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Features of ceruloplasmin in the cerebrospinal fluid of Alzheimer's disease patients

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Abstract The level of the apo-form of the copper enzyme ceruloplasmin (CP) is an established peripheral marker in diseases associated with copper imbalance. In view of the proposal that disturbances of copper homeostasis may contribute to neurodegeneration associated with Alzheimer's disease (AD), the present work investigates, by Western blot and non-reducing SDS-PAGE followed by activity staining, the features of CP protein, and the copper/CP relationship in cerebrospinal fluid (CSF) and serum of AD patients. Results show that only a fraction of total copper is associated with CP in the CSF, at variance with serum, both in affected and in healthy individuals. Furthermore, a conspicuous amount of apo-ceruloplasmin and a decrease of CP oxidase activity characterize the CSF of the affected individuals, and confirm that an impairment of copper metabolism occurs in their central nervous system. In the CSF of AD patients the decrease of active CP, associated with the increase in the pool of copper not sequestered by this protein, may play a role in the neurodegenerative process.

Keywords Alzheimer's disease · Copper · Ceruloplasmin · Serum · Cerebrospinal fluid

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Introduction

In the latest years consistent evidence has been obtained on the involvement of the dysmetabolism of the transition metal copper in the pathogenesis of Alzheimer's disease (AD) (Maynard et al. 2005; Pajonk et al. 2005).

In diseases related to altered copper metabolism, the peripheral molecular marker is the serum copper protein ceruloplasmin (CP) (Walshe 2003) that carries about 95% of serum copper. CP is a single chain glycoprotein of 132 kDa, containing six atoms of copper. The copper sites are essential for the oxidase activity of CP, with Fe(II) being the main substrate; but also aromatic amines such as catecholamines are substrates of CP (Bielli and Calabrese 2002; Hellmann and Gitlin 2002). Serum CP is provided by the liver but extra-hepatic CP gene expression is well documented in various tissues, including the epithelium of the choroid plexus (Aldred et al. 1987; Klomp et al. 1996).

Lesions of the CP gene, leading to aceruloplasminemia (Yoshida et al. 1995), or impairment of copper transfer to CP as in Wilson's disease, are

associated with oxidative damage and extensive degeneration of neural tissue (Miyajima et al. 2002). In addition, it has recently been reported that mutations of CP are associated with Parkinson's disease (Hochstrasser et al. 2005), thus reinforcing the concept that a lower CP activity is associated with neurodegeneration.

CP is routinely measured in clinical chemistry as the immunoreactive protein. Since this may comprise both the holo-CP and the copper-free protein, this type of analysis does not give unequivocal information on the copper content of the sample. Indeed, the need of evaluating CP enzymatic activity, which better describes the copper/CP relationship, has emerged as a topical issue for Wilson's disease (Walshe 2003). Despite the notion that cerebrospinal fluid (CSF) is a reliable and easily available mirror of the brain situation, no comprehensive study has been performed on the copper/CP relationship in AD CSF. The biochemical characterization of CP in AD has become important because indications of a lack of statistical correlation between serum copper content and immunoreactive CP have been obtained recently (Squitti et al. 2006).

The present work investigates the features of CP protein and the copper/CP relationship in CSF in comparison with serum of AD patients and age-matched control individuals. We combined copper content determinations with electrophoretic analyses of CP, to detect different forms of the protein and evaluate their enzymatic activities. A considerable amount of copper not accounted for by the immunoreactive CP level was found in CSF and a higher amount of inactive CP was detected in the CSF of AD patients. These results represent the first characterization of CP in the CSF and in AD, and support the hypothesis that an alteration of copper distribution in the central nervous system may occur in AD.

Materials and methods

Patients and controls

Ten AD patients and 10 sex- and age-matched control individuals were investigated in the current study. They were characterized as described (Squitti et al. 2006). They do not differ for the mean age and sex (see Table 1). Neither in the group of control nor in the AD group there was any family history of neurodegenerative disease. The control group consisted of individuals with no clinical evidence of neurological or psychiatric disease. The AD patient sample consisted of individuals with a diagnosis of probable AD (NINCDS-ADRDA criteria), and a Mini-Mental State Examination (MMSE) score of 25 or less (Table 1). AD patients underwent neurological, neuroimaging (brain MRI) and extensive neuropsychological evaluation, as well as routine laboratory tests. No evidence of blood brain barrier dysfunction was detected in these individuals (see Squitti et al. 2006 for details). The study was approved by the institution review board of the Fatebenefratelli Hospital, Isola Tiberina, Rome, Italy and all participants or their legal guardians signed an informed consent.

Table 1 Characteristics of the investigated groups

	AD patients	Controls
No. of subjects	10	10
Sex (male/female)	3/7	3/7
Age (years)	73.2 ± 8	74.6 ± 7.5
MMSE	16.9 ± 5.6	28.4 ± 0.9

Serum and cerebrospinal fluid (CSF) specimens

Serum samples and CSF samples obtained by lumbar puncture from fasting individuals were collected and immediately centrifuged, and the supernatants were rapidly frozen in liquid nitrogen and immediately stored at −80°C until analysis. Protein content was assayed according to Lowry et al. (1951).

Electrophoretic assays

Western blot analysis

Fully denatured samples were applied to 12% SDS-PAGE, followed by blotting on Protean nitrocellulose transfer membranes (Schleicher and Schuell, Dassel, Germany). Anti-human CP polyclonal antibody was obtained from Sigma (St. Louis, MO). Protein-antibody complexes were identified by the Super Signal Chemiluminescent Substrate (Pierce, Rockford, IL).

Non reducing SDS-PAGE

Samples were applied to 7.5% polyacrylamide gels, containing SDS (0.1%), without prior heating or reduction. Under these conditions, apo- and holo-CP show distinct electrophoretic mobilities (Hellman et al. 2002; Sato and Gitlin 1991). Throughout electrophoresis, the gel was maintained around 20°C. At the end of the run, the gel was either equilibrated with acetate buffer (pH 5.4) and then incubated with *o*-dianisidine (Sigma), substrate of the enzyme (Schosinsky et al. 1974), in order to detect active CP or, after treatment for 10 min at 90°C, processed for Western blotting, to detect the immunoreactive apo- or holo-protein. A CP standard was purified from human plasma and characterized as described (Musci et al. 1993).

Control of gel loading was performed by Coomassie staining, while control of transfer was carried out by staining the membrane with Ponceau Red.

The densitometric analyses of blots was performed by a computerized image processing system (Gel-Pro Analyzer).

Copper measurement

Samples of CSF or serum were added to an equal volume of 65% nitric acid. After at least 1 week at room temperature, copper content was assayed by atomic absorption spectrometry using an Analyst 300 Perkin Elmer instrument, equipped with a graphite furnace with platform (HGA800) and an AS-72 autosampler.

Statistic analysis

Data are expressed as mean ± S.D. and were analysed by the Student's *t* test for significance.

Results

The AD and control groups analysed don't differ for mean age (73.2 ± 8 vs 74.6 ± 7.5) and sex composition (three males and seven females for both groups), as shown in Table 1.

Copper concentration of CSF, as evaluated by atomic absorption spectrometry, was in average 0.39 ± 0.11 μM in AD samples ($n = 10$) and 0.52 ± 0.14 μM in controls ($n = 10$), showing no significant difference. The level of copper in this patients is close to the value obtained in other studies (Molina et al. 1998; Melo et al. 2003; Squitti et al. 2006).

Figure 1a shows a representative Western blot analysis, performed following complete denaturation of CSF and serum samples from control and AD individuals. The same amount of total protein was applied to each lane; since the total protein content of the CSF samples was about two order of magnitude lower than that of serum, this had to be diluted. The electrophoretic pattern of immunoreactive CP in CSF samples was analogous to that seen in serum, considering that the 132 kDa band, attributable to the intact polypeptide chain of the soluble form of CP, was evident in all samples.

Densitometric analyses of the bands allowed the calculation of the concentration of the immunoreactive protein in each sample, to be correlated with the copper content. On a molar basis, the ratio of CSF copper to CSF immunoreactive CP gave values

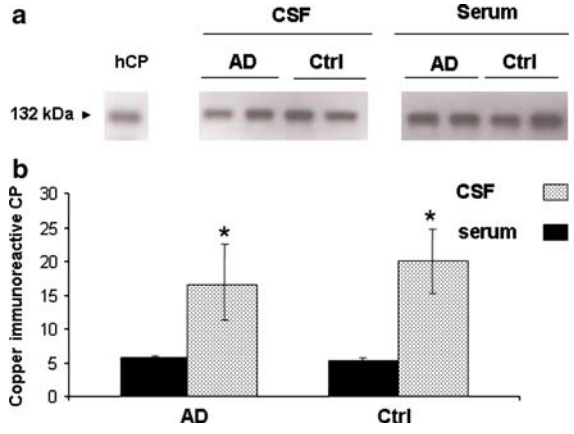

Fig. 1 *Copper/ceruloplasmin ratio is higher in CSF than in serum*. (**a**) Representative Western blot analyses of serum or CSF samples of two AD patients and two age-matched controls. Ten microgram protein was applied to each lane. Purified human ceruloplasmin (45 ng) was used as a standard. (**b**) Ratio between copper and CP. The concentration of CP (μM) was obtained by the densitometric analyses of bands with reference to purified human ceruloplasmin (hCP). Copper concentration (μM) was obtained by atomic absorption spectrometry analysis of serum or CSF samples. Data are the means (±S.D.) of the densitometric analysis of at least three electrophoreses performed on 10 samples for each group of individuals. $*P < 0.001$

around 15–20 for both AD and control subjects (Fig. 1b, 16.5 ± 5.6 for AD and 20.1 ± 4.8 for controls), at variance with serum samples, where a ratio of about 6 (5.7 ± 0.3 for AD and 5.3 ± 0.4 for controls) was always found, consistent with the notion that serum CP binds six copper atoms and carries 95% of copper in plasma.

It should be pointed out that the 132 kDa band of the fully denatured protein visible in Figure 1a may represent a pool of the two forms of the protein, the copper-free apo-CP and the copper-containing holo-CP. Due to conformational differences induced by copper binding, these can be separated under non-reducing SDS-PAGE without previous heat denaturation of the sample (Hellmann et al. 2002). In following immunoblot analysis, the copper-free apo-protein gives rise to a 132 kDa band, while holo-CP to an 80 kDa band; after activity staining, the oxidase-active band appears at 80 kDa. In Fig. 2 are shown the results obtained by non-reducing SDS-PAGE of serum or CSF samples. The immunoblot analysis of serum samples (Fig. 2a) reveals a pattern invariably characterized by the presence of 80–90% holo-CP for both AD patients and healthy subjects, consistent with the notion that only about 10% of circulating CP exists as the apo-protein (Bielli and Calabrese 2002; Hellmann and Gitlin 2002). As shown in Fig. 2c, the activity staining detects only the holo-CP form in serum; it appears as a strong band that matches the intensity of that of same species found by the immunoblot analysis (the 80 kDa immunoreactive protein) both in AD and in controls. The immunoblot analysis of CSF samples (Fig. 2b) reveals findings similar to serum only for controls, but outlines a different ratio of holo- to apo-CP in AD CSF, due to the presence of a conspicuous amount of apo-CP. The oxidase-active band is barely visible in these samples (Fig. 2d), revealing that holo-CP of CSF of AD patients is less active than that of healthy individuals.

On the basis of the densitometric data, it can be calculated that the immunoreactive 80 kDa protein of the AD CSF samples accounts for about 70% that of controls, while the oxidase-active band accounts for only 30% of that of control CSF samples (Fig. 3). This means that only about 50% of the immunoreactive 80 kDa CP is active in AD CSF.

Discussion

This study shows that only about 35% of total copper exists in association with CP in the CSF. The imbalance of copper to CP in CSF indicates that CP is not the major copper sequestering protein in this compartment, suggesting that copper is bound to either low molecular weight compounds or to other copper-binding proteins. Several protein candidates may be proposed to bind copper in CSF, like peptidyl-glycine α-amidating monooxygenase (PAM) (Wand et al. 1987), Cu, Zn superoxide dismutase (both SOD1 and SOD3) (Jacobsson et al. 2001), the Aβ polypeptide (Squitti et al. 2006).

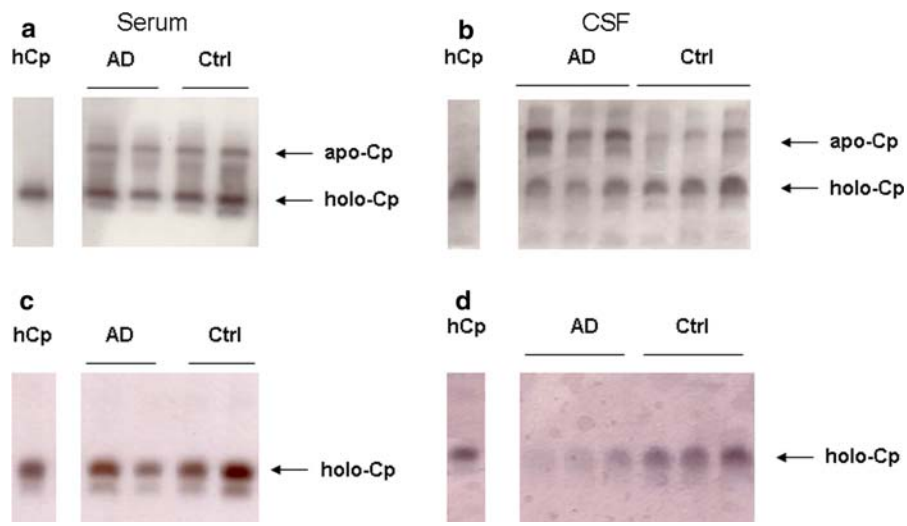

Fig. 2 *CSF samples of AD patients show lower amount of active ceruloplasmin*. Serum (**a** and **c**) or CSF samples (**b** and **d**) underwent non-reducing SDS-PAGE. (**a**) and (**b**) Western blot analyses. (**c**) and (**d**) activity staining by *o*-dianisidine. One representative analysis is shown. Ten microgram protein was applied to each lane. Purified human ceruloplasmin (hCP) (45 ng) was used as standard

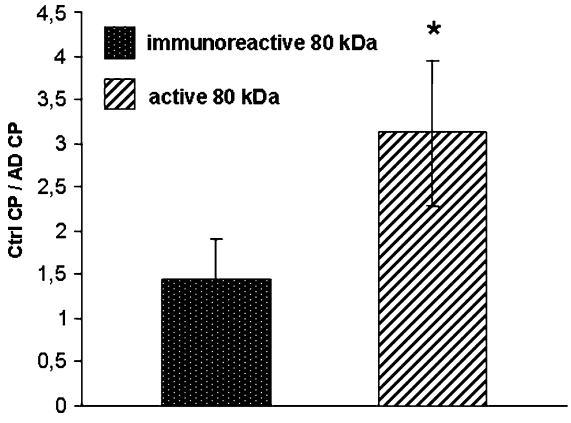

Fig. 3 *Oxidase-active ceruloplasmin is lower than the immunoreactive holo-protein in the CSF of AD subjects*. Average densitometric data of the 80 kDa bands of Western blotting following SDS PAGE under non-reducing conditions of control samples were divided by those of AD samples. The same procedure was applied to the densitometric data of the bands appearing after activity staining. Data are the means (±S.D.) of the densitometric analysis performed on at least three electrophoreses. Ten samples for controls and 10 for AD patients were analysed. $*P < 0.001$

However, due to their low amount in CSF (in the nM range or less), none of them can represent the major copper-binding protein element. Of note, the concentration of some copper-binding proteins such as PAM and Aβ have been reported to decrease in AD CSF (Wand et al. 1987; Squitti et al. 2006). On the contrary, albumin, which can bind exchangeable copper ion at its amino terminus (Linder and Hazegh-Azam 1996) and is present at the concentration of 4 μM (Aldred et al. 1995), might be a reasonable candidate for binding copper in the CSF.

While CSF CP is nearly 90% holo-protein and oxidase-active in healthy individuals, a lower amount of active CP is found in AD. The consequence is that the ratio copper/CP should be even higher in AD. Our results reinforce the belief that in diseases related to the alteration of copper homeostasis it is more relevant to assess the active form of CP as a reliable marker of copper status (Walshe 2003). The oxidase activity of CP is considered of utmost importance for iron metabolism and oxidative stress defense mechanisms in the brain; elevated iron content and lipid peroxidation have been detected in CSF of aceruloplasminemic patients and correlated to the severity of the disease, in particular to memory disturbance (Miyajima et al. 1998).

The finding that CP is partially present as apoprotein devoid of copper in AD CSF suggests that an impairment of copper distribution occurs in this pathology. In serum, the concentration of apo- and holo-protein is determined both by copper availability and by the differences in the half lives of the two protein forms. The six integral copper atoms are incorporated into CP during its biosynthesis through the copper-transporting ATPase ATP7B in the secretory compartment of liver cells (Terada et al. 1998). In Wilson's disease, the absence or impairment of ATP7B prevents copper translocation to the secretory pathway, resulting in the secretion of apo-CP (Terada et al. 1998; Shim and Harris 2003) that being unstable is rapidly degraded in the blood (Shim and Harris 2003). In healthy conditions, only about 10% of circulating CP exists as the apo-protein. In the present study, the finding of a very low apo- to holo-CP ratio in AD serum, similar to that of the control group, demonstrates that ATP7B should not be impaired in AD, at least in liver cells.

Given that serum CP does not cross undamaged blood brain barrier, the decreased amount of holo-CP in AD CSF could be due to an alteration of copper distribution in the epithelium cells of choroid plexus structures, which also synthesize CP (Aldred et al. 1987; Klomp et al. 1996), as a consequence of the impairment of copper chaperone(s) and/or of copper-ATPases. Indeed, impairment of synthesis, secretory and transportation functions of the choroid plexus is documented in aging, and worsens in AD (Serot et al. 2003). Another copper-transporting ATPase, ATP7A, which is homologous to ATP7B, is present in the choroid plexus and it is thought to control the overall copper supply to the brain (Iwase et al. 1996; Qian et al. 1998). Therefore, ATP7A may be affected in AD choroids plexus, thus resulting in decreased activity of secreted copper proteins, needs to be ascertained. The finding that holo-CP fraction in the CSF of AD patients seems not to be as active as in controls also requires further studies, for the identification of the causal mechanism.

In conclusion, the present study, though performed on a very small population, indicates that in CSF at difference with serum CP is not the major copper-binding protein. Furthermore, by finding that CP is less active in AD CSF, this work highlights the relevance of measuring CP activity rather than protein amount and supports the hypothesis that an

alteration of copper homeostasis occurs in AD central nervous system.

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