Neurotoxicity and Physicochemical Properties of Aβ Mutant Peptides from Cerebral Amyloid Angiopathy

IMPLICATION FOR THE PATHOGENESIS OF CEREBRAL AMYLOID ANGIOPATHY AND ALZHEIMER'S DISEASE*

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Kazuma Murakami‡, Kazuhiro Irie‡‡, Akira Morimoto‡, Hajime Ohigashi‡, Mayumi Shindo‡¶, Masaya Nagao, Takahiko Shirimizu**, and Takuju Shirasawa***

From the **Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, †Applied Biosystems Japan Ltd., Tokyo 104-0032, Japan, ‡Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan, and **Department of Molecular Gerontology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan.

Cerebral amyloid angiopathy (CAA) due to β-amyloid (Aβ) is one of the specific pathological features of familial Alzheimer’s disease. Aβ mainly consisting of 40- and 42-mer peptides (Aβ40 and Aβ42) exhibits neurotoxicity and aggregative abilities. All of the variants of Aβ40 and Aβ42 found in CAA were synthesized in a highly pure form and examined for neurotoxicity in PC12 cells and aggregative ability. All of the Aβ40 mutants at positions 22 and 23 showed stronger neurotoxicity than wild-type Aβ40. Similar tendency was observed for Aβ42 mutants at positions 22 and 23 whose neurotoxicity was 50–200 times stronger than that of the corresponding Aβ40 mutants, suggesting that these Aβ42 mutants are mainly involved in the pathogenesis of CAA. Although the aggregation of E22Q-Aβ42 and D23N-Aβ42 was similar to that of wild-type Aβ42, E22Q-Aβ42 and E22K-Aβ42 aggregated extensively, supporting the clinical evidence that Dutch and Italian patients are diagnosed as hereditary cerebral hemorrhage with amyloidosis. In contrast, A21G mutation needs alternative explanation with the exception of physicochemical properties of Aβ mutants. Attenuated total reflection-Fourier transform infrared spectroscopy spectra suggested that β-sheet content of the Aβ mutants correlates with their aggregation. However, β-turn is also a critical secondary structure because residues at positions 22 and 23 that preferably form two-residue β-turn significantly enhanced the aggregative ability.

Alzheimer’s disease (AD)† is neuropathologically characterized by the progressive deposition of amyloid in the brain parenchyma and cortical blood vessels (1). This deposition mainly consists of 40- and 42-mer β-amyloid peptides (Aβ40 and Aβ42) generated from amyloid precursor protein by two proteases, β- and γ-secretases (2, 3). Cerebral amyloid angiopathy (CAA) in familial Alzheimer’s disease is linked to missense mutations inside the Aβ-coding region in the amyloid precursor protein. The mutations of Aβ sequence are concentrated at positions 21–23 and are called Flemish (A21G) (4), Arctic (E22G) (5, 6), Dutch (E22Q) (7), Italian (E22K) (8), and Iowa (D23N) (9) mutations. These Aβ mutant peptides may play a pathological role in the CAA because wild-type Aβ peptides induce neuronal death in vitro (10). Neurotoxicity and formation of amyloid fibrils of some CAA-related Aβ40 mutants have been independently reported by several groups (11–15). However, there are no reports on the neurotoxicity and aggregation of the CAA-related Aβ42 mutants with the exception of Dutch mutation (E22Q) (11), the investigation of which is essential to reveal the mechanism of CAA because wild-type Aβ42 shows considerably stronger neurotoxicity and aggregative ability than wild-type Aβ40 (11). Moreover, it is indispensable to simultaneously compare neurotoxicity and aggregative ability of all of the CAA-related Aβ40 and Aβ42 mutants in the same conditions such as pH, peptide concentration, reaction buffer, and temperature.

It is difficult to synthesize Aβ42 with 14 hydrophobic and/or bulky amino acid residues at the C terminus in a highly pure form, because it easily aggregates even under weakly acidic and neutral conditions (16). We recently established a highly efficient method for synthesizing long peptides over 50 amino acid residues with a continuous flow-type peptide synthesizer (Pioneer™) using N-(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethylaminium hexafluorophosphate N-oxide (HATU) (17) as an effective coupling reagent for Fmoc chemistry (18–22). This enabled us to obtain the CAA-related Aβ42 peptides with high purity in combination with the purification under the alkaline condition (23). Our continuous research on the CAA-related Aβ42 peptides led to the synthesis of all of the CAA-related Aβ40 and Aβ42 mutants at positions 21–23 (Fig. 1). Because Lys-Asp (Italian), Glu-Asp (Dutch), Gly-Asp (Arctic), and Gln-Asp (Iowa) sequences at positions 22 and 23 of CAA-related Aβ40 mutants are frequently

1-ylmethylene]-N-methylmethylaminium hexafluorophosphate N-oxide; HPLC, high pressure liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PEG, polyethylene glycol; MT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Th-T, thioflavin-T; FS, polystyrene support.
found in a two-residue β-turn (24), we synthesized several Aβ40 and Aβ42 derivatives at position 22 substituted by the amino acid residues, which influence the formation of the two-residue β-turn as follows: 1) proline and serine residues in the first position as β-turn inducers and 2) valine and leucine residues as β-turn breakers (24). This paper describes a comprehensive study that shows the neurotoxicity in PC12 cells, aggregative ability, and secondary structure of a series of Aβ derivatives at positions 21–23 including all of the CAA-related mutants and discusses the contribution of the CAA-related mutation to the implication of the pathogenesis of CAA and AD and to the secondary structure at positions 22 and 23 of Aβ peptides.

**EXPERIMENTAL PROCEDURES**

**General**—The following spectroscopic and analytical instruments were used: peptide synthesizer, Pioneer® peptide synthesizer (Applied Biosystems, Foster City, CA); HPLC, Waters 600E multisolvent delivery system with 2487 UV dual λ absorbance detector; Waters 625 LC system with 486 UV tunable absorbance detector and 741 data module; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Applied Biosystems Voyager-DE PRO (Applied Biosystems); thioflavin-T (Th-T) fluorescence, SPECTRA max Gemini XRS-TR (Molecular Devices, Ashiya, Japan) micro-plate reader, MPR-A4 (TOSOH, Tokyo, Japan); attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), Jasco 480 plus; and electron microscope, JEOL JEM-2000EX. MALDI-TOF-MS was measured as reported previously (18, 20). HPLC was carried out on a Develosil-packed column ODS-UG-5 (20-mm inside diameter × 150 mm and 6.0-mm inside diameter × 100 mm) (Nomura Chemicals, Seto, Japan).

HATU, piperidine, Fmoc amino acids, Fmoc-Val-PEG-PS resin, Fmoc-Ala-PEG-PS resin, and N,N-diisopropylethylamine (DIPEA) were purchased from Applied Biosystems. N,N-Dimethylformamide (DMF), trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, m-cresol, diethyl ether (peroxide-free), and CH3Cl were purchased from Nacalai tesque (Kyoto, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagents were purchased from Sigma.

**Synthesis of Aβ Derivatives**—Each Aβ derivative (Fig. 1) was synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-Val-PEG-PS resin to examine their relation to the pathogenesis of these CAAAs (Fig. 1). Several Aβ derivatives in which the Glu-22 residue of wild-type Aβ was substituted by amino acid residues (proline, serine, valine, and leucine) that effectively influence the formation of the two-residue β-turn (24) were also prepared to investigate the secondary structure of Aβ derivatives at positions 21–23 (Fig. 1).

**RESULTS**

**Synthesis of Aβ Derivatives**—The CAA-related Aβ40 and Aβ42 mutants at positions 21–23 were synthesized to examine their relation to the pathogenesis of these CAAAs (Fig. 1). Several Aβ derivatives in which the Glu-22 residue of wild-type Aβ was substituted by amino acid residues (proline, serine, valine, and leucine) that effectively influence the formation of the two-residue β-turn (24) were also prepared to investigate the secondary structure of Aβ derivatives at positions 21–23 (Fig. 1).

**MTT Assay Using PC12 Cells**—Reduction of MTT by mitochondrial reductase was carried out by the protocols based on a previous report (25) with slight modifications. Each solution of Aβ derivatives (0.1% NH4OH) sterilized by the filter (0.22 μm) was diluted with 0.1% NH4OH at concentrations ranging from 0.12 to 120 μM. 10 μl of the resultant solution and 10 μl of 50 mM sodium phosphate buffer containing 100 mM NaCl were, respectively, added to the above-mentioned 100-μl cell culture, which was incubated at 37 °C under 5% CO2 overnight before experiment.

**Sedimentation Assay for Fibril Formation**—Each Aβ derivative was dissolved in 0.02% NH4OH at 250 μM. After a 10-fold dilution by 50 mM sodium phosphate containing 100 mM NaCl at pH 7.4, the resultant peptide solution (25 μM) was incubated at 37 °C for 4, 8, 16, 24, or 48 h. After centrifugation at 15,000 rpm in an Eppendorf microcentrifuge at 4 °C for 10 min, 25 μl of the supernatant was then analyzed by HPLC as reported previously (23). The area of the absorption at 220 nm was integrated and expressed as a percentage of the control.

**Th-T Fluorescence Assay**—Each Aβ derivative was dissolved in 0.02% NH4OH at 250 μM. The peptide solution (25 μM) diluted with the phosphate buffer solution described above was incubated at 37 °C for 4, 8, 16, 24, or 48 h. Ten microliters of each Aβ solution was added to 1 ml of 5 μl Th-T in 50 mM Gly-NaOH, pH 8.5. Fluorescence intensity was measured at 450-nm excitation and 482-nm emission as reported previously (26).

**Transmission Electron Micrographs of Negatively Stained Preparations of Aβ42 Fibrils**—The fibril formation of the Aβ42 derivatives was detected by electron microscope. Each Aβ42 derivative (25 μM) was incubated in 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl for 48 h at 37 °C. After centrifugation, the supernatant was removed and fed as pelleted Aggregates. Aggregates were then suspended in water by gentle vortex mixing. These suspensions were applied to a 400-mesh collodion-coated copper grid (Nissin EM, Tokyo, Japan) and allowed to dry in air before being negatively stained for 2 min with 2% uranyl acetate. Fibrils were examined with the JEOL JEM-2000EX electron microscope.

**ATR-FTIR Measurements to Estimate the Secondary Structure of Aβ42 Derivatives**—Each Aβ42 derivative was dissolved in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl at 250 μM. The Aβ42 microsolution was lyophilized or incubated at 37 °C for 48 h. The 48-h incubated sample was centrifuged at 15,000 rpm in an Eppendorf microcentrifuge at 4 °C for 10 min, and the supernatant was removed. The pellet was suspended in distilled water, centrifuged, and lyophilized to give a white powder, which was loaded on an ATR cell in the FTIR spectrometer (Jasco 480 plus). At the same time, each Aβ42 derivative without incubation was also subjected to the ATR-FTIR measurement. 50 scans were accumulated to improve the signal/noise ratio, and the spectra were recorded at a resolution of 4.0 cm⁻¹. The program IR-SSE (Jasco) was used to estimate the secondary structure of each derivative by principal component regression.
Wild-type Aβ40 did not inhibit the formazan formation even at 10\(^{-5}\) M, whereas wild-type Aβ42 considerably inhibited it at 10\(^{-6}\) M. All of the CAA-related Aβ40 mutants at position 22 inhibited significantly the formazan formation at 10\(^{-7}\) M. On the other hand, A21G-Aβ40 (Flemish) did not show any neurotoxic effect even at 10\(^{-5}\) M. The CAA-related Aβ42 mutants at position 22 (Dutch, Italian, and Arctic) also showed approximately 10-fold the neurotoxicity of wild-type Aβ42. A concentration of 50% inhibition of the formazan formation was approximately 10\(^{-7}\) M. D23N-Aβ42 (Iowa) mutant at position 23 showed 2–3-fold more potent cytotoxicity than wild-type Aβ42. A21G-Aβ42 (Flemish) showed a slightly weaker yet still significant neurotoxic effect on the PC12 cells compared with wild-type Aβ42 (Table II).

Aggregation Studies—The aggregative ability of Aβ can be estimated by sedimentation assay using HPLC after centrifugation of the Aβ peptide solution. Aβ aggregates are also detectable using Th-T, which binds specifically to the aggregates to show intense fluorescence (28). One drawback of the Th-T assay is that it is not always quantitative because Th-T fluorescence can vary depending on the structure and morphology of the fibrils. The sedimentation assay is more suitable for quantification of the aggregates.

Almost of all soluble wild-type Aβ40 was detected even after 24-h incubation by the sedimentation assay (Fig. 2A), indicating that wild-type Aβ40 hardly aggregated by 24-h incubation. This result is in good agreement with that of the Th-T assay (Fig. 2B). The fluorescent intensity of Aβ40 was almost equal to that of the control. On the other hand, soluble wild-type Aβ42 could not be detected after 24-h incubation by the sedimentation assay (Fig. 3A), indicating that wild-type Aβ42 aggregated completely after 24-h incubation. This result correlated very well with that of the Th-T assay where the fluorescent intensity already reached plateau after 16-h incubation (Fig. 6B). These results indicate that the aggregative ability of Aβ42 is far more potent than that of Aβ40, and their aggregative ability correlates very well with their neurotoxicity.

Among all of the CAA-related Aβ40 mutants at position 22, E22Q-Aβ40 (Arctic) aggregated faster than wild-type Aβ40 as shown in Fig. 2A and the aggregative rate of the Aβ40 mutants at position 22 was larger than that of the Aβ40 mutant at position 23. Reflecting the significant difference in the aggregative ability between Aβ40 and Aβ42, the aggregative ability of all of the Aβ40 mutants at position 22 failed to exceed that of wild-type Aβ42. E22G-Aβ40 showed a potent Th-T fluorescence while the Th-T intensity of E22Q and E22K-Aβ40 did not reflect their high aggregative rate in the sedimentation assay (Fig. 2B). The aggregative potency of A21G-Aβ40 (Flemish) was lowest among the Aβ40 mutants (Fig. 2A), and the Th-T fluorescence of this peptide was also weaker than that of wild-type Aβ40 (Fig. 2B). The aggregative ability of all of the Aβ40 mutants

| Aβ derivatives | Yield | Observed mass | Calculated mass (MH\(^{+}\)) |
|----------------|-------|---------------|-------------------------------|
| Aβ40           | 14.4  | 4331.62       | 4330.96                       |
| A21G-Aβ40      | 26.2  | 4316.94       | 4316.84                       |
| E22Q-Aβ40      | 9.8   | 4329.31       | 4329.88                       |
| E22K-Aβ40      | 9.6   | 4329.33       | 4329.92                       |
| E22G-Aβ40      | 8.0   | 4329.12       | 4329.88                       |
| E22P-Aβ40      | 10.9  | 4329.12       | 4329.88                       |
| D23N-Aβ40      | 15.9  | 4329.12       | 4329.88                       |
| Aβ42           | 3.2   | 4514.43       | 4515.20                       |
| A21G-Aβ42      | 22.7  | 4510.23       | 4510.08                       |
| E22Q-Aβ42      | 6.3   | 4514.39       | 4514.12                       |
| E22K-Aβ42      | 2.3   | 4514.42       | 4514.16                       |
| E22G-Aβ42      | 2.2   | 4443.35       | 4443.04                       |
| D23N-Aβ42      | 1.9   | 4513.94       | 4514.12                       |
| E22P-Aβ42      | 4.0   | 4483.61       | 4483.20                       |
| E22S-Aβ42      | 4.9   | 4473.56       | 4473.07                       |
| E22V-Aβ42      | 6.4   | 4485.49       | 4485.12                       |
| E22L-Aβ42      | 6.1   | 4499.89       | 4499.15                       |

**Table I**

| Aβ derivatives | Neurotoxicity in PC12 cells of Aβ derivatives estimated by the MTT assay |
|----------------|-------------------------------------------------------------------------|
| Aβ40 (Wild-type) | >100 |
| A21G-Aβ40 (Flemish) | >100 |
| E22Q-Aβ40 (Dutch) | 3.4 ± 0.91 |
| E22K-Aβ40 (Italian) | 11 ± 2.2 |
| E22G-Aβ40 (Arctic) | 11 ± 2.2 |
| D23N-Aβ40 (Iowa) | 78 |
| E22P-Aβ40 | 8.0 ± 0.72 |
| Aβ42 (Wild-type) | 0.97 ± 0.18 |
| A21G-Aβ42 (Flemish) | 1.7 ± 0.30 |
| E22Q-Aβ42 (Dutch) | 0.08 ± 0.011 |
| E22K-Aβ42 (Italian) | 0.14 ± 0.030 |
| E22G-Aβ42 (Arctic) | 0.14 ± 0.050 |
| D23N-Aβ42 (Iowa) | 0.38 ± 0.16 |
| E22P-Aβ42 | 0.08 ± 0.011 |
| E22V-Aβ42 | >100 |

**Table II**

**Fig. 1.** Structures of Aβ derivatives synthesized in this study.

**Fig. 2.** Yields and MALDI-TOF-MS data of Aβ derivatives synthesized in this study.
Neurotoxicity and Aggregation of CAA-related Aβ Mutants

estimated by the sedimentation assay correlated generally with their neurotoxicity (Table II).

Aggregative ability of the CAA-related Aβ42 mutants is shown in Fig. 3. E22Q-Aβ42 (Dutch) and E22K-Aβ42 (Italian) aggregated faster than wild-type Aβ42 in the sedimentation assay (Fig. 3A). Although the aggregative potency of E22G-Aβ42 (Arctic) and D23N-Aβ42 (Iowa) was slightly lower than that of wild-type Aβ42, the aggregative rate of E22G-Aβ42 after 4-h incubation was almost similar to that of wild-type Aβ42 and the aggregated amount of D23N-Aβ42 after 24-h incubation was nearly comparable with that of wild-type Aβ42 (Fig. 3A). Th-T fluorescence intensity of all of the Aβ42 mutants at positions 22 and 23 estimated by sedimentation assay correlated generally with their neurotoxicity (Table II). However, A21G-Aβ42 (Flemish) with a substantial neurotoxicity (Table II) aggregated hardly after 24-h incubation (Fig. 3A) and showed no Th-T fluorescence after 4-h incubation (Fig. 3B). Only this peptide did not follow the general pattern that the aggregative ability of the peptide correlates with its neurotoxicity.

Transmission Electron Micrographs of Negatively Stained Preparations of Aβ42 Fibrils—Fibril formation of the Aβ42 mutants was evaluated by transmission electron microscopy after a 48-h incubation at 37 °C (Fig. 4). Typical fibrils were formed in all of the CAA-related Aβ42 mutants with the exception of A21G-Aβ42 (Flemish), which did not aggregate at all as described above. The morphology of these fibrils resembled each other well. However, the length of the D23N-Aβ42 (Iowa) fibril was slightly shorter than that of the others.

ATR-FTIR Measurements of the Aβ42 Derivatives—Aβ aggregates are generally considered to contain a significant amount of the β-sheet structure (29). To estimate the secondary structure of the Aβ mutants, ATR-FTIR measurement was carried out on the CAA-related Aβ42 peptides. Each Aβ42 mutant lyophilized immediately after dissolving in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl or after a 48-h incubation in the same sodium phosphate buffer at 37 °C was subjected to the ATR-FTIR analysis (Fig. 5). The amid I profiles of the freshly prepared Aβ42 mutants at positions 22 and 23 exhibited a major absorbance between 1620 and 1640 cm⁻¹ (Fig. 5A) corresponding to the β-sheet structure (30). The secondary structures calculated from the amid I absorption in these spectra using the program IR-SSE (Jasco) are summarized in Table III. All of the CAA-related Aβ42 mutants at positions 22 and 23 had slightly higher β-sheet content (49–56%) than wild-type Aβ42 (48%) when freshly prepared. The β-sheet content of E22K-Aβ42 (56%) with potent

![Figure 2](image1.png)

Fig. 2. A, comparative study of the aggregation of CAA-related Aβ40 mutants at positions 21–23 estimated by sedimentation assay. ▲, wild-type Aβ40; ●, A21G-Aβ40 (Flemish); ○, E22G-Aβ40 (Arctic); ●, E22Q-Aβ40 (Dutch); □, E22K-Aβ40 (Italian); and △, D23N-Aβ40 (Iowa). B, comparative study of the aggregation of CAA-related Aβ40 mutants at positions 21–23 estimated by Th-T fluorescence method (26). The data show the Th-T fluorescence after 48-h incubation at 37 °C. *, p < 0.001 versus control.

![Figure 3](image2.png)

Fig. 3. A, comparative study of the aggregation of CAA-related Aβ42 mutants at positions 21–23 estimated by sedimentation assay. ●, wild-type Aβ42; ●, A21G-Aβ42 (Flemish); ○, E22G-Aβ42 (Arctic); ●, E22Q-Aβ42 (Dutch); □, E22K-Aβ42 (Italian); △, and D23N-Aβ42 (Iowa). B, comparative study of the aggregation of CAA-related Aβ42 mutants at positions 21–23 estimated by Th-T fluorescence method (26). The data show the Th-T fluorescence after 4-h incubation at 37 °C. *, p < 0.001 versus control.
aggregative ability was highest, whereas that of A21G-\(\alpha\beta\)42 (44%) with quite low aggregative potency was significantly lower than that of the other \(\alpha\beta\) mutants and 5% \(\alpha\)-helix was detected instead for \(\beta\)-sheet.

In all of the fibrils of the \(\alpha\beta\) mutants, intensity of the absorption between 1620 and 1640 cm\(^{-1}\) increased significantly (Fig. 5B), indicating that the \(\beta\)-sheet content increased by fibril formation. The \(\beta\)-sheet content of the fibrils of the \(\alpha\beta\) mutants reflected very well the aggregative potency estimated by the sedimentation assay. The \(\beta\)-sheet content of the E22K-\(\alpha\beta\)42 fibrils was especially high, whereas that of the E22G-\(\alpha\beta\)42 and D23N-\(\alpha\beta\)42 was slightly lower than that of wild-type \(\alpha\beta\)42.

**Neurotoxicity, Aggregation, Secondary Structure, and Transmission Electron Microscopy of Several \(\alpha\beta\) Derivatives at Position 22**—Because Lys-Asp (Italian), Gln-Asp (Dutch), Gly-Asp (Arctic), and Gln-Asp (Iowa) sequences are often found in the two-residue \(\beta\)-turn (24), high aggregative ability of these CAA-related \(\alpha\beta\)40 and \(\alpha\beta\)42 mutants suggests that \(\beta\)-turn formation at positions 22 and 23 might correlate with the aggregative ability of \(\alpha\beta\) peptides. To confirm this speculation, several \(\alpha\beta\)40 and \(\alpha\beta\)42 derivatives at position 22, substituted by amino acid residues that induce or suppress the formation of two-residue \(\beta\)-turn, were synthesized (Fig. 1). Proline and serine residues at the first position are known as \(\beta\)-turn inducers, whereas valine and leucine residues are known as \(\beta\)-turn breakers (24). E22P-\(\alpha\beta\)42 and E22P-\(\alpha\beta\)40 showed stronger neurotoxicity with potent aggregative ability than the corresponding wild-type \(\alpha\beta\)42 and \(\alpha\beta\)40, respectively (Table II and Fig. 6). The neurotoxicity and aggregative ability of E22P-\(\alpha\beta\)42 were considerably stronger than those of E22P-\(\alpha\beta\)40 and were comparable with those of E22Q-\(\alpha\beta\)42 (Dutch) and E22K-\(\alpha\beta\)42 (Italian) (Table II and Figs. 3 and 6).

**Fig. 4.** Electron micrographs of negatively stained preparations of \(\alpha\beta\)42 derivatives containing the CAA-related \(\alpha\beta\)42 mutants. Each \(\alpha\beta\) peptide (25 \(\mu\)m) was incubated in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl at 37 \(^\circ\)C for 24 h. A, wild-type \(\alpha\beta\)42; B, \(\alpha\beta\)42 (Dutch); C, \(\alpha\beta\)42 (Italian); D, \(\alpha\beta\)42 (Arctic); E, D23N-\(\alpha\beta\)42 (Iowa); and F, E22P-\(\alpha\beta\)42. Scale bar = 200 nm.

ATR-FTIR spectra of freshly prepared E22P-\(\alpha\beta\)42 indicated a major absorbance between 1620 and 1640 cm\(^{-1}\) along with a little absorbance between 1640 and 1700 cm\(^{-1}\) because of turn and random structure (Fig. 5A). Calculation of the spectra using the IR-SSE program showed higher \(\beta\)-sheet content (53%) than wild-type \(\alpha\beta\)42 (48%), correlating with its higher neurotoxicity and aggregative ability. E22P-\(\alpha\beta\)42 also formed typical fibrils quite similar to those of wild-type \(\alpha\beta\)42 as shown in Fig. 4. High \(\beta\)-sheet content (66%) of E22P-\(\alpha\beta\)42 fibrils was suggested by the calculation of the spectrum (Fig. 5B), indicating that the turn formation at positions 22 and 23 increased significantly \(\beta\)-sheet content of these \(\alpha\beta\) fibrils. On the other hand, the neurotoxicity and aggregative ability of E22V-\(\alpha\beta\)42 were quite low. Because a valine residue is scarcely found in the two-residue \(\beta\)-turn, turn formation at positions 22 and 23 seems to be critical for the neurotoxicity and aggregative ability of \(\alpha\beta\) peptides. Unfortunately, we failed to measure the neurotoxicity and aggregative ability of E22S-\(\alpha\beta\)42 and E22L-\(\alpha\beta\)42 because of their poor solubility in the sodium phosphate buffer.

**DISCUSSION**

Neurotoxicity of \(\alpha\beta\) is significantly implicated in the pathogenesis of neuronal degeneration in AD. Despite many previous studies on \(\alpha\beta\) peptide-derived neurotoxicity, the precise mechanism has not yet been clarified. Because it is generally accepted that wild-type \(\alpha\beta\) shows neurotoxicity in vitro mainly through aggregation, inhibition of the aggregative process might be a promising therapeutic strategy. To reach this goal, it is indispensable to reveal the pathological conformation of \(\alpha\beta\)42 because these mutations may be related to the neurotoxicity of the variant \(\alpha\beta\) peptides. According to the method recently established for long peptide synthesis (18–23), all of the variant forms of \(\alpha\beta\)40 and \(\alpha\beta\)42 were synthesized in a highly pure form.

The CAA mutations at positions 22 and 23 of \(\alpha\beta\) peptides enhanced their neurotoxicity, suggesting that these \(\alpha\beta\) mutants are involved in the pathogenesis of the CAAs. Especially, the neurotoxicity of the \(\alpha\beta\)42 mutants at positions 22 and 23 were considerably higher than that of the corresponding \(\alpha\beta\)40 mutants, which never exceeded that of the wild-type \(\alpha\beta\)42. These results indicate that the \(\alpha\beta\)42 mutants are crucial determinants in the pathogenesis of these CAAs. The neurotoxicity of E22Q-\(\alpha\beta\)42 and E22K-\(\alpha\beta\)42 was especially potent (Table II), supporting that Dutch and Italian mutations cause hereditary cerebral hemorrhage with amyloidosis. The Dutch and Italian patients develop cerebral hemorrhage, whereas parenchymal amyloid deposits are rare and neurofibrillary tangles are constantly absent. This appearance of the Dutch and Italian mutations clearly differs from that of the Arctic and Iowa patients (12) and coincides well with the remarkable aggregative ability of the corresponding \(\alpha\beta\) mutants.

It has recently been reported that E22Q-\(\alpha\beta\)40 rather than E22Q-\(\alpha\beta\)42 plays a significant role in Dutch-type CAA because E22Q-\(\alpha\beta\)42 did not show any cytotoxic effects (11). However, we could clearly demonstrate the most potent cytotoxicity of E22Q-\(\alpha\beta\)42 among the \(\alpha\beta\)42 mutants. In our assay system, wild-type \(\alpha\beta\)42 aggregated far more rapidly than wild-type
Aβ40, differing slightly from the data of other groups (11, 31–33). Establishment of a practical and reliable synthetic method of pure Aβ42 peptides (19, 22, 23) would be one of the major reasons for these discrepancies. Our synthetic method enabled precise estimation of the neurotoxicity and aggregative ability of all of the CAA-related Aβ mutants simultaneously in the same conditions for the first time.

It is quite noteworthy that A21G-Aβ42 (Flemish) showed a substantial albeit weak neurotoxicity in PC12 cells, although the aggregative ability of A21G-Aβ42 was significantly lower than that of wild-type Aβ42. A21G-Aβ42 is the only exception in which the neurotoxicity fails to correlate with the aggregation. It has recently been reported that the neurotoxicity of Aβ peptides might be the result of the oligomeric species, not the fibrils (34, 35). If the oligomeric A21G-Aβ42 peptides are attributable to the neurotoxicity, aggregation to form fibrils would not be a requisite condition for the Flemish-type CAA.

Tsubuki et al. (36) speculate the cause of AD as the catabolism rather than the anabolism of Aβ since the catabolic dysregulation of Aβ well accounts for the neuronal degeneration. Recently, they reported (36) that A21G-Aβ42 as well as other CAA-related Aβ mutants at position 22 is hardly decomposed by the degradation enzyme of Aβ peptides. This catabolic characterization of A21G-Aβ42 might be an alternative cause of Flemish-type CAA. The CAAs at positions 22 and 23 other than Flemish-type may also be explained by this degradation scheme. The lack of the cytotoxicity of A21G-Aβ40 is reasonable because the cytotoxicity of wild-type Aβ40 is quite low in our assay system.

There was a good correlation between the neurotoxicity in PC12 cells and the aggregative ability determined by the sedimentation assay of the CAA-related Aβ42 and Aβ42 mutants at positions 22 and 23 (Figs. 2A and 3A and Table II). This finding suggests that these Aβ mutants showed neurotoxicity through the aggregative process. Because Th-T recognizes a specific structure of amyloid fibrils, aggregative ability estimated by sedimentation would not always correlate with the Th-T fluorescence. We think that the aggregative ability itself can be more precisely estimated by the sedimentation assay than by the Th-T fluorescence assay. The discrepancies of the results between the sedimentation and Th-T assay would not imply that most of the aggregates generated by Aβ mutants do...
not correspond to amyloid fibrils since the FTIR spectra and morphology of these fibrils were similar to those of the wild-type Aβ42 fibrils (Figs. 4 and 5).

The electron microscope measurements clearly showed fibrillar materials for all of the CAA-related Aβ42 mutants at positions 22 and 23 (Fig. 4). Recently, Miravalle et al. (12) reported that the aggregates of E22K-Aβ40 (Italian) showed less fibrillar organization and a rather amorphous organization regardless of its strong neurotoxicity. Their report correlates well with lower Th-T fluorescence of E22K-Aβ40 compared with wild-type Aβ40 (Fig. 2B). Extremely rapid aggregative rate of E22K-Aβ40 peptide should result in amorphous rather than fibrillar organization. However, we could not detect the intrinsic difference in morphology between E22K-Aβ42 and wild-type Aβ42 (Fig. 4). This inconsistency might be because of the accidental failure of detection of the amorphous.

ATR-FTIR analysis of freshly dissolved Aβ42 derivatives indicated that β-sheet content of all of the CAA-related Aβ42 mutants at positions 22 and 23 (49–56%) was slightly higher than that of the wild-type Aβ42 (48%), supporting the previous hypothesis that β-sheet structure is closely related to the neurotoxicity and aggregative ability of Aβ peptides. The FTIR analysis of these mutant fibrils showed that aggregation of these Aβ42 peptides increased significantly their β-sheet content. Because the ATR-FTIR profiles of the fibrils of the CAA-related Aβ42 mutants are almost similar to that of wild-type Aβ42, these fibrils would adopt a similar tertiary structure as exemplified by the electron microscopic analysis. Although the shape of the fibrils derived from D23N-Aβ42 was a little bit different from the other Aβ42 mutants, the intrinsic difference was not detected by the ATR-FTIR measurement.

This work suggests that pathogenesis of the CAAs is related to the alteration of the amino acid residues at position 22 of the Aβ peptides. Miravalle et al. (12) and Melchor et al. (13) proposed that a change or loss of charge at position 22 enhanced the pathogenic properties of the Aβ40 peptides. However, our results using Aβ42 derivatives at position 22 contradict their hypothesis because E22V-Aβ42 without charge at position 22 showed very weak neurotoxicity in PC12 cells and aggregative ability. Moreover, the neurotoxicity and aggregative ability of E22A-Aβ42 were almost similar to those of E22D-Aβ42 and a little bit weaker than those of the wild-type Aβ42 as reported previously (23). These results led us to speculate that the steric factor of the side chain at position 22 would change the conformation of Aβ peptides to affect their neurotoxicity and aggregative ability.

It is noteworthy that the fibrils as well as freshly prepared Aβ42 mutant peptides contain a turn structure in addition to the β-sheet structure (Table III). Because Lys-Asp (Italian), Gln-Asp (Dutch), Gly-Asp (Arctic), and Glu-Asn (Iowa) sequences are frequently found in the two-residue β-turn (24), Aβ42 might form the two-residue β-turn at positions 22 and 23, which eventually leads to intramolecular and/or intermolecular β-sheet structure that causes the neurotoxicity and aggregative ability. Several Aβ derivatives at position 22 were prepared to confirm this speculation. The neurotoxicity and aggregative ability of E22P-Aβ42, E22P-Aβ40, and E22V-Aβ42 strongly supported this hypothesis because prolines most frequently occur in the first position of the two-residue β-turn as a Pro-X corner (24, 37) and bulky hydrophobic residues like valine and leucine are rarely found in the two-residue β-turn (24). D23N-Aβ42 peptides are only examples of the mutation at position 23. It is interesting that Asn residue most frequently occurs at the second position of the two-residue β-turn (24). The D23N mutation does not contradict the β-turn formation at positions 22 and 23. Moreover, our recent results on the proline replacement at positions 19–21 and 24–26 of Aβ42 suggest that these amino acid residues are involved in the β-sheet formation (38). This is a strong circumstantial evidence that β-turn at positions 22 and 23 of Aβ42 enhances the neurotoxicity and aggregative ability. On the basis of these considerations, we propose the pathological conformation of Aβ42 peptides at positions 21–24 as shown in Fig. 7, A and B.

After the completion of this study, a structural model for

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### Table III

| Aβ derivatives | α-Helix | β-Sheet | Turn | Random |
|----------------|--------|--------|------|--------|
| Fresh Aβ42     | 0      | 48     | 25   | 27     |
| A21G-Aβ42      | 5      | 44     | 24   | 27     |
| E22Q-Aβ42      | 0      | 49     | 24   | 27     |
| E22K-Aβ42      | 0      | 56     | 21   | 23     |
| E22G-Aβ42      | 0      | 40     | 24   | 27     |
| D23N-Aβ42      | 0      | 50     | 24   | 26     |
| E22P-Aβ42      | 0      | 53     | 22   | 25     |

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**Fig. 6.** A, comparative study of the aggregation of Aβ derivatives at position 22 estimated by sedimentation assay. B, comparative study of the aggregation of Aβ derivatives at position 22 estimated by Th-T fluorescence method (26). ▲, Wild-type Aβ40; ○, wild-type Aβ42; △, E22P-Aβ40; ◼, E22P-Aβ42; and ×, E22V-Aβ42.
β-amyloid fibrils derived from Aβ40 has been proposed using solid state NMR spectroscopy (Fig. 7C and Ref. 39). The model shows that the residues 25–28 contain a bend of the backbone that brings the two β-sheets in contact through side chain-side chain interactions. Turn formation at positions 26 and 27 is reasonable because Ser-Asn sequence is often found in the C-terminal residues of the residues Asp-23 and Lys-28. Mutation at positions 22 and 23 is the key secondary structure related to neurotoxicity and aggregative ability of Aβ peptides. This hypothesis could also explain why many CAA mutations are concentrated at positions 22 and 23 of Aβ peptides. The large difference in neurotoxicity and aggregative ability of Aβ42 and Aβ40 peptides also implies the importance of the C-terminal two residues. The present results unambiguously indicate that the presence of the bend structure, the positions are slightly different from the Tycko’s model (39). To examine the bend structure at positions 25–28, G25P-Aβ42, S26P-Aβ42, N27P-Aβ42, and K28P-Aβ42 peptides were synthesized and examined for their aggregative ability. However, these peptides hardly aggregated after 24 h of incubation, unlike wild-type Aβ42 estimated by the sedimentation assay, and were far less neurotoxic than wild-type Aβ42 (data not shown), suggesting that positions 25–28 of Aβ42 are involved in the β-sheet formation rather than the hairpin formation because prolines are rarely present in the β-sheets (24, 37). Because Aβ42 is highly neurotoxic than Aβ40, the pathological conformation of Aβ42 might be different from that of Aβ40. It is also possible that the pathological conformation of the CAA-related Aβ peptides, especially E22K-Aβ42 (Italian), is different from that of wild-type Aβ40. In the Tycko’s model (Fig. 7C), the turn at positions 26 and 27 is stabilized by an ionic interaction between side chains of the residues Asp-23 and Lys-28. Mutation at positions 22 and 23 of Aβ peptides, especially E22K-Aβ42 (Italian) and D23N-Aβ42 (Iowa), might destabilize this interaction to change the turn position from 26 and 27 to positions 22 and 23 (Fig. 7B). This conformational change, which increases the intermolecular parallel β-sheet region, would enhance the aggregative ability of Aβ peptides.

In summary, the present results using highly pure Aβ mutants indicated that Aβ42 mutants at positions 22 and 23 play a more crucial role in the pathogenesis of the CAA than the corresponding Aβ40 mutants. The secondary structure analysis of these Aβ derivatives and the systematic proline replacement of Aβ42 led to the new hypothesis that β-turns at positions 22 and 23 is the key secondary structure related to neurotoxicity and aggregative ability of Aβ peptides. This hypothesis could also explain why many CAA mutations are concentrated at positions 22 and 23 of Aβ peptides. The large difference in neurotoxicity and aggregative ability of Aβ42 and Aβ40 peptides also implies the importance of the C-terminal two residues. The present results unambiguously indicate that the residues at positions 22 and 23 and 41 and 42 should be taken into account to clarify the precise aggregation mechanism of Aβ peptides and to develop new medicinal leads, which inhibit the aggregation of Aβ. Systematic proline replacement of Aβ peptides, one of the promising approaches to this goal, along with the solid state NMR study of Aβ42 is under investigation.

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Note Added in Proof—Approximately 50–100 μg of each peptide sample was spread evenly on the surface of a horizontal single reflection diamond ATR plate. The data in Fig. 5 were collected using JASCO FTIR/IR-450 plus (Jasco) with a DILATOS detector in the following conditions: resolution, 4.0 cm⁻¹; scanning speed, 2 mm/s; apodization, cosine; and one level of zero filling. The conspicuous artifacts of the spectra would be ascribable to water in a sample, although all of the samples were lyophilized sufficiently. In estimation of the secondary structure of each Aβ42 derivative, the peak of the water vapor was subtracted. The difference between previously published spectra of wild-type Aβ42 (for example, Janek, K., Rothemund, S., Gast, K., Beyermann, M., Zipper, J., Fabian, H., Bienert, M., and Krause, E. (2001) Biochemistry 40, 5457–5463) and in this paper might be because of various measurement conditions (solvent, concentration, pH, and so on) and/or Aβ42 employed. We used highly pure Aβ42 prepared by high pressure liquid chromatography under the basic conditions. Commercially available Aβ42 is usually purified under the acidic conditions where the peak of Aβ42 is too broad to be purified strictly. The program IR-SSE (Jasco) for estimation of peptide secondary structure was made according to the previously reported method (Sarver, R. W., Jr., and Krueger, W. C. (1991) Anal. Biochem. 194, 89–100). In this paper, the IR data matrices were constructed from the normalized FTIR spectra from 1700 to 1600 cm⁻¹ of 17 proteins and the secondary structure matrices (α-helix, β-sheet, turn, and random) were constructed from the x-ray data of the 17 proteins. There is a possibility that the calculated β-sheet contents, especially turn and random coil, are not always true because there has never been demonstrated that estimation of the secondary structure of Aβ peptides can be obtained from the amide I spectra. However, we have another circumstantial evidence of increase of the β-sheet structure in several Aβ42 mutants by CD spectra with the exception of E22K-Aβ42, which aggregates very rapidly (23). The mean ± S.D. of the estimation in Table III was within 2% when the same sample was measured several times. When wild-type Aβ42 was prepared on another day by the same procedure, the mean ± S.D. was 1.5%. In ATR-FTIR measurements, there is an optical distortion due to dispersion that would affect absolute values of percentage of the secondary structures. This paper discusses only the relative increase of the β-sheet contents compared with wild-type Aβ42.

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Neurotoxicity and Physicochemical Properties of Aβ Mutant Peptides from Cerebral Amyloid Angiopathy: IMPLICATION FOR THE PATHOGENESIS OF CEREBRAL AMYLOID ANGIOPATHY AND ALZHEIMER'S DISEASE
Kazuma Murakami, Kazuhiro Irie, Akira Morimoto, Hajime Ohigashi, Mayumi Shindo, Masaya Nagao, Takahiko Shimizu and Takuji Shirasawa

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