Amyloid Basis of Alzheimer’s Disease

OUTLINE

HISTORICAL BACKGROUND OF AMYLOID AND PLAQUES

Amyloid is a somewhat infamous insoluble, fibrous protein; infamous because amyloid deposits are responsible for tissue damage in a fair number of genetic and inflammatory diseases and disorders. Of course in modern times, amyloid has become most commonly associated with Alzheimer’s disease (AD): as of November 2015, a Google search for “amyloid and Alzheimer’s” gets almost 11 million hits.

Before the relationship between amyloid and Alzheimer’s was discovered, there were accounts of the plaques in the brain that became associated with dementia. Early descriptions of nerve cell degeneration
in senile dementia were described as nodules of glial sclerosis, or round heaps of nerve cell degeneration. Subsequently in 1898, Emil Redlich named miliary sclerosis as “plaques” in two cases of senile dementia. He also described plaques of different sizes and forms like the smaller cotton-wool type suggesting they represented a modified glial cell.

Several years later in 1907, extracellular deposits (also known as senile or neuritic plaques) were described as miliary foci of dystrophic neuronal processes surrounding a “special substance in the cortex” by Alois Alzheimer in the autopsied brain of a 51-year-old patient, who presented a very unusual clinical picture with loss of short-term memory and odd behavioral symptoms. However, it would not be until the application of the Congo red stain that this “special substance” would be identified as amyloid creating an association between amyloid, dementia, and senile plaques. Using a silver staining method, Alzheimer also identified the presence of neurofibrillary deposits in sections of her autopsied brain tissues; these were subsequently determined to be composed of aggregates of the abnormally hyperphosphorylated tau protein.

At the same time in 1907, Oskar Fischer provided the first illustrations of the neuritic plaques that captured many of the features reported today (Fig. 1.1). Fischer studied a total of 275 brains from cases of psychosis, neurosyphilis, and controls of various ages, with 110 being over 50 years old at the time of death. He observed plaques in 56 cases, all of whom

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**FIGURE 1.1** Drawings of three neuritic plaques from the brains of patients with senile dementia. Compiled from the illustrations of Fischer’s 1907 paper. Note the abnormal, club-shaped neurites and the displacement of normal-looking fibrils in the space occupied by the plaques. *Source: Used with permission from Brain 2009;132:1102–11.*
were >50 years of age.\(^3\) Alzheimer and Fischer disagreed on the origin of tangles: Alzheimer believed tangles consisted of chemically modified neurofibrils, while Fischer thought that they represented fibril proliferation \textit{de novo}, and that the material was unrelated to neurofibrils.\(^3\) However, the origin of the plaque remains a matter of debate (see Chapter 5).

Despite these discoveries of plaques in the demented brain, it took additional effort to recognize amyloid as the component of these deposits. Amyloid itself was first discovered over 150 years ago through an iodine-sulfuric acid test that demonstrated the transformation of plant material to starch.\(^4,10\) This starch-like material was for the first time, referred to as amyloid, from the Latin word for starch, “amylym.”\(^11\)

Even though such amyloid deposits may have been observed as homogenous material in the liver and spleen back in 1693, it wasn’t until 1854 that amyloid was first described as small round deposits in the nervous system using the same iodine-sulfuric acid staining method.\(^12\) Although the iodine-sulfuric acid test produced similar results to the newly developed metachromatic stain in 1878, eventually the method of choice to detect amyloid in tissue sections would become the Congo red stain, initially produced to stain textile fibers.\(^13,14\) The application of the Congo red staining led to the discovery of cerebrovascular amyloid in 80–90% in the brains of patients with AD.\(^15\) Over time, the standard staining methods to describe AD pathology would be the silver stain to identify tangles, along with the Congo red to stain amyloid in microscopic sections of autopsied brain tissues.\(^4\)

Although several biochemical methods led to the discovery of several forms of amyloidosis, it was a new water extraction method that led to the discovery of different amyloid proteins.\(^4\) After years of describing various forms of amyloid, it was determined that the plaques in AD brains were composed of insoluble protein denaturants, and did not represent any known form of amyloid at that time.\(^16\) Then in 1984, the AD-associated A\(\beta\) form of amyloid (A\(\beta\)), 4.2-kDa peptide, primarily 40 or 42 amino acids in length, in the cerebrovascular tissue in Alzheimer’s tissues was also found in a Down syndrome patient, thereby providing a link between the two conditions.\(^4,17,18\) The next year, the same A\(\beta\) was described in the AD plaques, and later determined to be toxic (see Chapter 4).\(^4,19,20\)

### Amyloid Generation and Processing

Of all the neuropathological features reported in the AD brain, such as neuronal and synaptic loss, and NFTs, it is the extracellular aggregates of A\(\beta\) peptides as senile plaques that have occupied most of the AD research activity, especially concerning how A\(\beta\) is generated and
processed from APP. Such research has been in an effort to prevent aggregation, and spare neuronal death.

APP is a single-pass transmembrane protein, referred to as a type I integral membrane protein (Fig. 1.2), and is encoded by a single gene on human chromosome 21, containing 18 exons.\(^7,21,22\) APP has a signal peptide, a large extracellular N-terminal domain, a small intracellular

**FIGURE 1.2** Cleavage of APP and physiological roles of APP and APP fragments. APP can be cleaved via two mutually exclusive pathways. In the so-called amyloidogenic pathway APP is cleaved by BACE1 and \(\gamma\)-secretase enzymes (presenilin I is the catalytic core of the multiprotein \(\gamma\)-secretase complex). The initial \(\beta\)-secretase cleavage produces a large soluble extracellular domain, sAPP\(\beta\). The remaining membrane bound C99 stud is then cleaved by multiple sequential \(\gamma\)-secretase cleavages. These begin near the inner membrane at a \(\gamma\)-secretase cleavage site \(\varepsilon\) (the \(\varepsilon\)-site) to produce the AICD, and then subsequent sequential \(\gamma\)-secretase cleavages trim the remaining membrane bound component to produce different length A\(\beta\) peptides including A\(\beta\)\(_{43}\), A\(\beta\)\(_{42}\), A\(\beta\)\(_{40}\), and A\(\beta\)\(_{3}\). In the so-called nonamyloidogenic pathway APP is processed consecutively by \(\alpha\)- and \(\gamma\)-secretases to produce sAPP\(\alpha\), p3 (which is in effect A\(\beta\)\(_{17-40/42}\)), and AICD. The major \(\alpha\)-secretase enzyme is ADAM10. Cleavage via amyloidogenic and nonamyloidogenic pathways depends on the cellular localization of cleavage enzymes, and of full-length APP, which are expressed and trafficked in specific subcellular locations. **APP**, Amyloid Precursor Protein; **AICD**, APP intracellular domain; **BACE1**, \(\beta\)-site APP cleaving enzyme; **sAPP\(\beta\)**, secreted Amyloid Precursor Protein-\(\beta\); **ADAM10**, A Disintegrin and Metalloproteinase domain-containing protein 10. *Source: Has been slightly modified: Used with permission from Acta Neuropathol Commun 2014; 2:135.*
C-terminal domain, a single transmembrane domain, and an endocytosis signal at the C-terminal. 7 APP not only has a very wide distribution in the body, but is expressed at high levels in the brain, is produced in large quantities in neurons, and is rapidly metabolized (see Chapter 3). 5

APP undergoes enzymatic processing to produce fragments some of which are believed to play a crucial role in the pathogenesis of AD. This is not only because some of these fragments are located in the senile plaques, but also because mutations in the APP gene and the processing enzymes (eg, presenilin I, the catalytic core of $\gamma$-secretase complex) have been associated with rare cases of familial and inherited early onset of AD, respectively. 6,15,21,23–26 APP is cleaved sequentially by $\alpha$-, $\beta$-, and $\gamma$-secretases, which results in the generation of the large soluble NH$_2$-terminal ectodomain, small hydrophobic extracellular A$\beta$ (A$\beta$40- and A$\beta$42-residues) peptide, and APP intracellular domain (AICD, 57- and 59-residue-long COOH-terminal fragments). 5,19–21,27

The cleavage and processing of APP is divided into the nonamyloidogenic and amyloidogenic pathways (Fig. 1.2). 6,7,20,25 In the nonamyloidogenic pathway, APP is first cleaved by the $\alpha$-secretase producing the soluble APP-$\alpha$ (sAPP-$\alpha$) peptide that is secreted into the extracellular medium. 28 The intact membrane fragment is subsequently cleaved by $\gamma$-secretase at two areas in the remaining fragment to produce a short fragment (p3) and the AICD. Hence, this nonamyloidogenic pathway does not produce the A$\beta$ peptide.

The A$\beta$ peptide is produced through the processes of the amyloidogenic pathway (Fig. 1.2). APP is first cleaved by $\beta$-secretase yielding two products: the soluble APP-$\beta$ fragment and the membrane-retained fragment. This membrane product of APP is subsequently cleaved by $\gamma$-secretase to produce the A$\beta$ peptide and another AICD fragment. Mutations in these processes that are associated with AD are discussed in Chapter 4.

Fig. 1.3 shows how APP traffics in the neurons, and how APP is associated with several cellular organelles. After sorting in the endoplasmic reticulum and Golgi, APP is delivered to the axon and further processed in the membrane. 5,26

A$\beta$ States

Once released from the cell, the A$\beta$ peptides contain an amino acid sequence that favors A$\beta$–A$\beta$ binding and $\beta$-sheet formation. 29 A$\beta$ aggregates with other A$\beta$ peptides forming a variety of shapes and molecular weights (Fig. 1.4) that may impact its toxicity. 27,30 The formation and biological activities of A$\beta$ oligomers, protofibrils, and fibrils have been under intensive investigation in recent years. Although insoluble fibrillar A$\beta$ has been shown to be neurotoxic, compelling evidence also indicates that
oligomers and protofibrils contribute significantly to cellular cytotoxicity, inflammatory responses, synaptic dysfunction, and reduced neurogenesis. Hence, the aggregation of the $\text{A}\beta$ species is thought to play a pivotal role in the disease progression of AD through a cascade of events, as described in the amyloid cascade hypothesis (see Chapter 4).

The kinetic relationship is not clear among the two $\text{A}\beta$ species: $\text{A}\beta40$ and $\text{A}\beta42$. Although oligomers may be the intermediate in fibril formation (Fig. 1.4), it is possible that oligomers may actually represent a separate assembly pathway. For example, the aggregation conditions for $\text{A}\beta42$ did not compare to structural or functional species of $\text{A}\beta40$, even though the $\text{A}\beta42$ peptide is more fibrillogenic than the $\text{A}\beta40$ species. The differences may be attributed to aggregation time, assuming they follow the same pathways. The pH also affects the aggregation conditions of the $\text{A}\beta$ peptides. The metal binding sites of $\text{A}\beta$ contain three histidine residues of $\text{A}\beta$ that are involved in the interaction with metal ions, and the metal-His(Ntau) ligation is a common feature among the insoluble Zn(II)- and Cu(II)-$\text{A}\beta$ aggregates at pH 5.8-7.4 and 5.8-6.6, respectively. Interestingly, under normal physiological conditions, Cu(II) is expected to protect $\text{A}\beta$ against Zn(II)-induced aggregation by competing with Zn(II) for histidine residues of $\text{A}\beta$. 

**FIGURE 1.3** APP trafficking in neurons. Newly synthesized APP (purple) is transported from the Golgi down the axon (1) or into a cell body endosomal compartment (2). After insertion into the cell surface, some APP is cleaved by $\alpha$-secretase (6) generating the sAPP$\alpha$ fragment, which diffuses away (green), and some is reinternalized into endosomes (3), where $\text{A}\beta$ is generated (blue). Following proteolysis, the endosome recycles to the cell surface (4), releasing $\text{A}\beta$ (blue) and sAPP$\beta$. Transport from the endosomes to the Golgi prior to APP cleavage can also occur, mediated by retromers (5). APP, Amyloid Precursor Protein; sAPP$\alpha$, soluble APP$\alpha$. Source: Used with permission from Annu Rev Immunol 2011;34:185–204.
APP was reported in neurons over 30 years ago as the source of the extracellular amyloid in the AD brains, whereby the neurons secrete the Aβ to eventually form toxic senile plaques. These eventually

**FIGURE 1.4** Pathways of aggregation and observed Aβ-aggregate intermediates. Monomeric Aβ folds to the activated state and then exists in rapid equilibrium with LMWO, which aggregate over various transient high molecular weight intermediates to matured fibrils. The definition of LMWO and HMWO is related to the elution profile of Aβ-aggregates in size exclusion chromatography, revealing two predominant peaks at the exclusion limit (>60 kDa) and at the void volume (4–20 kDa), respectively. The HMW intermediates comprise pentamers, hexamers, and multiples thereof, finally forming protofibrils, which are the precursors for multistranded ribbons of matured fibrils. Further neurotoxic aggregate species for example AβO, ADDL, and ASPD are believed to aggregate over alternative pathways but preliminary data revealed that these are able to converge into the other pathways of aggregation (interconversion). Interestingly, every change in the experimental paradigm can provoke this aggregate conversion. Therefore, one might assume that many different aggregates coexist and, thus, neurotoxicity can be attributed to several pathogenic modes of action. Monomers and fibrils are believed to be biologically inert; however fibrils are able to collapse into protofibrils and then also reveal toxicity. The broad range of prefibrillar aggregates have been reported as pathophysiologically relevant in AD. AβO, Amyloid-β-oligomers; ADDL, Alzheimer-derived diffusible ligands; ASPD, amylospheroids; HMWO, high molecular weight oligomers; LMWO, low molecular weight oligomers. Source: Used with permission from Immun Ageing 2013;10:18.
lead to the demise of the neurons, as per the amyloid cascade hypothesis (see Chapter 4). Given these theories of events, the presence of Aβ in neurons was only viewed as the source of the extracellular amyloid.

But in the last 15 years, that attitude slowly changed as the attention turned back inside the neurons, perhaps due to the inaccuracies of the amyloid hypothesis. Reports began to demonstrate how Aβ accumulates in neurons, and how that may be considered one of the earlier pathological events leading to AD.\textsuperscript{26,34–42} Subsequently, these early observations led to hundreds of follow-up papers suggesting that “further investigations of intraneuronal Aβ could improve the understanding of early stage AD and the mechanistic links between intraneuronal Aβ and tau pathology, neurodegeneration and dementia.”\textsuperscript{43}

Before discussing the findings of these reports (the purpose of this book), it is important to review some of the technical details on how the data was generated. These data are typically based on the immunohistochemical (IHC) detection of Aβ in the cells. For the most part, the development of IHC has provided a wealth of contributions to help propel discoveries of the pathological processes leading to AD, and it was my expertise in this methodology that facilitated my contributions in this field.\textsuperscript{44} Essentially, IHC is a method of staining specific targets in tissues for microscopic analyses. Unlike typical slide-staining methods that stain tissue elements indiscriminately (e.g., haematoxylin and eosin stain), IHC utilizes target-specific antibodies to visualize their specific antigens in tissues.

The integrity of the method is dependent upon the specificity of the antibody to its antigen; otherwise, nonspecific binding can mislead the data, and therefore the interpretation of the results. In the case of using IHC to stain targets (e.g., Aβ) in AD tissues, other variables can also contribute to erroneous staining, such as the degree and type of fixation of the tissue, the use of antigen retrieval pretreatment methods, and quality of the detection reagents to produce the staining. Indeed, the proper uses of positive and negative controls are essential, but in addition, the antibody has to be validated to its antigen. Although Western blot data supports the molecular weight characteristics, preincubating the antibody with its specific antigen is another method used to validate the specificity of the antibody. Hence, if the antibody is preincubated with its specific antigen, and then placed on the tissue sections mounted on microscopic slides for detection, no immunolabeling should be observed since the antibody-specific antigen binding site on the antibody should be occupied with the antigen, leaving no free binding sites on the antibody to bind to the antigens in the tissue.
APP/Aβ-Related Antibodies

Antibodies are specific to epitopes of the target, or antigens (antibody generators). Hence, any data discussing the presence of APP and Aβ in tissues will depend on the antibody selected.

As an example, seven different commercial antibodies (4G8, 6E10, 82E1, 6F3D, Aβ40, Aβ42, 12F4) directed to the N- or C-terminus or mid-portion of the Aβ fragment (Fig. 1.5) were used to demonstrate that the selection of the primary antibody is critical to the interpretation of the study. All of these antibodies claim to recognize Aβ in the tissue according to their specifications. With three (4G8, 6E10, 82E1) of these seven antibodies, intracellular immunolabeling was detected in a...
variety of normal human tissues, including that of a 2-year-old, and AD brain tissues; similar immunolabeling was observed in transgenic mice brain tissues using the N-terminus antibodies (4G8, 6E10, 3D6). However, only intracellular Aβ was detected in the AD tissues using the C-terminal antibodies (Aβ40, Aβ42, 12F4), while the monoclonal antibody 6F3D did not label the intracellular compartment with any of the tissues. Hence, for some of the data, the detection of intracellular Aβ in all tissues using the N-terminus antibodies represented a product of normal neuronal metabolism. However, if only the antibodies directed to C-terminus of the Aβ had been used, the conclusion would have been that the intracellular Aβ is only detected in the brains of subjects with AD, and this would confirm the reports suggesting that the accumulation of intracellular Aβ is an event associated with the pathogenesis of AD. These results strongly emphasize that staining results and interpretations are strongly dependent on several variables that include the choice of antibodies as well as the methods employed (eg, pretreatment condition) when assessing the presence of intracellular Aβ.

Fixation and Pretreatment Factors

Tissue fixation is used to chemically preserve the natural state of the tissue or cells for subsequent histological analyses. Unfortunately, the type (eg, formalin, paraformaldehyde) and duration of fixation can hamper the ability of the antibody to bind to its target antigen, which could lead to false-negative immunolabeling. For example, some studies have only found intracellular Aβ in the brains of aged and AD individuals, but not in the brains of nonhuman primates, while others have detected intracellular Aβ in cortical neurons of monkeys of various ages. The author noted that the inconsistent data could be attributed to differences in tissue fixation, as the anti-Aβ antibodies produced a stronger immunolabeling signal when tissues were fixed with paraformaldehyde than formalin, which may be due to the cross-linkages in paraformaldehyde-fixed tissues that are much weaker than those in tissues fixed with neutral-buffered formalin.

To combat the issues of fixation, several methods were designed to pretreat the specimens before the IHC assay.

Pretreatment methods on the tissue slides include the use of formic acid, periodic acid, enzymatic digestion, or an antigen retrieving/restoration method using heat to assist antibody penetration into the targets in the tissues or cells. In one example, a study compared three conditions (heat or enzymatic pretreatment to no pretreatment) on their effect to detect Aβ42 in formalin-fixed, paraffin-embedded human AD tissues. Although all three protocols produced Aβ42 immunolabeling...
in amyloid plaques using four commercially obtained Aβ42 specific antibodies, only the heat pretreatment protocol consistently detected prominent intracellular Aβ42 in pyramidal neurons suggesting that consistent detection of intracellular Aβ42 is dependent on the IHC protocol using controlled heat.

The use of the formic acid pretreatment method was also evaluated on its effect to detect intracellular Aβ42 in control and AD brain tissues. Both methods detected amyloid in plaques, neurons, ependymal cells, circulating monocytes, vascular smooth muscle, and endothelial cells. Although there were no observable differences in the intensity of the amyloid labeling in these cell types using both pretreatment methods, there were considerable differences in the intensity of amyloid immunolabeling in the plaques. The formic acid produced much more intense amyloid labeling in the plaques than the heat method perhaps overshadowing the relatively less intense and less relevant intraneuronal Aβ immunolabeling. With the heat method, the intensity of the amyloid labeling in the plaques was similar to that detected in nearby neurons, suggesting a neuronal origin of plaques. These data suggest that the obvious benefits of formic acid for increasing the intensity of amyloid plaque immunolabeling may unintentionally emphasize plaques over amyloid-containing cells during analyses especially considering that plaque load was typically the objective of the stain.

In another study, when formic acid was used in conjunction with heat pretreatment, the formic acid treatment counteracted such effects of heat pretreatment via autoclaving. Thus, intraneuronal Aβ42 accumulation may have been underestimated by conventional methods using formic acid only. However, contrary findings were reported noting that formic acid was preferred for the staining of highly aggregated Aβ peptides in fixed frozen or paraffin tissues of the AD transgenic mouse brain.

The use of formic acid and/or heat pretreatment can also affect the intensity of immunolabeling by specific antibodies. Intracellular immunolabeling with clones 6E10 and 82E1 (Fig. 1.5) was only seen when the sections were both heated and incubated in formic acid. In another study, heat pretreatment alone increased immunolabeling the 4G8 and AβPP antibodies, and so the need to properly annotate each step in the methods is so vital not only for reproducibility, but to help in the interpretation of the data.

Critical technical factors, such as the type of tissue fixation, selection of the primary antibody, the type of pretreatment method (if any), and the detections system (although not discussed) will affect immunolabeling intensity, and not only affect the interpretation and reproducibility, but can make it challenging to compare data among studies, a bane that continues to impact IHC methods.
SUMMARY

Before delving into the biological properties of amyloid, the focus of this chapter is to provide a brief background of the origin of amyloid and plaques as they relate to AD. Not only is it important to understand the historical association of amyloid and AD, but it is also informative to understand how amyloid is produced, processed, and detected in cells, which is the basis of the data presented in this book.

References

REFERENCES


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