

# Levels of $\beta$ -Secretase (BACE1) in Cerebrospinal Fluid as a Predictor of Risk in Mild Cognitive Impairment

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**Context:** Elevated  $\beta$ -secretase ( $\beta$ -site amyloid precursor protein–cleaving enzyme 1 [BACE1]) activity has been found in the brains of patients with sporadic Alzheimer disease (AD) compared with controls. Now we are particularly interested in whether BACE1 can be identified in the cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI), a population at high risk for AD. The possible presence of BACE1 in the CSF of patients with AD and MCI has so far gone unreported.

**Objective:** To examine whether BACE1 can be identified in the CSF of patients with MCI.

**Design:** We evaluated CSF BACE1 levels using 2 sandwich enzyme-linked immunosorbent assays, BACE1 enzymatic activities by means of synthetic fluorescence substrate, and total amyloid- $\beta$  peptide levels using a sandwich enzyme-linked immunosorbent assay.

**Setting:** Two independent research centers.

**Participants:** Eighty patients with sporadic AD, 59 patients with MCI, and 69 controls.

**Main Outcome Measures:** BACE1 levels and enzymatic activities and amyloid- $\beta$  peptide levels.

**Results:** Increased CSF levels of BACE1 protein were associated with increased risk ratios (RRs) for patients with MCI compared with controls (RR, 2.08; 95% confidence interval [CI], 1.58-2.58) and patients with AD (RR, 1.65; 95% CI, 1.19-2.03). Similarly, patients with MCI showed increased levels of BACE1 activity compared with controls (RR, 2.17; 95% CI, 1.66-2.71) and patients with AD (RR, 3.71; 95% CI, 2.74-4.36). For total amyloid- $\beta$  peptide and tau, increased CSF levels were associated with a higher risk of MCI compared with controls. The BACE1 activity was significantly correlated with BACE1 protein level ( $\rho=0.23$ ;  $P<.001$ ) and amyloid- $\beta$  peptide level ( $\rho=0.39$ ;  $P<.001$ ), with amyloid- $\beta$  peptide correlated with BACE1 protein level ( $\rho=0.30$ ;  $P<.001$ ).

**Conclusion:** Significant elevation of BACE1 levels and activity in CSF is an indicator of MCI, which could be an early stage of AD.

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**A**LZHEIMER DISEASE (AD) IS characterized by the progressive formation of insoluble amyloid plaques and vascular deposits consisting of the 4-kDa amyloid- $\beta$  peptide (A $\beta$ ) in the brain.<sup>1</sup>  $\beta$ -Secretase ( $\beta$ -site amyloid precursor protein–cleaving enzyme 1 [BACE1])<sup>2</sup> is one of the 2 key enzymes in amyloid precursor protein (APP) processing. Amyloid- $\beta$  peptide results from cleavage of APP initially by BACE1 to produce a C99 fragment and release soluble APP $\beta$ ; C99 is then further cleaved by  $\gamma$ -secretase, leading to A $\beta$ . Increased BACE1 activity and elevated levels of insoluble A $\beta$  have been shown in the brains of patients with sporadic AD.<sup>3,4</sup>

Because cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the central nervous system, biochemical changes in the brain could potentially

be reflected in CSF. The CSF-based detection of BACE1 levels and activity might be valuable in aiding the early diagnosis of AD, especially in patients with mild cognitive impairment (MCI), who show a higher risk of AD.<sup>5</sup> Several recent studies<sup>6-8</sup> showed that BACE1 activity can be detected in the CSF. However, whether changes could occur in BACE1 activity or protein levels in the CSF of patients with AD or MCI remains unknown.

In the present study, we quantitatively analyzed the enzymatic activities and protein levels of BACE1 and total A $\beta$  levels in CSF samples from 208 individuals. We aim to determine whether BACE1 levels and activity can be detected in CSF, whether they are altered in AD compared with healthy aging, and whether levels of BACE1 protein and activity may be useful to discriminate patients with AD or MCI from healthy individuals.

**Table 1. Descriptive Statistics for Each Study Group\***

Group	Sample Size, No.	Age, y	MMSE Score	BACE1 Activity, pmol/min- $\mu\text{L}^{-1}$	BACE1 Level, ng/mL	Total A $\beta$ , ng/mL	Tau, pg/mL
AD	80	69.78 $\pm$ 8.89	20.25 $\pm$ 4.30†	0.33 $\pm$ 0.12	1.07 $\pm$ 0.65	63.37 $\pm$ 33.88	645.16 $\pm$ 349.70
MCI	59	70.93 $\pm$ 7.42	26.49 $\pm$ 2.17†	0.49 $\pm$ 0.19	2.0 $\pm$ 1.26	100.03 $\pm$ 73.50	623.18 $\pm$ 474.50
Control	69	62.58 $\pm$ 10.15†	29.28 $\pm$ 0.91	0.28 $\pm$ 0.08†	0.94 $\pm$ 0.86†	42.03 $\pm$ 37.55†	356.44 $\pm$ 144.20†

Abbreviations: A $\beta$ , amyloid- $\beta$  peptide; AD, Alzheimer disease; BACE1,  $\beta$ -site amyloid precursor protein–cleaving enzyme 1; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination.

\*Data are given as mean  $\pm$  SD.

†Statistically significant compared with all other groups ( $P < .001$ ).

## METHODS

### PARTICIPANT SELECTION AND CSF SAMPLING

A total of 208 individuals were recruited from 2 independent research centers for AD, including 50 patients with probable AD, 45 with MCI, and 19 controls from the Alzheimer Memorial Center, Department of Psychiatry, Ludwig-Maximilian University. The other 30 patients with AD, 14 patients with MCI, and 50 controls were recruited at the Department of Clinical Neuroscience, University of Göteborg, Sahlgren's University Hospital. Demographics of the patients are given in **Table 1**. Diagnosis of AD was made according to the National Institute of Neurological and Communicative Disorders and Stroke criteria.<sup>9</sup> Mild cognitive impairment was diagnosed according to the method of Petersen et al<sup>5</sup>; that is, patients with MCI had memory performance 1.5 SD below the age-adjusted reference average, as assessed using the Consortium to Establish a Registry for Alzheimer's Disease cognitive battery.<sup>10</sup> This battery included verbal learning, recognition, and recall tests; global cognitive function and activities of daily living were unimpaired. Controls were cognitively healthy individuals who underwent spinal anesthesia for surgery of the urinary tract or lower extremities. Psychiatric comorbidity was excluded by means of history, clinical examination, and Composite International Diagnostic Interview.<sup>11</sup> All the controls were cognitively normal according to Consortium to Establish a Registry for Alzheimer's Disease cognitive battery performance (within 1 SD in all subtests), and the participants had no complaints of cognitive impairment. To avoid spinal anesthesia as a potential confounding factor when collecting CSF, CSF was obtained immediately after inserting the needle and just before application of the anesthetic drug. All the procedures were approved by the institutional review boards of the respective institutions, and consent forms were signed by the patients before sample collection.

### WESTERN BLOT AND ENZYME-LINKED IMMUNOSORBENT ASSAY

For Western blot analysis, CSF from each group was mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer and separated using an 8% SDS polyacrylamide gel. The protein was then transferred to a Hybond polyvinyl difluoride (PVDF) membrane (Hybond-P; Pharmacia, Uppsala, Sweden) by semidry transfer according to the standard procedure. The membrane was then probed with the following antibodies: SECB1 and SECB2 for the N-terminus of BACE1 and C-15 for the C-terminus of BACE1 (Amgen Inc, Thousand Oaks, Calif). Antibodies targeted different sites inside the Asp domain: B278 (aa366-378), B279 (aa295-310), and B280 (aa191-207) (gifts from Riqian Yan, PhD, Cleveland Foundation, Cleveland, Ohio). Other commercially available anti-BACE1 antibodies used include anti-BACE1 polyclonal antibody (BioSource, Camarillo,

Calif) and anti-BACE1 monoclonal antibody (R&D Systems Inc). For detecting tubulin, antitubulin polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, Calif) was used. Optical densitometry analysis was performed using FluorChem8900 (Alpha Innotech, San Leandro, Calif).

Two BACE1 protein sandwich enzyme-linked immunosorbent assays (ELISAs) were established: one used a combination of anti-BACE1 polyclonal antibody SECB2 as the capture antibody and biotinylated anti-BACE1 polyclonal antibody SECB1 as the detection antibody.<sup>3,4</sup> The other ELISA was established by using the anti-BACE1 polyclonal antibody B280<sup>12</sup> as the capture antibody and anti-BACE1 monoclonal antibody (R&D Systems Inc, Minneapolis, Minn) as the detection antibody. Recombinant BACE1 (Amgen) was used as the standard and was assayed under the same conditions. The BACE1 concentration was calculated from the standard curve and is expressed in micrograms per milliliter. Total A $\beta$  levels in CSF were measured by means of sandwich ELISA with anti-A $\beta$  monoclonal antibody 4G8 (aa17-24) as the capture antibody and biotinylated anti-A $\beta$  monoclonal antibody 7N22 as the detection antibody, whose epitope has been mapped to aa8-13 (data not shown). The CSF total tau level was determined using a sandwich ELISA constructed to measure total tau levels, including normal tau and hyperphosphorylated tau.<sup>13</sup>

### BACE1 ENZYMATIC ACTIVITY ASSAY

Activity assays of BACE1 were performed by using synthetic peptide substrates containing the BACE1 cleavage site (MCA-Glu-Val-Lys-Val-Asp-Ala-Glu-Phe-[Lys-DNP]-OH) at a 50mM concentration in reaction buffer (50mM acetic acid, pH 4.1, 100mM sodium chloride). Ten microliters of CSF from each sample was used to examine BACE1 activity. Fluorescence was observed by using a fluorescent microplate reader with an excitation wavelength at 320 nm and an emission wavelength at 383 nm.

### DEGLYCOSYLATION OF BACE1 PROTEIN IN CSF

Samples of CSF, 200  $\mu\text{L}$ , from patients with MCI or AD and controls were immunoprecipitated by anti-BACE1 antibody (R&D Systems Inc). The mixture was then incubated with protein G agarose (Sigma-Aldrich Corp, St Louis, Mo) for 3 hours. The beads were washed 4 times with washing buffer, and immunoprecipitates were eluted by boiling in 40  $\mu\text{L}$  of 0.1% SDS, 0.1M  $\beta$ -mercaptoethanol for 5 minutes. For deglycosylation, 12  $\mu\text{L}$  of eluent were added to 1.8  $\mu\text{L}$  of 1M Tris (pH 8.6), 9  $\mu\text{L}$  of water, 2.4  $\mu\text{L}$  of 10% NP-40, and 5  $\mu\text{L}$  of 1-mU/mL peptide N-glycosidase F (Sigma-Aldrich Corp). Reaction mixtures were incubated at 37°C for 16 hours, resolved by means of 8% SDS polyacrylamide gel, and then transferred to the PVDF membrane. The BACE1 was detected by using the anti-BACE1 polyclonal antibody from BioSource.

## TRANSIENT TRANSFECTION

The BACE1 complementary DNA was subcloned into pcDNA3.1, and the Kozak sequence was added in front of translation start ATG codon. The 293T cells were maintained in Dulbecco modified eagle medium with 10% fetal bovine serum. The 293T cells on 6-well plates were transfected with 0.1 and 0.5  $\mu$ g of BACE1 expression plasmid by lipofectamine (Invitrogen Corp, Carlsbad, Calif) according to the manufacturer's instructions. Cells were harvested 48 hours after transfection. Cells and brain tissue were homogenized in 1% TritonX-100 lysis buffer with phenylmethylsulfonyl fluoride and protease inhibitor mix (Sigma-Aldrich Corp). The BACE1 Western blot was performed by loading 25  $\mu$ g of cell lysate/brain lysate onto a 6% SDS polyacrylamide gel. Western blot was performed as described previously herein, and optical densitometry was analyzed by using FluorChem8900. The BACE1 enzymatic activity assay and deglycosylation were performed as described previously herein.

## STATISTICAL ANALYSIS

Separate 1-way analyses of covariance were computed for the comparisons between diagnostic groups for each dependent variable, including BACE1 levels, BACE1 activities, total A $\beta$  levels, and total tau levels. In addition to the variable group, sex was added as a predictor and age as a covariate in the analysis of covariance models. Simple main effects were followed up by using Tukey post hoc tests. The risk ratios (RRs) and associated 95% confidence intervals for each predictor were computed on the basis of the corrected odds ratios derived from logistic regression analysis.<sup>14</sup> For ease of interpretation, the RRs were computed for binarized values of the predictors. Cutoff values for each predictor were obtained from the logistic regression analysis. In addition to the basic prediction models for each CSF-based predictor, logistic regression models combining BACE1 protein, BACE1 activity, total tau level, total A $\beta$  level, and age, entered in a feed-forward manner, were built.

To investigate the relationships among BACE1 protein levels, BACE1 enzymatic activity, total A $\beta$  levels, and total tau levels, Spearman  $\rho$  correlations were computed. All analyses were performed using a software program (SPSS version 11.5.1; SPSS Inc, Chicago, Ill).

## RESULTS

### CSF BACE1 LEVELS AND ACTIVITIES

To examine whether there was any BACE1 in the CSF of patients with AD, Western blotting was performed by using different antibodies against BACE1. When using the anti-N-terminus antibody, 70-kDa and approximately 60-kDa BACE1 proteins were detected in all the CSF groups (**Figure 1A**). Tubulin was the most abundant protein in the CSF and could be used as loading control.<sup>6</sup> When normalized to the tubulin level (**Figure 1A**), we found that the approximately 70-kDa band and the approximately 60-kDa band were significantly higher in patients with MCI than in those with AD and controls ( $P < .001$  for both), whereas no significant difference between patients with AD and controls was observed ( $P = .53$ ) (**Figure 1A**). To examine whether they were full-length BACE1 proteins, the antibody that recognizes the C-terminus of BACE1, C-15 (Amgen), was used. The same bands were detected (**Figure 1A**). Similar to the result obtained using the anti-BACE1 N-terminus antibody, op-

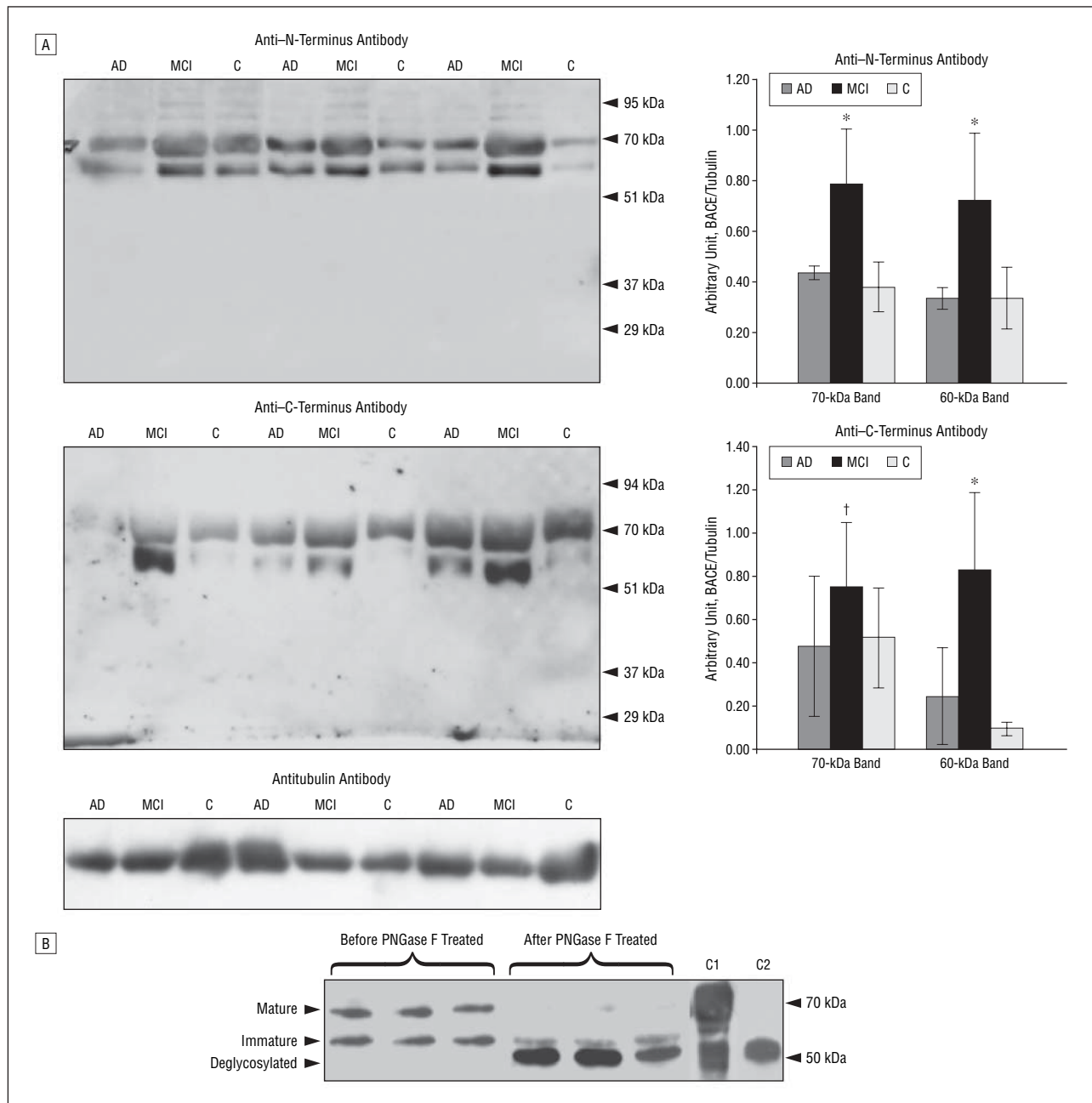
tical densitometry analysis showed a significant increase in the approximately 70-kDa and the approximately 60-kDa BACE1 bands in patients with MCI compared with those with AD and controls ( $\sim 70$  kDa: MCI vs AD,  $P = .04$ ; MCI vs controls,  $P = .029$ ;  $\sim 60$  kDa: MCI vs AD,  $P = .002$ ; MCI vs controls,  $P < .001$ ), whereas there was no significant difference between patients with AD and controls in either band ( $\sim 70$  kDa,  $P = .43$ ;  $\sim 60$  kDa,  $P = .27$ ) (**Figure 1A**). Similar results were also found by using antibodies raised against recombinant BACE1 protein, including anti-BACE1 polyclonal (BioSource) and anti-BACE1 monoclonal antibodies (R&D Systems Inc) as well as antibodies raised against different sites inside the Asp domain: B278 (aa366-378), B279 (aa295-310), and B280 (aa191-207) (data not shown). Both bands of BACE1 protein could be deglycosylated by peptide N-glycosidase F and resulted in a single 50-kDa species, which corresponds to the size of the deglycosylated form of BACE1 protein, indicating that mature and immature forms of BACE1 protein exist in the CSF (**Figure 1B**).

Because we found that the BACE1 protein level is significantly increased in patients with MCI compared with patients with AD and controls in a few samples in Western blot analysis, in the next step we quantitatively measured BACE1 protein levels in a large number of CSF samples by means of 2 BACE1 ELISAs (**Figure 2A**). The mean concentration of BACE1 in the CSF differed significantly between groups ( $F_{2,185} = 23.4$ ;  $P < .001$ ). The BACE1 protein levels in the CSF of patients with MCI were significantly higher than those in patients with AD and controls ( $P < .001$  for both), but the difference in BACE1 levels between patients with MCI vs AD was not significant ( $P = .79$ ). The interaction between sex and diagnosis was not significant ( $P = .52$ ), suggesting that the differences between diagnostic groups were not dependent on sex. Logistic regression analysis showed that increased levels of BACE1 were associated with increased risk of MCI compared with controls or AD and of AD compared with controls (**Table 2**).

To examine whether CSF BACE1 displayed enzymatic activity, we used fluorescent-labeled APP-derived peptide as the BACE1 substrate.<sup>3,4</sup> The main effect of the group comparison was significant ( $F_{2,198} = 32.04$ ;  $P < .001$ ). The post hoc test showed that BACE1 activity in the CSF of patients with MCI was significantly higher than that in patients with AD and controls ( $P < .001$  for both), but no significant difference between AD and controls was observed ( $P = .15$ ) (**Figure 2C**). The interaction between sex and diagnosis was not significant ( $P = .61$ ). Increased levels of BACE1 activity were associated with an increase in RR by a factor greater than 2 for patients with MCI compared with controls and patients with AD. However, elevated levels of BACE1 activity were not associated with increased risk of AD compared with controls (**Table 2**).

### CSF TOTAL A $\beta$ AND TOTAL TAU LEVELS

To determine whether increased levels of BACE1 correlate with increased CSF total A $\beta$  levels, we further examined CSF total A $\beta$  levels by means of sandwich ELISA (**Figure 3A**). Differences in CSF total A $\beta$  levels among patients with AD, those with MCI, and controls were sig-

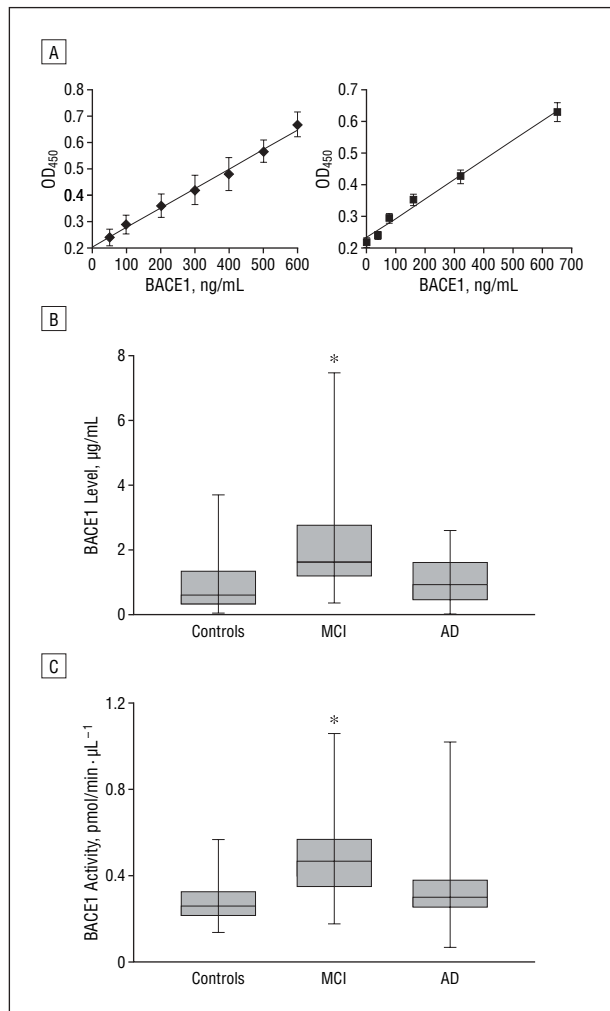


**Figure 1.** Cerebrospinal fluid (CSF)  $\beta$ -site amyloid precursor protein-cleaving enzyme 1 (BACE1) Western blot and deglycosylation. A, The CSF samples from controls (C), patients with mild cognitive impairment (MCI), and patients with Alzheimer disease (AD) were analyzed by means of Western blot. Equal volumes of CSF sample were separated on an 8% sodium dodecyl sulfate–polyacrylamide gel, and the proteins were then transferred onto a polyvinylidene difluoride membrane. Two different antibodies were used to detect BACE1 protein: anti-BACE1 N-terminus polyclonal antibody and anti-BACE1 C-terminus polyclonal antibody. Both antibodies detected the approximately 70-kDa and approximately 60-kDa BACE1 protein bands. Antitubulin was used as a non-AD loading control. Optical densitometry from anti-BACE1 N- or C-terminus blot was normalized to tubulin. B, Deglycosylation of CSF BACE1. The BACE1 was detected by the anti-BACE1 antibody (BioSource, Camarillo, Calif). Mature, immature, and deglycosylated forms of BACE1 are indicated. The BACE1 protein standard was used as a control. C1 indicates untreated recombinant BACE1 protein control; C2, recombinant BACE1 protein treated with peptide N-glycosidase F (PNGase F). Error bars represent SD. \* $P < .001$ . † $P < .05$ .

nificant ( $F_{2,156} = 11.94$ ;  $P < .001$ ), with a higher level in patients with MCI compared with patients with AD and controls ( $P < .001$  for both). The effect of diagnosis was not dependent on sex ( $P = .48$ ). No significant difference was observed between patients with AD and controls ( $P = .37$ ) (Figure 3B). Increased  $A\beta$  levels were associated with a higher RR to exhibit MCI compared with controls or AD. No elevated RR associated with levels of  $A\beta$  was present when patients with AD were compared with controls.

To study whether elevated BACE1 levels in CSF were related to neuronal death, we measured neuronal skeleton protein tau as a surrogate marker of neurodegeneration. We found that CSF total tau protein concentrations in patients with AD and MCI were significantly higher than in controls ( $F_{2,146} = 7.47$ ;  $P = .001$ ), with the difference between AD and MCI not reaching significance ( $P = .99$ ). The interaction between sex and diagnosis was not significant ( $P = .71$ ) (Figure 3C). Increased tau levels were associated





**Figure 2.** Cerebrospinal fluid (CSF)  $\beta$ -site amyloid precursor protein-cleaving enzyme 1 (BACE1) levels and activities. A, Two sandwich enzyme-linked immunosorbent assays (ELISAs) were established to measure BACE1 protein levels in CSF. Left, The capture antibody is anti-BACE1 N-terminus antibody SECB2, and the detection antibody is biotinylated anti-BACE1 polyclonal antibody SECB1.<sup>3</sup> Right, The capture antibody is anti-BACE1 polyclonal antibody B280, and the detection antibody is anti-BACE1 monoclonal antibody. Error bars represent SD. OD<sub>450</sub> indicates optical density at 450 nm. B, The CSF BACE1 protein levels in controls, patients with mild cognitive impairment (MCI), and patients with Alzheimer disease (AD). The mean  $\pm$  SD level of BACE1 in controls is 0.94  $\pm$  0.86  $\mu$ g/mL, in patients with MCI is 2.0  $\pm$  1.26  $\mu$ g/mL, and in patients with AD is 1.07  $\pm$  0.65  $\mu$ g/mL. C, The activity of BACE1 was measured by using synthetic peptide substrates containing the BACE1 cleavage site. The mean  $\pm$  SD BACE1 activity in controls is 0.28  $\pm$  0.08 pmol/min  $\cdot$   $\mu$ L, in patients with MCI is 0.49  $\pm$  0.19 pmol/min  $\cdot$   $\mu$ L<sup>-1</sup>, and in patients with AD is 0.33  $\pm$  0.12 pmol/min  $\cdot$   $\mu$ L<sup>-1</sup>. Horizontal lines represent medians; boxes, 25th and 75th percentile boundaries; and error bars, range. \**P* < .001.

with higher risk of AD compared with controls and for MCI vs controls. No elevated risk of AD compared with MCI was observed for tau (Table 2).

#### ASSOCIATION BETWEEN CSF MEASURES

Across groups, BACE1 levels significantly correlated with BACE1 activity ( $\rho=0.23$ ; *P* < .001). Levels of CSF total A $\beta$  were significantly correlated with CSF BACE1 protein levels ( $\rho=0.30$ ; *P* < .001) and CSF BACE1 activity ( $\rho=0.39$ ; *P* < .001).

**Table 2. Relative Risk Ratios for Group Comparisons Based on Univariate and Multivariate Prediction Models Controlled for Age**

Group Comparison	Predictor Model	Risk Ratio (95% CI)	Classification Accuracy, %
MCI vs controls	BACE1 level	2.08 (1.58-2.58)	76.0
	BACE1 activity	2.17 (1.66-2.71)	79.2
	Total A $\beta$ level	2.04 (1.62-2.44)	76.9
	Total tau level	1.28 (1.03-1.50)	63.5
	Combined	2.90 (2.15-3.65)	83.5
AD vs controls	BACE1 level	1.21 (1.03-1.40)	66.2
	BACE1 activity	1.17 (0.97-1.31)	67.8
	Total A $\beta$ level	1.19 (0.99-1.39)	65.7
	Total tau level	1.24 (1.08-1.40)	69.6
	Combined	1.24 (1.08-1.40)	69.6
MCI vs AD	BACE1 level	1.65 (1.19-2.03)	63.1
	BACE1 activity	3.71 (2.74-4.36)	76.3
	Total A $\beta$ level	1.86 (1.33-2.28)	65.0
	Total tau level	1.00 (0.99-1.00)	57.1
	Combined	4.77 (3.33-5.63)	80.4

Abbreviations: A $\beta$ , amyloid- $\beta$  peptide; AD, Alzheimer disease; BACE1,  $\beta$ -site amyloid precursor protein-cleaving enzyme 1; CI, confidence interval; MCI, mild cognitive impairment.

#### RRs FOR COMBINED PREDICTION MODEL

When combining all 4 CSF-based measures (levels of BACE1 protein, BACE1 activity, total A $\beta$ , and total tau), only BACE1 protein and BACE1 activity levels remained in the age-controlled model for discrimination between patients with MCI and controls. The combined model was associated with an almost 3-fold increase in RR for MCI, with a classification accuracy of 83.5% (Table 2). For the comparison between AD and controls, only tau level remained as a significant predictor in the model, with an RR of 1.24 (95% confidence interval, 1.08-1.40) and a classification accuracy of 69.6% (Table 2). All 4 CSF-based predictors contributed independently to the differentiation of MCI and AD, with an increase in the associated RR by a factor of almost 4.77 (95% confidence interval, 3.33-5.63) and an overall classification accuracy of 80.4% (Table 2).

#### IMMATURE BACE1 PROTEIN EXHIBITS LOW ENZYMATIC ACTIVITY

The BACE1 enzymatic activity in CSF was significantly correlated with its protein level; however, we noticed that the correlation is relatively low ( $\rho=0.23$ ; *P* < .001). This could be due to different enzymatic activity from these 2 forms of BACE1 protein. It has been reported that glycosylation is critical for BACE1 protein activity and that increased BACE1 maturation contributes to the increased activity.<sup>15</sup> Therefore, immature BACE1 could possess less enzymatic activity than the fully glycosylated mature protein. To find out whether immature BACE1 protein has less activity, we transfected 293T cells with a BACE1 expression construct. We expected to be able to detect 2 forms of BACE1 protein: mature and immature. We found that the pattern of BACE1 expression in cells transfected with a higher amount of pcDNA-

BACE1 (0.5  $\mu\text{g}$  per plate) is different from that in cells transfected with a lower amount of pcDNA-BACE1 (0.1  $\mu\text{g}$  per plate). Within 48 hours of transfection, cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 produced 2 major bands: approximately 60 kDa and approximately 50 kDa (**Figure 4A**); in contrast, in cells transfected with a lower amount of pcDNA-BACE1 (0.1  $\mu\text{g}$  per plate), 2 major bands appeared: approximately 70 kDa and approximately 60 kDa (**Figure 4A**). The band at approximately 70 kDa is consistent with the mature BACE1 protein in brain lysate (**Figure 4A**). In addition, the approximately 70- and approximately 60-kDa bands were consistent with the mature and immature forms, respectively, of BACE1 proteins reported by Huse et al.<sup>16</sup>

Optical densitometry analysis showed that the total BACE1 protein expression (including both bands) in cells transfected with 0.1  $\mu\text{g}$  per plate of pcDNA-BACE1 was approximately 3-fold lower than that in cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 (**Figure 4A**). Whereas the band at approximately 70 kDa was dramatically increased in cells transfected with 0.1  $\mu\text{g}$  per plate of pcDNA-BACE1 compared with cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 (**Figure 4A**), the band at approximately 60 kDa in cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 was 3.2-fold higher than the cells transfected with 0.1  $\mu\text{g}$  per plate of pcDNA-BACE1 (**Figure 4A**).

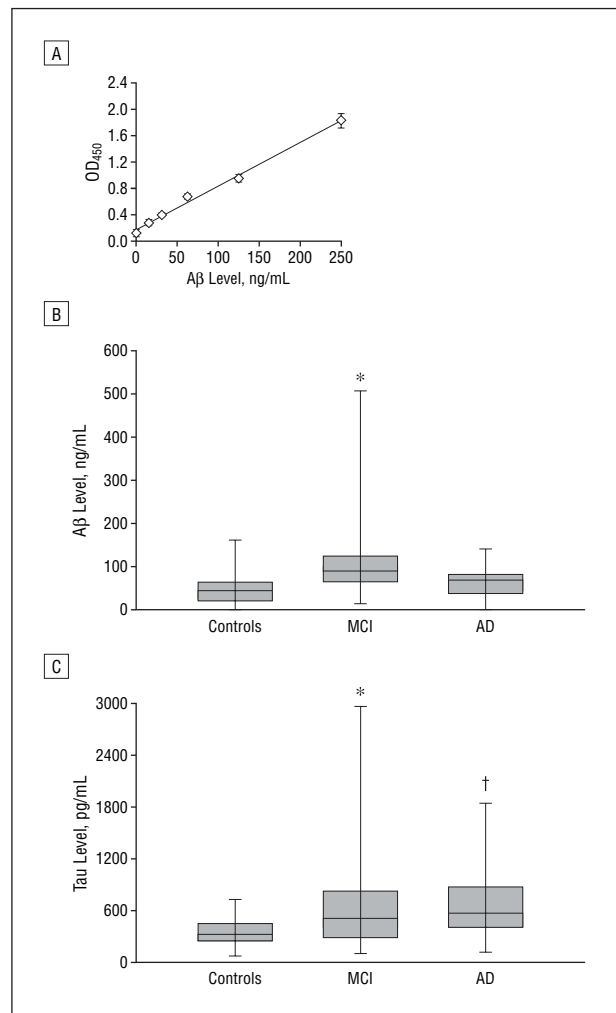
We next analyzed the BACE1 activity between these 2 cell systems with different expression levels of BACE1. After normalizing to the total BACE1 input as in the Western blot, BACE1 activity in cells transfected with 0.1  $\mu\text{g}$  per plate of pcDNA-BACE1 was almost 10-fold higher than that in cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 (**Figure 4B**). Therefore, BACE1 activity in transfected cells correlated with the appearance of the approximately 70-kDa mature form of BACE1 but not with the approximately 60-kDa immature form.

The origin of the approximately 50-kDa BACE1 band in cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 is not known. Deglycosylation analysis of cells transfected with 0.5  $\mu\text{g}$  per plate of pcDNA-BACE1 showed only 1 band at approximately 50 kDa, which is at the same position compared with the 50-kDa band shown in nonglycosylated samples (**Figure 4C**).

## COMMENT

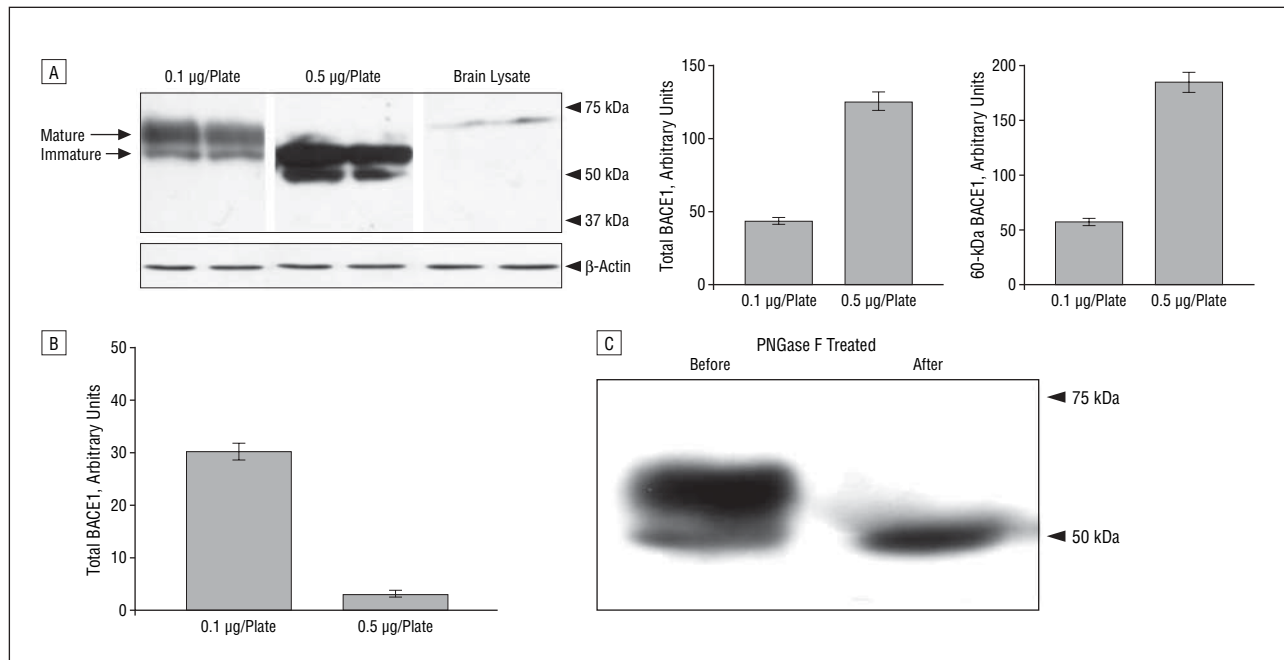
The BACE1 activities have been reported to be detectable in human CSF.<sup>6-8</sup> However, before this study, systematic analysis of possible changes in BACE1 level and activity in patients with MCI and AD had not yet been performed. In the present study we detected BACE1 levels and activities in the CSF of patients with AD or MCI and in healthy elderly individuals. We found that CSF BACE1 levels and activity were significantly altered in patients with MCI but not in those with AD compared with controls.

The CSF BACE1 levels were statistically correlated with its activity. However, the correlation was not very strong, possibly because (1) other  $\beta$ -secretase-like enzymes could exist in the CSF, which could affect the BACE1 activity assay, or (2) glycosylation interferes with the biological



**Figure 3.** Cerebrospinal fluid (CSF) total amyloid- $\beta$  peptide (A $\beta$ ) and total tau levels. A, The CSF total A $\beta$  concentration was measured by means of sandwich enzyme-linked immunosorbent assay (ELISA) with anti-A $\beta$  monoclonal antibody 4G8 as the capture antibody and biotinylated anti-A $\beta$  monoclonal antibody 7N22 as the detection antibody. Error bars represent SD. OD<sub>450</sub> indicates optical density at 450 nm. B, Total A $\beta$  levels in controls, patients with mild cognitive impairment (MCI), and patients with Alzheimer disease (AD) are shown. The mean  $\pm$  SD total A $\beta$  levels in controls is 42.03  $\pm$  37.55 ng/mL, in patients with MCI is 100.03  $\pm$  73.50 ng/mL, and in patients with AD is 63.37  $\pm$  33.88 ng/mL. C, The CSF total tau levels were measured using a sandwich ELISA constructed to measure total tau, including normal tau and hyperphosphorylated tau.<sup>13</sup> The mean  $\pm$  SD total tau level in controls is 356.4  $\pm$  144.2 pg/mL, in patients with MCI is 623.18  $\pm$  474.5 pg/mL, and in patients with AD is 645.16  $\pm$  349.7 pg/mL. Horizontal lines represent medians; boxes, 25th and 75th percentile boundaries; and error bars, range. \* $P$  < .001. † $P$  < .01.

activity of proteins and affects their folding and stability.<sup>17</sup> Recent proteomic studies showed glycosylation changes in AD.<sup>18</sup> The BACE1 has been identified as a 54-kDa transmembrane protein with 4 potential glycosylation sites<sup>16,19</sup>; 3 of the 4 potential sites were found to be glycosylated in the endoplasmic reticulum<sup>16</sup> and further processed in the Golgi complex, giving rise to a glycoprotein with heterogeneous oligosaccharides contributing to an apparent molecular weight of approximately 70 kDa of the mature Endo-H-resistant form of BACE1.<sup>19,20</sup> Glycosylation has been reported to play an important role in BACE1 enzymatic activity.<sup>15</sup> Increased BACE1 maturation contributes to increased BACE1 enzymatic activ-



**Figure 4.** Immature form of  $\beta$ -site amyloid precursor protein–cleaving enzyme 1 (BACE1) protein exhibits trivial activity. **A**, The BACE1 expression levels from 293T cells transfected with 0.5 and 0.1  $\mu$ g per plate of pcDNA-BACE1. Brain lysate was used as a control. Two major bands were found in cells transfected with 0.1  $\mu$ g per plate of pcDNA-BACE1, at approximately 70 and approximately 60 kDa, which represent the mature and immature BACE1, respectively. Two major bands were found in cells transfected with 0.5  $\mu$ g per plate of pcDNA-BACE1, at approximately 60 and approximately 50 kDa. In brain lysate, BACE1 protein migrated to the approximately 70-kDa position. Optical densitometry of the Western blot was measured by using FluorChem8900 (Alpha Innotech, San Leandro, Calif) software. Total BACE1 levels (including both detected bands) and the band at approximately 60 kDa were measured. Error bars represent SD. **B**, The BACE1 enzymatic activity was measured as described in the “BACE1 Enzymatic Activity Assay” subsection of the “Methods” section. BACE1 activity was normalized to the BACE1 protein level based on the densitometry analysis shown previously herein. Error bars represent SD. **C**, Deglycosylation of BACE1 protein in 293T cells transfected with 0.5  $\mu$ g per plate of pcDNA-BACE1. PNGase F indicates peptide N-glycosidase F.

ity and increased A $\beta$  production. When treated with tunicamycin, which inhibits N-glycosylation, BACE1 activity was reduced,<sup>21</sup> which is consistent with the present results showing that immature BACE1 protein exhibited much lower enzyme activity than the mature BACE1 protein. Glycosylation can also affect protein folding. This reminded us that, in an experiment and previous studies,<sup>22,23</sup> nonglycosylated BACE1 protein expressed in the *Escherichia coli* system could easily form insoluble inclusion bodies, suggesting that full glycosylation in mammalian cells could be critical for the solubility and stability of BACE1 protein. In the present study, BACE1 ELISAs detected total BACE1 levels. On the other hand, this study shows that BACE1 enzymatic activity correlates with mature BACE1 level instead of total BACE1 level; this might be why the correlation between BACE1 levels and activity was low in the present assay.

Note the different patterns present in 293T cells transfected with different amounts of pcDNA-BACE1. How the approximately 50-kDa band is produced is not yet known. Nonglycosylated BACE1 cannot be detected in cells under normal conditions because glycosylation occurs during translation, suggesting that the approximately 50-kDa band might not be the nonglycosylated form of BACE1. However, it only appeared in the higher amount of pcDNA-BACE1 transfection and disappeared in the lower amount of pcDNA-BACE1 transfection (Figure 4A), suggesting that above-normal expression in 293T cells transfected with 0.5  $\mu$ g per plate of pcDNA-BACE1 could be responsible for this. Huse et al<sup>16</sup> mentioned that, because of the presence of

SV40T in 293T cells, above-normal expression could occur. Deglycosylation and Western blot showed that, after treatment with peptide N-glycosidase F, only 1 band at approximately 50 kDa was detected in cells transfected with 0.5  $\mu$ g per plate of pcDNA-BACE1, suggesting that (1) the approximately 50-kDa band migrates to the same position as nonglycosylated BACE1 and therefore cannot be distinguished by Western blot or (2) above-normal expression of BACE1 could dramatically increase the burden of the glycosylation machinery and, therefore, under such conditions some newly translating BACE1 protein chains could be skipped by this machinery and produce such abnormal nonglycosylated BACE1 protein in cells. This explains why in cells transfected with 0.1  $\mu$ g per plate of pcDNA-BACE1 the approximately 50-kDa band disappeared.

Although the mechanism of generation of the approximately 50-kDa BACE1 remains to be further investigated, it provided a method to compare the activity of the immature and mature forms of BACE1. In the next BACE1 activity assay, BACE1 activity in cells expressing approximately 60- and approximately 50-kDa immature forms of BACE1 showed significantly lower activity than cells expressing approximately 70- and approximately 60-kDa BACE1 bands. The BACE1 activity increased as the approximately 70-kDa band increased, whereas the level of the approximately 60-kDa immature form decreased significantly in the low-amount pcDNA-BACE1 transfected cell, indicating that most BACE1 activity in transfected cells is correlated with the approximately 70-kDa mature form of BACE1,

whereas the approximately 60-kDa immature form of BACE1 exhibits only trivial activity.

Another concern is the possible interference of BACE2<sup>24</sup> and other  $\beta$ -secretase-like enzymes in CSF. The BACE2 is a protein homologous to BACE1<sup>24</sup>; however, we did not detect any BACE2 immunoreactivity in CSF by Western blot (data not shown), consistent with the previous study<sup>25</sup> that very low expression of BACE2 in the brain is nondetectable. In addition, although BACE1 and BACE2 are highly homologous, as demonstrated in recent studies on BACE2 structure, the structure of BACE2 revealed differences in the S3, S2, S1', and S2' active site substrate pockets compared with BACE1,<sup>26</sup> indicating that BACE1 and BACE2 show different substrate specificity. Also, Sun et al<sup>27</sup> found that an increase in BACE2 expression did not entail an increase in C99 production, such as that produced by BACE1. Although BACE2 could process APP, this occurs at a site other than that targeted by BACE1.<sup>27</sup> In the present experiment, up to 80% of CSF  $\beta$ -secretase activity could be inhibited in the presence of a BACE1 inhibitor (data not shown), indicating that the interference of BACE2 and other  $\beta$ -secretase-like enzymes can be ruled out.

The absence of full-length APP in the CSF by Western blot (data not shown) indicates that A $\beta$  in the CSF is not the result of CSF BACE1 activity. The A $\beta$  levels were significantly increased in the CSF of patients with MCI compared with patients with AD and controls. Similarly, the previous study showed that levels of CSF soluble APP $\beta$  were significantly increased in patients with MCI compared with controls, and no difference was detected between patients with AD and controls,<sup>28</sup> suggesting that BACE1 levels and activities inside the brain could already be starting to increase during the MCI stage.

Total tau and phosphorylated tau protein are well-characterized CSF markers for AD.<sup>29-35</sup> Because tau is a structural protein that normally enriches axons,<sup>30</sup> higher amounts of tau protein found in CSF would suggest increased neuron permeability and damage. However, although tau levels in patients with AD and MCI were higher than in controls, there was no significant difference between patients with AD and those with MCI (Figure 3C). In addition, no significant correlation between total tau and BACE1 levels was found (data not shown), indicating that permeability or cell damage is not a major contributor to the release of BACE1 protein into the CSF.

The BACE1 is a membrane-bound aspartyl protease, the release of which into CSF is still under investigation. Recently, Murayama et al<sup>36</sup> reported that a holoprotein of BACE1 was released extracellularly. Treatment with the metalloprotease inhibitor TAPI-1 increased BACE1 holoprotein release, suggesting that the release of BACE1 holoproteins may be a physiologically relevant cellular process.<sup>36</sup> In the present study, we found an increase in BACE1 levels in the CSF of patients with MCI compared with those with AD and controls on Western blot with no significant difference in tubulin levels, suggesting that the increase in CSF BACE1 levels could be specific to BACE1 rather than the result of a general protein-releasing mechanism.

On the other hand, it has also been reported that a higher inflammatory response was observed in patients with MCI compared with patients with AD<sup>37,38</sup> and that

inflammatory cytokines and free radicals can up-regulate BACE1 expression.<sup>39-43</sup> Moreover, some magnetic resonance imaging studies<sup>44-47</sup> show that ventricular size in patients with AD is enlarged owing to reduction in regional brain volume in patients with AD compared with patients with MCI, which could result in an increased volume of CSF so that protein concentration could be more diluted. However, because little has been reported about BACE1 levels or activities in the brains of patients with MCI, further research on BACE1 protein, activity, and messenger RNA levels in the MCI brain compared with other stages of AD is anticipated.

There is interest in MCI as a possible early stage of AD.<sup>48</sup> Although it has been shown that not all patients with MCI will finally convert to AD, it has been reported that more than 75% to 84% of the patients who are clinically diagnosed as having MCI convert to AD,<sup>49</sup> indicating that MCI is associated with an increase in the risk of progressing to AD and may represent an early stage of AD. Inability to discriminate AD from controls might compromise this application in diagnosing AD. However, early diagnosis of AD is important so that preventive therapy can be initiated as early as possible. The present assays show that BACE1 levels and activities in the CSF of patients with MCI are significantly increased, which may help diagnose MCI. This may provide an alternative method for the diagnosis of early-stage dementia.

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