A Proteomic Approach for the Involvement of the GAPDH in Alzheimer Disease in the Blood of Moroccan FAD Cases

Nadia El Kadmiri · Raquel Cuados ·
Bouchra El Moutawakil · Ilham Slassi · Jesus Avila ·
Sellama Nadifi · Ahmed Hachem · Abdelaziz Soukri

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Abstract Several articles have highlighted the potential involvement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in neurodegeneration by showing a non-glycolytic activity of GAPDH specifically in the brains of subjects with Alzheimer's disease (AD). The novel aim of this study was to elucidate the critical role of GAPDH and its interaction with β -amyloid in the blood of Moroccan patients with familial AD (FAD) carrying presenilin mutations and in sporadic late onset AD (LOAD). Our results show a significant decrease in the activity of GAPDH in blood samples from patients with FAD as compared to sporadic cases and healthy controls. The expression level of GAPDH in brain specimens from mutant *tau* transgenic mice and patients with FAD was unchanged as compared to healthy controls. In contrast, the expression level of GAPDH in blood samples from mutant *tau* transgenic mice and patients with FAD was decreased as compared to sporadic cases and healthy controls. Moreover,

there is an accumulation of β -amyloid aggregates in the blood samples of patients with FAD and an increase in amyloid fibrils in both the blood and brain samples of these patients. Our study adds new insight to previous ones by showing the involvement of GAPDH in AD, which may influence the pathogenesis of this neurodegenerative disease.

Keywords Moroccan FAD · GAPDH

Introduction

Recent proteomic studies aimed at determining altered levels of a multitude of proteins in the brains of individuals with Alzheimer's disease (AD), which could be involved in AD pathogenesis (Butterfield et al. 2010). The identification of proteins affecting the degree of neurodegeneration could contribute to the development of biomarkers or new drug targets in the management of AD.

AD is the most common neurodegenerative disease in the elderly, accounting for 50 to 56 % of cases as assessed by autopsy and clinical series, with an annual incidence of approximately 3 % in the 65–74 age group (Castellani et al. 2010). AD is clinically characterized by progressive impairment in memory and cognitive decline. In Morocco, AD affects almost 30,000 individuals, and this number could increase to 75,000 by 2020 (projections of the World Health Organization (WHO)). However, this disease remains poorly understood by the Moroccan general public. To our knowledge, the genes predisposing individuals to AD and predicting disease incidence remain elusive, and no proteomic studies have been carried out in this area. AD is pathologically characterized by extracellular deposition of β -amyloid ($A\beta$) in senile plaques and formation of intracellular neurofibrillar tangles, mainly composed of the hyperphosphorylated microtubule-associated protein *tau* (Ling et al. 2003;

N. El Kadmiri (✉) · B. El Moutawakil · I. Slassi · S. Nadifi
Laboratory of Medical Genetics and Molecular Pathology, Faculty of
Medicine and Pharmacy, University Hassan II, 19 Rue Tarik Ibnou
Ziad, B.P. 9154, Casablanca 20000, Morocco
e-mail: elkadmiri1979@gmail.com

N. El Kadmiri · A. Soukri
Laboratory of Physiology and Molecular Genetics, Faculty of
Sciences Aïn Chock, University Hassan II, Casablanca, Morocco

B. El Moutawakil · I. Slassi
Department of Neurology, CHU IBN ROCHD, Casablanca,
Morocco

R. Cuados · J. Avila
CIBERNED—Investigations Center of Neurodegenerative
Diseases—the Centro de Biología Molecular “Severo Ochoa”,
Madrid, Spain

A. Hachem
Laboratory of Thrombosis and Hemostasis, Montreal Heart Institute,
Montreal, Quebec, Canada

Gomez-Ramos et al. 2004). Several articles have highlighted the potential involvement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) in neurodegeneration by reporting a non-glycolytic activity of GAPDH specifically in brains of AD cases. GAPDH strongly interacts with the β -amyloid precursor protein (β -APP) with high affinity in vitro and it is found as a protein component of neurofibrillary tangles (NTFs). Whether GAPDH is also involved in the initiation of programmed cell death cascades is still unknown (Ling et al. 2003; Schulze et al. 1993; Chen et al. 2000; Oyama et al. 2000; Mazzola and Sirover 2001; Cumming and Schubert 2005; Verdier et al. 2005; Nakajima et al. 2007; Naletova et al. 2008).

The aim of this study is to probe for the role of GAPDH and its interaction with A β amyloid aggregates especially in blood samples from Moroccan patient of FAD cases carrying frameshift mutations in the presenilin genes.

Methods

Sample Preparation

Human Blood

Peripheral blood was taken from three FAD cases (one woman (ID: F1) with a disease onset age of 63 years and two men with disease onset age of 60 (ID: F2) and 55 (ID: F3) years respectively), 17 sporadic cases (7 men and 10 women; age 66 $\pm$ 10,81), and two healthy controls (two women aged 58 and 55 years). All patients were seen by the Memory Consultation group at the CHU IBN ROCHD Neurology Department in Casablanca, Morocco. The protocol was approved by the Human Ethical Committee of the CHU IBN ROCHD in accordance with the Declaration of Helsinki for experiments involving humans, and written consent was obtained from the patients and their legal guardians prior to their enrollment in the study. A family history was obtained by a clinical interview of the patient and a “yes” or “no” self-reporting questionnaire from the legal guardian or other family member. The disease was considered familial if at least one additional first degree relative suffered from early onset AD-type dementia. All patients underwent standard somatic neurological examination, cognitive function assessment, brain imaging, and laboratory tests. The clinical, neuropathological, and genetic assessments of FAD cases have several shared features. For instance, there is a strong correlation between clinical symptoms and genetic factors in our patients with a family history of AD. Our mutational analysis (El Kadmiri et al. 2014) revealed that our FAD cases carry frameshift mutations located in the presenilin genes (F1/exon 11/PS1, F2/exon 5/PS2, and F3/Exon 9/PS2 respectively), whereas our sporadic cases do not carry mutations in the presenilin genes.

Human Brain Samples

Brain specimens were removed at autopsy from one patient with confirmed FAD and one healthy control. Brain specimens were frozen and stored at -80°C at CIBERNED—Investigations Center of Neurodegenerative Diseases—the Centro de Biología Molecular “Severo Ochoa” (Madrid, Spain). Written informed consents from the donors and their next of kin were obtained for using the brain samples in research in accordance with approved protocols by the local Ethical Committees of the CIBERNED—the Centro de Biología Molecular “Severo Ochoa” brain banks for nervous tissue donation.

Animals

Mutant *tau* transgenic mice were engineered, bred, and housed under pathogen-free conditions at the CIBERNED animal facility. Their wild type (WT) littermates were used as controls. Handling and caring of mice were in compliance with the guidelines established by the Animal Care and Ethical Committee of CIBERNED. Only the male mice were used for this study.

Mouse Blood Retrieval Murine blood was retrieved by cardiac puncture from WT and mutant *tau* transgenic-anesthetized mice into 1 mL syringes containing 50 μL of heparin (1,000 U/mL).

Mouse Brain Mice were sacrificed with an overdose of anesthetics to minimize suffering. After dissection, the brains from WT and mutant *tau* transgenic mice were quickly removed. The brains were then dissected on an ice-cold plate, and brain samples were placed in tubes on ice for immediate use.

Blood Protein Extraction

Protein extracts were obtained from red blood cells (RBC). Briefly, blood samples (human or mouse) were centrifuged, and the RBC layer was recovered after discarding the platelet-rich plasma and white blood cell layers. The RBC extracts were washed with isotonic NaCl (0.9 %) and then lysed with ice-cold lysis buffer (Hepes 20 mM pH 7.4, NaCl 100 mM, orthovanadate 1 mM, EDTA 5 mM, 1 % Triton X-100, 1 $\times$ protease inhibitor cocktail and okadaic acid 100 μM).

Brain Protein Extraction

Brain samples were homogenized four times for 25 s, centrifuged for 1 h at 10,000 RPM, and lysed with the abovementioned lysis buffer. Brain protein extracts were then recovered after centrifugation for 30 min at 22,000 RPM.

GAPDH Activity Assay

The phosphorylating activity of GAPDH was determined. The reaction was started by the addition of 10 µg of protein preparation to an assay mixture containing 50 mM Tricine buffer (pH 8.5), 10 mM sodium arsenate, 1 mM NAD⁺, and 2 mM D-glyceraldehyde-3-phosphate (D-G3P). The total volume of the reaction mixture was 1 ml. Absorbance changes at 340 nm were monitored. One minute of enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 µmol NAD⁺ per minute. All experiments and assays were carried out in triplicates.

GAPDH Protein Expression

The protein samples were assayed for their protein concentration via Bradford. The equivalent of 30–40 µg of proteins was mixed with Laemmli sample buffer, boiled for 3 min, resolved in 10 % SDS-PAGE gels, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked with 5 % non-fat dry milk for 1 h, washed three times with PBS/Tween (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20), and incubated 1 h at room temperature with the mouse monoclonal anti-GAPDH antibody (1:5,000 dilution) (Abcam, Spain). Following the washing steps, membranes were labeled with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-Mouse IgG HRP-conjugated, Santa Cruz biotechnology, Spain) for 1 h; washed and bound peroxidase activity was then detected by the enhanced chemiluminescence detection system (Amersham Biosciences, Arlington Heights, IL). To assess equal amounts of protein loading, membranes were stripped, blocked with 5 % milk, and blotted for β-actin (1:2,000 dilution) (mouse monoclonal anti-β-actin antibody, Sigma-Aldrich, Spain). Protein bands were analyzed with an imaging densitometer.

β-Amyloid Protein Aggregates

Aβ protein SC489 and its monoclonal HRP-conjugated antibody 4G8 were purchased from Covance. One microliter of Aβ protein SC489 was added per 10 µg of protein extracts. All samples were heated for 30 min at 37 °C. The samples were deposited onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked with 5 % non-fat dry milk at room temperature for 30 min, washed three times with PBS/Tween, and incubated for 1 h at room temperature with the 4G8 antibody followed by another 1 h of incubation with an HRP-conjugated IgG. Washed and bound peroxidase activity was then detected using the Immobilon™ Western chemiluminescent HRP substrate system. Spots were manually selected, and intensities of the spots

were analyzed as mean gray values corrected for the background value using Image J software (Abramoff et al. 2004).

EM

One microliter of Aβ protein was added per 10 µg of protein extracts. All samples were heated for 30 min at 37 °C. The samples were then stained with 2 % uranyl acetate for 1 min followed by electron microscopy (EM) imaging.

Results

We first sought to evaluate the activity of GAPDH in the blood and brain of FAD cases—the blood from sporadic cases and healthy individuals. As shown in Fig. 1, the activity of GAPDH in protein extracts from both the peripheral blood and brain specimens from Moroccan patients with FAD was significantly decreased as compared to that of healthy controls. The activity of GAPDH in protein extracts from peripheral blood from Moroccan sporadic cases was not altered (data not shown). This decrease in activity was evaluated as a loss of absorbance, which was present throughout the 1-min time interval of incubation of the protein extracts from the FAD samples with the assay mixture. To evaluate the relationship between the expression and the activity of GAPDH in the samples, the protein extracts were assayed for their content in GAPDH. As shown in Fig. 2, the expression of GAPDH in the brain specimens from mutant *tau* transgenic mice and FAD case was unchanged compared to the healthy controls. In contrast, the expression of GAPDH in protein extracts from the peripheral blood of mutant *tau* transgenic mice and FAD cases was decreased as compared to controls. The expression of GAPDH in protein extracts from the peripheral blood from sporadic cases was non-significant (data not shown).

To investigate the effect of the frameshift mutations on the structural properties of Aβ and the influence of GAPDH on Aβ protein aggregates formation, protein extracts from healthy subjects and individuals with FAD were assayed for their potential to induce Aβ aggregates and analyzed for the presence of amyloid fibrils. Interestingly, incubation of peripheral blood protein extracts with Aβ induces an increase in Aβ accumulation in samples from FAD cases but not from healthy controls (Fig. 3). These FAD cases each carry a single frameshift mutation. The F1 case has a frameshift mutation in 73683837-G at codon 378, which is caused by a single-nucleotide insertion located in exon 11 of PS1, whereas the F2 case has a frameshift mutation in 227073260-A, which is caused by a single-nucleotide insertion at codon 126 located in exon 5 of PS2 (El Kadmiri et al. 2014). Furthermore, EM examination of the protein extracts and Aβ incubations showed significant amyloid fibrils formation with brain

Fig. 1 Activity assay of GAPDH in protein extract from the peripheral blood and brain. Histogram represents the absorbance of the protein sample at 340 nm over the 1-min incubation period. *F1* and *F2* are protein extracts from the peripheral blood from two FAD cases, *BC* protein extracts from brain specimens of a healthy control obtained at autopsy, *BAD* protein extracts from brain specimens of a confirmed AD case at autopsy, and *C* protein extracts from peripheral blood from a healthy subject

extract from a confirmed AD case and from peripheral blood of our FAD cases, which carries the frameshift mutation. Conversely, no formation of amyloid fibrils was observed when protein extracts from sporadic cases (data not shown) or healthy subjects was used (Fig. 4).

Discussion

Our finding shows a significant decrease in GAPDH activity albeit unchanged protein expression in the brain of both mutant *tau* transgenic mice and FAD case, which may be due to post-translational alterations. This result is consistent with previous studies demonstrating a decrease in GAPDH glycolytic activity regardless of the change in its expression in brain specimens of AD cases (Schulze et al. 1993; Mazzola and Sirover 2001; Cumming and Schubert 2005). In contrast, the expression of GAPDH in the blood of mutant *tau* transgenic mice and our FAD cases was decreased in comparison to

controls, probably due to an alteration at the transcriptional level.

Several studies have confirmed the crucial role of GAPDH in influencing amyloid aggregation through post-translational alterations leading to a significant reduction in amyloid activity. These studies focused on the molecular mechanism by which GAPDH influences in amyloid aggregation. Moreover, GAPDH has previously been shown to bind to the cytoplasmic C-terminal domain of the amyloid precursor protein (APP) (Schulze et al. 1993). Recent studies confirmed that GAPDH could also bind a variety of A β isoforms, and it displays a greater affinity for the A β 1–42 form (Cumming and Schubert 2005; Verdier et al. 2005; Naletova et al. 2008). Indeed, GAPDH is locally deposited in aggregates at lesion sites in AD via the formation of intermolecular disulfide bonds. Several studies have demonstrated that only the oxidized and denatured forms of GAPDH are able to form highly stable complexes with A β , indicating the direct involvement of GAPDH in amyloid aggregation. Cumming and Schubert 2005 revealed an increase in GAPDH intermolecular disulfide

Fig. 2 Representative Western blots for GAPDH performed on protein extracts from the indicated samples. Blots for β -actin are from stripped GAPDH membranes

Fig. 3 Representative *dot blot* of formed A β aggregates. Protein extracts from peripheral blood from two FAD cases (F1 and F2) and two control individuals (C1 and C2) were incubated with A β and then transferred onto nitrocellulose membranes. Membrane were then blotted for A β to assess the potential of the protein extracts to induce accumulation of A β aggregates

bonding in all examined AD brain extracts as compared to age-matched controls. The authors also verified the activity of GAPDH and showed that the oxidant-induced disulfide bonding of GAPDH leads to a reduction in its enzymatic activity. On the other hand, *tau* has been shown to bind and promote the denaturation and inactivation of GAPDH in vitro, suggesting a direct involvement of GAPDH in *tau* aggregation and NFT formation in brains of individuals with AD (Chen et al. 2000; Wang et al. 2005; Yang et al. 2008).

All these findings are consistent with our proteomic results, which assess the aggregation of amyloid beta in patients carrying mutations located in presenilin genes. Dot blotting showed increased A β accumulation in peripheral blood from our FAD cases but not in healthy controls. EM examination revealed significant amyloid fibril formation both in brain specimens from confirmed AD case and in peripheral blood from our FAD cases, who carry frameshift mutations located in presenilin genes. Based on our results and results from other

studies, we can conclude that there is a link between amyloid aggregation and GAPDH through conversion of the normal conformation of GAPDH to an abnormal one, which leads to a decrease in its activity. Whether GAPDH is also involved in the initiation of the programmed cell death cascades remains to be determined. Moreover, ROS have been shown to play an important role in modulating the non-glycolytic functions of GAPDH, which may significantly contribute to loss of neuronal function and neurodegeneration in brains of AD patients (Butterfield and Lauderback 2002; Uttara et al. 2009).

Our mutational and proteomic analysis report a correlation between genotype and phenotype in our cases of EOAD involving the frameshift presenilin mutations and suggest that these mutations increase the risk of developing neurodegenerative diseases through A β -42 accumulation, which is due to modulation in the proteolytic processing of APP (De Strooper et al. 2012). This modulation is manifested by beta amyloid aggregates that directly interact with GAPDH and alter its function.

Identification of proteins affecting the degree of neurodegeneration could contribute to the development of biomarkers or new drug targets in the management of AD. Cerebrospinal fluid (CSF) and blood plasma are the most promising sources for AD biomarkers as compared to brain tissue. These fluids are more easily accessible, which was the aim of this study, differentiating it from several others ones to elucidate the critical role of GAPDH in the blood of AD individuals that may influence the pathogenesis of neurodegenerative diseases. However, it remains to clarify this decreased expression of GAPDH observed in the blood compared to brain. A study

Fig. 4 Representative *electron microscopy* images of formed A β aggregates. Protein extracts from **a** protein extracts from brain specimens of a confirmed AD case at autopsy, **b** protein extracts of peripheral blood from a FAD case (ID. F1), **c** protein extracts from the peripheral blood from a FAD case (ID.F2), **d** protein extracts from brain specimens from a healthy subject at autopsy, **e** protein extracts from peripheral blood from a healthy subject, and **f** PBS as control were incubated with A β and then visualized on an electron microscopic to assess amyloid fibril formation. **a**, **b**, and **c** show significant amyloid fibril formation, whereas **d**, **e**, and **f** show no abnormalities. The scale bar for images **a**, **b**, **c**, and **d** represents 70 nm, whereas that for images **e** and **f** represents 100 nm

is underway to eventually demonstrate the mechanism responsible for this decreased expression and its involvement in the neurodegenerative process of AD. These findings open prospects for diagnosis by using GAPDH as a biomarker for diagnostics and monitoring AD modification.

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