Analysis of Heterogeneous βA4 Peptides in Human Cerebrospinal Fluid and Blood by a Newly Developed Sensitive Western Blot Assay* 

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The βA4 peptide, a major component of senile plaques in Alzheimer's disease (AD) brain, has been found in cerebrospinal fluid (CSF) and blood of both AD patients and normal subjects. Although βA4 1–40 is the major form produced by cell metabolism and found in CSF, recent observations suggest that the long-tailed βA4 1–42 plays a more crucial role in AD pathogenesis. Here, we established new monoclonal antibodies against the C-terminal end of βA4 1–40 and 1–42, and used them for the specific Western blot detection. After optimizing the assay conditions, these antibodies detected low picomolar levels of βA4 1–40 and 1–42 in CSF could be determined by direct loading of the samples. Blood levels of βA4 1–40 and 1–42 were also determined by specific immunoprecipitation followed by Western blot detection. We found that CSF βA4 1–42 level is lower in AD patients compared with non-demented controls, although there was a significant overlap between the groups. The level of βA4 1–40 in CSF, and of βA4 1–40 as well as βA4 1–42 in plasma, were not different between AD patients and controls. Besides the 4-kDa full-length βA4 band, we could also detect several N-terminal variants of βA4 in CSF and plasma of both AD patients and controls. Two N-terminally truncated βA4 species migrating at the position of 3.3 kDa were found in CSF, while 3.7- and 5.0-kDa forms were found in plasma. The relative abundance of these various species were considerably different in the CSF and plasma, suggesting that the cellular source and/or clearance of βA4 is different in these two compartments. 

A major neuropathological feature of Alzheimer’s disease (AD)1 is the presence of senile plaques in the brain. These extracellular deposits of fibrillar aggregates are mostly composed of a 4-kDa peptide called βA4 or β-amloid (1, 2). βA4 is a proteolytic fragment derived from larger protein precursor called amyloid precursor protein (APP) (3). Although βA4 exists as an aggregated, poorly soluble form in brain deposits, it is secreted from cells by normal metabolism as a soluble molecule and this soluble βA4 is also detected in cerebrospinal fluid (CSF) and blood of both AD patients and healthy controls (4–6). 

There are two major C-terminal variants of βA4, “short-tailed” βA4 1–40 and “long-tailed” βA4 1–42, with Val-40 and Ala-42 as C-terminal residues, respectively. βA4 1–40 is the major species secreted from cultured cells (7, 8) and found in CSF (9), while βA4 1–42 is the major component of brain deposits (10–16), suggesting the importance of this more hydrophobic variant (17) in the development of AD pathology. Also from cell transfection experiments, clinical APP mutants associated with early-onset familial AD (APP Val-717 to Ile, Gly, or Phe mutation) have been shown to secrete increased amounts of βA4 1–42, suggesting a link of this long-tailed βA4 to AD pathogenesis (18, 19). 

On the other hand, several recent biochemical and immunohistochemical analyses indicated that plaques and brain homogenates of AD and Down’s syndrome patients contain considerable amounts of N-terminally truncated βA4 (12, 13, 19–23). These truncated forms of βA4 are especially abundant in diffuse plaques, which are assumed to represent a premature form of plaques, suggesting an important role of these species in the initial phase of AD pathogenesis. N-terminal heterogeneity was also detected in βA4 isolated from cell culture conditioned medium (24) and pooled CSF (5, 9), although their levels in individual CSF samples have not been reported. So far, there is no information about N-terminal variants of βA4 in blood, and it is not known whether the N-terminal raggedness of soluble βA4 is altered during preclinical or clinical AD. 

Total βA4 levels in CSF have been examined by several groups. One study showed increased level of βA4 in early-onset AD (25), while others showed no significant difference between AD and controls (26–28). Recently, an ELISA specific for βA4 1–42 was established and applied to CSF measurement, showing the somewhat surprising results that the CSF βA4 1–42 level is lower in AD cases (29). Taken together, the contribution of soluble βA4 occurring in body fluids to AD pathogenesis remains obscure. 

So far, βA4 levels in body fluid samples were mostly measured by sandwich ELISA method. Although ELISA is a sensitive and simple method suitable for routine clinical use, one drawback in βA4 measurement is that it can be affected by the presence of other proteins which may cross-react or mask the epitope of antibodies used in the assay. In this report, we...
established a sensitive Western blot assay to specifically measure various βA4 variants. By using newly developed monoclonal antibodies that recognize the C-terminal end of βA4 1–40 (C40) and 1–42 (C42) for detection, we could determine βA4 1–40 and 1–42 levels in CSF by direct loading of samples. This assay was also applied to analyze βA4 1–40 and 1–42 levels in plasma, as well as N-terminally truncated variants of βA4 in CSF and plasma of both AD patients and control individuals.

**EXPERIMENTAL PROCEDURES**

**Establishment of Monoclonal Antibodies—βA4 C-Terminal specific monoclonal antibodies (mAbs) were generated as follows.** Synthetic peptides corresponding to βA4 33–40 (GLMGVGVV) or βA4 35–42 (MVGVVVIA) were conjugated with keyhole limpet hemocyanin or bovine serum albumin (BSA) through a cysteine residue added at the N terminus of the peptides. BALB/c mice were immunized with 50–100 μg of the peptides three to four times with about 2-week intervals. Three days after the final boost, spleen cells were isolated and fused with SP2/0 myeloma cells using polyethylene glycol 1500. Fused cells were cultured in HAT (hypoxanthine/aminopterin/thymidine) selection medium supplemented with 15% fetal calf serum, growth promoting reagent (HFCs, Boehringer Mannheim) and 4 μg/ml of human interleukin-6. Antibody-producing hybridoma cells were screened by solid phase ELISA, and positive clones were cultured twice by limited dilution. Two mAbs, G2–10 and G2–11, were selected as C40- and C42-specific mAbs, respectively. mAbs against βA4 N-terminal region were obtained from mice immunized with full-length βA4 1–40 or 1–42 with the same procedure, and one clone designated W0-2 was selected for further use. Isotypes of mAbs were determined by using an ELISA kit (Bio-Rad).

**Western Blot Assay for the Determination of βA4 1–40 and βA4 1–42 Levels in CSF—**For the determination of βA4 1–40, 8 μl of CSF sample was mixed with 4 μl of 3× sample loading buffer (6% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.3 mg/ml bromphenol blue in 188 mM Tris-HCl, pH 6.8), heated at 90°C for 10 min, and separated by 16% Tris-Tricine SDS-PAGE. Separated proteins in the gels were electrophoretically transferred onto nitrocellulose membrane at 380 mA for 45 min (30). The blotted membrane was heated in boiling phosphate buffered saline (PBS; 8.1 mM sodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, 137 mM sodium chloride, and 2.7 mM potassium chloride, pH 7.4) for 5 min to enhance the signal (31), and blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) buffer for 1 h. After washing twice with PBS-T, bound protein was detected by horseradish peroxidase-conjugated anti-mouse Ig secondary antibody (Amersham Corp.) followed by ECL detection system (Amer- sham) according to the manufacturer’s instruction. For the detection of βA4 1–42, 100 μl of CSF sample was vacuum-dried (Speed Vac), dissolved in 15 μl of 3× sample loading buffer, heated at 90°C for 10 min, and loaded onto a 16% Tris-Tricine SDS-PAGE gel. Western blot detection was performed by the same procedure employed for the βA4 1–40 detection, except that the membrane was blocked with 0.25% BSA instead of skim milk and that G2–11 antibody (6 μg/ml in PBS containing 0.25% BSA and 0.05% Tween 20) instead of G2–10 was used as primary antibody with 2 h incubation at room temperature. For the detection of total βA4, W0-2 antibody (1 μg/ml) was used as primary antibody for staining. In some experiments, 10–20% gradient Tris- Tricine gel (Novex) was also used. Band density was quantitated by densitometric analysis using Mac Bas (Fuji) program. In each case, known amount of synthetic βA4 1–40 or 1–42 was loaded onto the same gel and measured in parallel to draw calibration curves for quantification. The amount of the standard βA4 was determined by amino acid analysis.

**Determination of βA4 1–40 and 1–42 Levels in Plasma—**Plasma βA4 was analyzed by two-step immunoprecipitation: 1) purification of total βA4 from plasma by W0-2-coupled gel beads and 2) specific precipitation with G2–10 (for βA4 1–40 detection) or G2–11 (for βA4 1–42 detection) followed by Western blot detection. First, 700 μl of plasma sample was mixed with 70 μl of 10× immunoprecipitation buffer (250 mM Tris-HCl, pH 8.0, containing 5% Triton X-100 and 5% Nonidet P-40) and centrifuged at 13,000 rpm for 3 min. W0-2 antibody covalently coupled to Affi-Gel 10 (Bio-Rad) (11 μg of antibody coupled to 7.5 μl of gel) was added and incubated at 4°C for 2 h with continuous rocking of the tubes. After centrifugation and washing the pellet three times with PBS-T, bound proteins were eluted by the addition of 300 μl of 8 M urea, 0.1 M HCl buffer, pH 1.5, containing 0.1% SDS, 0.5% Triton X-100, and the supernatant was transferred to fresh tube containing 30 μl of 1 × Tris-HCl, pH 8.0, and 0.5% Tween 20. G2–10 antibody (4 μg) and 20 μl of protein G-agarose beads (Boehringer Mannheim) was added and incubated for 4 h at room temperature. Beads were collected by centrifugation and washed twice with PBS-T. Immunoprecipitated protein was solubilized from the beads by adding 80 μl of 8 M urea, 0.1 M HCl buffer and heated at 90°C for 10 min. The sample loading buffer was loaded onto 10% Tris-Tricine SDS-PAGE, and βA4 band was detected by Western blot using W0-2 as primary antibody. For βA4 1–42 detection, the supernatant after G2–10 immunoprecipitation was used for the assay. First, 20 μl of protein G-agarose (without antibody) was added and incubated for 2 h at room temperature to remove any remaining antibody, and then G2–11 antibody (4 μg) and 20 μl of protein G-agarose were added to the supernatant. Tubes were incubated at 4°C overnight, centrifuged, and precipitated proteins were eluted by 30 μl of 3× sample loading buffer and analyzed as described above. Known amount of synthetic βA4 1–40 and 1–42 peptides, diluted in 1 ml of 0.1 M Tris-HCl, pH 7.4, containing 0.25% BSA and 0.05% Tween 20, were analyzed in parallel and used as standards to calculate βA4 concentrations.

**Detection of N-terminally Truncated Forms of βA4 from CSF—**CSF samples (1 ml) mixed with 200 μl of Tris-HCl, pH 7.4, containing 0.25% BSA and 0.05% Tween 20 were incubated with W0-2 (4 μg) and protein G-agarose (20 μl) at 4°C overnight. After centrifugation, supernatant was transferred to fresh tube and again incubated with W0-2 (4 μg) and protein G-agarose (20 μl) at room temperature for 4 h, followed by supernatant transfer and incubation with protein G-agarose (20 μl) alone at room temperature for 2 h. During these three cycles of immunoprecipitation, most of the full-length βA4 was removed. Then, G2–10 antibody (4 μg) and protein G-agarose (10 μl) were added to the supernatant and incubated at 4°C overnight. Beads were collected by centrifugation, washed twice with PBS-T, and bound protein was solubilized by incubation with 15 μl of 3× sample loading buffer at 90°C for 10 min. Samples were separated by 16% Tris-Tricine SDS-PAGE and detected by Western blotting using G2–10 (3 μg/ml) for detection.

**Detection of N-terminally Truncated Forms of βA4 from Plasma—**Plasma samples (1 ml) were mixed with 100 μl of 10× immunoprecipitation buffer and centrifuged at 15,000 rpm for 3 min. G2–10 coupled Affi-Gel 10 (7.5 μg of antibody coupled to 5 μl of gel) was added to the supernatant and incubated at 4°C overnight. Gels were collected by centrifugation, washed three times with PBS-T, and bound protein was eluted by incubating with 15 μl of 3× sample loading buffer at 90°C for 10 min. Samples were separated by 16% Tris-Tricine SDS-PAGE and detected by Western blotting with G2–10 (3 μg/ml) for detection.

**Subjects—**CSF samples were collected from 59 subjects, and they were separated into three groups as follows; AD patients (n = 39), demented non-AD patients (n = 14), and non-demented patients with other neurological diseases (n = 11). Clinical diagnosis of AD was based on NINCDS/ADRDA criteria (32). Among AD cases, 11 were also affected with cerebrovascular diseases and therefore should be classified as possible AD. Demented non-AD cases include vascular dementia (n = 10) and Pick’s disease (n = 4). Non-demented cases include major depression (n = 6), schizophrenia (n = 2), mania (n = 1), personality disorder (n = 1), and Parkinson’s disease (n = 1). Samples were obtained by lumbar puncture and stored frozen until use.

Plasma samples (heparinized plasma) were collected from a different group of patients. Total number of samples measured was 63, consisting of early-onset AD (onset age < 65) (n = 20), late-onset AD (onset age ≥ 65) (n = 12) and healthy controls (n = 31). Summarized profiles of patients are listed in Table I (CSF samples) and Table II (plasma samples).

**RESULTS**

**Establishment and Characterization of Monoclonal Antibodies against βA4—**In order to obtain mAbs which are specific for the different C termini of short-tailed and long-tailed βA4, we immunized two mice with synthetic βA4 33–40 peptide and four mice with synthetic βA4 35–42 peptide, both conjugated to carrier protein. After ELISA screening and cloning of positive wells, we established 10 different clones (seven IgG class and three IgM class) specific for the C terminus of βA4 1–42 (C42) and five clones (four IgG class and one IgM class) specific for the C terminus of βA4 1–40 (C40). Unexpectedly, three out of five clones specific for C40 were obtained from mice immunized...
with 35–42 peptide. We focused on IgG class mAbs and evaluated their affinity and specificity. One of each mAbs from C40-specific clones and C42-specific clones was selected for further use and designated G2–10 (class IgG2b,κ) and G2–11 (class IgG1,κ), respectively. We also obtained eight mAbs that recognize both βA4 1–40 and 1–42 (all IgG class) from mice immunized with βA4 1–40 or 1–42 (two mice each). Epitopes of these eight clones were all mapped to the residues 1–16 of βA4.

All of these mAbs also reacted with secreted APP cleaved at the α-secretase site (APP\textsubscript{sec}) (34). The clone designated W0-2 (class IgG2a,κ), which showed the highest affinity, was selected for further analysis. Epitope of W0-2 was further examined using shorter peptides and the major recognition site was mapped to residues 5–8 of βA4. The specificities of the selected three antibodies were evaluated by solid phase ELISA and are demonstrated in Fig. 1. W0-2 reacted with βA4 1–40, 1–42, and 1–43 with nearly same affinity, as expected from its epitope. G2–10 was found to be completely specific for 1–40 in this assay. G2–11 did not cross-react with 1–40, but showed 1–2% of cross-reactivity with 1–43 peptide.

The three mAbs were next evaluated for their use in Western blot detection of βA4 (Fig. 2). After optimizing the assay conditions such as blotting time, membrane heat treatment, blocking reagent, antibody concentrations, and incubation times, G2–10 could detect 3 pg (0.7 fmol) of βA4 1–40 without any detectable cross-reactivity with 1–42. Using G2–11, we could visualize 12 pg (3 fmol) of βA4 1–40 without cross-reactivity with 1–40. W0-2, which recognizes both 1–40 and 1–42, turned out to be superior over the other mAbs in terms of the sensitivity and detected 0.4 pg (0.1 fmol) of βA4 1–40 and 1.6 pg (0.4 fmol) of βA4 1–42. The staining for 1–40 peptide was always about four times higher compared with 1–42, when the same amounts of peptide (determined by amino acid analysis) were loaded on the gels.

**Analysis of βA4 in CSF**—Direct loading of 8 μl of CSF onto Tris-Tricine SDS-PAGE gel followed by Western blot detection with G2–10 or W0-2 mAb gave one clearly detectable βA4 band (Fig. 3). APP\textsubscript{sec} was also detected by W0-2. When G2–11 was used, we could not see staining of a 4-kDa band of βA4 from direct loading of 8-μl samples (data not shown). However, when 100 μl of CSF was concentrated by vacuum-drying (Speed Vac) and loaded onto the gel, a βA4 1–42 band became visible (Fig. 3). We analyzed 59 CSF samples (34 AD patients, 14 demented non-AD patients, and 11 non-demented controls) and measured the levels of both βA4 1–40 and 1–42. βA4 concentrations were determined by densitometric analysis of the βA4 bands in comparison with the known amount of synthetic βA4 peptides. As shown in Fig. 4 and Table 1, there were no significant differences in βA4 1–40 levels between AD patients, demented non-AD patients, and non-demented control groups. For βA4 1–42 levels, control (non-demented patients) showed slightly higher levels, and the differences between the control group and the AD group, and between the control group and the demented non-AD group, were both statistically significant (p < 0.05, t test). However, there was no difference between the AD and the demented non-AD group, and considerable overlap was seen between control and demented groups. There was no obvious correlation between βA4 levels and disease severity.


**FIG. 4. βA4 1–40 and 1–42 levels in CSF samples.** βA4 levels were determined by densitometric analysis of the bands in comparison with known amounts of synthetic βA4 1–40 or 1–42 peptides loaded onto the same gel. AD, Alzheimer's disease patients; nonAD dem., demented non-Alzheimer's disease patients (10 vascular dementia and 4 Pick's disease); non-dem., non-demented patients with other neurological diseases. Bars indicate mean ± S.D. value of each group.

**TABLE I**  
*Patient profiles and βA4 levels of CSF samples*

|                      | AD            | Non-AD demented | Non-demented (other diseases) |
|----------------------|---------------|-----------------|------------------------------|
| **n**                | 24            | 14              | 14                           |
| **Age (year)**       | 73.6 ± 8.2a   | 72.4 ± 13.2     | 66.6 ± 11.1                  |
| Male/female (n)      | 13/21         | 6/9             | 3/8                          |
| MMSEb                | 15.7 ± 6.3    | 16.8 ± 6.3      |                              |
| Total protein (mg/dl)| 41.9 ± 15.5   | 35.8 ± 15.7     | 45.6 ± 14.0                  |
| βA4 1–40 (ng/ml)     | 2.65 ± 1.25   | 2.14 ± 0.77     | 3.04 ± 1.29                  |
| βA4 1–42 (ng/ml)     | 0.277 ± 0.105c| 0.282 ± 0.066d  | 0.501 ± 0.266                |

a Values represent means ± S.D.  
b Mini-mental state examination (33).  
c p < 0.05 compared with non-demented group.

(9-mini-mental state examination score) or age (data not shown).

**Analysis of βA4 in Plasma**—The Western blot assay was next applied to blood specimens. We found that plasma βA4 was not detectable by direct loading of samples, because of the low content of βA4 and of high total protein concentration that limit the possible loading volume onto the gels. Therefore, we used immunoprecipitation treatment before Western blot analysis. First, total βA4 was purified by immunoprecipitation with W0-2-coupled gel followed by acid elution. Then βA4 1–40 and 1–42 were precipitated with G2–10 and G2–11 antibody, respectively, and stained with W0-2 in Western blotting. Representative pictures of the exposed films are shown in Fig. 5. We applied the assay to 63 plasma samples (20 early-onset AD, 12 late-onset AD, and 31 normal controls) and determined the levels of βA4 1–40 and 1–42. Results are shown in Fig. 6 and Table II. In this study, we did not find significant differences between each groups for both βA4 1–40 and 1–42 levels. When analyzing βA4 1–40, besides full-length βA4 1–40, one additional minor band at 5 kDa was detected that was not observed in CSF samples. From longer exposure of the films, this 5-kDa band was detected from all of the samples, and the amount of the 5-kDa band seemed to be proportional to the 4-kDa βA4 band.

**Detection of N-terminally Truncated Forms of βA4**—We next tried to detect N-terminally truncated forms of βA4 in CSF and plasma samples in order to find out whether there is any difference in their levels between AD patient and controls. From direct loading of CSF samples, we could not detect any band which migrate below full-length βA4 (Fig. 3). Immunoprecipitation with G2–10 followed by the detection with the same antibody in Western blotting was also not successful, because the strong signal of full-length βA4 1–40 on the exposed film interfered with the detection of the closely migrating N-terminally truncated βA4 species. Therefore, we used W0-2 for immunodepletion of the full-length βA4 prior to the immunoprecipitation and Western blot detection with G2–10. From 1 ml of CSF, two bands below the full-length βA4 were visualized in this assay (Fig. 7). From longer exposure of the film, these
two bands were detected in all of the nine samples analyzed. The molecular weight of the upper and the lower bands were estimated to be 3.7 and 3.3 kDa, respectively. The 3.3-kDa band migrated slower than “p3” band derived from conditioned medium of SY5Y cells transfected with SPA4CT fragment (100 amino acid peptide of APP C-terminal plus signal peptide) (35). Because the “p3” peptide comprises residues 17–40 of βA4, it suggests that both the 3.7- and 3.3-kDa species are N-terminally extended from Leu-17. Although the amount of these truncated βA4 differed considerably between each samples, these peptides were detected both in AD and control CSF samples, and their amount did not correlate with the AD diagnosis. When G2–11 instead of G2–10 was used to precipitate N-terminally truncated βA4 species ending at Ala-42 (C42), no band below 4 kDa was detected (data not shown).

In contrast to the results of CSF, some plasma samples were shown to contain N-terminally truncated βA4 with comparable amounts to full-length βA4. When 700 μl of plasma (12 samples, 6 AD, and 6 controls) were analyzed by G2–10–coupled gel immunoprecipitation (without immunodepletion of full-length βA4) and Western blot detection, the 3.7-kDa band was found in five samples (three AD and two controls) (Fig. 8). The amount of this band varied considerably between each sample and did not correlate with the amount of full-length βA4 1–40, which is in contrast to the 5-kDa band in Fig. 5, the intensity of which was proportional to that of the 4-kDa βA4 band. There was no obvious correlation between the amount of the 3.7-kDa band and the diagnosis.

**Discussion**

Although amyloid deposition in the form of senile plaques is a central feature of AD pathology, the source of βA4 in the deposits is not known. Since the amyloid exists in extracellular spaces which are in direct contact with CSF, one possible model for plaque formation is that increased production (or decreased clearance) of βA4 in AD causes accumulation of soluble βA4 in CSF, which then is converted to aggregated forms or directly deposits onto a nucleating aggregate of βA4. There are also observations that suggest the possibility of a hematogenic origin of βA4 in brain deposits (15, 36). In order to address these issues, it is essential to analyze βA4 levels in body fluids of individual patients. Especially, when considering the recent observations that suggest a critical role of long-tailed βA4 1–42 in plaque formation, it is becoming more desirable to measure the levels of βA4 1–40 and 1–42 separately. For this purpose, we, first, developed several mAbs which are specific for the C termini of βA4 1–40 (C40) and βA4 1–42 (C42). Unexpectedly, three of the five clones specific for C40, including G2–10, were obtained from the mice immunized with 35–42 peptides. These clones were obtained from two mice, both of which also produced C42 specific clones. Although we do not have an explanation for this unexpected outcome, we assume that there is a possibility that the 35–42 peptide was processed in vivo in those mice, and the newly generated peptides with C40 end may have served as immunogen.

mAbs with the highest affinities were selected and applied for Western blot detection of βA4. In order to increase the sensitivity of our detection system, we optimized the assay conditions such as membrane blotting time, blocking reagents, antibody concentrations, and staining times. Blocking reagent was one of the critical factors for high sensitivity. For example, we found that for the staining with G2–11, BSA was better compared with the commonly used skim milk, which caused much higher backgrounds in this assay (data not shown). We also found that the heat treatment of the nitrocellulose membrane after the blotting is essential for high sensitivity. For heating the membranes, we used boiling PBS instead of water, which is used in the initial report of this technique (31), since the signal enhancement was much more pronounced in PBS compared to water (data not shown). Although the mechanism of this signal enhancement is not clear, we found that this heat treatment is effective for several other antigen-antibody combinations (data not shown), indicating the general usefulness of this method for sensitive Western blot analysis. After these assay conditions were optimized, both C40-specific mAb (G2–10) and C42 specific mAb (G2–11) could detect βA4 peptides in the low picogram range. The sensitivity obtained with W0-2 (0.4 pg for βA4 1–40, 1.6 pg for 1–42) was especially remarkable and is more than 1000 times higher compared with the previous reports on βA4 detection by Western blot (6, 25). The reason why the βA4 1–40 synthetic peptide always gives a stronger signal compared with the same amount of βA4 1–42 is not clear. One possible explanation is that 1–40 and 1–42 peptides have different conformations and the binding affini-
ties to the antibody is not the same. However, we think that it is more plausible that 1–40 and 1–42 peptides bind to nitrocellulose membranes with different efficiency, since this difference in signal intensity between 1–40 and 1–42 was also seen by other antibodies directed against N-terminal part of βA4 (data not shown) and since W0-2 reacted with 1–40 and 1–42 peptides with the same efficiency by the ELISA evaluation (Fig. 1).

By using this Western blot assay, we could directly quantitate both βA4 1–40 and 1–42 in CSF samples. We found that there was no need for an immunoprecipitation step. We argue that this direct detection has the advantage to measure total βA4 levels, since βA4 in CSF is reported to bind to carrier proteins such as apolipoprotein δ (apoJ) (37, 38), apoE (39), and transthyretin (40), and if antibody epitopes of βA4 are masked by such binding proteins, it cannot be detected by sandwich ELISA or Western blot analysis which depends on a prior immunoprecipitation step.

Our results showed that there is no difference in the βA4 1–40 levels among AD patients, demented non-AD patients, and non-demented cases. In contrast, we found that some of the non-demented CSF samples contain higher levels of βA4 1–42 compared with demented cases. The mean values of CSF βA4 1–42 in non-demented cases (0.501 ng/ml) was 1.8 times higher compared with AD cases (mean = 0.277 ng/ml) or non-AD demented cases (0.282 ng/ml). This result is consistent with the recent ELISA measurement by Motter et al. (29), which showed similar 1.7 times increase when non-demented controls (mean = 0.632 ng/ml) were compared with AD patients (mean = 0.383 ng/ml). In our assay, however, more considerable overlapping of the values between control and AD samples was observed, and we found no difference between AD and non-AD demented groups, suggesting that the measurement of βA4 1–42 levels in CSF may not be useful for diagnostic purpose. A correlation with disease severity (mini-mental state examination score) or age of patients was also not clear, although we think that this must be further examined with larger number of samples.

As to the blood levels of βA4, there are not many studies in the literature, probably because of the difficulty to detect the low levels of βA4 in the blood. Very recently, one group measured βA4 1–40 and 1–42 levels in the plasma by an ELISA method and reported that familial Alzheimer’s disease patients of a Swedish family having a clinical APP mutation (APP Ly5-670 to Asn, Met-671 to Leu double mutation) have two to three times higher plasma concentration of both βA4 1–40 and 1–42 compared with non-carrier of the mutant gene (41). Furthermore, they also reported that a clinical presenilin-1 (S182) gene mutation in another familial Alzheimer’s disease case also had about two times higher βA4 1–42 levels in plasma (42). In sporadic AD cases, this increase of blood βA4 levels was less prominent, but still about 10% of patients are reported to have elevated levels of βA4 1–42 (41). In our study, which includes mainly sporadic AD patients, no such increase was observed both for 1–40 and 1–42 levels. It must be clarified in future studies whether this discrepancy is simply because our assay for blood samples was not accurate enough to detect this subtle increase of 1–42 levels or because of some other reason such as different severity of the disease in the two patient groups or due to the different assay methods used in these two studies.

With our Western blot assay, we were also able to detect N-terminally truncated forms of βA4 from CSF and blood. The measurement of N-terminally truncated βA4 in individual samples was of considerable interest, since recent studies indicated that brain deposits contain large amounts of these shortened βA4 species along with full-length βA4, and especially the diffuse plaques, which are assumed to be a premature form of senile plaques, are reported to contain N-terminally ragged forms as the major component, suggesting its contribution in early stages of AD pathogenesis (21). In CSF, we detected two different forms of N-terminally truncated βA4 species, which are ending at C40 and migrating at a positions corresponding to 3.3 and 3.7 kDa. In comparison with the results of former biochemical analyses using pooled CSF (9), we assume these two band are most likely to be βA4 11–40 and βA4 6–40. Although βA4 ending at Leu-34 was also identified as one of the major components (9), we could not detect this C-terminally truncated form in our assay using W0-2 for staining (Fig. 3). We were also unable to detect the p3 peptide (βA4 17–40), which is abundant in cell culture conditioned medium, suggesting either the ratio of various βA4 species produced in vivo and in the cultured cells are different or the in vitro clearance of p3 peptide is faster than that of the longer βA4 species. The amount of truncated βA4 species was very low and visible only after the immunodepletion of full-length βA4. There was no obvious correlation between their amount and the diagnosis of AD, in this measurement of a relatively limited number of samples. However, since we could not detect N-terminally truncated βA4 species ending at C42, there is still the possibility that the levels of this more hydrophobic peptide is different between AD patients and controls and that this long-tailed βA4 species could contribute to AD pathogenesis. Further studies will be needed to examine this possibility.

There were several differences between the N-terminally truncated variants of βA4 found in CSF and in plasma. In plasma, we detected only a 3.7-kDa form and relative amount of this molecule (p3.7/full-length βA4 ratio) was much higher compared with CSF. We also detected a 5-kDa band only in plasma. This band presumably represents an APP fragment generated by cleavage at the C40 end of βA4 and at N-terminal upstream of βA4 residue Asp-1 (43). These differences between plasma and CSF suggest that the cellular sources and/or metabolism of βA4 are different in these two compartments.

The results of this study do not support the hypothesis that the βA4 deposition in the brain of AD patients is caused by extracellular accumulation of soluble βA4. There still remains a possibility that minor structural variants of βA4, such as Glu3-pyroglutamated βA4 (23), βA4 isomerized at Asp or Ser residues (44, 45), or conformational variant of βA4 (46) are increased in CSF of AD patients and contribute to the pathogenesis, since our assay may not distinguish these species from unmodified forms. However, we rather assume that soluble βA4 in body fluids is neither a useful marker of the disease without additional information, nor does it play a direct role in the pathogenesis, at least in cases of sporadic AD. Whether the mechanism of βA4 deposition between familial AD and sporadic AD is the same or not is an important issue to be addressed in future studies.

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