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In vitro studies of amyloid β-protein fibril assembly and toxicity provide clues to the aetiology of Flemish variant (Ala^{692} → Gly) Alzheimer's disease

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In a Flemish kindred, an Ala^{692} → Gly amino acid substitution in the amyloid β-protein precursor (AβPP) causes a form of early-onset Alzheimer's disease (AD) which displays prominent amyloid angiopathy and unusually large senile plaque cores. The mechanistic basis of this Flemish form of AD is unknown. Previous *in vitro* studies of amyloid β-protein (Aβ) production in HEK-293 cells transfected with cDNA encoding Flemish AβPP have shown that full-length [Aβ(1–40)] and truncated [Aβ(5–40) and Aβ(11–40)] forms of Aβ are produced. In an effort to determine how these peptides might contribute to the pathogenesis of the Flemish disease, comparative biophysical and neurotoxicity studies were performed on wild-type and Flemish Aβ(1–40), Aβ(5–40) and Aβ(11–40). The results revealed that the Flemish amino acid substitution increased the solubility of each form of peptide, decreased the rate of formation of thioflavin-T-positive assemblies, and increased the SDS-stability of peptide oligomers. Although the kinetics of peptide assembly were altered by the Ala^{21} → Gly substitution, all three Flemish variants formed fibrils, as did the wild-type peptides. Importantly, toxicity studies using cultured primary rat cortical cells showed that the Flemish assemblies were as potent a neurotoxin as were the wild-type assemblies. Our results are consistent with a pathogenetic process in which conformational changes in Aβ induced by the Ala^{21} → Gly substitution would facilitate peptide adherence to the vascular endothelium, creating nidi for amyloid growth. Increased peptide solubility and assembly stability would favour formation of larger deposits and inhibit their elimination. In addition, increased concentrations of neurotoxic assemblies would accelerate neuronal injury and death.

Key words: fibrillogenesis, neurotoxicity.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent dementia in the developed world [1] and is on a par with cerebrovascular diseases as the third leading cause of death in the United States [2]. Characteristic neuropathological lesions of AD include intracellular neurofibrillary tangles and extracellular amyloid deposits [3]. The principal protein component of amyloid deposits is the amyloid β-protein (Aβ) [4], produced through proteolytic processing of the amyloid β-protein precursor (AβPP) [5]. A substantial body of genetic and biochemical evidence suggests that Aβ plays a seminal role in the development of AD [6]. Mutations which increase the total plasma or cerebrospinal-fluid concentration of Aβ, or selectively increase the concentration of a 42-residue form of the peptide [Aβ(1–42)], are linked to AD [7]. In addition, studies of five hereditary diseases characterized by cerebrovascular amyloid deposition and dementia provide strong evidence that alterations in Aβ structure may directly produce peptide-mediated pathogenetic effects. Here, a causal relationship between Aβ structure and disease is clear; mis-sense mutations in the AβPP gene produce Aβ peptides containing single amino acid substitutions and these peptides form extensive amyloid deposits. Prominent examples of these diseases are a Dutch kindred in which mutant AβPP produces Aβ containing a Glu^{22} → Gln substitution [8] and a Flemish kindred expressing an Ala^{21} → Gly substitution [9]. Two other kindreds produce Aβ peptides with amino acid substitutions at position 22: a Swedish family expressing a Glu^{22} → Gly substitution [10,11] and an Italian family expressing a Glu^{22} → Lys substitution [12]. Recently, a new mutation, causing an Asp^{23} → Asn substitution, has been discovered in an Iowa family [13].

The disease manifested by the Dutch kindred, hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D), displays pronounced deposition of Aβ in the microvasculature of the brain, accompanied by predominately diffuse parenchymal plaques and only rarely by neurofibrillary tangles [14,15]. Differences in the post-translational processing of AβPP into Aβ have been observed between Dutch and wild-type proteins [16], but the most significant effects of the glutamine substitution appear to be accelerated fibrillogenesis kinetics [17–22] and increased fibril stability [23]. In addition, [Gln^{22}]Aβ(1–40), but not wild-type Aβ(1–40), has been shown to assemble on the surfaces of cultured human smooth-muscle cells and to kill these cells [24]. Similar biological activity has also been demonstrated for the 'Italian' peptide [25]. The HCHWA-D phenotype may thus be a result of the 'Dutch' peptide's increased propensity to aggregate, to interact with vascular tissue or to resist elimination from tissues.

The biochemical and biophysical mechanisms responsible for producing the Flemish disease are less well understood. Although the Ala^{21} → Gly substitution responsible for the disease occurs immediately adjacent to the site of the Dutch substitution, the neuropathology of the Flemish disease is distinct. In particular, whereas HCHWA-D has characteristics of multi-infarct dementia with little senile-plaque formation [26], the Flemish disease presents as a more typical AD syndrome in which large senile plaques, neurofibrillary tangles and tau-reactive dystrophic neurites are all found, along with vascular amyloid deposits [14].

Abbreviations used: Aβ, amyloid β-protein; AD, Alzheimer's disease; AAA, amino acid analysis; AβPP, amyloid β-protein precursor; CHC, central hydrophobic cluster; HCHWA-D, hereditary cerebral haemorrhage with amyloidosis-Dutch type; ThT, thioflavin T; LDH, lactate dehydrogenase.

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Early *in vitro* studies of fibril aggregation have shown that the Aβ fragment [Gly^{21}]Aβ(13–26) forms large insoluble aggregates more slowly than does the wild-type homologue [18]. Later studies using full-length wild-type and Flemish Aβ(1–40) revealed similar effects on aggregate formation [20] and a decreased rate of protofibril formation by the Flemish homologue [27]. However, these studies did not examine early stages of fibril formation and fibril elongation. Another aspect of the pathogenesis of the Flemish disease, and of other Aβ-related amyloid deposition diseases, that may have clinical relevance is N-terminally truncated peptides [28,29]. For example, Pike et al. [30] reported a correlation between peptide length, solubility and toxicity; however, the relationship was imperfect, and a number of the peptides examined were not physiologically relevant. The effects of peptide truncation have not been studied with regard to the Flemish disease. The mechanism(s) by which the Flemish mutation produces disease thus remains elusive. In addition, phenomena other than altered fibrillogenesis kinetics may have pathogenetic relevance. These phenomena may include differences in AβPP processing, leading to the production of increased amounts of Aβ or of truncated Aβ peptide variants, differences in the stability of Flemish peptide assemblies or differences in the cytotoxic activity of the Flemish peptides and their assemblies.

Here we have studied the biophysical and toxic properties of the Flemish peptides [Gly^{21}]Aβ(1–40), [Gly^{21}]Aβ(5–40), and [Gly^{21}]Aβ(11–40). The truncated peptides were chosen on the basis of earlier cell-culture studies, which showed that increased amounts of these peptides were secreted by cells transfected with Flemish AβPP cDNA [31]. Our results reveal differences in peptide behaviour that are related to peptide length and are shared between Flemish and wild-type peptides, as well as differences which are associated specifically with the Ala^{21} → Gly mutation and may be of relevance to the pathogenesis of the Flemish disease.

EXPERIMENTAL

Chemicals and reagents

Chemicals were obtained from Sigma and were of the highest purity available. Water was double-distilled, and then deionized using a Milli-Q system (Millipore Corp., Bedford, MA, U.S.A.). Tissue-culture components were obtained from Gibco BRL (Grand Island, NY, U.S.A.).

Peptides

Aβ(1–40), [Gly^{21}]Aβ(1–40), and truncated analogues thereof, were synthesized and purified essentially as described in [27]. Peptide mass, purity and quantity were determined by a combination of matrix-assisted laser-desorption/ionization–time-of-flight ('MALDI–TOF') MS, analytical HPLC and quantitative amino acid analysis (AAA). Purified peptides were aliquoted, freeze-dried, and stored at −20 °C until used.

Solubility of peptides in physiological buffers

Peptides were dissolved directly in water to yield peptide concentrations of 200 μM. Solutions were vortex-mixed briefly and then diluted 1:1 with either PBS (10 mM sodium phosphate, pH 7.4, containing 137 mM NaCl and 27 mM KCl) or 100 mM Tris/HCl, pH 7.4. After brief vortex-mixing, samples were immediately centrifuged at 16000 ***g*** for 5 min and the supernatant removed, leaving ≈ 50 μl behind. Triplicate aliquots of the supernatant were subjected to AAA. All experiments were performed at ambient temperature. The significance of pairwise differences among the samples was determined by a one-way analysis of variance using a Tukey test, as implemented in SigmaStat v.2.0.3 (SPSS Inc., San Rafael, CA, U.S.A.).

Thioflavin T (ThT) binding assay

Peptides were dissolved in DMSO to yield protein concentrations of ≈ 4 mM and then spun through a 0.2 μm-pore-size filter (Millipore Corp., Bedford, MA, U.S.A.) to remove large pre-existing aggregates. Peptides prepared in this way produced no ThT fluorescence at zero time. However, if peptide stocks were prepared in water, as was done in the solubility experiments, a significant background of ThT fluorescence was observed. Aliquots were then taken for AAA and the stock solutions stored at −20 °C for up to 8 days prior to analysis. Stock solutions were thawed at ambient temperature and diluted with DMSO to produce solutions of 2 mM peptide concentration. To initiate reactions, 760 μl of 0.5 × PBS, containing 0.02 % (w/v) sodium azide, was added to 40 μl of peptide stock and vortex-mixed briefly. Samples were assayed immediately (see below) and then incubated at 37 °C. At intervals of 24, 48, 72 and 120 h, samples were removed and vortex-mixed for 10 s, then assayed. At time points greater than 24 h, aliquots of samples were diluted 2-fold prior to assay. Electron microscopy was performed on samples following 120 h of incubation.

ThT binding was assessed as described [32]. Briefly, 100 μl of sample was added to a 1 cm-square cuvette containing 800 μl of water and 1 ml of 100 mM glycine/NaOH, pH 8.5. The cuvette was vortex-mixed, then 50 μl of 100 μM ThT was added and the solution was vortex-mixeded again. Fluorescence was measured after ThT addition at 90, 100, 110 and 120 s. Measurements were made using a Perkin–Elmer LS-5B luminescence spectrometer with excitation and emission at 446 nm (slit width 5 nm) and 490 nm (slit width 10 nm) respectively.

Western blotting

Peptide solutions from the 0 and 120 h time points of the ThT assay were also examined by Western blotting. Samples were vortex-mixed and 10 μl aliquots were removed, mixed with an equal volume of 2 × sample buffer (Novex, San Diego, CA, U.S.A.), and then boiled at 100 °C for 10 min. Approx. 1 nmol of peptide/lane was electrophoresed on 16 % Tris/Tricine gels (Novex) and then transferred on to 0.2 μm nitrocellulose membranes (Schleicher and Schuell Inc., Keene, NH, U.S.A.) at 400 mA for 2 h at 4 °C. Membranes were then boiled for 10 min in PBS [33] and blocked overnight at 4 °C with 5 % (w/v) dried skimmed milk in 20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl and 0.05 % (v/v) Tween 20 (TBS-T). After washing the membrane in TBS-T, antibody 2G3 (2 μg/ml) was used as the primary antibody. This monoclonal antibody was produced using Aβ(31–40) as an immunogen and is specific for the C-terminus of Aβ species terminating at residue 40 [34]. Bound antibody was detected using alkaline phosphatase-conjugated anti-mouse Ig (1:2000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) and revealed by the addition of (5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium ('BCIP/NBT'; Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.).

Neurotoxicity experiments

Peptides were prepared for neurotoxicity experiments, essentially as described in [32]. Briefly, Aβ(1–40) and [Gly^{21}]Aβ(1–40) were dissolved at a concentration of ≈ 1 mM in 1 mM NaOH

containing 0.01 % (v/v) Phenol Red. NaOH (10 mM) was then added at a ratio of $\approx 120\ \mu$l/mg of peptide. This solution then was diluted with PBS to give a final Aβ concentration of ≈ 0.5 mM in $0.5 \times$ PBS, pH 7.0–7.4. Solutions were incubated for 2–3 days at 37 °C and diluted directly into tissue-culture medium to yield a final nominal peptide concentration of 25 μM. Electron microscopy showed fibrils were present in each preparation prior to addition to cells.

Cultured rat primary cortical cells were prepared as described in [35], with slight modifications. Briefly, brain cells were isolated from the neocortex of E15–17 rat embryos and plated at high density (1.3×10^5 cells/cm^2) in plating medium containing Dulbecco's minimal essential medium, 10 % (v/v) fetal-bovine serum, 10 % (v/v) Ham's F-12 medium, Hepes (20 mM), glutamine (2 mM) and penicillin/streptomycin (500 units/ml and 500 μg/ml respectively) on to poly-L-lysine-coated 48-well plates. Cultures were fed twice weekly with plating medium containing 5 % (v/v) fetal-bovine serum. At 3–4 weeks after plating, Aβ samples were added to cultures. Cell viability was monitored by optical microscopy and release of lactate dehydrogenase (LDH) [36].

Electron microscopy

Samples from the ThT and neurotoxicity experiments were centrifuged at 16000 ***g*** for 5 min and the resulting pellets resuspended in 20 μl of buffer. Negative-contrast electron microscopy was performed as described in [27]. Sample was applied to a carbon-coated Formvar grid (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.) and incubated for 60 s. The droplet was then displaced with an equal volume of 0.5 % (v/v) glutaraldehyde solution and incubated for an additional 60 s. The grid was then washed with two or three drops of water and 'wicked dry'. Finally, the peptide was stained with 2 % (w/v) uranyl acetate solution (Ted Pella, Inc., Reading, CA, U.S.A.) for 2 min. This solution was 'wicked off' and the grid air-dried. Samples were observed using either a JEOL 1200EX or a JEOL 100CX transmission electron microscope.

RESULTS

Peptide solubility

One of the most basic and clinically relevant physicochemical characteristics of peptides is solubility in an aqueous medium. If a peptide has a propensity to precipitate, either in ordered assemblies or disordered aggregates, cell and tissue damage usually results. The solubility of full-length and truncated wild-type and Flemish Aβ peptides was examined by solvating freeze-dried peptide preparations directly in water, adding 1 vol. of PBS, pH 7.4, centrifuging the solutions briefly at 16000 ***g*** to remove undissolved material and large aggregates, and then quantifying the protein content in the supernatant by AAA. Pretreatment of peptides with organic solubilizing agents such as DMSO, trifluoroethanol or trifluoroacetic acid, or with NaOH, was avoided so as not to confound analysis of the solubility characteristics of each peptide. A direct correlation was observed between peptide length and solubility for both the wild-type and Flemish peptides (Figure 1). For wild-type peptides, Aβ(5–40) and Aβ(11–40) were significantly ($P < 0.001$) less soluble than was Aβ(1–40), and Aβ(11–40) was significantly ($P = 0.002$) less soluble than was Aβ(5–40). The Flemish peptides displayed an identical trend. [Gly^{21}]Aβ(5–40) and [Gly^{21}]Aβ(11–40) were significantly ($P < 0.005$) less soluble than [Gly^{21}]Aβ(1–40), and the average solubility of [Gly^{21}]Aβ(11–40) was lower than that of [Gly^{21}]Aβ(5–40), although in this latter case the difference was not significant. For each peptide length, the Flemish peptides showed a trend towards higher solubility than did their wild-type homologues. The solubility differences between Flemish and wild-type Aβ(5–40) and between Flemish and wild-type Aβ(11–40) were statistically significant ($P < 0.002$ and $P < 0.005$ respectively). The full-length Flemish peptide showed a trend towards higher solubility relative to full-length wild-type peptide, but the difference was not significant. These results were not buffer-specific, in that similar data were obtained using 50 mM Tris/HCl, pH 7.4 (results not shown).

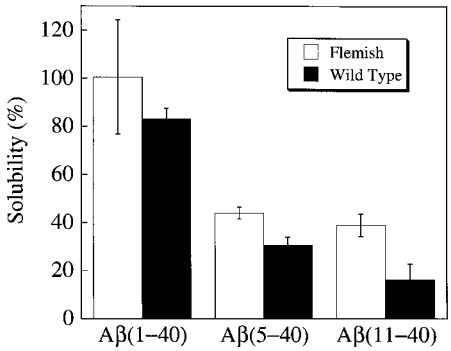

Figure 1 Peptide solubility

Freeze-dreid wild-type and Flemish peptides were dissolved in water and then mixed with PBS, pH 7.4, to produce neutral solutions with 100 μM Aβ concentration. The solubility of each peptide was then determined by AAA of aliquots of supernatant fluid obtained following centrifugation (see the Experimental section). Data are presented as normalized mean solubilities $\pm$ S.D. from triplicate AAAs of samples from three independent experiments. Normalization was done relative to total peptide present prior to centrifugation.

Peptide aggregation

The differences in solubility between wild-type and Flemish peptides suggested that the Flemish mutation might also affect the rate of peptide fibrillization. To examine this question, temporal changes in ThT binding were monitored periodically during 5 days of incubation of each peptide in PBS, pH 7.4, at 37 °C. Wild-type and Flemish Aβ(1–40) displayed a sigmoidal increase in ThT binding, typical of fibrillogenesis processes (Figure 2, upper panel). In addition, wild-type ThT binding decreased after 3 days, a phenomenon associated with the formation of suprafibrillar assemblies. Both wild-type and Flemish peptides produced equivalent levels of maximal ThT binding, but did so at different rates. As a means of comparing fibrillogenesis rates, the time at which half-maximal ThT binding occurred (t_{50}) was calculated for each peptide. t_{50} for the Flemish peptide was about 13 h later (43 versus 30 h) than that for the wild-type peptide. A 13 h, t_{50} difference (35 versus 22 h) was also seen with the Aβ(5–40) peptides (Figure 2, middle panel). However, t_{50} occurred earlier for the truncated relative to the full-length peptides. An additional difference between the truncated and full-length peptides was that the Flemish Aβ(5–40) peptide bound substantially greater amounts of ThT than did wild-type Aβ(5–40) or either full-length peptide. Wild-type and Flemish Aβ(11–40), the shortest peptides, behaved similarly to their full-length homologues with respect to the overall kinetics of fibrillogenesis, the comparatively slow fibrillogenesis of the Flemish peptide relative to wild-type peptide (t_{50} values of 50 versus 37 h), and the absolute maximal level of ThT binding (Figure 2, bottom panel). This behaviour was distinct from that displayed by the Aβ(5–40) peptides.

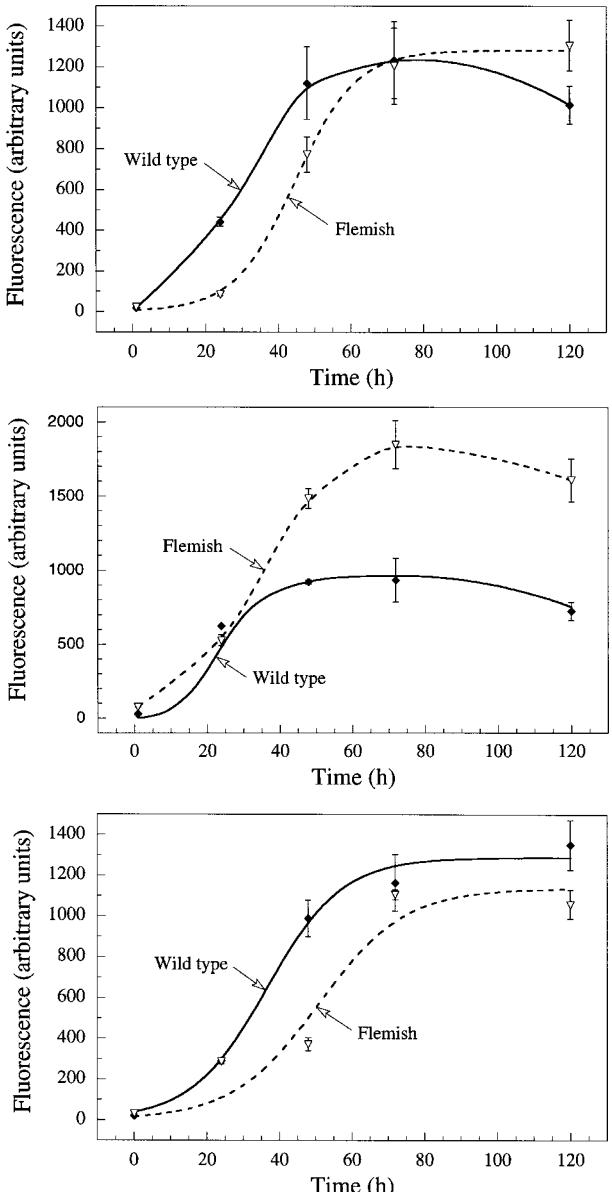

Figure 2 Temporal development of ThT binding

Flemish and wild-type Aβ(1–40) (upper panel), Aβ(5–40) (middle panel) and Aβ(11–40) (bottom panel) peptides were dissolved in PBS, pH 7.4, at a concentration of 100 μM, then incubated at 37 °C. Periodically, aliquots were removed and ThT binding was measured. Data are presented as mean ThT binding $\pm$ S.D. ($n = 3$). In some cases, the S.D. is smaller than the diameter of the diamond or triangle signifying the mean. For each peptide, the arrow points to the time at which half-maximal ThT binding was observed (t_{50}). The curves shown were derived using the curve-fitting option available in Kaleidagraph 3.5 (Synergy Software, Reading, PA, U.S.A.).

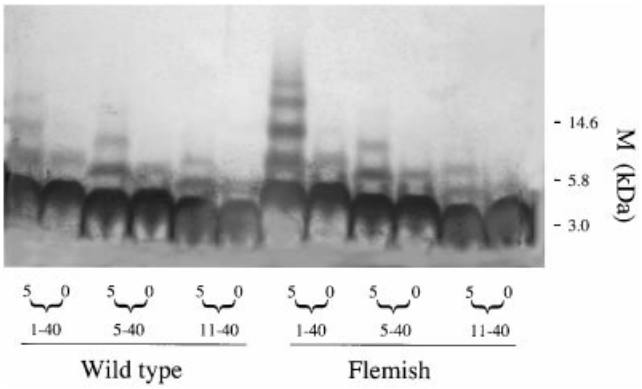

Figure 3 Stability of assemblies formed by Flemish and wild-type peptides

Peptide solutions (100 μM) were prepared and incubated at 37 °C (see the legend to Figure 2). Aliquots were removed immediately upon dissolution (signified by '0'), and then again after 5 days (signified by '5'). Each aliquot then was subjected to SDS/PAGE and the immunoreactive bands revealed by Western blotting using the Aβ C-terminal specific antibody 2G3. The blot shown is typical of those seen in each of a total of six experiments. It should be noted that this blot has been 'developed' for a relatively short period in order to maximize contrast. Longer exposure times result in decreased contrast, but typically produce oligomer distributions which extend to higher molecular masses ('M'), corresponding to oligomers containing one or two additional Aβ monomers. We note that no aggregates were observed at the interfaces between stacking and separating gels in any of the six experiments that were performed.

Formation of SDS-stable oligomers

Aβ peptides form non-covalent oligomers, some of which are stable during SDS/PAGE [27,37,38]. This stability may correlate with amyloidogenicity. For example, Aβ(1–42) forms abundant oligomers, whereas the less amyloidogenic peptide, Aβ(1–40), produces few of these species. To determine the effects of N-terminal truncation and the Ala$^{21} \rightarrow$ Gly substitution on the SDS-stability of peptide oligomers, wild-type and Flemish variant peptides were dissolved in PBS, pH 7.4, as was done for ThT assay, and then subjected to SDS/PAGE, either immediately or after incubation for 5 days at 37 °C. To enhance detection sensitivity, Western blotting was performed following electrophoresis using the Aβ C-terminal-specfic antibody 2G3 (Figure 3).

Immediately following dissolution and SDS/PAGE, finite distributions of oligomers were observed for all six peptides. Each distribution consisted of an intense band with an R_F consistent with that of monomer along with decreasing amounts of higher-order oligomers. Dimers were the highest-order oligomers readily seen from wild-type Aβ(1–40), Aβ(5–40), Aβ(11–40) samples, although a faint trimer band was occasionally visible in some samples. Flemish peptides displayed very similar distributions. Incubating the peptides for 5 days resulted in the broadening of all six distributions. Monomers remained the most abundant species in all cases, but discrete bands of higher-molecular-mass oligomers now appeared which were not seen initially. The ability to form SDS-stable oligomers correlated with peptide length. Bands corresponding to oligomers up to the size of tetramer were readily apparent in wild-type Aβ(1–40) samples. A similar distribution of oligomer size was seen with wild-type Aβ(5–40), but the tetramer band was lower in intensity. The trimer band was the highest-molecular-mass band readily visible in the wild-type Aβ(11–40) samples. Interestingly, broader distributions were observed with the Flemish peptides. Flemish Aβ(1–40) produced intense bands up to pentamer, although fainter hexamer and heptamer bands were visible in some experiments. A trimer band, and a faint tetramer band, were seen in the Flemish Aβ(5–40) samples. The Flemish Aβ(11–40) distribution extended up to trimer, with a faint tetramer band sometimes seen. With long exposure, one or two additional steps in each oligomer ladder could be observed. However, in all cases, the width of the oligomer distributions correlated with peptide length. In addition, Flemish Aβ(5–40), and to a lesser extent, wild-type Aβ(5–40), also produced a slower-migrating species

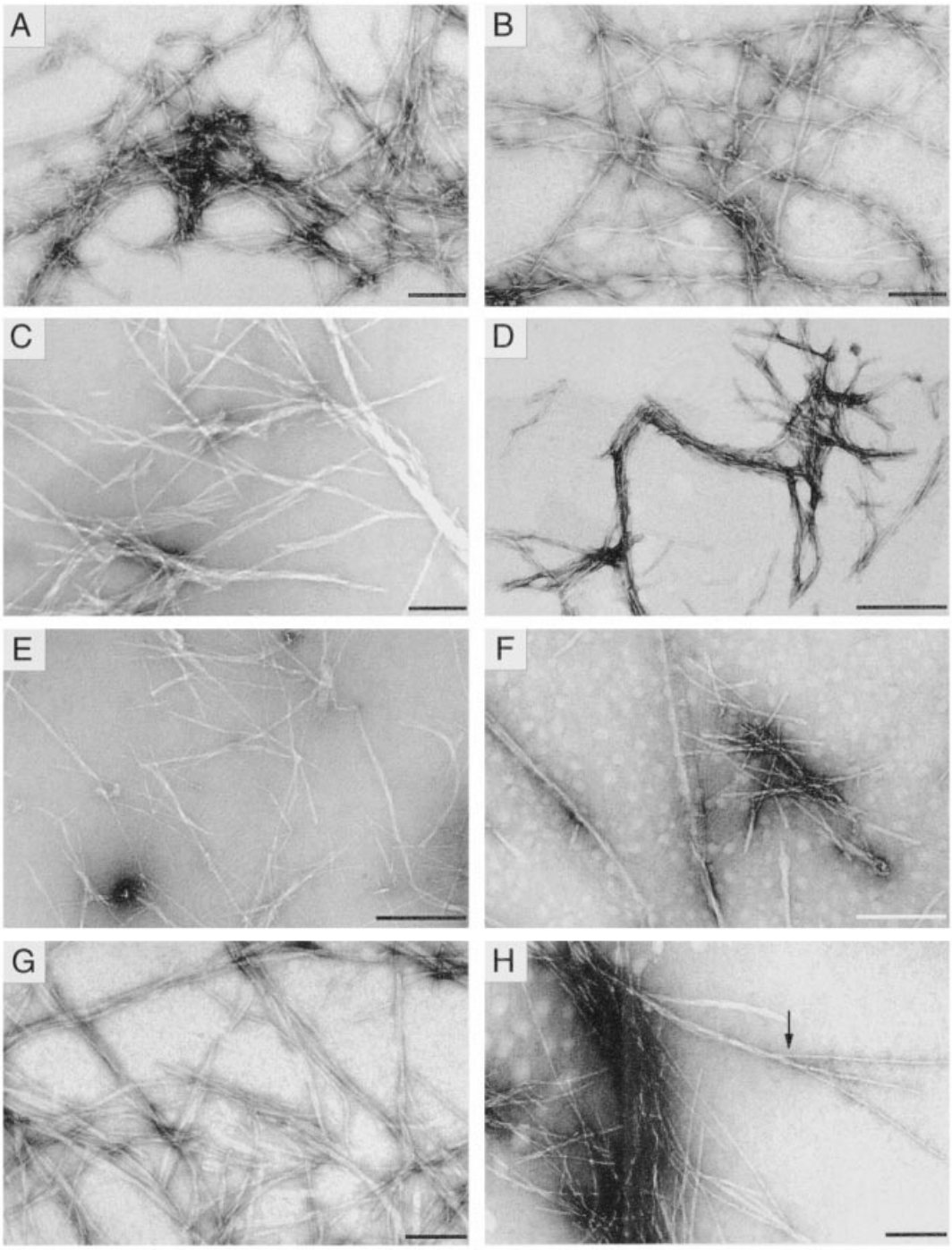

Figure 4 Morphologies of assemblies formed by Flemish and wild-type peptides

Assemblies formed during incubation of peptides at 37 °C in PBS, pH 7.4 (see the legend to Figure 2), were isolated by centrifugation and studied by electron microscopy following uranyl acetate staining. (**A**) Wild-type Aβ(1–40); (**B**) Flemish Aβ(1–40); (**C**) Wild-type Aβ(5–40); (**D**) Flemish Aβ(5–40); (**E** and **G**) Wild-type Aβ(11–40); (**F** and **H**) Flemish Aβ(11–40). The arrow in (**H**) points to the splayed ends of laterally associated fibrils. Scale bars represent 100 nm.

with an apparent molecular mass of ≈ 60 kDa (results not shown).

Morphologies of peptide assemblies

As shown above, truncations and amino acid substitution within Aβ alter peptide solubility, the kinetics of peptide assembly and assembly stability. To evaluate the effects of primary structure on assembly morphology, electron-microscopic studies were performed. All peptides assembled into filamentous assemblies (Figure 4). In general, fibrils were less abundant in Flemish peptide samples than in wild-type samples. However, globular or

amorphous aggregates were observed in greater abundance in the Flemish samples (results not shown). The ease of detection of fibrils correlated with peptide length, with the full-length peptides producing the most readily detectable assemblies. On average, short, relatively straight, fibrils were detected more frequently in samples from the truncated peptides. In addition, truncated peptides had an increased propensity for lateral aggregation.

Wild-type Aβ(1–40) produced many long, straight fibrils. These fibrils appeared bifilar in nature and displayed either smooth margins (unwound) or a clear helical twist (wound) (Figure 4A). Diameters of the bifilar structures ranged from about 5 to 7 nm. A number of protofibrils [27,39] appeared to be associated with the longer fibrils. The Flemish Aβ(1–40) fibrils displayed a more prominent rope-like morphology. Although bifilar structures were common, the Flemish assemblies often were composed of three or more filaments, each about 3–4 nm in diameter, which were laterally associated and wound together into helical arrays (Figure 4B).

Prominent multi-filament, helical assemblies were also observed in the wild-type Aβ(5–40) samples (Figure 4C), except that their lengths were substantially smaller, on average, than those of their full-length homologues. The most common form of these assemblies was composed of two, tightly wound $\approx$ 5 nm-diameter filaments. Lateral arrays of these assemblies were frequent and often formed large coiled-coil structures. Short, laterally-associated fibril assemblies were also observed in Flemish Aβ(5–40) samples (Figure 4D). The tendency of these arrays to arrange into helical superstructures appeared lower than that of their wild-type homologues.

Wild-type Aβ(11–40) produced fields in which many short, straight, thin filaments were visible (Figure 4E). These filaments appeared to associate into loosely wound bi- or multi-filar arrays, often displaying splayed ends. The bifilar structures had diameters of $\approx$ 7–11 nm. In other fields, denser fibril meshes were seen in which loosely associated lateral arrays of thin ($\approx$ 3–4 nm) filaments were apparent (Figure 4G). These arrays had the appearance of narrow, flat ribbons; however, a number exhibited irregular helical segments. Short, straight fibril arrays were also seen interspersed among the longer arrays. The Flemish Aβ(11–40) peptides produced abundant short fibrils (Figure 4F). The simplest of these structures appeared bifilar in nature and were often wound into helices with diameters of $\approx$ 10–13 nm and with somewhat indistinct margins. In some fields, many of these simple fibrils associated laterally to form long, longitudinally oriented, multifibril assemblies (Figure 4H). A particularly clear example of lateral association and end splaying is illustrated in Figure 4H (arrow), which shows an assembly composed of three wound fibrils, each of which contains two filaments $\approx$ 3–4 nm in diameter.

Taken together, these data demonstrate that all six peptides form fibrils and that a key determinant of fibril morphology is peptide length. Superimposed on these basic length effects are more subtle, but observable, alterations in the structures of fibril assemblies mediated by the Gly^{21} substitution.

Peptide neurotoxicity

An important biological activity of Aβ assemblies is neurotoxicity. To examine the activity of wild-type and Flemish assemblies, LDH-release assays, which measure cell death, were performed on cultured primary rat cortical cells. These cultures contain both glia and neurons. However, previous studies have shown that Aβ kills primarily neurons [36]. Prior to addition to cells, Aβ(1–40) peptides were incubated for 2–3 days at 37 °C.

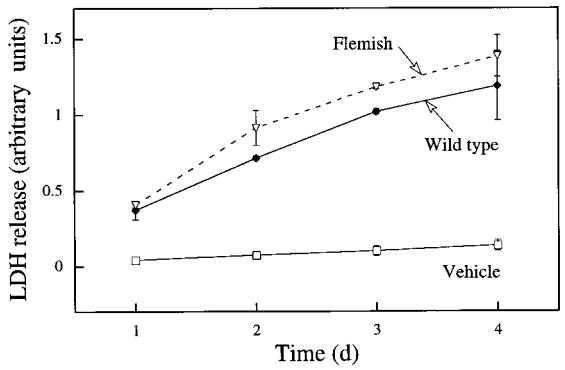

Figure 5 Neurotoxicity of Flemish Aβ(1–40)

Flemish and wild-type Aβ(1–40) peptides were incubated for 2–3 days at 37 °C and then added to cultures of primary rat cortical cells. The concentrations of the Flemish and wild-type peptides in the cultures were 24 and 23 μM, respectively. Cultures were examined daily and cell loss was assessed by release of the cytosolic enzyme LDH. Data are presented as the mean LDH release $\pm$ S.D. ($n = 3$). The results are representative of those obtained from a total of three independent experiments. In all experiments, treatment with vehicle alone did not significantly alter LDH release.

Subsequent addition of either wild-type or Flemish Aβ(1–40) peptides to rat neurons caused significant LDH release (Figure 5). LDH release increased over time and the amounts of LDH released were similar for both peptides. In the experiment illustrated in Figure 5, the Flemish peptide appears slightly more toxic than does the wild-type peptide. However, in other experiments, this difference is either not observed or the wild-type peptide appears slightly more toxic (results not shown). We note also that the toxicity of [Gly^{21}]Aβ(1–40) did not depend on the method of solvation, as similar results were obtained with peptides prepared from DMSO stocks (results not shown). We conclude from these results that both peptides are equipotent neurotoxins. In fact, in each case, optical-microscopic examination of the treated cultures revealed massive neuronal-cell loss relative to cultures treated with vehicle alone (results not shown).

DISCUSSION

Amyloid deposition occurs when the balance between production and elimination of amyloidogenic proteins is perturbed. [In this discussion, amyloidogenic peptides are included under the rubric of 'proteins'.] Perturbations may result from mutations in genes encoding amyloid proteins, which alter protein structure or the levels of protein expression, or from changes in the production or activity of molecules or cells participating in the metabolism of amyloid proteins. In a Flemish kindred, a mutation in the gene encoding the AβPP results in the production of Aβ peptides containing an Ala^{21} $\rightarrow$ Gly substitution [9] and leads to a form of AD with prominent amyloid angiopathy [14]. Although the linkage between the AβPP mutation and Flemish variant AD is clear, the mechanistic basis for the disease is not entirely understood.

One explanation for the Flemish disease is altered proteolytic processing of AβPP, which could result in the production of different quantities and types of peptides, particularly those with increased amyloidogenic propensities. Studies of AβPP processing in cultured HEK-293 cells have, in fact, shown that

expression of the Flemish AβPP gene, relative to the wild-type AβPP gene, results in the secretion of proportionately greater amounts of Aβ peptides beginning at Val^{-3}, Arg^{5}, Glu^{11} and Leu^{17} [31]. In addition, a two-fold increase in Aβ beginning at Asp^{1} and a decrease in α-secretase processed APP relative to wild-type transfectants was observed. Subsequent work confirmed and extended these results by demonstrating that increased secretion of both Aβ(1–40) and Aβ(1–42) occurs in CHO-K1 and H4 cells transfected with Flemish AβPP cDNA [40]. Moreover, in a recent report [41], [Gly^{21}]Aβ(19–35) was found to form sedimentable aggregates more readily than the corresponding wild-type peptide. However, it should be noted that the peptides used in this latter study were C-terminally amidated and contained norleucine in place of Met^{35}. In addition, such highly truncated peptides have not been detected in the brain or in cell-culture models *in vitro*.

Here we elucidated basic aspects of the physicochemical and biological behaviours of the physiologically relevant Flemish peptides [Gly^{21}]Aβ(1–40), [Gly^{21}]Aβ(5–40) and [Gly^{21}]Aβ(11–40). Our studies revealed that truncation of either the wild-type or Flemish peptides substantially decreased their solubility. However, the Flemish variant peptides were consistently more soluble than were the wild-type peptides. All six peptides exhibited time-dependent increases in ThT binding, suggesting all formed assemblies containing β-sheet structure. Electron-microscopic examination of the assemblies revealed that all had fibrillar morphology, consistent with their high β-sheet content, and that the Gly^{21} substitution affected assembly morphology. The rates of fibril formation, as measured by ThT binding, were slower among the more soluble Flemish peptides than among the homologous wild-type peptides. These data are consistent with earlier studies on a peptide fragment, Aβ(13–26), and on Aβ(1–40), which showed that the $Ala^{21} \rightarrow Gly$ substitution decreased the rate of formation of large, insoluble aggregates [18,20]. The magnitude of the effect of the $Ala^{21} \rightarrow Gly$ substitution on the solubility and fibrillogenesis behaviour of both full-length and truncated Aβ peptides emphasizes the importance of the central hydrophobic cluster (CHC; $Leu^{17} \rightarrow Ala^{21}$) in controlling Aβ assembly [42,43].

In addition to its role in controlling the kinetics of fibrillogenesis, the CHC also affects the intermolecular associations of the Aβ peptide which mediate formation of fibrillar assemblies. This conclusion evolves from the electron-microscopic examination of the constellations of peptide assemblies formed from the wild-type and Flemish peptides, which showed that the Gly^{21} substitution caused variations in the diameters of the filaments, the extent of lateral filament association, the helicity of the fibrils, and the density of filament packing. *In vivo*, these differences could affect the catabolism of the fibrils by affecting the thermodynamic stability of the fibrils or their ability to be metabolized by phagocytic cells. In addition, the structural organization of the fibrils could affect their biological activities, resulting in differences in target-cell specificity and cytotoxicity.

One aspect of Aβ assembly stability that is demonstrable *in vitro* is SDS-stability. Studies have shown that assemblies of Aβ(1–42) dissociate to form low-molecular-mass oligomers during gel electrophoresis [37,38,44]. Oligomers can also be detected in experiments with Aβ(1–40) if sensitive detection methods are employed [36]. Comparative analysis of SDS-gel-stability of wild-type and Flemish peptide assemblies revealed that the $Ala^{21} \rightarrow Gly$ substitution actually increased the SDS-stability of these assemblies. In the light of the increased solubility and decreased fibrillogenesis rates of the Flemish peptides, this result was surprising. However, this type of experiment measures more than simply the stability of Aβ fibrils. Any assembly which is present in the incubation mixture can be electrophoresed, and the structures of these may range from dimer to fibrils or large aggregates. The broadened distributions of oligomers observed with the Flemish peptides may thus have resulted from the increased stability of the oligomers or other peptide assemblies. Recent studies of the fibrillogenesis of the Parkinson's-disease-associated protein α-synuclein support this contention [45]. Here, an $Ala^{30} \rightarrow Pro$ α-synuclein mutant associated with early-onset Parkinson's disease was found to fibrillize slower than did the wild-type protein, even though its assembly into oligomers proceeded more rapidly. These observations suggested that increased oligomer stability resulted in decreased fibrillogenesis rates. In this regard, it is intriguing that electron-microscopic examination of the Flemish samples revealed concentrations of globular and amorphous aggregates that were higher than those observed in wild-type samples. In addition, for Flemish Aβ(5–40) peptides in particular, a band of molecular mass ≈ 60 kDa was often observed in the SDS-stability experiments (results not shown).

The solubility characteristics and the aggregation propensity of full-length and truncated Flemish Aβ peptides may be relevant to the unique plaque morphology and prominent vascular component of the Flemish disease. Conformational changes in Aβ induced by the $Ala^{21} \rightarrow Gly$ substitution could facilitate its association with vessel walls by exposing portions of the peptide not normally capable of interacting with endothelial-cell surface molecules. If the increased solubility of the Flemish peptides and their increased propensity to form stable aggregates (seeds) are recapitulated *in vivo*, then the basic laws governing nucleation-dependent polymerization processes [46,47] would predict increases in the numbers and sizes of vascular amyloid deposits in Flemish relative to wild-type patients. Increased concentrations of soluble Aβ in the cerebrospinal fluid also should result in larger parenchymal plaque sizes. Interestingly, incubation of Flemish Aβ(5–40) produced assemblies that bound substantially greater amounts of ThT than did the other peptides studied, suggesting that this peptide (whose production is elevated in cultured cells transfected with Flemish AβPP constructs [31]) had a greater propensity to form amyloid-like aggregates. In fact, extensive amyloid angiopathy and unusually large senile plaque size are two characteristics of the Flemish disease [14]. Interestingly, recent results have suggested that many plaque cores may consist in part of blood vessels clogged with amyloid [48].

Once formed, Aβ assemblies have biological effects. In addition to tissue injury caused by mechanical damage resulting from amyloid deposition, ordered Aβ assemblies have neurotoxic activity [32,38,49]. We found that assemblies formed by Flemish Aβ(1–40) killed rat neurons in culture and that the toxic activity was equivalent to that of wild-type peptide. To our knowledge, this is the first report demonstrating that Flemish Aβ peptides have neurotoxic activity. The structural nature of the neurotoxic assembly is currently unclear. Both Flemish and wild-type peptide preparations contained fibrils; however, associated oligomeric or protofibrillar species could also have caused neuronal injury. The truncated wild-type and Flemish peptides also displayed significant toxicity in a number of experiments (results not shown). However, as noted previously (Figure 1), truncation of Aβ after Phe^{4} or Tyr^{10} causes decreased peptide solubility, and this phenomenon made systematic comparisons of toxicity among the truncated peptides and their full-length homologues difficult. This notwithstanding, our findings that Flemish Aβ peptides are neurotoxic is consistent with a recent report that transgenic mice overexpressing human Flemish AβPP display signs of brain injury, including gliosis, white-matter micro-

spongiosis, apoptotic neurons and behaviour disturbances [50]. Interestingly, *in vitro* studies of the cytotoxic activities of Aβ(1–40) peptides towards human smooth-muscle cells derived from brain microvessels or aorta have revealed that Flemish peptides are not toxic, whereas Dutch peptides are [51]. These results suggest that the cytotoxic activity of assemblies of Flemish Aβ are cell-type-specific. Our demonstration that assemblies of Flemish Aβ are neurotoxic suggests a direct route by which Flemish Aβ peptides may cause neuronal loss and dementia *in vivo*.

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