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Research report

Amyloid-β42 protofibrils are internalized by microglia more extensively than monomers

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ABSTRACT

One pathological hallmark of Alzheimer's disease (AD) is the accumulation of amyloid-β peptide (Aβ) in the affected brain. While there are numerous deleterious effects of Aβ accumulation, there is general agreement that a sustained inflammatory response to aggregated Aβ contributes to progressive neurodegeneration in AD and microglial cells play a significant role in this process. Our laboratory and others have shown that small soluble aggregates of Aβ activate a microglia-mediated inflammatory response. One component of the response involves internalization of extracellular Aβ, and this process is likely very sensitive to Aβ structure. In this study we analyzed the proclivity of microglia for internalization of Aβ42 monomers and protofibrils using fluorescently-labeled Aβ. Both Aβ42 species were labeled directly via amino linkage with an Alexa Fluor 488 tetrafluorophenyl ester (AF488-TFP) and then isolated individually by chromatography. Aβ42 protofibrils retained their size and morphological properties after labeling but monomers had a much higher stoichiometry of labeling compared to protofibrils. Primary murine microglia internalized AF488-Aβ42 protofibrils rapidly and in significant amounts compared to AF488-Aβ42 monomers. Microglial internalization of protofibrils was dependent on time and concentration, and corresponded with tumor necrosis factor α secretion. In competition studies, unlabeled Aβ42 protofibril internalization, detected by immunostaining, did not diminish AF488-protofibril uptake. Internalized AF488-Aβ42 protofibrils were found widely dispersed in the cytosol with some lysosomal accumulation but little degradation. These studies highlight the sensitivity that microglia exhibit to Aβ structure in the internalization process and emphasize their affinity for soluble Aβ protofibrils.

1. Introduction

Microglial cells play an important role in Alzheimer's disease-related (AD) neuroinflammation by responding to accumulations of amyloid-β peptide (Aβ) (Heneka et al., 2015). Activated microglia and proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1-β (IL-1β) can be observed clustering around extracellular Aβ plaques in the human AD brain and in transgenic AD mice (Apelt and Schliebs, 2001; Dickson et al., 1993; McGeer et al., 1987). One key problem in AD is that the phagocytic microglia are not completely efficient at removing the plaques, however they may work in some manner to restrict plaque growth (Meyer-Luehmann et al., 2008). The form of Aβ present in, or surrounding, the deposits likely affects the way microglia respond to the peptide accumulation.

Soluble Aβ peptides, primarily 40 or 42 residues in length, are released via enzymatic cleavage of the amyloid precursor protein (APP). Both peptides are ultimately found in the plaque deposits with the dense plaque core comprised of the more aggregation-prone Aβ42. The biophysical mechanisms by which plaque formation occurs in the brain are not fully understood however significant *in vitro* work has increased our understanding of the Aβ aggregation process. These studies have shown that unstructured Aβ monomer will undergo non-covalent self-assembly (Jarrett et al., 1993) to form a polydisperse mixture of soluble oligomers (Bitan et al., 2003; Kayed et al., 2003; Mittag et al., 2014) and/or protofibrils (Harper et al., 1999; Mittag et al., 2014; Walsh et al., 1997, 1999) and ultimately insoluble fibrils (Harper et al., 1997a). Aβ assembly occurs via a nucleation-dependent polymerization process (Jarrett and Lansbury, 1993) and the rate-limiting nucleation step is characterized by a lag phase followed by rapid polymerization. More recent information on plaque composition suggests that there is greater structural diversity within the plaques

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β protein; aCSF, artificial cerebrospinal fluid; HFIP, hexafluoroisopropanol; PBS, phosphate-buffered saline; SEC, size exclusion chromatography; TEM, transmission electron microscopy; TNFα, tumor necrosis factor α

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than previously thought. Hyman and colleagues observed a halo of oligomeric Aβ surrounding Aβ plaques after immunostaining with the oligomer-selective NAB61 antibody (Koffie et al., 2009). Although the plaques contain fibrillar Aβ at the core (Terry et al., 1964), these data suggest that microglial cells may interact with multiple Aβ species within the plaque environment.

Numerous studies have demonstrated the ability of microglia (primary and cell lines) to internalize and traffic Aβ to the lysosome (Ard et al., 1996; Chu et al., 1998; Halle, et al., 2008; Li et al. 2012; Mandrekar et al., 2009; Paresce et al., 1997; Sheedy et al., 2013). Multiple receptors appear to regulate the Aβ internalization process including scavenger receptor (Paresce et al., 1996), complement receptor type 3 (CR3, Mac-1), signal regulatory protein-β1 (SIRPβ1) receptor (Gaikwad et al., 2009), $P2Y_4$ receptor (Li et al., 2013), and a receptor complex of CD36, $\alpha_6\beta_1$ integrin and CD47 (Koenigsknecht and Landreth, 2004). Many of these, and other, studies utilized either fibrillar, "soluble", or both forms of Aβ (Chung et al., 1999; Fu et al., 2012; Gaikwad et al., 2009; Koenigsknecht and Landreth, 2004; Mandrekar et al., 2009). The term "soluble" likely encompasses mixtures of aggregates and monomers. Cumulatively, the results indicate microglia internalization of Aβ is sensitive to structure or oligomerization state, and that different intracellular degradation pathways are utilized for different Aβ species.

Aβ protofibrils are well-recognized soluble intermediates in Aβ fibrillogenesis (Harper et al., 1997b, 1999; Walsh et al., 1997, 1999). We have previously demonstrated Aβ42 protofibril formation in several physiological buffering systems including artificial cerebrospinal fluid (aCSF) (Paranjape et al., 2012, 2013). These structures possess a classic curvilinear morphology with lengths < 100 nm, and are robust stimulators of tumor necrosis factor α (TNFα) secretion in microglia. Our earlier work utilized cytokine production, immunochemistry, and confocal microscopy imaging to show preferential activation of microglia by protofibrils (Paranjape et al., 2012) and BV-2 microglia cell-surface binding of protofibrils (Paranjape et al., 2013) compared to monomers and fibrils. In the current study, we expand the biophysical characterization of Aβ-fluorophore conjugation and demonstrate that soluble fluorescently-labeled Aβ42 protofibrils are taken up rapidly by primary murine microglia, and in much greater quantity, than Aβ42 monomers.

2. Results

2.1. Characterization of fluorophore-labeled Aβ

Aβ42 protofibrils and monomers were labeled simultaneously using a unique adaptation of a previously-described procedure by LaDu and colleagues (Jungbauer et al., 2009). Aβ42 protofibrils were allowed to form in aCSF as outlined in the Methods. This Aβ42 preparation typically produces a roughly equal distribution of protofibrils and monomers. The Aβ solution mixture was then incubated with amine-reactive Alexa Fluor® 488 (AF488) TFP ester dye (Fig. 1A). Potential Aβ conjugation sites for AF488 were the primary amines at K16, K28, and the N-terminus. Since the typical protofibril preparation also contains monomers, both species can be labeled with fluorophore simultaneously. Size exclusion chromatography (SEC) separation of the Aβ42 labeling solution on Superdex 75 yielded AF488-labeled Aβ42 protofibrils and monomers (Fig. 1B). Monitoring of the elution in-line with UV absorbance ($A_{280\ nm}$) produced three peaks: Aβ42 protofibrils, Aβ42 monomers, and the unconjugated AF488 dye peak. AF488 by itself has a contribution to the $A_{280\ nm}$ absorbance that is equal to 11% of the AF488 $A_{494\ nm}$ absorbance. This contribution was taken into account in subsequent Aβ concentration determinations. Both Aβ42 protofibrils and monomers were labeled with AF488 as fluorescence measurements of collected fractions showed the

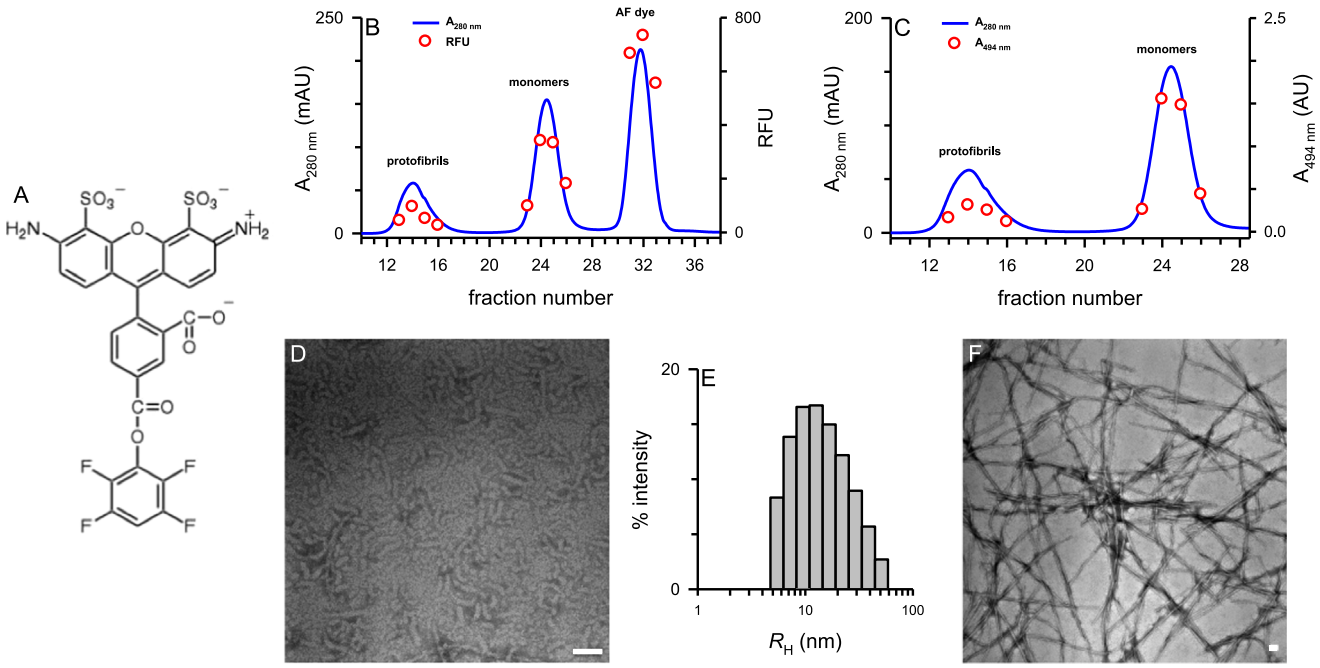

Fig. 1. Preparation and characterization of AF488-Aβ42. Panel A. Structure of the AF488 TFP compound structure (Life Technologies). Panel B. AF488-labeled protofibrils, monomers and unincorporated dye were separated on Superdex 75 and eluted in aCSF. The elution trace (blue line) was acquired from in-line $Abs_{280\ nm}$ readings, and peak fraction fluorescence (red circles) was determined by integrating the AF488 emission from 505 to 550 nm. Panel C. SEC elution trace (blue line) overlaid with peak fraction $Abs_{494\ nm}$ measurements (red circles) subsequently used in stoichiometry calculations. Panel D. Protofibrils (10 μL, 38 μM) applied to a copper formvar grid and imaged by TEM at a magnification of 59,000, scale bar = 50 nm. Panel E. AF488-Aβ42 protofibrils were analyzed by DLS directly after SEC isolation and a representative regularized histogram of percent intensity *versus* R_H is presented. Panel F. TEM of AF488-Aβ42 fibrils (10 μL, 37 μM) that formed during incubation of AF488-Aβ42 monomer fractions for 53 days at 4 °C. Magnification = 18,000, scale bar = 50 nm.