Identification and quantification of amyloid beta-related peptides in human plasma using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract: Proteolytic processing of the amyloid precursor protein (APP) by β -secretase and γ -secretase leads to the generation and deposition of amyloid β (A β) in Alzheimer's disease (AD). N-terminally or C-terminally truncated A β variants have been found in human cerebrospinal fluid and cultured cell media using immunoprecipitation and mass spectrometry. Unfortunately, the profile of plasma A β variants has not been revealed due to the difficulty of isolating A β from plasma. We present here for the first time studies of A β and related peptides in human plasma. Twenty-two A β -related peptides including novel peptides truncated before the β -secretase site were detected in human plasma and 20 of the peptides were identified by tandem mass spectrometry. Using an internal standard, we developed a quantitative assay for the A β -related peptides and demonstrated plasma dilution linearity and the precision required for their quantitation. The present method should enhance the understanding of APP processing and clearance in AD progression.

Keywords: amyloid precursor protein, amyloid β , Alzheimer's disease, immunoprecipitation, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, plasma

Introduction

Amyloid β (A β) peptides are centrally involved in the pathogenic early events of Alzheimer's disease (AD).¹⁾ A β peptides are generated by proteolytic processing of the amyloid precursor protein (APP). APP is a single pass transmembrane protein comprised of 770 amino acids. According to the amyloid cascade hypothesis,²⁾ the accumulation of A β peptides in the cerebral cortex initiates cellular events that lead to neurodegeneration and AD.^{3),4)} In the amyloidogenic β -secretory pathway, β -secretase cleaves on the amino side of Asp672, generating two fragments: an N-terminal fragment (β -sAPP) that is secreted and a C-terminal fragment (β -CTF or C99) that remains in the plasma membrane. β -CTF is further cleaved at the carboxyl side of Val711 or

Ala713 by γ -secretase, which releases soluble A β 1-40 or A β 1-42, respectively. In the nonamyloidogenic α -secretory pathway, APP is first cleaved between Lys687 and Leu688 by α -secretase, releasing the larger soluble APP fragment (α -sAPP). The remaining C-terminal fragment (α -CTF or C83) undergoes cleavage by γ -secretase to release the short p3 peptide.⁵⁾ A β generation is believed to occur mainly in the trans Golgi network and endocytic compartments and endocytic dysfunctions have been reported in the early stages of AD and are likely to be relevant to AD pathogenesis.^{6)–9)}

Under normal conditions, A β that accumulates in the brain is degraded and cleared by numerous proteases such as neprilysin, insulin-degrading enzyme, matrix metalloproteinase, plasmin and other proteases that are able to cleave multiple sites of A β .^{10)–13)} On the other hand, a portion of the soluble A β peptide is secreted and hence found in extracellular fluids including cerebrospinal fluid (CSF) and peripheral blood. A decrease of the A β 1-42 peptide or the A β 1-42/A β 1-40 ratio in CSF is reported to be associated with AD and as such is a possible candidate for an AD marker.^{14)–16)} However, CSF collection is a time-consuming procedure and rela-

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tively invasive as a screening trial for early diagnosis. Other methods show promise as diagnostic tools for AD, including magnetic resonance imaging (MRI) assessment of medial temporal lobe atrophy, and positron emission tomography (PET) imaging of glucose metabolism and A β deposits. In particular, amyloid imaging with PET allows early diagnosis of AD. However, few elderly people without symptoms of AD can be examined using amyloid imaging with PET. Therefore, a simple, noninvasive and practical detection method is needed for early diagnosis of AD, such as a blood marker test. However, such a test is currently unavailable. Many studies have examined the plasma concentration of A β peptides in AD patients using a sandwich enzyme-linked immunosorbent assay (ELISA). Unfortunately, a consistent association between plasma A β 1-40 or A β 1-42 and AD has not been demonstrated so far.¹⁷⁾

Quantification of A β in a biological sample is typically performed using a sandwich ELISA. This method is limited because the targeted A β peptide is detected by two monoclonal antibodies (capture and detection antibodies) that simultaneously bind to the N-terminus and C-terminus of the peptide. Thus, A β forms quantified in earlier studies were limited to A β 1-40 and A β 1-42. Importantly, other A β variants have been found in cultured cell media and human CSF. These variants were identified by enriching A β variants and by depleting of non-target molecules contained in these biological fluids using immunoprecipitation (IP) followed by mass spectrometry (MS).^{18)–23)} N-terminal and C-terminal truncated A β variants have been detected in human CSF using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).^{18),19)} Peptides starting prior to the β -secretase site and ending at the α -secretase site have also been identified in CSF from AD patients and control subjects.²²⁾ Unfortunately, A β variants have not been found in human plasma due to the difficulty of conducting IP of A β variants in plasma. The reasons are as follows: 1) the concentrations of A β 1-40 and A β 1-42 in human plasma are approximately 50-fold lower than those in human CSF²⁴⁾ and 2) the total protein concentration is 100-fold higher in plasma compared with CSF.^{25),26)} Therefore, the detection of plasma A β variants by immunoprecipitation coupled to mass spectrometry (IP-MS) remains a major challenge.

In this paper, we present an IP-MS method that makes it possible to detect and identify A β -related peptides in human plasma. Furthermore, we developed a quantitative assay using two different

isotopically labeled human peptides (A β 1-38 and A β 1-40) as internal standards. The precision of the quantitative assay was demonstrated by inter-well, as well as intra- and inter-day assays.

Materials and methods

Preparation of stable isotopically-labeled A β peptide solutions. Stable isotopically-labeled (SIL) human A β 1-38 (¹³C-labeled at Phe and Ile) and A β 1-40 (¹³C, ¹⁵N-labeled at Arg and Lys) were used for internal standards. They were purchased from AnaSpec (San Jose, CA). The lyophilized stocks of SIL-A β 1-38 and SIL-A β 1-40 were dissolved in 50 mM NaOH and subsequently applied to a COSMOSIL(R) 5Diol-120-II [7.5 mm I.D. $\times$ 600 mm] column (Nacalai Tesque, Kyoto) attached to a Prominence HPLC System (Shimadzu Corp., Kyoto, Japan). Mobile phase, flow rate, column temperature, and detection wavelength were as follows: 40 mM Tris-HCl, pH 8.0, one mL/min, 25 °C, and 214/280 nm, respectively. Portions of the fractions were analyzed using 15–20% Tricine-SDS-PAGE under non-reducing conditions. Peptides were visualized using a silver staining kit (Invitrogen, Carlsbad, CA). The eluates were collected in fractions with bands corresponding to the molecular weight of monomers and were diluted with 40 mM Tris-HCl, 150 mM NaCl, pH 8.0 containing one mg/mL bovine serum albumin. Monomeric SIL-A β 1-38 and SIL-A β 1-40 were dispensed in tubes and stored at –80 °C.

Preparation of the antibody-coated beads. Mouse monoclonal anti-A β antibodies (6E10 IgG1 and 4G8 IgG2b) were purchased from Covance (Princeton, NJ). The buffers containing the antibodies were exchanged using Zeba Desalt Spin Columns (Pierce, Rockford, IL, USA). Specifically, ten mM citrate buffer, pH 6.0, containing five mM EDTA-2Na and four mM L-cysteine was used for 6E10 whereas 50 mM Tris-HCl, pH 8.8 was used for 4G8. 6E10 IgG1 (500 μ g) was incubated with 600 μ L immobilized ficin (Pierce) at 37 °C for 24 h. 4G8 IgG2b (500 μ g) was incubated with 1200 ng Lys-C (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for two h. Proteolytic fragments were concentrated in Amicon Ultrafiltration devices (Millipore, Cork, IR); 50 μ L of each concentrate was subsequently separated by size exclusion chromatography (SEC). SEC was carried out using a Prominence HPLC System (Shimadzu Corp.). Mobile phase, flow rate, column temperature, and detection wavelength used were as follows: 50 mM phosphate buffer, pH 6.5, containing 300 mM NaCl and five mM

EDTA, 0.5 mL/min, 25 °C, and 214/280 nm, respectively. The proteolytic fragments were applied to a TSKgel G3000SWXL [7.8 mm I.D. × 30 cm L] column (TOSOH Bioscience, Tokyo, Japan). Fractions corresponding to the F(ab')₂ fragment were concentrated in Amicon Ultra filtration devices (Millipore). F(ab')₂ fragments were reduced with 30 mM cysteamine at 37 °C for 30 min in 50 mM phosphate buffer, pH 6.5, containing five mM EDTA. To remove cysteamine from the solutions, 6E10 and 4G8 F(ab') fragments were dialyzed in 50 mM phosphate buffer, pH 6.5, containing five mM EDTA using a Slide-A-Lyzer MINI Dialysis Unit (Pierce). Dynabeads M-270 Amine (120 µL suspension containing 30 mg beads/mL, Invitrogen) were washed three times with 480 µL of 100 mM phosphate buffer, pH 7.4, and added to 240 µL of 2.25 mM SM(PEG)₂₄ Crosslinkers (Pierce) in 100 mM phosphate buffer, pH 7.4. After incubating at room temperature for 30 min in the dark, the beads were washed three times with 480 µL of 100 mM phosphate buffer, pH 7.4 and then resuspended in 120 µL of 100 mM phosphate buffer, pH 7.4. The dialyzed 6E10 and 4G8 F(ab') fragments (12 µg each) were mixed and then added to the bead solution, incubated at room temperature for two h in the dark, and washed three times with 480 µL of 100 mM phosphate buffer, pH 7.4. Unreacted maleimide groups of the beads were blocked with 0.4 mM L-cysteine in 100 mM phosphate buffer, pH 7.4.

Immunoprecipitation of A β -related peptides. Human EDTA plasma from a 44 years old donor, who was confirmed to be healthy by a diagnostic process with an interview, was purchased from C.C. Biotech Corp. (San Diego, CA). Using the antibody-coated beads with a mixture of 6E10 and 4G8 F(ab') fragments, A β -related peptides were purified from human plasma using the following method. Plasma (250 µL) was mixed with an equivalent amount of the binding buffer (100 mM Tris-HCl, 800 mM N-acetyl-D-glucosamine, 1% w/v n-octyl- β -D-thioglucoiside (OTG), 300 mM NaCl, adjusted to pH 7.4) with or without SIL-A β 1-38 and/or SIL-A β 1-40, and then added to five µL of 10% w/v PEG6000 (Nacalai Tesque). For the evaluation of plasma dilution linearity, 150, 100, 50 and 25 µL of plasma were diluted with phosphate buffered saline (PBS) up to 250 µL before mixing with the binding buffer. In the stability verification of A β -related peptides, 250 µL human plasma was mixed with 240 µL binding buffer and five µL of 10% w/v PEG6000. After incubation at 4 °C for zero or three

h, ten µL of SIL-A β 1-38 at 250 pM in the binding buffer was added to it. The diluted plasma sample was filtered using Ultrafree-MC, DV 0.65 µm centrifugal filter devices (Millipore). The plasma sample was subsequently pre-treated with 500 µL of Protein G Plus Agarose (50% slurry; Pierce) at 4 °C for one h. The antibody-coated beads (150 µg) were first washed twice with 50 mM glycine-HCl buffer, pH 2.8 containing 1% OTG and then three times with Tris-buffered saline (TBS) containing 0.5% OTG. They were then incubated with the plasma sample at 4 °C for one h. The beads were then washed five times with TBS-0.5% OTG. After washing twice with 50 mM ammonium acetate, pH 7.4 and once with H₂O, bound peptides were eluted with 2.5 µL acetonitrile/H₂O (7:3 v/v) containing five mM HCl. The eluate was immediately applied onto a µFocus MALDI plateTM 900 µm (Hudson Surface Technology, Inc., Fort Lee, NJ) as follows: each 0.5 µL of the eluate on four wells for quantification using a linear TOF or two µL of the eluate on one well for peptide identification using a quadrupole ion trap (QIT) reflectron TOF.

MALDI-TOF MS. Mass spectra were obtained using a MALDI-linear TOF mass spectrometer (AXIMA Performance, Shimadzu/KRATOS, Manchester, UK) equipped with a 337 nm nitrogen laser in the positive ion mode. To reduce signal variability for peptide quantification, a linear TOF MS spectrum was automatically acquired from the accumulation of 40 laser shots at each of 400 different spots in raster mode. MS/MS analysis for identification of the A β -related peptides was conducted using a MALDI-QIT reflectron TOF mass spectrometer (AXIMA Resonance, Shimadzu/KRATOS) in the positive ion mode and MS/MS fragments were generated by collision-induced dissociation (CID) with argon gas. α -cyano-4-hydroxycinnamic acid (CHCA) purchased from LaserBio Labs (Sophia-Antipolis Cedex, France) was used for the MALDI matrices for linear TOF MS, and 2,5-dihydroxybenzoic acid (DHB) purchased from LaserBio Labs for QIT reflectron TOF MS. The matrix solutions were prepared by dissolving one mg of CHCA or five mg of DHB in one mL 70% v/v acetonitrile containing 0.1% v/v trifluoroacetic acid. Methanediphosphonic acid (MDPNA; 0.4% w/v in 70% v/v acetonitrile) was used as a matrix additive. After the 20 µL CHCA or DHB matrix solution was mixed with an equivalent amount of 0.4% w/v MDPNA, 0.5 µL of the matrix containing MDPNA was added to the eluate on the µFocus MALDI plate.

The signal-to-noise ratio (S/N) was determined using Launchpad version 2.9.1 software (Shimadzu, Kyoto, Japan). The limit of detection (LOD) was established with an S/N of 3:1. The m/z reported in the linear TOF and the QIT-reflectron TOF represent the average and monoisotopic peak of the protonated signal $[M + H]^+$, respectively. The m/z value was calibrated with 50 fmol each of human angiotensin II and human ACTH fragment 18–39 and 250 fmol each of bovine insulin oxidized beta-chain and bovine insulin as external standards. Peak lists were created from the raw MS/MS spectra by Mascot Distiller (Matrix Science) as follows: a minimal signal-to-noise of ten and a maximal number of 100 masses. Peaks closer than 64 to the precursor were excluded. The generated peak list was followed by analysis using Mascot software Version 2.4 (Matrix Science). The Mascot search parameters were as follows: no enzyme, no missed cleavage, SwissProt database with species limitation (only human) that corresponded to 20,253 protein entries, and tolerances of 0.3 and 0.4 Da for the precursor and fragment ions, respectively. Variable modification of oxidation (M) was only used for the oxidized peptide.

ELISA measurement of A β . The concentrations of monomeric SIL-A β 1-38 and SIL-A β 1-40 solutions and the endogenous APP672-711(A β 1-40) in plasma were determined using a human A β (1-40) ELISA Kit II purchased from Wako Pure Chemical Industries and human A β (1-38) (FL) Assay Kit purchased from Immuno-Biological Laboratories (Gunma, Japan). The assays were performed according to each manufacturer's protocol. Each sample was assessed in duplicate or triplicate.

Data analysis. Quantification of the A β -related peptides in plasma was performed by normalizing the intensity ratios of an analyte peptide to an internal standard (SIL-A β 1-38 or SIL-A β 1-40) measured in a linear TOF. The peptide mass tolerance for quantification was set within 2.5 Da of the theoretical mass. To correct the signal variation of the MS peak intensities, we established the following analytical criteria. One immunoprecipitation preparation produced four MS spectra and consequently made four intensity ratio values per analyte peptide. The value of an analyte peptide peak was accepted for averaging if its intensity ratio was within the range of 0.7- to 1.3-fold median of four intensity ratios obtained from one preparation. Unacceptable values were eliminated from averages as outliers. An analyte peak with S/N < 3 was not used for averaging. If the number of intensity ratio values used for averaging was < 3, the

analyte peak was defined as “not detectable (N/D)” in the immunoprecipitation preparation. The standard curve of SIL-A β 1-38 or SIL-A β 1-40 was constructed using intensity ratios (SIL-A β 1-38/SIL-A β 1-40) or (SIL-A β 1-40/SIL-A β 1-38), respectively, versus various concentrations. The relative error (%RE) of IP-MS measurement to ELISA was calculated using the following formula: %RE = (|IP-MS measurement value – ELISA value| $\times$ 100)/ELISA value. The square of the correlation coefficient (R^2) was used to evaluate the fit of the standard curve to a quadratic function and the linearity of the intensity ratio of A β -related peptides in the plasma dilution series. The precision of the quantitative A β -related peptides assay was evaluated on each of four days by analyzing four replicates. Percent coefficient of variance (%CV) is expressed as the standard deviation (SD) divided by the mean. Inter-well precision was assessed using %CV values between wells from each of four replicates on each of four days. Intensity data with N/D were excluded from the assessment of inter-well, intra- and inter-assays. Intra-day precision was assessed using %CV values between replicates on each of four days. If the number of usable intensity ratio values of an analyte peptide was < three among four replicates on one day, the %CV value of the analyte peptide was not acceptable for intra-assay. The analyte peptide without intra-day %CV value on all four days is represented by “not acceptable (N/A)”. Inter-day precision was assessed using the %CV value between days. If usable values of an analyte peptide were obtained on < three of the four days, the inter-day %CV value of the analyte peptide is represented by “N/A”.

Results

Detection of peptides immunoprecipitated from human plasma. We immunoprecipitated 250 μ L aliquots of human plasma, followed by application of linear TOF and a QIT reflectron TOF (Fig. 1). Peaks at m/z corresponding to A β 1-40 and A β 1-42 (*i.e.*, APP672-711 and APP672-713) were observed in mass spectra in both the linear TOF and the QIT reflectron TOF. In addition to these major components found in senile AD plaques, molecular masses of other peaks corresponded to those of peptides truncated from APP. They included N-terminally or C-terminally truncated APP672-711(A β 1-40) peptides and novel truncated forms of peptides that started several amino acids N-terminally of the β -secretase site (Asp672 of APP), *i.e.*, longer N-terminal A β forms than previously known.

Fig. 1. MALDI-TOF mass spectra of A β -related peptides purified from human plasma by IP. A β -related peptides were immunoprecipitated from 250 μ L human plasma and eluted with 2.5 μ L acetonitrile/H₂O (7:3 v/v) with five mM HCl. The eluate (0.5 μ L) was measured by a linear TOF (A). Two μ L of the eluate was measured by QIT reflectron TOF (B). Symbols as follows: *: peak corresponding to molecular weight of peptide cleaved from APP, n: non-specific peak, f: fragment ion peak generated during mass spectrometry.

Four peaks represented by “f” in mass spectra from the QIT reflectron TOF were probably generated by peptide fragmentation in the ion trap because they were not observed in mass spectra of linear TOFs. Mass spectra of the linear TOF detected non-specific peaks from human plasma that were demonstrated by examining plasma molecules bound to (PEG)₂₄ beads without F(ab') (Fig. 2). To summarize the results, 22 peaks at m/z values corresponding to peptides truncated from APP were confirmed in linear TOF and QIT reflectron TOF (Table 1). Among the 22 peaks, the peak at m/z corresponding to APP672-711(A β 1-40) showed the highest intensity, which is consistent with results of A β variants in human CSF in previous reports.^{20),27)}

Fig. 2. The background peaks in the mass spectrum of a negative control. Plasma (250 μ L) was treated using (PEG)₂₄ beads without F(ab'), instead of 6E10/4G8 F(ab')-(PEG)₂₄ beads, as a negative control. Molecules bound to (PEG)₂₄ beads without F(ab') were eluted with 2.5 μ L acetonitrile/H₂O (7:3 v/v) with five mM HCl and 0.5 μ L of the eluate was measured by a linear TOF.

Characterization of the A β -related peptide peaks. To verify the identities of the A β -related peptides detected in IP-MS, we obtained MS/MS spectra from 20 of the 22 detected peaks using CID. The MS/MS peak lists were analyzed by Mascot searches to identify peptides truncated from APP (Table 1). Whereas Mascot scores of six peaks were more than 20, those of the other peaks showed relatively low scores due to their weak intensities. However, fragment ions from each corresponding A β -related peptide, especially those generated by preferred cleavage on the C-terminal side of aspartic acid or glutamic acid residues,²⁸⁾ were detected in all MS/MS spectra (Table 1). Furthermore, considering (1) the high selectivity of the antibodies, (2) the precision of the masses measured by QIT reflectron TOF MS and (3) the amino acid sequences including the epitope of antibody 6E10 or 4G8, it is highly probable that the proposed identities of the A β -related peptides in Table 1 are correct. For two of the 22 A β -related peptides, no MS/MS spectra were acquired because the two peptides were not detected in QIT reflectron TOF MS.

The development of quantitative assay for A β 1-38 and A β 1-40. Quantitative assays of plasma A β -related peptides were performed using SIL-A β 1-38 (m/z 4162.6) or SIL-A β 1-40 (m/z 4356.9) as internal standards. SIL-A β 1-38 and SIL-A β 1-40 peptides contained the epitope sequences for 6E10 and 4G8. Since an A β peptide and the corresponding SIL-A β peptide were chemically identical, they had the same ionization efficiency and the ratio between their peak intensities was a direct measurement of their relative abundance.²⁹⁾ Thus, concentrations of the endogenous APP672-709(A β 1-38) and APP672-711(A β 1-40) peptides could be estimated by constructing a standard curve for the intensity ratio of each corresponding SIL-A β . The binding buffer (containing SIL-A β 1-40 over a range from 2.5 to 100 pM and SIL-A β 1-38 at a constant concentration of ten pM) was mixed with an equivalent amount of plasma, which was followed by IP-MS (Fig. 3A). The intensity ratios of SIL-A β 1-40/SIL-A β 1-38 are plotted versus the concentration of measured SIL-A β 1-40 in Fig. 3B. The standard curve could be fitted to a quadratic function and its R^2 value was 0.999. We used the standard curve of SIL-A β 1-40 to estimate the concentration of endogenous APP672-711(A β 1-40) in tested plasma, determining a final value of 57.87 ± 0.77 pM. To support the quantitative IP-MS assay data, a commercially available ELISA for A β 1-40 was performed, indicating its concentration was

51.70 ± 2.35 pM in the same sample. The relative error of IP-MS measurement to ELISA was 11.9% RE. There was little difference between the value obtained by the quantitative assay of IP-MS and that obtained by ELISA, which demonstrated that the quantitative assay using SIL-A β 1-38 as an internal standard could be used as a substitute for a generally used ELISA for A β . In a similar manner, a standard curve for the intensity ratio of SIL-A β 1-38/SIL-A β 1-40 versus the concentration of measured SIL-A β 1-38 was obtained by IP-MS of plasma mixed with SIL-A β 1-38 at concentrations ranging from one to 25 pM and SIL-A β 1-40 at a constant concentration of 25 pM (Fig. 3C and D). The standard curve of SIL-A β 1-38 could also be fit to a quadratic function and its R^2 value was 1.000. A concentration of endogenous APP672-709(A β 1-38) in tested plasma was calculated to be 2.82 ± 0.17 pM using the standard curve of SIL-A β 1-38. To date, determining the plasma concentration of APP672-709(A β 1-38) has not been easy owing to the lack of a commercially available ELISA. However, our method can provide an effective approach in determining the endogenous APP672-709 (A β 1-38) concentration in human plasma.

Validation of the quantitative assay for A β -related peptides. We addressed the quantification of all of the A β -related peptides detected in IP-MS by using SIL-A β 1-38 as the internal standard. Table 2 shows the intensity ratio values of analyte peptides to SIL-A β 1-38 obtained from a mixture of 250 μ L human plasma and the equivalent amount of 10 pM SIL-A β 1-38. To validate the quantitative assay for A β -related peptides, we first evaluated the linearity of the intensity ratio of each A β -related peptide to SIL-A β 1-38 for dilutions of plasma. Figure 4 presents the intensity ratio of each A β -related peptide obtained by measuring plasma samples at dilutions from 10% to 100% with PBS, that is, plasma volumes over the range 25–250 μ L. Intensity ratios of 15 A β -related peptides demonstrated excellent linearities ($R^2 = 0.912$ – 0.999). APP677-711(A β 6-40) peptide showed a relatively low R^2 value of 0.861. R^2 values of six other A β -related peptides were not calculated owing to their detection limits of 150 or 250 μ L plasma (Table 2). This result indicated that the analyzed volume of plasma was acceptable in volumes of 250 μ L or less.

To validate the precision of the quantitative IP-MS assay, inter-well, intra-day and inter-day assays were conducted using SIL-A β 1-38 as an internal standard. We performed IP-MS on four replicates on each of four different days and then calculated %CV values between the four wells, the four replicates or

Table 1. A β -related peptides observed in mass spectra of immunoprecipitation from human plasma

A/ β -related peptides	Sequence	Theoretical	Measured	Measured	Observed fragmentation at C-terminal side of Asp or Glu in MS/MS spectrum	Mascot score	
	<u>TEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA</u> 663	average	average	Theoretical			monoisotopic
		mass	mass in linear TOF	monoisotopic mass			mass in QIT reflectron TOF
APP682-711(A/ β 11-40)	EVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	3152.7	3153.5	3150.68	3150.89	b ₁₂ , b ₁₃ , y ₁₇ , y ₁₈	5
APP677-709(A/ β 6-38)	HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	3514.0	3513.5	3511.74	3511.92	b ₁₇ , b ₁₈ , y ₁₅ , y ₃₁	27
APP677-710(A/ β 6-39)	HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV	3613.1	3613.1	3610.81	3610.93	b ₁₇ , b ₁₈ , y ₁₆	22
APP672-704(A/ β 1-33)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG	3675.0	3677.0	3672.78	3672.85	b ₂₂ , b ₂₃ , y ₂₂ , y ₂₆	5
APP677-711(A/ β 6-40)	HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	3712.2	3711.8	3709.88	3709.99	b ₁₇ , b ₁₈ , y ₁₇ , y ₁₈ , y ₃₃	33
APP676-711(A/ β 5-40)	RHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	3868.4	3868.1	3865.98	3866.06	b ₁₉ , y ₁₈ , y ₃₃	11
APP672-706(A/ β 1-35)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM	3919.4	3918.1	3916.91	3916.95	b ₂₂ , b ₂₃ , y ₁₂ , y ₂₈	6
APP672-708(A/ β 1-37)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG	4075.6	4074.8	4073.00	4072.93	b ₂₂ , b ₂₃ , y ₁₄ , y ₃₀ , y ₃₆	14
APP672-709(A/ β 1-38)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	4132.6	4132.4	4130.02	4129.89	b ₁₁ , b ₂₂ , b ₂₃ , y ₃₁ , y ₃₅ , y ₃₇	22
APP674-711(A/ β 3-40)	EFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4144.7	4144.7	4142.09	4142.08	b ₉ , b ₂₀ , b ₂₁ , y ₁₇ , y ₃₃	9
APP672-710(A/ β 1-39)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV	4231.8	4231.7	4229.09	4229.20	b ₁₁ , b ₂₂ , y ₃₂	13
APP672-711(A/ β 1-40)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4330.9	4330.6	4328.16	4328.11	b ₁₁ , b ₂₂ , b ₂₃ , y ₁₇ , y ₂₉ , y ₃₃ , y ₃₇ , y ₃₉	90
OxAPP672-711(Ox A/ β 1-40)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4346.9	4346.6	4344.16	4343.99	b ₁₁ , b ₂₂ , b ₂₃ , y ₃₃ , y ₃₉	14
APP671-711	MDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4462.1	4462.0	4459.20	4459.40	b ₂₃ , b ₂₄ , y ₁₇ , y ₂₉ , y ₃₃ , y ₃₉	8
APP669-709	VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	4491.1	4491.6	4488.22	4488.15	b ₁₀ , b ₁₄ , b ₂₆ , y ₃₅	15
APP672-713(A/ β 1-42)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	4515.1	4514.8	4512.28	4512.35	b ₁₁ , b ₂₂ , b ₂₃ , y ₃₅	17
APP669-710	VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGV	4590.3	4590.2	4587.29	4587.06	b ₉ , b ₁₃ , b ₂₅ , y ₃₃ , y ₃₉	11
APP669-711	VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4689.4	4689.0	4686.36	4686.19	b ₁₀ , b ₁₄ , b ₂₅ , b ₂₆ , y ₃₃ , y ₃₇	17
APP666-709	ISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	4820.5	4818.2	4817.38	4817.27	b ₁₃ , y ₃₁ , y ₃₇	N/I
APP666-711	ISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	5018.7	5018.7	5015.52	5015.28	b ₁₃ , b ₂₈ , b ₂₉ , y ₃₉	17
APP664-711	EEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	5277.0	5276.9	5273.60	N/D	N/A	N/A
APP663-711	TEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	5378.1	5378.7	5374.65	N/D	N/A	N/A

Peptides observed in linear TOF and QIT reflectron TOF are listed. OxAPP672-711/Ox β 1-40 represents APP672-711/A β 1-40 peptide with the oxidized Met696. N/D = Not detectable. N/A = Not applicable in MS/MS analysis. N/I = Not identified by Mascot search.

Fig. 3. Standard curves for A β 1-38 and A β 1-40 determination using stable isotopically labeled peptides. Binding buffer (250 μ L) containing SIL-A β 1-40 at concentrations ranging from 2.5 to 100 pM plus SIL-A β 1-38 at a constant concentration of 10 pM (A) or SIL-A β 1-38 at concentrations ranging from one to 25 pM and SIL-A β 1-40 at a constant concentration of 25 pM (C) were added to the equivalent amount of plasma, which was followed by IP-MS. These mass spectra were acquired by a linear TOF. The standard curves were generated for A β 1-40 (B) and A β 1-38 (D) determination over the range 2.5–100 pM and 1–25 pM, respectively.

the four days (Table 3). The inter-well precision of the 22 A β -related peptides had means ranging from 4.96–10.43%CV, which showed that these measurements provided high reproducibility between four spectra obtained from one preparation. In intra-day assays carried out by using four replicates, 17 of 20 analyzable peptides achieved adequate precisions of 3.43–17.59%CV. In inter-day assays over a period of four days, 15 of 19 analyzable peptides indicated appropriate precisions of 7.39–18.26%CV, demonstrating that the quantitative assay of peptides can be used for differential analysis and biomarker eval-

uation. However, APP677-710(A β 6-39), APP672-704(A β 1-33) and APP677-711(A β 6-40) had higher %CV values in both intra-day and inter-day assays. These three peptide peaks appeared in the vicinity of the highest intensity nonspecific peak (m/z 3765.0) observed in Fig. 1, decreasing the precision and dilution linearity of the three peptide analyses. In the plasma tested for this validation, the peak signals of APP682-711(A β 11-40), APP671-711 and APP663-711 appeared near the LOD. Hence, the amount of data for the three peptides was insufficient for validation, appearing as N/A in intra- and inter-assays.

Fig. 4. Evaluation of plasma dilution linearity. The intensity ratio of each A β -related peptide was obtained by measuring plasma at a dilution from 10% to 100%, that is, plasma volumes over the range 25–250 μ L. Plasma dilution linearity was evaluated with the correlation coefficient (R^2) obtained from the intensity ratio of A β -related peptide versus plasma concentration. The R^2 value is presented in the graph of each A β -related peptide. A β -related peptides with only two measured points are represented by N/A and those with less than two measured points were eliminated from this evaluation.

It is possible that the generation of A β -related peptides was due to degradation during IP completed over a three h period. To verify the stability of A β -related peptides, we allowed human plasma to incubate at 4°C for three h before IP-MS and analyzed the change of 16 A β -related peptide intensities compared to a plasma sample without the three h pre-incubation step (Table 2). The other A β -related peptides were eliminated from this evaluation due to their LOD near 250 μ L plasma. The pre-incubation at 4°C for three h did not change the intensity ratios as indicated in Fig. 5. This result demonstrated that the A β -related peptides were not artifacts produced from IP.

Discussion

The present study is, to our knowledge, the first report describing the use of MS to detect many A β -

Fig. 5. Comparison of the A β -related peptides in plasma samples with or without pre-incubation at 4°C for three h. Human plasma (250 μ L) was mixed with 240 μ L binding buffer and five μ L 10% w/v PEG6000, allowed to incubate at 4°C for three h. The plasma samples with or without pre-incubation were analyzed by IP-MS after ten μ L of SIL-A β 1-38 solution at a concentration of 250 pM was added to it.

Table 2. Intensity ratios and LODs of A β -related peptides of the tested plasma sample

A β -related peptides	Intensity ratio in plasma 250 μ L [Analyte/SIL-A β 1-38] ^{a)}	LOD (μ L)
APP682-711(A β 11-40)	0.058 $\pm$ 0.003	250
APP677-709(A β 6-38)	0.410 $\pm$ 0.026	250
APP677-710(A β 6-39)	0.178 $\pm$ 0.021	100
APP672-704(A β 1-33)	0.571 $\pm$ 0.068	50
APP677-711(A β 6-40)	1.601 $\pm$ 0.078	$\leq$ 25
APP676-711(A β 5-40)	0.290 $\pm$ 0.015	$\leq$ 25
APP672-706(A β 1-35)	0.141 $\pm$ 0.013	50
APP672-708(A β 1-37)	0.173 $\pm$ 0.008	50
APP672-709(A β 1-38)	0.371 $\pm$ 0.035	$\leq$ 25
APP674-711(A β 3-40)	0.152 $\pm$ 0.006	50
APP672-710(A β 1-39)	0.191 $\pm$ 0.016	50
APP672-711(A β 1-40)	4.114 $\pm$ 0.327	$\leq$ 25
OxAPP672-711(Ox-A β 1-40)	0.339 $\pm$ 0.044	50
APP671-711	0.040 $\pm$ 0.007	250
APP669-709	0.098 $\pm$ 0.007	100
APP672-713(A β 1-42)	0.067 $\pm$ 0.007	100
APP669-710	0.083 $\pm$ 0.011	100
APP669-711	0.273 $\pm$ 0.026	50
APP666-709	0.045 $\pm$ 0.004	150
APP666-711	0.054 $\pm$ 0.007	100
APP664-711	0.027 $\pm$ 0.002	150
APP663-711	0.019 $\pm$ 0.001	250

The intensity ratio values of A β -related peptides to SIL-A β 1-38 were obtained from 250 μ L plasma mixed with the equivalent amount of 10 pM SIL-A β 1-38. LOD of each A β -related peptide was evaluated using the plasma volumes of 25, 50, 100, 150 and 250 μ L.

^{a)}Intensity ratios are represented by means $\pm$ SD that were obtained in four spectra from one preparation.

related peptides from human plasma. Twenty-two A β -related peptides including the well-known A β peptides were observed, and 20 of them were identified by MS/MS analysis. Surprisingly, of the 22 A β -related peptides found in this study, eight were previously unknown forms that extended N-terminally from the β -secretase site to the γ -secretase site. Although many researchers have demonstrated A β variants in human CSF using MS, no one had previously succeeded in detecting them in human plasma (except for A β 1-40 and A β 1-42). Therefore, it has not been clear which forms are present in human plasma. In earlier studies of CSF A β peptides, IP-MS analyses have employed $\geq$ 940 μ L human CSF, in which A β 1-40 is present at low nanomolar concentrations and A β 1-42 is at subnanomolar concentra-

tions.^{20)-22),27),30)} Our approach achieved the detection of A β 1-40 (51.70 ± 2.35 pM) and A β 1-38 (2.82 ± 0.17 pM) from as little as 25 μ L human plasma (Table 2). Additionally, the standard curve of SIL-A β 1-38 showed the LOD of A β 1-38 at concentrations as low as one pM (Fig. 3D). These results demonstrated that our method has high detection sensitivity in comparison with conventional IP-MS methods. We have further developed the quantitative aspect of this assay for A β -related peptides by adding SIL-A β 1-38 or SIL-A β 1-40 as an internal standard. The replacement of ^{12}C and ^{14}N with ^{13}C and ^{15}N led to a mass shift of 30 Da and 26 Da for SIL-A β 1-38 and SIL-A β 1-40, respectively. Although the peaks of the internal standards did not interfere with the peaks of the corresponding peptides naturally present in plasma, the peak of SIL-A β 1-40 appeared in the vicinity of the oxidized APP672-711(A β 1-40) peak (Fig. 3A, C). In addition, SIL-A β 1-40 peptides readily aggregate due to their high hydrophobicity compared to SIL-A β 1-38. Hence, SIL-A β 1-38 was preferred as the internal standard over SIL-A β 1-40. The means of the %CV values in intra- and inter-day assays were $< 20\%$ for 15 A β -related peptides, demonstrating that the quantitative assay for most peptides can be used for differential analysis and biomarker evaluation. However, the non-specific peak with high intensity caused a decrease of the precision and the plasma dilution linearity of any A β -related peptides in its vicinity. To overcome this, future improvement of the method is needed to reduce the non-specific binding to the antibody-coated beads. Whereas the assay precision of IP-MS is not comparable with that of sandwich ELISA, our approach has a distinct advantage of simultaneously quantifying A β -related peptides, including peptides for which there is no commercially available ELISA.

Sandwich ELISA measurements have shown that the concentration of APP672-713(A β 1-42) is 10–30% of that of APP672-711(A β 1-40).^{24),31)} However, in the present study, the signal intensity of APP672-713(A β 1-42) was $< 10\%$ of that of APP672-711(A β 1-40) (Table 2). In other word, the ratio of APP672-713(A β 1-42) to APP672-711(A β 1-40) determined by IP-MS was low relative compared to that obtained by ELISA. In MALDI-TOF MS, the signal intensity of hydrophobic peptides is generally lower than that of hydrophilic peptides.³²⁾ Since APP672-713(A β 1-42) has a higher hydrophobicity than APP672-711(A β 1-40), the signal intensity of APP672-713(A β 1-42) appears lower than that of

Table 3. Evaluation of the assay precision by inter-well, intra- and inter-day assays

A β -related peptides	Precision (%CV)		
	Inter-well (4 wells) ^{a)}	Intra-day (4 replicates) ^{b)}	Inter-day (4 days) ^{c)}
APP682-711(A β 11-40)	5.20 ^{d)}	N/A	N/A
APP677-709(A β 6-38)	6.52 $\pm$ 2.73	16.43 $\pm$ 5.15	13.74
APP677-710(A β 6-39)	10.43 $\pm$ 3.44	18.85 $\pm$ 7.63	26.72
APP672-704(A β 1-33)	8.34 $\pm$ 4.08	34.53 $\pm$ 18.82	36.46
APP677-711(A β 6-40)	6.13 $\pm$ 2.21	25.48 $\pm$ 11.12	21.95
APP676-711(A β 5-40)	5.27 $\pm$ 2.60	13.96 $\pm$ 6.13	14.92
APP672-706(A β 1-35)	8.59 $\pm$ 3.25	15.12 $\pm$ 1.77	14.78
APP672-708(A β 1-37)	5.26 $\pm$ 3.04	3.89 $\pm$ 1.87	7.39
APP672-709(A β 1-38)	4.96 $\pm$ 3.21	3.43 $\pm$ 2.50	9.20
APP674-711(A β 3-40)	5.45 $\pm$ 2.90	9.04 $\pm$ 4.01	8.47
APP672-710(A β 1-39)	6.36 $\pm$ 3.13	8.75 $\pm$ 5.67	10.90
APP672-711(A β 1-40)	5.08 $\pm$ 3.69	17.59 $\pm$ 4.78	8.84
OxAPP672-711(Ox A β 1-40)	7.69 $\pm$ 4.69	5.95 $\pm$ 1.58	7.48
APP671-711	7.97 $\pm$ 2.73	24.50 ^{d)}	N/A
APP669-709	7.27 $\pm$ 2.84	12.52 $\pm$ 4.20	13.72
APP672-713(A β 1-42)	8.61 $\pm$ 2.40	14.36 $\pm$ 7.17	18.26
APP669-710	8.98 $\pm$ 3.30	13.37 $\pm$ 6.77	12.52
APP669-711	7.88 $\pm$ 5.07	17.45 $\pm$ 6.19	11.27
APP666-709	8.67 $\pm$ 3.47	15.24 $\pm$ 8.83	17.96
APP666-711	7.15 $\pm$ 4.03	13.96 $\pm$ 7.06	16.75
APP664-711	7.31 $\pm$ 2.86	15.49 $\pm$ 10.38	23.74
APP663-711	8.52 $\pm$ 3.89	N/A	N/A

IP-MS of four replicates were performed on each of four different days for evaluating the precision between four wells, four replicates or four days.

^{a)}The means $\pm$ SD calculated from all of %CV values between four spectra of each of four replicates on each of four days.

^{b)}The mean $\pm$ SD calculated from all of %CV values between four replicates on each of four days.

^{c)}The %CV values between four days.

^{d)}The SD value was not calculated because the only one %CV value was obtained.

N/A = Not applicable in the evaluation of Intra- or Inter-day assay.

APP672-711(A β 1-40). In addition, it is likely that the differences in the affinities of the used antibody-coated beads for APP672-711(A β 1-40) and APP672-713(A β 1-42) caused a decrease in the recovery rate of APP672-713(A β 1-42) during IP. Although the sensitivity of different peptides in IP-MS could depend on their hydrophobicity and the used antibody applied, the plasma dilution linearity of each A β -related peptide was demonstrated in Fig. 4. Therefore, our method is able to quantify each A β -related peptide using the intensity normalized to an internal standard.

The A β -related peptides showed one of the following three features: (1) C-terminally truncated A β , (2) N-terminally truncated A β or (3) APP peptides truncated before the β -secretase site. γ -secretase has some cleavage sites at the C-terminus

of A β , generating A β X-33, A β X-37, A β X-38, A β X-39 and A β X-40.^{33)–35)} On the other hand, N-terminally truncated A β s such as A β 3-X, A β 5-X and A β 6-X could be cleavage products of neprilysin, metalloendopeptidase, coagulation factor XIa or plasmin.^{12),36)} The presence of the short C- and N-terminal A β s found in human plasma was in agreement with the features of A β variants reported for CSF.^{20),21)} However, to date, there has been no persuasive evidence of the production of APP peptides truncated before the β -secretase site. In the amyloidogenic β -secretory pathway, β -secretase first cleaves at the amino side of Asp672 of APP and γ -secretase cleavage of β -CTF subsequently occurs.^{1),37)} Since cleavage occurs in a sequential fashion, the release of APP fragments starting prior to the β -secretase site might suggest processes

independent of the amyloidogenic β -secretory pathway. In the nonamyloidogenic α -secretory pathway, APP is cleaved between Lys687 and Leu688 by α -secretase, releasing α -sAPP from cells. IP-MS analysis of human CSF has revealed A β -related peptides that start from the N-terminus of the β -secretase site, all of which end at Gln686 of APP.²²⁾ Ida *et al.* noted the presence of an A β -related peptide generated by cleavage at the γ -secretase site and at an N-terminal site upstream from the β -secretase site by a detection of a five kDa band using Western blotting for A β .³⁸⁾ However, no report has demonstrated a terminal amino acid sequence of A β -related peptides that start N-terminally of the β -secretase site and end at the γ -secretase site. Secretases or proteases that have the ability to cleave N-terminally of the β -secretase site of APP sequence remain unknown. The novel eight A β -related peptides (APP671-711, APP669-709, APP669-710, APP669-711, APP666-709, APP666-711, APP664-711 and APP663-711), which were generated by cleaving at an amino acid before the β -secretase site and at the γ -secretase site, might suggest a previously unknown pathway independent of the α - and β -secretases. Hence, further studies using cultured cells or human CSF are needed to elucidate the production mechanism of these novel A β -related peptides.

Early diagnosis of AD is necessary for treatment initiation at the earliest stage. A blood marker is suitable for screening for early AD because it is more practical for routine use than collection of CSF or imaging approaches. Unfortunately, APP672-713(A β 1-42) and APP672-711(A β 1-40) in blood are reportedly unusable as AD diagnostic markers due to the lack of differences between AD patients and controls, although a decrease in the [APP672-713(A β 1-42)/APP672-711(A β 1-40)] ratio in CSF has shown potential as an AD biomarker.^{39)–41)} However, our method is able to measure A β -related peptides except APP672-713(A β 1-42) and APP672-711(A β 1-40), which could be a valuable tool for finding new blood markers for AD.

Here, we have shown the use of MS to detect 22 A β -related peptides from human plasma, eight of which were found for the first time. We have further developed a quantitative assay for A β -related peptides by adding SIL-A β 1-38 as an internal standard. In the future, applying this method to subjects with AD, mild cognitive impairment and healthy controls will provide insights into the relationship between plasma A β -related peptides and AD progression.

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