Verdict in Neurodegeneration: Murder by Amyloid Protofibrils

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Summary

Neurodegenerative diseases are devastating human disorders characterized by neuronal cell death. Each disease is linked to the misfolding of specific proteins that accumulate and cause intracellular or extracellular brain inclusions. However, the mechanism by which neuronal death develops is still unknown. Since these protein aggregates are typically fibrillar, one hypothesis identifies such fibrils as neurotoxic. My lab primarily focused on biochemical analysis of misfolded and aggregated proteins in Prion disease, Alzheimer's disease (AD), and Parkinson's disease (PD). In Prion disease, we developed in vitro assays that recapitulated how a protease-resistant Prion form of the PrP protein causes the change in shape of the healthy form, leading to dangerous accumulation of the toxic, infectious Prion shape. In AD and PD, we discovered prefibrillar oligomers, which we named protofibrils. We believe this to be the true toxic conformer, as one of its properties is to permeabilize lipid membranes. In contrast, the fibrillar aggregates are likely protective. We have also investigated how polymorphisms in an ubiquitin hydrolase (UCHL-1) involved in PD can be either protective or a risk factor. Thus, our insight into protein misfolding of diverse proteins provides new avenues for developing treatments and cures against these neurodegenerative diseases.

Introduction

Our society is afflicted by a variety of human brain disorders. Amongst them are a group of disorders classified as neurodegenerative diseases. Neurodegeneration involves the deterioration or death of neurons in the brain, specific to the disease. This degeneration is devastating because regeneration in the central nervous system is extremely restricted[1].

Neurodegenerative diseases affect millions of people worldwide and unfortunately, no cure currently exists. Understanding the molecular and cellular mechanisms by which these diseases operate, might eventually lead to more effective treatments and ultimately a cure. By 2040, neurodegenerative diseases will surpass cancer as one of the leading causes of death[2]. The effect on the human population provides the necessary motivation to study their pathogenesis.

The best-studied neurodegenerative diseases include, Alzheimer's disease (AD), Lou Gehrig's disease, Creutzfeldt-Jacob disease, Huntington disease, Parkinson's disease (PD), and Transmissible Spongiform Encephalopathies (TSEs), or Prion diseases. These all vary in symptoms and time of onset, but one common and shared characteristic is the presence of an abnormal protein accumulation unique to each disease. In each specific disease, a particular protein misfolds, aggregates, and usually leads to accumulation as intracellular or extracellular inclusions in the brain of patients[3]. These inclusions are often fibrillar in nature[4]. In most cases, these proteins are thought to gain a toxic new function, leading to pathogenesis, but data is not yet conclusive.

This review focuses on studies that have attempted to provide a relationship between protein aggregation and neuronal cell death. We focus on three neurodegenerative diseases: Prion disease, AD, and PD. Studies with humans are difficult because symptoms do not arise until later in the disease, but some studies with animals have proven to be effective. My lab, however, focuses on characterizing the early stages of this protein aggregation process, by specifically isolating the aggregates from post-mortem brains and performing kinetic studies of in vitro protein aggregation[4]. Additionally, by understanding the in vitro effects of the protein aggregates, we can establish a link between aggregation and toxicity[5].

We have advanced the understanding of protein aggregates by characterizing a special case of proteins called prions (here on referred to as PrP). These are different from other aggregation prone proteins in that they are transmissible. A protease-resistant Prion form of PrP causes the change in shape of the healthy form, leading to dangerous accumulations of the toxic infectious prion shape[6].

We also have contributed to the field by discovering prefibrillar intermediates in AD and PD, referred here as protofibrils, which are precursors to amyloid formation. We also believe these are the true toxic species conformer, because one of their properties is to permeabilize lipid membranes. In contrast, the fibrillar aggregates are likely protective[7,8]. Moreover, we have also investigated how polymorphisms in an ubiquitin hydrolase (UCHL-1) involved in PD can either provide protection or increase risk for the onset of the disease[9].

Prion Disease

In my lab, we first characterized Prion disease and the Prion protein (PrP) as the culprit protein. Prion diseases include scrapie, bovine spongiform encephalopathy (BSE), or more commonly known as mad cow disease, and human Creutzfeldt-Jakob disease (CJD). Prion diseases are different than other neurodegenerative diseases because they are transmissible across individuals of one species, and in certain cases, between different species as well4. Symptoms of Prion diseases vary, but holes in post-mortem brains remain to be the hallmark symptom[10].

The Unique Case of the Prion Protein

The question of which protein accumulates in Prion diseased brains was puzzling. Recently, PrP was determined to have more than one conformation, and one of these conformations were found in individuals with Prion disease, linking it as a possible disease-causing shape. Non-disease causing PrP (here on referred to as PrP^{c}) is usually a protease-sensitive protein. What is interesting is that the possible disease-causing PrP (here on referred to as PrP^{sc}) is resistant to proteases. Proteases or proteinases are enzymes that break down proteins by hydrolyzing the

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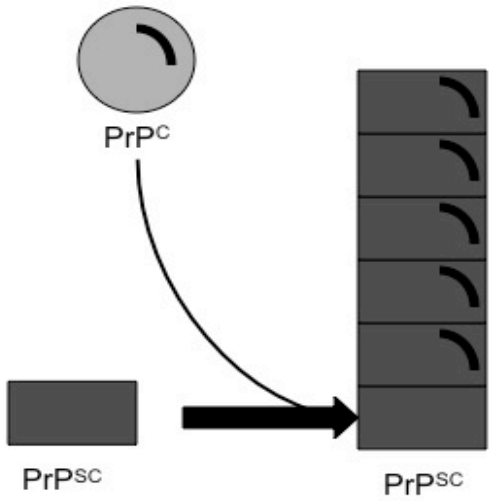

Figure 1: Shape change of PrPC by PrPSC
In this model, PrPC (light gray circle) is interacting with PrPSC under certain cellular conditions; the interaction will cause a conformation change in PrPC to the PrPSC shape. The semi-circle structure at the top right of the light gray circle and the five dark gray squares demonstrate the conformation change from one state to another.

peptide bonds that link amino acids together. A gap in the field was whether or not Prp^{sc} converted Prp^{c} into Prp^{sc}.

My lab was the first to report this conversion *in vivo.* Using this cell-free system was effective because the reactions occured quickly and we could use PrP^{sc} from infected brains and PrP^{c} from uninfected tissue[11]. We started the reaction with PrP^{sc} from infected hamster brains and added radioactively labeled PrP^{c}. After incubation for two days, protease was added and radioactively labeled PrP exhibited fragments of comparable size to infected PrP^{sc} sources. These bands were not observed when PrP^{sc} was not present, but was observed when PrP^{sc} was highly denatured (but not completely). In fact, partial denaturation of PrP^{sc} was optimal for conversion. Therefore, PrP^{c} converted into PrP^{sc} only when the latter is present, even at small amounts, which was demonstrated by determining its sensitivity to protease after the interaction[6] (Figure 1).

Furthermore, the mechanism by which normal PrP^{c} is converted into the protease-resistant PrP^{sc} depends on a variety of factors. Firstly, increasing the concentration of PrP^{sc} always increases the conversion rate of PrP^{c}, which also increases with time. Secondly, in an experiment where PrP^{sc} was centrifuged and the pellet and supernatant were tested for converting ability, we showed that the converting ability was only apparent in the pellet. This coincides with the idea that conversion is accelerated by aggregates[12].

We further analyzed the species selectivity of Prion diseases, since this was an important question for understanding the basis of species barriers in transmission. We used PrP^{c} and mouse, hamster and chimeric PrP^{sc} to study this barrier. Results demonstrated that mouse PrP^{sc} effectively converts hamster PrP^{c}. Interestingly, mouse PrP^{c} did not convert to protease-resistant PrP^{sc} when hamster PrP^{sc} was present. We further studied the primary sequence of PrP^{c} to determine the conversion regions. Using mouse and hamster PrP^{c} chimeras, we observed an area in the PrP sequence that seems to be critical in PrP^{sc} species interaction, especially since mouse and hamster PrP sequences differ at residues 139, 155, and 170[13].

Analyzing species-specificity of transmission is relevant since the infectious protein has the ability to cross species barriers. This is important when analyzing the infectivity to humans from eating infected meat from cows, as was demonstrated in the 1986 British BSE epidemic[10]. We provided evidence that the efficacy of transmission is much greater for homologous conversions than for cross-species conversions. Thus the ability of the infectious agent in BSE to affect humans is very low. The experiment tested the conversion rate of PrP^{c} of and among human, sheep, mouse, hamster and bovine PrP^{sc}, and little to no reaction was seen in non-homologous PrP combinations[14].

Lastly, beyond species selectivity of PrP transmission, my lab has demonstrated the varying conversion of PrP^{c} from different PrP^{sc} strains. This was first observed in mice with drowsy and hyper strains, because their PrP^{sc} were cleaved at different sites with protease. We demonstrated that when PrP^{c} is taken from infected tissue and put into the culture with PrP^{c} (of either strain), the PrP^{c} converts into two distinct forms of PrP^{sc}. This conversion is dependent upon what PrP^{sc} strain is present. This suggests that there are different scrapie strains and pre-existing PrP^{sc} can determine the conversion shape of PrP^{c} [15].

My years of research with PrP have provided evidence of the necessity of PrP^{sc} to be present in order for PrP^{c} to be converted in a cell-free system, identifying a relationship to Prion disease infectivity. This work could further our understanding of cell death and protein aggregation in pathogenesis. Now, let's examine AD and PD and how my lab discovered an intermediate in fibril formation that may be leading to cell death.

Alzheimer's Disease

Continuing to identify the toxic agents in neurodegenerative diseases is important, and our work with AD has furthered our understanding of these culprit proteins. AD is the most prevalent neurodegenerative disease and is characterized by the loss of cognitive function4. Brains infected with AD are characterized by the presence of extracellular fibrillar amyloid plaques and intraneuronal neurofibrillary tangles. This is exhibited in the memory and learning brain areas. The plaques are mostly composed of the amyloid β-protein (Aβ), which is derived from the amyloid precursor protein (APP)16. Secretase enzymes break down Aβ in three ways: a harmless Aβ1-40, and two toxic Aβ1-42 and Aβ1-43 cuts17. Currently, data remains inconclusive about whether the presence of the protein will lead to pathogenesis.

Much research has been done to understand the Aβ protein. Fibrillar amyloid has been shown through X-ray diffraction data to contain the cross- β conformation. Prior to my work, one of the questions in the field was to determine the factors that lead to aggregation of the Aβ protein at the molecular level[18]. Therefore, we first focused on addressing the role of the hydrophobic C-terminal region of the protein in the stabilization and formation of fibrils. Conformational properties as well as solubility studies demonstrated that the C-terminal sequence, in particular the β 34-32 sequence, possesses a stable anti-parallel β-sheet. This is responsible for the insolubility of the β-protein, which also could implicate its role in amyloid plaque deposition[18, 19].

Furthermore, the peptide NAC (non- Aβ component of AD) was identified as a component of the amyloid plaque, comprising of 10% of protein concentration. NAC is a fragment of α-synuclein, a protein involved at the pre-synaptic nerve terminal. Since this fragment is associated with AD plaques, it was necessary to understand its mechanism in amyloid formation. My lab answered this question by demonstrating that NAC amyloid can seed Aβ

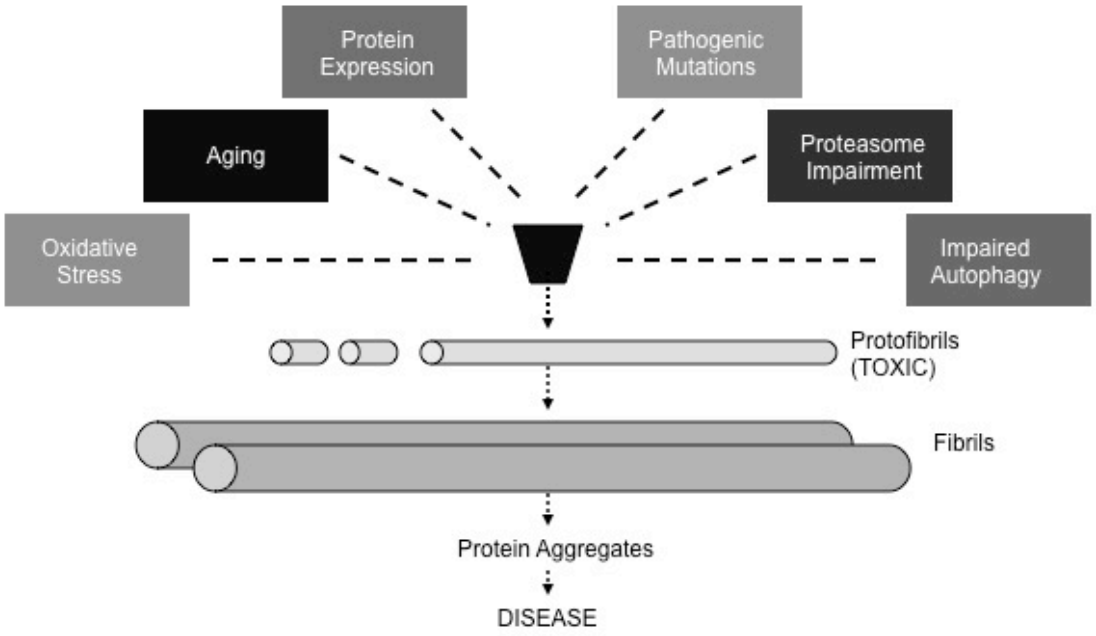

Figure 2: Disease and protofibril formation may have a common cause
A number of factors including oxidative stress, aging, protein overexpression, pathogenic mutations, and impaired autophagy or proteasome, have been proposed to trigger protofibril formation. We propose a strong correlation between protofibril formation and neurodegenerative disease in patients' brains. On the other hand, fibrils have been discussed as having a possible protective component.

protein fibril formation and that Aβ protein and PrP can seed NAC amyloid. This links the NAC precursor, part of α-synuclein, to neurodegeneration[20]. By further analyzing this possibly important component in amyloid deposition, we see that NACP is representative of a class of "natively unfolded" proteins from its unusual conformation properties. These proteins often have abilities in regulating protein-to-protein interactions[21].

One important characteristic of Aβ is its location. In order to further understand Aβ, we wanted to test where it is mostly found within the brain. We demonstrate that the compartment in which Aβ resides in is a detergent-insoluble glycolipid membrane (DIG). This finding was important because it might be possible that APP makes the Aβ cuts there[22].

Additionally, the apolipoprotein E (ApoE) allele is one risk factor for the development of late onset AD, because it is also involved in amyloid formation. Depending on the ApoE genotype, there is a strong correlation between it and the level of amyloid deposition. For example, ApoE4 is correlated with most deposition. My lab demonstrated that neither ApoE3 nor ApoE4 inhibited the seeded growth of amyloid fibrils. Therefore, when looking at inhibitors of amyloid formation it is important to examine the ApoE alleles[23].

The Identification of a New Toxic Conformer

Now that fibril formation was further understood, the field now wanted to test if inhibition of the formation of amyloid fibrils had a therapeutic benefit. This would only be the case if fibril formation is toxic. If the pathogenic species were actually a precursor or an alternate to fibril formation, inhibiting fibrils would only increase toxicity. Therefore, it was essential to examine the mechanisms of fibril assembly and determine the true pathogenic species. Using atomic force microscopy (AFM) my lab was able to identify an intermediate in fibril formation, which we termed protofibril. AFM is a powerful technique because it has been used to determine early events in the process and is able to provide 10-20 nanometer resolutions of a particular species. Data demonstrates that Aβ protofibrils become longer and less numerous over time. Moreover, protofibrils disappear upon fibril formation. The elongation of protofibrils is also dependent on concentration[7]. Once these fibril intermediates were discovered, the question that still puzzled the field is if they are the true pathogenic species, which my lab strongly supports.

Lashuel et al. (2003) examined a mutation of the amyloid β-protein (Artic variant-E22G), demonstrating that Aβ40ARC accelerates fibril formation but also promotes the formation of annular protofibrils. Although the mechanism of protofibrillar toxicity is unknown, annular protofibrils have been implicated in forming ion-permeable pores in other neurodegenerative diseases. Therefore, membrane disruption by pore formation may be a causal factor that leads cell death[24]. Finally, to understand the role of protofibrils, we characterized their secondary structural features. We found that protofibrils had a very stable H-bonded core structure, possibly demonstrating their role in amyloid formation[25] (Figure 2).

The work with AD performed in my lab has furthered the field's understanding of Aβ in addition to the existence of the fibrillar intermediates named protofibrils, which have been implicated in amyloid formation. Further work with PD and protofibrils has increased our knowledge of the link between protofibrils and cell death, which is our next area of discussion.

Parkinson's Disease

PD has furthered our understanding of the protofibril hypothesis in protein aggregation and it has been my latest area of focus in recent years. Classic PD symptoms include tremors, muscular rigidity, slowness of movement, and impaired balance and coordination. Similar to other neurodegenerative diseases, PD is linked to the misfolding

and accumulation of the protein, α -synuclein, into structures called Lewy bodies located in the substantia nigra region of the midbrain. These individuals experience loss of dopaminergic neurons[2,26,27,28,29]. PD, like, AD also forms fibrils. Studies in my lab have demonstrated that mutant forms of α-synuclein (A30P and E46K) also form Lewy body-like fibrils in vitro[30]. Work with PD and α-synuclein has also demonstrated that α-synuclein forms anti-parallel β-sheets with fibrils, just like Aβ. Interestingly, researchers also saw protofibrils, but in a "sphere" shape. Overall, the data in the field links α-synuclein characteristics to other amyloid proteins[31,32]. Cytoplasmic concentrations of dopamine have been demonstrated to promote and stabilize protofibrillar intermediates, linking the dopaminergic selectivity of α-synuclein[33].

Another radical hypothesis in the field is that synuclein membrane binding causes cell toxicity, as opposed to fibrillization in yeast. Studies show that α-synuclein disrupts normal membrane processes and eventually leads to toxicity[31]. Further work with yeast has demonstrated that the N-terminus has an important role in α-synuclein's normal function. Therefore, its deletion causes toxicity.

Confirmation of the Protofibril Testimony

In order to answer the question of whether protofibrils are pathogenic or not, my lab continued to assess protofibril characteristics. Volles et al. (2001) provided clearer evidence of protofibrillar effects on cells. Data demonstrated that these protofibrillar intermediates binded tightly to synthetic vesicles via their β-sheet structure. This process causes a transient permeabilization of membranes, a potentially toxic event. This event could also lead to cellular stress and possibly apoptosis4. With this study, we have made a stronger case for the protofibrillar toxic hypothesis. Surprisingly, we have demonstrated that α-synuclein protofibrils did not bind to plasma membranes nor permeabilize synthetic vesicles. Ultimately, α-synuclein inhibits the protofibril and fibril formation of A53T, one of α-synuclein's familial gene mutations[36].

Examining USCH-L1

One important question that my lab has dealt with are the α-synuclein degradation routes. This is important because altered degradation of α synuclein has been implicated in the pathogenesis of PD. Data demonstrates that WT α-synuclein is degraded and internalized by the cell through chaperone-mediated autophagy (CMA)37. Dopamine-modified α-synuclein, on the other hand, has been demonstrated in my lab to be poorly degraded by CMA. Furthermore, this activity also blocks degradation of other common CMA substrates. Since an important degradation pathway is compromised, we saw an increase in cellular stressors. The link between dopamine modified α-synuclein and deficient CMA explains the selective degradation of dopaminergic cells[38].

Further work with PD and degradation routes led my lab to studies involving an ubiquitin C-terminal hydrolase-L1 (UCH-L1), an enzyme that is critical for protein degradation. An interesting characteristic of UCH-L1 mutants is their ability to have both a protective component (S18Y), and to be a risk factor for the onset of the disease (I93M). My lab's results demonstrated that the UCH-L1 mutation increases PD susceptibility through ligase activity[9]. UCH-L1 is also interesting because its hydrolase activity is tightly regulated. This allows it to only be active upon binding with a specific substrate, which induces a shape change[39]. Past data had suggested a role of UCH-L1 in proteasome activity. Liu et al. (2009) demonstrated that UCH-L1 exists in membrane-associated forms, which were enhanced by farnesylation. Moreover, this study demonstrated that UCH-L1 has no effect on the proteasome, perhaps linking lysosome degradation involvement. More importantly, farnesyltransferase inhibitors reduce α-synuclein levels and increase cell viability[40].

Conclusion

It's important to study neurodegenerative diseases because of their affliction on the human population. One characteristic shared by all diseases is the presence of an abnormally misfolded protein, and consequential cell death in a specific area of the brain. In most cases, these proteins are thought to gain a toxic new function, leading to pathogenesis, but data is not yet conclusive. My lab focuses on in vitro studies of PrP, Aβ, and α-synuclein.

Overall, we have characterized the shape change of the healthy PrP form to the protease-resistant form through interactions with the protease-resistant form, which, leads to pathogenesis. Furthermore, we discovered fibrillar intermediates, or protofibrils, which we believe are the true toxic species in amyloid formation. Their role has been associated with membrane permeabilization.

A limitation in our study is that we only dealt with assessing the molecular environment in vitro, which is not representative of other factors present in organisms or live cells. Understanding the role of amyloid formation in neurodegenerative diseases is a worldwide concern and need.

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