Research Report

Alzheimer disease: correlation of cerebro-spinal fluid and brain ubiquitin levels

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Abstract

Neurofibrillary degeneration is one of the histopathological hallmarks of Alzheimer disease (AD). Previous studies have shown an association of ubiquitin with the cytoskeletal protein pathology in AD. In the present study, we report (i) the measurement of ubiquitin levels in cerebrospinal fluid (CSF) from histopathologically confirmed AD and control cases, using a new rapid immunoassay, the competitive enzyme-linked immunoflow assay (CELIFA), (ii) the determination of ubiquitin levels in brain tissue taken from the same cases, using a competitive enzyme-linked immunosorbent assay (ELISA), and (iii) an evaluation of the correlation between levels of ubiquitin in CSF and in brain tissue. Ubiquitin levels in CSF of AD and neurological control groups are significantly higher than those of non-neurological aged controls. Ubiquitin levels in brain homogenates of the AD group are significantly higher than those of both non-neurological aged and neurological control groups. The source of this increase in brain ubiquitin in AD is the particulate fraction, because ubiquitin levels in the brain cytosol fraction are the same among the three groups. In AD and non-neurological aged controls, there is a significant positive correlation between ubiquitin levels in CSF and in homogenate of cerebral white matter. In contrast, the correlation in the non-AD neurological control group has a negative tendency. These studies suggest that in AD, elevated levels of ubiquitin in the CSF reflect the increased amount of the protein in the brain and, therefore, can serve as a biomarker of the neuropathology in this disease.

Key words: Alzheimer's disease; Biomarker; Ubiquitin; Cerebrospinal fluid; Enzyme-linked immunosorbent assay; ELISA; Competitive enzyme-linked immunoflow assay; CELIFA

1. Introduction

Alzheimer disease (AD) is a neurodegenerative disorder with progressive dementia. It is the most common cause of dementia in middle-aged and elderly individuals. To date, neither the etiology nor the pathogenesis of this disease is known. Moreover, definitive diagnosis of AD can be made only by histopathological examination of brain tissue.

The two histopathologic hallmarks of AD are the neurofibrillary changes of paired helical filaments (PHFs) and the neuritic (senile) plaques of dystrophic neurites surrounding either a core or wisps of extracellular amyloid. Thus, amyloid and PHF-associated pro-

teins can serve as markers of AD. The biochemical composition of PHF includes abnormally phosphorylated tau as the major protein subunit [12–14,18,20] and, as a minor component (unpublished results), ubiquitin [15,27,31]. Ubiquitin has also been observed in various intermediate filamentous inclusion bodies specific for a number of neurological diseases [11,21]. In the non-demented aged brain granular deposits of ubiquitin reactivity have been observed in degenerated neurites in the cerebral cortex and in white matter myelin [7,29,38]. The 39–43 amino acid amyloid β protein, the A β protein, which is the major polypeptide of amyloid in senile plaques [26], is a fragment of a large transmembrane glycoprotein designated amyloid β -protein precursor, or β PP (for review, see ref. #26). In addition, α_1 -antichymotrypsin immunoreactivity has been found frequently intermixed with the plaque amyloid [1]. Tau [41], α_1 -antichymotrypsin [22], and β PP

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and its degradative products [19,28,33] have also been detected immunochemically in the CSF of AD patients, with the levels of ubiquitin and α_1 -antichymotrypsin reactivity being significantly increased in comparison to controls [19,33].

Previously, using a competitive enzyme-linked immunosorbent assay (ELISA), we demonstrated the high immunoreactivity of ubiquitin in the lumbar cerebrospinal fluid (CSF) of clinically diagnosed patients [39]. In a separate study, we also showed that conjugated ubiquitin is markedly elevated in the AD brain and that the increased ubiquitin levels correlated with the increased number of neurofibrillary tangles in the tissue [40]. Subsequently it was demonstrated that the levels of not only conjugated but also of monomeric ubiquitin are significantly increased in the AD brain [2,37].

In the present study, we describe (i) the measurement of ubiquitin levels in postmortem ventricular CSF from histopathologically confirmed AD and control cases, using a new rapid immunoassay, the competitive enzyme-linked immunoflow assay (CELIFA), (ii) the determination of the ubiquitin levels in brain tissue taken from the same cases, using a competitive ELISA, and (iii) an evaluation of the correlation between levels of ubiquitin in CSF and brain. These studies were carried out using monoclonal antibody (mAb) 5–25 as the primary antibody. This antibody, raised against isolated PHF [38], reacts with the carboxyl-terminal sequence of ubiquitin, the amino acid residues 64–76 [32], including the carboxyl-terminal glycine, through which ubiquitin is conjugated via an isopeptide link to the ϵ -amino group of other proteins. The mAb 5–25 is several-fold more reactive with conjugated ubiquitin than with free ubiquitin [15].

2. Materials and methods

2.1. Cerebrospinal fluid

Postmortem ventricular CSF samples were obtained from the Netherlands Brain Bank. The samples were from 17 histopathologically confirmed AD patients, 17 patients with neurological diseases other than AD (neurological controls), and 12 aged individuals without neurological symptoms (non-neurological controls) (Table 1). The CSF employed for this study were stored at -75°C before use. Because the red blood cells contain ubiquitin, samples contaminated with blood were excluded.

2.2. Diagnosis

The patients were clinically assessed, and diagnosis of 'probable AD' was made by excluding other possible causes of dementia by history, physical examination, and laboratory tests. The diagnosis 'probable AD' was based on the NINDS-ARDRA criteria [23]. All patients with this diagnosis had a global deterioration scale (GDS) of 6–7 for severity of dementia [35]. The clinical diagnosis was verified

Table 1
Profiles of Alzheimer disease and control cases

Group		n	Mean age (year)	Sex (m/f)
Alzheimer disease	CSF	17	78 $\pm$ 15	5/12
	brain	9	77 $\pm$ 16	4/5
Neurological control	CSF ¹	17	81 $\pm$ 13	7/10
	brain ²	9	81 $\pm$ 10	4/5
Non-neurological control	CSF	12	73 $\pm$ 14	9/3
	brain	8	79 $\pm$ 5	5/3

¹ Parkinson's disease: 6 cases; Pick disease: 3 cases; Diffuse Lewy body disease: 2 cases; multiple sclerosis: 1 case; multi-infarct dementia: 4 cases; no known diagnosis: 1 case.

² Parkinson's disease: 2 cases; multi-infarct dementia: 3 cases; diffuse Lewy body disease: 2 cases; Pick disease: 2 cases.

by neuropathological examination on formalin-fixed specimens and was based on the presence and distribution of plaques and tangles in sections stained by histopathological staining procedures.

2.3. Brain homogenates

Temporal cortices were used from cases for whom CSF samples were available — 9 AD, 9 neurological control, and 8 non-neurological control cases (Table 1). Brain tissues were frozen after autopsy and stored at -75°C till use. Tissue pieces were chiseled off, allowed to thaw, and separated into gray and white matter. Tissue homogenates (20%, w/v) were prepared in homogenate buffer (10 mM Tris-HCl, pH 7.2, 0.2 mM PMSF, 1 mM EDTA, 1 mM EGTA) by using a motor-driven glass-Teflon homogenizer (2,000 rpm, 25 strokes) as described previously [40]. Some homogenates were centrifuged at $100,000 \times g$ for 30 min to separate cytosol and particulate fractions. The particulate fractions were re-homogenized to the original volume in the above buffer, followed by bath sonication for 5 min. Protein concentrations were determined by the method of Bensadoun and Weinstein [3].

2.4. Antibodies

Mouse mAb 5–25 to PHF [38], ascites (~ 30 mg IgG/ml), was used at a final dilution of 1:800,000 for the CSF assay and 1:2,000,000 dilution for the brain tissue assay. This antibody recognizes the C-terminal end of ubiquitin [32]. Because ubiquitin is conjugated to other proteins through the C-terminal glycine, mAb 5–25 preferentially recognizes ubiquitin conjugates [15]. Therefore, all data obtained with this antibody have been expressed as percent inhibition instead of protein units.

For the CSF study, the secondary antibody, alkaline phosphatase-conjugated immunoaffinity purified goat anti-mouse IgG from Pierce (Rockford, IL), was used at a dilution of 1:1,000. For the brain tissue study, alkaline phosphatase-conjugated anti-mouse IgG from TAGO (Burlingame, CA) at a dilution of 1:1,000 was used as the secondary antibody.

2.5. CSF ubiquitin assay

CSF was centrifuged at $12,000 \times g$ for 10 min to remove cell debris. Three hundred μl of supernatant was incubated with 60 μl of mAb 5–25 at a dilution of 1:133,000 in 3% BSA/TBS (3% bovine serum albumin in 10 mM Tris-HCl, pH 7.2, 0.85% NaCl) for 2 h at room temperature. After incubation, the mixture was diluted with the same amount of 3% BSA/TBS and then assayed by the competitive enzyme-linked immunoflow assay, using the Easy-Titer ELIFA

System (Pierce, Rockford, IL), as described elsewhere (Kudo et al., in preparation). This system overrides the limiting diffusion by sucking the solution through a membrane, thereby considerably shortening the assay time. The nitrocellulose membrane was coated with 200 μ l/well of 0.01 mg/ml purified ubiquitin (Sigma, St. Louis, MO) in the coating buffer (0.1% glutaraldehyde, 0.02% NaN_3 , 0.1 M Na_2CO_3 , pH 9.0). After washing with TBS buffer, the remaining protein binding sites were blocked with 5% BSA/TBS. Two hundred μ l/well of the pre-incubation mixture (see above) was added, followed by the alkaline phosphatase-labeled antibody to mouse IgG (Pierce, Rockford, IL). After washing, the membrane was incubated with 0.5 mg/ml p-nitrophenyl phosphate (PNPP) (Sigma, St. Louis, MO) solution (in 0.1% diethanolamine, pH 9.5) for 1 h at room temperature. The colored product was collected to the wells of a microtiter plate, and its absorption was read at 405 nm.

Instead of CSF, 0.2 mg/ml BSA/TBS as a negative control (no inhibition) and varying amounts of ubiquitin for establishment of the standard curve also were incubated with mAb 5–25. All assays were carried out in triplicate, and the mean of the concordant values was used to calculate the ubiquitin immunoreactivity.

2.6. Brain ubiquitin assay

Ubiquitin levels in brain homogenates of AD and control cases were estimated by the competitive ELISA, as described previously [40]. A 90- μ l aliquot of mAb 5–25 diluted 1:1,000,000 with solution A (10 mM Tris-HCl, 2% bovine serum albumin, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, and 4 μ g/ml pepstatin A) was pre-incubated at room temperature for 2 h with equal volumes of brain tissue samples diluted with homogenate buffer (12.5 μ g of homogenate, 4 μ g of cytosol fraction). Solution A without antibody was also pre-incubated with brain tissue samples under the same conditions as the background. Monoclonal Ab 5–25 at a dilution of 1:1,000,000 was pre-incubated with TBS buffer with bovine serum albumin as a negative control and with varying amounts of ubiquitin as positive controls. The wells of round-bottom microtiter plates (Fisher, Pittsburgh, PA) were coated for 18 h at 4°C with 50 μ l of 10 μ g/ml ubiquitin (0.001% sodium dodecyl sulfate) in 35 mM NaHCO_3 , pH 9.6. The remaining protein-binding sites were blocked with 150 μ l/well of 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h at room temperature. After the wells were washed, 50 μ l of the incubation mixture was added to the pre-coated plates. The plates were incubated for 3 h at 22°C, washed as above, and then incubated with 50 μ l alkaline phosphatase-conjugated antibody to mouse IgG (TAGO). After 18 h at 4°C, the plates were washed and incubated with 1 mg/ml PNPP (Sigma) for 0.5–1.0 h at room temperature. Absorption was read at 405 nm. Each sample was assayed in triplicate. A background without primary antibody of each sample was deducted from each sample value.

2.7. Statistical analysis

The Student *t*-test was used for comparison of ubiquitin levels among different groups. Pearson product-moment correlation coefficient was used for evaluation of the correlation between CSF and brain values. The level of significance was $P < 0.05$.

3. Results

3.1. Ubiquitin immunoreactivity in ventricular CSF

Ubiquitin levels in postmortem CSF revealed significant differences between the AD group and the non-

Fig. 1. Ubiquitin immunoreactivity of CSF measured by CELIFA using mAb 5–25. Plots of the CSF ubiquitin levels as measured as percent inhibition showed significant differences between the AD group and the non-neurological control group and between the neurological control group and the non-neurological control group. Each value is the mean of percent inhibition $\pm$ SD. *** $P < 0.02$, * $P < 0.05$.

neurological control group, and between the neurological control group and the non-neurological control group (Fig. 1). The difference between the inhibition values of the AD and the neurological control groups was not statistically significant.

3.2. Ubiquitin immunoreactivity in brain

Levels of ubiquitin in AD and control brain samples were estimated by competitive ELISA (Table 2). The ubiquitin levels of temporal white matter tended to be higher than temporal gray matter in non-neurological control, and as well as in AD and neurological cases. However, in the number of cases tested, a statistically significant increase was observed only in white matter homogenate of the neurological control group ($P < 0.05$).

Table 2

Ubiquitin level of brain tissue (% inhibition, mean $\pm$ S.D.)

	Temporal gray matter		Temporal white matter	
	Homogenate	Cytosol	Homogenate	Cytosol
AD (9)	49 $\pm$ 22	61 $\pm$ 13	55 $\pm$ 9	65 $\pm$ 9
Neurol. cont. (9)	34 $\pm$ 8	61 $\pm$ 11	45 $\pm$ 13	56 $\pm$ 12
Non-neurol. cont. (8)	27 $\pm$ 11	57 $\pm$ 13	39 $\pm$ 13	58 $\pm$ 10

Each value is the mean of percent inhibition $\pm$ S.D.

Difference in gray matter homogenates: between Alzheimer disease and neurological control groups, $P < 0.025$; between Alzheimer disease and non-neurological control groups, $P < 0.025$.

Difference in white matter homogenates: between Alzheimer disease and neurological control groups, $P < 0.05$; between Alzheimer disease and non-neurological control groups, $P < 0.01$.

Fig. 2. Correlation between ubiquitin levels of temporal white matter and CSF in AD and control cases. The correlation between ubiquitin levels of CSF and temporal white homogenate was evaluated by Pearson product-moment correlation coefficient. The combined AD, neurological control, and normal aged group had a significant correlation ($r = 0.4145$, $n = 26$, $P < 0.035$).

The ubiquitin levels in gray and white matter homogenates of the AD group were significantly higher than those of both the neurological control group and the non-neurological control group. However, there was no significant difference in ubiquitin levels in gray and white matters between the neurological and non-neurological control groups.

In the cytosol fractions, there was no statistically significant difference in ubiquitin levels among the three groups. An attempt was also made to measure the ubiquitin levels in the particulate fraction of the three groups, using the same method. In both gray and white matter, the ubiquitin levels in the particulate fraction of some AD cases were higher than those of the control cases (data not shown). However, the data from the particulate fraction had a large scatter; the presence of myelin and lipids in the particulate fraction probably interfered with the competitive reaction.

3.3. Evaluation of the relationship between CSF and brain ubiquitin levels

The correlation between ubiquitin levels of CSF and brain homogenate was evaluated by Pearson product-moment correlation coefficient. The combined AD, neurological, and non-neurological control group had a significant positive correlation between temporal white matter and CSF ubiquitin levels (Fig. 2). When each group was examined individually, both AD and non-neurological control groups had the same positive tendency (Fig. 3c,d). In contrast, the neurological control group had a negative tendency (Fig. 3b). The combined AD and non-neurological control group also had a significant positive correlation (Fig. 3a).

In gray matter, the correlation of the combined AD, neurological control, and non-neurological control

Fig. 3. Correlation between ubiquitin levels of temporal white matter and CSF in each group. a: the correlation in combined AD and non-neurological control cases ($r = 0.5853$, $n = 17$, $P < 0.014$). b: the correlation in neurological control cases ($r = -0.2381$, $n = 9$, $P < 0.537$). c: the correlation in AD cases ($r = 0.5693$, $n = 9$, $P < 0.110$). d: the correlation in non-neurological control cases ($r = 0.4025$, $n = 8$, $P < 0.323$). The correlations were evaluated by Pearson product-moment correlation coefficient.

groups had a positive tendency, but in the number of cases studied, the differences were not statistically significant. When the groups were examined individually, no group was found to have a significant correlation between ubiquitin levels in CSF and in brain tissue, in gray matter.

4. Discussion

The present study of CSF with CELIFA revealed that ubiquitin immunoreactivity in postmortem ventricular CSF is significantly greater in AD patients than in the non-neurological control group. This study also showed that the ubiquitin immunoreactivity of the neurological control cases was significantly higher than that of the non-neurological control cases. Previously, using the competitive ELISA, we demonstrated that the ubiquitin level in the lumbar CSF was significantly higher in AD patients than in neurological and non-neurological controls [39]. In both the previous and the present studies, the percent inhibitions of the AD and normal control groups were almost the same, even though methods were different and the CSF samples were collected from living patients in the former and postmortem in the latter study. These data demonstrate that ubiquitin is a useful marker for AD both in lumbar and postmortem ventricular CSF samples. To date, there are a few markers of AD that have been shown to work in both sources of the CSF [16,24].

In contrast to the present study, in a previous study, we observed significant differences in CSF ubiquitin levels between the AD group and the neurological control group. The cause of the discrepancy between the two studies is probably the different composition of the neurological control groups and the postmortem changes in the samples employed in the present study.

Employing a small number of samples, we reported that ubiquitin levels increased many-fold in the cerebral cortex of AD and that the increase in this protein was much less remarkable in the cerebral white matter [40]. The present study in which a large number of samples were evaluated confirmed that brain ubiquitin levels were significantly increased not only in gray but also in white matter homogenates compared with neurological and non-neurological control cases. Although ubiquitin immunoreactivity has been observed in the inclusion bodies characteristic amongst others for Parkinson's disease, Pick disease, and diffuse Lewy body disease [8,11], no striking and consistent increase in brain (or CSF) ubiquitin reactivity in any of these neurological control groups was observed.

The increased ubiquitin reactivity in AD cerebral cortex might have derived from PHF. In AD white matter, however, where few PHF are present, the increased ubiquitin may be due to an accelerated depo-

sition of various ubiquitin positive heterogenous inclusions and aggregates within glial cells and the lamellae of myelin as they have been described to occur in increasing numbers in the white matter during normal aging [7,29].

What is the source of elevated ubiquitin in the CSF of AD patients? The hypothesis that the CSF changes in Alzheimer disease patients reflect marked neuropathological changes in the brain provides the most likely answer. Examination of the correlation between ubiquitin levels in CSF and cerebral white matter from the same patients revealed a significantly positive value in the combined AD, neurological control, and non-neurological control group. These findings suggest that the increased level of ubiquitin in the CSF reflected its increase in the brain. However, upon examination of each group, the neurological control cases appeared to have a different mechanism for elevated ubiquitin level in CSF because of their tendency for negative correlation. Because each neurological disease has its own pathology, a unified explanation about the correlation among the diseases is difficult. Like white matter, the gray matter had a positive correlation in the combined group, but the correlation was not statistically significant in the number of cases examined. The reason for this discrepancy is unclear. Nevertheless, our results suggest that the increased ubiquitin in AD CSF is a reflection of increased levels in the brain.

To date, most studies of AD have focused on gray matter changes. However, some reports [6,10,36] have shown that the white matter also undergoes pathological changes in AD. Our results confirm that the AD brain has pathological changes in both the gray and the white matter in terms of ubiquitin levels.

The role of ubiquitin that has been studied most is its function as a co-factor for ATP-dependent non-lysosomal proteolysis [34]. This system of protein degradation is believed to be particularly important in the removal of damaged or abnormal proteins and in the degradation of short-lived proteins in cells [17]. Recent findings suggest an extended role of ubiquitin: that ubiquitinated proteins are also directed into the lysosomal system [9]. Protein ubiquitination may, therefore, have the central role of directing proteins into two major catabolic systems in cells. It is well-known that ubiquitin is induced in cells as part of the cell stress response [4,5,30]. The first indication that ubiquitin might have significant clinical impact came from studies of AD. The presence of ubiquitin in isolated PHF and the immunostaining of PHF with antibodies to ubiquitin have been demonstrated [15,27,31]. In AD, endogenous or exogenous stress may trigger the ubiquitin system in the brain and influence elevated ubiquitin levels in the CSF. The involvement of ubiquitin has been demonstrated not only in AD but also in Parkinson's disease, Pick disease, diffuse Lewy body disease,

and motor neuron disease [7,21]. Thus, as mentioned above, there may be the possibility that each neurological disease has its own different mechanism of elevating the ubiquitin level in CSF.

The present study suggests that in AD, the increased levels of ubiquitin in CSF reflect elevated levels of this protein in brain and can therefore be considered diagnostic markers of the elevated levels in the brain. Although there may be some apprehension that some non-AD neurological cases could be evaluated as false positive with this marker, the measurement of ubiquitin level in CSF might be helpful for diagnosis if increased ubiquitin in CSF is understood as a reflection of neurodegeneration in the brain. The patient whose CSF has high level of ubiquitin should be checked carefully by more specific diagnostic methods. CELIFA is a quick procedure because the entire assay requires only 4 h. Therefore, it is possible that this ubiquitin assay can be used as a marker of brain pathology in AD.

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