formation generally and with its likely sequelae.

Amyloid has always intrigued the medical profession, perhaps because it possesses an esthetic quality that many other tissue deposits lack. Amyloid's demonstration by microscopy requires the use of specific dyes, such as Congo red or Thioflavin T, and then its visualization with polarized or fluorescent light, respectively. The amyloid then glows. Congo red in particular imparts to it the characteristic red/green birefringence that has fascinated pathologists for decades. This staining property also indicated that there was an underlying organization to this deposit that became clearer with the advent of the electron microscope and the use of spectroscopic techniques. Amyloid was shown to be composed of arrays of fine fibrils usually 7 to 10 nm in diameter, which when examined by infrared or X-ray diffraction techniques revealed spectra characteristic of proteins with a predominance of crossed β-pleated sheets.

Such unique tinctotrial, ultrastructural, and protein conformational features led to the conclusion that amyloid was a single entity. The isolation and denaturation of the offending fibrils became a primary goal so that the responsible protein could be purified. Once this was available the protein sequence and its gene would be identifiable. Until 10 to 15 years ago relatively few laboratories were interested in this problem because amyloid when seen in tissue was thought to represent an uncharacterized "tombstone" of previous tissue injury with little influence on surrounding cells. Furthermore, relatively few patients had extensive systemic deposits that were thought to be required for fatal outcomes. These conclusions were premature and wrong.

The development of Pras' technique for the isolation of amyloid fibrils (Pras et al., 1968) led to the first surprise. The protein responsible for amyloid fibrils varied from one disorder to another, and each disease characterized by amyloid deposits exhibited a unique fibril protein. Where there was a common protein responsible for amyloid among several different clinical conditions, a common pathologic process became apparent, as, for example, acute inflammation in lung abscesses, cystic fibrosis, osteomyelitis, and rheumatoid arthritis, which are all antecedents of the AA form of amyloid. The first amyloid protein was isolated identified and partly sequenced (AL amyloid) by Glenner et al. in 1970 (Glenner et al., 1970; Glenner et al., 1971b). The Aβ form of amyloid was discovered, isolated, and partially sequenced also by Glenner and co-workers in 1984 (Glenner and Wong, 1984; Glenner and Wong, 1984).

II. AMYLOID AS A GENERIC ENTITY

A. Definition

Amyloid is defined by its tinctotrial, ultrastructural, and protein conformational features. These include: (1) an amorphous appearance by light microscopy when using

routine protein stains; (2) positive staining with Congo red, which when viewed in polarized light imparts to the amyloid a red/green birefringence (i.e., changing the orientation of the polarized light by 90° reverses the two colors); (3) a fibrillar appearance ultrastructurally with the fibrils being on average 10 nm in diameter and of varying length; and (4) the fibrils examined by infra-red or X-ray diffraction techniques exhibit spectra characteristic of proteins with a predominance of crossed β-pleated sheets.

B. Composition

Although all amyloids appear the same, and notwithstanding the fact that *in vitro* pure peptides may aggregate into fibrils with amyloid characteristics, *in vivo* amyloids are composed of two broad categories of constituents. The first is a disease, or process, specific protein, and the second a set of common structural components the majority of which are the building blocks of basement membranes. The former is now the basis for the classification of the amyloids (Kazatchkine et al., 1993; Husby, 1994). Among the latter are a heparan sulfate proteoglycan (HSPG), usually perlecan, laminin, type IV collagen, and serum amyloid P (SAP), all structural entites of basement membranes. How these may be related to amyloidogenesis *in vivo* is dealt with later. An additional component found in all amyloids examined to date (not just Aβ) is apolipoprotein E (apo E) (Gallo et al., 1994).

C. Classification

The classification of the amyloids is based on the disease- or process-specific protein rather than the clinical setting. The clinical classifications of the past are no longer useful. A modification of the World Health Organization classification (Table 1) illustrates the diversity of proteins and clinical settings involved. It further indicates that amyloid deposits are not based on an immunologic disorder, a notion that guided amyloid research in the past and that has unfortunately persisted in some circles.

III. CRITICAL PATHOGENETIC FACTORS

A. An Adequate Amyloidogenic Protein Precursor Pool

By far the best investigated animal model of amyloidogenesis is that which occurs in relation to acute inflammation (i.e., inflammation-associated or AA-Amyloid). This form of amyloid can be induced in a variety of rodents where it mimics the human condition closely. These animal models have also served as useful vehicles to investigate features that are common to all, or most, amyloids.

During any acute inflammatory process, regardless of the inciting agents, cytokines, such as IL-1, IL-6, and TNF, produced by activated inflammatory cells, in conjunction with hepatic nuclear regulatory elements, act as inducers of acute phase protein synthesis by the liver (Kushner and Rzewnicki, 1994). Among these acute phase proteins is serum amyloid A (SAA), the precursor of AA amyloid. In less than 24 h the quantity of hepatic SAA mRNA increases sufficiently so that 2 to 5% of total liver protein synthesis is devoted to the making of this protein (Sasaki et al., 1988), and its plasma concentration increases from 1 μg/ml to 1 mg/ml (in molar terms 80 n*M* to 80 μ*M*) (a 1000-fold increase) (Tape et al., 1988; Brissette et

TABLE 1
Classification of Amyloids

Amyloid Protein	Protein Precursor	Protein Variant	Clinical Setting
AA	SAA	SAA1/SAA2	Persistent acute inflammation
AL	κ or λ light chain		Multiple myeloma, plasma cell dyscrasias, and primary amyloid
AH	γ chain		Waldenstom's macroglobulinemia
ATTR	Transthyretin (TTR)	60 mutants normal TTR	Familial amyloid polyneuropathy (FAP) Senile systemic amyloid
AApoAI	apoAI	Arg 26	FAP Iowa
AgeI	Gelsolin	Asn 187	Familial amyloid, Finnish
Acys	Cystatin C	Gln 68	Hereditary cerebral hemorrhage with amyloid (HCHWA), Icelandic
Alys	Lysozyme	Thr 56	Hereditary systemic amyloid, Ostertag-type
Afib	Fibrinogen	Leu 554	Hereditary renal amyloid
Aβ	β-Protein precursor	Several mutants	Alzheimer's disease Down's syndrome, HCHWA Dutch
APrP	Prion protein	Several mutants	CJD,[a] scrapie, BSE,[a] GSS,[a] Kuru
Apro	Prolactin		Pituitary amyloid in the aged
Acal	(Pro)calcitonin		Medullary carcinoma of the thyroid
AANF	Atrial naturetic factor		Isolated atrial amyloid
AIAPP	Islet amyloid polypeptide		Type II diabetes, insulinomas
Ains	Insulin		Islet amyloid in the degu (a rodent)
AApoAII	ApoAII (murine)	Gln 5	Amyloid in senescence accelerated mice

[a] CJD, Creutzfelt Jacob Disease; BSE, bovine spongiform encephalopathy; GSS, Gerstmann Straussler Sheinker syndrome.

al., 1989). SAA is clearly being made for some role related to the process of inflammation (Kisilevsky et al., 1996), but it inadvertently becomes the pool from which AA amyloid is drawn.

Because inflammatory processes are so common during one's lifetime and if the enormous quantity of precursor were the only requirement for amyloid formation, virtually every individual would develop this form of amyloid at some point during their existence. However, few people develop this disorder. Furthermore, it can be shown with specific inhibitors of liver protein synthesis that

AA amyloid will not occur in the absence of SAA (Kisilevsky et al., 1979). Clearly, an adequate pool of SAA is required for AA amyloidogenesis, but this pool, even when large, is not sufficient for amyloid deposition to occur. In other forms of amyloid, such as ATTR in senile systemic, or senile cardiac amyloid, the precursor pool of constitutively synthesized normal transthyretin decreases with age (Skinner et al., 1985). Nevertheless, this reduced pool is still sufficient for amyloidogenesis to occur in later life in some individuals.

These findings with forms of amyloid precursors other than Aβ indicate that (1) an adequate precursor pool is required, but (2) increased synthesis of precursors is not a uniform feature of the pathogenesis of all amyloids, and (3) even a 1000-fold increase in the concentration of a precursor may be insufficient for amyloid deposition to occur. These findings set in relief the concentrations of Aβ (1 to 5 ng/ml, 0.25 to 1.25 p*M*) seen in most patients with Alzheimer's disease (Seubert et al., 1992; Kuo et al., 1996; Erken et al., 1996), and the 2- to 4-fold increase in Aβ concentrations associated with presenilin mutations (Martins et al., 1995; Scheuner et al., 1996; Lemere et al., 1996; Duff et al., 1996). This increase in the Aβ pool may play a role in the early development of AD, but additional factors that influence the local concentration and/or conformation of Aβ may be as, or more, relevant to the formation of Aβ tissue deposits.

B. Precursor Protein Processing and Proteolysis

Many amyloid proteins/peptides, when isolated from various forms of human amyloid deposits, represent specific stretches of sequence of larger precursor molecules. These observations together with the early demonstration by Glenner and co-workers that in vitro tryptic attack on the Bence-Jones protein (i.e., urinary L-chains in multiple myeloma) would convert it into an AL amyloid (Glenner et al., 1971a) led investigators to conclude that proteolytic processing of amyloid precursors was a critical pathogenetic factor in the initial formation of these deposits. This perspective has also guided work in Aβ amyloid formation (Greenberg et al., 1991; Tateishi et al., 1992; Evin et al., 1994).

Although there may be specific instances in which precursor processing and proteolysis play a role in a specific form of amyloidogenesis, there are cogent reasons for thinking this is not a general phenomenon, or process, in all forms of amyloid formation.

Human amyloid deposits, which usually serve as the initial source for amyloid peptide identification, have invariably been present in the patient's tissues for months to years. Ample time is therefore available for these deposited proteins to be attacked by cell or tissue proteases after, rather than before, the formation of the amyloid. Once in a fibril configuration, the region of the amyloid precursor involved in fibril structure may be less prone to proteolytic attack than other regions, leaving, over time, a peptide residue that corresponds to the peptides subsequently isolated. Conclusive proof that proteolysis of amyloid precursors is generally a prefibrillogenic event is wanting. Significant evidence, in the form of the presence of the complete protein, indicates that proteolysis is not involved in ATTR, Aβ2M, ACys, AApo A-II, and AIns (Kazatchkine et al., 1993; Husby, 1994 and see Table 1). Furthermore, in human and animal AL and AA amyloid deposits, although one peptide predominates, there is a spectrum of peptide sizes generally having common N-termini but ranging in size from intact molecules to fragments half the size of the parent precur-

sor (Westermark et al., 1987; Westermark et al., 1989; Klafki et al., 1992). In AA amyloid the spectrum of peptides may vary from one anatomic location to another in the same patient (Westermark et al., 1987; Westermark et al., 1989), possibly reflecting the complement of tissue proteases. Moreover, proteolysis of SAA has now been shown to be a post-fibrillogenic event in the genesis of murine AA amyloid (Kisilevsky et al., 1994). The *in vivo* events that "extract" the β-protein from the β-protein precursor (βPP) may be just as complicated. Three apparent protease activities have been implicated. An α-secretase, which generates protease nexin-II, cuts within the β-protein segment of βPP and thus precludes the products as being involved in Aβ amyloidogenesis (Esch et al., 1990; Sisodia, 1992; Haass and Selkoe, 1993). A β-secretase cleaves at the N-terminus of the β-protein segment of βPP releasing a large extracellular fragment and a C-terminal 100 residue fragment possessing the β-protein sequence (Haass and Selkoe, 1993), which is then internalized by the cell (Haass and Selkoe, 1993). It should be noted, however, that the N-terminal amino acid of the β-protein when isolated from Aβ amyloid plaques can vary considerably with starts at residues 1, 2, 4, 8, 11, as well as blocked peptides starting with glutamate at residue 3 (Selkoe et al., 1986; Mori et al., 1992; Miller et al., 1993; Roher et al., 1993; Lalowski et al., 1996; Tjernberg et al., 1997). The γ-secretase purportedly acts on the C-terminal fragment releasing the 1-40/42/43 peptides (Evin et al., 1994). These fragments can be identified in brain tissue fluid, cerebro-spinal fluid, plasma, and under appropriate conditions (discussed below) are thought to assemble into Aβ amyloid fibrils. The precise nature of the γ-secretase has not yet been identified, or whether it is a single protease. The variable residues at the C-terminus of the β-protein, and the intracellular compartment in which this proteolytic cut is thought to occur (endosome/lysosome) suggests that more than one protease may be involved (Joachim and Selkoe, 1992). Furthermore, recent evidence suggests that the β-protein regions of the C-100 fragments may begin to assemble in these endo-cellular compartments before the γ-secretase(s) excercise its/their function (Tjernberg et al., 1997), an intracellular reenactment of the manner in which other amyloid precursors are handled.

C. Amyloidogenic Aminoacid Sequences and *In Vitro* Fibrillogenesis

Because amyloid is a proteinaceous tissue deposit that has such a unique set of defining characteristics, it was anticipated that the amyloid protein, when isolated, would prove to be a unique protein. To date, 18 proteins have been identified as having amyloidogenic properties, and few, if any, are related to one another from the perspective of primary structure (Table 1). These observations have raised several fundamental questions. What is it about the sequence of individual proteins that endows them with amyloidogenic properties? What factors other than the primary sequence influences these proteins to take on the secondary, tertiary, and quaternary conformations that characterize an amyloid deposit?

In relation to the first question, a wealth of information has been obtained from studies examining individual amyloidogenic proteins, their mutant forms, synthetic fragments, and their propensity to form "amyloid" fibrils *in vitro* (Castano et al., 1986; Gorevic et al., 1987; Kirschner et al., 1987; Glenner et al., 1971a; Wisniewski et al., 1991; Fraser et al., 1992b; Wood et al., 1995; Hilbich et al., 1992; Kelly and Lansbury, 1994; Caputo et al., 1993; Greenberg, 1995; Ashburn et al.,

1992; Westermark et al., 1996; Campistol et al., 1996; Helms and Wetzel, 1996). These data have provided significant insights as well as confounding issues. Several examples are illustrative.

SAA, in reality, represents a family of 4 to 5 genes (Uhlar et al., 1994). Of these, SAA1 and SAA2 are acute phase proteins produced by the liver and that serve as the precursors to AA amyloid (Kushner and Rzewnicki, 1994). In mice only SAA2 is the precursor (Meek et al., 1986; Hoffman et al., 1984), and it differs from SAA1 by only 9 residues in 103. Substitutions at residues 6 and 7 seem to confer the amyloidogenic properties on this protein, as shown with synthetic 15mers corresponding to the N-termini of each protein (Westermark et al., 1992). Furthermore, strains of mice exist in which the acute phase forms of SAA are hybrids of 1 and 2 with the amino-terminal residues corresponding to SAA1 (Sipe et al., 1993). These animals are resistant to AA amyloid formation. In man, both SAA1 and SAA2 are amyloidogeneic and they differ in sequence toward the C-terminus (Liepnieks et al., 1995). These observations point out the need for an amyloidogenic sequence but do not indicate exactly how this sequence is responsible for AA amyloidosis. Furthermore, the amyloidogenic sequence in SAA is unique to SAA and is different in other amyloid precursors. Moreover, as indicated above, the simple presence of a large and adequate pool of SAA is necessary but not sufficient for amyloid to occur.

A similar scenario exists in adult onset diabetes (type II diabetes), where 90% or more of patients with this disorder have amyloid deposits in the Islets of Langerhans (Cooper and Tse, 1996). This form of amyloid is characterized by the presence of islet amyloid polypeptide (IAPP) (Cooper and Tse, 1996), also called amylin. IAPP is a 37mer whose amyloidogenic properties reside in residues 25 to 29, the sequence being -GAILS- (Westermark et al., 1990a). There is a strong correlation between the presence of this sequence in a species' IAPP, their susceptibilty to the development of type II diabetes, and the fibrillogenic properties of synthetic IAPP fragments possessing this sequence (Ashburn and Lansbury, 1993). All human IAPP possesses this sequence, no mutant forms have been reported. However, only a small proportion of people develop this type of pancreatic amyloid, indicating that factors in addition to the permissive amyloidogenic sequence are required for amyloid formation *in vivo*.

Transthyretin (TTR) is the plasma protein responsible for the transport of thyroxine and the retinol-binding protein (Kanda et al., 1974; Robbins and Rall, 1960; Kanai et al., 1968), and thus vitamin A. Human TTR is composed of four identical subunits of 127 residues (Kanda et al., 1974). TTR is responsible for senile systemic and senile cardiac amyloid and a large variety of familial amyloidotic polyneuropathies (FAP) (Benson and Uemichi, 1996; Saraiva, 1996). At present, of the order of 60 different TTR mutations are associated with FAP syndromes (Benson and Uemichi, 1996), and "wild-type" TTR is associated with both senile systemic and senile cardiac amyloid (Cornwell et al., 1988; Westermark et al., 1990b). The specific amyloidogenic sequence(s) of TTR has not been identified. The mutations are scattered along the length of the primary sequence, but no specific location seems to be strategically associated with its amyloidogenic properties (Benson and Uemichi, 1996). Nevertheless, there is a correlation between the location of the mutations and the effect they have on TTR tetramer stability (Mccutchen et al., 1993; Mccutchen et al., 1995; Miroy et al., 1996). These, as well as additional studies, have indicated that there are conformational intermediates (i.e., denaturation states) that exist between the native conformation of an amyloid precursor and the conformation as

found in an amyloid fibril (Kelly, 1996; Lai et al., 1996). The equilibria between these forms may be influenced by "nucleating agents" and a variety of factors, which in chemical terms may lower an energy barrier over which a conformation must pass to reach a new minimum energy state — the amyloid fibril (Jarrett and Lansbury, 1992; Jarrett and Lansbury, 1993; Kelly and Lansbury, 1994; Kelly, 1996; Suarez and Kelly, 1993; Evans et al., 1995; Lansbury, 1995). Similar processes appear to be operating in Aβ amyloidogenesis and are considered in greater detail below.

Many of the above-mentioned studies and their interpretation should be viewed with caution. Useful in establishing principles that are likely operative in amyloid formation, the protein concentrations, solvents, and pH conditions used in gathering these data are frequently extreme and far from physiological.

D. Nucleating Particles (Amyloid-Enhancing Factor [AEF], and Prions)

In vitro fibrillization studies have been useful not only in identifying "amyloid permissive" sequences, but also kinetic properties of fibril aggregation. As indicated above, the kinetic data are consistent with the native precursor being in equilibrium with intermediate conformational states, and that the shift in equilibrium toward fibrils can be significantly influenced by "seeding" the process with a preexisting fibril or protofibril. The process is reminiscent of crystal formation in salt solutions. These observations likely explain *in vivo* data with AA amyloidosis and prion disorders that have been known for some time (Kisilevsky and Boudreau, 1983).

Amyloid-enhancing factor (AEF), recently reviewed (Kisilevsky et al., 1995), was originally described in the context of AA amyloidosis (Werdelin and Ranlov, 1966). It is defined in functional terms as a factor that dramatically shortens the induction time for AA amyloid development during inflammatory processes, from 2 to 3 weeks to 36 h or less. With standard AA induction protocols, AA amyloid appears in tissues such as spleen and liver in 14 to 21 d, and AEF appears in these tissues 24 to 48 h before the amyloid is seen (Axelrad et al., 1975; Axelrad et al., 1982). If an AEF preparation is passively transferred by intravenous injection to recipient mice and is followed by an inflammatory stimulus (to induce the hepatic synthesis of SAA, the AA precursor) AA amyloid appears in spleen and liver in 18 to 36 h (Axelrad et al., 1982; Graether et al., 1996). Furthermore, the kinetics of AA amyloid deposition in these tissues has a sigmoid curve identical in shape to that seen with the standard induction regimens (Kisilevsky and Boudreau, 1983). These features are consistent with AA amyloidogenesis requiring a nucleating event that if passively introduced significantly shortens the onset of the process. The longer period of induction with standard protocols represents the lag phase for AEF/nucleating events to occur endogenously.

Ample evidence indicates that AEF is not a specific amyloid fibril, as tissue extracts from at least four forms of amyloid possess AEF activity, these being AA, AL, ATTR, and Aβ (Varga et al., 1986; Ali-Khan et al., 1988). Furthermore, synthetic IAPP and Aβ have AEF activity but only if they are in a fibril conformation (Ganowiak et al., 1994).

Many of the characteristics of AEF are reminiscent of exogenously delivered prions that when injected apparently serve as a template for endogenously synthesized prion proteins to reconfigure into a pathologically active agent (Prusiner, 1996; Coria and Rubio, 1996; Ghetti et al., 1996; Kisilevsky

et al., 1995; Axelrad et al., 1982). Both AEF and prions lose activity after denaturation and proteolytic digestion, and neither is sensitive to nuclease degradation. Injection of each of these factors into appropriate anatomic sites sets the stage for subsequent amyloid deposition, albeit of different types, AEF to systemic AA amyloid and prions to CNS APrP amyloid. In each case the amyloid is derived from an endogenous precursor rather than the exogenous nucleating protein. In contrast to prions, AEF exerts its effect in a matter of hours, whereas that of prions requires weeks to months. The latter may be a reflection of the relative availability of amyloid precursor in the two disorders.

E. Perlecan, Laminin, Collagen IV, Serum Amyloid P, Apo E, and the *In Vivo* Amyloidogenic Microenvironment

Over the last 15 years, it has become apparent that *in vivo* amyloid is composed of more than just the defining amyloidogenic protein. A set of common structural constituents has been identified in most forms of amyloid regardless of the amyloidogenic protein involved. These include serum amyloid P (SAP), perlecan (the basement membrane form of heparan sulfate proteoglycan [HSPG]), laminin, collagen IV, and apolipoprotein E (apo E) (Kisilevsky, 1991). The latter is a consituent of every form of amyloid examined to date, not just Aβ (Gallo et al., 1994). With the exception of apo E, the remaining components are all building blocks of extracellular basement membranes (ECBM). Thus, *in vivo* amyloid consists of a disease-specific (or pathologic process specific) protein and a set of common constituents. When first deposited, amyloid is usually adjacent to basement membranes (Schultz and Pintha, 1985; Schultz et al., 1985; Linke et al., 1989; Yamaguchi et al., 1992; Horiguchi et al., 1992). A significant body of evidence has accumulated, indicating that interactions between the common components on the one hand, and the amyloidogenic protein on the other, are important in amyloidogenesis. Of particular interest are the glycosaminoglycans (GAGs).

GAGs have been known to be part of amyloid deposits for more than 70 years (Boyd, 1932). Their potential significance in amyloidogenesis did not emerge until 10 to 12 years ago when the temporal appearance of GAGs was examined relative to the appearance of the AA protein in AA amyloid (Snow and Kisilevsky, 1985). The availability of conventional and rapid AA-amyloid induction protocols allowed one to examine whether the appearance of GAGs in amyloid preceded was coincident with or followed the deposition of the AA protein, and, furthermore, whether the presence of the GAGs was an epiphenomenon of the acute inflammation required for this type of amyloid induction. It became apparent that GAGs were deposited coincidentally with the AA protein no matter how the protocol was varied, what inflammatory stimulus was used, and what tissue was involved (Snow and Kisilevsky, 1985). Parenthetically, similar data are available for the involvement of SAP (Baltz et al., 1986). Inflammation on its own without the other elements of the induction protocol failed to elicit either AA amyloid or GAG deposition. Therefore, it became of interest to characterize the chemical nature of the GAGs and what influence they had on amyloidogenic protein/peptide conformation.

Light microscopic studies with immunohistochemical and histochemical techniques established that the GAG was heparan sulfate (HS) (Snow and Kisilevsky, 1985; Snow et al., 1987a), which was confirmed

chemically (Snow et al., 1987a). This HS was identified as part of perlecan (Snow et al., 1988; Snow et al., 1991), and the deposition of perlecan coincided anatomically, quantitatively, and temporally with the appearance of the AA fibrils (Snow et al., 1987a). This was also shown to be true for laminin and collagen IV (Lyon et al., 1991). Ultrastructural histochemical and immunogold studies revealed that HS and perlecan were constituents of the amyloid fibril *in situ* (Snow et al., 1991), as were laminin and collagen IV (Nass and Kisilevsky, unpublished data). High-resolution electron microscopy has suggested that the organization of the amyloid fibril *in situ* may well be different from that seen *in vitro* (Inoue and Kisilevsky, 1996). A preliminary model of the *in vivo* organization of the various AA amyloid structural components into AA amyloid has been proposed (Inoue and Kisilevsky, 1996). The general structure of the amyloid fibril *in situ* is similar to a microfibril and possesses a central core composed of SAP wrapped in a layer of chondroitin sulfate proteoglycan (CSPG). This in turn is covered by HSPG intimately associated with filaments of AA protein. The location of the laminin and collagen IV has not yet been determined. This structural organization is not unique to the AA form of amyloid, as a similar organization has been observed with the Aβ amyloid of Alzheimer's disease, the Aβ2M of dialysis associated amyloid, and the ATTR amyloid of FAP (Inoue, Ohashi, Saraiva, and Kisilevsky, unpublished data).

In mouse models, the quantity of tissue HS increases in parallel with the AA protein (Snow et al., 1987a). HS or HS-like GAGs have been shown to be part of every form of amyloid so far examined (Snow et al., 1987b; Magnus et al., 1989; Magnus et al., 1991; Stenstad et al., 1991), and perlecan has been found in forms as diverse as AA, Aβ, ATTR, and AIAPP (pancreatic amyloid in adult-onset diabetes) (Young et al., 1989; Young et al., 1991; Young et al., 1992).

Where studied, high-affinity binding (Kds in the 1 to 10 n*M* range) has been shown between individual ECBM proteins (such as perlecan or laminin) and amyloid proteins such as SAA, β-protein, or βPP (Narindrasorasak et al., 1991; Narindrasorasak et al., 1992; Narindrasorasak et al., 1995; Leveugle et al., 1994; Ancsin and Kisilevsky, 1997). Furthermore, consensus HS binding sequences have been identified in many amyloid precursors among which are SAA, apo A-I, apo A-II, apo E, pre-pro-IAPP, and βPP (Kisilevsky, 1989; Cardin and Weintraub, 1989; Multhaup, 1994; Clarris et al., 1997). In the latter one such sequence is found within the β-protein segment itself (Kisilevsky, 1989; Fraser et al., 1992a).

Of particular importance to the pathogenesis of amyloid is the influence of HS on amyloid protein/peptide conformation. Using circular dichroism HS and/or heparin when incubated *in vitro* with the β-protein or SAA rapidly increases their β-sheet content (McCubbin et al., 1988, and Fraser and Kisilevsky, unpublished data). In the case of the β-protein, this is followed rapidly (minutes) by the generation of Aβ fibrils (Fraser and Kisilevsky, unpublished data). More striking yet are the observations with murine SAA. HS, when incubated with isoforms 1, 2, or a nonamyloidogenic hybrid of the two, confers a marked increase in β-sheet structure only on isoform 2, the relevant precursor (McCubbin et al., 1988; De Beer et al., 1993). No other GAG examined possesses this property. Thus, in the case of SAA only the relevant isoform and the relevant GAG interact to increase the β-sheet content of SAA.

Further evidence implicating the ECBM proteins in amyloidogenesis comes from RT-PCR analyses of perlecan, laminin B2 chain, and the α_1-collagen IV chain splenic mRNA levels during rapid AA-amyloid induction (Ailles et al., 1993; Woodrow et al., 1994). In the mouse the spleen is the first organ to

manifest AA amyloid. The aforementioned splenic mRNAs all begin to increase within 18 to 24 h of the amyloid induction protocol and only in those animals receiving the full induction protocol. Amyloid is not visible histologically until 18 to 24 h later and again occurs only in those animals receiving the full induction protocol. None of the appropriate controls manifest this increase in mRNA or amyloid, suggesting that this change in ECBM protein gene expression is closely tied to, and may precede, AA amyloidogenesis. Preliminary data with laminin mRNA levels in AD patients also suggest that there is a significant increase in this mRNA that is not seen in age-matched controls (Murtomaki et al., 1992). Such changes have not been observed with CNS perlecan mRNA levels in AD (Maresh et al., 1996).

IV. REQUIREMENTS FOR *IN VIVO* AMYLOIDOGENESIS

The foregoing considerations define a set of requirements for amyloidogenesis *in vivo*.

1. An adequate precursor pool is necessary but is not sufficient.
2. A "permissive amyloidogenic sequence" is needed.
3. Precursor conformational instability may be initiated in some cases by mutations, protease processing, or microenvironmental factors, which in turn generate intermediate conformations from which fibrillogenesis may proceed.
4. A "nucleation or seeding process" helps shift the equilibrium in favor of fibril formation.
5. These latter two steps may involve structural components of the basement membrane and apo E.

V. AMYLOID AND ALZHEIMER'S DISEASE

Amyloid plaques (also referred to as senile or neuritic plaques) and neurofibrillary tangles have been recognized as cytopathological signposts of Alzheimer's disease (AD) since the turn of the century. Intensive investigations within the last decade have significantly advanced our understanding of the biochemical and biophysical pathways responsible for their formation. Amyloid occurs extracellularly as filamentous protein deposits often accompanied by dystrophic neurites. In contrast, the accompanying neurofibrillary tangles (NFT) are assembled intraneuronally and are the product of morphologically and biochemically distinct aggregates of paired helical filaments (PHF). AD is the most common form of dementia affecting a large percentage of the aging population, making its early diagnosis and treatment a growing concern. This now appears to be an attainable goal considering the current advances in understanding AD pathogenesis and the role of amyloid in this disorder.

A. The Amyloid β-Protein and Its Precursor

Mature amyloid accumulates extracellularly as senile plaques often surrounded by dystrophic neurites and as cerebrovascular amyloid (CVA) in the walls of cortical and meningeal vessels. Amyloid stability even under harsh conditions was one of the more insurmountable problems in the initial characterization of its constituent proteins. However, solubilization of isolated plaque cores in formic acid and sequencing of the principal component revealed that AD amyloid was

composed of a small 40 to 42 residue protein of novel sequence (Glenner and Wong, 1984; Suhr et al., 1995; Hucul et al., 1985; Masters et al., 1985b). Nomenclature for the AD-related amyloid protein has varied from the original A4 to β-amyloid protein (βAP), AP-β, A4/β and β/A4 and consensus on this issue has led to amyloid-β protein (Aβ) being the generally applied term (Husby et al., 1991).

Aβ sequencing provided a catalyst for Alzheimer research and quickly led to cloning of the encoding cDNA (Kang et al., 1987) and subsequently the gene. Remarkably Aβ represents an internal sequence derived from a large integral membrane Aβ precursor protein (βPP) having a receptor-like appearance with a large extracellular segment, a single transmembrane-spanning domain and a cytoplasmic tail ([Kang et al., 1987; Selkoe et al., 1988; Dyrks et al., 1988]; see Figure 1). Aβ constitutes 28 residues N-terminal to the extracellular-transmembrane interface as well as 12 to 14 residues of the intramembrane sequence. Amyloid-generating βPP appears to be provided locally by neurons rather than being delivered from systemic sources (Masters et al., 1985a; Probst et al., 1991). βPP contains a number of additional features, such as *O*- and *N*-glycosylation (Weidemann et al., 1989), and tyrosine sulfation sites (Schubert et al., 1989b), cysteine and acidic-rich domains, serine/threonine phosphorylation sites (Buxbaum et al., 1990) within the cytoplasmic region, and at least two lysosomal targeting consensus sequences (Chen et al., 1990; Harter and Mellman, 1992), which may be important in some aspects of βPP processing. βPP maps to chromosome 21 (St. George-Hyslop et al., 1987a), which explains the extensive amyloid deposition seen in Down's syndrome due to the consequences of gene dosage and increased Aβ levels (Royston et al., 1994). Unlike Down's syndrome, dosage effects do not appear to be relevant to the majority of sporadic AD cases (St. George-Hyslop et al., 1987b; Tanzi et al., 1987a; Podlisny et al., 1987).

Further investigations have determined that βPP_{695} is a member of a family of proteins including βPP_{751} and βPP_{770}, which arise from alternate transcription of a Kunitz-like protease inhibitor (KPI) domain (Tanzi et al., 1988; Kitaguchi et al., 1988; Ponte et al., 1988). In addition, a C-terminal truncated form, βPP_{563}, has also been cloned (Desauvage and Octave, 1989) that encodes a soluble βPP isoform in which an Alu-like repeat has been substituted for the C-terminal and transmembrane domains. Soluble extracellular ßPP derivative have been identified as heparin-activated inhibitors of coagulation factor XI_a (Smith et al., 1990), and as protease nexin II (PN-II) (Oltersdorf et al., 1989; Van Nostrand et al., 1989). Subsequent cloning of homologous βPP-like proteins (APLP1/APLP2) indicates that βPP is a member of a family of related integral membrane proteins (Sprecher et al., 1993).

The alternatively spliced βPP isoforms are expressed in a number of tissues throughout the body (Tanzi et al., 1987b), but their function is not fully understood. The release of the soluble, protease-inhibitor containing forms from platelet α-granules coupled with the inhibition of Factor XI_a is consistent with a role for systemic βPP in regulating coagulation (Van Nostrand et al., 1990; Gardella et al., 1990). Its action in the CNS is less evident, but it has been reported to have a number of potential functions such as a growth factor (Saitoh et al., 1989; Araki et al., 1991), a meditator of cell adhesion (Schubert et al., 1989a; Breen et al., 1991), a regulator of neurite outgrowth (Milward et al., 1992), a neuroprotective agent that functions by controlling intracellular calcium levels (Mattson et al., 1993), a modulator of Cu^{2+} homeostasis (Multhaup et al., 1996), and/or a G_o-coupled receptor regulating second messenger signaling (Nishimoto et al., 1993). Elimination of βPP function either by the introduction of antibodies to the extracel-

lular domain or via antisense constructs (Breen et al., 1991; Ancsin and Kisilevsky, 1992) decreases the adhesiveness of neuroblastoma cells by reducing both cell-substrate and cell-cell interactions. This may reflect an *in vivo* function of promoting neurite extension by controlling interactions with extracellular matrix proteins, such as laminin and heparan sulfate proteoglycan, for which βPP has high-affinity binding sites (Narindrasorasak et al., 1991; Narindrasorasak et al., 1992; Williamson et al., 1996; Beher et al., 1996). In addition, metal ions such as zinc and copper have been inplicated in mediating in βPP function and metabolism (Bush et al., 1994a; Multhaup et al., 1996). An impairment of βPP function in AD may also occur as shown by the reduced substrate adhesion of fibroblasts derived from AD patients (Ueda et al., 1989). In addition, fibroblasts transfected with antisense constructs displayed poor growth characteristics that could be reversed by the addition of βPP-containing conditioned medium that has led to speculation that βPP may act as a growth factor (Saitoh et al., 1989).

B. βPP Processing and the Amyloidogenic Aβ Pathway

Proteolysis of βPP culminating in the release of Aβ is a critical step in the development of amyloid plaques. Cleavage of βPP with consequences for amyloid formation occurs at three principal locations, termed the α-, β-, and γ-secretase sites (Figure 1). The α- and β-secretases (Sisodia et al., 1990; Esch et al., 1990; Seubert et al., 1993) are responsible for the N-terminal cleavage events with α-secretase activity at Lys-16 of Aβ (Lys-687 using $βPP_{770}$ numbering), resulting in the release of a 3-kd truncated Aβ fragment termed p3. This is referred to as the nonamyloidogenic pathway. The amyloidogenic pathway is mediated by β-secretase, which cleaves at Met^{671}-Asp^{672} site (using $βPP_{770}$ numbering) which releases the full-length 4 kd Aβ peptide.

Initally, the α- or β-cleavages result in the release of soluble βPP species (sβPP) and the residual C-terminal fragment containing the transmembrane and cytoplasmic domains. βPP C-terminal fragments are found in both glia and neurons and serve as the source of Aβ peptides. This amyloidogenic fragment is heterogeneous and can exist as five distinct species within the brain (Leblanc et al., 1997). Final processing of the βPP is mediated by the γ-secretase, which cleaves within the transmembrane domain. Sites of γ-cleavage are heterogeneous and can produce Aβ fragments varying from 39 to 43 residues in length. This has considerable significance for the pathogenesis of amyloid plaques, as the longer Aβ isoforms (1 to 42 and 1 to 43) display a greater tendency to aggregate and may be the "seed" for fibril formation. The metabolic origins of these different γ-secretase activities is unclear, but protease inhibitor studies have indicated that separate pathways are responsible for the generation the 39 to 40 and 42 to 43 isoforms (Higaki et al., 1995; Citron et al., 1996).

In addition, the α- and β-secretases may be responsible for the appearance of morphologically distinct amyloid plaques. Aβ deposits occur as two classes, the non-fibrillar 'diffuse' plaques, which do not have the characteristics of amyloid, and the mature amyloid fibril containing senile plaques (Yamaguchi et al., 1989; Joachim et al., 1989; Rozemuller et al., 1989; Wisniewski et al., 1989). Diffuse plaques are only recognized using immunocytochemical techniques, often accompanied by formic acid treatments to expose epitopes, while mature plaques are easily identified using standard amyloid histochemical procedures (e.g., Congo red staining). It has been proposed, without adequate data, that this represents a linear progression involving successive steps from

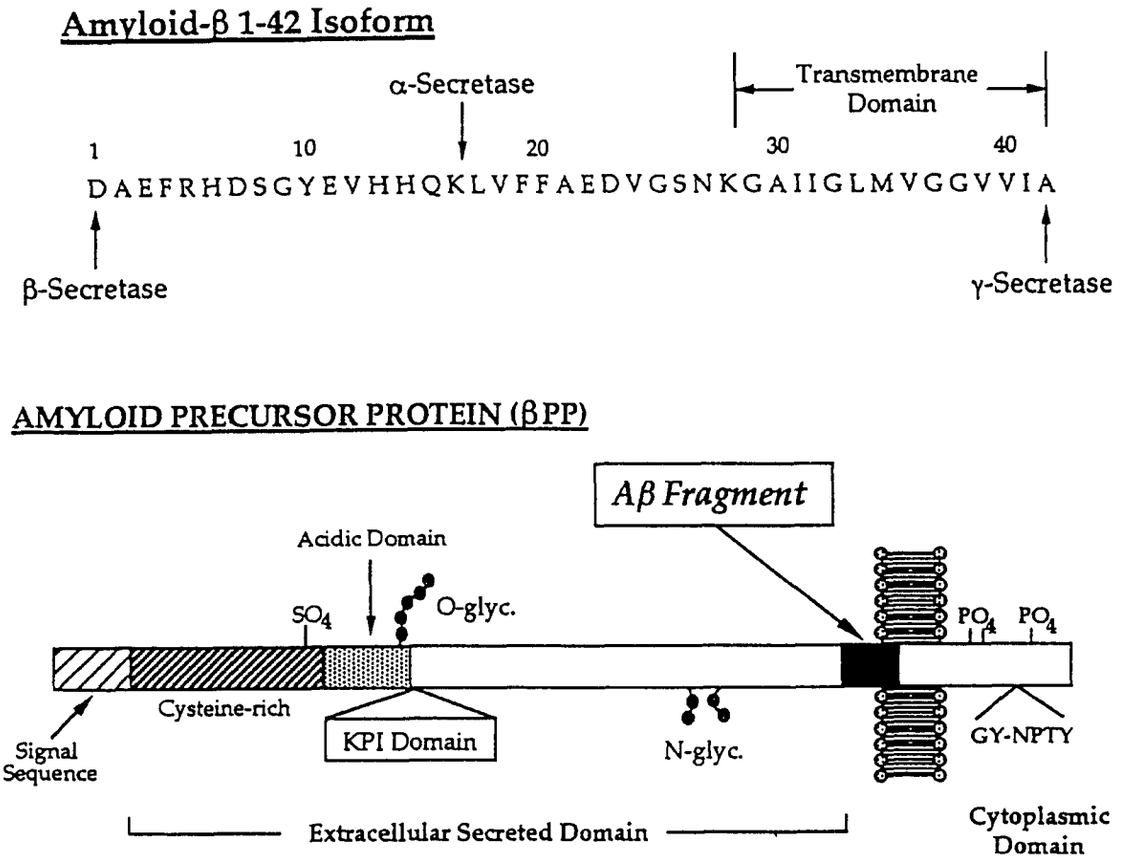

FIGURE 1. Sequence of the Aβ 1-42 protein indicating the secretase sites and the membrane boundary. Schematic diagrams of the amyloid precursor protein indicating its general feature and the location of the Aβ fragment within the precursor sequence.

βPP cleavage to form amorphous deposits that undergo a transition to fibrillar structures. However, an alternate unexplored possibility may be that diffuse plaques are merely the aggregated p3 byproduct of the secretory pathway, which would make it unlikely that they are precursors of mature amyloid (Gowing et al., 1994; Lalowski et al., 1996). In this case, diffuse and mature plaques would then be the end-stages of the distinct α- and β-secretase pathways.

The actual secretases have not been isolated, although a number of possibilities, for example, chymotrypsin-like (clipsin) (Nelson and Siman, 1990), calcium-dependent (Abraham et al., 1991; Razzaboni et al., 1992; Saito et al., 1993), and metalloproteases (Mcdermott and Gibson, 1991; Miyazaki et al., 1993) have been shown to cleave βPP. The cellular locations of the proteases responsible for βPP secretion are not known but may be facilitated by a membrane-bound proteases at the cell surface (Sisodia, 1992), or, considering the insensitivity of βPP secretion to exogenous protease inhibitors, proteolysis may be controlled by an intracellular secretase (De Strooper et al., 1992; Haass et al., 1993). This is supported by the observation that Aβ accumulates within intracellular compartments (Haass et al., 1992a; Shoji et al., 1992; Wertkin et al., 1993; Leblanc and Gambetti, 1994).

βPP undergoes a complex metabolism, and its processing may involve a number of routes, including regulated secretory and amyloidogenic pathways. Evidence such as the colocalization of lysosomal proteases with amyloid plaques, inhibition of Aβ and Aβ-containing fragments by lysosomal inhibitors (e.g., chloroquine and $NHCl_4$) (Cataldo and Nixon, 1990; Cataldo et al., 1991; Haass et al., 1992a; Golde et al., 1992; Estus et al., 1992; Caporaso et al., 1992a; Caporaso et al., 1992b) has implicated the endosomal/lysosomal pathway. However, this may not necessarily be a required step, as βPP secretion and Aβ production is observed in truncated βPP constructs that lack the lysosomal targeting sequences (Haass et al., 1993; De Strooper et al., 1993). On this basis, proposals have been made that βPP processing may also be occurring in late Golgi compartments. Alternatively, Aβ may be generated by βPP trafficking to the lysosome considering that intact protein is observed in lysosomal compartments (Benowitz et al., 1989). Although it is not clear if this is the result of a direct βPP shuttling to lysosomes or the internalization of intact protein from the cell surface (Weidemann et al., 1989; Haass et al., 1991).

Phosphorylation of the βPP C-terminus has been implicated in the processing pathways, as shown by treatment with either by phorbol esters or okadaic acid, stimulated βPP release (Caporaso et al., 1992a; Caporaso et al., 1992b). This is accompanied by a concomitant decrease in Aβ formation, indicating that the α-secretory pathway is favored. A number of kinases have been shown to be active in this pathway (Suzuki et al., 1994; Aplin et al., 1996), including protein kinase C, calmodulin kinsase II, cyclin-dependent kinases (p34cdc2), and glycogen synthesis kinase-3β. From these observations it is apparent that shifts from one pathway to another determines the likelihood of Aβ and presumably senile plaque formation. Thus, identification of Aβ-specific proteases and their modulators may be key therapeutic targets for controlling amyloid formation.

C. Aβ Structure and Fibrillogenesis

Structural studies of Aβ have been advanced through the use of *in vitro* synthetic peptide models (Castano et al., 1986; Kirschner et al., 1987; Gorevic et al., 1987; Halverson et al., 1990; Fraser et al., 1991; Hilbich et al., 1992; Barrow and Zagorski, 1991; Barrow et al., 1992; Burdick et al., 1992; Fraser et al., 1992b; Vinters, 1991), which spontaneously assemble into amyloid-like fibers (Figure 2A,B). Comparative spectrocopic and X-ray diffraction studies between synthetic and native AD amyloid have indicated they are folded into β-pleated sheet conformations with the individual strands arranged in a cross-β configuration. A number of models can be constructed for the fibrils, but it is apparent that the major β-sheet regions are provided by the hydrophobic core (residues 17 to 21) and tail (residues 29 to 42). Further studies (Inouye et al., 1993; Blake and Serpell, 1996), which fit these models to the X-ray diffraction data and the utlilization of solid-state NMR (Lansbury et al., 1995), are continuing to realize a more defined atomic resolution structure of *ex vivo* Aβ fibrils.

A similar organization of other amyloid fibrils is observed from a number of unrelated proteins, which raises the possibility that they represent a structural superfamily of proteins. Given the substantial differences in physical and chemical characteristics among amyloidogenic proteins (e.g., wide range of molecular weights (3 to 30 kd) and no apparent sequence similarity), it is surprising that the end-point fibers are morphological-

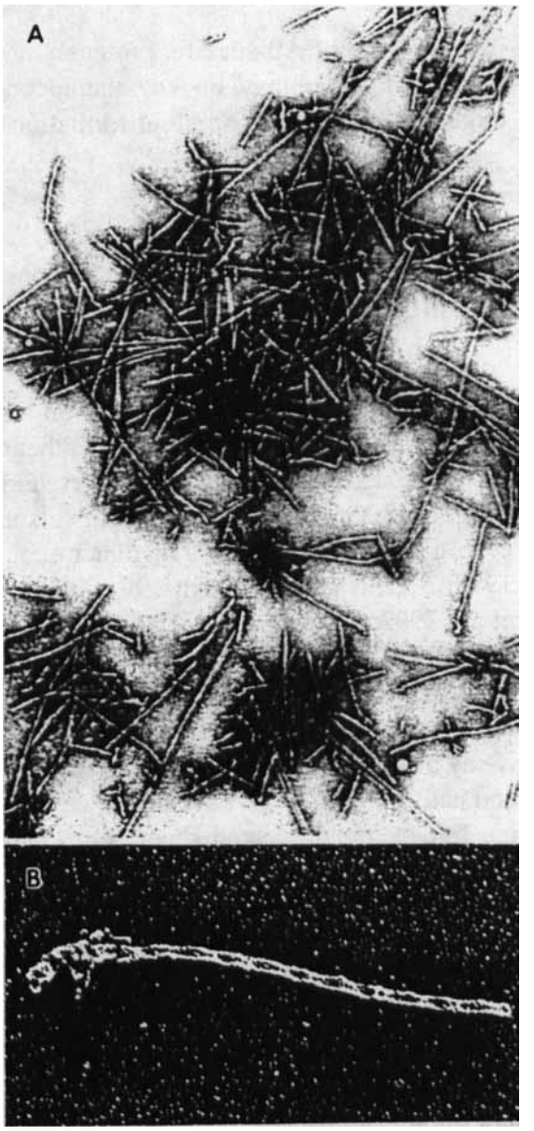

FIGURE 2. (A) A negative-stain electron micrograph of the Aβ 1-40 protein containing the Dutch mutation and indicating the regular amyloid fibril morphology. (B) Higher-resolution platinum/carbon shadowing electron micrography of the Aβ 1-40 protein showing the helical twisting of the fibril.

ly indistinguishable from one another. Electron microscopic studies (Cohen et al., 1982) have revealed that irrespective of their origin amyloid deposits consistently appear as densely packed fibrillar structures 7 to 10 n*M* in diameter of indeterminate length, which *ex vivo* are assembled from 2.5- to 3.5-n*M* diameter protofilaments (Serpell et al., 1995; Fraser et al., 1991). X-ray diffaction analyses of synthetic and *ex vivo* amyloid fibrils have indicated a striking similarlity in protofilament structure (Sunde, Serpell, Bartlam, Fraser, Pepys and Blake, unpublished data). Based on these findings, it appears that these highly elongated, β-pleated sheet protofilaments may be the basic building blocks of amyloids *ex vivo*.

The kinetics of amyloidogenesis is essentially a two-stage reaction similar to the polymerization of actin fibrils (Lomakin et al., 1996) and has been compared to a two-dimensional protein crystallization (Jarrett and Lansbury, 1993; Jarrett et al., 1993). This involves a slow, lag period that reflects the thermodynamic barrier to the formation of a nucleation center. Once a nucleus or 'seed' is formed it rapidly propagates Aβ aggregation into fibrils. The slow kinetics of nucleation is the most probable explanation for why plaques are not a more widespread occurrence. It has been estimated that for less amyloidogenic species, such as Aβ residues 1 to 40, the physiological nucleating lag phase is on the scale of 100 years (Jarrett et al., 1993). Proposals have also been made that nucleation may involve a conformational change from a nonamyloidogenic, α-helical to aggregated β-sheet (Soto et al., 1995). As recently shown for lysozyme (Booth et al., 1997), this certainly is the case for the formation of fibrils from larger proteins of defined structure, but a similar transition for Aβ is less apparent, as conformations other than the amyloidogenic β-sheet are not seen under physiological conditions. Whereas the precise nature of the Aβ "seed" is being investigated, it is clear that a number of possible factors may accelerate its formation: (1) increased Aβ concentrations that promote nucleation; (2) changes in Aβ primary sequence that create thermodynamically stable aggregates; and (3) interactions with other elements that stimulate Aβ aggregation. All of these processes may be

important *in vivo* factors in the development of amyloid plaques.

For example, βPP missense mutations associated with a Swedish pedigree results in a Lys-Met → Asn-Leu substitution at the Aβ N-terminus (Mullan et al., 1992). These mutations promote β-secretase processing and increase Aβ circulating concentrations 4- to 5-fold (Citron et al., 1992). This phenomenon may be due to the introduction of a more favorable cleavage site and/or subtle changes in βPP trafficking (De Strooper et al., 1995). The end result of increasing the physiological Aβ levels of 5 ng/ml to 20 to 25 ng/ml decreases the age of onset by an average of 20 years. In addition, changes in primary sequence can produce a similar response, as demonstrated by βPP codon 717 mutations (Goate et al., 1991; Chartier-Harlin et al., 1991; Murrell et al., 1991). The 717 mutations — Val → Ile, Phe or Gly — occur within the transmembrane domain outside of the normal Aβ sequence but are capable of altering βPP processing (Suzuki et al., 1994). The result is the creation of longer Aβ sequences, ranging from 1 to 42 and 1 to 43, as opposed to the more abundant 1 to 40 variant. This slight change in sequence dramatically decreases both the peptide nucleation lag time and solubility, as shown by *in vitro* light-scattering techniques (Jarrett et al., 1993). Although the intrinsic properties and processing of Aβ 1 to 42 may be more widely relevant to the pathogenesis of sporadic AD, at present they are known contributors to only a few familial cases. Therefore, other factors are likely to be contributing to amyloid plaque formation.

D. Modulation of Aβ Fibril Assembly by Plaque-Associated Proteins

Early thoughts on AD pathogenesis considered that an AD-specific aberrant βPP cleavage was responsible for Aβ biosynthesis (Esch et al., 1990; Golde et al., 1992; Haass et al., 1992a). It has been demonstrated since that Aβ is a naturally secreted product of many cells. With the exception of some familial forms of AD, it has also been shown that sporadic AD cases and unaffected individuals have comparable Aβ levels in both plasma and CSF (Shoji et al., 1992; Haass et al., 1992b; Seubert et al., 1992). These observations have led to a revised mechanism of amyloid formation and raised the possibility that Aβ may have some normal cellular function. More importantly, perhaps it raised as a key question: What factors are responsible for the transition of Aβ from a normal, soluble protein into an amyloid fibril?

Although it has been demonstrated that synthetic full-length Aβ and its peptide fragments assemble into amyloid-like fibrils *in vitro* (Fraser et al., 1992b; Vinters, 1991), the concentrations used in these studies were several orders of magnitude higher than the nanomolar Aβ found in the CSF. Little is known concerning the behavior of Aβ at low concentrations, but one possible mechanism for the AD-related plaque formation is interaction with one of the several proteins that consistently localize with amyloid fibrils. These amyloid-associated elements include complement factors (Mcgeer et al., 1989), serum amyloid P component (Coria et al., 1988), the serine protease inhibitor α1-antichymotrypsin (ACT) (Abraham et al., 1988; Abraham et al., 1990; Picken et al., 1990), apolipoproteins E and J (Wisniewski and Frangione, 1992; Mcgeer et al., 1992), a novel neurotrophic factor — midkine (Yasuhara et al., 1993), metal ions such as zinc (Bush et al., 1994b; Esler et al., 1996), the non-amyloid component (NAC) peptide (Yoshimoto et al., 1995), and sulfated proteoglycans (Snow et al., 1987b; Snow et al., 1990b; Young et al., 1989; Perry et al., 1991). How these various proteins may affect Aβ

production, fibrillogenesis, and/or neuronal death is still under investigation, but several lines of evidence suggest that they may play important roles in AD pathology.

Sulfated proteoglycans are common to all types of amyloid studied to date and could be key factors in the formation of mature plaques (Snow et al., 1987b). A number of subtypes are associated with AD plaques and tangles, including heparan (HSPG), dermatan (decorins), keratin, and chondroitin sulfate proteoglycans (Snow et al., 1996). Perhaps the most common amyloid-associated proteoglycan is perlecan, which is a member of the HSPG family whose name is derived from its 'pearl-like' structure, as seen by electron microscopy (Laurie et al., 1988). HSPGs are a diverse class of proteins (e.g., syndecans, glypicans, betaglycans, and perlecan) that contain highly sulfated heparan sulfate glycosaminoglycan (GAG) chains linked to large protein cores (typically 400 to 450 kD). The perlecan protein core is organized into well-defined domains with the N-terminal region containing a series of Ser-Gly-Asp repeats that provide the Ser-O-linkage GAG attachment sites (for review see Iozzo et al., 1994). Perlecan is a major component of the basement membrane and assists in maintaining its structural integrity and architecture. This is accomplished by a combination of protein-protein and protein-carbohydrate interactions. The ability of perlecan to stabilize large protein aggregates such as those found in the basement membrane is consistent with their participation in the deposition of amyloid plaques.

In vitro studies have shown that sulfated GAG chains can induce extensive Aβ aggregation via electrostatic interactions (Fraser et al., 1992a) and is known to increase the β-sheet content of other amyloidogenic proteins, such as SAA (McCubbin et al., 1988). This action was mediated by a specific sulfate-binding site (His-His-Gln-Lys; residues 13 to 16) as originally proposed by Kisilevsky (Kisilevsky, 1989). *In vivo* studies that employed a continuous infusion of Aβ peptide and purified perlecan (Snow et al., 1994) demonstrated that congophilic plaque-like structures were maintained only in the presence of proteoglycan. This could reflect the enhanced aggregation induced by perlecan-Aβ interactions, as well as a reduced degradation of the amyloid fibrils that is created by the protective effects of the GAG chains (Gupta-Bansal et al., 1995). Aβ-perlecan interactions likely account for the targeting of amyloid to the endothelium basement membrane HSPG of cerebral vessels (Perlmutter et al., 1991). The source of senile plaque HSPG is not known but astrocytes (Johnson-Green et al., 1992) and neurons (Buee et al., 1993) synthesize proteoglycans capable of interacting with Aβ. In addition, it has been shown that GAGs and proteoglycans may have much wider reaching effects in the development of AD pathology, as demonstrated by their association with the intraneuronal neurofibrillary tangles (Snow et al., 1987c; Snow et al., 1996). The importance of proteoglycans to tangle formation has been supported by *in vitro* investigations, where combinations of the tau protein and heparin resulted in the formation of PHF-like structures (Goedert et al., 1996). Based on these observations, it is possible that Aβ-proteoglycan interaction is a driving force in the assembly and deposition of AD-related tangles and amyloid plaques.

As with proteoglycans, apolipoproteins are also associated with all forms of systemic (Charge et al., 1996; Gallo et al., 1994) and CNS amyloid (Strittmatter et al., 1993), suggesting a similar universality in their role for fibril formation. The most common form in plaques, apolipoprotein E (apoE), is a 299-residue protein that exists as three allelic variants, termed apoE2, 3, and 4. In Alzheimer's disease, the apoE4 allele is a risk factor that is associated with an earlier age of onset for sporadic cases (Strittmatter et al., 1993). A number of *in vitro* investigations have demonstrated that Aβ-apoE com-

plexes result in enhanced fibril formation, possibly of some novel structures (Sanan et al., 1994; Naslund et al., 1995). The process by which this occurs remains speculative, and some investigations into the molecular mechanisms have indicated that apoE has complex effects on Aβ assembly and may under some circumstances act as a barrier to aggregation (Evans et al., 1995). However, the genetic evidence strongly supports a significant contribution to the progression of Alzheimer's disease and the development of amyloid plaques (Strittmatter et al., 1993).

In contrast to other amyloid-associated proteins, α_1-antichymotrypsin (ACT) is AD-specific and is not found in any of the other cerebral (e.g., prion) or systemic amyloidoses, which implies an Aβ sequence-dependent binding site (Abraham et al., 1988; Abraham et al., 1990; Picken et al., 1990). It has been proposed that the Aβ sequence Asp-Ser-Gly (residues 7 to 9) may be sufficient for recognition of ACT, based on its similarity to the catalytic domain of serine proteases (Potter et al., 1991). Small synthetic peptides containing these residues are capable of binding ACT even under gel electrophoresis conditions (Dressler et al., 1989). However, the contribution of ACT to Aβ assembly appears to be condition dependent. For example, at high concentrations it has been shown ACT binding destabilizes amyloid fibrils *in vitro,* which is also dependent on the presence of the proposed ACT binding site (Fraser et al., 1993; Lukacs and Christianson, 1996). In contrast, light-scattering studies have indicated that ACT binding to the Aβ 1 to 42 isoform resulted in an enhancement of aggregation and fibril formations (Ma et al., 1994). Based on these observations, it is difficult to assign a consistent role for ACT in amyloid pathogenesis.

The remaining elements that colocalize with amyloid plaques may also play roles in the stabilization of Aβ aggregates and/or the cellular consequences of their pathophysiology. For example, markers of the classic complement pathway (C1q, C3d, and C4d) have been well characterized in AD and may represent a mechanism for neuronal damage via the membrane attack complex (MAC) (Mcgeer et al., 1989). This role has been bolstered by the observation that Aβ itself can activate the classic pathway in the absence of antibody (Rogers et al., 1992a; Haga et al., 1993). Finally, other amyloidogenic peptides, such as the nonamyloid component (NAC), may assist in the seeding or nucleation of Aβ aggregates (Iwai et al., 1995; Weinreb et al., 1996). Taken together, a strong case can be made for critical contributions by Aβ-associated elements in the evolution of amyloid plaques.

VI. AMYLOIDS AS TOMBSTONES

Over the last quarter century or more, accumulated evidence indicates that amyloid can no longer be viewed simply as an inert tissue marker of past cell injury. Supporting this view are clinical observations with AA, ATTR, AL, Aβ2M, ALys, and APrP. In each of these cases organ function deteriorates as a direct result of amyloid infiltration. Where it has been possible to treat the underlying disease that serves as the antecedent to amyloid deposition and where the organ has regenerative properties regression of amyloid coincides with reappearance of organ function. These features are not consistent with amyloid being an inert deposit without physiological or pathological effects. Details of several of the above examples are illustrative.

A. Aβ2M

Aβ2M is the form of amyloid found in patients with kidney failure who have under-

gone long periods (8+ years) of hemodialysis (Gal et al., 1994). This form of amyloid is a relatively new disease, because hemodialysis was not available as a therapeutic modality until approximately 25 to 30 years ago. Prior to such dialysis, the patients requiring this form of therapy did not survive for long periods of time. The majority of patients undergoing long-term hemodialysis develop this problem because the membranes used for dialysis are unable to adequately eliminate β-2-μglobulin (Gaucher et al., 1992), a circulating naturally occurring cell membrane protein that normally can be excreted by the kidney. Aβ2M amyloid is deposited in the gastrointestinal tract, spine, and major joints of the body, where it is directly responsible for a destructive arthropathy (Gaucher et al., 1992).

B. ATTR

Amyloid associated with transthyretin falls into two broad categories, the familial amyloid polyneuropathies (FAP) (usually associated with a mutant form of TTR) and the senile systemic or cardiac forms in which no mutations have been identified. In each case the clinical features are a result of amyloid deposition in the relevant organs. In FAP the patients present with peripheral neuropathies, whereas in the senile forms usually with cardiac failure. The clinical features are the direct result of the amyloid deposits. They do not appear until amyloid is deposited, although in FAP the mutant protein has been present since birth. Robust evidence that this conclusion is valid comes from FAP patients who have undergone liver transplantation (Ericzon et al., 1995). Circulating TTR is made primarily in the liver (Kanda et al., 1974). Following liver transplantation with "normal" livers the mutant TTR disappears from the plasma (Holmgren et al., 1991), is replaced by the "wild-type" TTR (Holmgren et al., 1991), ATTR amyloid load decreases as measured by scintigraphy (Hawkins, 1994a), and there is partial improvement in the neuropathy (Bergethon et al., 1996). These observations are not consistent with an inert, innocuous deposit.

C. AA and AL Amyloid

AA and AL amyloid are systemic forms that follow on persistent acute inflammatory processes and plasma cell dyscrasias, respectively. In both forms amyloid infiltration of the kidneys and heart are common and lead to the patient presenting with either heart or kidney failure (Walley et al., 1995; Kyle, 1994). Sufficient cases have now been reported where the underlying infection (inflammation) or plasma cell disturbance has been treated effectively with reduction of the relevant circulating amyloid precursor. This in turn has been correlated with the resolution of amyloid in the affected organ (as proven by biopsy) and the reappearance of heart/kidney function (Karsenty et al., 1985; Falck et al., 1979; Wegelius, 1982; Edwards et al., 1988; Eulitz, 1992; Hawkins et al., 1993; Perfetti et al., 1994; Hawkins, 1994b; van Buren et al., 1995; Dubrey et al., 1996).

D. Evidence from *In Vitro* Cultures

Viability of cells in the presence of amyloid has also been examined in tissue culture. In both Aβ and AIAPP the peptides are rela-

tively non-toxic unless they take on the conformation associated with amyloid (Pike et al., 1993; Lorenzo and Yankner, 1994; May et al., 1993; Lorenzo et al., 1994; Mirzabekov et al., 1996). In the case of Aβ, multiple reports of the neuronal "toxicity" of this peptide have appeared in the literature. Similar results have been reported with AIAPP and the β-cells of the islets of Langerhans (May et al., 1993; Lorenzo et al., 1994; Mirzabekov et al., 1996) and APrP and neuroblastoma cells (Forloni et al., 1993; Priola et al., 1994; Forloni et al., 1996). The precise mechanism of toxicity awaits clarification.

Given the overwhelming clinical data indicating that amyloid is noxious at sites outside the nervous system, it would be surprising indeed if the Aβ deposits in the CNS were an exception. In addition to the effects of Aβ on neuronal cell viability in culture, the genetics of AD provides further evidence that Aβ amyloid is a significant factor in the pathogenesis of this disease.

E. Genetics of AD

The genetic evidence may be divided into two categories, those mutations and polymorphisms that affect proteins directly present in the Aβ amyloid deposit and those that likely affect steps "upstream" of Aβ processing or amyloid formation.

In the first group are the mutations associated with βPP and identified near the β- and γ-secretase sites. A double mutation at codons 670 to 671 (the β-secretase site) characterizes the Swedish pedigree of familial AD (Lannfelt et al., 1994; Axelman et al., 1994). Several different mutations at codon 717 (three residues beyond the putative γ-secretase site) are associated with several other forms of FAD (Naruse et al., 1991; Yoshizawa et al., 1993; Mullan et al., 1993; Lantos et al., 1992; Cairns et al., 1993; Mann et al., 1992; Hanger et al., 1992; Ghetti et al., 1992). Hereditary cerebral hemorrhage associated with amyloid angiopathy and presenile dementia is linked to a mutation at codon 692 (Hendriks et al., 1992). A substitution at residue 693 is the cause of hereditary cerebral hemorrahge with amyloid — Dutch type without dementia (Levy et al., 1990; Bakker et al., 1991; Rozemuller et al., 1993). Thus, some early-onset FAD is associated with substitutions that flank the β-protein, whereas those within the β-protein are associated with cerebral hemorrhage ± dementia. Furthermore, Down's syndrome, in which there is a significant over expression of the βPP gene because of complete or partial trisomy of chromosome 21 (the chromosomal site of the βPP gene), is invariably associated with dementia as these patients live into their 4th decade and beyond (Delabar et al., 1987; Takashima et al., 1990; Lejeune, 1990). The neuropathologic findings in such patients are typical of AD (Rafalowska et al., 1988; Miyakawa and Kuramoto, 1989; Mann, 1989; Phelps, 1989; Snow et al., 1990a; Yoshimura et al., 1990; Cork, 1990; Snow, 1990).

One may also include the apo E gene (chromosome 19) in this first category, as apo E has been identified as a constituent of all forms of amyloid examined to date (Gallo et al., 1994). Apo E "knock-out" mice develop AA amyloid when they receive an appropriate induction regimen (M. Kindy, personal communication). However, in these apo E –/– mice the onset of AA amyloidogenesis is delayed and the rate of amyloid deposition is slower when compared with their apo E +/+ counterparts. These findings indicate that apo E is not critical for amyloidogenesis per se, but it does influence the onset and speed of progression of this deposit. These latter findings are similar to observations made in humans with different apo E polymorphisms (Strittmatter and Roses,

1996; Roses, 1996). Human apo E4 is similar to murine apo E in that the pertinent arginines are present at the homologous locations (Kindy et al., 1995; Rajavashisth et al., 1985). Apo E4 is associated with an earlier onset and more rapid progression of AD than patients with other apo E isoforms (Strittmatter and Roses, 1996; Roses, 1996).

The presenilin genes (located on chromosomes 1 and 14) are likely involved in steps "upstream" of Aβ amyloid formation. Presenilin-1 (PS1) is located on the long arm of chromosomes 14 and encodes an ~470 residue multispanning transmembrane protein that is responsible for the majority of early onset AD cases (Sherrington et al., 1995). To date, more than 35 different mutations have been discovered in the PS1 gene. The majority of these mutations are missense mutations giving rise to the substitution of a single amino acid. These mutations are predominantly located either in highly conserved transmembrane domains, at or near putative membrane interfaces, or in the N-terminal hydrophobic or C-terminal hydrophobic residues of the putative TM6-TM7 loop domain. PS1 is highly conserved in evolution, with homologues being present in *C. elegans*, and based on sequence similarities it could potentially function in the control of protein trafficking and/or signal transduction (Sherrington et al., 1995; Levitan and Greenwald, 1995), both of which are relevant to amyloid pathology.

Subsequent to the cloning of the presenilin 1 gene, a similar sequence was identified via database searching (Rogaev et al., 1995; Levy-Lahad et al., 1995). Further analysis revealed that this sequence was derived from a gene on chromosome 1 and encodes a polypeptide whose open reading frame contained 448 amino acids with substantial amino acid sequence identity with that of the presenilin 1 protein (overall identity approximately 60%). Mutational analyses uncovered two different missense mutations in the presenilin 2 (PS2) gene in families segregating early onset forms of Alzheimer disease. The first mutation (Asn141Ile) was detected in a proportion of families of Volga German ancestry (Rogaev et al., 1995; Levy-Lahad et al., 1995), in which the FAD locus had been independently mapped by genetic linkage studies to chromosome 1. The second mutation (Met239Val) was discovered in an Italian pedigree (Rogaev et al., 1995). However, in contrast to the frequency of presenilin 1 mutations, screening of large data sets reveal that presenilin 2 mutations are likely to be rare (Sherrington et al., 1995).

The functional significance of the presenilins remains to be determined, but several lines of evidence suggest a direct link to amyloid formation. Studies of βPP processing in transfected cell lines and in PS1 transgenic mice have revealed a significant increase in the levels of the highly amyloidogenic Aβ 1-42 isoform (Duff et al., 1996; Citron et al., 1997; Borchelt et al., 1996; Scheuner et al., 1996). The biochemical processes that are responsible for this phenomenon are currently under investigation, but PS1/2 mutations could, for example, cause improper handling of βPP, leading to a preferential trafficking along the amyloidogenic pathway.

The foregoing examples illustrate that amyloid generally is not an innocuous deposit or an "end point" pathogenetically. It is responsible for significant pathophysiology and ultimately leads to a fatal outcome in most circumstances. The *in vitro* findings with Aβ and the genetic evidence regarding AD clearly focus attention on Aβ amyloid as being directly involved in the pathogenesis of AD. It is frequently argued that mutations related to βPP primary structure, or its processing, represent only 1% of AD cases, and thus the genetics of Aβ amyloid is involved in few cases of AD. This small figure is due to the exclusion of the apo E data. Because apo E is a constituent part of all

amyloids and also plays a role in the onset and rate of progression of AA amyloid, there is no reason to exclude the apo E data. When included, these data raise the genetic involvement of amyloid constituents in AD to 30 to 40% of cases, a significant percentage indeed. Given the general noxious properties of the amyloids, and evidence for the direct involvement of Aβ in AD, interference with Aβ amyloid formation is a worthy target for AD therapy.

VII. ANTI-AMYLOIDS AS A TARGET OF ALZHEIMER THERAPY

In subdividing the pathogenetic steps of amyloidogenesis as described above, we have perforce identified targets for anti-amyloid therapy. These may be summarized as follows.

A. Reducing the Precursor Pool

Successful attempts at inhibiting/reducing amyloidogenesis by reducing the precursor pool have been made with AA and ATTR. In the former, murine AA amyloid has been inhibited by the use of a liver-specific protein synthesis inhibitor (Kisilevsky et al., 1979). This blocked the formation of SAA, the AA precursor, and thus AA amyloid. The latter is a clinical example where mutant TTR is responsible for FAP. Liver transplants in such patients remove the offending source of mutant TTR. As described above, this results in the disappearance of this form of TTR from the plasma, its replacement by "wild-type" TTR, and a reduction in amyloid load systemically, as measured by scintigraphy. Additional examples have been reviewed recently (Kisilevsky, 1996).

B. Interfering with Precursor Processing

Some forms of amyloidogenesis may require proteolytic modification of the precursor as part of the precursor's normal processing. In the course of doing so a pool of amyloidogenic peptide is produced (e.g., Aβ). Many investigators have been actively pursuing the design and synthesis of protease inhibitors for these purported specific proteases, which have yet to be identified with confidence. The objective is to reduce the pool of amyloidogenic peptide and thus amyloid formation. Such an approach is theoretically possible, but concrete data substantiating this approach in any amyloid model, or form of amyloid, have yet to be published.

C. Inhibiting Nucleation or Seeding Steps

Part of the pathogenesis of amyloid formation appears to be the occurrence/generation of a "seeding" event, as, for example, AEF. In familial Mediteranean fever, an inherited disorder characterized by recurrent episodes of pleural and serosal inflammation, such episodes inevitably lead to systemic AA amyloidosis and eventual death from kidney failure (Reimann, 1979; Zemer et al., 1993; Woo, 1994). Through trial and error, colchicine has been shown to prevent the development of amyloid in such patients (Better and Zemer, 1991; Livneh et al., 1992; Livneh et al., 1994). In experimental studies colchicine apparently exerts its therapeutic effect by

preventing the generation of AEF (Brandwein et al., 1985).

D. Inhibiting Amyloid Precursor/ ECBM Protein Interactions

In vivo amyloid deposits are composed of more than just the specific amyloid protein. Among the components that have been consistently found in most, if not all, amyloids are the HSPG perlecan, laminin, collagen IV, SAP, and apo E. With the exception of apo E, all are structural components of ECBM. *In vitro* studies with several different amyloid proteins, including βPP and Aβ, have demonstrated high-affinity binding interactions between the amyloidogenic proteins and purified ECBM proteins free of other ECBM proteins (Narindrasorasak et al., 1991; Narindrasorasak et al., 1992; Narindrasorasak et al., 1995; Leveugle et al., 1994; Ancsin and Kisilevsky, 1997). It is during these binding interactions that several of the amyloidogenic proteins take on a conformation characteristic of an amyloid. These findings have suggested that amyloidogenesis involves nascent ECBM proteins, or those that have dissociated themselves from the ECBM and are no longer part of the structure of a basement membrane (Narindrasorasak et al., 1995; Ancsin and Kisilevsky, 1997). They further suggest that amyloid formation may also be responsible for the failure to form organized basement membranes at sites of amyloid deposition despite the availability of the necessary ECBM proteins (Narindrasorasak et al., 1995; Lyon et al., 1991). Moreover, they suggest why initial amyloid deposits tends to be seen anatomically close to basement membranes (Schultz and Pintha, 1985; Schultz et al., 1985; Linke et al., 1989; Yamaguchi et al., 1992; Horiguchi et al., 1992). These considerations logically suggest that prevention of these interactions, or interference with the synthesis of common components may interfere with amyloid deposition. The first approach has proven successful and has been reviewed recently (Kisilevsky, 1996). The second is still in the conceptual stage.

E. Aβ Amyloid, AD, and Antiinflammatory Agents

Recent epidemiologic data have suggested that prolonged use of non-steroidal antiinflammatory agents may prove useful in preventing or delaying the appearance of AD clinically (Mcgeer and Rogers, 1992; Breitner, 1996). The basis for these findings is thought to be the ability of Aβ amyloid to act as a scaffold for the activation of complement, which in turn sets in motion a local inflammatory reaction (Rogers et al., 1992b; Rogers et al., 1992a; Lue and Rogers, 1992; Jiang et al., 1994; Schultz et al., 1994; Snyder et al., 1994; Webster et al., 1995; Valazquez et al., 1997). The inflammation may have a local adverse effect on the surrounding neurons. In this scenario Aβ amyloid occurs first with inflammation, although a secondary event, compounding and extending existing pathology (Korotzer et al., 1995).

F. Amyloid Removal

As alluded to above, amyloid is not an inert substance physiologically, nor is it inert metabolically. Experimental and clinical data show that amyloid deposits may be resorbed once the underlying mechanisms for deposition are removed (Karsenty et al., 1985; Falck et al., 1979; Wegelius, 1982; Edwards et al., 1988; Eulitz, 1992; Hawkins et al.,

1993; Perfetti et al., 1994; Hawkins, 1994b; van Buren et al., 1995; Dubrey et al., 1996). Unfortunately, the mechanisms that remove amyloid have not been cleary identified. On theoretical grounds, displacement of the interacting components that make up an amyloid may make the remainder more susceptible to degradation (Gupta-Bansal et al., 1993; Gupta-Bansal et al., 1995; Tennent et al., 1995).

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