in patients with iNPH. We performed PIB-PET study in patients with "pure" iNPH without any clinical signs of AD. We found that small fraction of pure iNPH patients had cortical PIB retention, but with quite a different distribution pattern from that seen in AD.

Subjects and Methods

Subjects

Ten patients with iNPH (six men and four women, aged 79.2 ± 5.0 years, 24.5 ± 1.9 in Mini-Mental State Examination (MMSE) scores (mean ± standard deviation)) were enrolled in this study (Table 1). Among the iNPH patients, none had obvious dementia with impairment of usual activities of daily living, but seven of them were diagnosed as having mild cognitive impairment (MCI) because of their cognitive concern reported by themselves or their caregivers and cognitive tests (Table 1). Cerebral retention of PIB in those iNPH patients was compared with those in seven age-matched AD patients (four men and three women, aged 70.4 ± 11.3 years). The iNPH patients consisted of those with probable (n = 8) or definite (n = 2) iNPH, diagnosed according to the Japanese guidelines for iNPH [7]. The AD patients were diagnosed as probable AD according to the standard clinical criteria [8]. Before diagnosis, all the iNPH subjects underwent clinical examinations including neurological and neuropsychological examinations, routine blood analyses, brain magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT) cerebral blood flow imaging. This study included only those iNPH patients confirmed to have neither AD-like brain atrophy in medial temporal lobes by MRI nor AD-like hypoperfusion in parietotemporal, posterior cingulate or precuneus cortices by SPECT imaging.

All the examined subjects provided written informed consent to participate in the study, which was approved by the University Ethics Committee (Kyoto Prefectural University of Medicine, Kyoto, Japan).

Acquisition and Analysis of PIB-PET Data

Pittsburgh compound B (PIB: *N*-Methyl-[^{11}C]2-(4-methylaminophenyl)- 6-hydroxybenzothiazole) was prepared at Nishijin Hospital (Kyoto). PIB radiosynthesis was performed using a simplified method reported by Wilson et al. [9]. PIB was injected intravenously (555 ± 185MBq, 15 ± 5mCi) as a slow bolus for 10-20 seconds. PET scanning was performed using a Siemens ECAT ACCEL scanner (Siemens Medical Systems, Erlangen, Germany) equipped with LSO crystals (3D mode, 16.2 cm field of view, 47 planes) with 35 dynamic frames acquired over 70 min. The ECAT ACCEL has a transaxial spatial resolution of 6.3 mm full width at half maximum (FWHM) measured at 1 cm off-axis, and of 6.8 mm measured at 10 cm off-axis. The axial resolution is 4.7 mm at the centre of the system. Attenuation correc-

Table 1 Demographics and clinical characteristics of patients with iNPH.

Case No.	Diagnosis	Age (y) / Sex	MMSE	FAB	Symptoms G	Symptoms D	Symptoms U	3mTUG Time (sec)	3mTUG Steps	Cortical retention of PIB
1	Probable	78 / F	27	13	+	–	–	12.5	19.5	–
2	Definite	80 / F	24	9	+	±	–	15.4	27.4	–
3	Probable	84 / F	26	16	+	±	+	20.6	26.6	+
4	Probable	82 / M	28	12	+	–	+	10.7	16.0	–
5	Probable	81 / M	24	12	+	±	+	30.2	56.8	–
6	Probable	88 / M	22	11	+	±	+	17.4	30.2	+
7	Probable	73 / M	24	12	+	±	–	13.6	18.2	–
8	Probable	72 / M	23	16	+	±	+	23.5	43.3	–
9	Definite	75 / F	24	15	+	±	–	14.2	21.0	+
10	Probable	79 / M	23	14	+	±	+	21.8	27.0	–

MMSE = Mini-Mental State Examination; *FAB* = Frontal assessment battery; *PIB* = Pittsburgh compound B; *G* = gait disturbance; *D+* = dementia; *D±* = mild cognitive impairment; *U* = urinary disturbance; *TUG* = timed up-and-go test. Required time and steps of 3mTUG were the mean values of 3 tests.

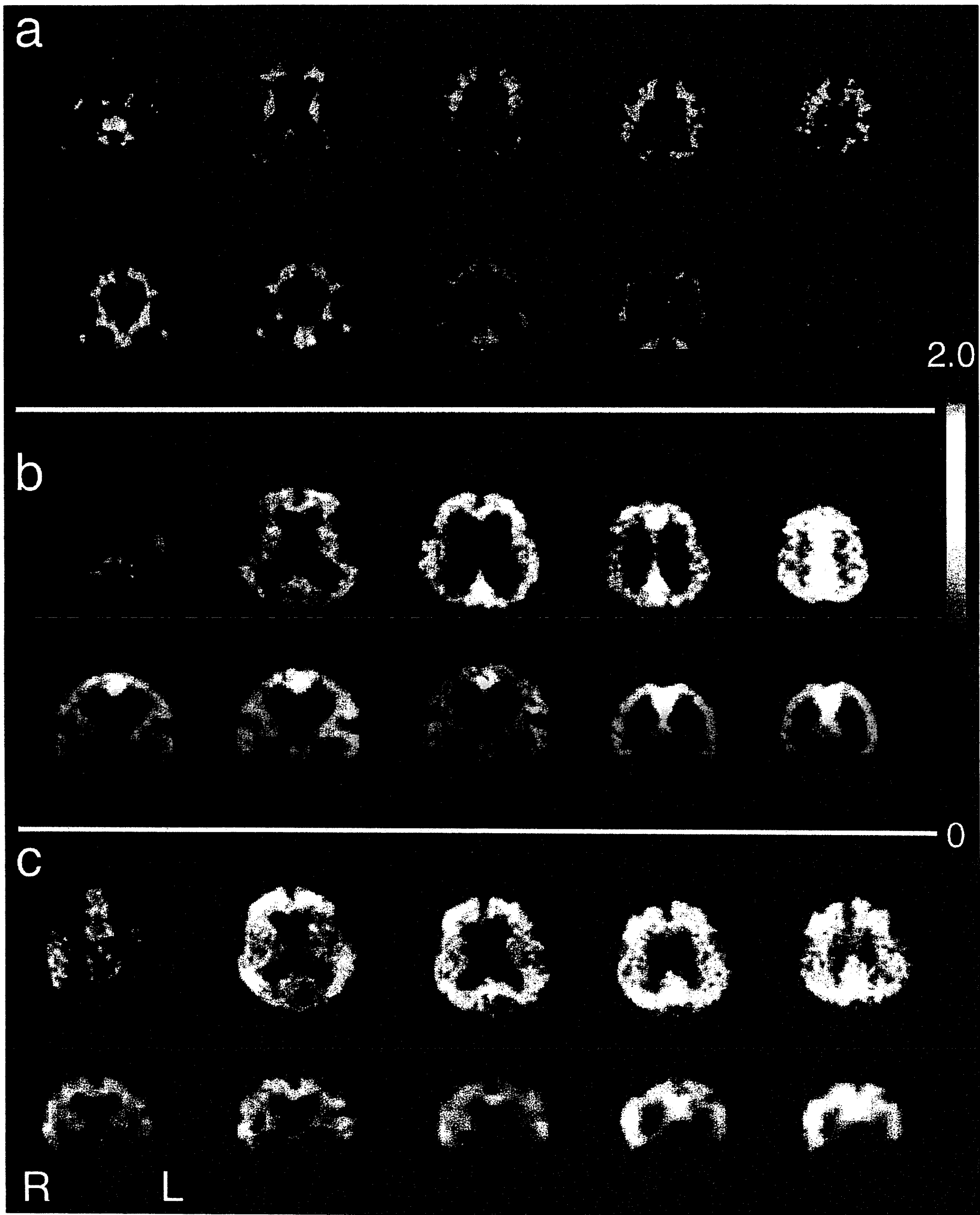

Figure 1 PIB-PET in patients with iNPH and AD. Representative axial and coronal images of PIB-PET in the iNPH patient without (A, patient 1 in Table 1) and with (B, patient 9) cortical PIB retention and AD (C) showing different patterns of cortical PIB retention between the two diseases. The color bar on the right indicates the standardized uptake values.

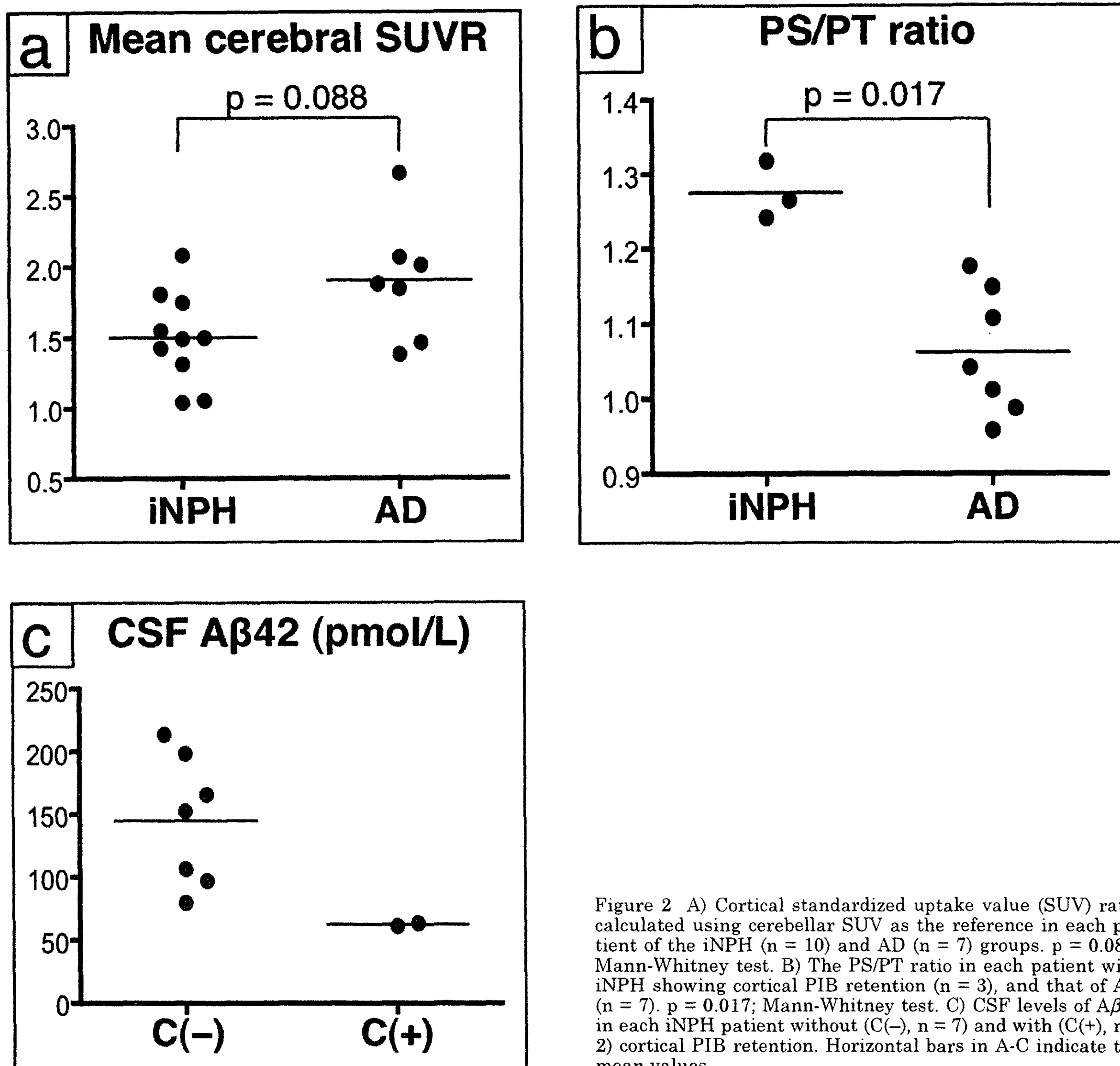

Figure 2 A) Cortical standardized uptake value (SUV) ratio calculated using cerebellar SUV as the reference in each patient of the iNPH (n = 10) and AD (n = 7) groups. p = 0.088; Mann-Whitney test. B) The PS/PT ratio in each patient with iNPH showing cortical PIB retention (n = 3), and that of AD (n = 7). p = 0.017; Mann-Whitney test. C) CSF levels of Aβ42 in each iNPH patient without (C(−), n = 7) and with (C(+), n = 2) cortical PIB retention. Horizontal bars in A-C indicate the mean values.

tion was performed using transmission scans, obtained using three rotating germanium-68 rod sources. PET data were reconstructed using the ordered subsets expectation maximization (OSEM) algorithm, with six iterations and 16 subsets. The reconstructed images were displayed in coronal, transverse and sagittal planes for analysis.

Regions of interest (ROI)s were manually generated for 18 regions on each PET image by one experienced neurologist and one experienced radiologist familiar with the neuroanatomy (MK and SM). The locations of the ROIs included bilateral anterior cingulate gyri, frontal cortices (dorsal and ventral), lateral temporal cortices, medial temporal cortices (amygdala and hippocampus), lateral parietal cortices, occipital cortices, precuneus and cerebellum, while parasagittal areas included anterior cingulate gyri and precuneus, which were modifications of a previous method to concentrate ROIs in cortical areas [10]. PMOD software (version 3.2, PMOD Technologies Ltd., Zurich, Switzerland) was used to extract count data for specified ROI. A mean cortical PIB retention measurement was also computed across cortical ROIs without including the cerebellum. The PIB retention outcomes were evaluated

based on the standardized uptake value (SUV) measures. SUVs were determined over 50-70 minutes post injection intervals and normalized to injected dose and body mass. SUV ratio (SUVR) was generated using the cerebellum as reference.

Coronal sections of PIB-PET images across precuneus areas were analyzed using an arbitrary index, SUVR measured in parasagittal (PS) and parietotemporal (PT) areas (the PS/PT ratio). The PS/PT ratio was calculated using the formula:

$$PS/PT \text{ ratio} = (\text{right } PS/PT + \text{left } PS/PT)/2.$$

Measurement of Aβ42 in Cerebrospinal Fluid

To evaluate the extent of amyloid burden in each patient's brain, cerebrospinal fluid (CSF) levels of β-amyloid 1-42 peptide (Aβ42) which are known to decrease inversely with the extent of cerebral amyloid deposition were determined by sandwich ELISA according to the manufacturer's instructions (Human/Rat β Amyloid (42) ELISA Kit, Wako, Osaka, Japan) in nine of the ten patients with iNPH who agreed to undergo the test.

Statistical Analysis

We compared iNPH and AD groups using the Mann-Whitney U test. The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS (Dr SPSS II version, SPSS Japan Inc., Tokyo, Japan).

Results

Three of the ten patients with iNPH, all of whom were diagnosed as MCI in cognitive tests, showed increased cortical PIB retention, especially in the bilateral high-convexity parasagittal areas (Table 1, Figure 1B). The mean cortical SUVRs, calculated using cerebellar SUV as the reference, did not significantly differ between iNPH and AD patients (Figure 2A). However, the distribution of cortical PIB retention was clearly different between the two groups. PIB retention was limited to the high-convexity parasagittal areas in iNPH patients, while it spread over the frontal and parietotemporal areas in AD (Figure 1B). This was confirmed by the mean PS/PT ratio that was significantly higher in iNPH patients with cortical PIB retention than in AD patients ($p=0.017$) (Figure 2B). The mean cortical SUVR had no significant correlation either with MMSE score or measures of timed up-and-go (TUG) tests (required time or steps) in iNPH patients (data not shown).

The levels of CSF Aβ42 were measured in nine patients with iNPH, including two cases with higher cortical PIB retention; those two patients showed the lowest levels of CSF Aβ42 among the nine iNPH patients (Figure 2C). This low CSF level indicates that there is an actual deposition of Aβ amyloid in the brain, and PIB retention in iNPH would not therefore reflect a delay in the clearance of PIB from the brain tissue but its real binding to cerebral Aβ amyloid.

Discussion

We have demonstrated here that three out of ten (30%) patients with iNPH without any clinical signs of AD had obvious cortical retention in PIB-PET, indicating that iNPH is one of the PIB-positive diseases. We also revealed that the pattern of cortical PIB retention seen in iNPH patients was characteristic of iNPH, limited to the high-convexity parasagittal areas showing a high PS/PT ratio (Figure 2B). This pattern was apparently different from that in AD with high retention in parietotemporal cortices showing a low PS/PT ratio. All three iNPH patients with positive cortical PIB retention were diagnosed as having MCI in cognitive tests. It will be very interesting to see whether or not and how fast their cognitive functions will deteriorate in the course of their iNPH, compared with the usual course of AD. We are therefore planning a longitudinal follow-up on these patients.

Our data also suggest that differentiation between cognitive decline due to iNPH alone and due to comorbid AD pathology may be possible, not by the CSF Aβ42 levels, but by the distribution pattern of PIB-positive cortical amyloid. This was most effectively judged based on coronal images rather than conventional transverse images. These results indicate the possible role of PIB-PET in the diagnosis of the etiology of dementia in iNPH, which could be valuable in considering a shunt indication.

Aβ-amyloid deposition depends on the local balance between production and clearance. It has been reported that local Aβ production increases in specific brain regions correlating with synaptic activity or aerobic glycolysis [11,12]. In AD patients, some of those regions belong to

a group of brain areas called the default mode network [13]. Thus, the deposition of Aβ amyloid in AD brain is caused by the combination of both increased local Aβ production in parieto-temporal cortices and decreased Aβ clearance due to aging.

On the other hand, in the iNPH patients' brains, cortical Aβ deposition is conceivably attributed mainly to the decrease in local Aβ clearance. It is well-known that CSF turnover is much slower in iNPH [14]. Since the upper half of the brain is passively lifted cranially in these patients, the high-convexity parasagittal regions will be compressed and there will be less interstitial fluid [15]. This could lead to a decline in local clearance of Aβ, which would ultimately lead to local Aβ-amyloid depositions.

There is a possibility that the cortical PIB retention in iNPH might be attributable to other causes. One possibility is that it does not reflect a real accumulation of Aβ amyloid but merely picks up the delay in the clearance of administered PIB from the brain due to the slower turnover of CSF [14]. However, we consider PIB retention in iNPH would reflect the actual accumulation of Aβ amyloid because the iNPH patients with high cortical PIB retention showed lower CSF levels of Aβ42, indicating the presence of actual amyloid deposition in the cerebral cortex.

Conclusion

iNPH is one of the diseases that may present cortical PIB retention. However, the distribution of PIB retention differs widely from that in typical AD patients and is limited to the high-convexity parasagittal areas, thus distinguishing the two conditions.

Acknowledgements

TT received research support from the Ministry of Health, Labour and Welfare of Japan (Grants-in-Aid from the Research Committee of CNS Degenerative Diseases).

References

1 Bech-Azeddine R, Høgh P, Juhler M, et al. Idiopathic normal-pressure hydrocephalus: clinical comorbidity correlated with cerebral biopsy findings and outcome of cerebrospinal fluid shunting. J Neurol Neurosurg Psychiatry. 2007; 78: 157-161.
2 Hamilton R, Patel S, Lee EB, et al. Lack of shunt response in suspected idiopathic normal pressure hydrocephalus with Alzheimer disease pathology. Ann Neurol. 2010; 68: 535-540.
3 Klunk WE, Engler H, Nordberg A, et al. Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann Neurol. 2004; 55: 306-319.
4 Rowe CC, Ng S, Ackermann U, et al. Imaging β-amyloid burden in aging and dementia. Neurology. 2007; 68: 1718-1725.
5 Golomb J, Wisoff J, Miller DC, et al. Alzheimer's disease comorbidity in normal pressure hydrocephalus: prevalence and shunt response. J Neurol Neurosurg Psychiatry. 2000; 68: 778-781.
6 Leinonen V, Koivisto AM, Alafuzoff I, et al. Cortical brain biopsy in long-term prognostication of 468 patients with possible normal pressure hydrocephalus. Neurodegener Dis. 2012; 10: 166-169.
7 Ishikawa M, Hashimoto M, Kuwana N, et al. Guidelines for management of idiopathic normal pressure hydrocephalus. Neurol Med Chir (Tokyo). 2008; 48: S1-S23.
8 McKhann G, Drachman D, Folstein M, et al. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology. 1984; 34: 939-944.
9 Wilson AA, Garcia A, Chestakova A, et al. A rapid one-step radiosynthesis of the β-amyloid imaging radiotracer N-methyl-[^{11}C]2-(4β-methylaminophenyl)-6-hydroxybenzothiazole ([^{11}C]-6-OH-BTA-1). J Label Compd Radiopharm. 2004; 47: 679-682.
10 Rosario BL, Weissfeld LA, Laymon CM, et al. Inter-rater reliability of manual and automated region-of-interest delineation for PiB PET. Neuroimage. 2011; 55: 933-941.
11 Cirrito JR, Yamada KA, Finn MB, et al. Synaptic activity regulates interstitial fluid amyloid-β levels in vivo. Neuron. 2005; 48: 913-922.
12 Vlassenko AG, Vashnavi SN, Couture L, et al. Spatial correlation between brain aerobic glycolysis and amyloid-β (Aβ) deposition. Proc Natl Acad Sci USA. 2010; 107: 17763-17767.
13 Buckner RL, Snyder AZ, Shannon BJ, et al. Molecular, structural, and functional characterization of Alzheimer's disease: evidence for a relationship between default activity, amyloid, and memory. J Neurosci. 2005; 25: 7709-7717.
14 Johanson CE, Duncan JA 3rd, Klinge PM, et al. Multiplicity of cerebrospinal fluid functions: New challenges in health and disease. Cerebrospinal Fluid Res. 2008; 5: 10.
15 Hashimoto M, Ishikawa M, Mori E, et al. Diagnosis of idiopathic normal pressure hydrocephalus is supported by MRI-based scheme: a prospective cohort study. Cerebrospinal Fluid Res. 2010; 7: 18.

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Neuroscience Letters 551 (2013) 17–22

Contents lists available at ScienceDirect

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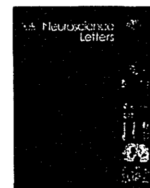

Correlation of Aβ oligomer levels in matched cerebrospinal fluid and serum samples

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HIGHLIGHTS

- We measured levels of HMW Aβ oligomers in matched samples of serum and CSF with the BAN50 ELISA.
- HMW Aβ oligomers were detectable in 60% of serum samples and in 80% of CSF samples.
- There was no interference by heterophilic antibodies.
- There was a positive correlation between the levels of Aβ oligomers in CSF and those in matched serum.

ARTICLE INFO

Article history:
Received 2 February 2013
Received in revised form 23 May 2013
Accepted 15 June 2013

Keywords:
Amyloid-β
Oligomer
Matched serum and CSF
ELISA
Heterophilic antibody

ABSTRACT

We recently reported a newly developed enzyme-linked immunosorbent assay (ELISA) for high molecular weight amyloid-β (Aβ) oligomers in which the same Aβ monoclonal antibody, BAN50, was used for both capture and detection in a single antibody sandwich enzyme linked immunosorbent assay (ELISA) system. Although our previous data have suggested that this assay will be useful for the early diagnosis of Alzheimer disease (AD) in cerebrospinal fluid (CSF) samples, the invasive CSF sampling procedure, with associated potential complications, limits use of these samples in routine clinical practice. In this study, we have demonstrated that our ELISA can detect signals in 60% of serum samples and in 80% of CSF samples obtained from non-demented subjects. Heterophilic antibodies that are reported to be a primary confounding factor in this type of ELISA system did not affect the signals obtained. Although the levels of serum Aβ oligomers were unexpectedly high, suggesting the possible detection of non-pathological Aβ complexes associated with serum carrier proteins, they did show a significant positive correlation with the levels obtained from matched CSF samples. This correlation between CSF and serum Aβ oligomer levels implies that the levels of serum Aβ oligomers measured with our ELISA might be useful as a marker for AD that reflects an intact system of Aβ transport across the blood brain barrier.
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1. Introduction

Alzheimer disease (AD) is the most common cause of dementia and is a rapidly growing social and medical problem throughout the world. The two major pathological hallmarks in the brains of patients with AD are the senile plaques and neurofibrillary tangles. The main protein component of the senile plaques is amyloid-β (Aβ) and that of the neurofibrillary tangles is hyperphosphorylated tau protein [1,7]. Aβ1–42 is more prone to aggregation than Aβ1–40 and undergoes accelerated formation of Aβ oligomers, larger intermediate assemblies such as protofibrils, and, finally, insoluble amyloid fibrils. The "amyloid cascade hypothesis" originally regarded these insoluble amyloid fibrils as the primary molecular culprit responsible for AD. However, growing evidence supports the hypothesis that soluble Aβ oligomers (or ADDLs/protofibrils) are more toxic than the larger Aβ fibrils [8].

Such pathological molecules, which can leak into human body fluids, or be secreted into human body fluids from the brain, can be regarded as potential candidate biomarkers to predict the risk of developing AD. For example, decreased levels of Aβ1–42 and

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http://dx.doi.org/10.1016/j.neulet.2013.06.029

increased levels of either 'total tau' or phosphorylated tau in cerebrospinal fluid (CSF) are now widely accepted as early diagnostic biomarkers for AD.

Recently, we reported a novel enzyme linked immunosorbent assay (ELISA) system for the detection of Aβ oligomers in which the same anti-Aβ monoclonal antibody, BAN50, was used for both capture and detection of the oligomers, in a single antibody sandwich ELISA (SAS-ELISA) system. This BAN50 SAS-ELISA cannot detect Aβ monomers because the capture antibody occupies the only antibody-binding site available, but it can detect Aβ oligomers because they have multiple binding sites. In our previous study, the BAN50 SAS-ELISA specifically detected high molecular weight (HMW) Aβ oligomers of 10–20mer in size, and gave signals in CSF samples from patients with AD or mild cognitive impairment (MCI) that were significantly higher than those from age-matched controls, as well as having a negative correlation with Mini-Mental State Examination (MMSE) scores. These facts suggest our SAS-ELISA can identify a useful molecular diagnostic marker for AD, and also a potential surrogate marker for disease severity [5].

However, there are problems with using any CSF-based measurement in routine clinical practice due to the invasive sampling procedure, with its associated potential complications. Therefore, we have been keen to attempt to apply the BAN50 SAS-ELISA to a blood-based measurement of Aβ oligomers.

Some research groups, including ourselves, have already developed techniques for the selective detection of Aβ oligomers in CSF [5,6,15]. Moreover, there are also a few reports regarding the detection Aβ oligomers in blood samples [11,12,15]. However, there is no previous study which has examined the levels of Aβ oligomers in matched samples of blood and CSF, and so the relationship between these two different measures, which would have important implication for biomarker development, is unknown. To address these issues, we investigated the following two points in the present study. Firstly, we determined whether the BAN50 SAS-ELISA can detect HMW Aβ oligomers in human serum. Secondly, we examined the possible relationship between the levels of HMW Aβ oligomers in matched samples of CSF and serum collected at the same time, from the same individuals.

2. Materials and methods

2.1. Sample collection

This study complied with the Declaration of Helsinki and was approved by the University Ethics Committee (Kyoto Prefectural University of Medicine, Kyoto, Japan). All subjects provided written informed consent to participate in this study. Twenty subjects with no dementia who were required to give CSF and blood samples for routine clinical diagnosis or treatment [12 men/8 women, aged 53–89, mean ± standard deviation (SD), 70.8 ± 9.3 yr] were enrolled (the main group). Unless otherwise stated, the data referred in this study were obtained from the main group. At the time of diagnosis, a full clinical history was taken, and physical and neurological examinations, and brain imaging (magnetic resonance imaging or computed tomography) were performed for all subjects. The subjects comprised neurologically normal individuals ($n = 3$) and cases with peripheral neuropathy ($n = 8$), cranial nerve dysfunction ($n = 4$), cervical myelopathy ($n = 2$), epilepsy ($n = 1$), cerebellar ataxia ($n = 1$) and vitamin B1 deficiency ($n = 1$). CSF was obtained through a lumbar puncture at the L3/L4 or L4/L5 interspace, according to a standard protocol. CSF samples were collected in polypropylene vials. Blood samples were obtained within one hour of those from CSF. Serum samples were taken through venous puncture and 10 ml of total blood was collected in blood collection tubes with clot activator and gel separator (Terumo, Tokyo, Japan). After being allowed to clot for 15 min at room temperature, serum was separated by centrifugation for 10 min at 3000 rpm and distributed in polypropylene vials. Fresh samples obtained from the enrolled subjects were immediately stored at −80 °C until used for immunoassays.

To obtain reference data regarding well-to-well signal variability in our ELISA, we used an additional 35 serum samples and 14 CSF samples (the supplementary group) where serum and CSF were not matched. All of these samples were collected and stored in the same way as for the main group, according to the same ethical approval and informed consent as above.

2.2. Measurement of HMW Aβ oligomers with BAN50 SAS-ELISA

The BAN50 SAS-ELISA was used in duplicate (unless otherwise stated) to measure the signal for HMW Aβ oligomers in the human biological fluids. The buffers and assay procedures were similar to those described previously [5]. As a standard for inter-plate calibration, we used a synthetic 'multiple antigenic' (MAP) peptide containing 16 copies of the BAN50 epitope linked to a branching lysine core. The signal per 1 arbitrary unit of this standard has been estimated to correspond to 1.54–5.0 pM of HMW Aβ1–42 oligomers [9]. The CSF samples were not diluted, whereas the serum samples were diluted 5-fold with the standard diluent for this ELISA to avoid any effect of interfering substances. To detect very small signals from the human body fluids, we used an auto-injector and a highly sensitive chemiluminescence substrate (Thermo Fisher Scientific, IL, USA), with luminescence detection on a multi-function microtiter plate-reader (Synergy 2, Biotek, VT, USA).

2.3. Blocking of heterophilic interactions in the ELISA

To make sure that the positive ELISA signals were not affected by non-specific interactions of heterophilic antibodies (HA) [13], we used a HA inhibitor (super chemiblock heterophile blocking reagent, Chemicon-Millipore, Livingston, UK) designed to inhibit signals from heterophilic antibodies and/or Rheumatoid Factor (RF) in human body fluids. We added 4 μg/well of this reagent to duplicate wells containing each of the serum or CSF samples before the assay, and then compared the final signals to those obtained without any anti-HA treatment.

2.4. Measurement of total Aβ (Aβx–40 and Aβx–42)

Sandwich ELISAs were carried out to measure CSF and serum levels of Aβx–40 (hereafter referred to Aβ40) and Aβx–42 (Aβ42) using human/rat β amyloid 40 ELISA kit and human/rat β amyloid 42 ELISA kit, respectively (Wako, Osaka, Japan), as described by the manufacturer.

2.5. Statistics

Mann–Whitney's *U* test was used for comparison between two independent groups. The correlation analysis was performed using Spearman's rank correlation coefficient test. Normality of distribution in groups was tested by Kolmogorov–Smirnov test. The level of significance was set at $P < 0.05$. All analyses were performed with SPSS, version 11 for Windows (SPSS Inc., Tokyo, Japan).

3. Results

In advance of measuring the levels of HMW Aβ oligomers in human body fluids, we first determined the profile of total Aβ in our matched samples. The mean concentrations of CSF Aβ42 and Aβ40 were 275.0 ± 100.7 and 2975 ± 995 pM (mean ± SD),

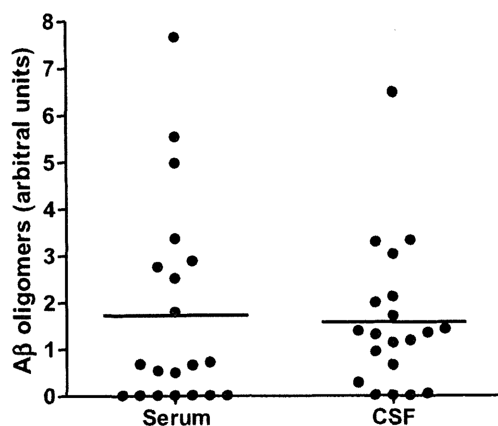

Fig. 1. Scatter plots for levels of HMW Aβ oligomers in serum and CSF. The scale of the y-axis represents the standard concentration of MAP 16-mer peptide. Bars indicate mean values. Values of P obtained by 2-sided Mann–Whitney U test are indicated.

respectively. In the serum samples, the mean levels of Aβ42 and Aβ40 were 13.28 ± 4.45 pM and 83.49 ± 39.9 pM, respectively. (Detailed profiles were shown in the Supplementary Table.)

We then measured the levels of HMW Aβ oligomers in the same matched serum and CSF samples, using the BAN50 SAS-ELISA. These Aβ oligomers were detectable in 60% (14/20) of the serum samples and in 80% (16/20) of the CSF samples. In the following statistical analyses, we have dealt with values below the detection limit (0.19 arbitrary units) by assigning them a value of zero [9]. The levels of HMW Aβ oligomers in the subjects are shown in Fig. 1. The mean levels of HMW Aβ oligomers were 0.439 ± 2.19 in serum and 1.071 ± 1.554 in CSF.

To confirm that HA or RF in human body fluids does not affect signal strength in the BAN50 SAS-ELISA, we chose the samples having positive signals (14 serum and 16 CSF samples) and reanalyzed them after adding the HA inhibitor. The comparisons between signals from samples treated with and without the HA inhibitor are shown in Fig. 2. Apparently, blocking of HA did not affect the signals obtained from either serum (Fig. 2A), or CSF (Fig. 2B). However, some of the signals did increase or decrease by more than 30% after HA blocking. To evaluate the possibility that such slight changes after anti-HA treatment could be explained by well-to-well heterogeneity in the ELISA, we calculated a parameter of "relative signal change" following HA inhibition as well as another parameter of "well-to-well signal variation" in the duplicate measurements, for reference. These parameters were obtained from the formulae indicated in the legend of Fig. 2. In order to obtain reliable mean and SD values from the reference groups, sample sizes greater than 25 are generally needed. Therefore, we combined the main group and the supplementary group and then used these raw data from the Aβ HMW oligomer assays, obtained from 55 serum and 34 CSF samples in total, to calculate "well-to-well signal variability", also ensuring that this value was normally distributed by employing the Kolmogorov–Smirnov test, for both CSF and serum samples ($P > 0.200$ and $P = 0.123$, respectively). As shown in Fig. 2C, there was no significant difference between "relative signal change" and "well-to-well variability" in either serum or CSF. Most of "relative signal change" in each group fell into the range of mean ± 2SD of "well-well signal variability" in the corresponding columns. Such resilience against undesirable effects from immunoglobulin species in the ELISA was also observed in an experiment involving immunoglobulin depletion from serum where no apparent difference was found between signals from serum with/without protein G treatment (Supplementary Fig. 1A).

To clarify whether the levels of serum Aβ oligomers could reflect the levels of CSF Aβ oligomers, we investigated the correlation of these measures. The levels of serum Aβ oligomers had a significant positive correlation with the levels of CSF Aβ oligomers (Spearman's $\rho = 0.505$; $P = 0.023$, Fig. 3A). On the other hand, the levels of Aβ42 in serum did not correlate with those in CSF (Spearman's $\rho = 0.066$; $P = 0.782$, Fig. 3B) and there was no relationship between Aβ40 levels in serum and CSF (Spearman's $\rho = 0.159$; $P = 0.504$, Fig. 3C). Nor was there any significant correlation between Aβ oligomer levels and either Aβ42 or Aβ40 in serum or CSF (data not shown).

4. Discussion

Our first major finding is that the BAN50 SAS-ELISA could detect signals from more than half of the serum samples. This rate of signal detection in our ELISA (60% of serum samples) is better than that reported in two previous blood-based ELISA systems (48% [15] or 34% [12] in plasma). The detectability of Aβ HMW oligomers in CSF (80%) was a little lower than that found in one of our previous reports [5]. This could be explained by the fact that here we have estimated the lower detection limit using the newly developed MAP standard [9] and have eliminated any values below that limit.

Recently, interference by HA has been argued as being a primary confounding factor in SAS-ELISAs. HA are polyreactive antibodies that recognize antibodies from other species. When using such an ELISA for analyses of body fluids, especially plasma and serum, false positive signals may be obtained when HA cross-bind to the assay antibodies. In fact, plasma signals of Aβ oligomers in a SAS-ELISA developed by another group completely disappeared after anti-HA treatment [13]. However, as shown in Fig. 2, our ELISA was not affected by anti-HA treatment. Presumably, this is because our ELISA uses the Fab′ fragment of the antibody as the detector, which is less susceptible to positive interference by HA [4].

The second finding is that the levels of HMW Aβ oligomers in serum were much higher than we expected. Signals from fivefold diluted serum were apparently similar to those obtained for CSF with no dilution. In our samples, the mean concentration of total Aβ40 in serum was no more than 1/50th of that in CSF and, therefore, we had anticipated that the serum signals for Aβ oligomers would be much lower than those obtained from CSF. It is possible that sample dilution might increase signals. However, the preliminary experiment showed that sample dilution did not cause such serious influence in our ELISA (Supplementary Fig. 1B). The reason why the BAN50 SAS-ELISA gave such a high signal for Aβ oligomers in serum remains to be elucidated. However, hypothetically, we suppose that this could be because Aβ species in serum are bound to carrier proteins and these complexes could be detected by the SAS-ELISA. This ELISA can theoretically detect complexes composed of multiple copies of the Aβ peptide in association with other components, in addition to HMW complexes composed solely of oligomeric Aβ [5] (Supplementary Fig. 2A). Several proteins or protein complexes have been reported to associate with Aβ in vivo (e.g. albumin, high density lipoprotein particles containing apolipoprotein E (Apo E) or Apo J). The vast majority of such proteins are present in serum, and not in CSF [2]. Therefore, we suppose these 'carrier' complexes could account for a considerable amount of the Aβ present in serum, and that a large proportion of the signal obtained from serum by our SAS-ELISA might be derived from such non-pathological Aβ complexes.

The third novel finding is the positive correlation between the levels of Aβ HMW oligomers detected in serum and those in matched samples of CSF. This result suggests that Aβ oligomers can diffuse or be efficiently transported across the blood–brain or

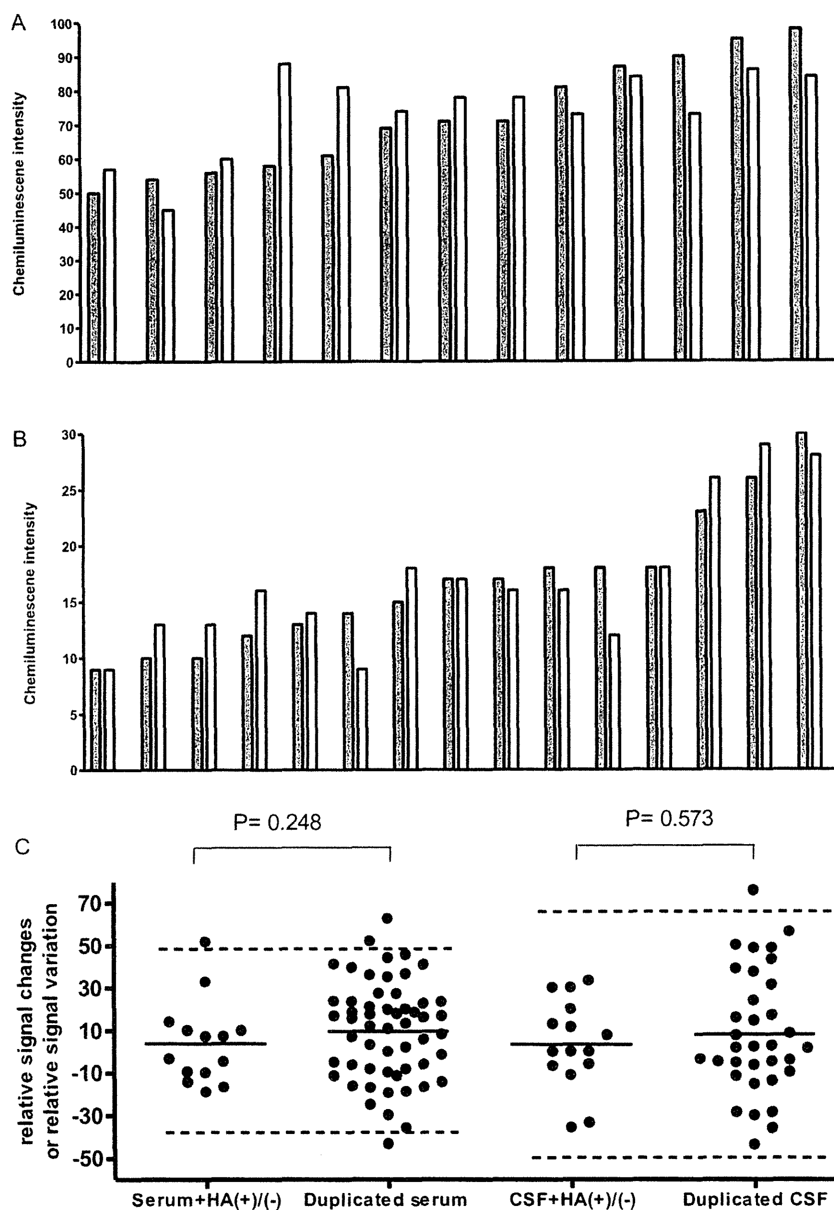

Fig. 2. HA interference in HMW Aβ oligomer analysis of serum and CSF. ELISA signals obtained with (white bars) and without (gray bars) anti-HA treatment are shown in bar graphs of serum samples (A) and CSF samples (B). Based on the raw data, relative signal changes caused by anti-HA treatment are shown in (C): columns of "Serum with HA inhibition" and "CSF with HA inhibition". The parameter is calculated by the formula of $(B-A)/A$ (%) (A: signal without HA treatment, B: signal with HA treatment). For reference, well-to-well signal variations for duplicate measurements are shown in columns of "duplicated Serum" and "duplicated CSF" in the same graph, which are also calculated by the same formula as above, although signal A and signal B are arbitrarily chosen from each duplicated data. Bars indicate mean value. Dotted lines represent the ranges of mean ± 2SD in "duplicated Serum" and "duplicated CSF." Note, measurements with or without anti-HA treatment are single values in order to enable statistical comparison between these two parameters. Values of P are obtained by 2-sided Mann–Whitney U test.

the blood–CSF barrier (collectively referred to as the BBB) and/or that the BBB allows Aβ carrier proteins (discussed above) to be transferred between serum and CSF, which would transfer Aβ peptides to blood in the form of 'pseudo Aβ oligomer' complexes. It has already been determined that some proteins (e.g. low-density lipoprotein receptor protein 1 (LRP-1) and receptor for advanced glycation end products) can function as Aβ transporters across the BBB [14]. However, many studies, including ours, have failed to observe any correlation between 'total Aβ' levels in serum (or plasma) and those in CSF [10] and yet, as reported here, the 'Aβ HMW oligomer' levels do appear to correlate. This selective correlation of Aβ oligomer levels between serum and CSF suggests that the transport of Aβ complexes (and not Aβ monomer) across the BBB is a likely explanation for our findings. Since we examined non-demented subjects, the 'Aβ HMW oligomers' detected in serum (and possibly in CSF) by our ELISA might be derived mostly from non-pathological Aβ complexes. On the other hand, we have found significantly higher levels of 'Aβ HMW oligomers' in CSF taken from AD patients than in neurological controls [5], suggesting that, in this case at least, the CSF Aβ HMW oligomers detected in CSF from AD patients are likely to be due to genuine 'pathological' Aβ oligomers (consisting of only Aβ) (Supplementary Fig. 2B). Regardless of the identity of the molecular form(s) of the HWM Aβ oligomers that our ELISA detects, the correlation between levels of serum and

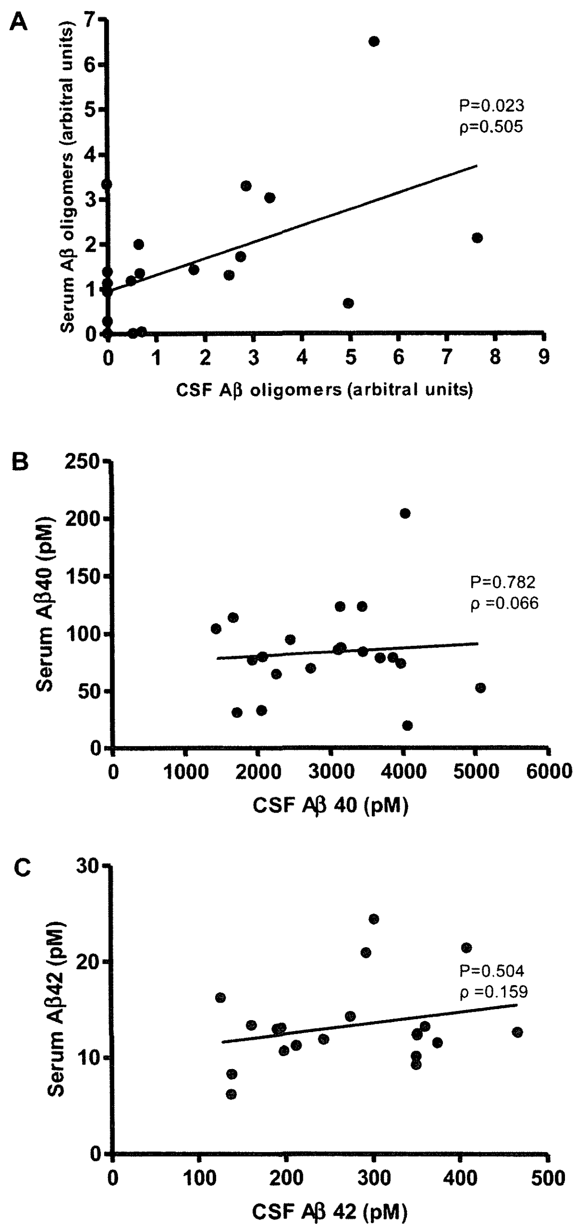

Fig. 3. Correlations between CSF and plasma levels of Aβ40 (A), Aβ42 (B), and Aβ oligomers (C). Lines indicate regression line. Values of P and correlation efficient "ρ" were obtained by Spearman's rank correlation coefficient test.

CSF HMW Aβ oligomers could well reflect the existence of Aβ clearance mechanisms [3].

We should also comment on the reason why we have chosen serum samples instead of plasma. In general, plasma Aβ is preferable for biomarker studies because it avoids the potential confounding effect of Aβ release upon platelet activation. Although plasma Aβ was also detectable by the BAN50 SAS-ELISA in our preliminary experiments, the data obtained from plasma samples were difficult to interpret properly due to an outlier observed in one of 19 subjects examined, as shown in the Supplementary Fig. 3. Although the signal detection rate in serum is lower than that in plasma, no outlier was detected in serum (Fig. 1). We suppose that such an outlier might be derived from improper sampling or storage procedures, which often cause invisibly small fibrin clots to form in plasma. It is possible that some Aβ species could be trapped within these fibrin clots, and the BAN50 SAS-ELISA would then recognize clots containing multiple Aβ peptides as 'pseudo Aβ oligomers' which could generate extremely strong false positive signals. In addition, such clots were thoroughly removed from our serum samples, with clot activator and gel separator being present in the collection tubes. We should bear in mind that, even so, a certain proportion of Aβ oligomers might be absorbed into clots during the serum sampling procedure which might explain why the detectability in serum was a little worse than that in plasma. However, such a signal loss would equally affect each serum sample, while the occurrence of the 'pseudo positive' interference in plasma would occur more randomly. In order to avoid difficulties of data analysis caused by such a random effect, we focused on serum in this study, and not on plasma.

In summary, we have demonstrated that the BAN50 SAS-ELISA for HMW Aβ oligomers could detect signals from more than half of the enrolled subject plasma samples. This signal detection rate is better than those of the previous blood-based ELISAs for Aβ oligomers. Moreover, our ELISA was not affected by HA. Cross-sectional, well-controlled studies are necessary to validate the application of our ELISA to blood-based AD diagnosis. We have also shown that the levels of serum HWM Aβ oligomers had a significant correlation with those found in matched CSF samples. This correlation might reflect the operation and intactness of Aβ clearance mechanisms, and, if this holds true in AD, has positive implications for development of a blood-based biomarker for this disease in future studies.

Acknowledgements

This work was supported by a grant from Takeda Pharmaceutical Company Limited (to TT), The Japan Heart Foundation/Bayer Yakuhin Research Grant Abroad, Takeda Science Foundation (to TK) and The Great Britain Sasakawa Foundation (to DA). The authors wish to thank Takeda Pharmaceutical Company Limited for the generous gifts of the monoclonal antibody BAN50 and its SAS-ELISA kit.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.06.029.

References

[1] D. Allsop, M. Landon, M. Kidd, The isolation and amino acid composition of senile plaque core protein, Brain Res. 259 (1983) 348–352.
[2] A.L. Biere, B. Ostaszewski, E.R. Stimson, B.T. Hyman, J.E. Maggio, D.J. Selkoe, Amyloid beta-peptide is transported on lipoproteins and albumin in human plasma, J. Biol. Chem. 271 (1996) 32916–32922.
[3] P.E. Cramer, J.R. Cirrito, D.W. Wesson, C.Y. Lee, J.C. Karlo, A.E. Zinn, B.T. Casali, J.L. Restivo, W.D. Goebel, M.J. James, K.R. Brunden, D.A. Wilson, G.E. Landreth, ApoE-directed therapeutics rapidly clear beta-amyloid and reverse deficits in AD mouse models, Science 335 (2012) 1503–1506.
[4] G. Csako, B.D. Weintraub, M.H. Zweig, The potency of immunoglobulin G fragments for inhibition of interference caused by anti-immunoglobulin antibodies in a monoclonal immunoradiometric assay for thyrotropin, Clin. Chem. 34 (1988) 1481–1483.
[5] H. Fukumoto, T. Tokuda, T. Kasai, N. Ishigami, H. Hidaka, M. Kondo, D. Allsop, M. Nakagawa, High-molecular-weight beta-amyloid oligomers are elevated in cerebrospinal fluid of Alzheimer patients, FASEB J. 24 (2010) 2716–2726.
[6] C.M. Gao, A.Y. Yam, X. Wang, E. Magdangal, C. Salisbury, D. Peretz, R.N. Zuckermann, M.D. Connolly, O. Hansson, L. Minthon, H. Zetterberg, K. Blennow, J.P. Fedynyshyn, S. Allauzen, Abeta40 oligomers identified as a potential biomarker for the diagnosis of Alzheimer's disease, PLoS ONE 5 (2010) e15725.
[7] M. Goedert, Tau protein and the neurofibrillary pathology of Alzheimer's disease, Trends Neurosci. 16 (1993) 460–465.

[8] J. Hardy, D.J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297 (2002) 353–356.
[9] T. Kasai, T. Tokuda, M. Taylar, M. Nakagawa, D. Allsop, Utilization of a multiple antigenic peptide as a calibration standard in the BAN50 single antibody sandwich ELISA for Aβ oligomers, Biochem. Biophys. Res. Commun. 422 (2012) 375–380.
[10] N. Le Bastard, L. Aerts, J. Leurs, W. Blomme, P.P. De Deyn, S. Engelborghs, No correlation between time-linked plasma and CSF Abeta levels, Neurochem. Int. 55 (2009) 820–825.
[11] A.N. Santos, A. Simm, V. Holthoff, G. Boehm, A method for the detection of amyloid-beta1-40, amyloid-beta1-42 and amyloid-beta oligomers in blood using magnetic beads in combination with flow cytometry and its application in the diagnostics of Alzheimer's disease, J. Alzheimers Dis. 14 (2008) 127–131.
[12] N. Schupf, M.X. Tang, H. Fukuyama, J. Manly, H. Andrews, P. Mehta, J. Ravetch, R. Mayeux, Peripheral Abeta subspecies as risk biomarkers of Alzheimer's disease, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 14052–14057.
[13] D. Sehlin, S. Sollvander, S. Paulie, R. Brundin, M. Ingelsson, L. Lannfelt, F.E. Pettersson, H. Englund, Interference from heterophilic antibodies in amyloid-beta oligomer ELISAs, J. Alzheimers Dis. 21 (2010) 1295–1301.
[14] M. Ueno, T. Nakagawa, B. Wu, M. Onodera, C.L. Huang, T. Kusaka, N. Araki, H. Sakamoto, Transporters in the brain endothelial barrier, Curr. Med. Chem. 17 (2010) 1125–1138.
[15] W. Xia, T. Yang, G. Shankar, I.M. Smith, Y. Shen, D.M. Walsh, D.J. Selkoe, A specific enzyme-linked immunosorbent assay for measuring beta-amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease, Arch. Neurol. 66 (2009) 190–199.