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Common proteomic profiles of induced pluripotent stem cell-derived three-dimensional neurons and brain tissue from Alzheimer patients

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ABSTRACT

We established a unique platform for proteomic analysis of cultured three-dimensional (3D) neurons and brain tissue from Alzheimer's disease (AD) patients. We collected peripheral blood mononuclear cells (PBMC), converted PBMC to induced pluripotent stem cell (iPSC) lines, and differentiated the iPSC into human 3D neuro-spheroids. The postmortem brain tissue from the superior frontal cortex, inferior frontal cortex and cerebellum area of the AD patients was compared to the same regions from the control subjects. Proteomic analysis of 3D neuro-spheroids derived from AD subjects revealed the alteration of a number of proteins involved in axon growth, mitochondrial function, and antioxidant defense. Similar analysis of post-mortem AD brain tissue revealed significant alteration in proteins involved in oxidative stress, neuro-inflammation, along with proteins related to axonal injury. These results clearly indicate that the dysfunction of 3D neurons from AD patients in our *in vitro* environment is comparable to the post-mortem AD brain tissue *in vivo*. In conclusion, our study revealed a number of candidate proteins that have important implications in AD pathogenesis and supports the notion that the iPSC-derived 3D neuronal system functions as a model to examine novel aspects of AD pathology.

Significance: In this study, we present a unique platform for proteomic analysis of induced pluripotent stem cell-derived three dimensional (3D) neurons and compare the results to those from three regions of post-mortem brain tissue from Alzheimer's disease patients and normal control subjects. Our results show that the dysfunction of 3D neurons from AD patients in our *in vitro* environment is comparable to the post-mortem AD brain tissue *in vivo*. Our results revealed several candidate proteins that have important implications in AD pathogenesis.

1. Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia and is characterized by multiple cognitive deficits including early memory loss, impaired language skills, and a compromised ability to focus and reason [1,2]. The classic AD pathological features include the extracellular deposition of misfolded amyloid-β (Aβ) peptide, the accumulation of hyperphosphorylated tau containing neurofibrillary tangles, and massive neuronal cell and synapse loss [3–5]. Extensive work has attempted to correlate the aforementioned AD cognitive impairments with these biological processes [6–13]; however, this relationship remains inconclusive.

Efforts have been made to model AD brains in cultured dishes. One major limitation of the conventional 2D cell cultures is that secreted Aβ peptides diffuse quickly from the cells to the media and are removed once the media is changed [14]. With the intention of improving cell culture models of disease and solving this limitation, a three-dimensional (3D) neuronal system was created which aims to mimic the aged AD brain environment in an effort to better reproduce the full AD pathology [15]. Due to the heterogeneity and multifactorial nature of AD, a 3D culture may represent a better model to study molecular pathways contributing to AD *in vitro*. It was hypothesized that 3D hydrogels would provide suitable conditions for accelerating Aβ deposition by limiting the circulation into the cell culture medium [15]. Choi et al.

Abbreviation: AD, Alzheimer's disease; CRLM, cerebellum; DAVID, the Database for Annotation Visualization and Integrated Discovery; ELISA, the enzyme-linked immunosorbent assay; FDR, false discovery rate; GO, Gene Ontology; IF, inferior frontal cortex; iPSC, induced pluripotent stem cells; LC-MS/MS, liquid chromatography mass spectrometry; PBMC, peripheral blood mononuclear cells; SF, superior frontal cortex; TMT, tandem mass tag

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have reported the presence of Aβ aggregates and phosphorylated Tau accumulation in 3D neuronal cultures, indicating that both of these processes are accelerated in 3D conditions [15]. One source of cells for developing 3D neurons is induced pluripotent stem cells (iPSC) [16]. Continuous advancements in molecular technologies have allowed us to successfully reprogram cells from peripheral blood mononuclear cells (PBMCs) derived from AD patients to generate iPSCs and subsequently, 3D human neurons [16]. Previous work has proven the efficacy of this new technology by showing that fibroblast-derived iPSCs from AD patients can be used as an *in vitro* model to mimic AD pathology [17–26], and pathological proteins, like tau, have been characterized in iPSC-derived 3D neuronal culture [27,28].

The use of proteomic profiling on AD pathologic processes could serve as an indicator of disease presence and progression. The development of mass spectrometry (MS)-based proteomics has been driven by the growth of new technologies for peptides/protein fractionation, advancements in MS instruments, and new labeling reagents. MS-based proteomics possess the advantage of having no requirement of prior knowledge of the proteins being identified, allowing for unbiased, hypothesis-free biomarker discovery in complex biological samples such as plasma and tissue extracts. This approach also meets the requirement for discovery-level proteomics, which is to measure multiple targets simultaneously in a multiplexing manner. Therefore, MS has been increasingly applied to the study of neurodegenerative diseases including AD. Previous MS-based proteomic studies in plasma from AD patients have revealed alterations in proteins linked to inflammation, vascular dysfunction, disturbed metal homeostasis and lipid metabolism [29–31]. Further, profiling of post-mortem brain tissues from AD patients suggested abnormal phosphorylation and O-GlcNAcylation of many proteins [32–34].

The present study is the first reporting global quantitative MS-based proteome analysis of human 3D neurons derived from AD subjects. So far, there are only a few MS-based proteome studies using 2D cultured mouse neurons [35–37]. Here we describe a proteomic assessment of isobaric tags for relative quantitation using 3D cultures and post-mortem brain tissues from AD patients with the goal of exploring disease mechanisms and discovering proteins associated with AD pathology. Specifically, proteomic profiles of 3D neurons were obtained, and the ratio changes in protein expression associated with each AD relative to the average of the healthy subjects were calculated. Similarly, the changes in protein expression associated with the three regions of post-mortem brain tissues from AD subjects were analyzed in the same way. Differentially expressed proteins were analyzed using the bioinformatics tools Database for Annotation, Visualization, and Integrated Discovery (DAVID) and STRING (a biological database and web resource of known and predicted protein–protein interactions) to determine functional relationships altered in AD. Changes in protein expression between AD and control subject-derived 3D neurons and brain tissues were compared, allowing us to evaluate the similarities in proteomic profiles from *in vitro* 3D neurons and *in vivo* brain tissue.

2. Materials and methods

2.1. Materials and reagents

Reagents used for biochemical methods and cell culture preparation were purchased from Sigma–Aldrich (St Louis, MO, USA) unless otherwise indicated. Reagents for BCA protein assay and sample preparation kits for liquid chromatograph (LC)-mass spectrometer (MS) analysis were purchased from Thermo Scientific (Rockford, IL, USA), including Pierce top twelve abundant protein depletion spin columns, Pierce protein concentrators (3 kDa) and Tandem Mass Tag (TMT) reagent 10-plex kits.

2.2. Subjects

The human study was approved by the Bedford VA Hospital Institutional Review Board, and the signed informed consents were obtained before the initiation of the study. Ten subjects including 5 healthy controls and 5 diagnosed with AD were enrolled from the Bedford VA Hospital Dementia Care Special Unit. The average ages of the AD patients were 69.4 ± 11.8 years (two female and three male subjects) and the controls were 71.2 ± 4.9 years (five male subjects). Controls scored over 27 on the Montreal Cognitive Assessment, which is a validated screening tool for subjects with mild cognitive impairment or AD [38]. Blood from these 10 subjects was obtained for iPSC-differentiated 3D neuronal culture.

The postmortem brain tissue from 5 AD patients and 5 healthy controls were obtained from the Bedford Brain Bank. Among all subjects, two of the five AD cases were the same subjects described above who donated blood for the generation of 3D neuronal cultures. The areas collected were the superior frontal cortex, inferior frontal cortex and cerebellum. The post-mortem interval between the donor's death and brain autopsy/specimen collection was ≤24 h. The average age of patients from whom we had postmortem brain tissue was 75.6 ± 8.8 for AD patients and 83.0 ± 6.0 for the controls (three males and two females for both groups). Alzheimer's disease “ABC” scoring system was used for neuropathological assessment of the post-mortem human brains, showing a high degree of Alzheimer's neuropathological change according to the NIA Alzheimer Association's guidelines and is sufficient to account for the patient's dementia [39].

2.3. Generation of human 3D neuro-spheroids from blood-derived iPSCs

Blood was collected in Vacutainer cell tubes (CPT, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged at 1500 ×*g* for 20 min at room temperature shortly after collection. After centrifugation, the plasma was separated and kept at −80 °C and the peripheral blood mononuclear cell (PBMC) layer was transferred to a new 15 mL Falcon tube with 10 mL of sterile PBS. The PBMCs were centrifuged at 300 ×*g* for 10 min at room temperature and the supernatant was discarded. The cell pellet was resuspended with 6 mL of PMBC medium with 10% DMSO (Invitrogen), aliquoted in 2 mL tubes, and frozen at a controlled rate of −1 °C/min in −80 °C then transferred to a liquid nitrogen cryogenic tank for storage.

Induced pluripotent stem cells (iPSC) were derived from PBMC following the integration-free CytoTune-iPS Sendai Reprogramming Kit (Invitrogen) [40,41] and characterized, as previously reported [16]. Generation of 3D neuro-spheroids from iPSCs was accomplished using a modified protocol [42]. Briefly, the iPSCs in E8 medium with a ROCK inhibitor (Thiazovivin, 1 uM, MiltenyiBiotec, San Diego, CA) were transferred into 100 mm ultra-low-attachment plastic plates (Corning, Tewksbury, MA). On the day following formation of the spheroid, the medium was replaced with neural induction medium (Invitrogen) for 6 days. After the sixth day, the media on the floating spheroids was exchanged with Neural Medium (NM) containing Neurobasal (Invitrogen), B-27 serum substitute without vitamin A (Invitrogen), GlutaMax (Invitrogen), penicillin and streptomycin (Invitrogen). The NM was supplemented with 20 ng/mL FGF2 and 20 ng/mL EGF (R&D Systems, Minneapolis, MN). The cells were grown in this medium for 21 days with daily replacement during the first 10 days, and replacement every other day for the subsequent 11 days. To promote differentiation of the neural progenitors into neurons, FGF2 and EGF were replaced with 20 ng/mL BDNF and 20 ng/mL NT3 (Peprotech, Rocky Hill, NJ) starting at day 27. From day 48 onwards, NM without growth factors was used and replaced every 4 days.