Mild Cognitive Impairment and Alzheimer Patients Display Different Levels of Redox-Active CSF Iron

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Abstract. Oxidative stress constitutes a hallmark of Alzheimer’s disease (AD). Recent studies also point to redox active metals such as iron, copper and zinc in mediating oxidative stress in AD pathogenesis. However, the reactivity of cerebrospinal fluid (CSF) iron and its possible correlation with the severity of cognitive decline in both Alzheimer’s patients and subjects with mild cognitive impairment (MCI) is still unknown. Here we show that different stages of cognitive and functional impairment are associated with changes in CSF reactive iron. In this work, we compared CSF samples from 56 elders, classified into 4 groups according to their scores on the Clinical Dementia Rating scale (CDR). Total CSF iron was analyzed by atomic absorption spectrometry. Redox-active iron was analyzed by a novel fluorimetric assay. One-way ANOVA was used to test differences in mean values, and Newman-Keuls Multiple Comparison Test was used for multi group comparisons. No difference in total CSF iron was found between different groups. Significant amounts of redox-active iron were found in CSF and their levels correlated with the extent of cognitive impairment. Redox-active CSF iron levels increased with the degree of cognitive impairment from normal to MCI subjects, while AD patients showed an abrupt decrease to levels close to zero.

Given the relevance of oxidative damage in neurodegeneration, it might be possible to associate the development of cognitive and functional decline with the presence of redox-active iron in the CSF. The decrease in redox-active iron found in AD patients may represent a terminal situation, whereby the central nervous system attempts to minimize iron-associated toxicity.

Keywords: Alzheimer disease, cerebrospinal fluid, iron, oxidative stress

INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia and will reach epidemic proportions if a cure is not found within the next decade. The neuropathological features of AD are a gradual and widespread neuronal loss, extraneuronal amyloid-β (Aβ) deposits or senile plaques (SP), alterations in cerebral blood vessels, and the presence of neurofibrillary tangles mainly composed of hyperphosphorylated tau protein [1]. These neuropathological changes are also found several years before the dementia stage in individuals with mild cognitive impairment (MCI), a fraction of which represents a preclinical stage of AD [2]. Thus, comparisons between subject with MCI and AD are important to understand the possible role and behavior of biochemical markers during the progression
Oxidative stress is a major pathological aspect of several neurodegenerative conditions and results from the generation of large amounts of reactive oxygen species (ROS), which alter the structure of proteins, lipids and nucleic acids, ultimately leading to cell degeneration and death. Several reports have implicated redox-active transition metals such as iron and copper in different neurodegenerative disorders such as AD [3–5] and Parkinson’s disease (PD) [6–8]. High levels of both copper [9] and iron [10] have been found in the blood plasma of people with AD. Iron and copper catalyze the production of reactive oxygen species (ROS) via the Fenton reaction, transforming the mild oxidant hydrogen peroxide into the highly reactive hydroxyl radical [11]. The labile iron pool in blood plasma correlates with the appearance of oxidation products and decreased plasma antioxidant capacity [12]. Neurodegenerative disorders [13–15] and aging [16,17] have been linked with molecular and tissue damage due to metal reactivity.

Alterations between labile and non-labile iron have been suggested to play a pivotal role in metal-evoked oxidative stress [18]. In that respect, iron has been found to accumulate in selected areas of the brain of AD [3,19] and of PD [20] patients, while neurons from Alzheimer brains display properties associated with oxidative stress [21,22]. This information suggests that the etiopathogenesis of AD is associated with a change in the redox balance of selected areas of the brain in which iron shows demonstrable levels of accumulation. However, a causal relationship between oxidative stress and iron demands the presence of redox-active iron in brain cells and/or fluids, and those hitherto have not been demonstrated in neurodegenerative disorders. Previous studies have provided useful tools to evaluate biomarkers in the cerebrospinal fluid (CSF) [23–25]. To address the relationships of iron accumulation in the CSF and AD, we analyzed the levels of total and redox-active iron in CSF from elderly people with different cognitive and functional status from normal to dementia.

**MATERIALS AND METHODS**

**Patients**

Fifty six elderly subjects were included in this study. Participants were recruited through the print media and underwent a multistage screening procedure. To be included in the study, participants needed: (i) to be more than 60 years old; (ii) to be free of significant underlying medical, neurological, or psychiatric illness, (iii) to have a reliable collateral source, and to be willing to participate in the study procedures. The age of the patients was between 65 and 73 years old, with a mean age of $69.7 \pm 5.6$ years. The education level of the entire population was analyzed and data are shown in Table 1. Gender was analyzed and 54% were women and 46% were men. Controls were selected among relatives of similar ages and gender distribution, among those giving informed consent for the patients, and for themselves. Patients were from the residential metropolitan area in Santiago, and corresponded to 41% of Amerindian population and 59% Caucasian ancestors. These subjects underwent a comprehensive medical and neurological examination to ascertain that they were free of any significant medical condition, including brain Magnetic Resonance Imaging or brain Computed Axial Tomography to exclude cerebral vascular lesions. Subjects could be using psychoactive medications, and disabilities and co-morbid illnesses could be present, but the neurologists did not judge that these factors were causing clinically significant cognitive impairments. This study was part of a long-term project on Alzheimer’s disease biomarkers based in the Department of Neurological Sciences, Faculty of Medicine, Universidad de Chile and protocols on clinical and neuropsychological studies as well as sample processing were as indicated in Lavados et al. [23] and Maccioni et al. [25]. All the experimental protocols were approved by the Committee on Ethical Issues of the Faculty of Medicine, University of Chile, and all subjects provided informed consent prior to the initiation of the study. In the cases of demented subjects, the informed consent for participation in the study was also obtained from their caregivers.

**Application of semi-structured interview**

Application of the Clinical Dementia Rating Scale (CDR) ratings were obtained using a Spanish translation of the English version of semi-structured interview specially adapted by Daly et al. [26] from the previously validated original version of Hughes et al. [27], whose details were described [25]. The CDR is a dementia staging instrument used to rate cognitive function and functional state along 5 levels of impairment from none to maximal (rated as 0, 0.5, 1, 2, or 3) in each of 6 domains: memory, orientation, judgment and problem solving, function in community affairs, home
and hobbies, and personal care (Personal care has no 0.5 impairment level). Based on the collateral source and participant interviews, a global CDR score is derived from individual ratings in each domain such that CDR 0 indicates no dementia, CDR 0.5 indicates MCI, and CDR 1, 2, and 3 represent mild, moderate, and severe dementia, respectively. The individual ratings can be totaled to yield the sum boxes, or the total box score (TBS), which is a more quantitative rating that ranges from 0 (no impairment in any of the 6 domains) to 18 (maximal impairment in each of the 6 domains). Into the CDR 0.5 category a higher TBS represent a higher level of impairment

In a preliminary study, we assessed the reliability of our Spanish adaptation of the English version of the semi-structured interview. To that end, the reliability of the instrument was evaluated in a sample of 20 subjects, distributed across a range of cognitive functions from no impairment to mild impairment, by 3 independent raters. The rating of the overall CDR scores and the ratings of the each six CDR domains were analyzed with a kappa index. A very high concordance was obtained. For the overall CDR the kappa was 0.957, and for Memory, Orientation, Judgment, Problem Solving, Community Affairs, Home and Hobbies, and Personal Care this index were 0.924, 0.964, 0.966, 0.929, 0.926 and 1.00 respectively. The CDR ratings were completed with the interviewers blinded to the results of the neuropsychological test. The written interviews were scored by a reviewer who made his own rating judgment, blinded to the interviewer. The interviewers had a weekly consensus conference in order to reach an agreement regarding the rating of each of the CDR subcategories. In this open discussion an explicit reference was made to CDR ratings coded by Daly et al. (2000) to ensure that the final rating of each of the CDR subcategories adhered as closely as possible to the CDR expanded criteria of Daly et al. [26]. According to their overall CDR and TBS scores the subjects were divided into four different groups for this study: 1) 12 Normal Subjects (NS), CDR 0/TBS 0.0; 2) 21 early stage of MCI (m-MCI), CDR 0.5/TBS 0.5–1.5; 3) 10 moderate MCI (M-MCI), CDR 0.5/TBS 1.5–3; and 4) 13 Alzheimer Disease (AD), CDR 1-2/TBS ≥ 6. All demented patients fulfilled the NINCDS-ADRDA diagnostic criteria for Alzheimer’s disease [28].

### Neuropsychological battery of tests

Once the interview was completed and rated, the subjects in the study were given a neuropsychological battery. The neuropsychological evaluation consisted of the Neuropsychological Battery of CERAD that includes Folstein’s MiniMental Test, Verbal Fluency, Boston Nomination Test (15 items), Learning Word List (10 items), and Constructional Praxis. The cognitive assessments were completed with the neurophysiologist blinded to the results of the CDR ratings. Statistical comparisons on age, education and cognitive measures were performed using a 1-way analysis of variance with each measure as the dependent variable comparing the groups. Relevant pair-wise comparisons were made between adjacent groups using Tukey honestly significant difference with a level of significance being set at the 0.05 level.
CSF samples

Samples were obtained by lumbar punctures performed early in the morning. CSF samples were stored in polypropylene tubes without preservative and frozen on dry ice at bedside within minutes of withdrawal. Samples were kept at \(-80^\circ C\). Blood contaminated samples were excluded from this study.

Measurement of total iron

Total iron was determined by atomic absorption spectroscopy. 100 \(\mu l\) of CSF samples were mixed with 100 \(\mu l\) of 5\% ultrapure nitric acid and incubated at 60\% C for 12 hrs. The digest was cooled, centrifuged at 12,000 g for 2 min and the supernatant diluted to 1 mL with 0.2\% ultrapure nitric acid. The final sample was colorless and transparent. Fe contents were determined in an atomic absorption spectrometer with graphite furnace (SIMAA 6100, Perkin Elmer, Shelton CT). MR-CCHEN-002 (\textit{Venus antiqua}) and DOlt-2 (\textit{Dogfish liver}) preparations were used as reference materials to validate the mineral analysis. CSF iron levels were expressed as mean \(\pm\) SEM (standard error of the mean).

Measurement of redox-active iron

Redox-active CSF iron was determined as described for labile plasma iron \cite{29}. Quadruplicates of 20 \(\mu l\) of CSF were transferred to clear-bottom, 96-well plates (Maxisorp 96, Nunc, Rotskilde, Denmark). 180 \(\mu l\) of iron-free HEPES-buffered saline (HBS; HEPES 20 mM, NaCl 150 mM, pH 7.4) containing 40 \(\mu M\) ascorbate and 50 \(\mu M\) DHR (dihydrorhodamine 123, dihydrochloride salt, Biotium, Hayward, CA, USA) was added to two of the wells. 180 \(\mu l\) of the same solution containing 50 \(\mu M\) of the iron chelator deferiprone (L1, Apotex, Weston, Ont., Canada) was added to the other two wells. Immediately following reagent addition the kinetics of fluorescence increase were followed at 37 \(^\circ\) C in a BMG Galaxy Fluostar microplate reader (BMG Lab Instruments, Germany) with a 485/538 nm excitation/emission filter pair, for 40 min, with readings every 2 min. The slopes \(r\) of DHR fluorescence intensity with time were calculated from measurements taken between 15-40 minutes and are given as F.U./min (fluorescence units per min). The duplicate values of \(r\) in the presence and absence of L1, \(r_{L1}\) and \(r\), respectively, were averaged and the labile CSF iron concentration (\(\mu M\)) was determined from calibration curves relating the difference in slopes with and without L1 against Fe concentration: \(\text{LPI} = \Delta r/r_{st} = (r - r_{L1})/r_{st}\), where \(\Delta r\) and \(r_{st}\) denote the L1 sensitive component of \(r\) and the calibration factor relating \(\Delta r\) to the Fe concentration, respectively. Calibration curves were prepared by serial dilutions of Fe:nitrilotriacetate, (1:7, mol:mol) to give final concentrations of 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 \(\mu M\) Fe in HBS buffer containing 0.3 mg/ml bovine serum albumin (Fraction V, Sigma Chem. Co., St. Louis, MO). Quadruplicates of 20 \(\mu L\) of these samples were assayed for labile iron as described above. CSF redox-active levels were expressed as mean \(\pm\) SEM (standard error of the mean).

Statistical analysis

One-way ANOVA was used to test differences in mean values, and Student-Newman-Keuls post-hoc test was used for comparisons (In Stat program from GraphPad).

RESULTS

Cognitive performance

Demographic and neuropsychological data among the 4 groups are displayed in Table 1. No significant differences were found on the average age of groups, while minor differences were observed when compared the AD group with normal subjects. As expected AD patients were significantly impaired on all measures relative to NS. On MMSE, a measure of general cognitive status, there were differences between AD patients and the three others groups, and as expected the MMSE score of the two MCI groups were in the normal range. On delayed word recall, the two MCI groups performed in an intermediate range between NS and AD consistent with their CDR score. In addition the MCI TBS \(>1.5\) group performed more poorly than NS and MCI with TBS \(\leq 1.5\) on constructional praxis, showing the impairment of another cognitive domain in addition of memory, as expected in a group with higher TBS. On measures of language, as verbal fluency and nomination, no differences were found between NS and the MCI groups.
Total CSF iron levels

Analysis of total CSF iron by atomic absorption spectrometry showed a broad range of concentrations from 0.12 µM to 2.00 µM (0.75 ± 0.048, mean ± SEM). No statistic differences were found between total CSF iron and the severity of dementia. Based on the reported CSF concentration for transferrin (Tf) [11] and the values for total iron reported above, Tf in CSF could be sat-

Correlations between labile CSF iron and early and moderate stages of the neurodegenerative process

As shown in Fig. 2, important quantities of CSF reactive iron were found both in normal and cognitive-impaired individuals. NS had redox-active CSF iron of 0.29 ± 0.04 µM; (mean ± SEM) significantly lower that M-MCI individuals (0.46 ± 0.04) (p < 0.05). Data shows an abrupt decrease in redox-active iron in AD patients (0.26 ± 0.05 µM) respect to M-MCI (P < 0.05).

Negative correlation between redox-active CSF iron and cognitive damage in AD patients

Further analysis of redox-active CSF iron levels in AD patients revealed that the degree of cognitive de-cline, quantified by TBS, negatively correlated with redox-active CSF iron (R²: 0.7839), showing an abrupt decrease to levels close to zero during the latest stages of neurodegeneration. (Fig. 3).

DISCUSSION

Analysis of total CSF iron by atomic absorption spectrometry showed a broad range of concentrations (0.75 ± 0.048 µM, mean ± SEM). The source of this variation is unknown, but it could be due to dietary habits, environmental conditions or genetic factors. No statistic differences were found between total CSF iron and the severity of dementia. Based on the reported CSF concentration for transferrin (Tf) [11] and the values for total iron reported above, Tf in CSF could be sat-

Values of redox-active CSF iron were plotted against the Total Box score of patients diagnosed as AD (n = 13). A strong negative correlation (R²: 0.7839) was found. The 95% confidence interval for the slope was −0.1057 to −0.05110.
High Redox-active CSF Iron levels → Brain damage

Increase in Aβ soluble peptide → Cognitive impairments

Amyloid plaque formation and Fe sequestration

Low Redox-active CSF Iron levels → Alzheimer disease

Fig. 4. Schematic representation of a possible mechanism for redox active CSF iron changes. High levels of redox active CSF iron could mediate brain toxicity generating cognitive impairments. As a homeostatic mechanism, those high levels could enhance the expression of AβPP, thus augmenting the concentration of Aβ in the CSF which could interact with Fe generating the senile plaques and diminishing the levels of redox active CSF iron and inhibiting its toxicity. According to this model, final stages of AD are characterized by high cognitive impairment, presence of Fe enriched senile plaques and low redox active CSF iron concentration.

urated and reactive iron could be present. Thus, we analyzed redox-active CSF iron of the different groups under study. Important quantities of CSF reactive iron were found both in normal and cognitive-impaired individuals. Values of redox-active iron found in normal subjects (0.27 ± 0.04 µM) agree with a previously reported value of 0.55 ± 0.27 µM, determined using a bleomycin-based assay [11]. In a paired-sample analysis we found no correlation between total CSF iron and redox-active iron in m-MCI, M-MCI or AD patients (data not shown). Hence, contrary to intuition, total and redox-active CSF iron pools seem to be independently regulated. It was nevertheless surprising to find such large amounts of redox-active iron in CSF since it is essentially absent from sera of healthy individuals [29]. It is possible that the presence of redox-active iron is due to the highly reductive property of CSF contributed by ascorbate concentrations of approximately 160 µM [30]. Thus, the predominant Fe species in CSF is the Fenton-active Fe^{2+} form, to which Tf does not have high affinity [31]. Moreover, Fe^{2+} is readily available for direct incorporation into the cell by the Fe^{2+} transporter DMT1 [32,33] in a process known as non-transferrin bound iron uptake. Thus, hippocampal neurons that express DMT1 [32] should be particularly susceptible to iron accumulation. Redox-active CSF iron increased in initial and middle stages of cognitive impairment. This increased redox-active CSF iron could promote neuronal damage in several ways. Redox-active CSF iron could act together with Aβ peptide to induce oxidative stress in an oxidative redox cycle [34]. Increased redox-active CSF iron should also result in increased cell iron accumulation, with an outcome of increased oxidative stress and oxidative damage [8,35]. Indeed, both iron accumulation and oxidative damage has been reported in hippocampus and substantia nigra pars compacta neurons from AD and PD patients [3,17,36]. Moreover, iron available for non-transferrin bound iron uptake could affect glial cells that play a key role in many physiological and pathological process of the central nervous system [37–39]. Thus, increased redox-active CSF iron should result in a series of events leading to deterioration of the redox capacity of cells and to neurodegeneration. An abrupt decrease in redox-active iron was found in AD disease (0.26 ± 0.05 µM). In this group, redox-active iron was significantly lower than in m-MCI and M-MCI. This result was contrary to expected, given the mild increase in total iron found in AD patients. Further analysis of redox-active CSF iron levels in AD patients revealed that the degree of cognitive decline, quantified by TBS, negatively correlated with redox-active CSF iron (R^2: 0.7839). These results indicate that development of the dementia stage of AD is accompanied by a progressive decline in redox-active CSF iron. This decrease suggests the induction of a mechanism to reduce reactive iron and its associated toxicity. A possible mechanism to explain the decrease of Redox-active CSF iron during the course of the disease could be the sequestration of this metal by the aggregation of Aβ peptides into the senile plaques, where 1 µM Fe has been found [40]. High redox-active CSF iron levels could increase the production of amyloid-β protein precursor (AβPP) [41] and the soluble form of AβPP (Aβ peptide) could act as an iron chelator promoting Aβ peptides aggregation into the senile plaques [42–44]. This mechanism is someway similar to the proposed “entombment” of toxic Aβ peptide into the senile plaques [45]. The suggested mechanism could represent a homeostatic process to regulate the CSF iron levels and its toxicity. Our observations suggest that the decrease in redox-active CSF iron may correspond to a final late effort by brain cells to decrease Fe-mediated oxidative stress. Our suggested interpretation of data is summarized in Fig. 4.
In summary, total CSF iron was not different in controls and cognitively impaired subject in accordance with previous reports from Molina et al. [46]. On the other hand, nor redox-active CSF iron was found to be correlated with cognitive impairment in preclinical stages of the neurodegenerative process associated with AD, while at the most advanced stages of the disease there was a profound drop in this iron. These findings suggest that redox-active CSF iron should not only be considered a risk factor in the development of cognitive disorders, but as a potential biomarker for early AD. To the extent that reactive CSF iron is present in AD and is involved in biological damage, one might advocate application of appropriate iron chelators [44] as a means to reduce progression of this and other neurodegenerative disorders.

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References


