

A Seed for Alzheimer Amyloid in the Brain

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A fundamental question about the early pathogenesis of Alzheimer's disease (AD) concerns how toxic aggregates of amyloid β protein (A β) are formed from its nontoxic soluble form. We hypothesized previously that GM1 ganglioside-bound A β (GA β) is involved in the process. We now examined this possibility using a novel monoclonal antibody raised against GA β purified from an AD brain. Here, we report that GA β has a conformation distinct from that of soluble A β and initiates A β aggregation by acting as a seed. Furthermore, GA β generation in the brain was validated by both immunohistochemical and immunoprecipitation studies. These results imply a mechanism underlying the onset of AD and suggest that an endogenous seed can be a target of therapeutic strategy.

Key words: Alzheimer's disease; amyloid; amyloid β protein; seed; ganglioside; raft

Introduction

The lifelong expression of genetic mutations responsible for familial Alzheimer's disease (AD) appears to induce the formation of neurotoxic aggregates of amyloid β protein (A β) by accelerating cellular A β generation (Selkoe, 1997). However, there is currently no evidence that A β generation is enhanced in sporadic, late-onset AD, the principal form of the disease. Thus, it is reasonable to assume that A β aggregation in conventional AD may be induced by unknown posttranslational modification(s) of A β and/or by altered clearance mechanisms.

We previously identified a novel A β species, characterized by its tight binding to GM1 ganglioside (GM1), in human brains that exhibited early pathological changes associated with AD (Yanagisawa et al., 1995, 1997; Yanagisawa and Ihara, 1998). On the basis of the molecular characteristics of the GM1-bound A β (GA β), including its altered immunoreactivity and a strong tendency to form aggregates of A β , we hypothesized that A β adopts an altered conformation by binding to GM1 and initiates the

aggregation of soluble A β by acting as a seed (Yanagisawa et al., 1995, 1997; Yanagisawa and Ihara, 1998). Evidence that supports our hypothesis is growing from *in vitro* studies by our group and other groups (McLaurin and Chakrabarty, 1996; Choo-Smith and Surewicz, 1997; Choo-Smith et al., 1997; McLaurin et al., 1998; Matsuzaki and Horikiri, 1999; Ariga et al., 2001; Kakio et al., 2001, 2002).

In the present study, we directly characterize GA β at the molecular level using a novel monoclonal antibody raised against GA β purified from an AD brain with the aim of validating our hypothesis. Here we show that GA β has a conformation distinct from that of soluble A β and initiates A β aggregation by acting as a seed. Importantly, we successfully verified GA β generation in the brain by both immunohistochemical and immunoprecipitation methods.

Materials and Methods

Preparation of seed-free A β solutions. A β solutions were prepared essentially according to a previous report (Naiki and Gejyo, 1999). Briefly, synthetic A β (A β 40 and A β 42) (Peptide Institute, Osaka, Japan) was dissolved in 0.02% ammonia solution at 500 μ M for A β 40. A β 42 was dissolved at 250 μ M because it has a higher potential to form aggregates rapidly than A β 40. To obtain seed-free A β solutions, the prepared solutions were centrifuged at 540,000 $\times g$ for 3 hr using the Optima TL Ultracentrifuge (Beckman Instruments, Fullerton, CA) to remove undissolved peptide aggregates, which can act as preexisting seeds. The supernatant was collected and stored in aliquots at -80°C until use. Immediately before use, the aliquots were thawed and diluted with Tris-buffered saline (TBS) (150 mM NaCl and 10 mM Tris-HCl, pH 7.4). In the present study, we used the seed-free A β solutions, except in the preparation of A β fibrils used as the seeds (see below).

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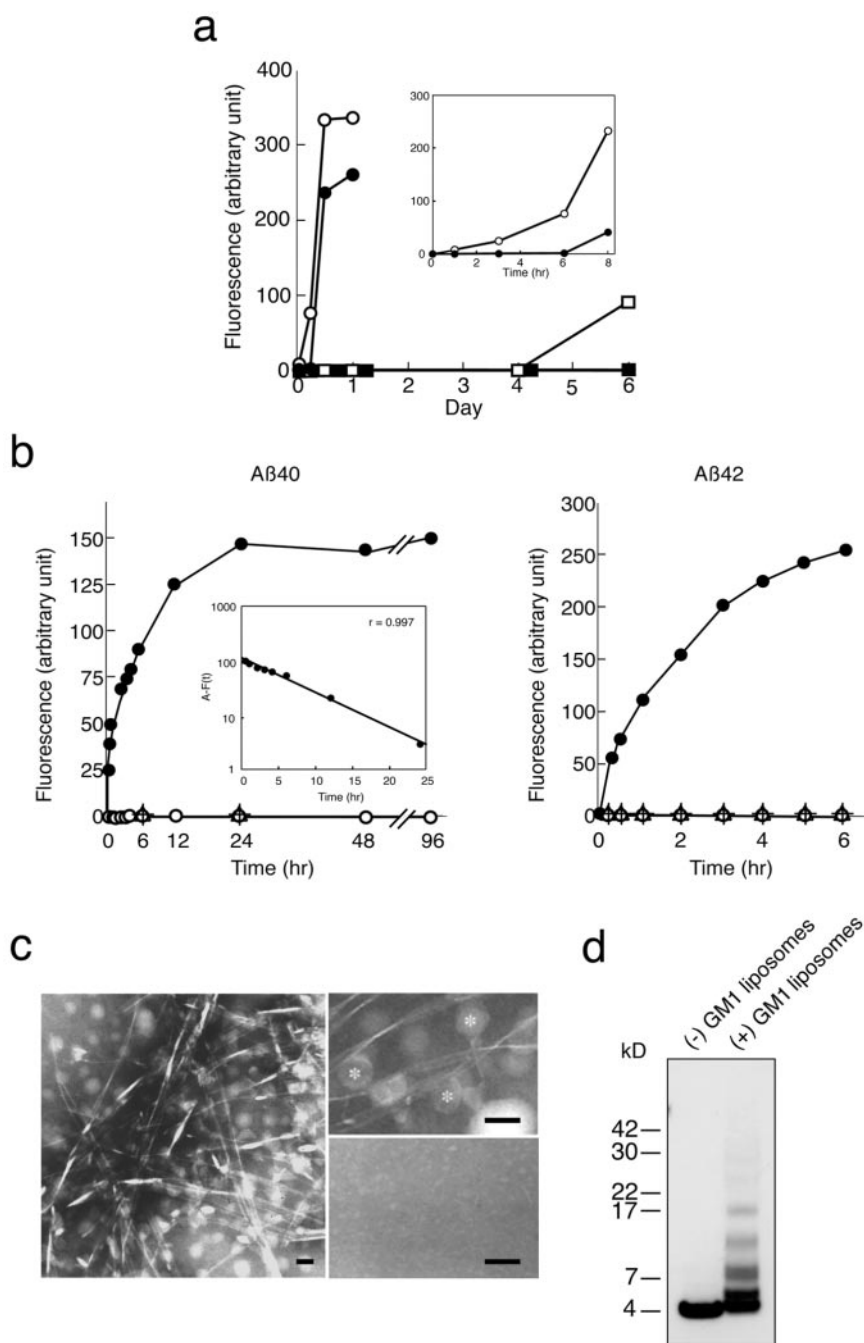


Figure 1. Amyloid fibril formation from soluble A β in the absence or presence of GM1-containing liposomes. *a*, Kinetics of A β fibrillogenesis using A β (A β 40 and A β 42) solutions, with or without removing undissolved peptide aggregates, which can act as preexisting seeds. A β solutions were incubated at 50 μ M and 37°C. Open and filled circles indicate ThT fluorescence intensities of A β 42 solutions without and with removing undissolved peptide aggregates, respectively. Open and filled squares indicate ThT fluorescence intensities of A β 40 solutions without and with removing undissolved peptide aggregates, respectively. *b*, Kinetics of A β fibrillogenesis. A β (A β 40 and A β 42) solutions, after removal of undissolved peptide aggregates, were incubated at 50 μ M and 37°C in the presence of GM1-containing liposomes (filled circles) or GM1-lacking liposomes (plus signs) or were incubated in the absence of liposomes (open circles). The GM1-containing liposomes alone were also incubated in the absence of A β (triangles). The fluorescence intensity of thioflavin T was obtained by excluding background activity at 0 hr. Inset, Semilogarithmic plot of the difference, $A - F(t)$, versus incubation time (0–24 hr). $F(t)$ represents the increase in fluorescence intensity as a function of time in the case of A β incubated with GM1-containing liposomes, and A is tentatively determined as $F(\infty)$. Linear regression and correlation coefficient values were calculated ($r = 0.997$). $F(t)$ is described by a differential equation: $F'(t) = B - CF(t)$. *c*, Electron micrographs of the A β 40 solutions incubated at 50 μ M and 37°C for 24 hr with GM1-containing liposomes (left and right top panels) or without liposomes (right bottom panel). The liposomes are indicated by asterisks. Scale bars, 50 nm. *d*, Western blot of A β 40 solutions incubated at 50 μ M and 37°C for 24 hr in the presence or absence of GM1-containing liposomes. The incubated A β solutions were centrifuged at 100,000 $\times g$ for 15 min. Ten nanograms of A β in the supernatant were subjected to SDS-PAGE (4–20%) after glutaraldehyde treatment. The A β in the gel was detected by Western blotting using BAN052. A β oligomers were detected in the A β solution incubated in the presence of GM1-containing liposomes but not detected in the incubation mixture containing A β alone.

Preparation of A β 40 seeds. A β 40 fibrils used as exogenous seeds were prepared essentially according to the method reported previously (Naiki and Nakakuki, 1996). Briefly, A β 40 solution was incubated at 500 μ M and 37°C for 72 hr without previous removal of the preexisting seeds. After incubation, newly formed A β 40 fibrils were precipitated by ultracentrifugation. The resultant pellets were subjected to sonication. Protein concentrations of the solutions containing A β 40 fibrils were determined using a protein assay kit (Bio-Rad, Hercules, CA) as described previously (Bradford, 1976). The A β 40 solution quantified by amino acid analysis was used as the standard. Aliquots of the solution were stored at -80°C until use.

Preparation of liposomes. Cholesterol and sphingomyelin (Sigma, St. Louis, MO) and GM1 (Wako, Osaka, Japan) were dissolved in chloroform/methanol (1:1) at a molar lipid ratio of 2:2:1 to generate GM1-containing liposomes. GM1-lacking liposomes were prepared by mixing cholesterol and sphingomyelin at a molar lipid ratio of 1:1. The mixtures were stored at -80°C until use. Immediately before use, the lipids were resuspended in TBS at a GM1 concentration of 2.5 mM and subjected to freezing and thawing. The lipid suspension was centrifuged once at 15,000 $\times g$ for 15 min, and the resultant pellet was resuspended in TBS. Finally, the suspension was subjected to sonication on ice.

Thioflavin T assay. The assay was performed according to a method described previously (Naiki and Gejyo, 1999) on a spectrofluorophotometer (RF-5300PC; Shimadzu, Tokyo, Japan). Optimum fluorescence measurements of amyloid fibrils were obtained at the excitation and emission wavelength of 446 and 490 nm, respectively, with the reaction mixture (1.0 ml) containing 5 μ M thioflavin T (ThT) (Sigma) and 50 mM glycine-NaOH buffer, pH 8.5. Fluorescence was measured immediately after making the mixture. The A β (A β 40 and A β 42) solution described above was incubated at 37°C with liposomes at an A β concentration of 50 μ M at a GM1/A β molar ratio of 10:1.

Detection of A β oligomers by SDS-PAGE. The A β 40 solution described above was incubated with liposomes at an A β concentration of 50 μ M at a GM1/A β molar ratio of 10:1 for 24 hr at 37°C and then centrifuged at 100,000 $\times g$ for 15 min. The supernatant was subjected to SDS-PAGE after glutaraldehyde treatment (LeVine, 1995) to stabilize oligomeric A β during SDS-PAGE. The A β in the gel was detected by Western blotting using BAN052, a monoclonal antibody specific to the N terminus of A β (Suzuki et al., 1994), as reported previously (Yanagisawa et al., 1995).

Experiments using animal models. All experiments using animals were performed in compliance with existing laws and institutional guidelines. For experiments using nonhuman primates, animals were anesthetized with pentobarbital (25 mg/kg, i.p.) and killed by draining blood from the heart.

Cell culture. Cerebral cortical neuronal cultures were prepared from Sprague Dawley rats

at embryonic day 17 as described previously (Michikawa and Yanagisawa, 1998). The dissociated single cells were suspended in a feeding medium and plated onto poly-D-lysine-coated 12-well plates at a cell density of 2.5×10^5 /well. The feeding medium, N2, consisted of DMEM-F-12 containing 0.1% bovine serum albumin fraction V solution (Invitrogen, Gaithersburg, MD) and N2 supplements. The reagents examined, including TBS, A β 40, GM1-lacking liposome, and GM1-containing liposome in the absence or presence of A β 40, were prepared as described above. For treatment, at 24 hr after plating, the culture medium was changed with fresh N2 medium diluted with the same volume of each reagent solution. At 48 hr after the commencement of the treatment, phase-contrast photomicrographs of each culture were taken, and the cells were stained with propidium iodide, which selectively permeates the broken membranes of dying cells and stains their nuclei, and with a viable cell-specific marker, calcein AM, as described previously (Michikawa and Yanagisawa, 1998). Photomicrographs were taken by laser confocal microscopy (Zeiss, Oberkochen, Germany) and the number of viable neurons on each micrograph was determined in each microscope field (40 \times objective). Two hundred to 500 cells were counted for each determination of cell viability. Statistical analysis was performed using ANOVA.

Production of 4396C. The IgG monoclonal antibody 4396C was produced by the genetical class-switch technique (Binding and Jones, 1996) from IgM hybridomas that were raised against GA β purified from an AD brain. The procedures for the generation and characterization of the original IgM monoclonal antibody 4396 were reported previously (Yanagisawa et al., 1997).

Immunoelectron microscopy. GM1-containing and GM1-lacking liposomes were mixed with soluble A β 40 on ice for 5 sec at a weight ratio of lipid/A β 40 at 100:3. The mixtures were immediately ultracentrifuged to remove unbound A β 40, and liposome pellets were fixed for immunoelectron microscopy of 4396C or isotype-matched control IgG staining. They were then incubated with gold-tagged goat anti-mouse IgG. A β 40 fibrils formed by the extension reaction of A β 40 seeds (10 μ g/ml) with seed-free A β 40 (50 μ M) as described above, were also subjected to immunoelectron microscopy of 4396C, isotype-matched control IgG, or 4G8 (Kim et al., 1988) staining. The first antibodies and control IgG were diluted at 1:100 using PBS containing 1% bovine serum albumin (PBS-BSA).

Quantitative binding assay. GM1-containing liposomes and GM1-lacking liposomes were mixed with soluble A β 40 at various concentrations (2.5–25 μ M) by vortexing for 60 sec, and the mixtures were ultracentrifuged at $540,000 \times g$ for 10 min to remove unbound A β 40. Then, 4396C and isotype-matched control IgG, at a concentration of 10 μ g/ml in PBS-BSA, were incubated with the liposomes at room temperature for 60 min. After incubation, the mixtures were ultracentrifuged at $540,000 \times g$ for 20 min, and the resultant pellets were washed with PBS-BSA to remove the unbound antibody. The levels of IgG bound to the liposomes were determined after the incubation of liposomes with peroxidase-conjugated goat anti-mouse IgG using cyanogen bromide Sepharose CL4B-bound 4396C as the standard.

Dot blot analysis. Liposomes carrying GA β were prepared by mixing GM1-containing liposomes with soluble A β (A β 40 and A β 42) on ice for 5 sec at a weight ratio of lipid/A β at 100:3. The liposomes, and A β and GM1, in amounts equal to those contained in blotted liposomes (300 and 600 ng of A β ; 2 and 4 μ g of GM1), were blotted. The blots were incubated with 4396C (1:1000), BAN052 (1:5000), HRP-conjugated cholera toxin subunit B (CTX) (1:20,000), or the isotype-matched control IgG (1:1000). The blots incubated with 4396C, BAN052, or control IgG were then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Invitrogen). The bound-enzyme activities were visualized with an ECL system (Amersham Biosciences, Buckinghamshire, UK).

Inhibition assay of A β aggregation. Soluble A β 40 (50 μ M) was incubated at 37°C with GM1-containing liposomes (GM1/A β molar ratio of 10:1) or preformed A β 40 seeds (10 μ g/ml), which were prepared as described above, and an antibody (4396C or 4G8) at various concentrations. A β 40 aggregation levels in the mixtures were determined by ThT assay.

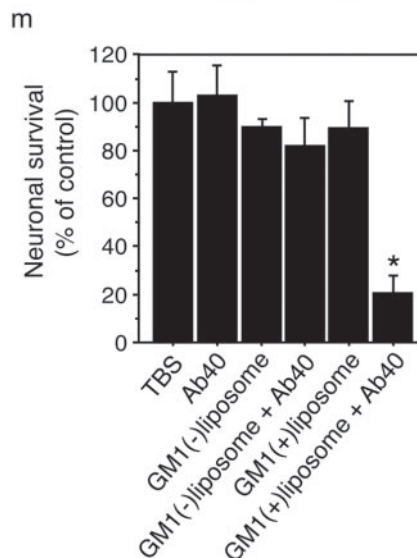
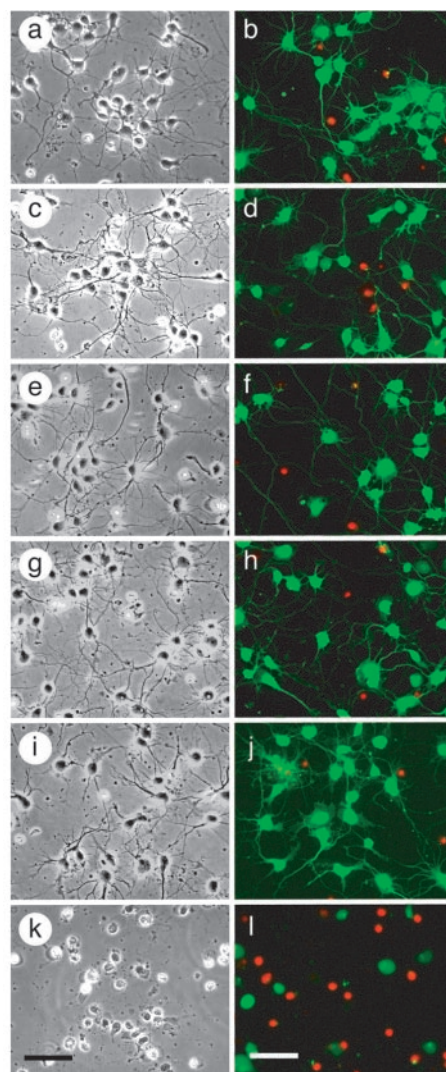


Figure 2. Viability of neurons treated with A β 40 incubated in the presence of GM1 ganglioside. Phase-contrast and calcein AM–ethidium homodimer-stained photographs of cultured neurons were taken after treatment with TBS (*a, b*), A β 40 (*c, d*), GM1-lacking liposomes (*e, f*), GM1-lacking liposomes plus A β 40 (*g, h*), GM1-containing liposomes (*i, j*), and GM1-containing liposomes plus A β 40 (*k, l*). *m*, Viable cells stained with calcein AM were counted. The data represent means \pm SE for triplicate samples. * $p < 0.003$ versus other treatments. Scale bars, 20 μ m.

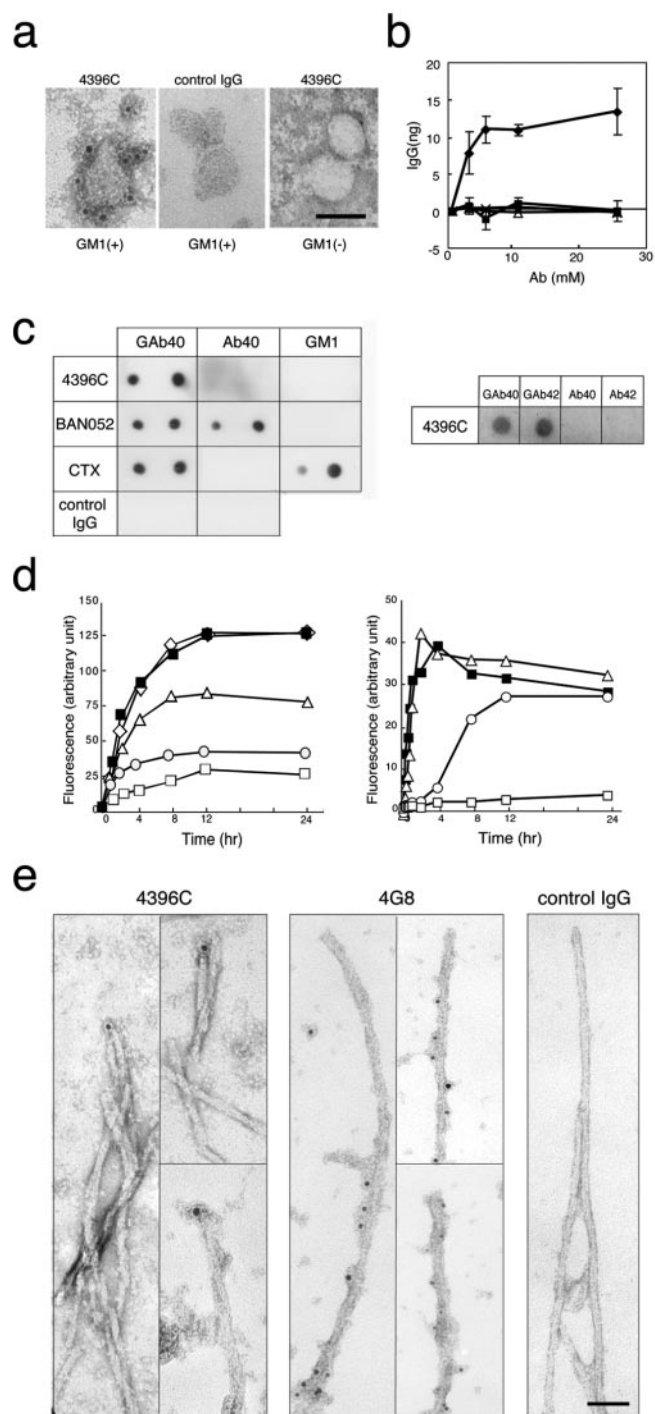


Figure 3. Characterization of the binding specificity of 4396C. *a*, Immunoelectron micrographs of liposomes. GM1-containing and GM1-lacking liposomes were subjected to immunoelectron microscopy of 4396C or isotype-matched control IgG staining after incubation with soluble A β 40. GM1(+), GM1-containing liposomes; GM1(-), GM1-lacking liposomes. Scale bar, 50 nm. *b*, Quantitative assay of binding of 4396C to liposomes. GM1-containing liposomes were incubated with 4396C (diamonds) or isotype-matched control IgG (filled squares) after their mixing with soluble A β 40 at indicated concentrations. GM1-lacking liposomes were also incubated with 4396C (triangles) or isotype-matched control IgG (\times symbols). Ab, Antibody. *c*, Dot blot analysis. Left, Liposomes carrying GAB (GA β 40), A β 40, and GM1 in amounts equal to those contained in blotted liposomes (300 and 600 ng of A β 40; 2 and 4 μ g of GM1) were blotted. The blots were incubated with 4396C, BAN052, HRP-conjugated CTX, or isotype-matched control IgG. Right, Liposomes carrying GAB (GA β 40 and GA β 42), prepared using A β 40 or A β 42, and A β 40 and A β 42 in amounts equal to those contained in GA β 40 and GA β 42 (600 ng of each peptide) were blotted. The blots were incubated with 4396C. *d*, Inhibition of amyloid fibril formation from soluble A β 40 by 4396C. Left, Soluble A β 40 was incubated

Immunohistochemistry. Sections of cerebral cortices of human brains, which were fixed in 4% formaldehyde or Kryofix (Merck, Darmstadt, Germany) and embedded in paraffin, were immunolabeled with 4396C (10 μ g/ml) or 4G8 (1 μ g/ml) after pretreatment with formic acid (99%) or SDS (4%). Sections of cerebral cortices of nonhuman primates at various ages (4, 5, 17, 19, 20, 30, and 36 years old) were fixed with 1% paraformaldehyde and subjected to immunohistochemistry with 4396C (10 μ g/ml). For double staining with 4396C and BAN052, we first labeled 4396C with FITC to distinguish its reaction from that of BAN052. The binding of BAN052 was detected using Alexa 568-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR). For double staining with 4396C and CTX, we used a xenon one-mouse IgG2a labeling kit (Molecular Probes) for the previous conjugation of 4396C with Alexa 568. For GM1 detection, we used FITC-conjugated cholera toxin subunit B (Sigma). Autofluorescence was blocked by pretreatment with Sudan Black B (Tokyo Kasei Kogyo Company, Tokyo, Japan).

Immunoprecipitation. Immunoprecipitation of GAB from nonhuman primate brains was performed as described previously (Yanagisawa and Ihara, 1998). Briefly, cerebral cortices of primates were Dounce homogenized with 9 vol of TBS, pH 7.6. Homogenates were subjected to sucrose density gradient fractionation to obtain the membrane fraction. The membrane fraction was dried and then directly labeled with 4396C (5 μ g/ml) after sonication on ice. After 1 hr incubation, the mixtures were centrifuged at 15,000 \times g for 10 min to remove the unbound antibody. The pellets were washed with TBS and solubilized in radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% deoxycholic acid, and 1% NP-40) for 5 min and then centrifuged at 15,000 \times g for 10 min. Supernatants were collected and diluted with TBS. Protein G Sepharose (PGS) (Amersham Biosciences, Piscataway, NJ), which had been precoated with goat anti-mouse IgG, was added to the supernatants. Finally, PGS pellets were thoroughly washed with TBS containing 0.05% Tween 20.

Results

Kinetics of A β fibrillogenesis in the presence of GM1 ganglioside

As reported previously (Naiki et al., 1998; Ding and Harper, 1999), the removal of preexisting seeds is critical in the kinetic study of A β fibril formation *in vitro*. The ThT fluorescence intensity of A β 42 solutions without removing undissolved peptide aggregates, which can act as preexisting seeds, started to increase as early as 1 hr of incubation at 50 μ M and 37°C (Fig. 1*a*). In contrast, the ThT fluorescence intensity of seed-free A β 42 solutions did not increase as long as 6 hr of incubation under the same conditions (Fig. 1*a*). The ThT fluorescence intensity of A β 40 solutions without removing undissolved peptide aggregates started to increase after 4 d of incubation at 50 μ M and 37°C (Fig. 1*a*); however, the seed-free A β 40 solutions incubated under the same conditions did not show any increase in the ThT fluorescence intensity at this point (Fig. 1*a*). Thus, to investigate the molecular process of GAB-initiated A β aggregation, we incubated the seed-free A β (A β 40 and A β 42) solutions at 50 μ M and 37°C under various conditions, as long as 48 and 6 hr for A β 40 and A β 42, respectively, in the following experiments.

We incubated A β 40 solutions in the presence or absence of GM1-containing liposomes. The ThT fluorescence intensity in-

with GM1-containing liposomes in the absence (filled squares) or presence of an antibody (4396C or 4G8). The molar ratios of 4396C to soluble A β 40 were 0.3:50 (triangles), 1.3:50 (circles), and 4:50 (open squares) and that of 4G8 to A β 40 was 4:50 (diamonds). Right, Soluble A β 40 was incubated with preformed A β 40 fibrils in the absence of an antibody (filled squares) or in the presence of 4396C. The molar ratios of 4396C to soluble A β 40 were 0.3:50 (triangles), 1.3:50 (circles), and 4:50 (open squares). *e*, Immunoelectron micrographs of preformed A β 40 fibrils. A β 40 fibrils were formed by the extension reaction of A β 40 seeds (10 μ g/ml) with seed-free A β 40 (50 μ M), as described in Materials and Methods, and subjected to immunoelectron microscopy of 4396C, 4G8, or isotype-matched control IgG staining. Scale bar, 50 nm.

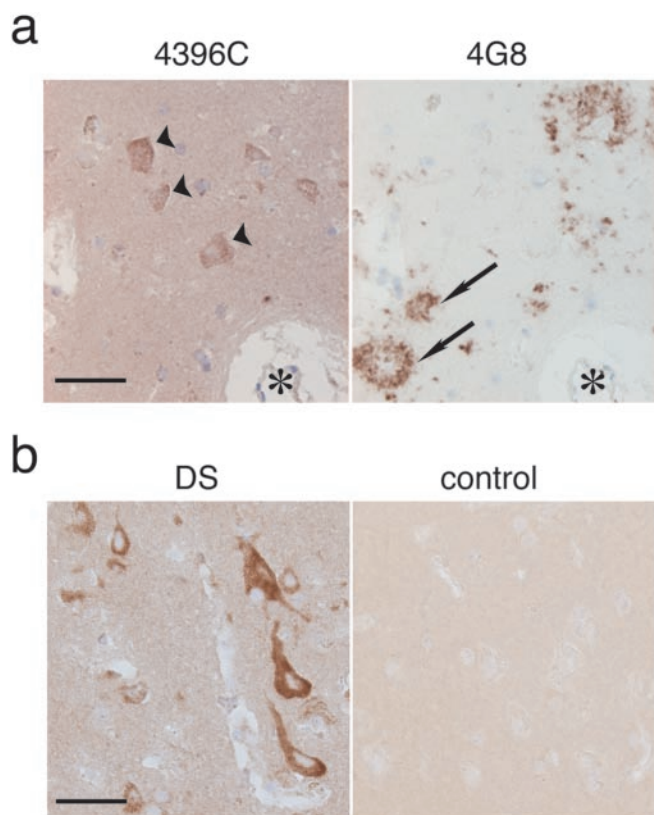


Figure 4. Immunohistochemistry of GAB in sections of human brains. *a*, Immunostaining of serial sections of the cerebral cortex of an AD brain fixed in Kryofix and pretreated with SDS. Neurons (arrowheads) were immunostained by 4396C but not by 4G8, whereas plaques (arrows) were immunostained by 4G8 but not by 4396C. The asterisks indicate the same blood vessel in the serial sections. Scale bar, 50 μm . *b*, Immunostaining by 4396C of sections of cerebral cortices of DS (left) and control (right) brains fixed in Kryofix and pretreated with SDS (DS, 47 years old; control, 65 years old). Scale bar, 50 μm .

Table 1. Neuronal immunoreactivity to 4396C in human cerebral cortices

	Score				
	0	1	2	3	4
Control (22)	4	5	12	1	0
AD (5)	0	2	2	1	0
DS (2)	0	0	0	0	2

Intensities of neuronal immunoreactivity to 4396C were semiquantitatively assessed in cerebral cortices obtained from nondemented individuals (Control), patients with AD, and those with DS. The numbers in parentheses indicate the number of cases. 0, Absent; 1, weak; 2, moderate; 3, intense; 4, most intense. Difference in the neuronal immunoreactivities between control and AD plus DS was significant ($p < 0.05$, Mantel-Haenszel χ^2 test; $p < 0.05$, Cochran-Armitage trend test).

creased without a lag phase after the addition of GM1-containing liposomes to the A β 40 solutions (Fig. 1*b*). In contrast, there was no increase in the ThT fluorescence intensity of the solutions containing A β 40 alone or A β 40 plus GM1-lacking liposomes during an incubation period of as long as 96 hr (Fig. 1*b*). By a semilogarithmic calculation, a perfect linear plot ($r = 0.997$) was obtained for the experiment using GM1-containing liposomes (Fig. 1*b*, inset). The fluorescence intensity of ThT also increased in seed-free A β 42 solution after the addition of GM1-containing liposomes (Fig. 1*b*). Under an electron microscope, typical amyloid fibrils were observed in the A β 40 solution after 24 hr incubation at 50 μM and 37°C in the presence of GM1-containing liposomes (Fig. 1*c*). These results suggest that A β binds to GM1, leading to the generation of GAB, and then induces A β fibrillogenesis in the manner of a first-order kinetic model (Naiki and

Nakakuki, 1996) by acting as a seed; that is, the extension of fibrils is likely to proceed via consecutive binding of soluble A β first onto GAB and then onto the ends of growing fibrils.

We then investigated whether the formation of A β oligomers is also accelerated in the presence of GM1 ganglioside. We performed SDS-PAGE of the A β 40 solutions incubated at 50 μM and 37°C for 24 hr. Notably, A β oligomers were detected by Western blotting of the SDS-PAGE in the incubation mixture with GM1-containing liposomes but was not detected in the incubation mixture containing A β 40 alone (Fig. 1*d*).

Neurotoxicity of A β incubated in the presence of GM1 ganglioside

We then investigated whether A β aggregates formed in the presence of GM1-containing liposomes are neurotoxic. We incubated A β 40 solutions in the presence or absence of GM1-containing liposomes for 24 hr and then applied them to a primary neuronal culture after dilution with N2 media. In this experiment conducted 48 hr after the commencement of the treatment, significant neuronal death was observed only in the culture treated with the A β 40 solution incubated in the presence of GM1-containing liposomes (Fig. 2).

Molecular characterization of GAB

To characterize GAB at the molecular level and to clarify the process of A β aggregation in the presence of GM1, we raised a monoclonal antibody against natural GAB purified from an AD brain. In an experiment using immunoelectron microscopy, the antibody (4396C), but not the isotype-matched control IgG, recognized artificially generated GAB on liposomes (Fig. 3*a*). The specificity of 4396C to GAB was confirmed by quantitative binding assay (Fig. 3*b*) and dot blot analysis (Fig. 3*c*, left). Notably, 4396C did not recognize the unbound forms of A β 40 and GM1, whereas BAN052, a monoclonal antibody specific to the N terminus of A β (Suzuki et al., 1994), recognized both GAB40 and A β (Fig. 3*c*, left). The 4396C antibody reacted with GM1-bound forms of two A β isoforms (A β 40 and A β 42) (Fig. 3*c*, right). In the inhibition assay of A β fibrillogenesis, the increase in the fluorescence intensity of ThT of the A β 40 plus GM1-containing liposomes was suppressed by 4396C in a dose-dependent manner (Fig. 3*d*, left). In contrast, the increase in the fluorescence intensity of ThT was not affected by 4G8, a monoclonal antibody specific to amino acid residues 17–24 of A β (Kim et al., 1988) (Fig. 3*d*, left). We then examined the possibility that A β fibrillogenesis proceeds with consecutive conformational alteration of A β at the ends of growing fibrils; that is, GAB and A β at the ends of growing fibrils share a specific conformation that is required for A β fibrillogenesis in the manner of a first-order kinetic model. We incubated A β 40 solutions with 4396C in the presence of preformed A β 40 seeds instead of GM1-containing liposomes. Notably, 4396C inhibited the increase in the fluorescence intensity of ThT under this condition in a dose-dependent manner (Fig. 3*d*, right). To further examine this possibility, we performed immunoelectron microscopy using preformed A β 40 fibrils. In this experiment, 4396C bound only to the ends but not to the lateral sides of the fibrils, whereas 4G8 bound only to the lateral sides (Fig. 3*e*).

GAB generation in the brain

Having established the specificity of 4396C, we then aimed to detect GAB in the brain by immunohistochemistry. We first performed routine immunohistochemistry: that is, fixation in formaldehyde and enhancement of immunoreactivity by formic acid

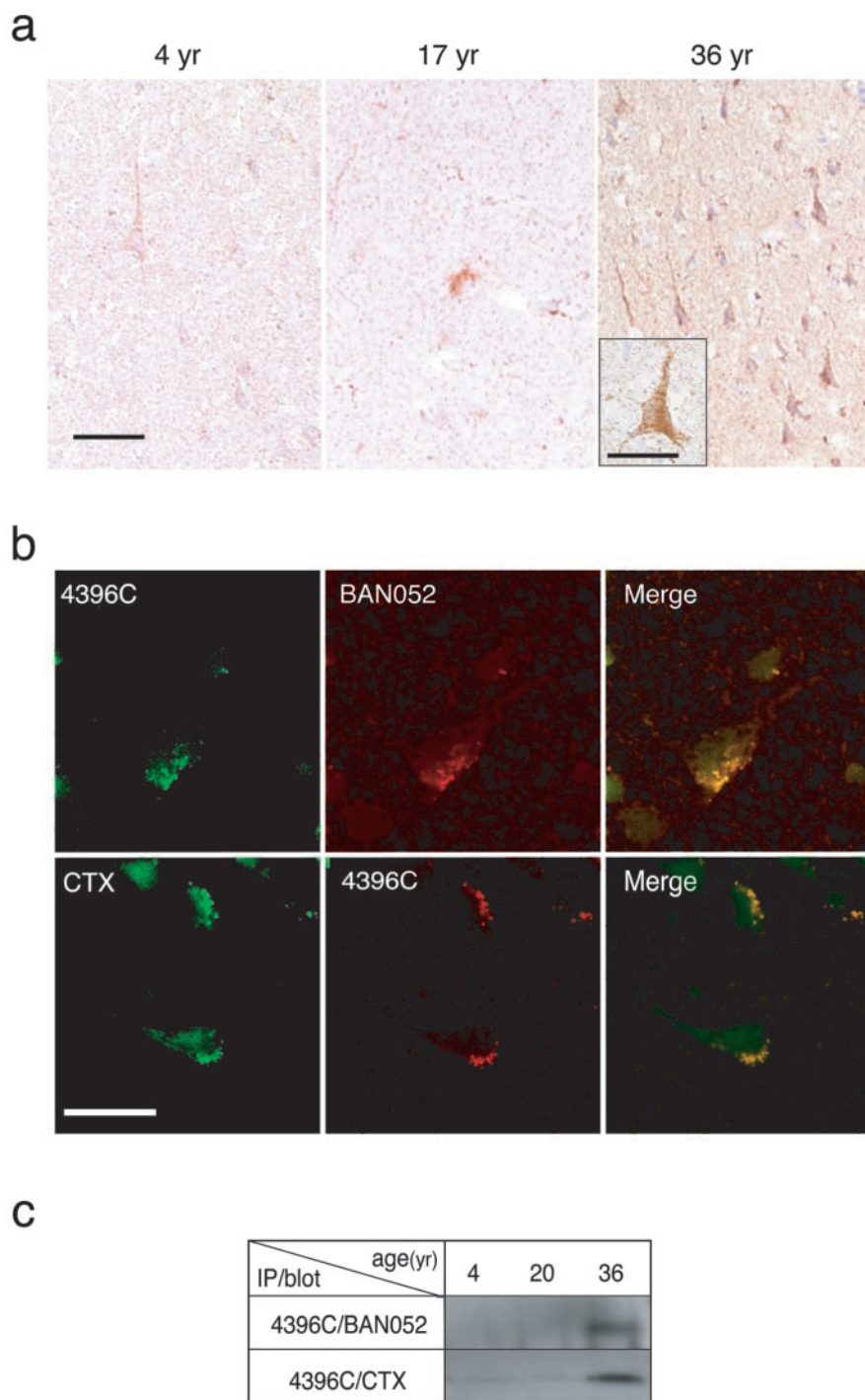


Figure 5. Immunohistochemistry and immunoprecipitation of GAβ in sections of nonhuman primate brains. *a*, Immunostaining by 4396C of sections of the cerebral cortices of primate brains, which were fixed in paraformaldehyde, from animals of different ages. Scale bar, 50 μm. Inset, Higher magnification. Scale bar, 20 μm. *b*, Double immunostaining of sections of the cerebral cortex of a 36-year-old primate brain, which was fixed in paraformaldehyde, after the blocking of autofluorescence by pretreatment with Sudan Black B. Colocalization of immunostaining by 4396C and that by BAN052 or CTX is shown in the merged image. Scale bar, 25 μm. *c*, Immunoprecipitation of GAβ by 4396C from cerebral cortices of primates at different ages. Immunoprecipitates were blotted and reacted with BAN052 or HRP-conjugated CTX.

or microwave treatment of brain sections. Under these conditions, no immunostaining by 4396C was observed in AD brains (data not shown), suggesting that the conformation of GAβ is sensitive to the procedures of conventional immunohistochemistry. Thus, we used an alternative fixation procedure using Kryofix, because it eliminates the possibility of obtaining false-

negative results that usually occur when using formaldehyde-fixed sections (Boon and Kok, 1991), and we also pretreated sections with SDS, which is known to improve immunostaining (Barrett et al., 1999). With these procedures, neurons in the cerebral cortices of AD brains were immunostained by 4396C (Fig. 4*a*). Although neuropil staining was rather strong, plaques were not recognized by 4396C (Fig. 4*a*). In contrast to staining by 4396C, 4G8 immunostained plaques but did not react with neurons (Fig. 4*a*). We performed immunohistochemistry of cerebral cortices of 22 nondemented control individuals (36–91 years old), which included one amyotrophic lateral sclerosis patient (44 years old) and two Down’s syndrome (DS) patients (47 and 52 years old), in addition to five AD patients (65–96 years old). The most intense neuronal staining by 4396C was observed in the brains of both DS patients (Fig. 4*b*). In the sections of control brains, neuronal staining was absent (Fig. 4*b*) or at comparable levels with those of AD brains (Table 1). These results suggest that GAβ can be detected by immunohistochemistry, but neuronal staining by 4396C under these conditions can also be nonspecifically induced, probably because of changes that occur during the agonal and/or postmortem state. Thus, to confirm the immunohistochemical detection of GAβ, we then examined fresh nonhuman primate (*Macaca fascicularis*) brains, which naturally and consistently develop Aβ deposition at ages >25 years (Nakamura et al., 1998). Cerebral cortices of seven animals at different ages (4, 4, 5, 17, 19, 30, and 36 years old) were fixed with paraformaldehyde, because it also preserves tissue and cell surface antigens (Smit et al., 1974), and were subjected to 4396C immunostaining without pretreatment with SDS. In the sections obtained from the two older animals, that is, 30 years old (data not shown) and 36 years old (Fig. 5*a*), a number of neurons were strongly immunostained by 4396C with a granular pattern (Fig. 5*a*, inset). In these sections, plaques were immunostained by 4G8 but not by 4396C (data not shown). In the sections of cerebral cortices of the five animals at ages <20 years old, which showed no plaques, the neuronal staining by 4396C was generally at negligible levels, and the strong staining was only occasionally observed in the sections from the 17-year-old (Fig. 5*a*) and 19-year-old (data not shown) animals. In double immunostaining of the sections obtained from the 36-year-old animal, intraneuronal staining by 4396C was distinctly colocalized with that by BAN052 or cholera toxin, a natural ligand specific to GM1 (Fig. 5*b*). To verify GAβ generation in the brain, we performed an

immunoprecipitation study using 4396C. GA β was immunoprecipitated by 4396C only from the cerebral cortex of the older animal (Fig. 5c). These results indicate that GA β is generated in the brain.

Discussion

In regard to the formation of pathogenic aggregates of constituent proteins, including A β and prion proteins, the seeded polymerization theory was proposed previously (Harper and Lansbury, 1997). For the transition of the nontoxic monomeric form of A β to its toxic aggregated form, the template-dependent dock-lock mechanism was reported previously (Esler et al., 2000). The present study supports these possibilities and also indicates that a seed can be endogenously generated by the binding of an aggregating protein to another molecule as was suggested in the mechanism underlying the aggregation of prion proteins (Telling et al., 1995; Deleault et al., 2003).

To understand the early events in the development of AD and also to develop therapeutic strategies, clarification of the time course of A β fibrillogenesis is fundamentally important. Previously, Harper et al. analyzed the process of *in vitro* A β assembly using an atomic force microscope at a fine resolution. They reported that protofibrils, transient species of A β assembly, are formed during the first week of incubation of A β 40 before mature fibrils are generated (Harper et al., 1997a,b, 1999; Ding and Harper, 1999). This model of A β assembly was supported by a recent study of Nichols et al. (2002). In the present study, we examined the acceleration of A β aggregation in the presence of GM1 ganglioside. In the EM examination of the present study, we observed mature fibrils in A β 40 solutions incubated at 50 μ M and 37°C for 24 hr, but protofibrils were hardly recognized (Fig. 1c). This discrepancy is likely to mainly stem from the presence or absence of GM1-containing liposomes and the differences in incubation period and peptide concentrations. Additional careful examination at a much greater resolution is required; however, the molecular process of A β fibrillogenesis in the presence of GM1 ganglioside may be different from that initiated by spontaneous nucleation from seed-free A β solution.

From the results of molecular characterization of GA β in the present study, it seems likely that A β adopts an altered conformation at its midportion through binding to GM1 because GA β was readily recognized by BAN052, an antibody specific to the N terminus of A β , and 4396C comparably recognized two A β isoforms with different lengths at their C termini. This possibility is supported by the following: first, 4G8, an antibody specific to the midportion of A β , failed to react with GA β in the inhibition assay of A β aggregation; and second, BAN052, but not 4G8, immunoprecipitated GA β from the cerebral cortex of an AD brain in our previous study (Yanagisawa and Ihara, 1998). The conformational epitope for 4396C on the A β molecule remains to be determined; however, the results of the present study suggest that the 4396C-reactive conformation, which is shared by GA β and A β at the ends of fibrils, is necessary for A β fibrillogenesis. Because amyloid fibrils composed of various proteins share a common structure that is readily recognized by Congo red, it may be interesting to study in the future whether the 4396C-reactive conformation is shared by seed or oligomer of other amyloidogenic proteins. Regarding this, we must draw attention to a recent report by Glabe and his colleagues (Kayed et al., 2003): they have successfully generated an antibody that potentially recognizes the common structure of soluble amyloid oligomers. Their results suggest that the oligomers have a conformation that is distinct from those of soluble monomers and amyloid fibrils.

In this study, only careful immunohistochemistry allowed us to visualize seed molecules, suggesting that we may likely fail to detect some population of seed molecules unless their conformations are preserved during immunohistochemistry. This may also be the case with other neurodegenerative diseases in which seed molecules are likely to play a critical role in the initiation of aggregation of soluble proteins. In this study, it remains to be clarified why 4396C did not immunostain plaques in which the ends of amyloid fibrils were supposed to exist. Possible explanations for this failure are as follows; first, the number of epitopes that can be recognized by the antibody may be limited, as was clearly indicated by immunoelectron microscopy using fibrils; second, we may have lost their immunoreactivities because of their higher susceptibility to treatment in immunohistochemistry than GA β ; and third, the ends of amyloid fibrils may have been masked or modified in the brain (Shapira et al., 1988; Roher et al., 1993).

A challenging subject of studies in the future is determining how and where GA β is generated in the brain. Regarding this issue, we favor the possibility that GA β is generated in GM1- and cholesterol-rich microdomains such as lipid rafts (Parton, 1994; Simons and Ikonen, 1997), because of the following: (1) lipid rafts contain soluble and insoluble A β s under physiological (Lee et al., 1998; Morishima-Kawashima and Ihara, 1998) and pathological (Sawamura et al., 2000) conditions, respectively; (2) amyloidogenic processing of the amyloid precursor protein is associated with lipid rafts (Ehehalt et al., 2003); and (3) the aggregation of soluble A β is readily induced by its interaction with lipid raft-like model membranes (Kakio et al., 2003). We found recently that A β binding to GM1 is markedly accelerated in a cholesterol-rich environment and that, in such an environment, GM1 forms a cluster that can be recognized by soluble A β as a receptor (Kakio et al., 2001). The alteration of cholesterol content in the AD brain is a controversial issue; however, it is noteworthy that cholesterol content in the outer leaflet of the synaptic plasma membrane can be increased in association with risk factors for the development of AD, including aging (Igbavboa et al., 1996) and the expression of apolipoprotein E allele ϵ 4 (Hayashi et al., 2002). Thus, altogether, one possible scenario may be as follows: GA β is generated in the lipid rafts or lipid raft-like microdomains in the neuron because of an increase in the local concentration of cholesterol, and then, GA β initiates its seed activity to accelerate A β aggregation after its transport to the neuronal surface and/or shedding into the extracellular space. Alternatively, GA β itself can be noxious per se because it has been reported previously that the disruption of membranes (McLaurin and Chakrabarty, 1996) and alteration of bilayer organization (Matsuzaki and Horikiri, 1999) can be induced by the generation of GA β on the membranes. Thus, it may also be worthy to examine in future studies whether GA β causes impairment of neuronal, particularly lipid raft-related, functions before extraneuronal A β deposition.

Several studies have suggested that therapeutically useful antibodies can be generated (Solomon et al., 1997; Bard et al., 2000; Hock et al., 2002; McLaurin et al., 2002; Lombardo et al., 2003). Together with the finding that GA β has a conformation distinct from that of soluble A β , it may be possible to develop a novel therapeutic strategy to specifically inhibit the initiation of oligomerization–polymerization of A β in the brain.

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