別紙４

研究成果の刊行に関する一覧表

書籍

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雑誌

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道川　誠	アルツハイマー病とコレステロール代謝	生化学	78	831-839	2006
道川　誠	Alzheimer病と脂質	Clinical Neuroscience	25	162-164	2007
道川　誠	アルツハイマー病:ベッドサイドからベンチへ	医療	60	752-759	2006
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Michikawa M.	Role of cholesterol in amyloid cascade: cholesterol-dependent modulation of tau phosphorylation and mitochondrial function.	Acta Neurol Scand Suppl	185	21-26	2006
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Role of cholesterol in amyloid cascade: cholesterol-dependent modulation of tau phosphorylation and mitochondrial function

Michikawa M. Role of cholesterol in amyloid cascade: cholesterol-dependent modulation of tau phosphorylation and mitochondrial function. Acta Neurol Scand 2006: 114 (Suppl. 185): 21–26. © Blackwell Munksgaard 2006.

Apolipoprotein E (apoE) alleles are important genetic risk factors for Alzheimer's disease (AD), with the ε4 allele increasing and the ε2 allele decreasing the risk of developing AD. ApoE is the major apolipoprotein that modulates cholesterol transport in the central nervous system, cholesterol being an essential component of membranes for maintaining their structure and functions. Epidemiological studies have suggested a link between serum cholesterol levels and AD development and the potential therapeutic effectiveness of statins for AD; and furthermore, biological studies have shown that amyloid β-protein (Aβ) secretion is modulated by cellular cholesterol level. However, other lines of evidence show controversial results. In addition to the role of cholesterol in Aβ generation, different interactions of cholesterol with Aβ and its role in AD pathogenesis have been shown, i.e. Aβ affects cholesterol dynamics in neurons, and altered cholesterol metabolism in turn leads to neurodegeneration with abnormally phosphorylated tau (tauopathy). In this review, the reciprocal interactions between cholesterol and Aβ, and the role of cholesterol in tauopathy are discussed. The isoform-specific involvement of apoE in this cascade, in which high-density lipoprotein-like particles are generated and supplied to neurons to maintain cholesterol homeostasis, is also discussed.

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Key words: Alzheimer's disease; amyloid β-protein; apolipoprotein E; cholesterol; high-density lipoprotein; Niemann–Pick type C1 disease; statin; tau phosphorylation

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Cholesterol transport system in central nervous system

Studies of cholesterol metabolism in the systemic circulation have been performed for decades. However, knowledge of the cholesterol transport system in the central nervous system (CNS), the most lipid-rich organ that accounts for 20–25% of the total body cholesterol, is very limited. As CNS is segregated by the blood–brain barrier from the systemic circulation, the regular lipid transport system mediated by plasma lipoproteins is not generally available. Accordingly, the cholesterol transport in the CNS is postulated to be regulated independent of the systemic circulation. For example, several types of lipoproteins are identified in the systemic circulation including chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL), whereas only HDL-like lipoproteins are found in the cerebrospinal fluid (CSF) (1, 2). Demonstration that the original apolipoprotein E (apoE) phenotype of a recipient in the CNS remained unchanged even after liver transplantation, which changed the plasma apoE phenotype (3), suggests that apoE in the CSF is produced within the CNS and is unexchangeable with the apoE associated with the plasma lipoproteins.

Under conditions in which cholesterol supply from the blood is not available, how do neurons obtain cholesterol? To answer this fundamental question, previous studies have shown a new type of neuron–glia interaction in the CNS, by which cholesterol is supplied to neurons for synapse formation and neurite outgrowth. It has been shown that cholesterol is a synapse-promoting

factor secreted from glia cells in apoE-containing lipoproteins (4), and that glial-derived lipoproteins containing apoE stimulate axon extension (5). In accordance with these findings, glial cells produce two- to threefold more cholesterol than neurons and secrete it as apoE-lipoprotein particles (6, 7), which are supplied to neurons via apoE receptors. The morphology of neurons is quite different from that of other cells in that neurons have a large number of long complicated processes, which have a membrane area 10- to 100-fold that of the cell body (8). In addition, more than 20% of the clusters of post-synaptic densities turn over within 24 h in hippocampal neurons (9). These may explain why neurons import cholesterol rather than synthesize it, which would require them to have a whole battery of enzymes distributed in different subcellular organelles and to consume large amounts of energy substrates.

Association of serum total and LDL cholesterol levels with risk of Alzheimer's disease: why and how?

Recent epidemiological studies have revealed the plausibility of a link between Alzheimer's disease (AD) and cholesterol metabolism based on the associations of an elevated serum total cholesterol level with a high prevalence of AD and mild cognitive impairment (MCI) (10, 11). These associations correlate well with the finding that the apoE allele ε4 is a strong risk factor for AD development, because there is a stepwise increase, as a function of alleles (ε2 to ε3 to ε4), in serum total and LDL cholesterol levels (12–16). The decreased prevalence of AD in individuals treated with statins (17) seems to strengthen this notion. Although the cause and effect relationship between high serum cholesterol levels and enhanced Aβ generation or secretion has not yet been demonstrated, another explanation has been proposed, i.e. a decreased cellular cholesterol level suppresses the synthesis of Aβ in neurons (18) and the secretion of Aβ from cells into the CSF *in vivo* (19). These lines of evidence imply that increased levels of serum total cholesterol and LDL cholesterol lead to an increased cellular cholesterol level in the CNS through the blood–brain barrier. However, the relationship between cholesterol levels in serum and in the CSF or CNS has not yet been established, and this notion has been partly challenged by recent studies showing that a reduced cholesterol level in detergent-resistant membrane domains of AD brains (20) and that a slight decrease in cellular cholesterol levels enhance Aβ generation (21). All these results indicate that major issues still remain to be resolved as follows: how cholesterol metabolism in the CNS and in the systemic circulation are associated beyond the blood–brain barrier; how cholesterol metabolism within the CNS is regulated; and how altered cholesterol metabolism in neurons or glia cells is associated with AD development and progression.

Relationship between serum and CSF cholesterol levels

The most fundamental and thus critical concern is that there is currently no explanation for how serum cholesterol levels are involved in brain diseases beyond the blood–brain barrier. Therefore, the above-mentioned data must be interpreted with caution and several issues need to be resolved before the idea that high serum cholesterol levels and, if it is the case, high cholesterol levels in CNS cells are responsible for AD development can be fully accepted. It has been shown that there is no significant correlation between CSF and serum cholesterol levels (22, 23), and that serum cholesterol level has no effect on the level of HMG-CoA reductase mRNA and its activity in the brain (24). These lines of evidence cannot explain why significantly higher levels of serum total cholesterol lead to a higher prevalence of AD, because of the differences between serum and CNS cholesterol metabolism.

Association of plasma HDL cholesterol level with risk of AD

If serum total and LDL cholesterol levels have no correlation with CSF cholesterol levels, does it mean that CNS cholesterol is not involved in AD pathogenesis? Thus, I propose an alternative interpretation of previous data. In previous reports, the authors mainly discussed the relationship between serum cholesterol levels and AD development; they did not focus on HDL cholesterol levels (10, 11, 25, 26). As has been reported, the LDL and total cholesterol levels are highest in those with apoE4, intermediate in those with apoE3 and lowest in those with apoE2 (12–16). However, it has also been shown that HDL cholesterol levels are highest in those with apoE2, intermediate in those with apoE3 and lowest in those with apoE4 (14–16), which can be explained by the isoform-dependent apoE ability to release cholesterol from cells to generate HDL particles (27, 28). This apoE-isoform-dependent HDL cholesterol level is also found in AD patients in whom serum HDL cholesterol levels inversely correlate with the dose of the apoE allele ε4 (29). It has also been shown that serum HDL cholesterol levels of AD patients is lower than that of controls,

decreased serum HDL cholesterol levels being correlated with AD severity (30), and HDL cholesterol levels are lower in the CSF of AD patients than in that of controls (31). A previous study showed that there is a strong correlation between CSF and serum HDL cholesterol levels, but not between CSF and serum total or LDL cholesterol levels (22). These lines of evidence led us to hypothesize that a low cholesterol level in serum and CSF is a risk factor for AD development. In accordance with this notion, recent studies have shown that cholesterol level decreases in the white matter (32) and detergent-resistant membrane domains (20) of AD brains. Moreover, a slight reduction in cellular cholesterol levels has been demonstrated to enhance Aβ generation (21). These results are contrary to what had been reported and accepted, indicating that further studies are required to better understand how lipoprotein cholesterol levels are associated with AD pathogenesis including Aβ generation or secretion.

Role of cholesterol in amyloid cascade

Oligomeric Aβ impairs cholesterol metabolism in neurons

How is altered cholesterol metabolism involved in AD pathogenesis? Previous studies have focused on cholesterol-mediated Aβ synthesis. However, here, I propose a different notion explaining the role of cholesterol in the amyloid cascade by promoting AD pathologies as follows: with respect to the neurotoxic effect of Aβ, it is widely accepted that oligomerization is a critical step for Aβ to impair neuronal functions (33). We have also found that Aβ, when it forms oligomers, affects cholesterol metabolism in neurons. Oligomeric Aβ causes cholesterol and phospholipid release to generate an Aβ–lipid complex whose density is similar to that of HDL. This Aβ–lipid complex is not taken up by neurons, whereas HDL generated by apoE can be taken up via apoE receptors and used by neurons (34). In addition, the oligomeric Aβ impairs cholesterol synthesis and finally reduces cholesterol levels in neurons (35). In contrast, Aβ monomers do not have such an effect; rather, they have a strong neuroprotective function as radical scavengers (36) (Fig. 1B).

Decreased cellular cholesterol levels promote tau phosphorylation and impair mitochondrial functions

Aβ-mediated alteration in cellular cholesterol homeostasis presumably leads to synaptic dysfunction, because cholesterol levels in neurons, derived

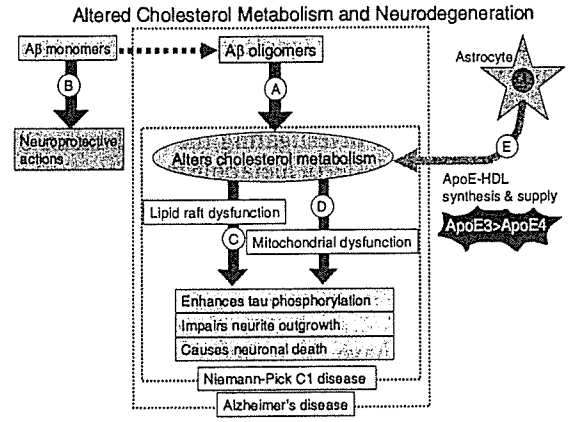

Figure 1. Hypothetical schema showing putative role of cholesterol in amyloid cascade that promotes AD pathogenesis. Increased extracellular Aβ levels with aging result in the formation of soluble oligomers and insoluble assemblies such as fibrils. (A) Oligomeric Aβ has been shown to affect cholesterol metabolism in neurons and to finally reduce cellular cholesterol levels in neurons by promoting cholesterol release from neurons to generate HDL–Aβ complexes (34), inhibiting cholesterol synthesis (35). (B) In contrast, Aβ monomers protect neurons (36). (C) The disruption of cholesterol homeostasis in neuronal membranes caused by oligomeric Aβ may induce AD pathological alterations including enhanced tau phosphorylation (38, 39), inhibition of dendrite outgrowth (37), impairment of synaptogenesis and synaptic plasticity (4, 5), and neurodegeneration (38, 52). (D) Interestingly, an altered cholesterol level in the mitochondrial membrane impairs mitochondrial functions, which results in a reduced cellular ATP level in neurons (47). (E) ApoE is involved in this cascade as an HDL generator and an HDL cholesterol supplier to neurons via apoE receptors in an apoE-isoform-dependent manner (27, 28).

from glial cells as apoE-containing lipoproteins, have been shown to play a critical role in the formation of mature synapses (4) and in neurite outgrowth (5, 37). Our findings and that of others suggest that cellular cholesterol levels modulate neurite outgrowth by modulating cellular kinases and phosphatases, and a decrease in cholesterol levels result in a dendrite-specific inhibition of neurite outgrowth (37, 38). In addition, it has been shown that reduced cellular cholesterol levels promote tau phosphorylation in cultured neurons (39) and *in vivo* (38). It is suggested that this enhanced tau phosphorylation in cholesterol-deficient neurons is mainly induced by enhanced MAPK/ERK1/2 activity, which is induced by an altered function of lipid rafts because of cholesterol deficiency in such rafts (40). Our recent study has shown that not only cholesterol deficiency but also sphingolipid deficiency promotes MAPK/ERK1/2 activity (41), indicating that an altered raft function, but not cholesterol itself, is responsible for enhanced ERK1/2 activity, leading to enhanced tau phosphorylation. With respect to the association between cholesterol deficiency and tauopathy, it is

of note that a perturbation of cholesterol metabolism and neurofibrillary tangle formation coexists in brains with Niemann–Pick type C1 (NPC1) disease (42–44). This suggests that NPC1 disease is a suitable model for investigating the mechanism by which altered cholesterol metabolism causes tauopathy (Fig. 1).

AD and NPC1 disease share a common pathway leading to tauopathy

Although there have been many different hypotheses for explaining the molecular basis of neurodegeneration in NPC1 disease, the continuous defective use of cholesterol in NPC1 neural tissues has been suggested to cause tauopathy (45, 46). Our recent studies have shown that tau in the brains of NPC1 model mice is hyperphosphorylated in a site-specific manner, which is accompanied by enhanced MAPK/ERK1/2 activity (45), and that reduced cholesterol levels in the lipid raft domain because of the lack of NPC1 activate MAPK/ERK1/2, which subsequently promotes tau phosphorylation (40). In support of this, enhanced tau phosphorylation accompanied by the focal deregulation of cdk5/p25 in the NPC1 mouse cerebrum has been reported (46). As free-cholesterol levels in NPC1-deficient cells markedly increase, these findings indicate that the state of tau phosphorylation is modulated not by total cellular cholesterol levels but by cholesterol levels in specific cellular compartments such as lipid rafts, leading to a change in intracellular signaling (Fig. 1C).

Recently, we have demonstrated that the amount of cholesterol in mitochondrial membranes is significantly elevated in NPC1 mouse brains, which reduces mitochondrial membrane potential, ATP synthase activity, and henceforth ATP levels, leading to neurodegeneration (47) (Fig. 1D). Our study also demonstrated that there is an optimal concentration of cholesterol in mitochondria, i.e. low and high cholesterol levels impair mitochondrial functions. These results suggest that in addition to enhanced tau phosphorylation, mitochondrial dysfunctions and the subsequent ATP deficiency may be responsible for neuronal impairment in NPC1 disease. As has been discussed above, it is suggested that altered cholesterol metabolism is associated with AD development. Importantly, it is now established that mitochondrial dysfunction is involved in AD development (48–50). Therefore, AD and NPC1 disease may share a common pathway involving cholesterol metabolism leading to neurodegeneration via mitochondrial dysfunction (51) (Fig. 1B).

Although many unclarified issues remain, particularly regarding various downstream events in the amyloid cascade, it is possible that cholesterol plays a key role in this cascade, modulating the processes that induce AD pathologies.

ApoE contributes to the maintenance of cholesterol homeostasis in neurons by generating and supplying HDL in an apoE-isoform-specific manner

The final question is how apoE is involved in this cascade. Our previous studies demonstrated that the apoE ability to generate HDL particles is isoform dependent; the amount of cholesterol released as HDL particles from apoE3-expressing astrocytes is ~2.5-fold greater than that from apoE4-expressing astrocytes with similar numbers of molecules of each apoE isoform (28). The apoE-isoform-dependent promotion (apoE4) or prevention (apoE3) of AD development may be explained by the apoE-isoform-dependent ability (apoE3 > apoE4) to generate HDL-like particles, which could supply cholesterol to neurons (27, 28). As it has been suggested that lipoprotein cholesterol plays a critical role in the repair of neurons including synaptogenesis (4) and neurite outgrowth (5), it is possible that when brain cholesterol homeostasis is impaired with aging by increased levels of oligomeric Aβ or oxysterols, both of which reduce cellular cholesterol level, HDL supply to neurons from glia cells of *apoE3* genotype is greater than that of *apoE4* genotype. The lower ability of apoE4 to generate and supply HDL may result in earlier disruption in cholesterol homeostasis in neurons, leading to tauopathy (Fig. 1E). The different viewpoints presented here may help in elucidating the cholesterol-dependent promotion of AD pathogenesis, in which key molecules such as Aβ and cholesterol, tauopathy, and neuronal death are significantly integrated in one schema (Fig. 1).

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A Ganglioside-induced Toxic Soluble Aβ Assembly

*ITS ENHANCED FORMATION FROM Aβ BEARING THE ARCTIC MUTATION**

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The mechanism underlying plaque-independent neuronal death in Alzheimer disease (AD), which is probably responsible for early cognitive decline in AD patients, remains unclarified. Here, we show that a toxic soluble Aβ assembly (TAβ) is formed in the presence of liposomes containing GM1 ganglioside more rapidly and to a greater extent from a hereditary variant-type ("Arctic") Aβ than from wild-type Aβ. TAβ is also formed from soluble Aβ through incubation with natural neuronal membranes prepared from aged mouse brains in a GM1 ganglioside-dependent manner. An oligomer-specific antibody (anti-Oligo) significantly suppresses TAβ toxicity. Biophysical and structural analyses by atomic force microscopy and size exclusion chromatography revealed that TAβ is spherical with diameters of 10–20 nm and molecular masses of 200–300 kDa. TAβ induces neuronal death, which is abrogated by the small interfering RNA-mediated knockdown of nerve growth factor receptors, including TrkA and p75 neurotrophin receptor. Our results suggest that soluble Aβ assemblies, such as TAβ, can cause plaque-independent neuronal death that favorably occurs in nerve growth factor-dependent neurons in the cholinergic basal forebrain in AD.

The poor correlation between amyloid load in the brain and the degree of neurological deficits in patients with Alzheimer disease (AD)[2] (1) or animal models of AD (2, 3) argues against amyloid fibrils being the primary toxic Aβ species. Recently, soluble Aβ assemblies, also referred to as Aβ oligomers (4), protofibrils (5, 6), or Aβ-derived diffusible ligands (7), have attracted attention because of their potency to impair neuronal function or induce neuritic degeneration (7–13). Several possibilities have been proposed in regard to the toxicities of soluble Aβ assemblies (*e.g.* the binding of assemblies to target molecules on neuronal membranes (7, 14) and the ubiquitous disruption of the plasma membrane in association with the perturbation of ionic homeostasis (15)). It is also noteworthy that neurotoxicities induced by soluble Aβ assemblies are mediated, at least in part, by the activation of signal transduction pathways, including those involving Src family kinases, extracellular signal-regulated kinase, or sphingomyelinases (7, 11, 16, 17). Notably, the level of soluble Aβ assemblies increases in the brain and cerebrospinal fluid of AD patients (18, 19, 20, 21, 22), and oligomer-specific immunoreactivity is readily observed in the AD brain (23). Furthermore, the inhibition of long term potentiation and the impairment of cognitive function *in vivo* can be induced by natural Aβ oligomers (9, 24) or a specific Aβ assembly called Aβ*56, which has recently been isolated from *Tg2576* mice (expressing a human amyloid precursor protein variant-linked familial AD) (25). Additionally, recent studies using AD mouse models revealed that soluble Aβ assemblies may play a role in the induction of tau pathology (26) and that the genetic deletion of β-secretase, which is responsible for Aβ production, rescues temporal memory deficit in conjunction with the suppression of the increase in the levels of cerebral Aβ-derived diffusible ligands (27). These lines of evidence indicate the pathological relevance of these soluble Aβ assemblies in AD development. However, it remains to be elucidated how these assemblies are formed *in vivo*.

Several mutations within the Aβ sequence have been reported to be responsible for the development of familial AD and hereditary cerebral amyloid angiopathy (28–32). Among these mutations, the Arctic mutation, unlike other mutations, accelerates the development of clinical and neuropathological features indistinguishable from those of sporadic AD, although it does not increase Aβ42 level or Aβ42/Aβ40 ratio (30). The pathological features induced by the Arctic mutation, including predominant Aβ deposition in the brain parenchyma, have also been confirmed in transgenic mice (33). Notably, Aβ bearing the Arctic mutation shows a propensity to form neurotoxic nonamyloid assemblies, including protofibrils, amyloid pores, and small nonfibrillar assemblies (13, 30, 34). Thus, researchers have focused on the Arctic mutation in terms of the mechanisms underlying the formation of soluble and insoluble Aβ assemblies.

In regard to the assembly of wild-type and hereditary variant-type Aβs, we have recently observed that Arctic-type Aβ, unlike other hereditary variant-type Aβs (*i.e.* Dutch-type, Italian-type,

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[2] The abbreviations used are: AD, Alzheimer disease; TAβ, toxic soluble Aβ assembly; NGF, nerve growth factor; LDH, lactate dehydrogenase; siRNA, small interfering RNA; AFM, atomic force microscopy; GM1, Galβ1,3GalNAcβ1,4(Neu5Ac-α2,3)Galβ1,4Glcβ1,1-ceramide; ThT, thioflavin-T; NTR, neurotrophin receptor.

and Flemish-type Aβs), preferably assembles in the presence of GM1 ganglioside, as does wild-type Aβ (35, 36). We also reported that GM1 ganglioside level increases in synaptosomes prepared from aged, human apolipoprotein E4 knock-in mice (37). Thus, it is possible that an alteration in the expression or distribution of GM1 ganglioside is the background to the assembly and deposition of Aβ in the brain parenchyma. This possibility has been supported by findings of recent studies as follows: 1) GM1 ganglioside level increases in membrane microdomains isolated from the frontal cortex but not from the temporal cortex, reflecting earlier and later stages of AD pathology, respectively (38), and 2) GM1 ganglioside level also increases in amyloid-positive nerve terminals obtained from the AD cortex (39).

In this study, we aimed to characterize the toxicity of assemblies formed from Arctic-type Aβ in the presence of GM1 ganglioside. We found that a toxic soluble Aβ assembly (TAβ) is formed more rapidly and to a greater extent from Arctic-type Aβ in the presence of GM1 ganglioside than from wild-type Aβ. Furthermore, our results suggest that TAβ induces nerve growth factor (NGF) receptor-mediated neuronal death. Thus, we propose that soluble Aβ assemblies, such as TAβ, are responsible for plaque-independent neuronal death that favorably occurs in NGF-dependent neurons in AD.

MATERIALS AND METHODS

Preparation of Seed-free Aβ Solutions and Liposomes—Synthetic wild-type Aβ (Aβ40) and Arctic-type Aβ (Aβ40) (Peptide Institute, Osaka, Japan) were dissolved in 0.02% ammonia solution at 500 μM. To obtain seed-free Aβ solutions, the prepared solutions were centrifuged at 540,000 × *g* for 3 h using an Optima TL ultracentrifuge (Beckman) to remove undissolved peptides that 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 (150 mM NaCl and 10 mM Tris-HCl, pH 7.4). To prepare liposomes, cholesterol (Sigma), sphingomyelin (Sigma), and GM1 ganglioside (Matreya LLC) were dissolved in chloroform/methanol at a molar lipid ratio of 50:50:0, 45:45:10, 42.5:42.5:15, or 40:40:20. The mixtures were stored at −80 °C until use. Immediately before use, the lipids were resuspended in Tris-buffered saline at a ganglioside concentration of 2.5 mM, and the suspension was subjected to freezing and thawing and sonication.

Cell Culture—Cerebral cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats and cultured in a serum-free medium consisting of Dulbecco's modified Eagle's medium nutrient mixture and N2 supplement. Rat pheochromocytoma PC12 (PC12) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated horse serum (Invitrogen) and 5% fetal bovine serum (Invitrogen). For their differentiation, PC12 cells were plated on 2-cm^2 poly-L-lysine-coated (10 mg/ml) dishes at a density of 20,000 cells/cm^2 and cultured for 6 days in Dulbecco's modified Eagle's medium supplemented with 100 ng/ml NGF (PC12N) (Alomone Laboratories, Jerusalem, Israel). Human neuroblastoma SH-SY5Y (SY5Y) cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum. All of the cells were cultured in humidified 5% CO_2 at 37 °C.

Aβ Incubation in the Presence of GM1 Ganglioside—A seed-free Aβ solution was incubated at 37 °C and 50 μM, unless otherwise indicated, in the presence or absence of GM1 ganglioside-containing liposomes, as previously reported (40). The concentration of GM1 ganglioside in the incubation mixtures was 500 μM, and the molar ratio of GM1 ganglioside in the liposomes varied, as indicated in each figure.

ThT Assay—Aβ solutions were incubated in the presence of liposomes at 50 μM and 37 °C for various durations. The ThT fluorescence intensity of the incubation mixtures was determined using a spectrofluorophotometer (RF-5300PC) (Shimadzu Co., Kyoto, Japan). The optimum fluorescence intensity of amyloid fibrils was measured at excitation and emission wavelengths of 446 and 490 nm, respectively, with the reaction mixture (1.0 ml) containing 5 μM ThT and 50 mM glycine-NaOH at pH 8.5. The fluorescence intensity was measured immediately after preparing the mixture.

LDH Release Assay—The LDH assay was performed on medium using an LDH assay toxicity kit (Promega, Madison, WI). The degree of LDH release in each sample was assessed by measuring absorbance at 490 nm using an Emax precision microplate reader (Molecular Devices Corp., Sunnyvale, CA). Background absorbances, as assessed using cell-free wells, were subtracted from the absorbances of each test sample. Absorbances measured from three wells were averaged, and the percentage degree of LDH release was calculated by dividing the absorbance measured from each test sample following treatment with 1% Triton X-100 to induce the release of intracellular LDH according to instructions provided by the manufacturer.

Electron and Atomic Force Microscopies—For electron microscopy, the samples were diluted with distilled water and spread onto carbon-coated grids. The grids were negatively stained with 2% uranyl acetate and examined under a JEM-2000EX transmission electron microscope (Tokyo, Japan) with an acceleration voltage of 100 kV. Atomic force microscopy (AFM) assessment was performed as described elsewhere (41). Briefly, the samples were dropped onto a freshly cleaved mica. After leaving them to stand for 3 min and then washing with water, the samples were assessed in a solution using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) set in the tapping mode (42). OMCL-TR400PSA (Olympus, Japan) was used as a cantilever. The resonant frequency was ~9 kHz.

Size Exclusion Chromatography—The molecular mass of TAβ was determined using a Superose 12 size exclusion column (1 × 30 cm; GE Healthcare) equilibrated with phosphate-buffered saline (pH 7.4) at a flow rate of 0.5 ml/min. Thirty-five fractions were collected and analyzed by dot blotting using anti-Oligo.

Preparation of Synaptosomes—Synaptosomes were prepared as previously described (43). A hippocampus or a whole brain minus the hippocampus was homogenized in 0.32 M sucrose buffer containing 0.25 mM EDTA. The homogenate was centrifuged at 580 × *g* for 8 min. The supernatant was centrifuged at 145,000 × *g* for 20 min. The resulting pellet was suspended in 0.32 M sucrose buffer without EDTA and layered over Ficoll in sucrose buffer. Following centrifugation at 87,000 × *g* for 30

min, the synaptosome-rich interface was removed and recentrifuged to remove any remaining Ficoll.

RNA Interference—Stealth™ small interfering RNA (siRNA) duplex oligoribonucleotides against PC12 cell TrkA (GenBank™ number NM_021589) and the p75 neurotrophin receptor ($p75^{NTR}$) (GenBank™ number NM_012610) were synthesized by Invitrogen. The siRNA sequences used were as follows: rTrkA-siRNA (position 1370) sense (5′-GCCCUCCUCCUAGUGCUCAACAAAU-3′) and antisense (5′-AUUUGUUGAGCACUAGGAGGAGGGC-3′); rTrkA-siRNA-control sense (5′-GCCCUCCGAUCUCGUCAACAUCAAU-3′) and antisense (5′-AUUGAUGUUGACGAGAUCGGAGGGC-3′); rp75-siRNA (position 1212) sense (5′-CAGCCUGAACAUAUAGACUCCUUUA-3′) and antisense (5′-UAAAGGAGUCUUAUAUGUUCAGGCUG-3′); rp75-siRNA-control sense (5′-CAGGUAAACAUAUAGUCCCUCCUUA-3′) and antisense (5′-UAAGGAGGGACUAUAUGUUUACCUG-3′). The control siRNA had a random sequence. siRNA oligonucleotides were transfected into PC12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

RESULTS

Toxicity of Aβ Assembly Formed from Arctic-type Aβ—We treated primary neurons with seed-free wild- or Arctic-type Aβ, which had been preincubated for 2 h in the absence or presence of GM1 ganglioside (10 or 20% molar ratio in the lipids composing liposomes). Unexpectedly, extensive neuronal death was observed in the culture treated with Arctic-type Aβ, which had been preincubated for 2 h in the presence of GM1 ganglioside at a 10% molar ratio in liposomes (Fig. 1*A*). The extent of neuronal death under this condition was greater than that under any other conditions examined in this study (Fig. 1, *A* and *B*).

To quantitatively characterize the toxic Aβ assembly, we examined its toxicity against NGF-treated PC12 cells (PC12N cells). We found that PC12N cells are also sensitive to the toxic Aβ assembly formed from Arctic-type Aβ (Fig. 1*C*). We performed an LDH release assay of cultures of PC12N cells under various conditions. The level of LDH released from the PC12N cells, which were treated with the toxic Aβ assembly, increased depending on Aβ dose (Fig. 1*D*), GM1 ganglioside dose (Fig. 1*E*), and the duration of the exposure of the cells to the toxic Aβ assembly (Fig. 1*F*). In regard to the time course of Aβ preincubation with GM1 ganglioside, the level of released LDH increased with peak value at 2 h and then decreased in conjunction with an increase in the ThT fluorescence intensity of the incubation mixtures (Fig. 1*G*).

The Toxic Aβ Assembly Is Soluble—Importantly, the toxicity of the Aβ incubated in the presence of GM1 ganglioside was observed exclusively in the supernatant obtained by ultracentrifuging the incubation mixture (Fig. 2*A*), suggesting that the toxic Aβ assembly is soluble. To examine the possibility that a TAβ is formed in the presence GM1 ganglioside, we performed dot blotting using an oligomer-specific antibody (anti-Oligo) (23). TAβ in the incubation mixtures was readily recognized by anti-Oligo (Fig. 2*B*). The specificity of TAβ recognition by anti-Oligo was confirmed by the finding that TAβ toxicity was significantly neutralized by coincubating the mixtures with anti-Oligo in the cultures of PC12N cells and primary neurons (Fig. 2*C*). However, coincubation with a monoclonal antibody (4396C), which inhibits Aβ fibrillogenesis through binding to GM1 ganglioside-bound Aβ as a seed (40), failed to inhibit the induction of TAβ toxicity (Fig. 2*D*).

TAβ Formation from Wild-type Aβ—We then examined whether TAβ is also formed from wild-type Aβ (Aβ40). We first investigated how TAβ is formed from wild-type Aβ in the presence of liposomes containing GM1 ganglioside. Interestingly, TAβ is favorably formed from wild-type Aβ in the presence of GM1 ganglioside at a 15% molar ratio in liposomes (Fig. 3*A*). TAβ toxicity was not significant in the nanomolar range of Aβ (Fig. 3*B*).

Biophysical and Structural Features of TAβ—To determine the biophysical and structural features of TAβ, we performed SDS-PAGE of the incubation mixtures containing TAβ. However, no high molecular weight bands corresponding to possible Aβ assemblies were detected. Bands were observed only after cross-linking pretreatment with glutaraldehyde (Fig. 4*A*), consistent with previous findings showing that soluble Aβ assemblies are probably degraded by denaturing gel electrophoresis (6) unless they are cross-linked (44, 45). A morphological analysis of TAβ by electron microscopy failed to detect any definite structure under conditions in which protofibrils, which had been prepared as previously reported (30), were readily detectable (Fig. 4*B*). In contrast, spherical particles with diameters of 10–20 nm, along with rod-shaped structures, were observed by AFM in the supernatant obtained by ultracentrifuging the incubation mixtures containing TAβ (Fig. 4*C*). We then determined the molecular mass of TAβ by size exclusion chromatography, which was followed by dot blotting using anti-Oligo. The immunoreactivity was recovered as a single peak with relative molecular masses of 200–300 kDa (Fig. 4*D*). The recovery of TAβ immunoreactivity in the same fraction was also observed in the incubation mixture containing wild-type Aβ (Aβ40) and GM1 ganglioside at a 15% molar ratio in liposomes (Fig. 4*D*). Furthermore, the collected peak showed a significant toxicity against PC12N cells (Fig. 4*E*).

TAβ Formation in the Presence of Natural Neuronal Membranes—Next, we tested whether TAβ can be formed in the presence of natural neuronal membranes. We incubated Arctic-type Aβ in the presence of synaptosomes prepared from brains of mice from three different age groups. The degree of TAβ formation was significantly higher in the incubation mixture containing synaptosomes prepared from the hippocampus of aged (2-year-old) mouse brains than in any other incubation mixtures, including those containing synaptosomes from the hippocampus or the whole brain minus the hippocampus from younger (1-month-old and 1-year-old) mouse brains (Fig. 5*A*). To determine the possibility that an alteration in the lipid composition of neuronal membranes, particularly GM1 ganglioside, underlies the acceleration of TAβ formation, we determined the levels of GM1 ganglioside, cholesterol, and phospholipids in synaptosomes prepared from hippocampi of young (1-month-old) and aged (2-year-old) mouse brains. Notably, the GM1 ganglioside level significantly increased, whereas cholesterol level significantly decreased with age (Fig. 5*B*).

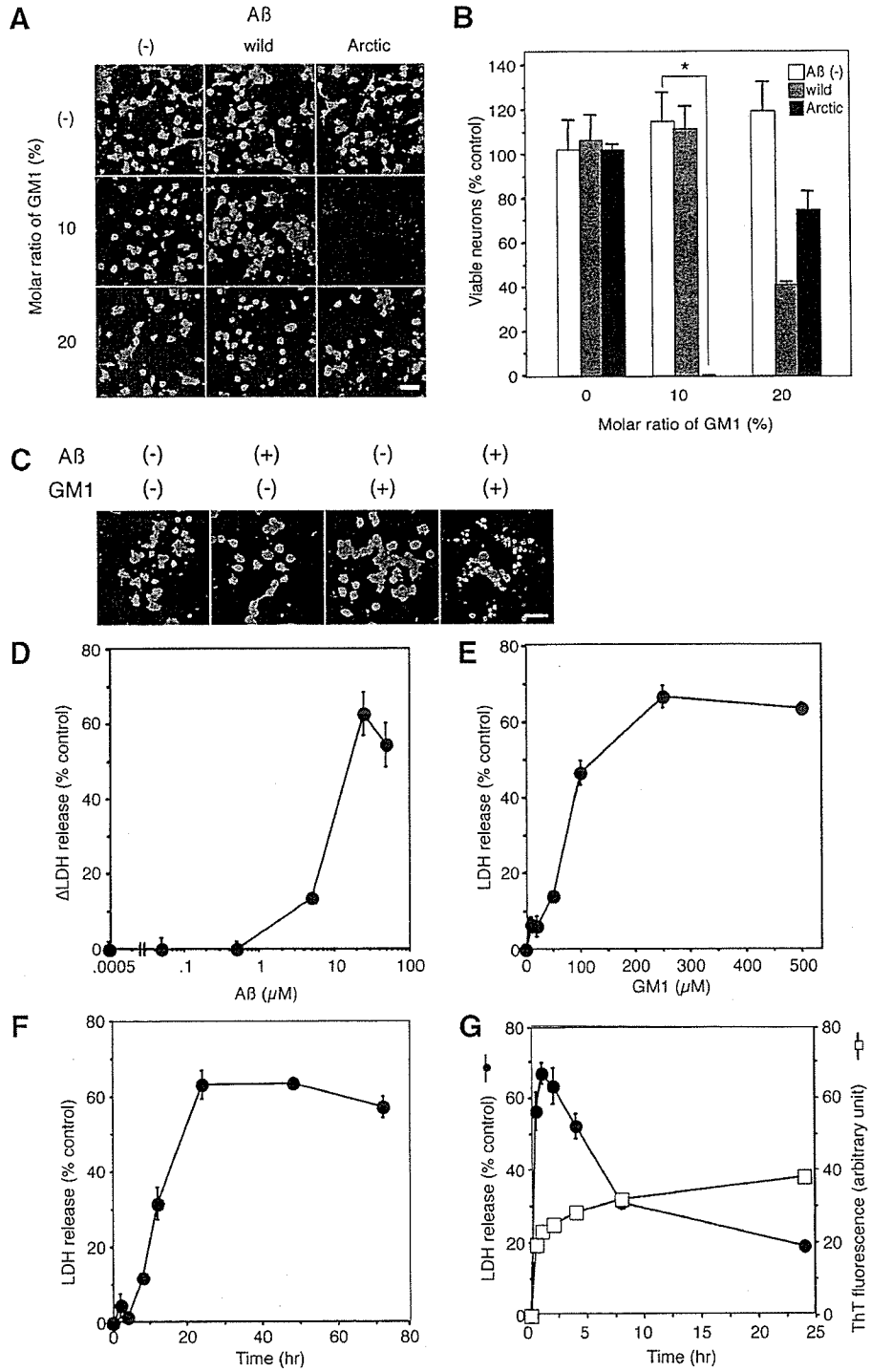

FIGURE 1. **Toxicity of Aβ assembly formed in the presence of GM1 ganglioside against primary neurons and PC12N cells.** *A*, primary cortical neurons cultured for 48 h in serum-free N2-supplemented medium were treated at 37 °C for 48 h with incubation mixtures containing seed-free wild-type Aβ (Aβ40) or Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of GM1 ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM; the molar ratio of GM1 ganglioside in liposomes varied as indicated. Neurons were stained with calcein AM (Invitrogen)/ethidium homodimer, showing green staining for viable cells and *red* staining for dead cells. *Bar*, 50 μm. *B*, the number of viable neurons in the culture shown in *A* was determined. Each *column* indicates the average of three percentages ± S.D. relative to that of control cultures in which neither Aβ nor GM1 ganglioside was added. *, $p < 0.0001$ (one-way analysis of variance combined with Scheffe's test). *C*, representative images of NGF-treated PC12 (PC12N) cells treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of GM1 ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM, and the molar ratio of GM1 ganglioside in liposomes was 10%. *Bar*, 50 μm. *D* and *E*, dose-response curves for the level of LDH released from cells treated with incubation mixtures containing Aβ, which had been preincubated as described in *C*. The concentrations of Aβ and GM1 ganglioside varied as indicated. The LDH value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. *D*, the points indicate LDH levels in the incubation mixtures containing GM1 ganglioside minus those lacking GM1 gangliosides, which were negligible below 25 μM Aβ. *F* and *G*, time course curves for level of LDH released from the cells treated with incubation mixtures containing Aβ, which had been preincubated as described in *A*. The durations of cell treatment (*F*) and Aβ preincubation in the presence of GM1 ganglioside (*G*) varied as indicated. ThT fluorescence intensities in the incubation mixtures are also shown in *G*. *D–G*, each point indicates the average of four values ± S.D.

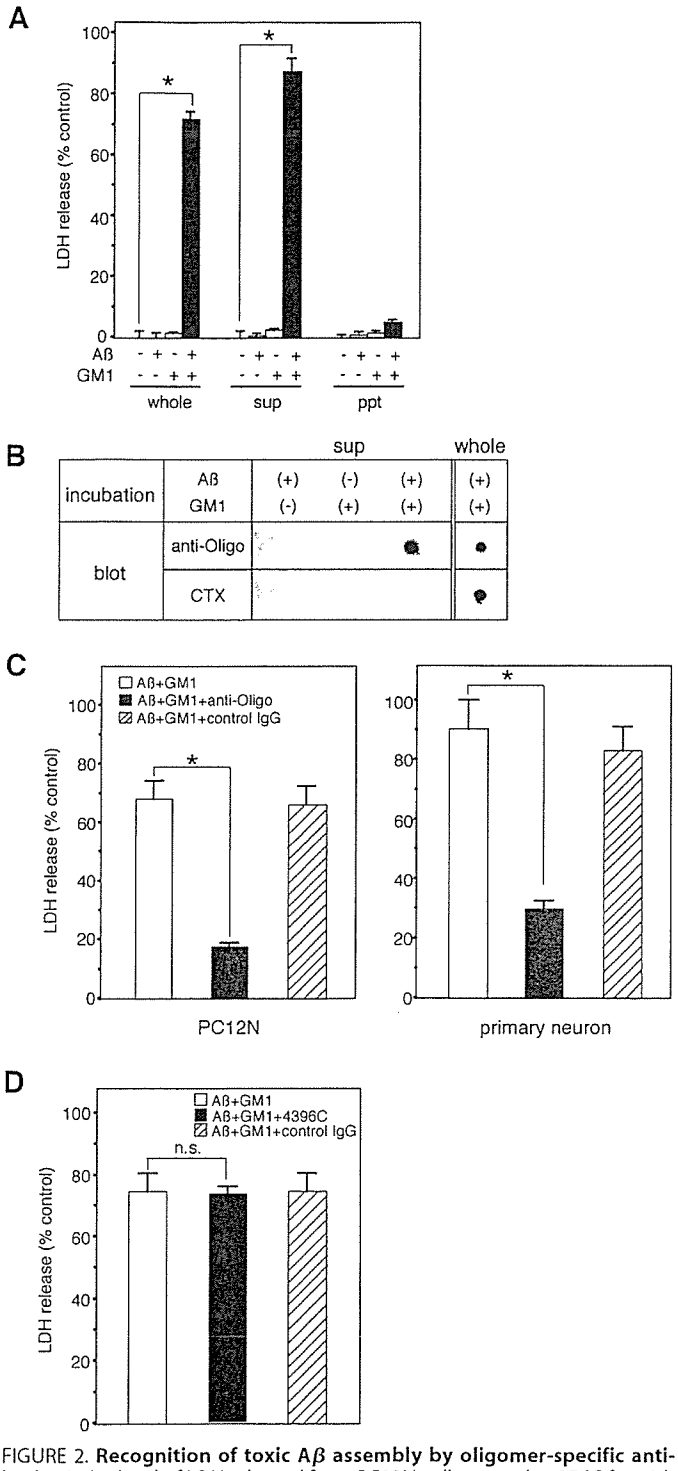

FIGURE 2. **Recognition of toxic Aβ assembly by oligomer-specific antibody.** *A*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with supernatant (*sup*) or precipitate (*ppt*) obtained by ultracentrifuging (540,000 × *g*, 15 min) incubation mixtures (*whole*) containing Arctic-type Aβ (*Aβ40*) at final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). Each value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$. *B*, dot blot analysis of supernatant (*sup*) obtained by ultracentrifuging incubation mixtures (*whole*) containing Arctic-type Aβ alone, GM1 ganglioside alone, or Arctic-type Aβ plus GM1 ganglioside. The blots were reacted with anti-Oligo (BIOSOURCE Inc., Camarillo, CA) or cholera toxin subunit B-horseradish peroxidase conjugate (Sigma) (*CTX*). *C*, the level of LDH released from PC12N cells and primary neurons treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the presence of GM1 ganglioside and anti-Oligo. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$. *D*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with Arctic-type Aβ, which had been preincubated in the presence of GM1 ganglioside and 4396C. Each column indicates the average of three values ± S.D. *n.s.*, not significant.

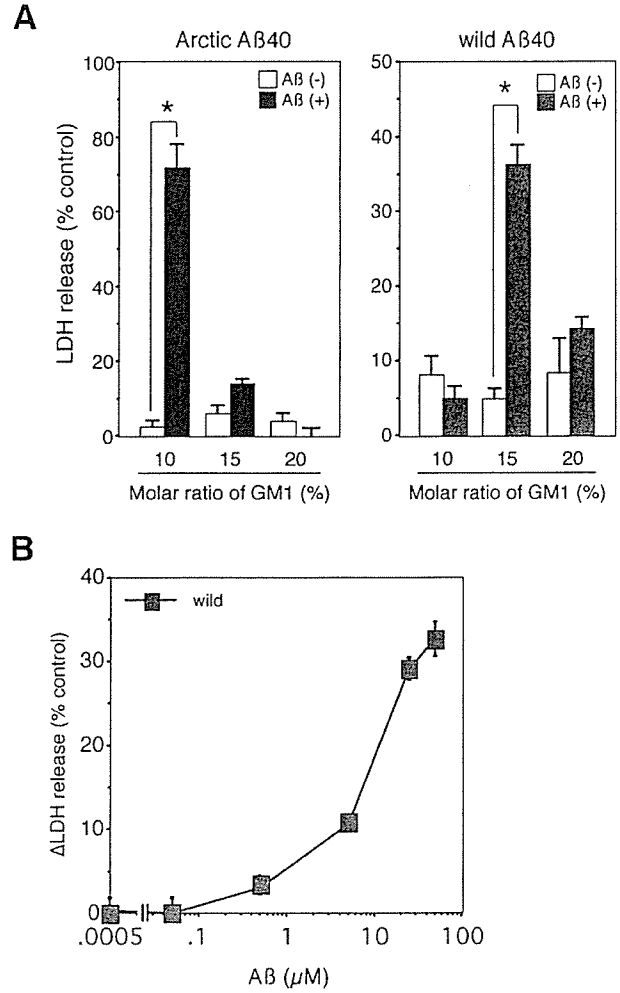

FIGURE 3. **TAβ formation from wild-type Aβ.** *A*, the level of LDH released from NGF-treated PC12 (PC12N) cells treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40), wild-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM for 2 h at 37 °C in the presence of GM1-ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM, and the molar ratio of GM1 ganglioside in liposomes varied as indicated. Each value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. Each column indicates the average of three values ± S.D. *, $p < 0.0001$. *B*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with incubation mixtures containing wild-type Aβ at various concentrations, which had been preincubated in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 15%). Each point indicates the LDH level in the incubation mixtures containing GM1 ganglioside minus that of the incubation mixtures lacking GM1 gangliosides, which was negligible below 25 μM for wild-type Aβ.

Putative Mechanism Underlying TAβ-induced Neuronal Death—To characterize cell death induced by TAβ, we performed nuclear staining with a membrane-permeable dye, Hoechst 33258. PC12N cells, which were treated with incubation mixtures containing TAβ for 12 h, showed characteristics of apoptotic changes, including retracted neurites, shrunken

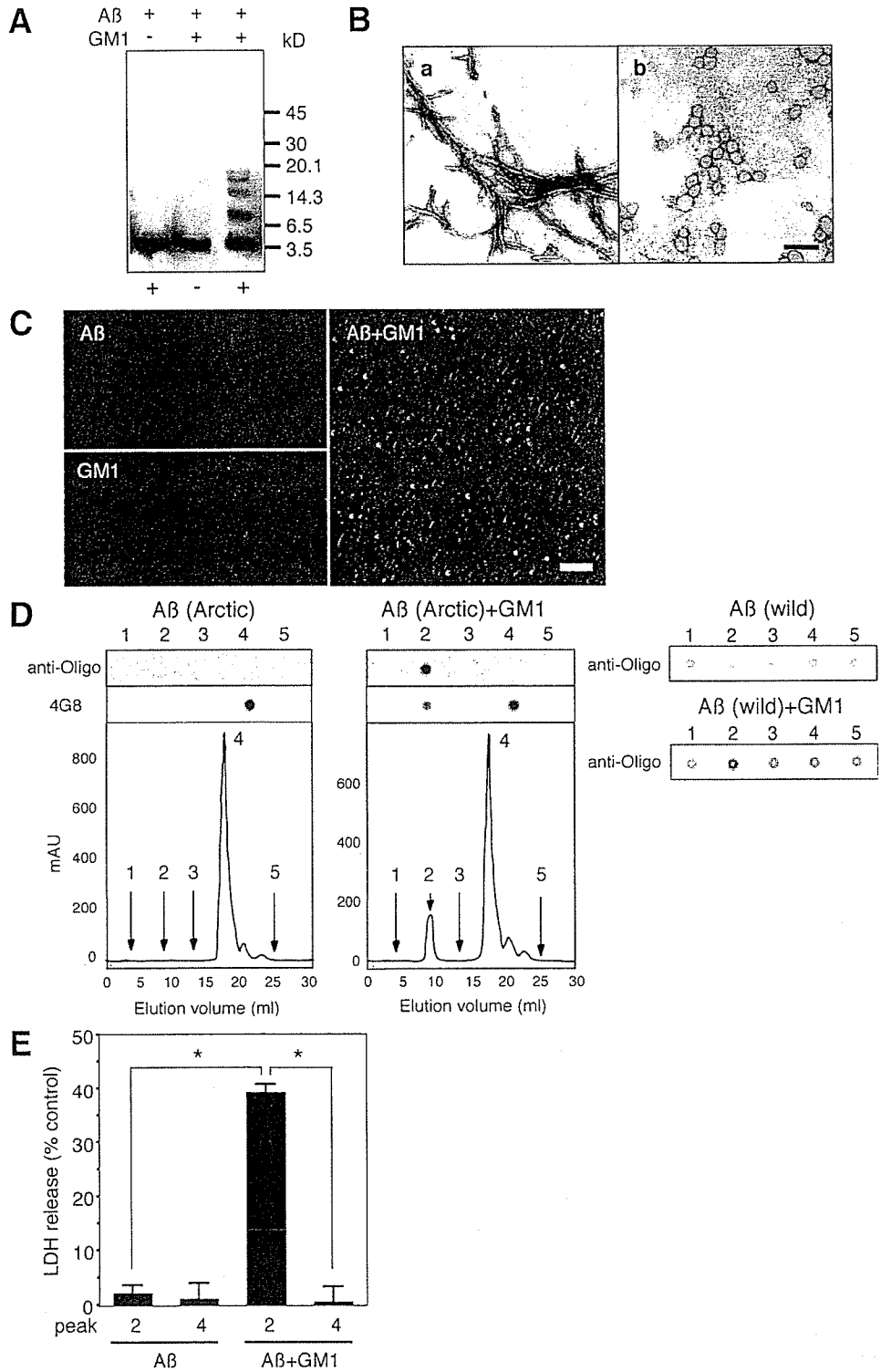

FIGURE 4. **Biophysical and structural analyses of TAβ.** *A*, Western blot of supernatants of incubation mixtures containing Arctic-type Aβ (Aβ40), which had been incubated at 50 μM and 37 °C for 24 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). Ten nanograms of Aβ in the incubation mixtures was subjected to SDS-PAGE (4–20% gradient gel) with (+) or without (−) cross-linking pretreatment using glutaraldehyde. The blot was reacted with 4G8. *B*, electron micrographs of incubation mixture containing Arctic-type Aβ preincubated to allow protofibril formation (*a*) or of incubation mixture containing TAβ formed from Arctic-type Aβ (*b*). Typical protofibril structures were observed in *a*; however, no definite structures aside from liposomes were observed in *b*. *Bar*, 100 nm. *C*, AFM image of fraction containing TAβ formed from Arctic-type Aβ. The supernatant obtained by ultracentrifuging (540,000 × *g*, 3 h) the incubation mixture containing TAβ was subjected to AFM. Spherical particles along with rod-shaped structures were observed. No definite structures were observed in the supernatants of incubation mixtures containing Arctic-type Aβ alone or GM1 ganglioside alone. The amplitude range is 0.1 V. *Bar*, 200 nm. *D*, size exclusion chromatography of incubation mixtures containing Aβ, which had been preincubated in the absence or presence of GM1 ganglioside, on a Superose 12 column. Elution samples from 35 fractions were dot-blotted on nitrocellulose membranes. The blot was reacted with anti-Oligo or 4G8. The immunoreactivity with anti-Oligo was recovered as a single peak with an apparent molecular mass of 200–300 kDa. Five representative fractions are shown. *Peaks 2* and *4* correspond to fractions containing TAβ and monomeric Aβ, respectively. *mAU*, milli-absorbance unit. *E*, toxicities of peaks (*2* and *4*) collected from incubation mixtures containing Arctic-type Aβ (shown in *D*) against PC12N cells. Each *column* indicates the average of three values ± S.D.*, $p < 0.0001$.

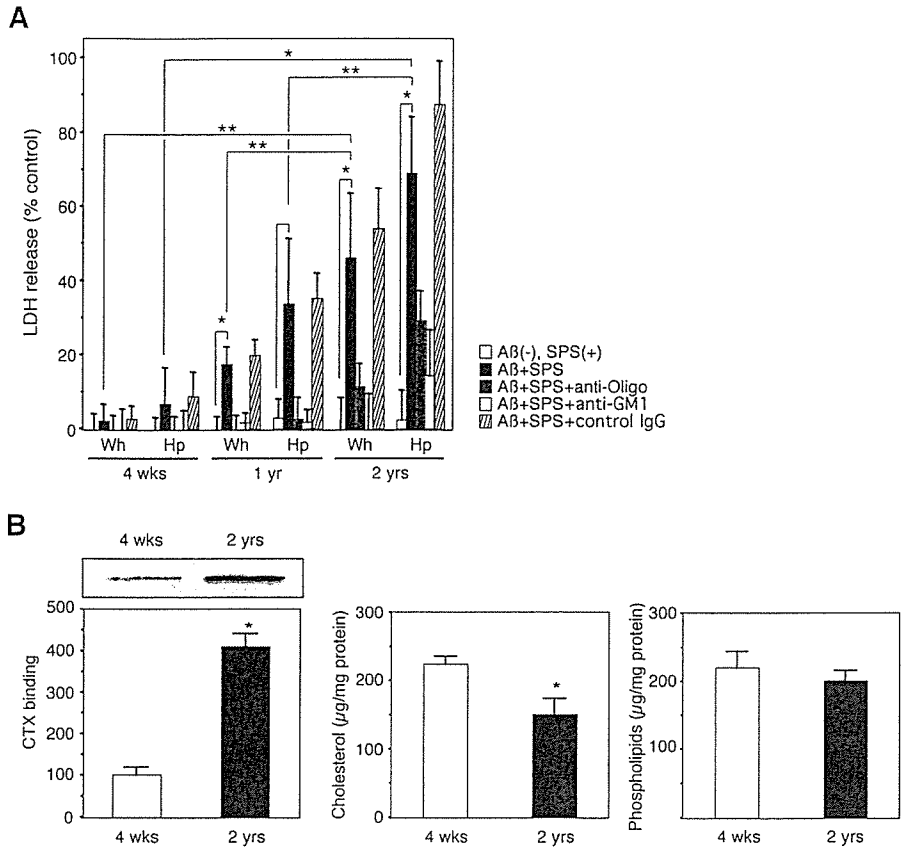

FIGURE 5. **TAβ formation from Arctic-type Aβ incubated in the presence of synaptosomes.** *A*, TAβ formation was assessed by LDH release assay of PC12N cell cultures treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of synaptosomes (*SPS*) prepared from brains of mice of three different age groups with or without anti-Oligo or an antibody specific to GM1 ganglioside (Calbiochem). *Wh*, whole brain minus hippocampus; *Hp*, hippocampus. Each *column* indicates the average of four values ± S.D. *, $p < 0.0001$; **, $p < 0.005$. *B*, lipid composition of synaptosomes prepared from young (1-month-old) and aged (2-year-old) mouse brains. GM1 ganglioside levels were determined by densitoscanning the blot following incubation with cholera toxin. Levels of cholesterol and phospholipids were determined using Determiner L (Kyowa, Tokyo, Japan) and phospholipids C (Wako, Osaka, Japan), respectively. Each *column* indicates the average of four values ± S.D. *, $p < 0.0001$.

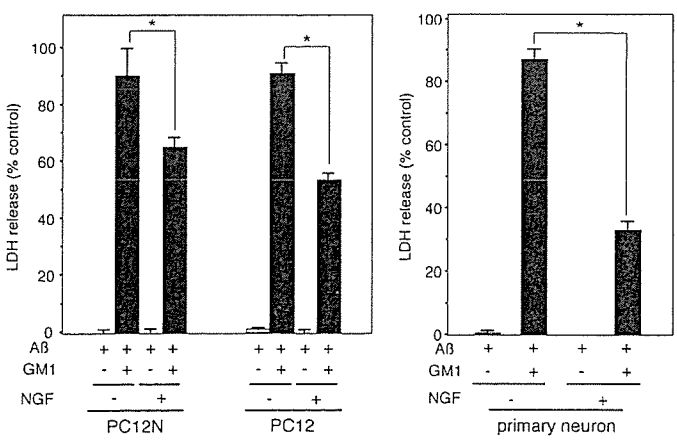

FIGURE 6. **Suppression of TAβ toxicity by the addition of exogenous NGF.** NGF-treated PC12 (PC12N), native PC12 cells, and primary neurons were treated with the incubation mixture containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%) and exogenous NGF (100 ng/ml). TAβ toxicity was assessed by an LDH release assay in these cultures. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$.

cell bodies, and the condensation and fragmentation of nuclei in conjunction with an increase in the level of LDH released from TAβ-treated PC12N cells (data not shown). To determine if TAβ toxicity is mediated by NGF receptors, we first treated PC12N cells, native PC12 cells, and primary neurons with TAβ in the presence of exogenous NGF. In these cultures, cell death was markedly prevented (Fig. 6). We then knocked down the NGF receptors, including TrkA and $p75^{NTR}$, of PC12 cells, SY5Y cells, and primary neurons using specific siRNAs. The knockdown of $p75^{NTR}$ or TrkA markedly suppressed the cell death induced by TAβ in these cultures (Fig. 7).

DISCUSSION

Here, we show that a highly toxic soluble Aβ assembly (TAβ) can be formed more rapidly and to a greater extent from Arctic-type Aβ than from wild-type Aβ. Notably, TAβ formation requires GM1 ganglioside at certain densities. TAβ is probably formed via a pathway different from one that leads to amyloid fibril formation. Biophysical and structural analyses by AFM and size exclusion chromatography revealed that TAβ is spherical with diameters of 10–20 nm and molecular masses of 200–300 kDa. The most striking feature of TAβ is its unique toxicity. Our results suggest that TAβ induces the NGF receptor-mediated apoptosis of cultured cells.

Accumulating evidence suggests that soluble Aβ assemblies are formed as intermediates en route to amyloid fibril formation. This scenario is mainly supported by the formation of soluble Aβ assemblies early during the incubation period *in vitro*, which is frequently followed by the appearance of mature fibrils (5, 6, 8, 13). Indeed, certain inhibitors of Aβ fibrillogenesis are potent for blocking the generation of Aβ oligomers (46). In this study, TAβ was preferably formed in the presence of GM1 ganglioside at lower densities than those required for amyloid fibril formation (36). Furthermore, a monoclonal antibody specific to a seed for amyloid fibril formation (40) failed to inhibit TAβ formation. These results suggest that TAβ is formed via a pathway different from a straightforward pathway leading to amyloid fibril formation, as was previously suggested in the formation of other soluble Aβ assemblies (11, 12).

In this study, monomeric Arctic-type Aβ was converted to TAβ more rapidly and to a greater extent than wild-type Aβ. The propensity of Arctic-type Aβ to form toxic nonamyloid Aβ assemblies has recently attracted interest (13, 30, 34); however,

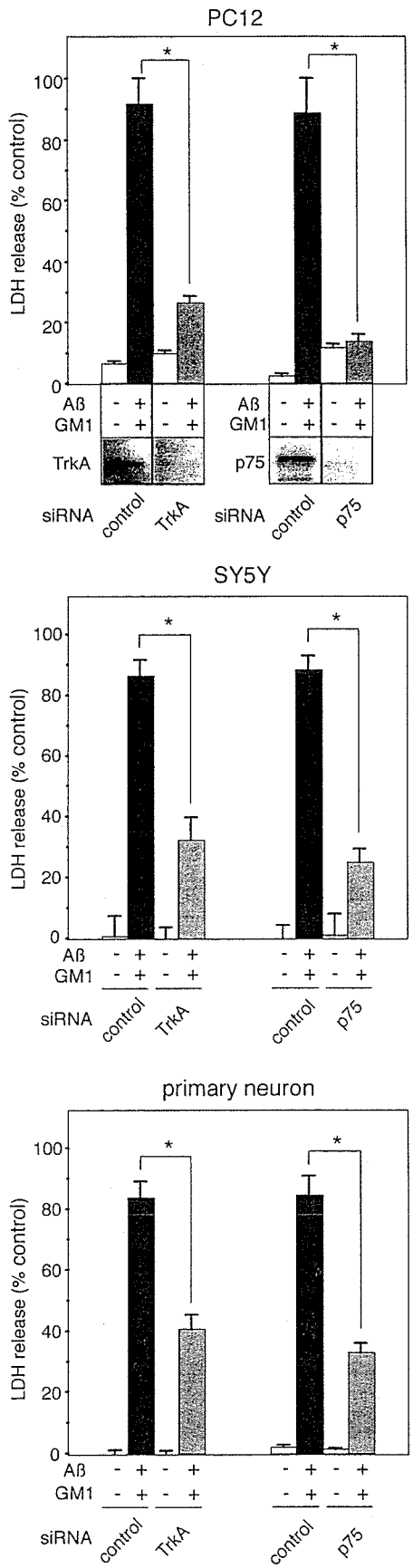

FIGURE 7. **TAβ toxicity mediated by NGF receptors.** PC12 cells, SY5Y cells, and primary neurons, which had been treated with siRNAs against TrkA or $p75^{NTR}$, were exposed to incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). TAβ toxicity, which was assessed by LDH release assay, was markedly suppressed by the knockdown of TrkA or $p75^{NTR}$. Decreases in TrkA and $p75^{NTR}$ expression levels were confirmed by Western blotting of cell lysates using anti-TrkA and anti-$p75^{NTR}$ antibodies, respectively. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$.

it remains to be clarified how the assembly of Arctic-type Aβ is accelerated compared with that of wild-type Aβ. We previously found that Aβ fibrillogenesis from Arctic-type Aβ is also enhanced in the presence of SDS as well as GM1 ganglioside (36). Thus, taken together with the results of this study, it is likely that the negatively charged membrane surface is a preferred environment for Arctic-type Aβ to form soluble and insoluble assemblies. A previous study suggested that the lateral distribution of GM1 ganglioside affects the spatial arrangements of the oligosaccharide chain of a molecule (47). Thus, the conformation of GM1 ganglioside may be modulated at certain densities, providing a favorable microenvironment for TAβ formation.

Results of this study imply that GM1 ganglioside potently accelerates the formation of not only amyloid fibrils but also the soluble Aβ assembly. It has recently been reported that Aβ oligomerization is induced in the presence of lipid rafts isolated from brain tissues and cultured cells in a ganglioside-dependent manner (48). Although further studies are necessary, it may be assumed that GM1 ganglioside-rich membrane microdomains, such as lipid rafts, provide a favorable environment that facilitates the formation of soluble Aβ assemblies, including Aβ oligomers and dimers (49).

In this study, the incubation of Arctic-type Aβ with synaptosomes prepared from aged mouse brains markedly induced TAβ formation. Furthermore, the level of GM1 ganglioside significantly increased, whereas that of cholesterol significantly decreased with age. Our observation of an age-dependent alteration in lipid composition of neuronal membranes is in agreement with the result of a recent study of cerebral cortices of AD brains (38). Taking this together with our recent observation that the level of GM1 ganglioside in synaptosomes increases not only with age but also with the expression of apolipoprotein E4 (37), it is possible that TAβ can be formed in the brain in association with the risk factors for AD development.

It was previously reported that Aβ-derived diffusible ligands potently alter NGF-mediated signaling in cultured cells (11). Moreover, many previous studies suggested that Aβ toxicities emerge through the association with $p75^{NTR}$ (50–56) (for a review, see Refs. 57–59). In particular, it is noteworthy that Aβ toxicity mediated by $p75^{NTR}$ depends on a death domain (60) in the cytoplasmic part of $p75^{NTR}$ molecules (56). Evidence indicates the dual function of $p75^{NTR}$: one for survival and the other for death (61) (for a review, see Refs. 57 and 58). Furthermore, a previous study revealed that heteromeric TrkA-$p75^{NTR}$ complexes have different functions from homo-oligomeric TrkA or $p75^{NTR}$ alone (62). Notably, the knockdown of either TrkA or $p75^{NTR}$ is sufficient for suppressing TAβ toxicity. Thus, it may be assumed that the function of heteromeric TrkA-$p75^{NTR}$ complexes is

perturbed by TAβ binding to $p75^{NTR}$ or TrkA, leading to apoptosis through the activation of the death domain of $p75^{NTR}$ (for a review, see Ref. 58). However, it should be noted that conflicting evidence also exists; the expression of $p75^{NTR}$ protects against the toxicity of soluble Aβ assembly or extracellular Aβ (63, 64). These opposite conclusions imply that the signaling pathways of $p75^{NTR}$ are complicated and that the functions of $p75^{NTR}$ vary depending on cell type and context (for a review, see Ref. 57).

To date, various soluble Aβ assemblies with diverse structural features have been detected in a broad range of *in vitro* and *in vivo* studies, which employed different techniques in preparing or isolating such assemblies. As previously reported (11, 65), Aβ assembles into multiple alternative structures. Thus, at this point, it is difficult to determine whether TAβ is identical to or distinct from previously identified soluble Aβ assemblies. However, on the basis of its biophysical features, including its SDS disaggregatability and unsuccessful detection on a carbon-coated grid by EM, TAβ probably differs from previously reported Aβ assemblies, particularly protofibrils, because most protofibrils appear to adsorb equally onto carbon-coated grids (65); moreover, no TAβ is detected by EM under conditions in which protofibrils are readily detected. One interesting soluble Aβ assembly is Aβ*56 (25). Aβ*56 may be a candidate Aβ assembly responsible for plaque-independent cognitive decline in AD; however, its biophysical features, including molecular mass and marked stability in SDS-PAGE, make it distinct from TAβ.

Finally, this study indicates a novel pathological implication of soluble Aβ assemblies. It is well documented that early and severe neuronal loss in the cholinergic basal forebrain in AD is probably responsible for cognitive decline in AD patients. Previous studies suggested that cholinergic phenotype alone is unlikely to be a sufficient condition for inducing neuronal death in AD. Certain cholinergic neurons, such as those in the pontomesencephalon, are unaffected in AD (66). Notably, cholinergic neurons in the pontomesencephalon are free of NGF receptors, whereas those in the basal forebrain, which are early and severely affected in AD, have NGF receptors (67). Taken together, our results suggest that soluble Aβ assemblies, such as TAβ, are responsible for the loss of NGF-dependent neurons in the cholinergic basal forebrain in AD. A future challenge is the production of a monoclonal neutralizing antibody against TAβ toxicity, which would provide promising therapeutic strategies, as suggested by *in vitro* and *in vivo* studies that selectively targeted Aβ oligomers (68, 69).

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