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Plaque array method and proteomics-based identification of biomarkers from Alzheimer's disease serum

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ABSTRACT

Background: Progressive accumulation of amyloid plaques in the regions of brain, carotid and cerebral arteries is the leading cause of Alzheimer's disease (AD) and related dementia in affected patients. The early identification of individuals with AD remains a challenging task relying on symptomatic events and thus the development of a biomarker-based approach will significantly aid in the diagnosis of AD.
Methods: Here we describe a flow cytometer-based serum biomarker identification method using plaque particles, and applying mass spectrometry based proteomic analysis of the isolated plaque particles for the identification of serum proteins present in the plaque particles.
Results: We identified 195 serum proteins that participate in the process of plaque particle formation. Among the 195 proteins identified, 68.2% of them overlapped in abeta-42, cholesterol, tau-275 and α-synuclein plaque particles. Significantly, 22.5% of the proteins identified as bound to abeta-42 plaque particles generated in AD serum were unique when compared with cholesterol, α-synuclein and tau plaque particles. In age-matched control experiments, 15% of them showed *in vitro* insoluble abeta-42 particle formation and 59% of the identified plaque particle constituents from AD serum were also present in the insoluble plaque particles derived from control.
Conclusions: We have developed an *in vitro* method for plaque particle detection and identified serum protein markers that are associated with AD-related plaque particle formation. With further clinical validation, this assay may provide a novel, non-invasive means for the early detection of AD.

1. Introduction

Amyloidoses are a group of more than twenty diseases characterized by protein aggregation including Alzheimer's disease (AD), a common neurodegenerative disease associated with progressive dementia [1]. Abnormal processing of the abeta amyloid protein is an early and causative event in the pathogenesis of AD [2]. Abeta amyloid peptides released from amyloid precursor protein by the action of β- and γ-secretases undergo structural transformation from a soluble monomer to oligomers and finally into amyloid fibrils/plaques [3]. Impaired clearance of the amyloid peptides produced in the brain and present in the circulation together contribute to the accumulation of senile plaques in the cerebral blood vessels and brain parenchyma [4]. The pathological consequences of such senile plaque accumulation are neuronal loss, cerebrovascular inflammation, reduction in the cerebrovascular space and cognitive decline [5,6].

Unfortunately, AD produces no symptoms until the damage to the brain is severe enough to show loss of memory in affected patients although some patients may display minor neurocognitive impairment which can be subtle. Identifying asymptomatic AD individuals is a challenging task and multiple tests involving neurophysiological, neuropathological and brain imaging techniques are currently used to diagnose these patients [7–9]. Identification of serum based novel biomarkers is imperative both for early diagnosis of AD and developing effective therapeutics [10,11]. Mass spectrometry-based proteomic, lipidomic and metabolomic approaches are being increasingly used for the identification of novel biomarkers from plasma/serum samples for AD diagnosis [12,13].

We describe the development of a novel plaque array method for inducing the formation of insoluble plaque particles *in vitro*. We also identify serum biomarkers with the potential for the diagnosis and

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stratification of AD subjects. The plaque array-based analysis of serum involves three steps: 1) incubation of soluble plaque forming constituents with serum samples — when incubated with serum from patients with AD, insoluble particles form in a time- and shape-specific manner; 2) particles are detected, quantified and sorted by flow cytometer; and 3) proteomic analyses of the sorted plaque complex is performed by mass spectrometry (Fig. 1). Rather than examining the proteome of serum and plasma of AD patients, we analyzed the composition and concentration of insoluble plaque particles upon which the associated serum components represented only a small subset of the approximately 10,500 human serum proteins.

2. Materials and methods

2.1. Reagents

Lyophilized powders of human abeta-42 peptide sequences (1–42), human abeta-42 peptide sequences abeta-28 (1–28), tau-275 (275–305 repeat 2 domain) and human recombinant α-synuclein protein (14.5 kDa) were commercially purchased (Anapec). Thioflavin S dye and phosphate buffered saline (PBS) tablets were purchased from Sigma (MI). TopFluor fluorescence cholesterol was purchased from Avanti Lipids. Serum samples of Alzheimer's disease and age-matched controls were purchased from DxBiosamples. Protease max solution and Trypsin/Lys C Mix were purchased from Promega.

2.2. Preparation of soluble amyloid and cholesterol aggregates

Plaque forming aggregates were prepared as described earlier [14,15]. Briefly, for preparing soluble abeta-42, tau (275–305) and α-synuclein aggregates/oligomers, 1 mg of each peptide was suspended separately in 1 mL of deionized water. Next, the samples were mixed with 1 mL of phosphate buffered saline (PBS) and incubated at 37 °C for 2 h. After centrifugation for 5 min at 5000 rpm, the supernatants containing soluble oligomers were used for protein measurement using Micro BCA protein assay kit (Pierce). The supernatants were also analyzed by flow cytometry for the presence of insoluble particles. The soluble amyloid oligomers/aggregates (5 μg) were stained with Thioflavin S, an amyloid binding fluorescent dye for detecting the presence of insoluble particles in the supernatants.

Fluorescence-labeled lyophilized cholesterol (1 mg) (excitation/emission = 495 nm/507 nm) was solubilized in 1 mL of 100% alcohol. After centrifugation for 5 min at 5000 rpm the supernatants were used as stock solution. From this stock solution, 100 μL was taken and mixed in 900 μL of PBS for preparation of fluorescent-labeled cholesterol aggregates. The aggregates (5 μg) were analyzed by flow cytometry for the presence of particles. For both the amyloid and cholesterol aggregates, the supernatants containing soluble aggregates with no insoluble particles or a minimum number of particles (~300 particles/mL) were used for screening serum samples.

2.3. Flow cytometer-based detection and quantitation of serum-derived plaque particles

For the detection of insoluble amyloid plaque particle formation in the presence of serum, each assay was performed in a 200 μL reaction mixture (100 μL of diluted serum 25% in PBS, and 100 μL of either abeta-42, tau and α-synuclein aggregates (5 μg)). The mixtures were incubated at 37 °C for 45 min without shaking to permit insoluble particle formation to occur. After incubation with diluted serum, 10 μL of Thioflavin S fluorescence dye (10 μg) was added to each sample and incubated for an additional 15 min at 37 °C. Following incubation, all samples were analyzed by flow cytometry (Guava EasyCyte 5HT, EMD Millipore) with the acquisition setting adjusted to count 2000 particles/min. Sample fluorescence was measured using 488 nm for excitation and 520 and 560 nm for emission.

For detection of insoluble cholesterol plaque particle formation in the presence of serum, each assay was performed in a 200 μL reaction (100 μL of diluted serum 25%, and 100 μL of the cholesterol aggregates (5 μg)). The mixtures were incubated at 37 °C for 45 min without shaking to permit insoluble particle formation to occur. Following incubation, all samples were analyzed by flow cytometry with the acquisition of 2000 particles/min. Diluted serum samples (25%) and aggregates/oligomers mixed in 200 μL PBS without serum were used as negative controls.

To determine lower and upper limits of insoluble plaque particle detection, soluble fluorescence-labeled cholesterol and abeta-42 aggregates were individually diluted (40 μg/mL) in PBS without the addition of serum and incubated for 36 h at 37 °C to allow formation of insoluble particles. The resulting self-formed insoluble abeta-42 particles were stained with Thioflavin S dye. Both the self-formed abeta-42 and cholesterol particles were serially diluted (1, 5, 10, 20 and 40 μg/mL) in triplicate and used for flow cytometer (Guava easyCyte 5HT, EMD Millipore) acquisition and quantitation of plaque particles. The lower and upper limits of detection were estimated using linear regression analysis.

For examining reproducibility of particle formation in the assay, soluble abeta-42, abeta-28 and cholesterol aggregates/oligomers were incubated in AD serum and the resulting insoluble particles were enumerated using flow cytometry. From each sample, 5000 and 10,000 particles were acquired separately and total particle counts were obtained using InCyte Guava Soft 2.6 software (EMD Millipore). The concentration of particles in the samples was calculated on the basis of the number of particles/volume with assays performed in triplicate. For examining specificity of the assay, 15 serum samples from age-matched normal (aged 60 to 90 y), 15 samples from patients with mild cognitive impairment (MCI) and 15 samples from severe AD subjects were screened against soluble abeta-42 aggregates.

Selection of the AD subjects were made based on multiple clinical tests: general cognitive decline (short and long-term memory loss), repeating conversation, clock drawing, Geriatric Depression Rating Scale, Wide-Range Achievement Test (reading subsets); Wechsler Memory—IV (selected subsets), Stroop Color-Word Interference, Trail

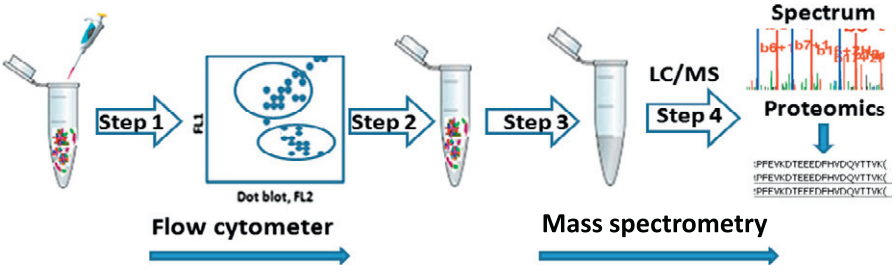

Fig. 1. Represents a schematic of the flow cytometry- and mass spectrometry-based detection, sorting and proteomic analysis of plaque particles. Step 1, *in vitro* formation of insoluble plaque particles in serum; step 2, cell sorter-based identification and isolation of fluorescent particles; step 3, trypsin digestion of the isolated plaque particles to prepare peptide fragments; and step 4, mass spectrometry (MS/MS)-based proteomics for the identification of proteins.