Cross-Sectional Characterization of Albumin Glycation State in Cerebrospinal Fluid and Plasma from Alzheimer's Disease Patients

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Abstract

We determined albumin post-translational modifications (PTMs) by mass spectrometry (MS) in plasma and cerebrospinal fluid (CSF) from 31 Alzheimer's disease (AD) patients (with 27 samples of paired plasma-CSF from the same patients). Results were cross-sectionally compared with healthy controls. For percentage of relative intensity of glycated isoforms, plasma albumin was globally more glycated in AD patients than in healthy controls ($P < 0.01$). MS results in plasma were confirmed by a quantitative enzymatic assay (Lucica GA-L) for albumin early-glycation detection. In CSF there were no global glycation differences detected by MS, although a different pattern of glycated isoforms was observed. Oxidized+glycated and cysteinylated+glycated isoforms were increased in both plasma and CSF of AD patients in comparison with healthy controls ($P < 0.001$). Furthermore, AD patients showed higher glycation in plasma than in CSF ($P < 0.01$). Our data support the role of glycation and oxidative stress in AD.

Key words: Albumin, glycation status, cerebrospinal fluid, plasma, Alzheimer's disease.

Introduction

Albumin is the most abundant protein in the human body. In addition to its well-known role as a plasma expander, albumin has a number of relevant physiologic functions such as being the main extracellular antioxidant and transporter, and exerting metal-binding and scavenger activities (1). In albumin, glycation-induced conformational changes can have a deleterious effect in both its binding capacity (2-5) and antioxidant capacity (6-8). Elevated levels of glycated albumin are also associated with aging (9) and related conditions such as retinopathy, nephropathy, neuropathy, cardiovascular diseases and Alzheimer's disease (AD) (4).

Serum albumin is thought to inhibit neuritic plaque formation tissue through regulating β -amyloid protein ($A\beta$) fibril growth in the brain (10), with glycated albumin being less effective than native albumin in preventing $A\beta$

aggregation (11). AD patients have been shown to have elevated levels of glycated proteins in serum (12, 13), plasma (14) and cerebrospinal fluid (CSF) (15, 16). More specifically, glycated albumin has been found increased in AD plasma and serum (11, 16), but no consistent results have been obtained in AD CSF (11, 16). Although a paired comparison of protein glycation in plasma and CSF from the same AD patient has already been assessed (17), the same approach for albumin glycation has not been studied. In addition, to the best of our knowledge, a quantitative approach to specifically assess early-glycated albumin in AD plasma has not been addressed, despite the fact that the early glycation process has been reported as playing a role in AD (16).

In a previous paper we reported that albumin of AD patients was significantly more oxidized than in healthy subjects, with this effect more marked in CSF than in plasma (18). These data supported the involvement of oxidative stress in AD, thus pointing out the need for further research to better understand the role of albumin in AD. The aim of this cross-sectional study was to extend the same approach to albumin glycation in plasma and CSF from AD patients, which was also characterized in comparison with age-matched healthy subjects.

Methods

Experimental design

Determination of albumin post-translational modifications (PTMs) for glycation in terms of their identification and relative quantification was performed by means of mass spectrometry (MS) technique. Albumin glycation levels were assessed in plasma ($n=28$) and CSF ($n=30$) samples from 31 patients with mild-moderate AD (there were 27 plasma and CSF samples drawn from the same individuals). Results were compared with albumin glycation levels assessed in plasma ($n=20$) and CSF ($n=10$) samples from healthy –not diagnosed with AD– age-matched subject controls. Additionally, quantification of early-glycated albumin in plasma ($n=34$ for AD

patients; n=31 for controls) was measured by quantitative enzymatic assay (Lucica GA-L). Complementarily, the paired plasma versus CSF comparison of albumin glycation was performed in the 27 samples available from the same AD patients.

Sampling

Plasma and CSF samples taken from mild-moderate AD patients (53 to 78 years of age) meeting NINCDS-ADRDA criteria for probable AD, and Mini-Mental State Examination (MMSE) scores between ≥ 18 and ≤ 26 were baseline specimens provided by the centers participating in the clinical trial EudraCT #2007-000414-36. Detailed patient population demographics, clinical profiles and inclusion/exclusion criteria are available in previous publications (19, 20).

Samples from control patients (48 to 81 years of age for plasma and 65 to 72 years of age for CSF) were kindly supplied by ACE Foundation, Catalan Institute of Applied Neurosciences (Barcelona, Spain) from their sample repository and purchased from Sera Laboratories International Ltd (West Sussex, UK), respectively.

All donors gave their informed consent in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Blood sampling (plasma) and lumbar puncture (CSF) were carried out following the standard techniques of each center (19). Albumin concentration in plasma and CSF from both AD patients and controls was similar (18).

Liquid chromatography-Mass spectrometry

PTMs of albumin were assessed on the intact mass protein (top-down) by using ultra high-performance liquid chromatography coupled to electrospray ionization mass spectrometer (LC-ESI-qTOF-MS) (21).

For plasma and CSF, albumin-enriched samples were obtained, separated by liquid chromatography and subsequently analyzed by MS on a Quadrupole Time of Flight analyzer with electrospray ionization source (ESI) (Agilent qTOF 6550 Jet Stream, Agilent Technologies, Santa Clara CA, USA). All procedures and mass spectrometry data analysis have been described in a previous publication (18). Albumin controls were processed in parallel to albumin-enriched samples to ensure sample stability during analysis.

Isoform relative intensity (%) was calculated by dividing the isoform absolute intensity by the sum of all isoform absolute intensities, expressed as a percentage. For every comparison, a filter of frequency was applied to consider only those isoforms that were present in all analyzed samples of at least one of the compared groups.

The identification of albumin PTMs was performed as previously described (22-24) by analyzing 60,000-75,000 Da mass range and comparing the theoretical mass with

the experimental value, allowing a mass drift of 10 Da (Agilent BioConfirm software).

Quantitative Enzymatic assay (Lucica GA-L)

Early-albumin glycation (fructosyl groups bound to albumin) in plasma samples was determined by an in vitro diagnostic assay, Lucica® GA-L (25) (Asahi Kasei Pharma Corp., Tokyo, Japan). This kit includes the measurement of glycated albumin by an enzymatic method using an albumin-specific protease and a ketoamine oxidase, and the measurement of total albumin using bromocresol purple reagent. Glycated albumin results were expressed in a percentage relative to the total albumin and corrected to match HPLC results: Glycated albumin (%) = (glycated albumin [g/dL] / total albumin [g/dL]) / 1.14 × 100 + 2.9, following the instructions provided by the kit manufacturer. CSF analysis was not performed because the method's sensitivity is insufficient for low albumin concentration samples, such as those typical in CSF.

Statistical analysis

Variables are expressed as the median and interquartile range (IQR). Unpaired and paired Student's t test and Mann-Whitney tests were used for the comparisons. Benjamini-Hochberg's correction for multiple comparisons was applied when comparing differences in albumin PTMs' relative abundance between groups (26).

To examine the magnitude of the differences in glycated albumin, the effect sizes based on standardized differences between mean scores were calculated (Cohen's delta) (27). Effect sizes of 0.20, 0.50 and 0.80 were considered small, medium and large, respectively (27).

Software used for charting and calculations was Graph-Pad Prism v6 (San Diego CA, USA) and GeneSpring 13.1 (Agilent Technologies, Santa Clara CA, USA).

Results

Albumin glycation in plasma and CSF: AD patients versus controls

Seventeen albumin PTMs were identified by intact mass protein analysis. In plasma, seven of these 17 PTMs corresponded to glycated PTMs: glycated (Alb+Glyc); oxidized+glycated (Alb+SO₃H+Glyc); cysteinylated+glycated+dehydroalanine (Alb+Cys+Glyc-DHA); cysteinylated+glycated (Alb+Cys+Glyc); two glycation modifications (Alb+2Glyc); cysteinylated+two glycation modifications (Alb+Cys+2Glyc); and three glycation modifications (Alb+₃Glyc). In CSF, four of these 17 albumin PTMs were identified as glycated PTMs: Alb+Glyc; Alb+SO₃H+Glyc; Alb+Cys+Glyc; and

Alb+2Glyc.

MS values for percentage of relative intensity of albumin glycation in plasma showed that AD patients had higher amount of glycosylated albumin isoforms than controls when considering the sum of the seven glycosylated PTMs ($P < 0.01$, Cohen's delta: 1.0) (Figure 1a). Separately analyzed, four out of seven glycosylated PTMs showed a statistically significant increase in AD versus controls: oxidized (Alb+SO₃H+Glyc [$P < 0.001$; Cohen's delta: 1.2]), and cysteinylated (Alb+Cys+Glyc-DHA [$P < 0.0001$; Cohen's delta: 1.0], Alb+Cys+Glyc [$P < 0.0001$; Cohen's delta: 1.7], and Alb+Cys+2Glyc [$P < 0.001$; Cohen's delta: 1.0]) (Figure 1b).

Figure 1. Albumin glycation in plasma as isoform relative intensity (%) identified in Alzheimer's disease (AD) patients and healthy controls. A) Sum of all 7 glycosylated post-translational modifications (PTMs). B) The 7 glycosylated PTMs separately (glycosylated [Alb+Glyc]; oxidized+glycosylated [Alb+SO₃H+Glyc]; cysteinylated+glycosylated+dehydroalanine [Alb+Cys+Glyc-DHA]; cysteinylated+glycosylated [Alb+Cys+Glyc]; two glycosylation modifications [Alb+2Glyc]; cysteinylated+two glycosylation modifications [Alb+Cys+2Glyc]; three glycosylation modifications [Alb+3Glyc])

Data are shown as median and IQR (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; AD versus control).

MS results in plasma were confirmed by the quantitative enzymatic assay (Lucica GA-L) for albumin early-glycation detection. The percentage of glycosylated plasma albumin in AD patients was 12.9% (12.3%-14.9%) versus 11.6% (10.5%-13.4%) in controls (median-IQR; $P < 0.001$; Cohen's delta: 0.7).

MS values of albumin glycation in CSF showed no differences in percentage of relative intensity when comparing the sum of the four glycosylated PTMs in AD patients versus controls (Figure 2a). However, if separately analyzed, three out of four glycosylated PTMs showed statistically significant differences in AD versus controls: a decrease in Alb+Glyc isoform ($P < 0.0001$; Cohen's delta: 1.7) and an increase in the oxidized and cysteinylated forms (Alb+SO₃H+Glyc [$P < 0.0001$; Cohen's delta: 3.0] and Alb+Cys+Glyc [$P < 0.0001$; Cohen's delta: 1.8]) (Figure 2b).

Figure 2. Albumin glycation in cerebrospinal fluid (CSF) as isoform relative intensity (%) identified in Alzheimer's disease (AD) patients and healthy controls. A) Sum of 4 glycosylated PTMs. B) The 4 glycosylated PTMs separately (glycosylated [Alb+Glyc]; oxidized+glycosylated [Alb+SO₃H+Glyc]; cysteinylated+glycosylated [Alb+Cys+Glyc]; two glycosylation modifications [Alb+2Glyc])

Data are shown as median and IQR (****, $p < 0.0001$; AD versus control).

Albumin glycation in AD patients: plasma versus CSF

When comparing albumin glycosylated PTMs in the subset of 27 AD samples consisting of paired plasma and CSF specimens both obtained from the same patient, plasma albumin was found significantly more glycosylated than in their paired CSF samples (Table 1). Moreover, the specific analysis showed that six out of seven glycosylated PTMs of albumin were statistically significantly different between compartments. The most relevant ones were Alb+Glyc and Alb+Cys+Glyc in plasma, and Alb+Glyc and Alb+SO₃H+Glyc in CSF. Details are shown in Table 1.

Discussion

In this study we observed that plasma albumin was overall more glycosylated in AD patients than in healthy age-matched controls, when analyzed as the percentage of relative intensity of albumin glycation isoforms assessed by MS. Conversely, in CSF albumin, there were no global glycation differences, although a different pattern of glycation isoforms was observed. Moreover, AD patients had their albumin significantly more glycosylated in plasma than in CSF.

In plasma samples from AD patients, when considering all the glycosylated albumin PTMs separately, statistical significance versus controls was shown with the oxidized+glycosylated albumin forms and especially the cysteinylated+glycosylated albumin forms, rather than

Table 1. Albumin glycation in paired samples of plasma and cerebrospinal fluid (CSF) of Alzheimer's disease (AD) patients (n=27). Compared relative intensity (%) of 7 individual glycated post-translational modifications (PTMs) (glycated [Alb+Glyc]; oxidized+glycated [Alb+SO₃H+Glyc]; cysteinylated+glycated+dehydroalanine [Alb+Cys+Glyc-DHA]; cysteinylated+glycated [Alb+Cys+Glyc]; two glycation modifications [Alb+2Glyc]; cysteinylated+two glycation modifications [Alb+Cys+2Glyc]; three glycation modifications [Alb+3Glyc]); and the sum of all PTMs are shown (median and IQR)

Glycated PTM	Plasma AD (%)	CSF AD (%)	p value	Cohen's delta
Alb+Glyc	5.4 [4.9-5.8]	6.0 [5.3-7.0]	<0.05	0.6
Alb+SO ₃ H+Glyc	1.2 [1.1-1.4]	2.7 [1.9-3.0]	<0.0001	2.2
Alb+Cys+Glyc-DHA	0.37 [0.27-0.42]	0.88 [0.67-1.1]	<0.0001	1.6
Alb+Cys+Glyc	4.5 [3.6-5.0]	1.8 [1.3-2.7]	<0.0001	2.3
Alb+2Glyc	0.90 [0.82-1.0]	0.77 [0.64-1.1]	>0.05	0.2
Alb+Cys+2Glyc	0.42 [0.37-0.56]	0.27 [0.18-0.38]	<0.0001	1.1
Alb+3Glyc	0.11 [0.06-0.14]	0.20 [0.15-0.28]	<0.05	1.2
All glycated PTMs	13 [12-14]	11 [9.8-14]	<0.01	0.4

with the purely glycated albumin. Since cysteinylated forms are considered to be reversibly oxidized albumin modifications (1), these findings would support the higher content of reversibly oxidized albumin (HNA1) detected by HPLC-FLD analysis in AD plasma samples compared to control plasma (18). Interestingly, cysteinylated albumin has been described as a sensitive plasma marker in oxidative stress-related chronic diseases (28).

In CSF, overall albumin glycation levels were similar between AD patients and controls but, interestingly, the glycation pattern was different. AD patients showed lower content of glycated albumin but increased content of both the oxidized+glycated and cysteinylated+glycated albumin forms. Again, since cysteinylated forms are considered to be reversibly oxidized albumin modifications while SO₃H forms are considered to be irreversibly oxidized albumin modifications (2), these findings are in accordance to a higher content in HNA1 and irreversibly oxidized albumin (HNA2) observed by HPLC-FLD analysis in AD CSF (18).

Furthermore, MS results obtained in plasma from AD patients and healthy controls were confirmed by a quantitative enzymatic assay for early glycation detection of albumin (Lucica GA-L).

Albumin glycation in AD patients has been previously addressed by a few other authors. Increased levels of early glycation products in all major proteins of CSF of AD patients, including albumin, apolipoprotein E and transthyretin, have been reported (16). Furthermore, targeted assessment of advanced glycation end products in albumin has been addressed in plasma (11) and serum (13), showing higher levels than in a control population, while presenting no differences in CSF (11). Our findings in CSF albumin glycation are in accordance with those obtained by Ramos-Fernández et al. (11). Differences in the AD study population, as well as in the experimental approach, might explain the lack of consensus with all of

the investigations.

Based on our literature search, albumin glycation in paired plasma and CSF samples taken from the same patient has not been previously reported. Our results showed higher overall albumin glycation in plasma compared to CSF, with both compartments being different in terms of PTMs relative quantification. Besides the glycated albumin form, in plasma the reversibly oxidized cysteinylated+glycated albumin was relevant, while in CSF the irreversibly oxidized SO₃H+glycated isoform gained predominance. These results suggest a restriction of albumin communication in both compartments, which would present different environments in terms of glycation and oxidation.

To summarize, accepting the limitations of this study, such as the sample size and the use of MS as a semi-quantitative method to analyze albumin glycation, the results presented here suggest that in both plasma and CSF samples taken from mild to moderate AD patients there is a marked increase in the cysteinylated+glycated and oxidated+glycated albumin forms, compared to healthy age-matched controls. Furthermore, AD patients showed higher glycation in plasma than in CSF. These data support the role of glycation and oxidative stress in AD and deserve further investigation.

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Conflict of interest: MC, AM, RH, AMO, AP and SG are employees of Grifols, a manufacturer of plasma derivatives. MB has consulted for Araclon, Avid, Grifols, Lilly, Nutricia, Roche and Servier. She received fees for lectures, and/or reimbursement of expenses for congresses attendance, and/or funds for research from Araclon, Grifols, Nutricia, Roche and Servier. She has not received personal compensations from these organizations. AR has consulted for Grifols. He is a member of the scientific advisory board of Landsteiner Genmed. He received funds for research from Araclon, Grifols, Nutricia, Roche and Servier. He received reimbursement of expenses for congresses attendance from Araclon, Grifols, and

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Ethical standards: All donors gave their informed consent in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

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