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 (Aβ) in Alzheimer’s disease (AD). N-terminally or C-terminally truncated Aβ variants have been found in human cerebrospinal fluid and cultured cell media using immunoprecipitation and mass spectrometry. Unfortunately, the profile of plasma Aβ variants has not been revealed due to the difficulty of isolating Aβ from plasma. We present here for the first time studies of Aβ and related peptides in human plasma. Twenty-two Aβ-related peptides including novel peptides truncated before the γ-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 Aβ-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 (Aβ) peptides are centrally involved in the pathogenic early events of Alzheimer’s disease (AD).1,2) Aβ 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 Aβ 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 (N-sAPP) that is secreted and a C-terminal fragment (C99 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 Aβ1-40 or Aβ1-42, respectively. In the nonamyloidogenic α-secretory pathway, APP is first cleaved between Lys687 and Leu688 by α-secretase, releasing the larger soluble APP fragment (N-sAPP). The remaining C-terminal fragment (α-CTF or C83) undergoes cleavage by γ-secretase to release the short p3 peptide.5) Aβ 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, Aβ 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 Aβ.10–13) On the other hand, a portion of the soluble Aβ peptide is secreted and hence found in extracellular fluids including cerebrospinal fluid (CSF) and peripheral blood. A decrease of the Aβ1-42 peptide or the Aβ1-42/Aβ1-40 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-
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 Aβ 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 Aβ peptides in AD patients using a sandwich enzyme-linked immunosorbent assay (ELISA). Unfortunately, a consistent association between plasma Aβ and AD has not been demonstrated so far.17) Aβ levels in plasma are approximately 50-fold lower than those in CSF.25),26) The concentrations of Aβ in human plasma due to the difficulty of conducting IP of Aβ variants in plasma. The reasons are as follows: 1) the concentrations of Aβ1-40 and Aβ1-42 in human plasma are approximately 50-fold lower than those in human CSF and 2) the total protein concentration is 100-fold higher in plasma compared with CSF.24) Therefore, the detection of plasma Aβ 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 Aβ-related peptides in human plasma. Furthermore, we developed a quantitative assay using two different isotopically labeled human peptides (Aβ1-38 and Aβ1-40) 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 Aβ peptide solutions. Stable isotopically-labeled (SIL) human Aβ1-38 (13C-labeled at Phe and Ile) and Aβ1-40 (15N-labeled at Arg and Lys) were used for internal standards. They were purchased from AnaSpec (San Jose, CA). The lyophilized stocks of SIL-Aβ1-38 and SIL-Aβ1-40 were dissolved in 50 mM NaOH and subsequently applied to a COSMOSIL(R) 5Diol-120-II [7.5 mm I.D. × 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-Aβ1-38 and SIL-Aβ1-40 were dispensed in tubes and stored at −80 °C.

Preparation of the antibody-coated beads. Mouse monoclonal anti-Aβ antibodies (6E10 IgG1 and 4G8 IgG2b) were purchased from Covance (Princeton, NJ). The buffers containing the antibodies were exchanged using a 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 µg) was incubated with 600 µL immobilized ficin (Pierce) at 37 °C for 24 h. 4G8 IgG2b (500 µg) was incubated with 1200 ng Lys-C (Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for two h. Proteolytic fragments were concentrated with Amicon Ultrafiltration devices (Millipore, Cork, IR); 50 µL 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 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). 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 Aβ-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-reflector 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 Da to the precursor and fragment ions, respectively. Variable modifications of oxidation (M) was only used for the oxidized peptide.

ELISA measurement of Aβ. The concentrations of monomeric SIL-Aβ1-38 and SIL-Aβ1-40 solutions and the endogenous APP672-711(Aβ1-40) in plasma were determined using a human Aβ (1-40) ELISA Kit II purchased from Wako Pure Chemical Industries and human Aβ (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 Aβ-related peptides in plasma was performed by normalizing the intensity ratios of an analyte peptide to an internal standard (SIL-Aβ1-38 or SIL-Aβ1-40) 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-Aβ1-38 or SIL-Aβ1-40 was constructed using intensity ratios (SIL-Aβ1-38/SIL-Aβ1-40) or (SIL-Aβ1-40/SIL-Aβ1-38), 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] × 100)/ELISA value. The square of the correlation coefficient (R²) was used to evaluate the fit of the standard curve to a quadratic function and the linearity of the intensity ratio of Aβ-related peptides in the plasma dilution series. The precision of the quantitative Aβ-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 “N/A”. Inter-day %CV value of the analyte peptide was 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 µL aliquots of human plasma, followed by application of linear TOF and a QIT reflectron TOF (Fig. 1). Peaks at m/z corresponding to Aβ1-40 and Aβ1-42 (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(Aβ1-40) 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 Aβ forms than previously known.
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 (Aβ1-40) showed the highest intensity, which is consistent with results of Aβ variants in human CSF in previous reports.  

Fig. 1. MALDI-TOF mass spectra of Aβ-related peptides purified from human plasma by IP. Aβ-related peptides were immunoprecipitated from 250 µL human plasma and eluted with 2.5 µL acetonitrile/H$_2$O (7:3 v/v) with five mM HCl. The eluate (0.5 µL) was measured by a linear TOF (A). Two µL 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.

Fig. 2. The background peaks in the mass spectrum of a negative control. Plasma (250 µL) 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 µL acetonitrile/H$_2$O (7:3 v/v) with five mM HCl and 0.5 µL of the eluate was measured by a linear TOF.
Characterization of the Aβ-related peptide peaks. To verify the identities of the Aβ-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 Aβ-related peptide, especially those generated by preferred cleavage on the C-terminal side of aspartic acid or glutamic acid residues,29 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 Aβ-related peptides in Table 1 are correct. For two of the 22 Aβ-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 Aβ1-38 and Aβ1-40. Quantitative assays of plasma Aβ-related peptides were performed using SIL-Aβ1-38 (m/z 4162.6) or SIL-Aβ1-40 (m/z 4356.9) as internal standards. SIL-Aβ1-38 and SIL-Aβ1-40 peptides contained the epitope sequences for 6E10 and 4G8. Since an Aβ peptide and the corresponding SIL-Aβ 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(Aβ1-38) and APP672-711(Aβ1-40) peptides could be estimated by constructing a standard curve for the intensity ratio of each corresponding SIL-Aβ. The binding buffer (containing SIL-Aβ1-40 over a range from 2.5 to 100 pM and SIL-Aβ1-38 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-Aβ1-40/SIL-Aβ1-38 are plotted versus the concentration of measured SIL-Aβ1-40 in Fig. 3B. The standard curve could be fitted to a quadratic function and its R² value was 0.999. We used the standard curve of SIL-Aβ1-40 to estimate the concentration of endogenous APP672-711(Aβ1-40) in tested plasma, determining a final value of 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-Aβ1-38 as an internal standard could be used as a substitute for a generally used ELISA for Aβ. In a similar manner, a standard curve for the intensity ratio of SIL-Aβ1-38/SIL-Aβ1-40 versus the concentration of measured SIL-Aβ1-38 was obtained by IP-MS of plasma mixed with SIL-Aβ1-38 at concentrations ranging from one to 25 pM and SIL-Aβ1-40 at a constant concentration of 25 pM (Fig. 3C and D). The standard curve of SIL-Aβ1-38 could also be fit to a quadratic function and its R² value was 1.000. A concentration of endogenous APP672-709(Aβ1-38) in tested plasma was calculated to be 2.82 ± 0.17 pM using the standard curve of SIL-Aβ1-38. To date, determining the plasma concentration of APP672-709(Aβ1-38) 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 (Aβ1-38) concentration in human plasma.

Validation of the quantitative assay for Aβ-related peptides. We addressed the quantification of all of the Aβ-related peptides detected in IP-MS by using SIL-Aβ1-38 as the internal standard. Table 2 shows the intensity ratio values of analyte peptides to SIL-Aβ1-38 obtained from a mixture of 250 µL human plasma and the equivalent amount of 10 pM SIL-Aβ1-38. To validate the quantitative assay for Aβ-related peptides, we first evaluated the linearity of the intensity ratio of each Aβ-related peptide to SIL-Aβ1-38 for dilutions of plasma. Figure 4 presents the intensity ratio of each Aβ-related peptide obtained by measuring plasma samples at dilutions from 10% to 100% with PBS, that is, plasma volumes over the range 25–250 µL. Intensity ratios of 15 Aβ-related peptides demonstrated excellent linearities (R² = 0.912–0.999). APP677-711(Aβ3-40) peptide showed a relatively low R² value of 0.861. R² values of six other Aβ-related peptides were not calculated owing to their detection limits of 150 or 250 µL plasma (Table 2). This result indicated that the analyzed volume of plasma was acceptable in volumes of 250 µL or less.

To validate the precision of the quantitative IP-MS assay, inter-assay, intra-day and inter-day assays were conducted using SIL-Aβ1-38 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. Aβ-related peptides observed in mass spectra of immunoprecipitation from human plasma

| Aβ-related peptides | Sequence | Theoretical average mass in linear TOF | Theoretical monoisotopic mass | Measured average mass in QIT reflectron TOF | Observed fragmentation at C-terminal side of Asp or Glu in MS/MS spectrum | Mascot score |
|---------------------|----------|----------------------------------------|------------------------------|--------------------------------------------|-----------------------------------------------------------------------|-------------|
| APP682-711(Aβ1-40)  | TEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVVVIA 663 | 3152.7 | 3153.5 | 3150.68 | b12, b13, y17, y18 | 5 |
| APP677-709(Aβ6-38)  | HDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 3514.0 | 3513.5 | 3511.74 | b12, b30, y10, y31 | 27 |
| APP677-710(Aβ6-39)  | HDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVGG | 3613.1 | 3613.1 | 3610.81 | b37, b49, y26 | 22 |
| APP672-704(Aβ5-33)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 3675.0 | 3677.0 | 3672.78 | b26, b29, y22, y26 | 5 |
| APP677-711(Aβ6-40)  | HDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVVV | 3712.2 | 3711.8 | 3709.88 | b17, b26, y17, y31 | 33 |
| APP676-711(Aβ5-40)  | RHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVVV | 3868.4 | 3868.1 | 3865.98 | b28, y30, y33 | 11 |
| APP672-706(Aβ1-35)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGG | 3919.4 | 3918.1 | 3916.91 | b22, b23, y12, y26 | 5 |
| APP672-708(Aβ1-37)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGG | 4075.6 | 4074.8 | 4073.00 | b16, b20, y17, y31, y32 | 22 |
| APP674-711(Aβ3-40)  | EFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGGVV | 4144.7 | 4144.7 | 4142.09 | b6, b28, b17, y31 | 9 |
| APP672-710(Aβ1-39)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGGVV | 4231.8 | 4231.7 | 4229.09 | b13, b22, y32 | 13 |
| APP672-711(Aβ1-40)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGGVV | 4330.9 | 4330.6 | 4328.16 | b11, b22, y17, y31, y32, y39 | 90 |
| OxAPP672-711(AoxAβ1-40) | MDAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGG | 4330.9 | 4330.6 | 4328.16 | b11, b22, y17, y31, y32, y39 | 90 |
| APP669-709  | VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLMVGG | 4462.1 | 4462.0 | 4459.20 | b13, b26, y20, y32 | 8 |
| APP666-711(Aα1-42)  | DAERHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVVVIA 663 | 4491.1 | 4491.6 | 4488.22 | b20, b26, y35 | 15 |
| APP669-710  | VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVGV | 4515.1 | 4514.8 | 4512.28 | b13, b26, y35 | 17 |
| APP669-711  | VKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVGV | 4515.1 | 4514.8 | 4512.28 | b13, b26, y35, y37 | 17 |
| APP666-709  | ISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 4820.5 | 4818.2 | 4817.38 | b13, y17 | N/I |
| APP666-711  | ISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 5018.7 | 5017.7 | 5015.52 | b13, b26, y20, y37 | 17 |
| APP664-711  | EEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 5277.0 | 5276.9 | 5273.60 | N/D | N/A |
| APP663-711  | TEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIGLVMGVG | 5378.1 | 5378.7 | 5374.65 | N/D | N/A |

Peptides observed in linear TOF and QIT reflectron TOF are listed. OxAPP672-711/OxAβ1-40 represents APP672-711/Aβ1-40 peptide with the oxidized Met696.
N/D = Not detectable. N/A = Not applicable in MS/MS analysis. N/I = Not identified by Mascot search.
the four days (Table 3). The inter-well precision of the 22 Aβ-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 evaluation. However, APP677-710(Aβ6-39), APP672-704(Aβ1-33) and APP677-711(Aβ6-40) 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(Aβ11-40), 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.
It is possible that the generation of Aβ-related peptides was due to degradation during IP completed over a three h period. To verify the stability of Aβ-related peptides, we allowed human plasma to incubate at 4 °C for three h before IP-MS and analyzed the change of 16 Aβ-related peptide intensities compared to a plasma sample without the three h pre-incubation step (Table 2). The other Aβ-related peptides were eliminated from this evaluation due to their LOD near 250 µL 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 Aβ-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 Aβ-related peptides was due to degradation during IP completed over a three h period. To verify the stability of Aβ-related peptides, we allowed human plasma to incubate at 4 °C for three h before IP-MS and analyzed the change of 16 Aβ-related peptide intensities compared to a plasma sample without the three h pre-incubation step (Table 2). The other Aβ-related peptides were eliminated from this evaluation due to their LOD near 250 µL 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 Aβ-related peptides were not artifacts produced from IP.
Analyses have employed plasma. In earlier studies of CSF Aβ has not been clear which forms are present in human plasma (except for Aβ previously succeeded in detecting them in human plasma). Twenty-two Aβ-related peptides from human plasma. Twenty-two Aβ-related peptides including the well-known Aβ peptides were observed, and 20 of them were identified by MS/MS analysis. Surprisingly, of the 22 Aβ-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 Aβ variants in human CSF using MS, no one had previously succeeded in detecting them in human plasma (except for Aβ-40 and Aβ-42). Therefore, it has not been clear which forms are present in human plasma. In earlier studies of CSF Aβ peptides, IP-MS analyses have employed ≥ 940 pM human CSF, in which Aβ-40 is present at low nanomolar concentrations and Aβ-42 is at subnanomolar concentration.

### Table 2. Intensity ratios and LODs of Aβ-related peptides of the tested plasma sample

| Aβ-related peptides | Intensity ratio in plasma 250µL [Analyte/SIL-Aβ-38] | LOD (µL) |
|---------------------|---------------------------------------------|---------|
| APP682-711(Aβ-1-40) | 0.055 ± 0.003                              | 250     |
| APP677-709(Aβ-3-38) | 0.410 ± 0.026                              | 250     |
| APP677-710(Aβ-3-39) | 0.178 ± 0.021                              | 100     |
| APP672-704(Aβ-3-33) | 0.571 ± 0.068                              | 50      |
| APP677-711(Aβ-3-40) | 1.601 ± 0.078                              | ≤ 25    |
| APP676-711(Aβ-5-40) | 0.290 ± 0.015                              | ≤ 25    |
| APP672-706(Aβ-3-35) | 0.141 ± 0.013                              | 50      |
| APP672-708(Aβ-3-37) | 0.173 ± 0.008                              | 50      |
| APP672-709(Aβ-3-38) | 0.378 ± 0.035                              | ≤ 25    |
| APP674-711(Aβ-3-40) | 0.152 ± 0.006                              | 50      |
| APP672-710(Aβ-3-39) | 0.191 ± 0.016                              | 50      |
| APP672-711(Aβ-3-40) | 4.114 ± 0.327                              | ≤ 25    |
| OxAPP672-711(OxAβ-3-40) | 0.339 ± 0.044 | 50      |
| APP671-711 | 0.040 ± 0.007 | 250     |
| APP669-709 | 0.098 ± 0.007 | 100     |
| APP672-713(Aβ-3-42) | 0.067 ± 0.007 | 100     |
| APP669-710 | 0.083 ± 0.001 | 100     |
| APP669-711 | 0.273 ± 0.026 | 50      |
| APP666-709 | 0.045 ± 0.004 | 150     |
| APP666-711 | 0.054 ± 0.007 | 100     |
| APP664-711 | 0.027 ± 0.002 | 150     |
| APP663-711 | 0.019 ± 0.001 | 250     |

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

*Intensity ratios are represented by means ± SD that were obtained in four spectra from one preparation.*

Our approach achieved the detection of Aβ-40 (51.70 ± 2.35 pM) and Aβ-38 (2.82 ± 0.17 pM) from as little as 25 µL human plasma (Table 2). Additionally, the standard curve of SIL-Aβ-38 showed the LOD of Aβ-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 Aβ-related peptides by adding SIL-Aβ-38 or SIL-Aβ-40 as an internal standard. The replacement of 12C and 14N with 13C and 15N led to a mass shift of 30 Da and 26 Da for SIL-Aβ-38 and SIL-Aβ-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-Aβ-40 appeared in the vicinity of the oxidized APP672-711(Aβ-40) (Fig. 3A, C). In addition, SIL-Aβ-40 peptides readily aggregate due to their high hydrophobicity compared to SIL-Aβ-38. Hence, SIL-Aβ-38 was preferred as the internal standard over SIL-Aβ-40. The means of %CV values in intra- and inter-day assays were < 20% for 15 Aβ-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 Aβ-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 Aβ-related peptides, including peptides for which there is no commercially available ELISA.

Sandwich ELISA measurements have shown that the concentration of APP672-713(Aβ-38) is 10–30% of that of APP672-711(Aβ-40). However, in the present study, the signal intensity of APP672-713(Aβ-42) was < 10% of that of APP672-711(Aβ-40) (Table 2). In other word, the ratio of APP672-713(Aβ-42) to APP672-711(Aβ-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. Since APP672-713(Aβ-42) has a higher hydrophobicity than APP672-711(Aβ-40), the signal intensity of APP672-713(Aβ-42) appears lower than that of...
In addition, it is likely that the differences in the affinities of the used antibody-coated beads for APP672-711(Aβ1-40) and APP672-713(Aβ1-42) caused a decrease in the recovery rate of APP672-713(Aβ1-42) 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 Aβ-related peptide was demonstrated in Fig. 4. Therefore, our method is able to quantify each Aβ-related peptide using the intensity normalized to an internal standard.

The Aβ-related peptides showed one of the following three features: (1) C-terminally truncated Aβ, (2) N-terminally truncated Aβ or (3) APP peptides truncated before the β-secretase site. γ-secretase has some cleavage sites at the C-terminus of Aβ, generating AβX-33, AβX-37, AβX-38, AβX-39 and AβX-40.33-35 On the other hand, N-terminally truncated Aβs such as Aβ3-X, Aβ5-X and Aβ6-X could be cleavage products of neprilysin, metalloendopeptidase, coagulation factor XIa or plasmin.12,36 The presence of the short C- and N-terminal Aβs found in human plasma was in agreement with the features of Aβ 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.

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

| Aβ-related peptides | Inter-well (4 wells)a) | Intra-day (4 replicates)b) | Inter-day (4 days)c) |
|---------------------|------------------------|---------------------------|----------------------|
| APP682-711(Aβ11-40) | 5.20±0.6d) N/A         | N/A                       | N/A                  |
| APP677-709(Aβ36-38) | 6.52±2.73              | 16.43±5.15                | 13.74                |
| APP676-710(Aβ36-39) | 10.43±3.44             | 18.85±7.63                | 26.72                |
| APP672-704(Aβ31-33) | 8.34±4.08              | 34.53±18.82               | 36.46                |
| APP677-711(Aβ36-40) | 6.13±2.21              | 25.48±11.12               | 21.95                |
| APP676-711(Aβ35-40) | 5.27±2.60              | 13.96±6.13                | 14.92                |
| APP672-706(Aβ31-35) | 8.59±3.25              | 15.12±1.77                | 14.78                |
| APP672-708(Aβ31-37) | 5.26±3.04              | 3.89±1.87                 | 7.39                 |
| APP672-709(Aβ31-38) | 4.96±3.21              | 3.43±2.50                 | 9.20                 |
| APP674-711(Aβ39-40) | 5.45±2.90              | 9.04±4.01                 | 8.47                 |
| APP672-710(Aβ31-39) | 6.36±3.13              | 8.75±5.67                 | 10.90                |
| APP672-711(Aβ31-40) | 5.08±3.69              | 17.59±4.78                | 8.84                 |
| OxAPP672-711(OxAβ31-40) | 7.69±4.69          | 5.95±1.58                 | 7.48                 |
| APP671-711         | 7.97±2.73              | 24.50±0d) N/A             | N/A                  |
| APP669-709         | 7.27±2.84              | 12.52±4.20                | 13.72                |
| APP672-713(Aβ13-42)| 8.61±2.40              | 14.36±7.17                | 18.26                |
| APP669-710         | 8.98±3.30              | 13.37±6.77                | 12.52                |
| APP669-711         | 7.88±5.07              | 17.45±6.19                | 11.27                |
| APP666-709         | 8.67±3.47              | 15.24±8.83                | 17.96                |
| APP666-711         | 7.15±4.03              | 13.96±7.06                | 16.75                |
| APP664-711         | 7.31±2.86              | 15.49±10.38               | 23.74                |
| APP663-711         | 8.52±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 ± SD calculated from all of %CV values between four spectra of each of four replicates on each of four days.
b)The mean ± 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.
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 Aβ-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 Aβ-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 Aβ.20 However, no report has demonstrated a terminal amino acid sequence of Aβ-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 Aβ-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 Aβ-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(Aβ1-42) and APP672-711(Aβ1-40) 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(Aβ1-42)/APP672-711(Aβ1-40)] ratio in CSF has shown potential as an AD biomarker.30–41 However, our method is able to measure Aβ-related peptides except APP672-713(Aβ1-42) and APP672-711(Aβ1-40), which could be a valuable tool for finding new blood markers for AD.

Here, we have shown the use of MS to detect 22 Aβ-related peptides from human plasma, eight of which were found for the first time. We have further developed a quantitative assay for Aβ-related peptides by adding SIL-Aβ38 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 Aβ-related peptides and AD progression.

Acknowledgements

This research was funded by a grant from the Japan Society for the Promotion of Science (JSPS) through the “Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program),” initiated by the Council for Science and Technology Policy (CSTP).

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(Received Dec. 9, 2013; accepted Jan. 16, 2014)