Protein Misfolding and Neurodegeneration

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A key molecular pathway implicated in diverse neurodegenerative diseases is the misfolding, aggregation, and accumulation of proteins in the brain. Compelling evidence strongly supports the hypothesis that accumulation of misfolded proteins leads to synaptic dysfunction, neuronal apoptosis, brain damage, and disease. However, the mechanism by which protein misfolding and aggregation trigger neurodegeneration and the identity of the neurotoxic structure is still unclear. The aim of this article is to review the literature around the molecular mechanism and role of misfolded protein aggregates in neurodegeneration and the potential for the misfolding process to lead to a transmissible form of disease by a prion-based model of propagation.
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Neurodegenerative diseases are some of the most debilitating disorders, affecting thinking, skilled movements, feelings, cognition, and memory. This diverse group of diseases includes common disorders such as Alzheimer disease (AD) and Parkinson disease (PD) and rarer disorders such as Huntington disease, spinocerebellar ataxia, transmissible spongiform encephalopathies, and amyotrophic lateral sclerosis. Despite the important differences in clinical manifestation, neurodegenerative disorders share some common features such as their appearance late in life, the extensive neuronal loss and synaptic abnormalities, and the presence of cerebral deposits of misfolded protein aggregates.[1] These deposits are a typical disease signature, and although the main protein component is different in each disease, they have similar morphological, structural, and staining characteristics. *Amyloid* is the name originally given to extracellular protein deposits found in AD and systemic amyloid disorders, but it is nowadays used to refer in general to disease-associated protein aggregates.[1] In this article, we use the term *amyloid-like deposits* to refer to these aggregates without necessarily meaning that they are structurally equivalent.

In each neurodegenerative disease, the distribution and composition of protein aggregates are different.[1] In AD, there are 2 types of protein deposits. Amyloid plaques are deposited extracellularly in the brain parenchyma and around the cerebral vessel walls, and their main component is a 40- to 42-residue peptide termed β-*amyloid protein* (Aβ).[2] Neurofibrillary tangles are located in the cytoplasm of degenerating neurons and are composed of aggregates of hyperphosphorylated tau protein.[3] In patients with PD, Lewy bodies are observed in the cytoplasm of neurons of the substantia nigra in the brain. The major constituents of these aggregates are fragments of a protein named α-*synuclein*.[4] In patients with Huntington disease, intranuclear deposits of a polyglutamine-rich version of huntingtin protein are a typical feature of the brain.[5] Patients with amyotrophic lateral sclerosis have aggregates mainly composed of superoxide dismutase in cell bodies and axons of motor neurons.[6] Finally, the brains of humans and animals with diverse forms of transmissible spongiform

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encephalopathy are characterized by accumulation of protease-resistant aggregates of the prion protein (PrP).[7]

Compelling evidence coming from biochemical, genetic, and neuropathological studies supports the involvement of protein misfolding and aggregation in the pathology of neurodegenerative diseases.[1] For example, the presence of abnormal aggregates usually occurs in the brain regions mostly damaged by the disease. Mutations in the gene encoding for the misfolded protein produce inherited forms of the disease, which usually have an earlier onset and more severe phenotype than the sporadic forms.[8] Transgenic animals expressing the human mutant gene for the misfolded protein develop some of the typical neuropathological and clinical characteristics of the human disease.[9] Finally, misfolded protein aggregates produced in vitro are neurotoxic, inducing apoptosis.[10]

MECHANISM AND INTERMEDIATES IN PROTEIN MISFOLDING AND AGGREGATION

The misfolding and aggregation of proteins implicated in neurodegenerative diseases has been modeled in vitro. There is no evident sequence or structural homology among the proteins involved in diverse neurodegenerative diseases. Low-resolution structural studies have shown in all cases a large structural rearrangement between the monomeric native protein and the aggregated material.[11] In most cases, the native monomeric protein is mainly composed of α-helical and unordered structure, whereas the misfolded polymers are rich in β-sheet conformation. Although high-resolution studies of aggregated proteins have been difficult with conventional methods because of their insolubility and noncrystalline nature, recent studies using nuclear magnetic resonance spectroscopy have confirmed the β-sheet–rich structure of protein aggregates implicated in neurodegenerative diseases.[11-13]

Although the detailed mechanism for the formation of fibrillar amyloid-like aggregates is not entirely clear, the initiating event is protein misfolding, which results in the formation of aggregation-prone structures that grow by an autocatalytic mechanism. Kinetic studies have suggested that the critical event is the formation of protein oligomers that act as seeds to further propagate protein misfolding.[14] This is the basis for the currently accepted nucleation-dependent polymerization model of amyloid formation.[14-16] Diverse proteins have been shown to follow this crystallization-like process, including Aβ, huntingtin, and α-synuclein. According to this model, aggregation starts after the protein concentration exceeds a point known as the critical concentration.[15] Unfavorable interactions between monomers determine a slow phase (termed *lag phase*) in which oligomers are formed, providing an ordered nucleus to catalyze the further growth of the polymers. Preformed nuclei (seeds) serve as templates for the reaction, and as a result, the initial, slow phase of primary nucleation is eliminated.[14,15]

In addition to mature fibrils, several other structures have been described as part of the protein misfolding and aggregation process, including soluble oligomers, pores, annular structures, spherical micelles, and protofibrils[17-19] (**Figure**). Interestingly, these diverse structures have been identified in the amyloidogenesis process of various disease-associated proteins, suggesting common misfolding pathways and perhaps common neurodegeneration mechanisms.[17-19] However, the biological relevance of these intermediates is currently not clear, and it is even questionable whether some of them exist in a meaningful quantity in the diseased brain. Furthermore, although it is likely that these metastable species assemble in a stepwise process, the relative importance of each is difficult to assess because they are too unstable to characterize.[17,20] Recent technological developments including the production of antibodies that recognize specifically different types of aggregated species such as oligomers, annular assemblies, protofibrils, and fibrils have led to important advances in understanding the role of these structures in neurodegeneration.[17,21] Strikingly, the intermediate species formed by different proteins are specifically recognized by the antibodies, suggesting that they display a common structural motif that is distinct from the other aggregated species.[17,21] These findings indicate that the antibodies recognize a generic polypeptide backbone epitope that is independent of the amino acid sequence but is shared among all types of polymers.[17,21] In summary, the biophysical studies of the intermediates in the amyloid formation process indicate that diverse species with progressive degrees of aggregation are present simultaneously and in dynamic equilibrium between each other.[17,18,20] This makes it very difficult to evaluate the relative contribution of different protein structures to neurodegeneration.

NEURODEGENERATION AND DISEASE

Selective neuronal loss, synaptic alterations, and neuroinflammation (in the form of reactive astrocytosis and activated microglia) are typical features of neurodegenerative diseases.[22] However, the region of the brain most affected differs among diseases and determines the distinct clinical symptoms of each. Although it was widely thought that neuronal apoptosis was the most important problem in neurodegeneration, recent evidence from different diseases suggests that extensive neuronal death may not be the initial cause of the disease.[19] Indeed, clinical symptoms have been clearly described before significant neuronal loss, and a better temporal and topographic correlation is found with synaptic dysfunction.[19]

As outlined earlier, although protein misfolding and aggregation are undoubtedly associated with neurodegeneration and disease, the mechanism by which misfolded aggregates produce synaptic dysfunction and neuronal death is unknown. It is also unknown which of the different polymeric structures formed in the process of amyloidogenesis is the triggering factor of brain damage[19,23] (Figure). For many years, it was thought that large amyloid-like protein deposits were the species responsible for brain damage.[1] However, the hypothesis that deposited aggregates are toxic has been challenged by results of histopathological, biochemical, and cell biology studies.[19,23] Neuropathological analysis of the brains of people with PD or AD has shown that neurons containing Lewy bodies or neurofibrillary tangles seem healthier

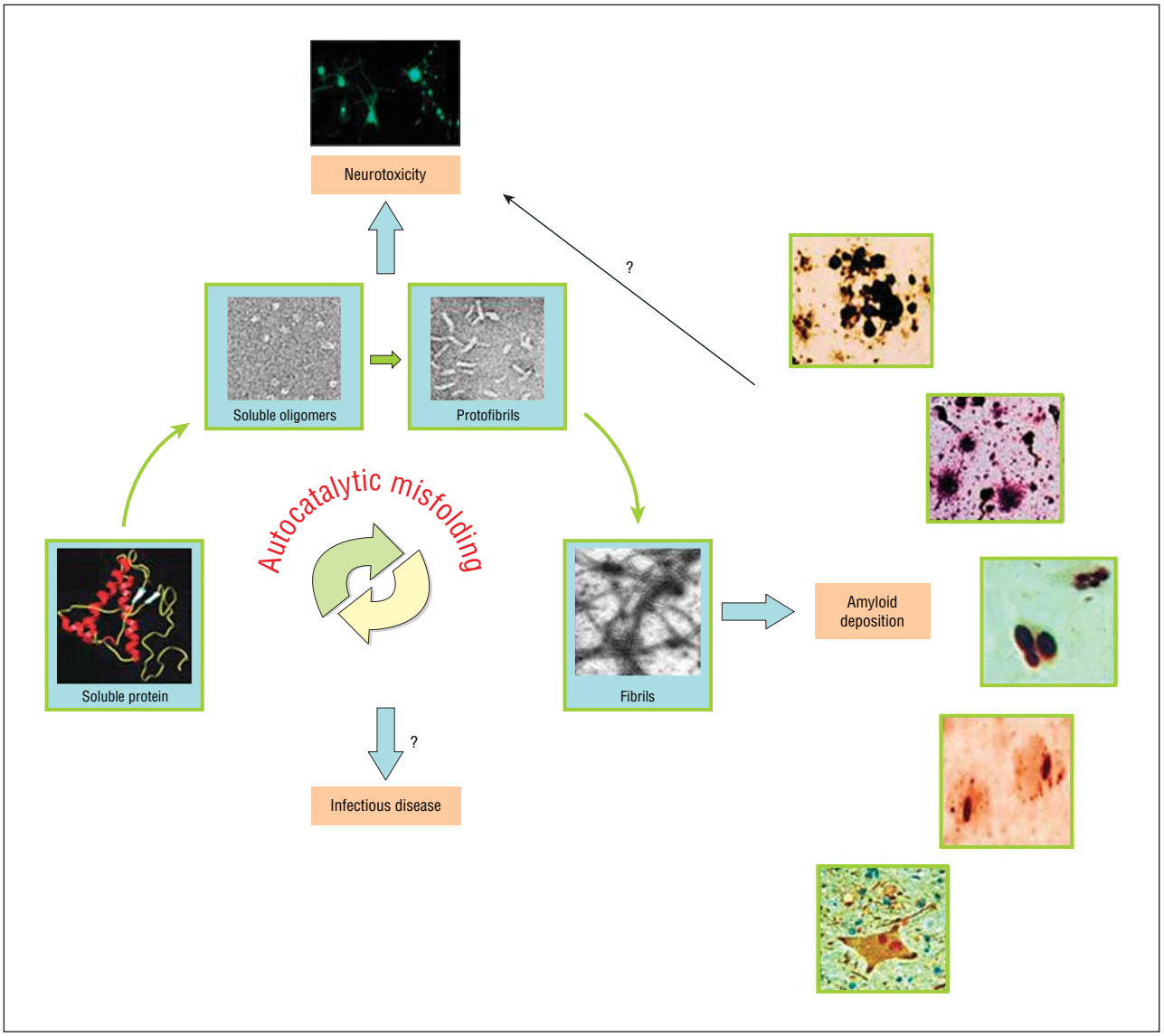

Figure. Molecular pathways in neurodegeneration. Compelling evidence suggests that a common cause of neurodegenerative diseases may be the misfolding of a protein to form toxic oligomeric structures that over time accumulate in large protein deposits in the brain. Neurodegeneration in all of these diseases is characterized by neuronal damage in the form of synaptic alterations, cellular apoptosis, and deposition of amyloid-like plaques. Protein misfolding and aggregation follow an autocatalytic seeding-polymerization mechanism that makes all of these diseases inherently capable to be transmitted by infection. Indeed, one of the members of this group of disorders, prion diseases, is well documented to be transmissible, and overwhelming evidence indicates that the infectious agent is the misfolded prion protein itself.

than neighboring cells by morphological and biochemical analysis.[24,25] In addition, amyloid-like plaques and Lewy bodies are found in people without evident neuronal loss or clinical signs of AD or PD.[26,27] Moreover, in some animal models of AD, transmissible spongiform encephalopathy, Huntington disease, and ataxias, cerebral damage and clinical symptoms have been detected before protein aggregates.[28,29] These findings have led to today's most accepted hypothesis that the process of misfolding and early stages of oligomerization, rather than the mature compacted aggregates deposited in the brain, are the real culprits in neurodegeneration.[17,19,23] This hypothesis is supported by results showing that purified oligomeric species and protofibrils are toxic to cultured neurons, inhibit hippocampal long-term potentiation, impair synaptic functions, and disrupt cognition and learned behavior in rats.[17,19,23] Some investigators have gone beyond to propose that the formation of amyloid-like fibrils could be a protective mechanism to sequester and isolate toxic misfolded intermediates.[23] Although this is theoretically an attractive hypothesis, it is likely that both soluble misfolded intermediates and amyloid-like fibril deposits are toxic, but perhaps by different mechanisms.[1] For example, soluble oligomeric species might induce a signaling pathway leading to apoptosis, whereas amyloid-like plaques might take up tissue space, break down neuronal connections, and recruit essential cellular factors. In addition, the concept that protein deposits are static and irreversible structures has been changing in the last several years to accommodate recent results showing that the protein component of aggregates as well as the associated proteins are in dynamic equilibrium with the soluble version of the proteins.[19,20,30] Therefore, the interesting possibility that large amyloid-like protein de-

posits act as a reservoir of toxic oligomeric species must be considered.

The most widely accepted theory of brain degeneration in neurodegenerative diseases proposes that misfolding and aggregation result in the acquisition of a neurotoxic function by the misfolded protein.[1] Several mechanisms have been proposed for the neurotoxic activity of misfolded aggregates, and it is likely that different pathways operate depending on whether the proteins accumulate intracellularly or extracellularly.[1] Extracellular aggregates might activate a signal transduction pathway leading to apoptosis by interacting with specific cellular receptors. Intracellular aggregates might damage cells by recruiting factors essential for cell viability into the fibrillar aggregates. Components of the proteosome, chaperones, cytoskeletal proteins, and transcription factors have been found in huntingtin and α-synuclein aggregates.[31,32] Another well-supported mechanism is membrane disruption and depolarization mediated by ion channel and pore formation, resulting in alteration of ion homeostasis and dysregulation of cellular signal transduction, leading to cell death.[17] Finally, protein aggregates could induce oxidative stress by producing free radical species, resulting in protein and lipid oxidation, elevation of intracellular calcium levels, and mitochondrial dysfunction.[33,34]

WHEN AN AMYLOID IS A PRION

The critical role of the protein misfolding process is perhaps mostly clear in the prion disorders,[35] also called transmissible spongiform encephalopathies, which are the only neurodegenerative disease transmissible by infection. The nature of the infectious agent and its mechanism of propagation are certainly some of the most debated and intriguing subjects in modern biology.[36] Initially, the infectious agent was thought to be a virus with an extraordinarily long incubation time and complicated properties that make it difficult to isolate. However, the facts that it resists conventional antiviral inactivation procedures[37] and that it is smaller than any other known viral particle[38,39] led to the hypothesis that the infectious agent is devoid of nucleic acid and instead consists of a self-replicating protein.[40] In 1982, Prusiner[41] and coworkers isolated a protease-resistant glycoprotein and proposed that it was the active component of the infectious agent, which they called *prion* (for proteinaceous infectious particle). The characterization of the gene encoding for the prion protein along with structural and biochemical studies started to reveal the unorthodox and fascinating aspects of prion biology.[42-44] During the last 20 years, compelling evidence has accumulated to support the prion hypothesis, including the finding that highly purified PrP^{Sc} produces the disease when injected into wild-type animals[41] and the discovery that PrP knockout mice are resistant to prion infection.[45] Nevertheless, skeptics argue that definitive proof consisting of the in vitro generation of infectivity by misfolding of the prion protein is still missing.[36,46] Recent reports have come tantalizingly close to such proof.[47,48]

The basic concept in the prion hypothesis is that the misfolded prion protein (PrP^{Sc}) is the only component of the infectious agent that can replicate in the brain in the absence of nucleic acid by converting the natively folded prion protein (PrP^{C}) into the misfolded form.[36,49] Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^{C}, catalyzing its conversion to the pathogenic form of the protein. The precise molecular mechanism of the conversion from PrP^{C} to PrP^{Sc} is not well understood. However, the available data support a model in which infectious PrP^{Sc} is an oligomer that acts as a seed to bind PrP^{C} and catalyze its conversion into the misfolded form by incorporation into the growing polymer.[50,51] At some point, the long PrP^{Sc} polymers break into smaller pieces either by a mechanical force or catalyzed by an as-yet-unknown process. This fragmentation allows the increase in the number of effective nuclei to direct further conversion of PrP^{C}.

The seeding-nucleation model provides a rational and plausible explanation for the infectious nature of prions. Infectivity lies on the capacity of preformed stable misfolded oligomeric proteins to act as a seed to catalyze the misfolding and aggregation process[14] (Figure). Indeed, in vitro conversion assays have been developed based on the assumption that prion replication depends on the formation of oligomeric seeds.[51,52] As discussed earlier, protein misfolding and aggregation in other neurodegenerative (and also systemic) disorders also follow a seeding-nucleation model; in fact, acceleration of protein aggregation by the addition of seeds has been convincingly reported in vitro for several proteins implicated in diverse diseases.[15,53] These findings suggest that protein misfolding processes have the inherent ability to be transmissible (Figure). Therefore, the key question is, why are other neurodegenerative diseases that are associated with protein misfolding and aggregation not transmissible? Or, perhaps a more appropriate question is, are other neurodegenerative diseases transmitted by infection through a prion-like phenomenon?

WHEN AN AMYLOID IS NOT A PRION

Transmissibility of amyloidosis and other protein misfolding disorders has not been thoroughly investigated,[14,54] but it is generally assumed, based on results from epidemiological studies, that they do not have an infectious origin. It should be emphasized that the mechanisms of conventional infectious diseases do not necessarily apply to this protein-only agent, which follows a complicated mechanism of transmission and requires special routes of infection. In addition, the putative long incubation times (up to several decades in humans) further complicate tracking a potentially infectious origin, which would be particularly difficult in much more prevalent disorders such as AD or PD.

Perhaps the best way to investigate the infectious propagation of a disease is by attempting to transmit it to experimental animals. Several attempts have been made to transmit AD, with intriguing but conflicting results.[55-57] Marmosets injected with AD brain homogenates developed scattered Aβ deposits in the brain parenchyma and cerebral vasculature 6 to 7 years after inoculation.[57] Interestingly, the resultant amyloid lesions were not limited to the injection site. However, other

studies have failed to transmit AD and other neurodegenerative diseases to primates.[56] More recent studies have used transgenic mice expressing the human mutant amyloid precursor protein gene. Infusion of diluted AD brain homogenates intracerebrally into 3-month-old transgenic mice showed no Aβ deposition in the brain 4 weeks after infusion; however, after 5 months, transgenic mice developed profuse Aβ-immunoreactive amyloid plaques and vascular deposits exclusively in the hemisphere injected.[58] After 12 months, abundant Aβ deposits were present bilaterally in the forebrain, but the plaque load was still clearly greater in the injected hemisphere.[59] A follow-up study from the same group found that the seeding activity of brain extracts was reduced or abolished by Aβ immunodepletion, protein denaturation, or Aβ immunization.[60] Interestingly, the phenotype of the exogenously induced amyloidosis depended on both the characteristics of the host and the source of the agent. These findings clearly show that preformed Aβ aggregates can enhance in vivo amyloid formation. However, because these transgenic animals developed AD pathology "spontaneously" later on, it is not possible to conclude that inoculation with AD brain acted as an infectious agent, but just as an accelerator of a process that was genetically programmed to occur. This is different from the prion phenomenon of disease transmission in which animals would not get sick unless exposed to the infectious agent. Other transmission studies have been done with systemic diseases, including amyloidosis associated with deposition of amyloid A and apolipoprotein A-II amyloid.[61,62] Again, the results clearly show that under certain experimental conditions, protein misfolding processes can be transmitted or at least accelerated by administration of oligomeric misfolded seeds.

Despite the fact that all protein misfolding and aggregation processes have the intrinsic possibility for transmissibility, it is likely that biological and pharmacokinetic barriers may prevent some amyloid aggregates from acting like prions.[14] For example, the "infectious" oligomeric seeds may not be able to reach the correct place of the tissue and the right subcellular compartment to propagate the misfolding. This is likely to be a problem especially for some of the intracellular aggregates, such as Lewy bodies in PD or intranuclear aggregates in Huntington disease. There could also be a problem of biological stability, determining that the clearance may be faster than the rate of polymer elongation. The high resistance of PrP^{Sc} to proteases and extreme conditions may be key in the efficiency of prions as infectious agents.[35] Finally, it is possible that some misfolded proteins form hyperstable aggregates that may be poor at propagating misfolding.[39] Indeed, from our findings with the in vitro amplification of mammalian prions[52] and from studies of the replication of yeast prions,[63] it seems clear that fragmentation of aggregates is essential for effective propagation.

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