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Original Contribution

An investigation of the molecular mechanisms engaged before and after the development of Alzheimer disease neuropathology in Down syndrome: a proteomics approach

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ABSTRACT

Down syndrome (DS) is one of the most common causes of intellectual disability, owing to trisomy of all or part of chromosome 21. DS is also associated with the development of Alzheimer disease (AD) neuropathology after the age of 40 years. To better clarify the cellular and metabolic pathways that could contribute to the differences in DS brain, in particular those involved in the onset of neurodegeneration, we analyzed the frontal cortex of DS subjects with or without significant AD pathology in comparison with age-matched controls, using a proteomics approach. Proteomics represents an advantageous tool to investigate the molecular mechanisms underlying the disease. From these analyses, we investigated the effects that age, DS, and AD neuropathology could have on protein expression levels. Our results show overlapping and independent molecular pathways (including energy metabolism, oxidative damage, protein synthesis, and autophagy) contributing to DS, to aging, and to the presence of AD pathology in DS. Investigation of pathomechanisms involved in DS with AD may provide putative targets for therapeutic approaches to slow the development of AD.

Down syndrome (DS), or trisomy 21, is the most frequent genetic disorder, most commonly caused by triplication of chromosome 21 [1]. DS is associated with developmental abnormalities of the central nervous system that lead to intellectual disability [2]. In addition to intellectual disability, virtually all DS brains over 40 years of age show Alzheimer disease (AD) neuropathology [3], including the presence of senile plaque (SP) and neurofibrillary tangles [3–5], and clinical signs of dementia become more frequent after 50 years of age [6,7]. Several studies support the hypothesis that the overexpression of genes present on chromosome 21 is responsible for the features of DS, including the development of AD [8,9]. The overexpression of amyloid precursor protein (APP), due to its location on chromosome 21, leads to an enhanced production of β-amyloid (Aβ), the principle component of SP. Mutations in the APP gene are related to familial AD [10], and therefore APP overexpression and subsequent Aβ formation may be a crucial event leading to the development of AD pathology in DS [11,12]. Aβ deposition in DS brains begins in the early years of life, as young as 8 years, and increases progressively with increasing age [13,14].

Further, oxidative damage appears to have a key role in DS and in the exacerbation of AD [15–17]. Among many genes located on chromosome 21 linked with oxidative damage and reactive oxygen species production, superoxide dismutase (SOD1) is the most relevant, because its upregulation without a similar increase in catalase leads to an accumulation of hydrogen peroxide [12,17] and consequently to increased oxidative stress levels. In addition, enhanced release of Aβ, both Aβ(1–40) and Aβ(1–42), also could contribute to oxidative damage [13,18]. As noted above, neuropathological features of dementia are manifested at a younger age in people with DS relative to the general population, but it appears delayed relative to AD neuropathology, suggesting the presence of compensatory mechanisms [19,20]. Therefore, a better understanding of these compensatory responses and an eventual manipulation of these mechanisms may be therapeutically beneficial for people with DS. Thus, individuals with DS could provide an understanding of the early alterations leading to AD and therefore to AD itself.

To clarify the cellular and metabolic pathways that could contribute to the pathomechanisms of the DS brain, in particular those responsible for the onset of AD neuropathology, we analyzed the

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frontal cortex of DS autopsy cases with or without AD pathology in comparison with age-matched non-DS controls, using a proteomics approach. Several studies from the past 10 years have already shown many proteins from different pathways to be dysregulated in DS fetal brains [21,22].

The aim of our study was to identify the proteins and associated pathways compromised in DS compared to people without DS to determine which pathways are different as a function of trisomy 21. Second, a comparison of younger individuals with DS and without AD to older DS cases with AD neuropathology was used to determine if specific pathways distinguish the two groups. Establishing novel cellular mechanisms and pathways that appear to contribute to the DS phenotype and additional development of AD neuropathology may provide putative novel targets for therapeutic intervention.

Materials and methods

Subjects

DS and young or nondemented older control cases were obtained from the University of California at Irvine Alzheimer Disease Research Center Brain Tissue Repository, the Eunice Kennedy Shriver NICHD Brain and Tissue Bank for Developmental Disorders, and the University of Kentucky Alzheimer Disease Center. Table 1 shows the characteristics of the cases used. DS cases were divided into two groups, with or without sufficient pathology for a neuropathologic diagnosis of AD. All cases with both DS and AD, referred to as DS/AD, were over the age of 40 years. Thus for the current study, controls were split into two groups, either less than or equal to 40 years or older than 40 years at death. The postmortem interval (PMI) was different across groups, with the DS/AD group overall having a lower PMI ($F(3,66)=7.30$; $p<0.0005$). A subset of these autopsy cases was used in previous experiments measuring insoluble Aβ as a function of age in DS [18], redox proteomics [23], and oxidative damage [24].

Sample preparation

Samples from the frontal cortex of non-DS controls, DS, and DS/AD were thawed in lysis buffer (pH 7.4) containing 320 mM sucrose, 1% 1.0 M Tris–HCl (pH 8.8), 0.098 mM $MgCl_2$, 0.076 mM EDTA, and proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 mg/ml), and phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). The brains were homogenized by 20 passes in a Wheaton tissue homogenizer, and the resulting homogenate was centrifuged at 14,000*g* for 10 min to remove cellular debris. The supernatant was extracted to determine the total protein concentration by the BCA method (Pierce, Rockford, IL, USA).

Two-dimensional electrophoresis

Proteins (150 μg) were precipitated in 15% final concentration of trichloroacetic acid for 10 min in ice. Each individual sample (six per group) was then spun down at 10,000*g* for 5 min and precipitates were washed in ice-cold ethanol:ethyl acetate (1:1) solution four times. The final pellet was dissolved in 200 μl of 8 M urea, 2% Chaps, 2 M thiourea, 20 mM dithiothreitol, 0.2% ampholytes (Bio-Rad, Hercules, CA, USA), and bromophenol blue and placed in agitation for 3 h. Solubilized proteins were then sonicated twice for 30 s. Samples were loaded on 110-mm, pH 3–10, immobilized pH gradient strips in a Bio-Rad isoelectric focusing cell system (Bio-Rad). After 18 h of active rehydration (50 V), isoelectric focusing was performed as previously reported [25]. The focused isoelectric focusing strips were stored at −80 °C until a second-dimension electrophoresis was performed. For the second dimension, thawed strips were sequentially equilibrated for 15 min in the dark in 375 mM Tris, pH 8.8, 6 M urea, 2% sodium dodecyl sulfate, 20% glycerol containing first 2% dithiothreitol and then 2.5% iodoacetamide. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in Criterion Tris–HCl gels 8–16% (Bio-Rad) at 200 V for 1 h.

Table 1
Case demographics.

Group	*n*	Gender (M/F)	Age (SD)	PMI (SD)
YC	6	3/3	13.10 (15.30)	17.50 (6.50)
OC	6	4/2	53.00 (8.50)	10.80 (5.90)
DS	6	5/1	11.01 (10.90)	17.00 (4.50)
DS/AD	6	3/3	45.60 (3.90)	9.39 (6.80)

M, male; F, female; SD, standard deviation; PMI, postmortem interval; YC, young control; OC, old control; DS, Down syndrome; AD, Alzheimer disease; DS/AD, Down syndrome with Alzheimer disease neuropathology.

Gel staining and image analysis

Gels were fixed for 45 min in 10% methanol, 7% acetic acid and stained overnight with SYPRO ruby gel stain (Bio-Rad). After destaining in deionized water, gels were scanned with a Storm UV transilluminator (λ_{ex} 470 nm, λ_{em} 618 nm; Molecular Dynamics, Sunnyvale, CA, USA). Images obtained were saved in Tagged Image File Format Gel and imaging was software-aided using PD-Quest (Bio-Rad) imaging software. Briefly, a master gel was selected followed by normalization of all gels according to the total spot density. Then, a manual matching of common spots that could be visualized among the differential two-dimension (2D) gels was performed. After a significant number of spots was obtained, the automated matching of all spots was initiated. Automated matching is based on user-defined parameters for spot detection. These parameters are based on the faint test spot, the largest spot, and the largest spot cluster that occur in the master gel and are defined by the user. This process generates a large pool of data, ~350 spots. Only proteins showing computer-determined significant differential levels between the two groups being analyzed were considered for identification. To determine significant differential levels of proteins, analysis sets were created using the analysis set manager software incorporated into the PD-Quest software. The numbers of pixels that occur in a protein spot, corresponding to an increase/decrease in protein level, were computed by the software. A quantitative analysis set was created that recognized matched spots with differences in the number of pixels that occur in each spot. Then, a statistical analysis set was created that recognized matched spots with differences in the number of pixels that occur in each spot and a statistical analysis set was created that used a Student *t* test at 95% confidence to identify spots with *p* values of <0.05. Spots with $p<0.05$ were considered significant.

In-gel trypsin digestion

Briefly, protein spots identified as significantly altered after PD-Quest analysis were excised from 2D gels with a clean, sterilized blade and transferred to Eppendorf microcentrifuge tubes. Gel plugs were then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min, followed by incubation with 100% acetonitrile at room temperature for 15 min. After solvent removal, gel plugs were dried in their respective tubes under a flow hood at room temperature (RT). Plugs were incubated for 45 min in 20 μl of 20 mM dithiothreitol (DTT) in 0.1 M NH_4HCO_3 at 56 °C. The DTT/NH_4HCO_3 solution was then removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and incubated with gentle agitation at room temperature in the dark for 30 min. Excess