Glutamine Synthetase in Cerebrospinal Fluid, Serum, and Brain

A Diagnostic Marker for Alzheimer Disease?

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Objectives: To determine whether the glutamine synthetase (GS) level in cerebrospinal fluid (CSF) is a useful biochemical marker in the diagnosis of Alzheimer disease (AD), and to assess the source of GS (brain vs blood derived) in CSF.

Methods: Sandwich enzyme immunoassay and immunoblotting were applied to detect GS in CSF and in serum from neurologically healthy control subjects and patients with neurodegenerative diseases, including AD. The origin of GS was estimated by the concentration gradients of CSF to serum and ventricular to lumbar CSF. In addition, postmortem brain tissue from controls and patients with AD was analyzed using immunohistochemistry for expression of GS.

Results: Levels of GS were significantly increased in lumbar CSF from patients with AD (20 ± 12 pg/mL; \( P = .01 \)) and to a lesser extent in patients with vascular dementia and amyotrophic lateral sclerosis. In CSF of controls, GS levels were 4 ± 3 pg/mL. The GS concentration gradients were less than 1:10 for CSF to serum and 2:1 for ventricular to lumbar CSF. Immunoreactivity of GS was most prominent in astrocytes from temporal neocortex of patients with AD, suggesting a relationship between astrocyte reactions and increased GS levels in CSF.

Conclusions: Level of GS in lumbar CSF of patients with AD is increased significantly but nonspecifically, probably related to the strong astrogliosis in brain. Glutamine synthetase in lumbar CSF is mainly brain derived.

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The search continues for a valid biological marker to support the diagnosis of Alzheimer disease (AD) during the lifetime of demented patients, as a definite diagnosis still depends on results of histopathological examination of brain tissue.

The enzyme glutamine synthetase (GS) was proposed as a potential diagnostic marker, since GS was detected exclusively in cerebrospinal fluid (CSF) of patients with AD but not in that of healthy control subjects or controls with other diseases. To our knowledge, this was the first study to report detection of GS in CSF using radiolabeled adenosine triphosphate and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Glutamine synthase (glutamate:ammonia ligase [adenosine diphosphate forming]) is localized in astroglial cells, and it protects neurons via converting the potentially neurotoxic glutamate and ammonia into glutamine. Changes of GS have been reported in various disorders, including AD. The enzymatic activity of GS is decreased in the brain of patients with hepatic encephalopathy, spinocerebellar atrophy, and AD.3-6 In patients with AD, reduced GS activity was localized to brain areas with increased oxidation products mostly present in the vicinity of amyloid plaques. It thereby may be involved in the pathogenesis of AD.6

The protein concentration of GS has not been reported yet in body fluids. We therefore tried to confirm the impressive findings of the previous study1 using a quantitative technique. Recently, GS was purified from human brain and characterized immunochemically as a prelude to developing a sensitive and specific sandwich enzyme immunoassay (EIA).7 The objectives of our study were to quantitate GS concentrations in CSF and in serum of controls; to determine the source of GS in CSF (blood vs brain); to evaluate its clinical relevance in neurodegenerative diseases, including AD; and to study GS immunoreactivity in postmortem brain of patients with AD and controls.
SUBJECTS AND METHODS

PATIENTS AND SAMPLES

Since it is notoriously impossible to obtain CSF from healthy volunteers, we selected 35 pairs of lumbar CSF and serum samples from patients of the Department of Neurology of the University Hospital of Göttingen, Göttingen, Germany, who had shown no evidence of neurologic disease. Informed consent was obtained from all patients to perform the lumbar puncture, which was part of the diagnostic workup. Exclusion of a central nervous system disease was judged by clinical criteria, by results of computed or magnetic resonance tomography, and by chemical criteria such as normal results of blood tests and absence of inflammatory signs in CSF (pleocytosis or intrathecally produced IgG, IgA, and IgM) and normal CSF-serum albumin ratio (<8 × 10^{-3}). The distribution of age and sex in the control group was as follows: mean age, 51 years; range, 13 to 87 years; females, n = 19; and males, n = 16. There were no sex-dependent significant differences between age and GS concentrations.

Lumbar CSF and serum samples (n = 45) from subjects in the different disease groups were obtained from the National Neurological Research Specimen Bank (NNRSB), Los Angeles, Calif. The groups included patients with sporadic AD (mean age, 62 years; range, 59-72 years), sporadic amyotrophic lateral sclerosis (ALS) (mean age, 58 years; range, 44-67 years), vascular dementia (mean age, 62 years; range, 55-83 years), Parkinson disease (mean age, 67 years; range, 58-75 years), and schizophrenia (mean age, 43 years; range, 30-57 years). The vascular dementia group consisted of patients with non-AD dementia confirmed by signs of multiple infarctions in the postmortem neuropathological examination. The lumbar CSF was obtained from a first diagnostic puncture at an early disease stage. The final classification of CSF samples into disease groups was made if clinical diagnoses were subsequently confirmed by postmortem examination according to the standards of the NNRSB. The ventricular CSF samples (n = 48) were obtained from autopsy cases and classified according to clinical and neuropathological criteria as well. These specimens were treated as a different subgroup and were not included in the diagnostic evaluation of lumbar CSF, since ventricular and lumbar CSF differ with regard to their protein content and sampling conditions. Although the samples obtained from the NNRSB were not collected specifically for our study, handling conditions followed a standard protocol. All CSF and serum samples from control and disease groups were stored at −70°C until analysis. Twenty-four serum specimens from healthy blood donors (mean age, 35 years; range, 21-49 years) were included as an additional control group.

Brain tissue was obtained from autopsy cases from the Department of Neuropathology at the University of Göttingen. Neuropathological diagnosis was made according to CERAD ( Consortium to Establish a Registry for Alzheimer’s Disease) criteria. Paraffin-embedded tissue was used from 12 patients (mean age, 72 years; 7 women and 5 men) with clinical and neuropathological AD and 6 controls (mean age, 69 years; 3 men and 3 women) who died of nonneurologic disease. From each subject, the following 4 brain regions were studied: hippocampus and temporal, frontal, and occipital neocortex.

SDS-PAGE AND WESTERN BLOTTING

The detailed procedures for SDS-PAGE and immunoblotting have been described previously. In brief, purified human brain GS (>93% purity, purified in our laboratory) in 10-mmol/L potassium phosphate (pH 7.2), CSF (30 µL, undiluted), and serum (30 µL, 1:200 diluted) were electrophoresed on a prestained 12% SDS polyacrylamide gel (Novex, San Diego, Calif) and transferred to nitrocellulose paper (0.2 µm; Bio-Rad, Hercules, Calif). After blocking with 10 g/L bovine serum albumin in a mixture of phosphate-buffered saline [PBS] and polysorbate 20 (Tweeen 20; Sigma-Aldrich Corporation, St Louis, Mo) for 1 hour, blots were incubated with mouse monoclonal antibody against sheep brain GS (0.1 µg/mL) (Chemicon, Temecula, Calif) for 3 hours at room temperature. After washing, blots were incubated for 1 hour with alkaline phosphatase–labeled goat anti–mouse IgG (Sigma-Aldrich Corporation) diluted 1:1000 in PBS–polysorbate 20. Immunoreactive bands were visualized using an alkaline phosphatase substrate kit (Bio-Rad). Low-molecular-weight markers (18.5-106 kd) (Bio-Rad) were used as standards. To show specificity of the monoclonal antibody against GS, control experiments included primary antibody preadsorbed with excess antigen (pure GS) and addition of conjugated secondary antibody without primary antibody. In both control experiments, a GS band was not observed.

ENZYMIE IMMUNOASSAY

In the EIA, the monoclonal antibody directed against sheep brain GS (1 µg/mL) (Chemicon) was used for coating the microtiter plates. After washing and blocking, serial dilutions of purified human brain GS (range, 2.5 to 80 pg/mL), CSF (undiluted), and serum (diluted 1:10) of serum showed recoveries of 95.9% ± 15.4% and 94.8% ± 5.1%, respectively. After up to 6 cycles of freezing (−70°C) and thawing to room temperature, no significant change in the concentration of purified GS or GS in CSF or in serum was observed. The specificity control experiments (omission of primary antibody and preadsorption of antigen with excess amounts of nonbiotinylated primary antibody) showed optical densities resembling those of 0 standards, indicating that the primary antibody against GS does not cross-react with other proteins.
patients and negative and positive control samples were plated and incubated for 2 hours. After removing the unbound components, biotin-labeled monoclonal antibody (0.1 pg/mL in PBS–polysorbate 20) against sheep brain GS was added and incubated 2 hours at room temperature. After removing the unbound conjugate, a streptavidin-peroxidase complex (Boehringer Mannheim, Mannheim, Germany) diluted 1:8000 in PBS–polysorbate 20 was added and incubated for 30 minutes. The following colormetric reaction was developed using 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide kit (Kirkegaard and Perry Labs, Gaithersburg, Md). The reaction was stopped by the addition of 0.5-mol/l sulfuric acid. The absorbance was read at 450 nm and corrected at 620 nm (Easy Reader 400 AT; Bio Whittaker, Walkerville, Md).

Unknown GS concentrations were calculated from the standard curve if the duplicates did not differ by more than 15%, and the measured optical density was within the linear portion of the curve. Serial dilutions of purified human brain GS were used to generate a standard curve in the range of 2.5 to 80 pg/mL. To ensure specificity of the monoclonal antibody against GS, control experiments included omission of biotin-labeled antibody against GS, preincubation of unknown CSF and serum with excess non-biotinylated monoclonal antibody to GS, and spiking/recovery assays. For the spiking/recovery assays, known amounts of standard antigen were added to previously assayed CSF and serum samples with known high and low concentrations of GS. Recovery was calculated as a percentage of measured vs expected concentrations of GS.

**IMMUNOHISTOCHEMISTRY**

The immunohistochemical procedure for detection of GS in human brain slices followed the protocol described earlier for rabbit brain. The monoclonal anti–sheep brain GS antibody (Chemicon) was used. An avidin-biotin complex technique was applied with 3-amino-9-ethylcarbazole (AEC) as chromogen. In the control experiments, the primary antibody was omitted and excess antigen was added before applying the primary antibody.

**STATISTICS**

Statistical evaluations and graphic illustrations were performed using commercially available software (Excel, Microsoft Corporation). We used analysis of variance and 2-tailed t test to calculate P values. Statistical significance was assumed in case of an α error of less than .05. Unless otherwise indicate, data are given as mean ± SD.

**GS CONCENTRATIONS IN SERUM AND CSF OF HEALTHY CONTROLS AND PATIENTS**

The mean concentrations of GS in control CSF and serum samples (n = 35) were 4 ± 3 pg/mL and 36 ± 27 pg/mL, respectively. The average concentration of GS in the serum of healthy blood donors (n = 24) was 50 ± 19 pg/mL.

There was no correlation between age and GS concentrations in CSF and in serum from control and disease groups.

**WESTERN BLOTTING**

To study the clinical utility of determining GS concentrations, CSF and serum samples from patients with neurodegenerative diseases and controls were examined. The mean GS concentrations were significantly increased in the CSF of patients with AD (20 ± 12 pg/mL; P = .01) and to a lesser extent in that of patients with vascular dementia (13 ± 7 pg/mL; P = .04) and ALS (13 ± 13 pg/mL; P = .047) (Figure 1). In serum samples, GS concentrations were increased in all disease groups, as follows: patients with ALS (n = 8), 116 ± 62 pg/mL (P = .01); patients with AD (n = 9), 111 ± 53 pg/mL (P = .12); patients with vascular dementia (n = 15), 72 ± 59 pg/mL (P = .01); patients with Parkinson disease (n = 5), 77 ± 32 pg/mL (P = .14); and patients with schizophrenia (n = 4), 74 ± 32 pg/mL (P = .08). In CSF of controls and patients with AD, no overlap of GS values was seen after adjustment for age (Figure 2).

To confirm the EIA-based difference of GS concentration between patients with AD and controls, we conducted immunoblotting experiments (Figure 3). The electrophoretic and immunochemochemical characteristics
of purified human brain GS are shown. The molecular weight of the denatured, monomeric form of GS was approximately 44 kd as determined using SDS-PAGE. An immunoreactive band of the same relative molecular weight was detected in the lumbar CSF (undiluted) from a patient with AD, but not in the corresponding (1:200 diluted) serum sample. Likewise, no GS band was detected in the lumbar CSF or serum from an age-matched, healthy control. The 1:200 dilution of serum was necessary to obtain equal amounts of total protein in CSF and serum and to ensure comparable electrophoretic separation conditions between both fluids. The nonspecific bands larger than 50 kd were less intensely stained than the GS band and did not appear if the substrate reaction time was kept at a minimum. Using Western blotting, we were able to detect a GS band from only 1 patient with AD who had the highest GS concentration in lumbar CSF (47 pg/mL) among a total of 8 patients. However, we observed GS-positive bands in all ventricular CSF samples from 5 patients with AD with known GS concentrations above 60 pg/mL.

GS IMMUNOREACTIVITY IN POSTMORTEM BRAIN TISSUE

In control brains, GS immunoreactivity was observed in astrocytes of the grey but not the white matter. A more prominent GS response was seen in tissue samples of patients with AD (Figure 4, top). The reactive gemistocytic astrocytes with their large cytoplasm stained intensively with a monoclonal antibody to GS. There was no immunoreactivity in the white matter of patients with AD, and the strongest GS response was seen in the temporal brain regions. Control experiments with omission of the primary antibody or preincubation with antigen gave negative results (Figure 4, bottom). Glutamine synthetase-positive astrocytes were diffusely distributed in the grey matter without any association with senile plaques or neurofibrillary tangles.

INTRATHECAL ORIGIN OF GS IN CSF

The CSF-serum concentration ratio of GS in healthy controls was about 1:10. The expected ratio, however, would be 1:500 (assumption is based on molecular size of GS and laws of diffusion across the blood-CSF barrier) if GS in CSF were to originate exclusively from blood. This is indicative of a mainly brain-derived source of GS in CSF. There was no relationship between GS concentration in CSF and CSF-serum albumin ratio (a representative measure for blood-CSF barrier function), which makes a barrier-dependent alteration of GS concentration in CSF unlikely.

The Table shows the concentrations of GS in lumbar and ventricular CSF from patients with AD, ALS, and Parkinson disease. There is a clear dropping gradient from ventricular to lumbar CSF (approximately 2-fold in AD, 3-fold in ALS, and 5-fold in Parkinson disease).
Glutamine Synthetase Concentrations in Lumbar and Ventricular CSF of Patients With Various Neurodegenerative Diseases*

<table>
<thead>
<tr>
<th>Disease</th>
<th>Lumbar CSF</th>
<th>Ventricular CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Patients</td>
<td>GS, pg/mL</td>
</tr>
<tr>
<td>Neurologically healthy controls</td>
<td>35</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>8</td>
<td>20 ± 12</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>9</td>
<td>13 ± 13</td>
</tr>
<tr>
<td>Parkinson disease</td>
<td>5</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SD. CSF indicates cerebrospinal fluid; ND, not determined.

COMMENT

NORMAL RANGE AND METHODS

To our knowledge, the normal ranges of GS concentrations in human CSF and serum are reported herein for the first time, with mean values of 4 and 36 pg/mL, respectively. The actual concentrations of GS protein could be higher than those presently reported, as the single monoclonal-based EIA used in our study would not detect monomeric GS in CSF. As judged from studies analyzing purified brain GS on native gel electrophoresis (nondenatured), the major part of total GS in CSF is expected to be the octameric form. The control experiments described herein suggest that the monoclonal antibodies specifically recognize GS, although cross-reaction to other proteins cannot be ruled out completely.

ORIGIN OF GS IN CSF

In addition to brain, GS occurs in kidney, liver, skeletal muscle, spleen, and heart. Systemic GS might be passively filtered across the blood-CSF barrier into the CSF space. The expected serum-CSF ratio for proteins originating from blood is dependent on the hydrodynamic size of the protein. Based on the relative molecular weight of the octameric GS (360 kd) and a corresponding hydrodynamic radius of 6.5 nm, the estimated CSF-serum ratio would be less than 1:500. Assuming an intact blood-CSF barrier, CSF concentrations of GS should be no greater than 0.2% of the serum concentration. However, the mean GS concentration in healthy lumbar CSF determined in our study was nearly 10% of the serum concentration. Therefore, approximately 98% of the GS in CSF should be of intrathecal origin. Further indirect evidence of the intrathecal origin of GS comes from the lack of correlation with increasing albumin CSF-serum quotient and from the dropping ventriculolumbar concentration gradient in CSF.

CLINICAL RELEVANCE

To evaluate the clinical utility of determining GS concentrations, CSF and serum samples from patients with neurodegenerative diseases were examined. High concentrations of GS were observed in lumbar CSF from patients with AD, confirming the results of a previous study. In addition, we observed elevated GS concentrations in the CSF from patients with ALS and vascular dementia. The authors of the previous study detected GS using photolabeled adenosine triphosphate binding. Presumably, this binding requires active enzyme, and indeed, increased enzymatic activity of GS in CSF of patients with AD was reported by the same authors. Since the EIA used in our study detects enzymatically active as well as inactive GS, we might have been able to detect GS in various disease groups. We could not, however, detect GS enzymatic activity in CSF and serum samples with the γ-glutamyl-transfer reaction as described previously.

The exact mechanisms leading to increased GS concentrations in CSF remain unknown. Overexpression of GS in reactive astrocytes and subsequent release into the extracellular space may be a possible cause. Our immunohistochemical data from brains of patients with AD showing increased immunoreactivity against GS in cortical astrocytes (Figure 4) support this view. Since reactive astrocytes are a common feature in various diseases of the central nervous system, the overall occurrence of elevated GS levels in different neurodegenerative diseases can be expected. Data of previous animal experiments (rabbit model of bacterial meningitis and rat model of cerebral ischemia) provide further evidence by demonstrating increased expression of brain GS in diseases of the central nervous system associated with reactive astrogliosis.

The marked elevation of GS protein in patients with AD deserves special attention. It has been shown that senile plaque-dense regions of the brain of patients with AD represent environments of elevated oxidative stress and that protein in the brain of patients with AD is more oxidized than that of controls. Likewise, the same authors reported decreased GS enzyme activity in brain regions associated with increased oxidation products (oxidized proteins) in patients with AD. Reactive microglia extensively present in senile plaque regions have been proposed as a source of oxyradicals in the brain. Glutamine synthetase is sensitive to oxidation because of its divalent cation site. A primary damage to the enzyme itself, leading to inactivation of GS, elevated turnover, and eventual shedding of the inactive GS protein into the extracellular space may be the explanation for the strong increase of GS protein levels in CSF of patients with AD.

Measurement of GS concentrations in serum is a more useful paraclinical marker, as blood tests are more appropriate for repeated serial analysis. The origin of the elevated concentrations in serum of patients with ALS remains to be determined, although muscles are the most likely candidate. Supporting evidence comes from an animal study where experimentally induced peripheral denervation caused a posttranscriptional increase in GS concentrations in rat skeletal muscle. More specific assays capable of differentiating between tissue-specific isoforms (muscle vs liver vs brain) are needed to determine the source of GS in serum.

In conclusion, elevated GS concentrations occur in CSF of patients with AD, but also in other diseases, such as vascular dementia and ALS. Glutamine synthetase might have an important role as a marker for some neu-
rodegenerative diseases associated with astroglial changes. A larger number of patients and intraindividual serial samples at different disease stages are needed to further substantiate and extend the present data.

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REFERENCES


