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Correlations of amyloid- β concentrations between CSF and plasma in acute Alzheimer mouse model

SUBJECT AREAS:
ALZHEIMER'S DISEASE
PEPTIDES
BIOCHEMICAL ASSAYS

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Received
4 July 2014

Accepted
7 October 2014

Published
27 October 2014

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Amyloid- β ($A\beta$) is one of the few neuropathological biomarkers associated with transporters of the blood-brain barrier (BBB). Despite the well-characterized clinical indication of decreasing $A\beta$ levels in the cerebrospinal fluid (CSF) during the development of Alzheimer's disease (AD), the link between the alternation of $A\beta$ level in the blood and the progress of the disorder is still controversial. Here, we report a direct correlation of $A\beta(1-42)$ levels between CSF and plasma in AD mouse model. We injected monomeric $A\beta(1-42)$ directly into the intracerebroventricular (ICV) region of normal adult mouse brains to induce AD-like phenotypes. Using sandwich enzyme-linked immunosorbent assays, we observed proportional elevation of $A\beta(1-42)$ levels in both CSF and plasma in a dose-dependent manner. Our findings that plasma $A\beta(1-42)$ reflects the condition of CSF $A\beta(1-42)$ warrant further investigation as a biomarker for the blood diagnosis of AD.

Blood $A\beta$ is an attractive biomarker of AD for the well-characterized efflux mechanism to pass the blood-brain barrier (BBB); the low-density lipoprotein receptor-related protein-1 in BBB allows brain $A\beta$ to be actively transported to blood^{1,2}. Thus, quantification of plasma $A\beta$ is considered as an emerging diagnostic tool for AD^{2,3}. Indeed, recent clinical studies have reported association of reduced $A\beta(1-42)$ level in plasma with cognitive decline and risk development of AD⁴⁻⁶. However, contrasting cross-sectional cases have claimed the medical use of blood-based $A\beta$ measurements to be debatable⁷⁻¹⁰; heterogeneity of patient groups and assay protocols may have contributed to the provocative results. Dissimilar aspects of plasma $A\beta$ analysis in varied subjects are also observed in amyloid precursor protein (APP) transgenic mouse models. Studies of two APP mouse models with different point mutations argue that the surrogate scale of plasma $A\beta$ reflected $A\beta$ abnormalities in CSF of AD brains^{11,12}. During the plaque formation, PDAPP mice, with Indiana APP mutation, lose the correlation between plasma and CSF $A\beta$; meanwhile, Tg2576 mice, with Swedish APP mutation, show a significant and simultaneous decrease in both CSF and plasma $A\beta$. Thus, it is critical to proceed a clinical investigation of plasma $A\beta$ for its validity as a surrogate marker of risk development of AD under controlled milieu. The $A\beta$ -infusion mouse model offers benefits to minimize neuropathological factors of AD over the use of APP over-expressing transgenic rodents¹³. In APP transgenic models, it is difficult to control isomer types, species and concentration of $A\beta$ in their brains. Such diversity would interfere targeted diagnosis of $A\beta$ in antibody-based measurements and may produce contrasting results depending on models. On the other hand, $A\beta$ -infusion animal model allows administration of defined quantity and species of $A\beta$ isomers and reduction of individual differences within each study group¹³. By mimicking certain aspects of AD with designated infusion of $A\beta$, researchers can bypass the aging process and acutely focus on downstream pathology of $A\beta$ abnormality between plasma and CSF. Here, we examined the dynamic equilibrium of $A\beta(1-42)$ between the brain and plasma after ICV injection of synthetic $A\beta(1-42)$ monomers to normal ICR mice.

Results

Development of $A\beta$ -infused mouse model with memory dysfunction. Despite the benefits to introducing $A\beta$ -focused environments, it is critical to assess if the $A\beta$ injection induces AD-like learning and memory



impairments in this animal model before studying the surrogate role of plasma A β to predict at-risk AD subjects. We performed Y-maze tasks to assess working memory alternations by measuring spontaneous alternation behaviors (Figure 1a,b)¹⁴. In a three-armed Y-shaped maze, the subjected animal must remember the order of arm entries, and the superior alternation rate indicates the better sustained cognition. Working memory ability and locomotion levels were determined as percent alternation and total arm entries, respectively, on the Y-maze. We found that single ICV injection of A β (1–42) monomers (0.25 nmole) significantly reduced cognitive behavioral performance on the Y-maze task when compared to vehicle-injected normal adult ICR mice (Figure 1c,d), without affecting locomotion levels.

Comparison of A β (1–42) levels in CSF and plasma. In AD brains, alternations of CSF A β (1–42) levels were reversely correlated with progressive deposition of insoluble A β -plaques and development of AD^{15,16}. To mimic alternations of CSF A β levels in AD, we injected diverse concentrations of A β (1–42) (0, 0.25, 0.5, 2 and 4 nmole) in parallel into the ICV region of normal adult ICR mice (male, n = 5 per group). We collected CSF by laboratory-produced capillary tubes with tapered tips as previously described¹⁷. In sandwich-ELISA utilizing two anti-A β antibodies with different epitopes, we confirmed that CSF A β (1–42) levels directly replicated dose-dependency of the injected peptides (Figure 2 and Table 1) (Kruskal-Wallis test, $P = 0.0076$). After the CSF collection, we transferred blood from vena cava directly to EDTA tubes and isolated plasma. In order to assess validity of plasma A β as an AD surrogate biomarker, we measured A β (1–42) levels in plasma samples of mice subjected to aforementioned CSF studies by the sandwich-ELISA. We found that the levels of plasma A β (1–42) correspondingly elevated as we increased the amount of ICV-injected A β (1–42) monomers in mice (Figure 3 and Table 1) (Kruskal-Wallis test, $P = 0.0008$). Collectively, these results indicated that plasma A β (1–42) levels acutely reflected alternations of the CSF A β (1–42) concentration (Figure 4).

Discussion

In this study, we compared A β (1–42) levels in CSF and plasma of at-risk AD model mice. We artificially raised amounts of A β (1–42)

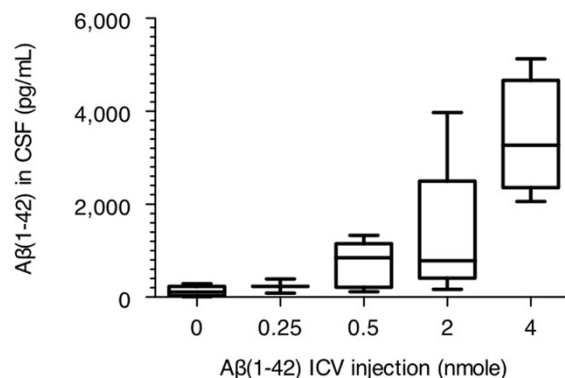


Figure 2 | Concentration in CSF A β (1–42) after ICV injection. CSF was collected 30 minutes after ICV injection of A β (1–42) in serial amounts; 0, 0.25, 0.5, 2 and 4 nmole. Concentrations of A β (1–42) in CSF were measured using sandwich-ELISA.

monomers in CSF of normal adult ICR mice by ICV injection and observed the corresponding increase of A β (1–42) levels in plasma. Our results correspond to two conflicting studies using APP transgenic mouse models; these studies conditionally agree on the significant association between CSF and plasma A β before the development of plaque deposition^{11,12}. Given that these transgenic mice have different genetic backgrounds and mutations, asymmetrical aspects of CSF-plasma A β ratio in these studies indicate strong needs for blood biomarker investigations to adopt A β specific animal models without additional neuropathological factors. Thus, we restricted neuropathology of subjected animals to abnormal increase of brain A β and measured A β levels considering the half-time for brain efflux of the peptide¹⁸. As we intended to create A β -focused environment in mice, our study lacks many pathophysiological mechanisms in AD such as chronic processing and clearance of A β . Thus, additional studies are warranted to determine whether the correlation between plasma and CSF A β will translate into AD diagnosis within diverse neuropathological conditions.

Our present study provides assurance that the A β in plasma is an important target candidate for AD diagnosis. The concept of A β blood test is an unprecedentedly convenient tool for both physicians

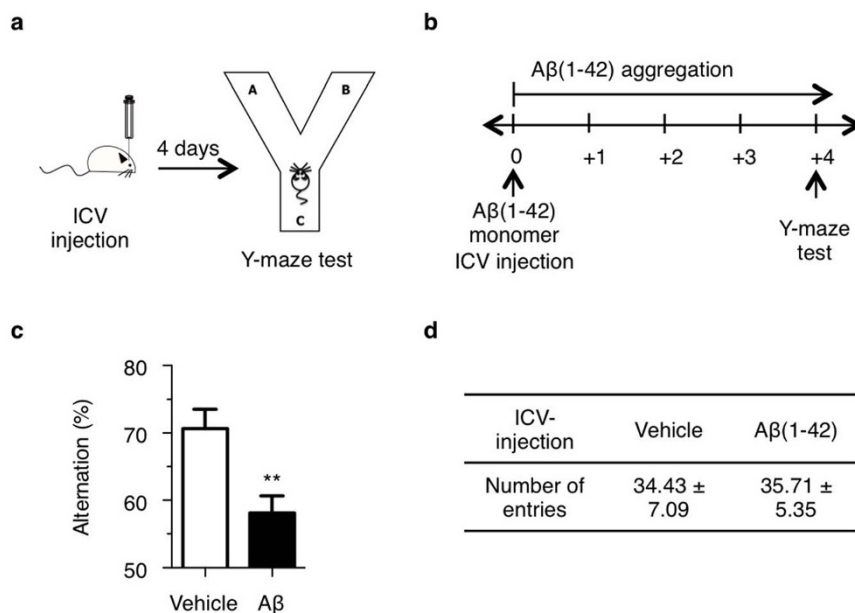


Figure 1 | Y-maze test of A β -infused mouse model. (a) Experimental scheme (drawn by SM Cho) and (b) schedule of Y-maze test. (c) Spontaneous alternations in percent (mean \pm SD, $p = 0.007$). (d) Number of arm entries (mean \pm SD)

Table 1 | Determined A β (1–42) concentration of CSF and plasma (mean \pm SD)

Injected A β (1–42) amount (nmole)	0 (vehicle)	0.25	0.5	2	4
CSF A β (1–42) conc. (pg/ml)	127.66 \pm 110.65	236.52 \pm 154.29	717.02 \pm 496.11	1,319.15 \pm 1516.58	3,431.91 \pm 1264.42
Plasma A β (1–42) conc. (pg/ml)	208.08 \pm 29.79	504.90 \pm 342.13	1,434.68 \pm 627.72	3,002.84 \pm 605.29	14,719.15 \pm 7,210.06

and patients to predict the pathological progress of AD. However, reliable measurements of plasma A β by overcoming cross-sectional diversity will subsequently confront the challenge as a valid surrogate biomarker for neurodegeneration of AD. Quantification of abnormal A β processing and clearance in the brain has been a major diagnostic method of AD. During the investigation of A β neuroimaging tracers, needs for additional biomarkers have been raised due to significant cases of non-demented, A β -positive individuals¹⁹. Thus, clinical evidences at present strongly support the view that diagnosis of AD requires detection of both A β and tau abnormalities²⁰. As A β deposition timely leads to hyperphosphorylated tau aggregation followed by cerebral atrophy, the former is considered useful for early diagnosis and the latter for neurodegeneration¹⁵. Given that plasma A β levels would limitedly reflect the status of A β abnormality in the central nervous system, further challenges of AD blood tests would be investigating additional blood surrogate markers of neurodegeneration²¹. Present findings propose that blood-based A β quantification should be investigated further as a possible surrogate biomarker for AD diagnosis.

Methods

Materials. PL-Wang resin (0.4 mmole/g) was obtained from Varian, Inc. (Shropshire, UK). Fmoc-Ser(tBu)-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gln(trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(pbf)-OH, N,N'-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP) and triisopropylsilane (TIS) were obtained from GL Biochem Ltd. (Shanghai, China). Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-His(trt)-OH and N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were obtained from CS Bio Co. (Menlo Park, USA). Dimethylformamide (DMF), dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were obtained from Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea). Anisole, piperidine and N,N-diisopropylethylamine (DIEA) were obtained from Sigma-Aldrich (St. Louis, USA). Trifluoroacetic acid (TFA) was obtained from Yakuri Pure Chemicals Co., Ltd. (Kyoto, Japan). Ether, Anhydrous was obtained from J.T. Baker (Center Valley, USA). Zoletil® (Virbac) and Rompun® (Bayer Parma) were obtained from SMP animal medicine (Suwon, Korea). Protease inhibitor cocktail was obtained from Roche Diagnostics (IN, USA). EDTA treated BD vacutainer® was obtained from Becton, Dickinson and Company (NJ, USA). Human A β 42 Ultrasensitive ELISA kit was obtained from Invitrogen (CA, USA).

A β (1–42) peptide synthesis. A β (1–42) was synthesized by Fmoc solid phase peptide synthesis. 0.4 M HBTU (3 : 2 DMSO/DMF, v/v) as a coupling solution and 20% piperidine (1 : 1 : 3 piperidine/DMSO/DMF, v/v/v) as Fmoc deprotecting solution

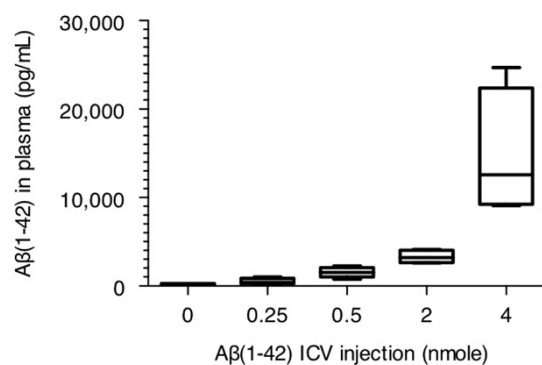


Figure 3 | Concentration in plasma A β (1–42) after ICV injection. Plasma was collected 30 minutes after ICV injection of A β (1–42) in serial amounts; 0, 0.25, 0.5, 2 and 4 nmole. Concentrations of A β (1–42) in plasma were measured using sandwich-ELISA.

were added DMSO. We synthesized A β (1–42) on 0.25 mmole of Wang resin with 1.1 mmole of every amino acid except for the first amino acid. First amino acid requires 2.2 mmole for symmetric anhydride activation. Before synthesis, resin should be swollen in DMF for an hour. For the symmetric anhydride activation, 2.2 mmole of first amino acid, Fmoc-Ala-OH, was dissolved in DMF (2 mL) and DIC (156 μ L) was added to amino acid dissolved solution. Until the clear DMF solution became unclear solution, it was placed in sonication. DMAP (0.051 g) was added to the unclear solution. Lastly the mixture was put into swollen resins and shaken for more than an hour. For the rest of amide couplings, CS336X peptide synthesizer (CS Bio, Menlo Park, USA) was used. After finishing synthesis, peptides were well cleaved from resin in 95% TFA (95 : 2.5 : 2.5 TFA/Anisole/TIS, v/v/v) within two hours. Using rotary evaporator, TFA was evaporated. Cold anhydrous ether (stored in -20° C) was added and centrifuged at 3000 rpm for 10 minutes twice. Finally, precipitated white powder was isolated, dissolved in 50% acetonitrile, lyophilized and purified using reverse-phase HPLC as previously reported²².

Animals. ICR mice (male, six weeks old) were purchased from Central Lab Animal Inc. (Seoul, Korea) and then bred in a laboratory animal breeding room at the Korea Institute of Science and Technology. They were housed in groups of five per cage and maintained at constant temperature with a 12/12 hour light/dark cycle. Water and food were available *ad libitum*. This work was carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, revised 1978). ICR mice (n = 29) were randomly divided into six groups (n = 5 each, except for the 0.25 nmole group; n = 4) depending on injection concentrations.

ICV injection of A β (1–42) peptide. Synthetic A β (1–42) was dissolved in sterile normal saline at 0, 0.25, 0.5, 2 and 4 nmole (5 μ L of 0, 50, 100, 400 and 800 μ M). Mice were anesthetized with a mixture of tiletamine·HCL, zolazepam·HCL (80 mg/kg, IP, Zoletil 50®, Virbac) and xylazine (20 mg/kg, IP, Rompun®, Bayer Parma) before ICV injection. Dissolved A β (1–42) was injected into the ICV region of mouse brains according to the previously reported protocols^{23,24}. In detail, we used a Hamilton syringe with a 26-gauge stainless-steel needle to inject the A β (1–42) stocks into the ICV region by 1.0 mm posterior to bregma, 1.8 mm lateral to saggital, 3.6 mm ventral and 2.4 mm depth.

Spontaneous alternation Y-maze after ICV injection A β (1–42). Subjected mice (n = 7 per group) received 0.25 nmole of A β (1–42) via ICV injection. The Y-maze task was carried out four days after A β (1–42) injection. This maze was composed of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at one arm area, and allowed to move freely through the maze during eight minutes. Alternation was defined a successive entry into the arms, on overlapping triplet sets. The alternation behavior (%) was calculated as

$$\text{alternation behavior}(\%) = (\text{actual alternations}) / (\text{possible alternations}) \times 100$$

CSF and plasma collection. Considering that the half-time of A β (1–40) efflux from the CSF to blood, of C57BL/6, was previously measured as 34.63 \pm 3.6 minutes¹⁸, we collected CSF and plasma 30 minutes after the ICV injection. CSF sampling was

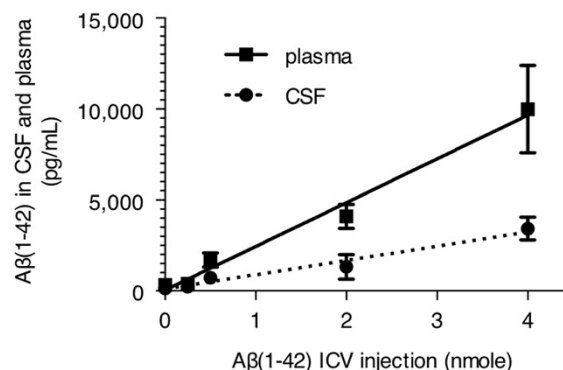


Figure 4 | Comparison of A β (1–42) levels between CSF and plasma.



performed according to the method described previously²⁵. Anesthetized mice were placed prone, and their *cisterna magna* were surgically exposed. The exposed meninges were penetrated with laboratory-produced capillary tube that had a tapered tip and obtained CSF. After CSF collection, the mice were placed supine, and their trunks were surgically opened. Blood collected from the vena cava was transferred to EDTA tube with protease inhibitor cocktail (Roche Diagnostics, cat# 11836170001) and shaken gently. Plasma was separated from EDTA treated blood. Collected CSF and plasma samples were frozen immediately on dry ice and then stored at -80°C freezer.

A β (1–42) analyzed by sandwich-ELISA in mouse CSF and plasma. Levels of A β (1–42) in CSF were quantified by using the Human A β 42 Ultrasensitive ELISA kit (Invitrogen, cat# KHB3544). Limit of detection of the kit is < 1.0 pg/mL. We added the protease inhibitor cocktail into the standard diluent buffer from the kit and, then, used the mixture to dilute the collected CSF samples to 100 or 400 folds depending on the amounts of ICV-injected A β (1–42): 0, 0.25 and 0.5 nmole of A β (1–42) to 100 folds and 2 and 4 nmole of A β (1–42) to 400 folds. The sandwich-ELISA was performed according to the manufacturer's instructions using the diluted samples. Briefly, we added diluted CSF into the 96-well plates coated with anti-A β (N-term) monoclonal antibody, and co-incubated with additional C-term targeting anti-A β (1–42) monoclonal antibody (rabbit) (3 hours) at RT. Then, we incubated the samples with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (30 minutes) at RT followed by the stabilized chromogen reagent. After addition of stop solution, we measured absorbance intensity at 450 nm to detect the amount of A β (1–42) in each sample. The concentration range of A β (1–42) standards was 0 to 100 pg/mL (8 points: 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 pg/mL). The data processing to obtain the concentration of A β (1–42) was based on the standard curve ($R^2 = 0.995$, %relative error = 7.658%).

Levels of A β (1–42) in plasma were quantified by using the Human A β 42 Ultrasensitive ELISA kit (Invitrogen, cat# KHB3544). Limit of detection of the kit is < 1.0 pg/mL. We diluted the collected plasma samples to 100 or 1,000 folds depending on the amounts of ICV-injected A β (1–42): 0, 0.25 and 0.5 nmole of A β (1–42) to 100 folds and 2 and 4 nmole of A β (1–42) to 1,000 folds. The sandwich-ELISA was performed according to the manufacturer's instructions using the diluted samples. The detail is identical to the aforementioned method measuring CSF A β (1–42). The concentration range of A β (1–42) standards was 0 to 100 pg/mL (8 points: 0, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 pg/mL). The data processing to obtain the concentration of A β (1–42) was based on the standard curve ($R^2 = 0.996$, %relative error = 11.824%).

Statistical analysis. A β (1–42) concentrations were expressed as mean \pm SD. Statistical analysis was performed with Graphpad Prism software (Graphpad Software, CA, USA). Significance was determined by using Student's *t*-test and *Kruskal-Wallis* test, followed by Dunn's post hoc tests for comparisons between vehicle control and treated samples.

All experimental protocols were approved by Korea Institute of Science and Technology and Institutional Animal Care and Use Committee of Korea Institute of Science and Technology (AP-2011L1015).

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Acknowledgments

This work was supported by KIST Institutional Program (Open Research Program 2E24582). The authors thank Mr. Jin Ho Yoon (MIT) for editing advices.

Author contributions

S.M.C., H.Y.K., W.K., T.S.K., D.J.K. and Y.K. designed the experiments. S.L. synthesized A β 42. H.V.K. performed animal preparation and Y-maze tasks. H.Y.K. and S.M.C. prepared CSF and plasma samples and performed sandwich ELISA. S.M.C., H.Y.K., H.V.K., S.L. and Y.K. wrote the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Cho, S.M. *et al.* Correlations of amyloid- β concentrations between CSF and plasma in acute Alzheimer mouse model. *Sci. Rep.* **4**, 6777; DOI:10.1038/srep06777 (2014).



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