これらは細胞質に分布する CLPPに取り込まれることがわかった．今回の研究ではapoA-Iによるアストロサイトの HDL 新生機構と細胞内コレステロール輸送機構をより理解するために、CLPP と微小管との相互作用における protein kinase C 依存性のリン酸化反応の役割を検討した．

B. 研究方法

(1)ラットおよびマウスアストロサイトの養培

胎生期 17 日目のラットあるいはマウス胎児脳より大脳を摘出し、血管、髄膜除去、脳細片後、1% trypsin 溶液で処理して、10%FCS 含有 F-10 培地で 1 週間培養し、primary culture とした。この細胞を再度 1% trypsin 溶液で処理し、ピペッティング後 6-well multitray あるいは petri dish (直径 10 cm)にはん種し、1 週間 secondary culture し、実験に供した。

(2)細胞質の調製

培養アストロサイトを 0.02M Tris-HCl buffer, pH 7.5, containing protease inhibitor (0.02M Tris) により回収し、5 分間毎に 20 回強く撹拌し、これを 3 回くり返した．90,000 rpm で 30 分間遠心し、得られた上清を細胞質画分とした．

(3) 細胞内リポタンパクの分析

ラットアストロサイトに 40 uCi/ml の 3H-acetate を２時間取り込ませてコレステロールを代謝的に3H 標識した．細胞を洗浄して、5 ug/ml のapoAI を90分作用させ、低張液で細胞を処理し、細胞質を得た．細胞質を1.175 g/ml の sucrose 溶液に重層して、49,000 rpm , 48 h 遠心した．これを12画分に分け、それぞれの画分より脂質を抽出し、TLC により分析した．

(3)微小管様フィラメントの再構成

細胞質画分に 100 uM GTP と 2 mM MgCl2 を加えて、室温で20分間インクベートした．これを、15,000 rpm, 30分間遠心沈澱を larger reconstituted microtubule-like filament とし、遠心上清をさらに 80,000 rpm 30分間の遠心し、その沈澱画分をshorter reconstituted filament とした．

（倫理面への配慮）

アストロサイトの培養に当たって、妊娠17日目のラットおよびマウスをドライアイスにより CO2 窒息させ、呼吸停止を確認した後、開腹し胎児を得た．

C. 研究結果

Protein kinase C の阻害剤である bisindolylmaleimide I (BIM) は apoA-I で誘導されるコレステロールの合成、細胞質へのコレステロール輸送および細胞外への搬出のすべての反応を抑制した．ApoA-I 誘導性コレステロール搬出に関わる細胞内コレステロール輸送反応が protein kinase C の機能に依存したものであることが示唆された．ApoA-I で5分間刺激されたラットアストロサイトの細胞質タンパク質をanti-caveolin-1 antibody-bound Protein G-Sepharose で免疫沈降すると、caveolin-1 以外に protein kinase Cα, α-tubulin, β–actin などのタンパク質も共沈し、これを ^{32}P-ATP でリン酸化するとapoA-I 刺激されたアストロサイトの細胞質では、52 kDa タンパク質のリン酸化が顕著に上昇した．このリン酸化反応は BIM によって強く抑制された．また、anti-α-tubulin antibody を用いた western blotting により52 kDa タンパク質がa-tubulin と同移動度を示した．一方、予めリン酸化された細胞質タンパク質は、もはやanti-caveolin-1 antibody-bound Protein G-Sepharose には結合しなかった．

ApoA-I で5分間刺激されたラットアストロサイトから細胞質を調製し、^{32}P-ATP でリン酸化反応した後に再構成されたreconstituted microtubule-like filaments (rMT) にはリン酸化

52 kDa タンパク質が認められた．さらに human tubulin-immobilized BioGel-10 を作成し、これにrMT を再構成した．この rMT-bound Sepharose は protein kinase Cα を結合しており、外来性 human tubulin もリン酸化した．このことから52 kDa タンパク質が tubulin であることがわかった．CLPP-related lipid (cholesterol, phosphatidylcholine, sphingomyelin) と rMT との結合はアストロサイトが apoA-I 刺激されると促進され、また BIM 処理により抑制された．

D. 考察

我々はこれまでの研究で、cytosolic lipid-protein particle (CLPP) が apoA-I 作用後のアストロサイトにおいて、コレステロール細胞内輸送に大きく関わることを示した．今回の研究では、CLPP と微小管細胞骨格と相互作用に対する protein kinase C の役割を検討した．得られた結果は以下の通りである．1）protein kinase C 阻害剤である bisindolylmaleimide I (BIM) が apoA-I 刺激後の細胞内コレステロール輸送反応を阻害した．2）caveolin-1 結合 52 kDa タンパク質のリン酸化が apoA-I により促進され、BIM 処理により阻害された．3）アストロサイト細胞質由来の reconstituted microtubule-like filaments (rMT) は内在性および外来性 52 kDa tubulin をリン酸化し、apoA-I 刺激により促進された．

これまでの研究で、アストロサイトのapoA-I 5分間刺激で、protein kinase Cα が CLPP 画分へ移行し、さらにセリンリン酸化され、活性化されることをすでに示している．今回の実験により apoA-I で刺激されたアストロサイト細胞質より再構成された rMTは、52 kDa tubulin のリン酸化が亢進され、BIM により抑制された．このことから、apoA-I 刺激により CLPP 上での caveolin-1 と protein kinase Cα との結合が促進され、活性化された protein kinase C が微小管の主要構成タンパク質である tubulin を特異的にリン酸化することが示唆された．今回の実験結果はtubulin リン酸化により微小管の脱重合を誘導しないことを示した．protein kinase C による微小管 tubulin リン酸化の意義は今のところ不明であるが、CLPP と微小管との解離会合に寄与することが考えられる．

E. 結論

apoAI は細胞骨格とcaveolin-1 、protein kinase C およびCLPPの結合を高め、微小管tubulinのリン酸化を特異的に促進した．

F. 健康危険情報

特になし．

G. 研究発表

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H. 知的財産権の出願・登録状況

1．特許取得

なし.

2. 実用新案登録

なし.

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分担研究報告書

アルツハイマー病発症の分子機構におけるコレステロールの役割の検討

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研究要旨

TKOマウスではDKOマウスに比べ、ほぼ生後4週間以内に死亡する。マウス直腸温の解析から、TKOマウスに見られる低生存率は著しい体温の低下によるものであることが明らかとなった。海馬ニューロンの初代培養系を用い、リポタンパク受容体を欠損したニューロンにおける血清_-VLDLの取り込みを解析した。VLDLR欠損ニューロンにおける β-VLDLの取り込みは、コントロールと比較して変化はなく、むしろLDLR欠損ニューロンで著しく減弱していた。ヒト・ApoE2、E3又はE4のcDNAを組み込んだアデノウイルスを作製し、アストロサイト初代培養系及びグリオーマ細胞系においてApoEの大量発現系を確立した。本培養上清中のApoEは脳内のものと同様に一部がシアル酸による修飾を受けていた。また、肝臓で合成されるApoEとは異なり２つの分子種として分泌されることが明らかとなった。この培養上清から各ApoEをヘパリンイオン、イオン交換カラム、ゲル濾過カラムにより精製した。

A. 研究目的

リポタンパク受容体ノックアウトマウスを用い、脳神経系のコレステロール代謝におけるリポタンパク受容体の機能解析を通して、ApoEの脳神経系における役割やアルツハイマー病との関連を明らかにする。

B. 研究方法

マウスApoE受容体2（ApoER2）遺伝子をジーンターゲッティング法により破壊し、ApoER2欠損マウスを作製した。また、VLDLR/ApoER2遺伝子を欠損したダブルノックアウト（DKO）マウス、更にApoE/VLDLR/ApoER2遺伝子を欠損するトリプルノックアウト（TKO）マウスを作製した。これらのマウスを用いて、表現型の解析を行った。また、各受容体ノックアウトマウスより初代培養・海馬ニューロンを調製し、ウサギ抹消血より調製したβ -VLDLの取り込みを蛍光法により観察した。

ヒトApoE2、E3、E4 cDNAをアデノウィルスベクターに組み込み、マウス・初代培養アストロサイト及びヒト・グリオーマ細胞に感染させる事により、ヒトApoEの大量発現系を確立した。この培養上清から一連のクロマトグラフィー操作により、各ApoEを均--にまで精製した。これら精製標品を用い、初代培養・海馬ニューロンに対する毒性及び取り込みなどを解析した。

（倫理面への配慮）

実験動物を用いる実験は、愛媛大学医学部動物実験倫理委員会の承認を受けて行われた。

C. 研究結果

TKO及びTKO2マウスではDKOマウス（VLDLR/ApoER2）に比べ、明らかに海馬の層構造形成異常が更に亢進しており、ほぼ生後4週間以内に死亡する。一方、DKOマウスでは約20%が3ヶ月以上生存することを見いだした。これらマウスの直腸温の解析から、TKO及びTKO2マウスに見られる低生存率は著しい体温の低下によるものであることが明らかとなった。TKOやTKO2マウスでは他の遺伝型を持つマウスと比較して著しく高い血中コレステロール値を示したが、血中リポタンパクの解析から、これらのマウスに見られる高コレステロール血漿は脳形成の異常によるものではないことが示された。一方、DKOマウスでは血中コレステロール値に異常は認められないことから、これらマウスに見られる体温の低下は脂質代謝異常によるものではないことが示唆された。

VLDLR(+/-)/ApoER2(-/-)では海馬CA1領域及びCA3領域のリボン部分の錐体細胞層が２層に分離しているが、ApoEがさらに欠損することで錐体細胞層が1層に回復することから、ApoEがCA1領域及びCA3領域のVLDLRに特異的に結合していることが示された。また、LDLR/ApoER2マウスの解析から、LDLRはVLDLRやApoER2に比べ弱いながらも大脳皮質と海馬に於いてreelin受容体としても機能しているが、ApoEに対する受容体としての機能は見いだせなかった。そこで、海馬ニューロンの初代培養系を用い、リポタンパク受容体を欠損したニューロンにおける血清_-VLDLの取り込みを解析した。VLDLR欠損ニューロンにおける_-VLDLの取り込みは、コントロールと比較して変化はなく、むしろLDLR欠損ニューロンで著しく減弱していた。

ヒト・ApoE2、E3又はE4のcDNAを組み込んだアデノウイルスを作製し、アストロサイト初代培養系及びグリオーマ細胞系においてApoEの大量発現系を確立した。本培養上清中のApoEは脳内のものと同様に一部がシアル酸による修飾を受けていた。また、興味深いことに、肝臓で合成されるApoEとは異なり２つの分子種として分泌されることが明らかとなった。しかし、培養上清中のApoEは非常にLipid-poorなため、ApoE-HDLとしては調製することは出来なかった。そこで、この培養上清から各ApoEをヘパリン、イオン交換カラム、ゲル濾過カラムにより精製した。これをリポソームに組み込んで調製したApoE-HDLを用いて神経細胞への取り込みや神経細胞に対する毒性を検討したが、ApoE単独の添加では神経細胞にほとんど毒性を示さなかった。

D. 考察

脳内におけるコレステロールはアストロサイトからApoE・コレステロールとして分泌され、神経細胞に発現するリポタンパク受容体によって取り込まれると考えられている。一方、VLDLR及びApoER2は神経細胞の移動及び配置決定を制御する分子、リーリンの受容体としても機能している。TKO及びTKO2マウスを用いた解析では、ApoEがCA1領域及びCA3領域の錐体細胞に発現するVLDLRに特異的に結合することを示した。海馬ニューロンを用いた血清β-VLDLの取り込み実験では、VLDLR欠損ニューロンではコントロールと比較して変化はなく、むしろLDLR欠損ニューロンで著しく減弱していた。すなわち、ニューロンにおけるVLDLR及びApoER2はApoE受容体としてほとんど機能していない事が示唆されてきた。一方、LDLRを欠損したニューロンではApoEを含むリポタンパク質の取り込み活性がほとんど見られなくなる事から、本受容体がニューロンに於ける主要なApoE受容体であると考えられた。

ApoEは様々な臓器・細胞で合成されるが、肝臓から分泌されるApoEは体循環のリポ蛋白質代謝に主要な役割を果たしている。近年、マクロファージで合成・分泌されたApoEを含むリポ蛋白質は肝臓には取り込まれないことが示され、マクロファージの産生するApoEが肝臓のリポタンパク受容体に結合できない事が示された。すなわち、肝臓以外の細胞から産生されるApoEは異なった修飾を受けている可能性が考えられる。事実、アデノウイルスを用いたアストロサイト初代培養系によるApoE発現系では、産生されたApoEは脳内のものと同様に一部がシアル酸による修飾を受けていると共に、肝臓で合成されるApoEとは異なり２つの分子種として分泌されることが明らかとなった。これらの結果は、アストロサイトで産生されたApoE・コレステロール複合体のリポタンパク受容体に対する結合特異性が血清リポ蛋白のものとは異なる可能性を示唆した。

E. 結論

海馬ニューロンを用いた血清_-VLDLの取り込み実験から、VLDLR欠損ニューロンではコントロールと比較して変化はなく、むしろLDLR欠損ニューロンで著しく減弱していた。すなわち、LDL受容体がニューロンに於ける主要なApoE受容体であると考えられた。一方、アストロサイト初代培養系によるApoE発現では、産生されたApoEはシアル酸による修飾を受けていると共に、2つの分子種として分泌されることが明らかとなった。これらの結果は、アストロサイトから分泌されるApoE・コレステロール複合体は血清リポ蛋白のものとはリポタンパク受容体に対する結合特異性が異なる可能性を示唆した。

F. 健康危険情報

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得
なし

2. 実用新案登録
なし

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Neurobiology of Disease

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 (Aβ) are formed from its nontoxic soluble form. We hypothesized previously that GM1 ganglioside-bound Aβ (GAβ) is involved in the process. We now examined this possibility using a novel monoclonal antibody raised against GAβ purified from an AD brain. Here, we report that GAβ has a conformation distinct from that of soluble Aβ and initiates Aβ aggregation by acting as a seed. Furthermore, GAβ 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 (Aβ) by accelerating cellular Aβ generation (Selkoe, 1997). However, there is currently no evidence that Aβ generation is enhanced in sporadic, late-onset AD, the principal form of the disease. Thus, it is reasonable to assume that Aβ aggregation in conventional AD may be induced by unknown posttranslational modification(s) of Aβ and/or by altered clearance mechanisms.

We previously identified a novel Aβ 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 Aβ (GAβ), including its altered immunoreactivity and a strong tendency to form aggregates of Aβ, we hypothesized that Aβ adopts an altered conformation by binding to GM1 and initiates the aggregation of soluble Aβ 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 Chakrabartty, 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 GAβ at the molecular level using a novel monoclonal antibody raised against GAβ purified from an AD brain with the aim of validating our hypothesis. Here we show that GAβ has a conformation distinct from that of soluble Aβ and initiates Aβ aggregation by acting as a seed. Importantly, we successfully verified GAβ generation in the brain by both immunohistochemical and immunoprecipitation methods.

Materials and Methods

Preparation of seed-free Aβ solutions. Aβ solutions were prepared essentially according to a previous report (Naiki and Gejyo, 1999). Briefly, synthetic Aβ (Aβ40 and Aβ42) (Peptide Institute, Osaka, Japan) was dissolved in 0.02% ammonia solution at 500 μM for Aβ40. Aβ42 was dissolved at 250 μM because it has a higher potential to form aggregates rapidly than Aβ40. To obtain seed-free Aβ solutions, the prepared solutions were centrifuged at 540,000 × *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 Aβ solutions, except in the preparation of Aβ fibrils used as the seeds (see below).

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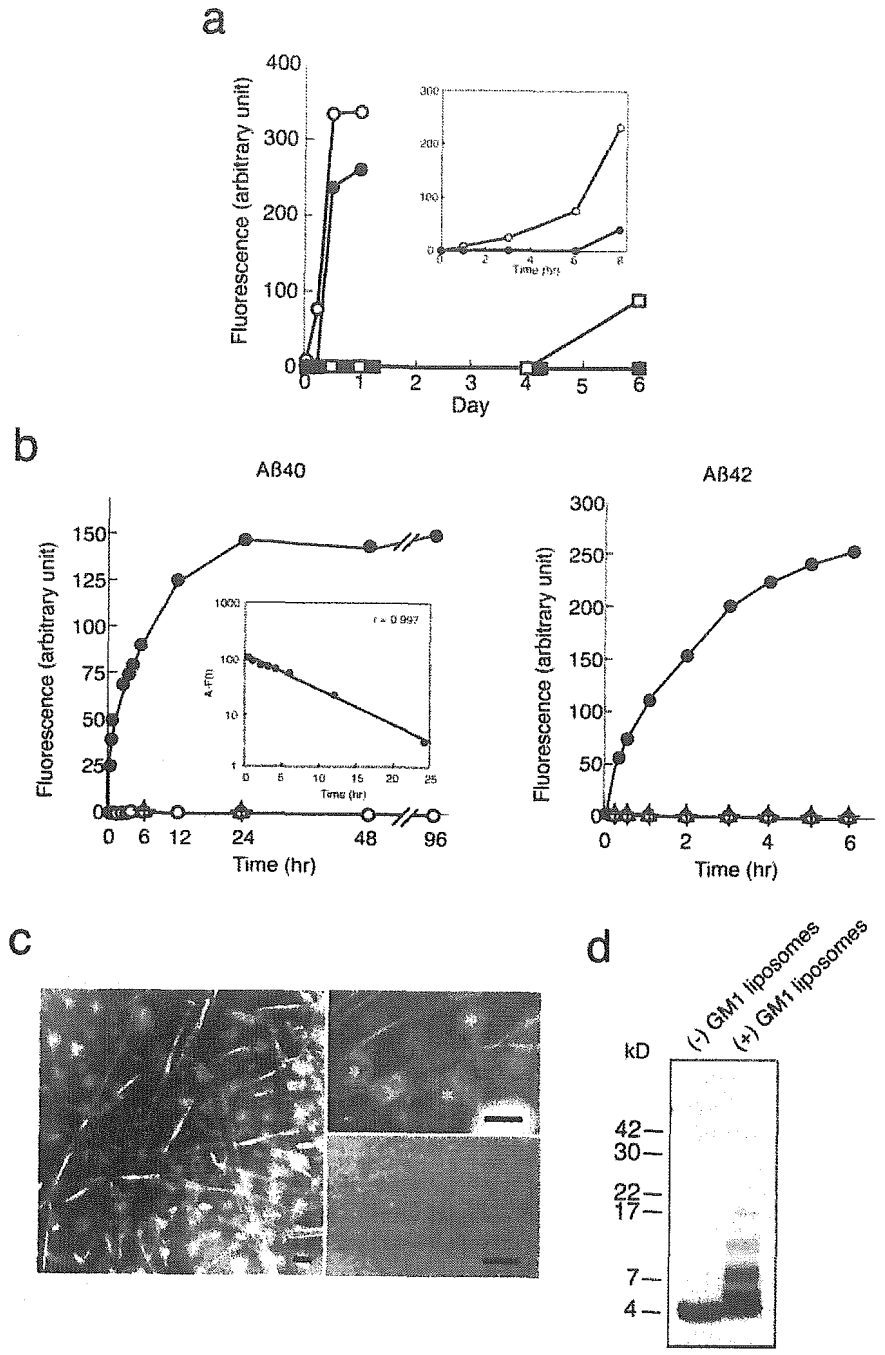

Figure 1. Amyloid fibril formation from soluble Aβ in the absence or presence of GM1-containing liposomes. *a*, Kinetics of Aβ fibrillogenesis using Aβ (Aβ40 and Aβ42) solutions, with or without removing undissolved peptide aggregates, which can act as preexisting seeds. Aβ solutions were incubated at 50 μM and 37°C. Open and filled circles indicate ThT fluorescence intensities of Aβ42 solutions without and with removing undissolved peptide aggregates, respectively. Open and filled squares indicate ThT fluorescence intensities of Aβ40 solutions without and with removing undissolved peptide aggregates, respectively. *b*, Kinetics of Aβ fibrillogenesis. Aβ (Aβ40 and Aβ42) solutions, after removal of undissolved peptide aggregates, were incubated at 50 μM 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 Aβ (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 Aβ incubated with GM1-containing liposomes, and A is tentatively determined as F (infinity). 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 Aβ40 solutions incubated at 50 μM 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 Aβ40 solutions incubated at 50 μM and 37°C for 24 hr in the presence or absence of GM1-containing liposomes. The incubated Aβ solutions were centrifuged at 100,000 × *g* for 15 min. Ten nanograms of Aβ in the supernatant were subjected to SDS-PAGE (4–20%) after glutaraldehyde treatment. The Aβ in the gel was detected by Western blotting using BAN052. Aβ oligomers were detected in the Aβ solution incubated in the presence of GM1-containing liposomes but not detected in the incubation mixture containing Aβ alone.

Preparation of Aβ40 seeds. Aβ40 fibrils used as exogenous seeds were prepared essentially according to the method reported previously (Naiki and Nakakuki, 1996). Briefly, Aβ40 solution was incubated at 500 μM and 37°C for 72 hr without previous removal of the preexisting seeds. After incubation, newly formed Aβ40 fibrils were precipitated by ultracentrifugation. The resultant pellets were subjected to sonication. Protein concentrations of the solutions containing Aβ40 fibrils were determined using a protein assay kit (Bio-Rad, Hercules, CA) as described previously (Bradford, 1976). The Aβ40 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 × *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 μM thioflavin T (ThT) (Sigma) and 50 mM glycine-NaOH buffer, pH 8.5. Fluorescence was measured immediately after making the mixture. The Aβ (Aβ40 and Aβ42) solution described above was incubated at 37°C with liposomes at an Aβ concentration of 50 μM at a GM1/Aβ molar ratio of 10:1.

Detection of Aβ oligomers by SDS-PAGE. The Aβ40 solution described above was incubated with liposomes at an Aβ concentration of 50 μM at a GM1/Aβ molar ratio of 10:1 for 24 hr at 37°C and then centrifuged at 100,000 × *g* for 15 min. The supernatant was subjected to SDS-PAGE after glutaraldehyde treatment (LeVine, 1995) to stabilize oligomeric Aβ during SDS-PAGE. The Aβ in the gel was detected by Western blotting using BAN052, a monoclonal antibody specific to the N terminus of Aβ (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, Aβ40, GM1-lacking liposome, and GM1-containing liposome in the absence or presence of Aβ40, 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× 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 GAβ 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 Aβ40 on ice for 5 sec at a weight ratio of lipid/Aβ40 at 100:3. The mixtures were immediately ultracentrifuged to remove unbound Aβ40, 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. Aβ40 fibrils formed by the extension reaction of Aβ40 seeds (10 μg/ml) with seed-free Aβ40 (50 μM) 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 Aβ40 at various concentrations (2.5–25 μM) by vortexing for 60 sec, and the mixtures were ultracentrifuged at 540,000 × *g* for 10 min to remove unbound Aβ40. Then, 4396C and isotype-matched control IgG, at a concentration of 10 μg/ml in PBS-BSA, were incubated with the liposomes at room temperature for 60 min. After incubation, the mixtures were ultracentrifuged at 540,000 × *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 GAβ were prepared by mixing GM1-containing liposomes with soluble Aβ (Aβ40 and Aβ42) on ice for 5 sec at a weight ratio of lipid/Aβ at 100:3. The liposomes, and Aβ and GM1, in amounts equal to those contained in blotted liposomes (300 and 600 ng of Aβ; 2 and 4 μg 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 Aβ aggregation. Soluble Aβ40 (50 μM) was incubated at 37°C with GM1-containing liposomes (GM1/Aβ molar ratio of 10:1) or preformed Aβ40 seeds (10 μg/ml), which were prepared as described above, and an antibody (4396C or 4G8) at various concentrations. Aβ40 aggregation levels in the mixtures were determined by ThT assay.

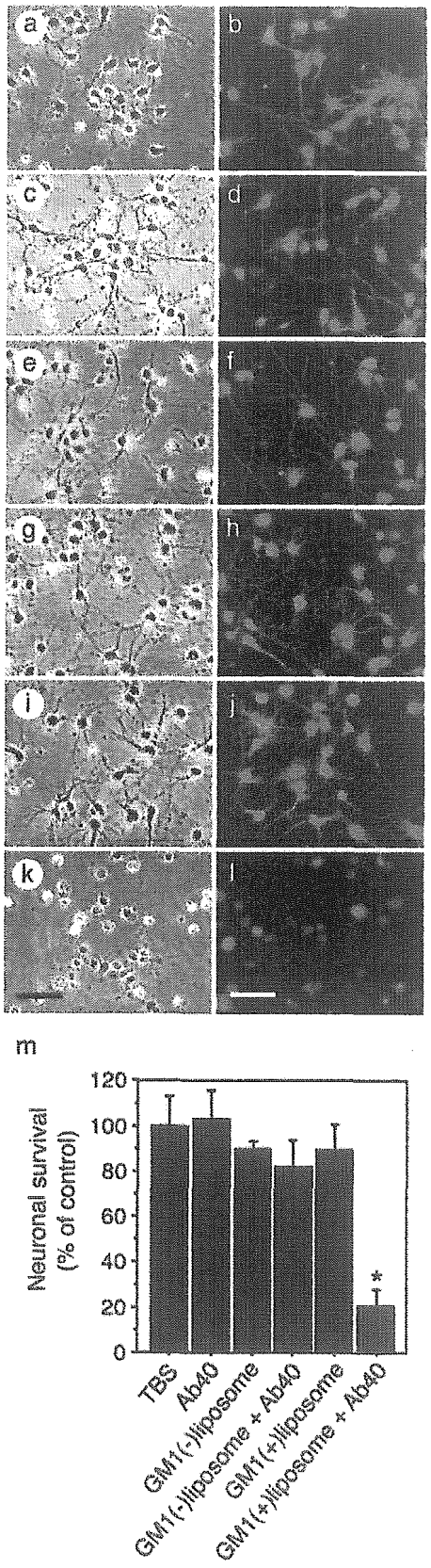

Figure 2. Viability of neurons treated with Aβ40 incubated in the presence of GM1 ganglioside. Phase-contrast and calsein AM– ethidium homodimer-stained photographs of cultured neurons were taken after treatment with TBS (*a*, *b*), Aβ40 (*c*, *d*), GM1-lacking liposomes (*e*, *f*), GM1-lacking liposomes plus Aβ40 (*g*, *h*), GM1-containing liposomes (*i*, *j*), and GM1-containing liposomes plus Aβ40 (*k*, *l*). *m*, Viable cells stained with calcein AM were counted. The data represent means ± SE for triplicate samples. $^*p < 0.003$ versus other treatments. Scale bars, 20 μm.

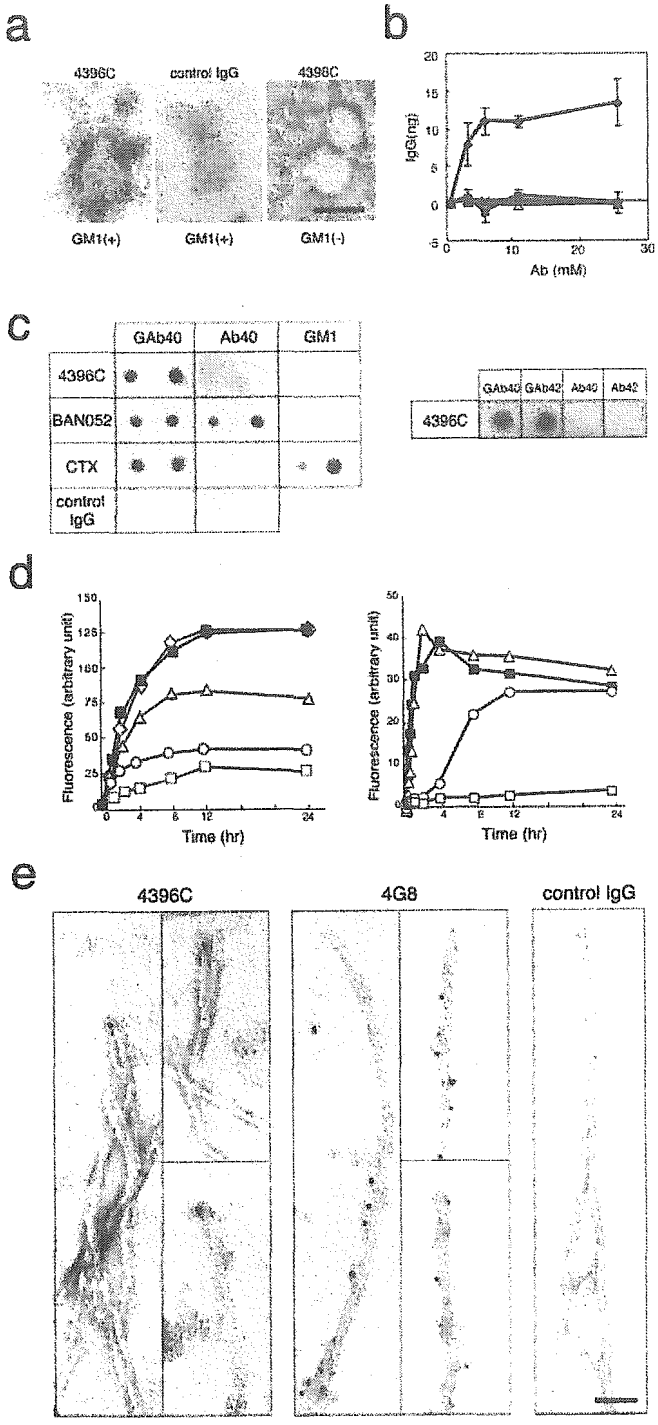

Figure 3. Characterization of the binding specificity of 4396C. *a*, Immunoelectron micrographs of liposomes. GM1-containing and GM1-lacking liposomes were subjected to immunoelectron microscopy of 4396C or isotype-matched control IgG staining after incubation with soluble Aβ40. GM1(+), GM1-containing 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 Aβ40 at indicated concentrations. GM1-lacking liposomes were also incubated with 4396C (triangles) or isotype-matched control IgG (× symbols). Ab, Antibody. *c*, Dot blot analysis. Left, Liposomes carrying GAβ (GAβ40), Aβ40, and GM1 in amounts equal to those contained in blotted liposomes (300 and 600 ng of Aβ40; 2 and 4 μg of GM1) were blotted. The blots were incubated with 4396C, BAN052, HRP-conjugated CTX, or isotype-matched control IgG. Right, Liposomes carrying GAβ (GAβ40 and GAβ42), prepared using Aβ40 or Aβ42, and Aβ40 and Aβ42 in amounts equal to those contained in GAβ40 and GAβ42 (600 ng of each peptide) were blotted. The blots were incubated with 4396C. *d*, Inhibition of amyloid fibril formation from soluble Aβ40 by 4396C. Left, Soluble Aβ40 was incubated with GM1-containing liposomes in the absence (filled squares) or presence of an antibody (4396C or 4G8). The molar ratios of 4396C to soluble Aβ40 were 0.3:50 (triangles), 1.3:50 (circles), and 4:50 (open squares) and that of 4G8 to Aβ40 was 4:50 (diamonds). Right, Soluble Aβ40 was incubated with preformed Aβ40 fibrils in the absence of an antibody (filled squares) or in the presence of 4396C. The molar ratios of 4396C to soluble Aβ40 were 0.3:50 (triangles), 1.3:50 (circles), and 4:50 (open squares). *e*, Immunoelectron micrographs of preformed Aβ40 fibrils. Aβ40 fibrils were formed by the extension reaction of Aβ40 seeds (10 μg/ml) with seed-free Aβ40 (50 μM), as described in Materials and Methods, and subjected to immunoelectron microscopy of 4396C, 4G8, or isotype-matched control IgG staining. Scale bar, 50 nm.

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 μg/ml) or 4G8 (1 μg/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 μg/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 zenon 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 GAβ 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 μg/ml) after sonication on ice. After 1 hr incubation, the mixtures were centrifuged at 15,000 × *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 × *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 Aβ 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 Aβ fibril formation *in vitro*. The ThT fluorescence intensity of Aβ42 solutions without removing undissolved peptide aggregates, which can act as preexisting seeds, started to increase as early as 1 hr of incubation at 50 μM and 37°C (Fig. 1*a*). In contrast, the ThT fluorescence intensity of seed-free Aβ42 solutions did not increase as long as 6 hr of incubation under the same conditions (Fig. 1*a*). The ThT fluorescence intensity of Aβ40 solutions without removing undissolved peptide aggregates started to increase after 4 d of incubation at 50 μM and 37°C (Fig. 1*a*); however, the seed-free Aβ40 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 GAβ-initiated Aβ aggregation, we incubated the seed-free Aβ (Aβ40 and Aβ42) solutions at 50 μM and 37°C under various conditions, as long as 48 and 6 hr for Aβ40 and Aβ42, respectively, in the following experiments.

We incubated Aβ40 solutions in the presence or absence of GM1-containing liposomes. The ThT fluorescence intensity in-

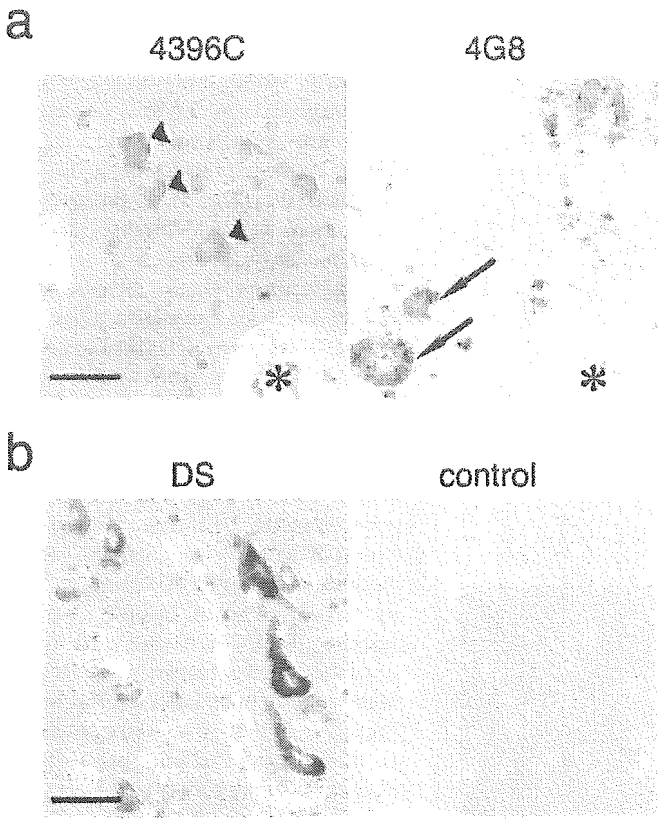

Figure 4. Immunohistochemistry of GAβ in sections of human brains. *a*, Immunostaining of serial sections of the cerebral cortex of an AD brain fixed in Kryofix and pretreated with SDS. Neurons (arrowheads) were immunostained by 4396C but not by 4G8, whereas plaques (arrows) were immunostained by 4G8 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 ($p < 0.05$, Mentel-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 Aβ40 solutions (Fig. 1*b*). In contrast, there was no increase in the ThT fluorescence intensity of the solutions containing Aβ40 alone or Aβ40 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 Aβ42 solution after the addition of GM1-containing liposomes (Fig. 1*b*). Under an electron microscope, typical amyloid fibrils were observed in the Aβ40 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 Aβ binds to GM1, leading to the generation of GAβ, and then induces Aβ 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 Aβ first onto GAβ and then onto the ends of growing fibrils.

We then investigated whether the formation of Aβ oligomers is also acclerated in the presence of GM1 ganglioside. We perfomed SDS-PAGE of the Aβ40 solutions incubated at 50 μM and 37°C for 24 hr. Notably, Aβ 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 Aβ40 alone (Fig. 1*d*).

Neurotoxicity of Aβ incubated in the presence of GM1 ganglioside

We then investigated whether Aβ aggregates formed in the presence of GM1-containing liposomes are neurotoxic. We incubated Aβ40 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 Aβ40 solution incubated in the presence of GM1-containing liposomes (Fig. 2).

Molecular characterization of GAβ

To characterize GAβ at the molecular level and to clarify the process of Aβ aggregation in the presence of GM1, we raised a monoclonal antibody against natural GAβ purified from an AD brain. In an experiment using immunoelectron microscopy, the antibody (4396C), but not the isotype-matched control IgG, recognized artificially generated GAβ on liposomes (Fig. 3*a*). The specificity of 4396C to GAβ 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 Aβ40 and GM1, whereas BAN052, a monoclonal antibody specific to the N terminus of Aβ (Suzuki et al., 1994), recognized both GAβ40 and Aβ (Fig. 3*c*, left). The 4396C antibody reacted with GM1-bound forms of two Aβ isoforms (Aβ40 and Aβ42) (Fig. 3*c*, right). In the inhibition assay of Aβ fibrillogenesis, the increase in the fluorescence intensity of ThT of the Aβ40 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 Aβ (Kim et al., 1988) (Fig. 3*d*, left). We then examined the possibility that Aβ fibrillogenesis proceeds with consecutive conformational alteration of Aβ at the ends of growing fibrils; that is, GAβ and Aβ at the ends of growing fibrils share a specific conformation that is required for Aβ fibrillogenesis in the manner of a first-order kinetic model. We incubated Aβ40 solutions with 4396C in the presence of preformed Aβ40 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 Aβ40 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*).

GAβ generation in the brain

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

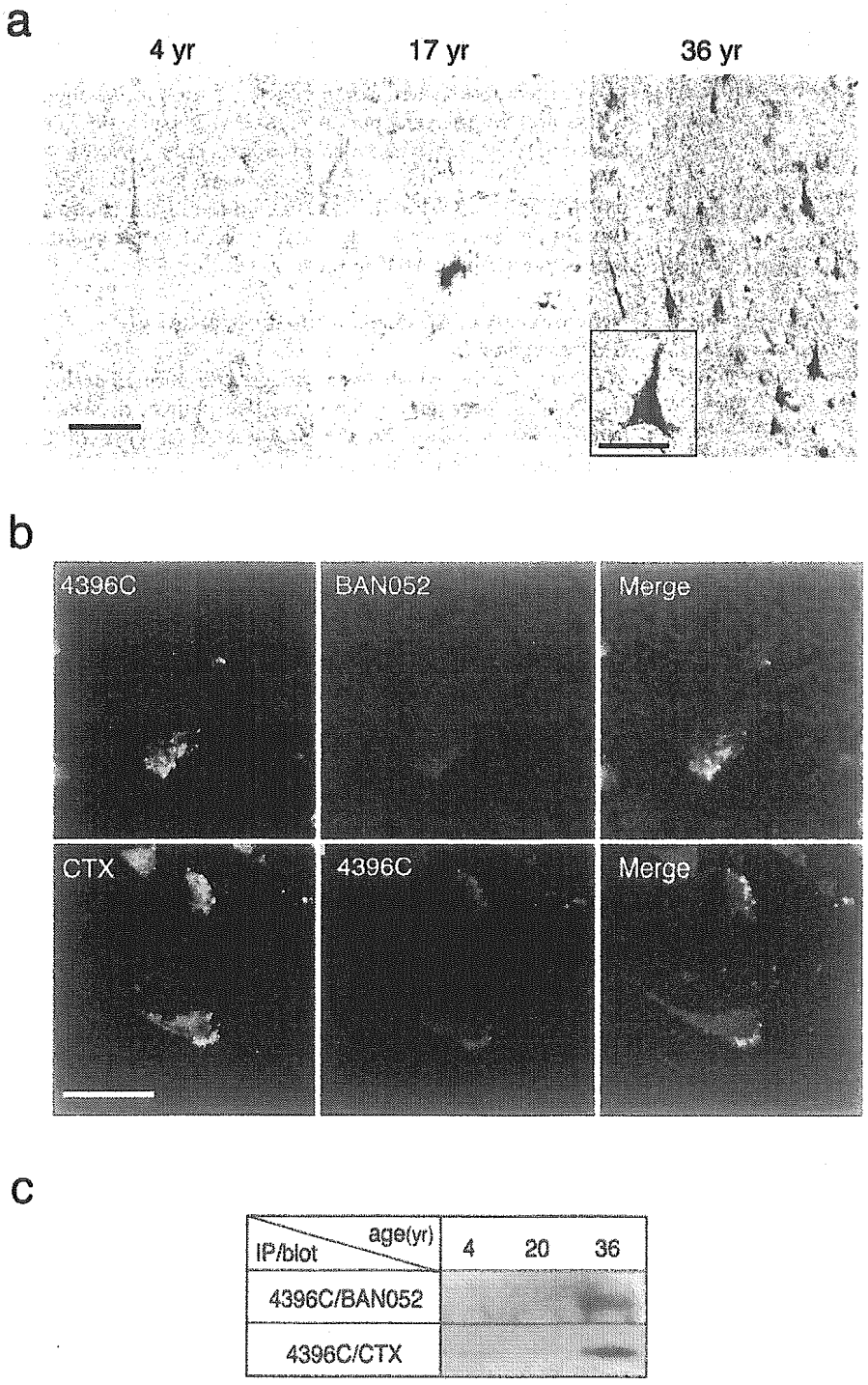

Figure 5. Immunohistochemistry and immunoprecipitation of GAβ in sections of nonhuman primate brains. *a*, Immunostaining by 4396C of sections of the cerebral cortices of primate brains, which were fixed in paraformaldehyde, from animals of different ages. Scale bar, 50 μm. Inset, Higher magnification. 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. GAβ was immunoprecipitated by 4396C only from the cerebral cortex of the older animal (Fig. 5*c*). These results indicate that GAβ is generated in the brain.

Discussion

In regard to the formation of pathogenic aggregates of constituent proteins, including Aβ and prion proteins, the seeded polymerization theory was proposed previously (Harper and Lansbury, 1997). For the transition of the nontoxic monomeric form of Aβ 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 tharapeutic strategies, clarification of the time course of Aβ fibrillogenesis is fundamentally important. Previously, Harper et al. analyzed the process of *in vitro* Aβ assembly using an atomic force microscope at a fine resolution. They reported that protofibrils, transient species of Aβ assembly, are formed during the first week of incubation of Aβ40 before mature fibrils are generated (Harper et al., 1997a,b, 1999; Ding and Harper, 1999). This model of Aβ assembly was supported by a recent study of Nichols et al. (2002). In the present study, we examined the acceleration of Aβ aggregation in the presence of GM1 ganglioside. In the EM examination of the present study, we observed mature fibrils in Aβ40 solutions incubated at 50 μM and 37°C for 24 hr, but protofibrils were hardly recognized (Fig. 1*c*). 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 Aβ fibrillogenesis in the presence of GM1 ganglioside may be different from that initiated by spontaneous nucleation from seed-free Aβ solution.

From the results of molecular characterization of GAβ in the present study, it seems likely that Aβ adopts an altered conformation at its midportion through binding to GM1 because GAβ was readily recognized by BAN052, an antibody specific to the N terminus of Aβ, and 4396C comparably recognized two Aβ isoforms with different lengths at their C termini. This possibility is supported by the following: first, 4G8, an antibody specific to the midportion of Aβ, failed to react with GAβ in the inhibition assay of Aβ aggregation; and second, BAN052, but not 4G8, immunoprecipitated GAβ from the cerebral cortex of an AD brain in our previous study (Yanagisawa and Ihara, 1998). The conformational epitope for 4396C on the Aβ molecule remains to be determined; however, the results of the present study suggest that the 4396C-reactive conformation, which is shared by GAβ and Aβ at the ends of fibrils, is necessary for Aβ 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 GAβ; 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 GAβ is generated in the brain. Regarding this issue, we favor the possibility that GAβ 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 Aβs 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 Aβ is readily induced by its interaction with lipid raft-like model membranes (Kakio et al., 2003). We found recently that Aβ 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 Aβ 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 ε4 (Hayashi et al., 2002). Thus, altogether, one possible scenario may be as follows: GAβ 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, GAβ initiates its seed activity to accelerate Aβ aggregation after its transport to the neuronal surface and/or shedding into the extracellular space. Alternatively, GAβ itself can be noxious per se because it has been reported previously that the disruption of membranes (McLaurin and Chakrabartty, 1996) and alteration of bilayer organization (Matsuzaki and Horikiri, 1999) can be induced by the generation of GAβ on the membranes. Thus, it may also be worthy to examine in future studies whether GAβ causes impairment of neuronal, particularly lipid raft-related, functions before extraneuronal Aβ 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 GAβ has a conformation distinct from that of soluble Aβ, it may be possible to develop a novel therapeutic strategy to specifically inhibit the initiation of oligomerization–polymerization of Aβ in the brain.

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Modulation of Amyloid Precursor Protein Cleavage by Cellular Sphingolipids*

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Lipid rafts and their component, cholesterol, modulate the processing of β-amyloid precursor protein (APP). However, the role of sphingolipids, another major component of lipid rafts, in APP processing remains undetermined. Here we report the effect of sphingolipid deficiency on APP processing in Chinese hamster ovary cells treated with a specific inhibitor of serine palmitoyltransferase, which catalyzes the first step of sphingolipid biosynthesis, and in a mutant LY-B strain defective in the LCB1 subunit of serine palmitoyltransferase. We found that in sphingolipid-deficient cells, the secretion of soluble APPα (sAPPα) and the generation of C-terminal fragment cleaved at α-site dramatically increased, whereas β-cleavage activity remained unchanged, and the ε-cleavage activity decreased without alteration of the total APP level. The secretion of amyloid β-protein 42 increased in sphingolipid-deficient cells, whereas that of amyloid β-protein 40 did not. All of these alterations were restored in sphingolipid-deficient cells by adding exogenous sphingosine and in LY-B cells by transfection with *cLCB1*. Sphingolipid deficiency increased MAPK/ERK activity and a specific inhibitor of MAPK kinase, PD98059, restored sAPPα level, indicating that sphingolipid deficiency enhances sAPPα secretion via activation of MAPK/ERK pathway. These results suggest that not only the cellular level of cholesterol but also that of sphingolipids may be involved in the pathological process of Alzheimer's disease by modulating APP cleavage.

The amyloid β-peptide (Aβ)[1] is the principle constituent of senile plaques found in Alzheimer's disease (AD) brains and is generated by proteolysis of an integral membrane protein, the amyloid precursor protein (APP). APP is metabolized via at least two post-translational pathways, one of which is a nonamyloidogenic pathway mediated by α-secretase proteolytically producing soluble APP (sAPPα), the dominant processing product; this cleavage generates the residual 10-kDa CTF (CTFα). Previous studies have shown that the activation of signal transduction pathways including protein kinase C (PKC) (1–3), mitogen-associated protein kinase (MAPK) (4), and growth factors (5) alter the relative amounts of sAPPα and Aβ production. The other cleavage is mediated by β-secretase, generating several proteolytic C-terminal fragments (CTFs), namely, CTFβ and CTFε, and γ-secretase, producing either a 40-residue protein (Aβ40) or a 42-residue protein (Aβ42) from CTFβ. The cleavages at residues 40–42 are referred to as γ-cleavage, and the cleavage at residues 49–52 are referred to as ε-cleavage (6).

Recent studies revealed that the prevalence of AD is linked to the serum cholesterol level and that sAPPα secretion and Aβ generation are modulated by the cellular cholesterol level (7–13). It is also suggested that APP processing and Aβ generation are associated with membrane microdomains, known as lipid rafts, that are rich in cholesterol and sphingolipids and are also the principal compartment in which Aβ is found (13–18).

The cholesterol content in lipid rafts has been shown to contribute to the integrity of the raft structure and the functions of the rafts in signaling and membrane trafficking (19–21). In addition, several studies showed that sphingolipids modulate raft functions; the reduction in the cellular sphingolipid level renders glycosyl phosphatidylinositol (GPI)-anchored proteins more sensitive to phosphatidylinositol-specific phospholipase C (22) and also enhances the solubility of GPI-anchored proteins in Triton X-100 (23). The blockade of ceramide synthesis was shown to inhibit folate uptake via GPI-anchored receptors (24) and to enhance the conversion of the prion protein to its scrapie form (25–27). These studies indicate that the cellular levels of cholesterol and sphingolipids modulate the functions of lipid rafts. Therefore, the evidence that the cholesterol level in lipid rafts can modulate APP processing reasonably raises the question of whether cellular sphingolipids also modulate APP processing and Aβ generation. However, the participation of sphingolipids in APP processing remains undetermined.

To address this issue, we examined the alterations in APP processing and Aβ generation in sphingolipid-deficient cells using ISP-1 (myriocin), a potent inhibitor of serine palmitoyltransferase (SPT) (28), and a CHO-K1-cell-derived mutant cell line, the LY-B strain, which has a defect in the LCB1 subunit of SPT and is therefore incapable of *de novo* synthesis of any sphingolipid species (29). Our findings indicate that not only

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[1] The abbreviations used are: Aβ, amyloid β-protein; AD, Alzheimer's disease; CHO, Chinese hamster ovary; FBS, fetal bovine serum; SPT, serine palmitoyltransferase; GPI, glycosylphosphatidylinositol; APP, amyloid precursor protein; CTF, C-terminal fragment; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-associated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C.