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Detection of Misfolded Aβ Oligomers for Sensitive Biochemical Diagnosis of Alzheimer's Disease

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SUMMARY

Alzheimer's disease (AD) diagnosis is hampered by the lack of early, sensitive, and objective laboratory tests. We describe a sensitive method for biochemical diagnosis of AD based on specific detection of misfolded Aβ oligomers, which play a central role in AD pathogenesis. The protein misfolding cyclic amplification assay (Aβ-PMCA), exploits the functional property of Aβ oligomers to seed the polymerization of monomeric Aβ. Aβ-PMCA allowed detection of as little as 3 fmol of Aβ oligomers. Most importantly, using cerebrospinal fluid, we were able to distinguish AD patients from control individuals affected by a variety of other neurodegenerative disorders or nondegenerative neurological diseases with overall sensitivity of 90% and specificity of 92%. These findings provide the proof-of-principle basis for developing a highly sensitive and specific biochemical test for AD diagnosis.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population and one of the leading causes of death in the developed world (Hebert et al., 2003). The disease is typically characterized by a progressive amnestic disorder followed by impairment of other cognitive functions and behavioral abnormalities associated with specific neuropathological changes, in particular accumulation of protein aggregates in the form of amyloid plaques and neurofibrillary tangles (Terry, 1994). Although the etiology of the disease is not yet clear, compelling evidence suggests that misfolding, oligomerization, and accumulation of amyloid aggregates in the brain is the triggering factor of the pathology (Selkoe, 2000; Haass and Selkoe, 2007; Soto, 2003). Amyloid aggregates are composed predominantly of a 42-residue peptide called amyloid-β (Aβ), which is the product of the enzymatic processing of a larger amyloid precursor protein (Selkoe, 2000). Aβ misfolding and fibrillar aggregation follow a seeding-nucleation mechanism that involves the formation of several intermediates, including soluble oligomers and protofibrils (Caughey and Lansbury, 2003; Soto et al., 2006; Jarrett and Lansbury, 1993). Recent findings have shown that Aβ oligomers, rather than large amyloid fibrils, might be the culprit of neurodegeneration in AD (Walsh and Selkoe, 2007; Haass and Selkoe, 2007; Glabe and Kayed, 2006; Klein et al., 2004).

AD belongs to a large group of diseases associated with misfolding, aggregation and tissue accumulation of proteins (Soto, 2003). These diseases, termed protein misfolding disorders (PMDs), include Parkinson's disease, type 2 diabetes, Huntington's disease, amyotrophic lateral sclerosis, systemic amyloidosis, prion diseases, and many others (Soto, 2003; Luheshi and Dobson, 2009). In all these diseases, misfolded aggregates composed of different proteins are formed by a similar mechanism resulting in the accumulation of toxic structures that induce cellular dysfunction and tissue damage (Caughey and Lansbury, 2003; Soto et al., 2006; Jarrett and Lansbury, 1993).

One of the major problems in AD is the lack of a widely accepted early, sensitive, and objective laboratory diagnosis to support neuropsychological evaluation, monitor disease progression, and identify affected individuals before they display the clinical symptoms (Parnetti and Chiasserini, 2011; Urbanelli et al., 2009). For diseases affecting the brain, a tissue with low regeneration capacity, it is crucial to intervene before irreversible neuropathological changes occur. Therefore, early diagnosis of AD is of utmost importance. Several lines of evidence point that the process of Aβ misfolding and oligomerization begins years or decades before the onset of clinical symptoms and substantial brain damage (Braak et al., 1999; Buchhave et al., 2012). Recent studies have shown that Aβ oligomers are naturally secreted by cells and circulate in AD biological fluids (Gao et al., 2010; Head et al., 2010; Walsh et al., 2002; Klyubin et al., 2008; Georganopoulou et al., 2005; Fukumoto et al., 2010). Thus, detection of soluble Aβ oligomers might represent the best strategy for early and specific biochemical diagnosis of AD. The challenge of this approach is that the quantity of Aβ

oligomers is likely very small in tissues other than the brain. An additional difficulty for specific detection of Aβ oligomers is that their sequence and chemical structure is the same as the native Aβ protein.

Our strategy to detect misfolded oligomers is to use their functional property of being capable of catalyzing the polymerization of the monomeric protein. For this purpose, we invented the protein misfolding cyclic amplification (PMCA) technology in order to achieve the ultrasensitive detection of misfolded aggregates through amplification of the misfolding and aggregation process in vitro (Saborio et al., 2001). So far, PMCA has been applied to detect minute quantities of oligomeric misfolded prion protein (PrP^{Sc}) implicated in prion diseases (Morales et al., 2012). Using PMCA, we were able to detect the equivalent of a single particle of misfolded PrP oligomer (Saá et al., 2006b) and strikingly to identify PrP^{Sc} in the blood and urine of infected animals at symptomatic and presymptomatic stages of the disease (Castilla et al., 2005; Saá et al., 2006a; Gonzalez-Romero et al., 2008). The basis for the PMCA technology is the fact that the process of misfolding and aggregation of Aβ, PrP, and the other proteins implicated in PMDs follow a seeding-nucleation mechanism (Soto et al., 2002, 2006). In a seeded-nucleated polymerization, the limiting step is the formation of stable oligomeric seeds that, depending on the conditions, may take a very long time to form or not occur at all. Once formed, oligomers grow exponentially by recruiting and incorporating protein monomers into the growing polymer. Addition of preformed seeds into a solution containing the monomeric protein accelerates protein misfolding and aggregation (Soto et al., 2006; Jarrett and Lansbury, 1993). Thus, measuring seeding activity could be used to estimate the presence and quantity of oligomers in a given sample. To increase the sensitivity of detection, PMCA combines steps of growing polymers with multiplication of oligomeric seeds to reach an exponential increase of misfolding and aggregation (Soto et al., 2002). In this study, we describe the implementation and optimization of PMCA for highly sensitive detection of misfolded Aβ oligomers and show its application to detect these structures in the cerebrospinal fluid (CSF) of AD patients.

RESULTS

Cyclic Amplification of Amyloid-β Misfolding

To implement the experimental conditions for Aβ-PMCA, we performed studies using in-vitro-produced oligomeric seeds. Because it is still unknown which of the different species of Aβ oligomers is most relevant for AD pathology, we decided to work with a mixture of oligomers of different sizes generated during the process of fibril formation. Aβ oligomers were prepared by incubation of monomeric (seed-free) synthetic Aβ1-42 (10 μM) at 25°C with stirring. After 5 hr of incubation, we observed an abundance of globular oligomers by electron microscopy with only a small amount of protofibrils and fibrils (Figure 1A). These aggregates were positive with the A11 oligomer-specific antibody (Kayed et al., 2003; data not shown). After longer incubation, protofibrillar and fibrillar structures were observed. The size of the aggregates was determined by filtration through filters of defined pore size and western blotting after SDS-PAGE separation. Oligomers formed by incubation for 5 hr migrated as ~170 kDa SDS-resistant aggregates, with a minor band at 17 kDa (Figure 1B).

Low concentrations of seed-free Aβ1-42 (2 μM) were incubated at 22°C with constant shaking (100 rpm) for different times alone or in the presence of distinct concentrations of synthetic Aβ oligomers, prepared by incubation during 5 hr as described above. Aβ aggregation was studied by the fluorescence emission of the amyloid-binding dye Thioflavin T (ThT) (LeVine, 1993; Soto et al., 1995). The peptide concentration, temperature, and pH of the buffer are critical to control the extent of the lag phase and reproducibility among experiments. Under these conditions, no spontaneous Aβ aggregation was detectable during the time in which the experiment was performed (125 hr). However, Aβ aggregation was observed in the presence of 0.3–8.4 fmol of Aβ oligomers (Figure 1C). To increase the efficiency of seeding, and thus the limit of detection of Aβ oligomers, we introduced cycles of amplification, combining phases of polymer growing with multiplication of seeds as in the PMCA assay. For this purpose, we subjected the samples to intermittent shaking, which has been previously shown to dramatically accelerate the seeded conversion of recombinant prion protein (Atarashi et al., 2008). Under these conditions, the kinetic of Aβ aggregation induced by 8,400, 300, 80, and 3 fmol of Aβ oligomers was clearly faster and easily distinguishable from that observed in the absence of Aβ seeds (Figure 1D). This result indicates that, using the Aβ-PMCA assay, we should be able to detect as little as 3 fmol of Aβ oligomers in a given sample.

Detection of Aβ Oligomers in the Cerebrospinal Fluid of AD Patients

To study the usefulness of the Aβ-PMCA assay to detect seeding-competent Aβ oligomers in biological fluids, we analyzed aliquots of CSF from 50 AD patients, 39 cognitively normal individuals affected by nondegenerative neurological diseases (NNDs), and 37 patients affected by non-AD neurodegenerative diseases (NANDs) including other forms of dementia. The experiments as well as the initial part of the analysis were done blindly, because the investigator was unaware of which samples were coming from AD or controls. Figure 2A shows the average kinetics of aggregation of five representative samples from the AD, NND, and NAND groups. The result indicates that CSF from AD patients accelerates significantly Aβ aggregation as compared to control CSF ($p < 0.001$). To determine the effect of individual samples on Aβ aggregation, we estimated the lag phase (Figure 2B), defined as the time required to get a ThT fluorescence larger than 40 arbitrary units (after subtraction of the blank). This value was selected considering that it corresponds to approximately five times the reading of the buffer alone. We also estimated the P90, which corresponds to the extent of Aβ aggregation at 90 hr (Figure 2C). By comparing both parameters among the experimental groups, a highly significant difference was observed between AD and control samples from individuals with nondegenerative neurological diseases or with non-AD neurodegenerative diseases. No correlation was detected between the aggregation parameters and the age of the AD patients, which indicates that the levels of the marker are not simply