Oxidative imbalance and cathepsin D changes as peripheral blood biomarkers of Alzheimer disease: A pilot study

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Abstract Markers of oxidative stress in peripheral blood from patients with Alzheimer disease (AD) were analyzed. Thirty-three AD patients were recruited. Plasma antioxidant power (AOP), plasma Cystatin C as well as Cathepsin D in PBL were evaluated. We found that the AOP levels were significantly decreased in AD patients if compared to healthy donors, while the plasma level of Cystatin C was significantly higher. Importantly, a significantly decreased expression of Cathepsin D in PBL was also observed. These results suggest that oxidative imbalance in the peripheral blood of AD patients could mirror oxidative changes previously described in the central nervous system.

Keywords: Alzheimer disease; Blood antioxidant power; Cathepsin D; Cystatin C; β-Amyloid precursor protein; Platelet; Peripheral blood lymphocyte

1. Introduction

Oxidative stress, resulting in excessive production of reactive oxygen species (ROS), is related to a variety of neuronal disorders caused by cerebrovascular injury, neuropathology and aging. One theory on the pathogenesis of Alzheimer's disease (AD) postulates that neurodegeneration is the result of oxidative stress and damage to vulnerable cerebral tissues [31]. The oxidative stress hypothesis in AD has been further strengthened by demonstration of (i) increased levels of trace elements such as iron, mercury and aluminum, which are potent catalysts for free radical generation in the brain of AD patients and (ii) beneficial effects of antioxidant treatment with α-tocopherol or selegiline in AD patients [14].

Signs of oxidative stress in AD are concomitant with β-amyloid deposition. This is a metalloprotein electrochemically active that binds copper and converts molecular oxygen into hydrogen peroxide by reducing copper or iron leading to Fenton chemistry. Hydrogen peroxide is a freely permeable pro-oxidant that may be responsible for many of the oxidative adducts that form in the Alzheimer-affected brain [26]. Moreover, in some neuropathological studies, markers of protein oxidation, DNA (mainly mitochondrial) oxidation and lipid peroxidation have been described, suggesting the occurrence of an oxidative stress in AD [9]. However, the co-occurrence of a systemic oxidative stress with brain oxidative stress during AD remains hypothetical. A major limitation in assessing systemic oxidative stress by examining markers in the peripheral circulation is that it gives a global assessment, with no indication as to the actual site of the oxidative stress. A confounding factor in the systemic oxidative stress evaluation is that aging itself seems to be associated with increased oxidative imbalance [2,43].

The aim of this work was to compare the oxidative stress markers in plasma, peripheral blood lymphocytes (PBL) and platelets from AD patients with age matched control subjects, in order to evaluate if the oxidative stress was not restricted to the brain but could also be recognized in the peripheral circulation. The following parameters were considered: (i) plasmatic antioxidant power (AOP), by evaluating Cu^{++} reduction on behalf of all present antioxidants, and (ii) plasma concentration of Cystatin C, a cathepsin inhibitor that was found to be co-localized with β-amyloid peptide [48]. Moreover, peripheral lymphocytes and platelets were also considered. In particular, (iii) concentration of Cathepsin D, an aspartyl protease, in PBL and (iv) the concentration of amyloid precursor protein A4 (APP) in platelets and PBL were evaluated.

2. Materials and methods

2.1. Subjects

We recruited 33 patients with Alzheimer disease (AD) according to the NINCDS-ADRDA criteria [32] and 33 healthy donors (HD) from the Day Hospital of the Department of Geriatrics, Gerontology and Physiatry of the Catholic University of Rome (Italy). Clinical classification of AD was determined according to the Diagnostic and

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Abbreviations: AD, Alzheimer disease; α-Fas, anti-human Fas IgM monoclonal antibody; AOP, plasma antioxidant power; APP, amyloid precursor protein A4; CRAOP, Cu^{+}-reducing antioxidant power; CT, cranial tomography; FT, fronto-temporal dementia; HD, healthy donors; IL-2, interleukin 2; IVM, intensified video microscopy; MCI, mild cognitive impairment; MMSE, mini mental state examination; MRI, magnetic resonance imaging; NAP, non-Alzheimer patients; PBL, peripheral blood lymphocytes; PD, Parkinson disease; PHA, phytohemoagglutinin; ROS, reactive oxygen species; SE, standard error

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Statistical Manual of Mental Disorders and the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria [38]. Healthy donors were recruited from subjects who underwent a day-hospital medical screening in order to follow a fitness program. A low number of additional patients ($n = 10$) displaying prodromal symptoms of dementia (apathy, disinhibition, irritability and aggressiveness) were also considered. These were diagnosed as patients with Parkinson Disease (PD, 4 patients), Frontotemporal dementia (2 patients), following the guidelines designed elsewhere McKhann et al. [33] or a neurodegenerative disorder characterized by initial symptoms resembling those of AD, i.e. mild cognitive impairment (MCI, diagnosed after a follow-up of at least 2.0 years, 4 patients). These patients were indicated as non-Alzheimer patients (NAP).

All AD, HD and NAP groups were matched for age and sex. A comprehensive physical examination, laboratory blood testing were undertaken for all subjects; CT scan or MRI were carried out in AD and NAP. No subject from the three groups was suffering from neurological or major medical diseases, including diabetes, except for moderate increment of blood pressure (140 ± 10/80 ± 5 mm Hg) or serum cholesterol (200 ± 20 mg/100 ml). No subject was malnourished or had medical history positive for psychiatric disorders, drug or alcohol abuse or brain injury. All AD patients received stable doses of cholinesterase inhibitors. All patients and normal donors had laboratory analysis of serum albumin, cholesterol, platelets and total level of Cu. No significant difference was however found among the different groups for what concerns laboratory parameters (see Table 1).

The nature and purpose of study was explained to all participants. The ethical committee of the Catholic University proved the study.

2.2. Sample collection

The sampling protocol was designed to minimize oxidation after collection. Venous blood (20 ml) was collected from subjects in heparinized tubes, maintained at room temperature throughout preparation.

2.2.1. Plasma samples. For plasma isolation, blood was centrifuged at $3000 \times g$ for 10 min at room temperature. Plasma was removed, aliquoted and frozen until analyses.

2.2.2. Platelet isolation. Platelet-rich plasma was prepared by centrifugation of whole blood at $100 \times g$ for 15 min at room temperature. The platelet-rich plasma supernatant was carefully removed and was used to isolate platelets by stepwise centrifugation as previously described [39].

2.2.3. Peripheral blood lymphocytes isolation. Human PBL from AD patients, NAP patients and HD were isolated from freshly heparinized blood through a Ficoll–Hypaque density gradient centrifugation and washed three times in phosphate-buffer saline (PBS), pH 7.4 (Lympholyte-H, Cedarlane Laboratories, Hornby, Ont., Canada). PBL were subcultured in 25 or 75 cm^2 Falcon plastic flasks at a density of approximately 1×10^6 cells/ml in RPMI 1640 (GIBCO-BRL, Life Technologies Milan, Italy) containing 15% fetal calf serum (Flow Laboratories, Irvine, Scotland), 1% non-essential aminoacids, 5 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37 °C in a humidified 5% CO^2 atmosphere. For apoptosis susceptibility analyses PBL were cultured for 72 h with phytohemoagglutinin (PHA 2 μg/ml, Boeringher Manneheim, Milan, Italy) and interleukin 2 (IL-2, 60 IU/ml, GIBCO) and than triggered for 48 h with 500 ng/ml of an anti-human Fas IgM monoclonal antibody (α-Fas, clone CH11, Upstate Biotechnology, Lake Placid, NY, USA).

Table 1
Main private and clinical data of AD patients and healthy donors

	AD patients	Healthy donors
Male/female	15/18	16/17
Age (years)	77.28 ± 4.06	74.82 ± 5.11
MMS E	19.22 ± 5.10	⩾ 26
Blood pressure (mm Hg)	140 ± 10/80 ± 5	130 ± 10/80 ± 5
Total cholesterol (mg/100 ml)	200 ± 20	190 ± 10
LDL cholesterol (mg/dl)	146.17 ± 32.67	130.71 ± 42.19
HDL cholesterol (mg/dl)	54.75 ± 33.81	49.87 ± 15.22
Total Cu (μg/100 ml)	60–160	60–160
Albumin (g/l)	4.02 ± 0.427	4.17 ± 0.78
Platelets (10^9/l)	235 ± 59.95	233 ± 62.59

2.3. Biochemical analyses in the blood plasma

2.3.1. Plasma antioxidant power. The evaluation of the plasma AOP was obtained by measuring the Cu^+-reducing antioxidant power (CRAOP) by means of a specific spectrophometric test (MED.DIA, Vercelli, Italy). This method provides a quantitative measurement of the antioxidant capability of a biological fluid, such as plasma, due to the non-enzymatic antioxidant system. To this system belong liposoluble (the more important is vitamin E) and water-soluble antioxidants (uric acid, vitamin C, bilirubin, reduced thiols and glutathione). Since the different diet followed by the patients can represent an important variable, e.g. for certain antioxidants such as vitamin C, the AOP was carried out by taking into account the action of the whole antioxidants present in the plasma. The antioxidant capability of our samples was obtained by evaluating Cu^+ derived by the reduction of Cu^{++}, which was added at known concentrations either to standard or to experimental samples. Cu^+ forms a stable complex with bathocuproine, which has a typical absorption to 480–490 nm and it is thus revealable by a spectrophotometer. For these reasons we selected healthy donors and pathological subjects with comparable amount of plasmatic Cu (see Table 1). Values detected in experimental samples were compared with the curve obtained from standard samples at known concentrations of uric acid used as typical riducent and expressed as μM [46,50]. To verify the results obtained by CRAOP we also indirectly measured the AOP of plasma by a spectrophotometric assay as reported by Valkonen and Kuusi [49]. In this assay we used H_2O_2 as oxidant compound and DCFH-DA as oxidable substrate. The oxidation of DCFH-DA by standard doses of H_2O_2 (3, 5, 10, 25, 50, 100 μM) converts it to dichlorofluorescein (DCF), which is highly fluorescent (Ex 480 nm, Em 526 nm) and also has absorbance at 504 nm. Following this protocol, the AOP of our plasma samples was negatively correlated with the absorbance values obtained. Importantly, results obtained by using this method, completely independent from the level of Cu in the plasma, overlapped those obtained by CRAOP. For this reason only these latter will be shown herein.

2.3.2. Cystatin C detection. Evaluation of Cystatin C in plasma of AD patients and HD was performed with a specific kit from BioVendor Laboratory Medicine (Brno, Czech Republic) by using 96 multi-wells. According to the manufacturer instructions standards and plasma samples from AD patients and HD were incubated for 2 h in microtitration wells coated with anti-human Cystatin C antibody. After washings, anti-human Cystatin C antibody labeled with horseradish peroxidase (HRP) was added to the wells and incubated for 2 additional hours with the immobilized antibody-Cystatin C complex. Following washing step, the remaining HRP-conjugated antibody was allowed to react with the substrate tetramethylbenzidine. The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product was measured spectrophotometrically at 450 nm. Standard curve was constructed by plotting absorbance values versus Cystatin C concentrations of the standards. Thus, concentrations of our samples were determined using this standard curves and reported as ng/ml.

2.4. Analytical cytology

2.4.1. Apoptosis evaluation. Quantitative evaluation of apoptosis was performed by the double staining with FITC-conjugated annexin V/propidium iodide (PI) by using apoptosis detection kit (Eppendorf, Milan, Italy), which allows discrimination between early apoptotic (annexin V single positive), late apoptotic (annexin V/PI double positive) and necrotic (single PI positive) cells.

2.4.2. Analyses of redox balance in freshly isolated lymphocytes. Immediately after isolation from peripheral blood, lymphocytes (5×10^5 cells) from AD, NAP and HD were incubated in 495 l of Hanks' balanced salt solution (HBSS, pH 7.4) containing 10 μM dihydrorhodamine 123 (DHR 123, Molecular Probes) or 1 μM dihydroethidium (DHE, Molecular Probes) in polypropylene test tubes for 15 min at 37 °C. DHE is a chemically reduced ethidium derivative. It is a non-fluorescent membrane-permeable dye that can be oxidized directly to the red fluorescent ethidium bromide by O_2 generated inside the cells after different treatments. DHR 123 is a dye freely diffusing into cells, oxidized primarily by H_2O_2 in a myeloperoxidase-dependent reaction to green fluorescence. Intracellular content of reduced thiols was explored by using 10 μM 5-chloromethyl-2′,7′-dichloro-dihydrofluoresceindiacetate (CM-H_2DCFDA, Molecular Probes). Cells exposed to the GSH depleting drug L-buthionine-[S,R]-sulfoximine 7.5 mM (BSO, Sigma) for 16 h were considered as negative controls. Samples were then analyzed with a FACScan flow cytometer (Becton