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Intracellular amyloid and the neuronal origin of Alzheimer neuritic plaques

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ABSTRACT

Genetic analysis of familial forms of Alzheimer's disease (AD) causally links the proteolytic processing of the amyloid precursor protein (APP) and AD. However, the specific type of amyloid and mechanisms of amyloid pathogenesis remain unclear. We conducted a detailed analysis of intracellular amyloid with an aggregation specific conformation dependent monoclonal antibody, M78, raised against fibrillar Aß42. M78 immunoreactivity colocalizes with Aß and the carboxyl terminus of APP (APP-CTF) immunoreactivities in perinuclear compartments at intermediate times in 10 month 3XTg-AD mice, indicating that this represents misfolded and aggregated protein rather than normally folded APP. At 12 months, M78 immunoreactivity also accumulates in the nucleus. Neuritic plaques at 12 months display the same spatial organization of centrally colocalized M78, diffuse chromatin and neuronal nuclear NeuN staining surrounded by peripheral M78 and APP-CTF immunoreactivity as observed in neurons, indicating that neuritic plaques arise from degenerating neurons with intracellular amyloid immunoreactivity. The same staining pattern was observed in neuritic plaques in human AD brains, showing elevated intracellular M78 immunoreactivity at intermediate stages of amyloid pathology (Braak A and B) compared to no amyloid pathology and late stage amyloid pathology (Braak 0 and C, respectively). These results indicate that intraneuronal protein aggregation and amyloid accumulation is an early event in AD and that neuritic plaques are initiated by the degeneration and death of neurons by a mechanism that may be related to the formation of extracellular traps by neutrophils.

Introduction

Alzheimer's disease (AD) has traditionally been defined by the presence of extracellular plaques containing aggregated amyloid ß (Aß) and intracellular tangles containing aggregated tau. However, accumulation of aggregated Aß in plaques does not correlate well with disease and many individuals with large amounts of amyloid deposits are not demented (Terry, 1996). This suggests that some form of unseen Aß which may accumulate prior to extracellular plaque deposition is more closely associated with pathogenesis. Soon after the discovery of Aß, it was observed that APP and Aß immunoreactivity accumulates in the perinuclear area of a subset of neurons and in the corona of dystrophic neurites surrounding neuritic plaques and it was proposed that this APP represents the penultimate source of Aß accumulating in the core of the neuritic plaques (Selkoe et al., 1988; Grundke-Iqbal et al., 1989; Ikeda et al., 1989; Perry et al., 1989; Stern et al., 1989; Arai et al., 1990; Cummings et al., 1992). However, with the discovery of soluble secreted Aß, this interpretation fell out of favor (Joachim et al., 1991). Many transgenic animal models of AD exhibit intracellular Aß and APP immunoreactivity (reviewed in (Wirths and Bayer, 2012)) that precedes cognitive decline and amyloid deposition or that is associated with neuronal loss (Schmitz et al., 2004) and synaptic dysfunction (Oddo et al., 2003). Many of the accumulating immunoreactive

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molecules appear to be APP and/or fragments of APP rather than Aß (Winton et al., 2011). This could represent the accumulation of normal APP and its metabolites, however the intracellular amyloid in at least two mouse models also reacts with conformation dependent antibodies that recognize pathologically misfolded oligomeric and fibrillar amyloid (Oddo et al., 2006; Ferretti et al., 2011; Kulic et al., 2012). Increasing evidence indicates that amyloids are structurally diverse and conformation dependent antibodies can detect these structural differences by the exposure or hiding of their epitopes depending on the particular aggregation state (Kayed et al., 2003, 2007, 2009; Glabe, 2008). To investigate whether this conformational diversity is relevant to intracellular amyloid pathogenesis we cloned a panel of conformation dependent antibodies raised against fibrillar Aß42 and carried out a detailed analysis of intracellular amyloid in 3XTg-AD mouse and human AD brains. We found that M78 immunoreactivity colocalizes with elevated intracellular Aß and APP-CTF immunoreactivity at early times in 3XTg-AD mice and human AD brains. M78 also stains neuronal nuclei at intermediate stages of plaque pathology, whereas at later stages the nuclear staining disappears and M78 immunoreactivity localizes with a subset of amyloid plaques that react only weakly with Aß or APP antibodies. Finally, we found that neuritic plaques display the same spatial relationships of perinuclear Aß, APP-CTF immunoreactivity and nuclear M78 immunoreactivity that are observed in neurons. Since these plaques also showed a diffuse DNA DAPI and neuronal marker NeuN positive core, we postulate that these plaques arise from the death of single neurons, releasing both chromatin and amyloid in the extracellular space.

Materials and methods

Antibodies

The antibodies used in this study and the working conditions are listed in Table 1.

M78 antibody production and characterization

Rabbit monoclonal antibody M78 was made under contract with Epitomics (Burlingame, CA) using fibrillar Aß42 as an antigen and immunizing New Zealand white rabbits, as previously described for preparing OC polyclonal serum (Kayed et al., 2007) and, more recently, monoclonal antibodies M64 and M87 (Kayed et al., 2007; Nussbaum et al., 2012). Approximately 10,000 pools of hybridomas were screened against Aß42 fibrils, prefibrillar oligomers or monomeric Aß, and 120 pools having an absorbance at least 3-folds above background in ELISA assays were selected for further analysis. Secondary screening consisted of probing blots of a medium density array of 130 different preparations of fibrils, prefibrillar oligomers and monomers of Aβ1–42, Aβ1–40, islet amyloid polypeptide (IAPP), polyQ40, overlapping 15 residue peptide segments of Aß and amyloid-forming random peptides. Hybridoma pools were also probed by immunohistochemistry on human AD and age-matched control brain tissues. Pools giving a unique pattern of immunoreactivity on the array or on immunohistochemistry were selected for cloning and further characterization by immunohistochemistry, western blotting and ELISA. Epitope mapping was performed as previously described (Nussbaum et al., 2012). A peptide array (PepSpotsTM) consisting of a series of overlapping 10 mers from the −4 position of the Aß sequence to residue 46 covalently bonded via the carboxyl terminus to a cellulose membrane was prepared by JPT Peptide Technologies GmbH (Berlin, Germany) and used according to the manufacturer's recommendations. 100 ng/ml of primary antibody was added to the membranes, followed by incubation with 1 μg/ml goat anti-rabbit secondary conjugated with alkaline phosphatase and TMB substrate detection (Promega, Madison, WI). The larger the number of contiguous immunopositive spots, the smaller the sequence that the spots have in common. For the antibody blocking experiment, Aß40 fibrils in HFIP/ddH_2O, prepared as described above, were coupled to M-270 Epoxy Dynabeads (Dynal/Invitrogen) at a peptide concentration of 0.2 mg/ml in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 M ammonium sulphate and incubated overnight at 37 °C under gentle tilting. Aß fibrils were cross-linked to magnetic beads with 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 for 5 min at room temperature, washed 3 times with PBS and used to affinity purify M78. Briefly, 200 μl of 0.14 mg/ml M78 in PBS was incubated with Aß40-cross-linked magnetic beads for 2 h at 4 °C. The sample was placed on a magnet and the resultant supernatant was retained as unbound fraction. Fibril bound M78 was eluted with 3 M glycine pH 2.0 and immediately neutralized with 1/10 (vol/vol) of 1 M Tris pH 8.0. Tissue sections from human AD and 12 month 3XTg-AD brains were then processed for immunohistochemistry as described below and probed with unfractionated, fibril bound and fibril unbound M78 at 1.4 μg/ml in TBS-B overnight at room temperature, followed by incubation with anti-rabbit biotinylated secondary antibodies (1:250) and DAB (ABC) detection kit (Vector, Burlingame, CA).

Preparation of Aß40/42, α-synuclein and IAPP fibrils and in vitro characterization of M78 immunoreactivity by immunoblot analysis

Lyophilized Aß40 or Aß42 peptides were resuspended in 50% acetonitrile/ddH_2O mixture and relyophilized. Fibrils were prepared by dissolving the peptide at 2 mg/ml in 50% hexafluoroisopropanol (HFIP)/ddH_2O in a siliconized Eppendorf tube and stirred at 500 rpm with a Teflon coated micro stir bar at room temperature, as previously described (Rasool et al., 2012). IAPP and alpha-synuclein were dissolved at a concentration of 420 μM in 10 mM NaOH, incubated for 30 min at room temperature and diluted to 70 μM in 100 mM phosphate buffer without stirring. Sample aliquots were taken at 0–24 h intervals to

Table 1
List of antibodies used in this study.

Antibody	Type	Epitope/Immunogen	Source	Antigen retrieval	Working dilution
M78	Rabbit mAb	Fibrillar Aß 8–11, 18–24 and 26–32 of Aß	Glabe lab	Not required (but stable IR under unmasking conditions listed below)	1–5 μg/ml
APP (C-term)	Rabbit pAb	751–770 of APP	Glabe lab	• Citrate and heat	1:200–1:500
OC	Rabbit pAb	Fibrillar Aß	Glabe Lab	Not required	1–5 μg/ml
6E10	Mouse mAb	1–16 of Aß	Covance, Princeton, New Jersey	• 70% formic acid	1:500
4G8	Mouse mAb	18–22 of Aß		• Citrate and heat • 2 N HCl in ddH_2O • EDTA and heat	1:500
NeuN	Mouse mAb	Purified cell nuclei from mouse brain	Merk Millipore, Darmstadt, Germany	Citrate and heat	1:100
GFAP	Rabbit pAb	GFAP isolated from cow spinal cord	Dako, Glostrup, Denmark	None	1:1000
MOG (D-10)	Mouse mAb	176–247 of MOG of human origin	Santa Cruz Biotechnology, Dallas, Texas	None	1:100
MAP2	Chicken polyclonal	MAP2	Millipore	None	1:1000