Abstract. Pseudotumor cerebri (PTC) is characterized by increased intracranial pressure and papilledema without a mass lesion. PTC predominantly affects obese women. Currently, the pathogenesis of PTC is obscure. Since cytoskeletal abnormalities are found in many neurodegenerative diseases, we hypothesized that some cytoskeletal protein might be involved in the pathophysiology of PTC. Western blotting with specific antibody probes was employed to evaluate ALZ-50 immunoreactive protein, cytoskeletal microtubule-associated protein (MAP), and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) samples from 8 PTC patients and 6 controls. Immunoblotting of ALZ-50 in CSF revealed intense staining of 50 kDa protein bands in 7 of 8 PTC patients, while weak staining was found in 4 of 6 controls. Moderate staining of ALZ-50 was seen in 1 of 8 PTC patients and in 2 of 6 controls. CSF blots with anti-ALZ-50 antibody also showed intense staining of a 65 kDa protein band in 3 of the 8 patients but in none of the controls. In anti-MAP CSF blots of the PTC patients and controls, weak staining of the MAP 60 kDa and 50 kDa protein bands was observed. Weak staining of 60 kDa bands was also observed in anti-GFAP CSF blots of all PTC patients and controls. In CSF blots reacted with anti-GFAP antibody, 65 kDa and 32 kDa bands were evident in some PTC patients, but in none of the controls. This study indicates that ALZ-50 immunoreactivity is elevated in CSF of PTC patients. The ALZ-50 immunoreactive protein, either normal tau protein or its phosphorylated variant, may be useful as a biomarker for the diagnosis of PTC. Since the ALZ-50 monoclonal antibody was generated against brain homogenate from Alzheimer's disease (AD) patients, this study suggests a possible link between PTC and AD.

Keywords: CSF, tau protein, glial fibrillary acidic protein, microtubule-associated protein, ALZ-50

Introduction

Pseudotumor cerebri (PTC), also known as benign intracranial hypertension, is a disorder of the cerebrospinal fluid (CSF) circulation characterized by elevated intracranial pressure without enlarged ventricles or a discrete mass [1,6,29]. Most victims of this disorder are obese women. PTC can be associated with incapacitating headache and may produce blindness. At present, the mechanism leading to PTC is unknown, but low CSF protein concentration has been implicated [1,24,29]. An inverse relationship between CSF protein concentration and intracranial pressure has been proposed [4], but this has not been confirmed [13]. The CSF protein composition has also been normal in some studies [1,4,6]. The conflicting results regarding CSF protein concentrations in PTC could arise from serum contamination of CSF during sample withdrawal.

Since increased intracranial pressure might be the result of a cytoskeletal abnormality in the brain or blood brain barrier (BBB), we investigated the possible presence of selected cytoskeletal proteins in the CSF of PTC patients and controls using Western blotting with specific polyclonal and monoclonal antibodies.
CSF proteins have been viewed as potential markers of neuronal damage for various diseases, such as acute brain infarction [26] and Alzheimer’s disease [7,9]. The proteins and peptides known to be involved in neurodegenerative diseases include beta-amyloid, normal tau, phosphorylated tau, synaptic proteins, amyloid precursor protein, and apolipoprotein E [7,28]. In this survey of cytoskeletal proteins in CSF samples from PTC patients and controls, we found increased immunoreactivity of ALZ-50 in CSF of the PTC patients.

Materials and Methods

CSF samples from 8 female patients with PTC, 4 control women, and 2 control men were collected and stored frozen at -80°C prior to use. All PTC patients had elevated intracranial CSF pressure, as indicated by spinal puncture, accompanied by headache and papilledema, while the controls had no such symptoms but suffered from other disorders, such as syphilis and AIDS. One control subject presented with headache only. The research was approved by the Adult Health Sciences Institutional Review Board. CSF samples were obtained as a regular treatment procedure with prior consent of the patients, and no serum contamination was observed in these samples.

For electrophoresis, CSF samples were concentrated by an Amicon apparatus and protein concentrations were determined by Lowry’s method [17]. Each CSF sample was treated with SDS sample buffer containing 65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 1% 2-mercaptoethanol, and then boiled in water for 5 min. Electrophoretic separation of CSF proteins (10 μg protein/lane) in the presence of SDS was performed using 10% polyacrylamide mini-gels as described by Laemmli [15]. For Western blotting [25], CSF proteins after separation by SDS-PAGE were electrotransferred in Tris buffer containing 20% methanol to a nitrocellulose membrane (NC), employing an American Bionetics semi-dry apparatus. The membrane was treated with 5% nonfat dry milk in PBS, pH 7.4 (blocking buffer) for 30 min, and sequentially treated with primary antibodies (1:100-1:200 dilution) at 4°C overnight, secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG, 1:200 dilution), and peroxidase-anti-peroxidase (PAP) (1:800 dilution). The latter two steps were done at room temperature for 1 hr. Between each step, blots were washed with blocking buffer 4 times, each for 5 min. At the end of incubation with PAP, blots were washed 3 times with sodium phosphate-buffered saline, once with 50 mM phosphate buffer, pH 7.4 (PB), and developed for 5 min with 0.05% 4-chloro-1-naphthol in PB containing 0.01% hydrogen peroxide. Blots were washed with deionized water and dried. Monoclonal ALZ-50 antibody was kindly provided by Drs. P. Davies and H. Ghanbari (Albert Einstein College of Medicine, New York, NY). Polyclonal rabbit anti-bovine brain microtubule-associated protein (MAP), and rabbit anti-human glial fibrillary acidic protein (GFAP) were obtained from Sigma Chemical Co (St. Louis, MO).

Results and Discussion

Western blotting showed that all CSF samples from PTC patients and controls reacted with anti-ALZ-50 (Figs. 1 & 2), anti-MAP (Fig. 3), and anti-GFAP (Fig. 4). One CSF sample from a control patient who suffered from headache without increased intracranial pressure was also included in the study but is not shown in Figs. 1-4. In the CSF blots treated with anti-ALZ-50, 7 of 8 patients (lanes 1 & 3-6 of Fig. 1 and lanes 1-7 of Fig. 2) showed intense staining, while 4 of 6 controls (Fig. 1, lanes 7-10) showed weak (lanes 7-9) or very weak (lane 10) staining. One control sample (lane 11, Fig. 1) showed moderate staining with anti-ALZ-50 that was comparable to the moderate staining in one of the PTC patients (lane 2, Fig. 1). Lane 5 of Fig. 1 and lanes 1 and 2 of Fig. 2 are CSF samples from the same PTC patient. Anti-ALZ-50 reacted with molecular weight (MW) 50 kDa protein bands on all samples tested. These bands probably represent tau protein, since ALZ-50 is a monoclonal antibody that recognizes normal tau and its phosphorylated variant [27] that is associated with neurofibrillary tangles of Alzheimer’s disease (AD) [23].

The results do not differentiate whether the anti-ALZ-50 stained bands represent normal tau protein or phosphorylated tau. The CSF tau was probably derived from the brains of PTC patients, since ALZ-50 has been stained immunohistochemically in many areas of normal brains as well as in brains of patients who had cerebral infarcts [26], trauma, grade 1 peri-ventricular hemorrhages, amyotrophic lateral sclerosis, Parkinson’s disease, multisystems atrophy, brain tumors, or Shy-Drager syndrome [23]. To date, the brains of PTC patients have not been tested for immunohistochemical reaction with ALZ-50 monoclonal antibody.

The finding that anti-ALZ-50 stained 65 kDa bands in 3 PTC samples (Figs. 1 & 2) is interesting, since it has been reported that paired helical filament-tau of AD samples contains 60, 64, and 68 kDa tau isoforms [16]. Whether or not the 65 kDa tau bands of PTC and AD samples are the same proteins merits further investigation. Tau isoforms have a mass between 50 and 70 kDa [5]. The ALZ-50 immunoreactive 50 kDa band in CSF of PTC patients remained elevated all the time,
Fig. 1. Analysis of ALZ-50 immunoreactive proteins in the CSF by Western blotting (lanes 1-6 = PTC patients; lanes 7-11 = controls; total protein = 10 μg/lane; antibody dilution 1:100).

Fig. 2. Analysis of ALZ-50 immunoreactive CSF protein from PTC patients collected at different times during a few years. Lanes 1-2 = CSF collected from patient #5 at two different visits. Lanes 3-5 = CSF collected from patient #7 at three different visits. Lanes 6-7 = CSF collected from patient #8 at two different visits. (Total protein = 10 μg/lane; antibody dilution = 1:100.)

Fig. 3. MAP immunoreactivity in the CSF (lanes 1-6 = PTC patients; lanes 7-11 = controls; total protein = 10 μg/lane; antibody dilution = 1:200).

Fig. 4. GFAP immunoreactivity in the CSF (lanes 1-6 = PTC patients; lanes 7-11 = controls; total protein = 10 μg/lane; antibody dilution = 1:100).
since multiple CSF samples drawn at different times from the same patient (P) (Fig. 2: P5: lanes 1-2; P7: lanes 3-5; P8: lanes 6-7) and compared in Western blots all displayed similar, intense staining, while the 65 kDa band did not stain every time (lane 2 of P5 & lanes 3-4 of P7; Fig. 2).

Anti-MAP and anti-GFAP show weak staining in the CSF blots (Figs. 3 & 4). In the case of anti-MAP (Fig. 3), the CSF blots from PTC patients (lanes 1-6) and the controls (lanes 7-11) express similar, weak MAP immunoreactivity that stains 60 kDa and 50 kDa bands. Some 60 kDa bands (lanes 1 & 5) of PTC patients show slightly denser staining than the remaining 60 kDa and all 50 kDa bands. The stained bands may be either tau isoforms or MAP2, since a previous report showed that the anti-bovine brain MAP cross-reacts with MAP2 and tau, but does not react with MAP1 or tubulin [14]. Anti-ALZ-50 and anti-MAP probably stain different tau isoforms judging from the different intensity of their stained bands (Figs. 1, 2, 3). If the stained bands are MAP2, then they probably are degradation products, since MAP2 protein has a molecular weight of >200 kDa [3].

Anti-GFAP cross-reacts weakly with 60 kDa protein bands of PTC patients and controls (lanes 1-11, Fig. 4). The 60 kDa bands in lanes 1-2 & 5-6 of PTC show slightly stronger staining. In addition, anti-GFAP also stains 65 and 32 kDa bands in some PTC patients (lanes 1 & 3-6, Fig. 4). The CSF blot of the control patient with headache also contained a 32 kDa band (Table 1). These MW figures are either higher or lower than a previous report that GFAP has a MW of 50-55 kDa [2]. Multiple GFAP isoforms with MW ranging from 42 kDa to 165 kDa have been observed in brains of multiple sclerosis patients and control subjects [18]. Citrullinated GFAP of varied MW was detected in hippocampal tissue extracts from AD patients but not in normal brain [12]. Peptide mapping studies by other investigators have also suggested the heterogeneity of this protein, with some variants having MW values of 60 and 80 kDa [8]. Molecular biological studies also found multiple GFAP isoforms derived from alternative splicing of the GFAP gene that could lead to the formation of hetero- and homo-dimers [19]. Experiments with a novel monoclonal antibody that mainly reacts with intact GFAP indicated that acidic and soluble GFAP isoforms are more susceptible to degradation [22]. Thus, the 32 kDa peptide that is stained with anti-GFAP (Fig. 4) could be a degradation product of the 65 kDa protein. Sequencing of these protein bands should answer these questions.

The results of this study are summarized in Table 1. The ALZ-50 cross-reactive tau protein (50

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>CSF protein concentrationa (mg/dl)</th>
<th>Molecular wt (KDa) &amp; cytoskeletal immunoreactivity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anti-ALZ-50</td>
<td>Anti-MAP</td>
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<td></td>
<td></td>
<td></td>
<td>65</td>
<td>50</td>
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<tr>
<td>1</td>
<td>F</td>
<td>PTC</td>
<td>36</td>
<td>-</td>
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<tr>
<td>2</td>
<td>F</td>
<td>PTC</td>
<td>49</td>
<td>-</td>
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<tr>
<td>3</td>
<td>F</td>
<td>PTC</td>
<td>40</td>
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<tr>
<td>4</td>
<td>F</td>
<td>PTC</td>
<td>24</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
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<td>PTC</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>F</td>
<td>PTC</td>
<td>28</td>
<td>+</td>
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<tr>
<td>9</td>
<td>F</td>
<td>Syphilis</td>
<td>44</td>
<td>-</td>
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<tr>
<td>10</td>
<td>F</td>
<td>?</td>
<td>117</td>
<td>-</td>
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<tr>
<td>11</td>
<td>M</td>
<td>Syphilis</td>
<td>57</td>
<td>-</td>
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<tr>
<td>12</td>
<td>F</td>
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<tr>
<td>13</td>
<td>M</td>
<td>AIDS</td>
<td>62</td>
<td>-</td>
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<tr>
<td>14</td>
<td>F</td>
<td>Headache</td>
<td>33</td>
<td>-</td>
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++ = strong immunoreactivity; + = weak immunoreactivity; - = no immunoreactivity.
a PTC patients: No. 1-8; n = 8, CSF protein (mean ± SD) = 36 ± 8 mg/dl; controls: No. 9-14; n = 6; CSF protein (mean ± SD) = 59 ± 30 mg/dl; CSF protein concentration in PTC patients vs controls: two-tailed p value = 0.0589.
kDa) was highly elevated in 7 of 8 PTC patients, while the remaining PTC patient showed a slight increase. Similar slight increase in the ALZ-50 reactive 50 kDa band was seen in a control woman with headache and one other control, while in 4 other controls the 50 kDa band was very faint. Some PTC patients expressed 65 and 32 kDa GFAP protein bands, and a faint 32 kDa band was seen in the control woman with headache. Overall, only one PTC patient had much lighter staining than the other 7 PTC patients in ALZ-50 and anti-MAP CSF blots (lane 2 of Figs. 1 & 3 and Table 1). The concentrations of CSF proteins of PTC patients (n = 8, mean ± SD = 36 ± 8, range 24–49 mg/dl) were lower than those of the controls (n = 6, mean ± SD = 59 ± 30; range 40 – 117 mg/dl). This difference, however, was not statistically significant (two-tailed p value = 0.0589, t-test).

CSF proteins are an important diagnostic tool in many neurological disorders. Pseudotumor cerebri is a poorly understood illness, in which, for unknown reasons, the intracranial pressure is increased and CSF volume is expanded [1,6]. It was suggested that PTC could result from low concentrations of CSF proteins, whose composition was considered to be normal [1,4,6]. Since it is difficult to detect subtle differences in CSF protein concentrations and profiles, we investigated cytoskeletal immunoreactivity in the CSF of PTC patients by employing specific anti-ALZ-50, anti-MAP, and anti-GFAP antibodies. These cytoskeletal proteins were selected for study because of their importance in maintaining the integrity of neurons and glia. Tau protein and MAP are microtubule associated proteins that are involved in microtubule assembly and stabilization in neurons, while GFAP is the main intermediate filament protein in astrocytes [20].

Tau protein has been a subject of intense scrutiny since it was found in AD brain [27]. Our analyses showed elevated levels of tau in the CSF samples of PTC patients. Similar high levels of tau have also been found in the CSF of AD [7]. In case of GFAP, we found an increase in GFAP isoforms in the CSF of PTC. In addition, high levels of GFAP were observed in blood serum of AD patients by Groppa and Chekhonin [11]. They suggested that the elevation was due to compromised integrity of the blood-brain-barrier (BBB), causing passage of brain proteins into the blood. Increased GFAP levels have not been reported in the CSF of AD patients. However, increased GFAP levels were found in the frontal, parietal, temporal, and occipital cortex of patients with Down’s syndrome or AD [10] and in the entorhinal cortex of patients with AD [22]. Another study did not show any increase in GFAP expression in AD or Pick’s disease [28]. The presence of GFAP in the CSF of MS patients was found to correlate with disability, and GFAP was considered a potential biomarker for irreversible damage [21]. These reports point to cytoskeletal abnormalities in the brain and BBB causing proteins to cross the BBB into the CSF.

We are cognisant of the problems in using CSF from patients suffering from other illnesses as the controls, but it is difficult to obtain CSF from healthy persons due to ethical reasons. Confirmation of our findings with normal CSF samples is needed. Additional quantitative data and larger numbers of CSF samples may elucidate the consistency and roles of cytoskeletal protein abnormalities in PTC.

In conclusion, increased ALZ-50 immunoreactivity in the CSF of PTC patients indicates that the CSF of PTC patients is abnormal. The increased presence of cytoskeletal proteins in the CSF could be a consequence of abnormal increase of the brain neuronal and glial cytoskeletal proteins, as well as an abnormal BBB. This study demonstrated that ALZ-50 immunoreactive protein is increased in CSF of PTC patients. Since the monoclonal antibody to ALZ-50 was generated against brain homogenate from AD patients (30), this study might indicate a possible link between PTC and Alzheimer’s Disease.

Acknowledgements

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References