Alzheimer’s disease (AD) is the most common cause of dementia in Western countries and in Japan. Numerous blood and cerebrospinal fluid (CSF) tests based on the disease pathology have been proposed for early detection of AD. By comparing the CSF proteome of AD patients and controls it might be possible to identify proteins that play a role in the disease process and thus study the pathogenesis of AD.

**BACKGROUND:** Alzheimer’s disease (AD) is the most common cause of dementia in Western countries and in Japan. Numerous blood and cerebrospinal fluid (CSF) tests based on the disease pathology have been proposed for early detection of AD. By comparing the CSF proteome of AD patients and controls it might be possible to identify proteins that play a role in the disease process and thus study the pathogenesis of AD.

**PATIENTS and METHODS:** Samples of CSF from normal (n=20) and AD patients (n=20) were collected by lumbar puncture. The total concentration of proteins in the CSF of normal subjects and AD patients was determined by Bio-Rad protein assay based on the Bradford dye binding procedure. The presence and level of NGF in the CSF of normal and AD patients was measured by enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and western blot.

**RESULTS:** The total protein concentration of all samples was within the normal range (0.10-0.44 g/L). A western blot analysis using anti-NGF antibody showed the presence of NGF in human CSF. By ELISA, the level of NGF in the CSF of AD patients was higher than in the CSF of normal subjects (81.5±15.03 pg/mL vs. 4.2±1.92 pg/mL, P<0.0001).

**CONCLUSION:** We suggest that the NGF level in the CSF may provide additional information in the differential diagnosis of Alzheimer’s disease. We also conclude that NGF could be significantly involved in the pathophysiology of AD.
AD exists in a genetically determined form, known as the familial form (with an autosomal dominant character), and a sporadic form. Postmortem studies of brains from AD patients show cortical atrophy with a loss of from 8% to 10% of brain weight every 10 years of disease progression and histopathologic lesions of two types: senile plaques and neurofibrillary tangles. The major component of senile plaques is \(\text{A}\beta\)-amyloid (AB) that is generated from amyloid precursor protein (APP), an integral membrane protein with one transmembrane domain, by enzymatic digestion involving \(\beta\)- and \(\gamma\)-secretase activities. Proper function and morphology of basal forebrain cholinergic neurons depends on the supply of NGF from the cortex and hippocampus. NGF plays an important role in stimulation, survival and phenotypic maintenance of adult BFCNs.

Since the cerebrospinal fluid (CSF) is in contact with the extracellular space of the brain, biochemical brain modifications could be reflected in the CSF and measurement of intrathecal peptides and amino acids might identify biomarkers of AD. Researchers have sought to highlight the correlation between the AD process and potential biochemical markers in the CSF. In this study the total concentration of proteins in CSF was determined by the Bio-Rad protein assay based on the Bradford dye binding procedure. For western blot analysis, aliquots of CSF from normal subjects and AD patients were mixed with a sample buffer containing 3.2% SDS, 15% glycerol, 2.8 M \(\beta\)-mercaptoethanol and 0.0015% bromophenol blue. Samples were applied to a 5% to 20% gradient SDS-PAGE gel (Bio-Rad, Italy) according to Laemly and the proteins obtained were transferred to nitrocellulose sheets, pore size 0.45 \(\mu\)m (Bio-Rad, Italy). After incubation for 2 hours at room temperature in the blocking solution (PBS containing 5% skimmed milk), the nitrocellulose sheets were exposed overnight, at 4°C, to anti-NGF monoclonal antibody (Abcam, Cambridge, UK) and identified with a peroxidase-labeled mouse IgM PK 4010 Vectastain Avidin Biotin complex kit (Vector laboratories, Peterborough, UK). The peroxidase activity was revealed with diaminobenzidine (0.5 mg/mL in PBS with 0.02% hydrogen peroxide).

NGF in CSF was measured using the sensitive two-site ELISA and the antiserum against human NGF. Microtiter plates (Dynatech, Canada) were first coated with 80 ng primary anti-NGF antibody per well in 0.1 M Tris buffer. After overnight incubation, the plates were blocked with EIA buffer (50 mM Tris pH 7.5, 0.3 M NaCl, 0.1% Triton X-100, 1% BSA and 1% gelatin). The samples and standards were placed in triplicate wells and incubated overnight at room temperature. After washing with phosphate buffered saline (PBS) a biotinylated secondary antibody (8 ng/mL) was added to each well and the incubation was carried out overnight at room temperature. \(\beta\)-galactosidase coupled to avidin was then added for 2 hours followed by washing. Finally, 200 \(\mu\)M 4-methylumbelliferyl-\(\beta\)-galactoside were added (Sigma-Aldrich, Poole, UK) in 50 mM sodium phosphate and 10 mM MgCl\(_2\) buffer was added and the amount of fluorescence was measured after 40 minutes incubation at 37°C using a fluorimeter (Dynatech, Canada).

Patients and methods

In this study the total concentration of proteins in the CSF of normal subjects and AD patients was determined by the Bio-Rad protein assay based on the Bradford dye binding procedure. The presence and level of NGF in the CSF of normal subjects and AD patients was measured by enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and western blot.

Samples of CSF from normal subjects and AD patients were collected by lumbar puncture. Samples were aged-matched between the two groups, who ranged in age between 60 and 75 years. CSF samples were subjected to routine analyses, protein electrophoresis, and analyses of total protein and NGF concentration. For control purposes, CSF samples from 60 to 70-year-old control individuals (n=20) without any neurological diseases or deficit were obtained to establish normal levels of protein and NGF concentration.

None of the patients had an earlier diagnosis of tumors of the nervous system or infection. Samples were taken from both male and female patients. For the lumbar puncture the skin was cleaned with 70% alcohol. One milliliter of CSF was collected and used for this study. The samples were centrifuged at 15 000 rpm for 10 minutes, the supernatant frozen immediately and stored at -20°C until used. Twenty samples from normal and AD patients were used for analysis of protein and NGF concentration. Three independent repeats of each analysis were carried out on each sample.

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All data presented are expressed as mean±standard error of the mean (SEM). In all experiments, a minimum of 20 measurements were taken in order to calculate a mean±SEM. Statistical analysis was performed using Student’s t-test and only values with P≤0.05 were considered as significant.

**Results**

The total protein concentration in CSF from AD patients and normal subjects was determined by the Bio-Rad protein assay. The total protein concentration of all CSF samples was within the normal range (0.10–0.44 g/L) (Figure 1).\(^1\) We also analyzed CSF from normal and AD patients using SDS-PAGE followed by silver staining. A difference on the gel was the presence of a low molecular weight protein (approximately 26 kDa) in the CSF from AD patients, which was not detectable in the CSF from the normal samples (Figure 2). A western blot analysis using anti-NGF antibody as a probe confirmed the presence of NGF (Figure 3). Our results show that NGF is present in human CSF. The level of CSF NGF in AD patients is more than that in normal CSF. Furthermore, since NGF was detected in all samples analyzed in this study, NGF appears to be a constant component of the CSF. Using ELISA, it was shown that the level of NGF in the AD patient CSF was higher than in normal CSF. The mean NGF concentration in the CSF of AD patients was 81.5±15.03 pg/mL, which was significantly higher than the 4.2±1.92 pg/ml of the normal subjects (Figure 4).

**Discussion**

The present study indicates that there are elevated concentrations of the neurotrophin NGF in the CSF of AD patients. NGF is the best-characterized neurotrophic polypeptide. We investigated NGF since it is one of the most important growth factors and changes in CSF NGF are seen in many neurological diseases.\(^1\) There are a number of in vivo studies showing neuroprotection by neurotrophic factors against focal ischemia,\(^13,14\) traumatic injury\(^15\) and free radical injury.\(^16\) It has been shown that brain derived neurotrophic factor (BDNF) promotes survival of rat embryonic septal cholinergic neurons in culture.\(^17\) Over the past decade, neurotrophic factors have generated much excitement for their potential as therapy for neurological disorders. In this regard, nerve growth factor (NGF), the founding member of the neurotrophin family, has generated great interest as a potential target for the treatment of AD.
This interest is based on the observation that cholinergic basal forebrain neurons, which provide the major source of cholinergic innervation to the cerebral cortex and hippocampus, undergo selective and severe degeneration in advanced AD and that these neurons are dependent upon NGF and its receptors for their survival.\(^\text{18}\)

The present study showed no significant difference in the total protein concentration in normal subject and AD patient CSF samples. NGF is important in cellular development, maintenance and regeneration in the brain. It is produced in the hippocampus and cerebral cortex, and is retrogradely transported to support basal forebrain cholinergic neurons.\(^\text{19}\) Proper function and morphology of BFCNs depends on the supply of NGF from the cortex and the hippocampus. A variety of brain injuries, including electrical stimulation and treatment with neurotoxins, can up-regulate NGF production, which plays an important role in neuronal repair of axonal damage.\(^\text{20}\) Since NGF is known to be involved in the regulation of survival and differentiation of neurons, it may play a role in the recovery of damaged nerve cells in AD patients.

It has also been shown that NGF is a neuroprotective factor and that peripheral neurons undergo apoptosis after NGF deprivation.\(^\text{21}\) In normal brain, neurons play a major role in the synthesis of NGF, while in injured brain, glial cells may produce NGF.\(^\text{22}\) It is possible that the elevation of NGF concentration in the CSF in our AD patients was caused by increased generation of glial cells that resulted from brain damage. It has been shown that if there is extensive damage of cortical neurons, reactive glial cells, rather than neurons, may be the major source of NGF in CSF.\(^\text{23}\) Production of NGF by glial cells in AD represents an active response to neurodegenerative changes.

Moreover, considering the major roles of NGF in the peripheral nervous system in the developing period, it is possible that a high level of NGF in CSF may be derived from the peripheral circulation. The blood brain or blood-CSF barrier is still immature in the early life of the rat\(^\text{24}\) and this may be the case in humans. The fact that there is a high penetration rate of injected protein into the brain supports this explanation. The comparison between plasma/serum and CSF levels for each neurotrophin should be included in future studies to better delineate the site of synthesis.

In summary, the CSF NGF level is increased in patients with AD, which suggests that it is involved in neurodegeneration. Thus, we conclude that NGF is not only a constant component of human CSF, but also could be involved in the pathophysiology of AD. Our data strengthen the association between NGF expression and AD. The results of our study indicate that NGF may have neuroprotective properties in AD and provide a basis for future studies related to neuroprotective mechanisms exerted by NGF in AD.

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**Figure 3.** A typical western blot for β-NGF protein. Immunoblot analysis of CSF was obtained in normal (Lanes 1, 2, 3) and AD patients (Lanes 4, 5, 6). The blot was probed with an anti-NGF monoclonal antibody.

**Figure 4.** Nerve growth factor concentrations (pg/mL) in the CSF of normal subjects and Alzheimer’s disease (AD) patients (20 samples each) (P<0.001)
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