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Original Contribution

Decreased expression and increased oxidation of plasma haptoglobin in Alzheimer disease: Insights from redox proteomics

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ABSTRACT

Alzheimer disease (AD) is one of the most disabling disorders of the elderly and the number of people worldwide facing dementia is expected to dramatically increase in the near future. Thus, one of the major concerns of modern society is to identify putative biomarkers that serve as a valuable early diagnostic tool to identify a subset of patients with increased risk to develop AD. An ideal biomarker should be present in blood before dementia is clinically confirmed, have high sensitivity and specificity, and be reproducible. Proteomics platforms offer a powerful strategy to reach these goals and recently have been demonstrated to be promising approaches. However, the high variability of technologies and studied populations has led to contrasting results. To increase specificity, we analyzed both protein expression profiles and oxidative modifications (carbonylation) of plasma proteins in mild cognitive impairment (MCI) and AD subjects compared with age-matched controls. Most of the proteins found to have differential levels in MCI and AD confirmed results already obtained in other cohort studies. Interestingly, we applied for the first time in MCI a redox proteomics approach to specifically identify oxidized proteins. Among them, haptoglobin, one of the most abundantly secreted glycoproteins with chaperone function, was found to be either increasingly downregulated or increasingly oxidized in AD and MCI compared with controls. We also demonstrated that in vitro oxidation of haptoglobin affects the formation of amyloid-β fibrils, thus suggesting that oxidized haptoglobin is not able to act as an extracellular chaperone to prevent or slow formation of amyloid-β aggregates. Another chaperone protein, α2-macroglobulin, was found to be selectively oxidized in AD patients compared with controls. Our findings suggest that alterations in proteins acting as extracellular chaperones may contribute to exacerbating amyloid-β toxicity in the peripheral system and may be considered a putative marker of disease progression.

Alzheimer disease (AD)[1] is a neurodegenerative disorder affecting more than 20 million people worldwide [1]. AD is characterized by progressive cognitive deficits that gradually lead to loss of memory and impaired activities in daily living. The pathological hallmarks of the disease are deposition of senile plaques resulting from the extracellular deposit of amyloid-β peptide (Aβ) and the intracellular neurofibrillary tangles caused by the aggregation of hyperphosphorylated tau protein [2,3]. Mild cognitive impairment (MCI) is considered to be an early stage of cognitive decline that precedes dementia [4]. MCI patients may progress to AD, thus suggesting MCI as a prodromal phase of the disease and a useful model to study the mechanisms of AD progression [5]. Even though the etiology of AD is still not clearly known, oxidative stress (OS) has been firmly established as one of the main pathogenic events. Numerous studies have demonstrated increased levels of OS markers including protein oxidation, lipid peroxidation, and DNA oxidation in the brain of AD subjects compared with healthy controls [6–8]. Most of these data support the view that Aβ plays a crucial role in AD pathogenesis and progression and it possibly triggers the oxidative stress-mediated damage that ultimately results in neuronal cell death. The amyloid cascade hypothesis of AD highlights that increased Aβ load is due not only to its increased release (i.e., APP mutations in familial AD) but also to the impairment of degradative systems, including

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[1] Abbreviations used: AD, Alzheimer disease; Aβ, amyloid-β peptide; APP, amyloid precursor protein; A2M, α2-macroglobulin; 2DE, two-dimensional electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; DNP, dinitrophenylhydrazone; Hp, haptoglobin; MCI, mild cognitive impairment; UCHL1, ubiquitin C-terminal hydrolase L1.

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proteasome, autophagy, and both intracellular and extracellular chaperones.

Up to now a diagnosis of AD is mainly based on clinical features, neuropsychological tests, and family history, thus resulting in a diagnosis of "probable" AD that sometimes groups together both AD and not-AD dementias. Only postmortem brain examination allow a diagnosis of "definite" AD [9]. To improve the diagnostic tools and early diagnosis of AD, it could be of great importance to identify sensitive and reliable biomarkers that are easy to collect by noninvasive and safe procedures. Body fluids are a great source for putative biomarkers, and cerebrospinal fluid (CSF), plasma, and serum are taken into consideration to search for biological molecules that could relate to AD. Though CSF is the most studied and validated, its collection requires lumbar puncture, which is not always feasible. Thus, increasing interest is currently devoted to the analysis of plasma, which reflects the physiological and pathological status of the organs [10]. Moreover about 500 ml of CSF is absorbed every day in plasma, providing a glimpse of brain content.

One of the major limitations using plasma to find reliable markers of AD, as well as of other pathologies, is related to the extreme complexity of its composition. Indeed, albumin itself represents about 60% of the total plasma protein content. The highly abundant proteins could mask the less abundant ones, which represent the potential biomarkers of diseases. Thus, depletion of abundant plasma proteins before analysis is considered an important strategy to increase the number of detectable proteins [11].

Recent studies have proposed possible biomarkers in plasma of patients with AD [12,13]. Many studies identified proteins differentially expressed in AD by a proteomics approach [10,14]. Bennet et al. analyzed plasma samples from AD patients and age-matched controls by iTRAQ technologies coupled with LC–MS/MS [11,15].

In recent years, increasing studies have been focused on establishing a direct link between tissue-specific oxidation and systemic oxidative damage, and correlations between total levels of markers of oxidation in the brain and in the periphery have been shown [16,17].

This study aimed at analyzing variations in the plasma proteome, both expression levels and posttranslational modifications, in subjects with AD and MCI compared to age-matched controls after depletion of the two most abundant proteins, albumin and IgG's. In particular, considering the role of oxidative stress in AD pathogenesis and progression, we focused our attention on oxidative modification of plasma proteins, by a redox-proteomics analysis, to obtain new insights into AD biomarker discovery.

Materials and methods

Sample collection

Blood samples were collected from 10 AD, 10 MCI, and 10 age-matched control subjects attending the Dementia Clinic, Department of Gerontology and Geriatrics, University Hospital of Perugia, Italy. In Table 1 the population demographics data are reported. Diagnosis of probable AD was accomplished according to the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's Disease (NINCDS-ADRDA) criteria [9]. MCI diagnosis was made according to the Petersen criteria [18]. Cognitively healthy subjects (CTR) were enrolled from among relatives of patients and subjects admitted to the Day Hospital of the same department for routine evaluation of the health status. All subjects underwent thorough clinical, neurological, and neuropsychological evaluation; the neuropsychological assessment included the Mini Mental State Examination (MMSE), whereas the CDR scale measured the severity of dementia. Functional status was evaluated on the basis of the activities of daily living and the instrumental activities of daily living. The presence of depressive symptoms was evaluated with the Geriatric Depression Scale, and the Hachinski Ischemic Score was used to consider ischemic risk. The investigation conformed to the principles outlined in the Declaration of Helsinki. All the patients or their relatives gave the written informed consent for blood donation. Blood was immediately centrifuged and plasma stored at $-80\ ^{\circ}C$.

Albumin and IgG plasma depletion

Plasma samples were depleted of these two most abundant proteins using a ProteoPrep Blue albumin and IgG depletion kit (Sigma–Aldrich). Briefly 50 μl of raw plasma sample was purified on a ProteoPrep column and eluted according to the manufacturer's instructions. Protein determination was performed on the eluate fraction with a Coomassie Protein Assay (Pierce).

Two-dimensional (2D) gel electrophoresis

Eluted proteins (100 μg) for each sample were diluted to a total volume of 200 μl with rehydration buffer (8 M urea, 20 mM dithiothreitol (DTT), 2.0% (w/v) Chaps, 0.2% Bio-Lyte, 2 M thiourea, and bromophenol blue). Isoelectric focusing was performed with ReadyStrip IPG Strips (11 cm, pH 3–10; Bio-Rad) at 300 V for 2 h linearly, 500 V for 2 h linearly, 1000 V for 2 h linearly, 8000 V for 8 h linearly, and 8000 V for 10 h rapidly. All the above processes were carried out at room temperature.

After the first-dimension run the strips were equilibrated two times, first for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol and again for another 10 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. The second dimension was performed using 12% precast Criterion gels (Bio-Rad). The gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min and then stained for 1 h in Bio-Safe Coomassie gel stain (Bio-Rad) and destained overnight in deionized water. The Coomassie gels were scanned using a GS 800 densitometer (Bio-Rad).

Table 1
General description of the population included in this study.

Patient group	*N*	Age (years)	MMSE	ADL	IADL	GDS	CDR	HIS	CHOL
CTR (female)	8	78 ± 5	27.7 ± 1.2	5.5 ± 0.5	7 ± 0.6	8 ± 1.7	0	1.5 ± 0.5	197 ± 22
CTR (male)	2	78 ± 9	28.5 ± 2.1	5 ± 0	6 ± 1.4	11.5 ± 3.5	0	1 ± 0	205 ± 5.7
MCI (female)	8	78 ± 5	23 ± 1.6	3.8 ± 0.2	4 ± 1.5	9 ± 2.7	0.5	2.2 ± 0.4	204 ± 38
MCI (male)	2	77 ± 1	22.5 ± 0.7	4 ± 1.4	6 ± 1.4	9 ± 0.7	0.5	1 ± 0	234 ± 49
AD (female)	6	83 ± 5	13.8 ± 4.9	3.8 ± 1.5	2 ± 1.4	3.8 ± 3.7	1.5 ± 0.8	1.5 ± 1.4	206 ± 31
AD (male)	4	77 ± 13	12.5 ± 2.1	2.5 ± 1.7	1 ± 2	4.5 ± 2.4	1 ± 0	1.8 ± 1.5	235 ± 26

N, number of samples for each group; age, average age ($\pm$ standard deviation) of patients; CTR, age-matched controls; AD, Alzheimer disease; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination (30 points total); ADL, activities of daily living (6 points total); IADL, instrumental activities of daily living (8 points total); GDS, Geriatric Depression Scale (15 points total); CDR, Clinical Dementia Rating; HIS, Hachinski Ischemic Score; CHOL, cholesterol.