Discussion

The clinical trials to prove the neuroprotective function of rasagiline and (−)deprenyl were reported, but the results are still contradicting, and biomarkers to estimate the progression of neuronal loss should be invented (Michell et al., 2004). The markers for the disease progression and treatment efficiency are based on clinical evaluation of symptoms, PET and SPECT imaging, transcranial ultrasound and some biochemical tests. However, blood tests for PD progression are limited to monitor the pathogenic factors, such as increased oxidative stress (malondialdehyde, superoxide radicals, 8-hydrox-2′-deoxyguanosine), or the reduced complex I (Schapira et al., 1990) and increased MAO-B activity in platelets (Zhou et al., 2001). (−)Deprenyl may reverse the increase in MAO-B and the subsequent reduction of β-phenylethylamine in plasma, but these markers represent MAO inhibitory function of (−)deprenyl, but not the neuroprotective activity. α-Synuclein and its phosphorylated proteins were proposed as the markers, but the recent results did not support this view. In CSF, increased levels of 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-guanosine and malondialdehyde were detected (Abe et al., 2003). However, these markers do not present information for progression of selective neuronal loss in PD.

At present, mechanistic makers for factors intervening the disease progress may be the only available markers to assess the neuroprotective potency. As described above, rasagiline induces GDNF in cultured cells, suggesting that the levels of neurotrophic factors specific for dopamine neurons may be used as markers. Indeed, we examined the change in neurotrophic factors in monkey CSF after systemic treatment of rasagiline (Maruyama et al., in preparation). The results proved the validity of our view, which was supported further by the analyses of the CSF from Parkinsonian patients before and after treatment of (−)deprenyl, even the limited number of the samples (Maruyama et al., in preparation). We are now examining the candidates of the biomarkers for the neuroprotective function in serum and CSF from Parkinsonian patients and primate models.

Recently, an increasing number of evidences indicate that rasagiline and related compounds can ameliorate pathogenic processes in AD and other neurodegenerative disorders. Rasagiline analogues with inhibitor potency to cholinesterase, TV 3326, and its *S* enantiomer TV 3279 were reported to regulate the processing of amyloid precursor protein (APP) and increase the soluble APP secretion through activation of α-secretase activity and the reduction of holo-APP protein (Youdim et al., 2003; Yogev-Falach et al., 2006). Their results suggest that propargylamines intervene the pathogenic processes in neurodegenerative disorders in general and ameliorate the disease process.

The stereo-chemical and enantiomeric specificity of the propargylamine for their neuroprotective activity suggests the occurrence of the target protein in mitochondria and other cell components. The identification of the binding site of neuroprotective propargylamines may give us a clue to find the most adequate chemical structure for the function, and develop new drugs that intervene the transcription of the cell death-regulating genes in the central nervous system.

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A Ganglioside-induced Toxic Soluble Aβ Assembly

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

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Naoki Yamamoto[‡§], Etsuro Matsubara[‡], Sumihiro Maeda[¶], Hirohisa Minagawa[‡], Akihiko Takashima[¶], Wakako Maruyama[‖], Makoto Michikawa[‡], and Katsuhiko Yanagisawa[‡1]

From the Departments of [‡]Alzheimer's Disease Research and [‖]Geriatric Medicine, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu 474-8522, Japan, the [§]Japan Society for the Promotion of Sciences, Tokyo 102-8472, Japan, and the [¶]Laboratory for Alzheimer's Disease, RIKEN Brain Science Institute, Wako 351-0198, Japan

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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[1] To whom correspondence should be addressed: Dept. of Alzheimer's Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu 474-8522, Japan. Tel.: 81-562-44-5651 (ext. 5002); Fax: 81-562-44-6594; E-mail: katuhiko@nils.go.jp.

[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*).

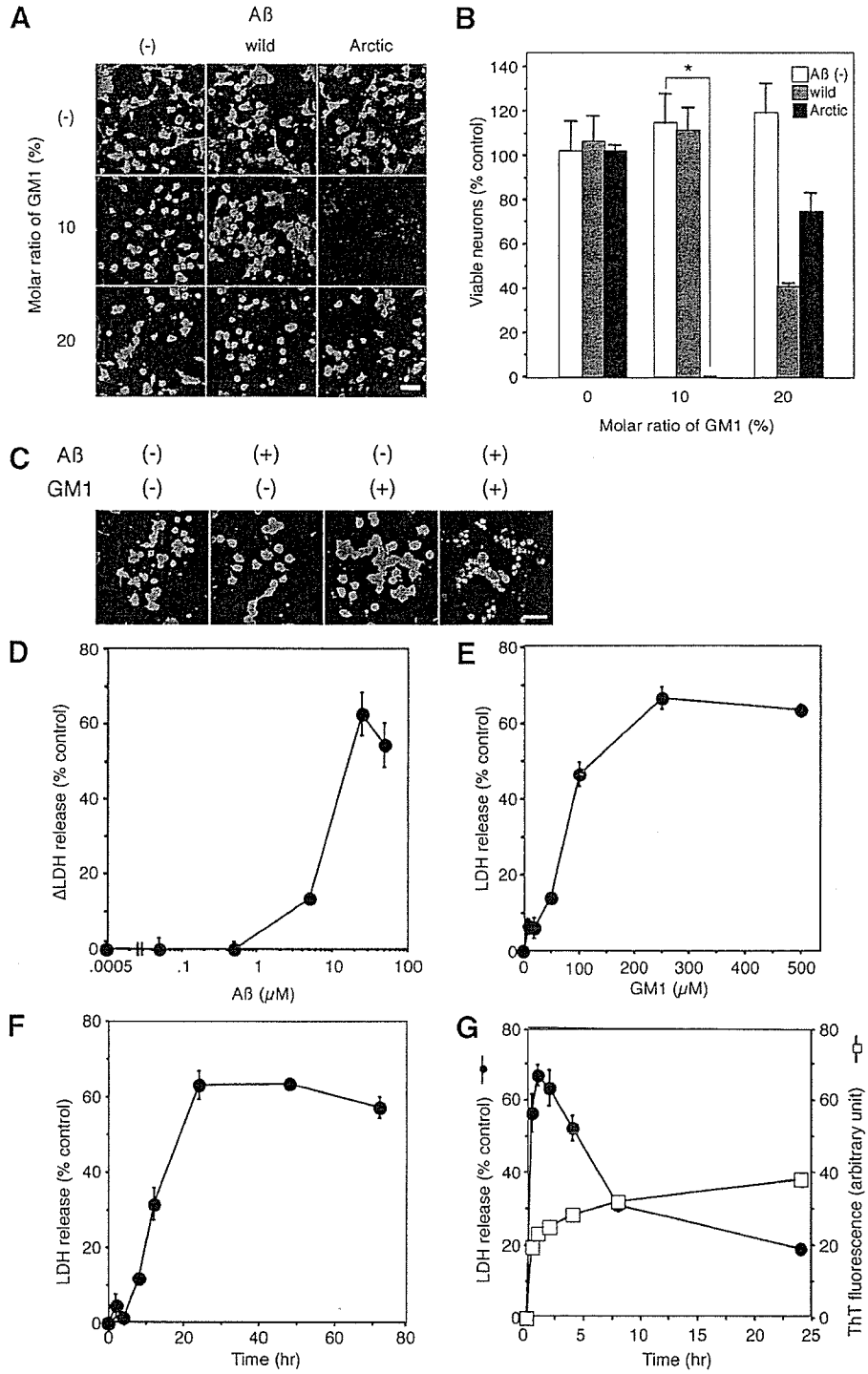

FIGURE 1. **Toxicity of Aβ assembly formed in the presence of GM1 ganglioside against primary neurons and PC12N cells.** *A*, primary cortical neurons cultured for 48 h in serum-free N2-supplemented medium were treated at 37 °C for 48 h with incubation mixtures containing seed-free wild-type Aβ (Aβ40) 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.

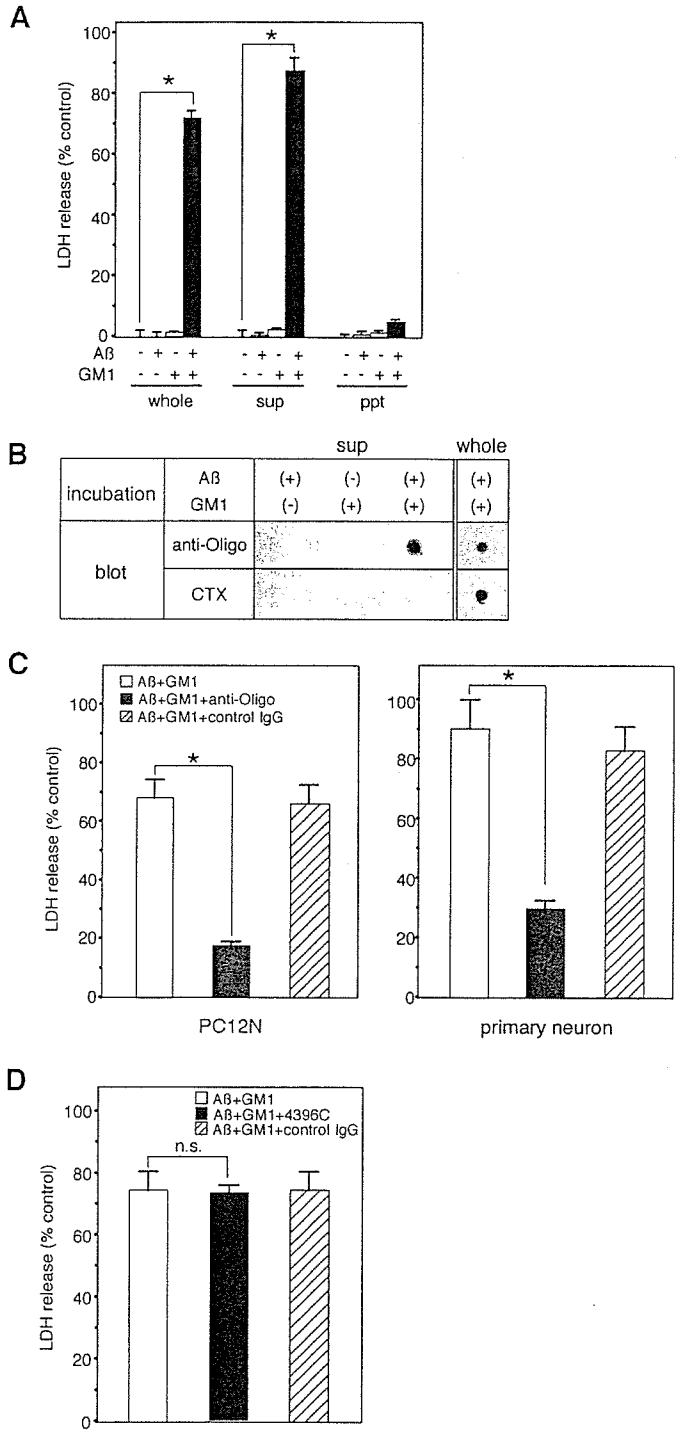

FIGURE 2. **Recognition of toxic Aβ assembly by oligomer-specific antibody.** *A*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with supernatant (*sup*) or precipitate (*ppt*) obtained by ultracentrifuging (540,000 × *g*, 15 min) incubation mixtures (*whole*) containing Arctic-type 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.

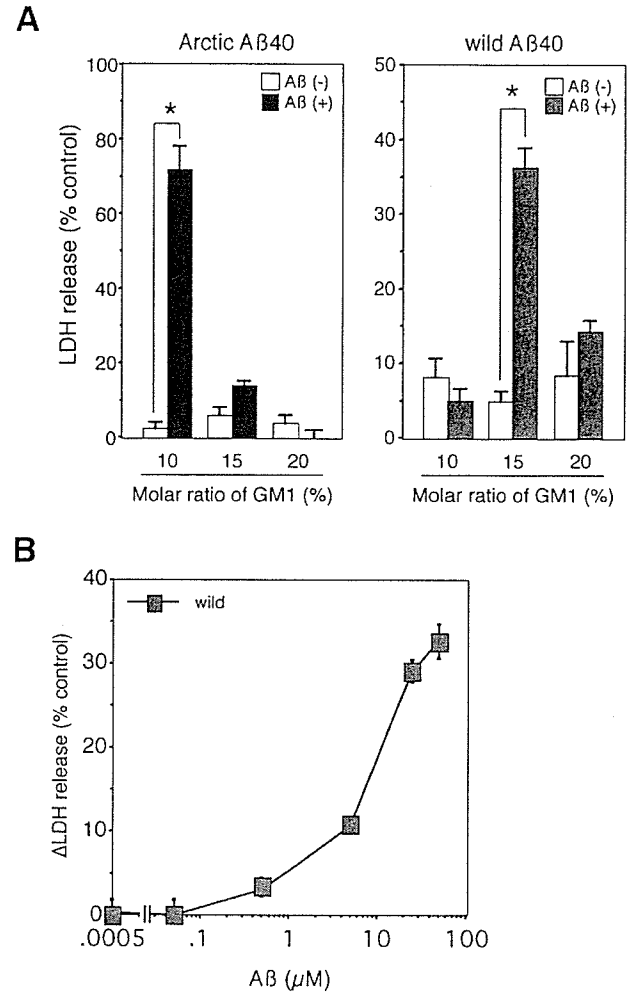

FIGURE 3. **TAβ formation from wild-type Aβ.** *A*, the level of LDH released from NGF-treated PC12 (PC12N) cells treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40), wild-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM for 2 h at 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

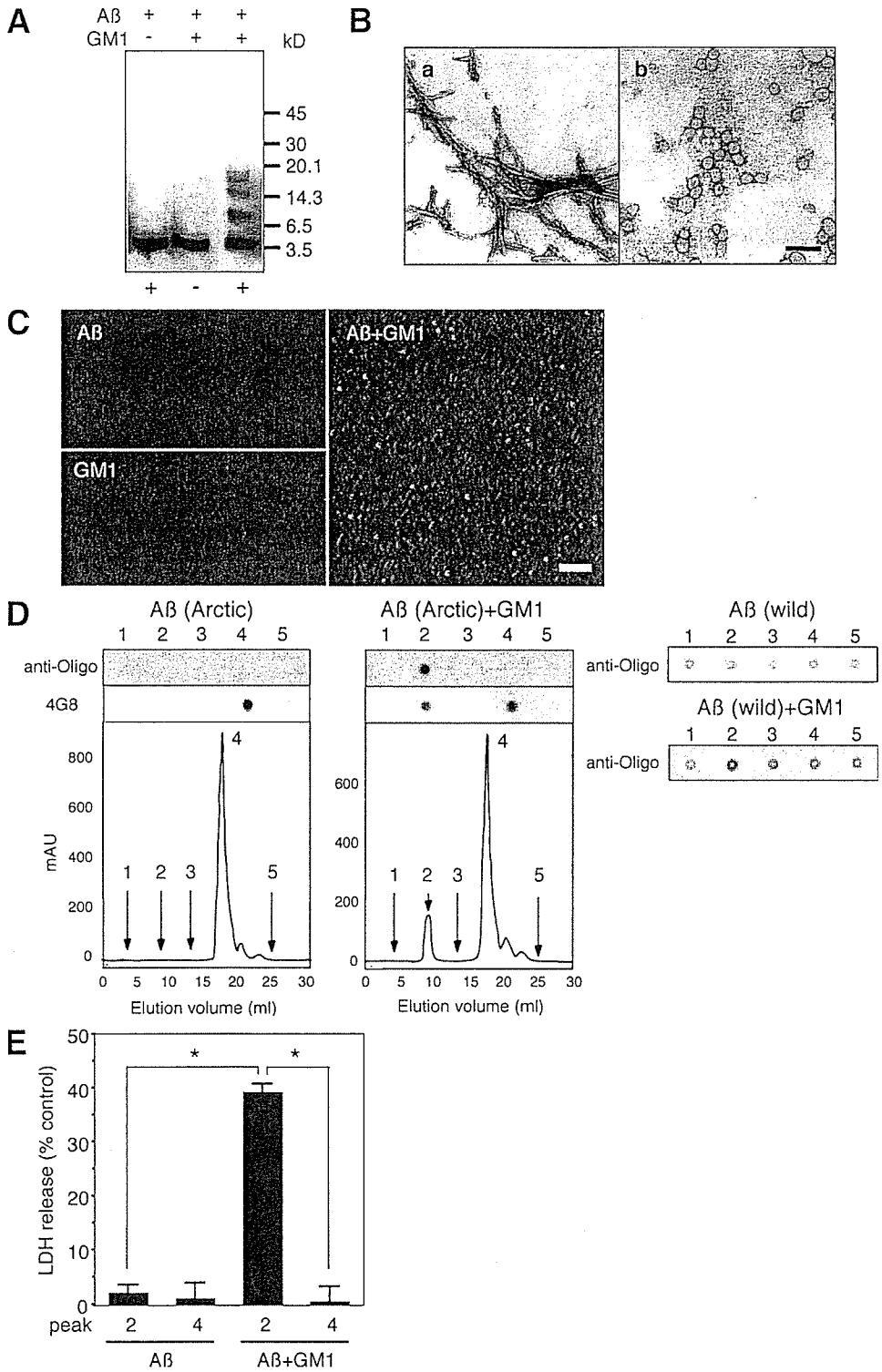

FIGURE 4. **Biophysical and structural analyses of TAβ.** *A*, Western blot of supernatants of incubation mixtures containing Arctic-type Aβ (Aβ40), which had been incubated at 50 μM and 37 °C for 24 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes 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.*, $p < 0.0001$.

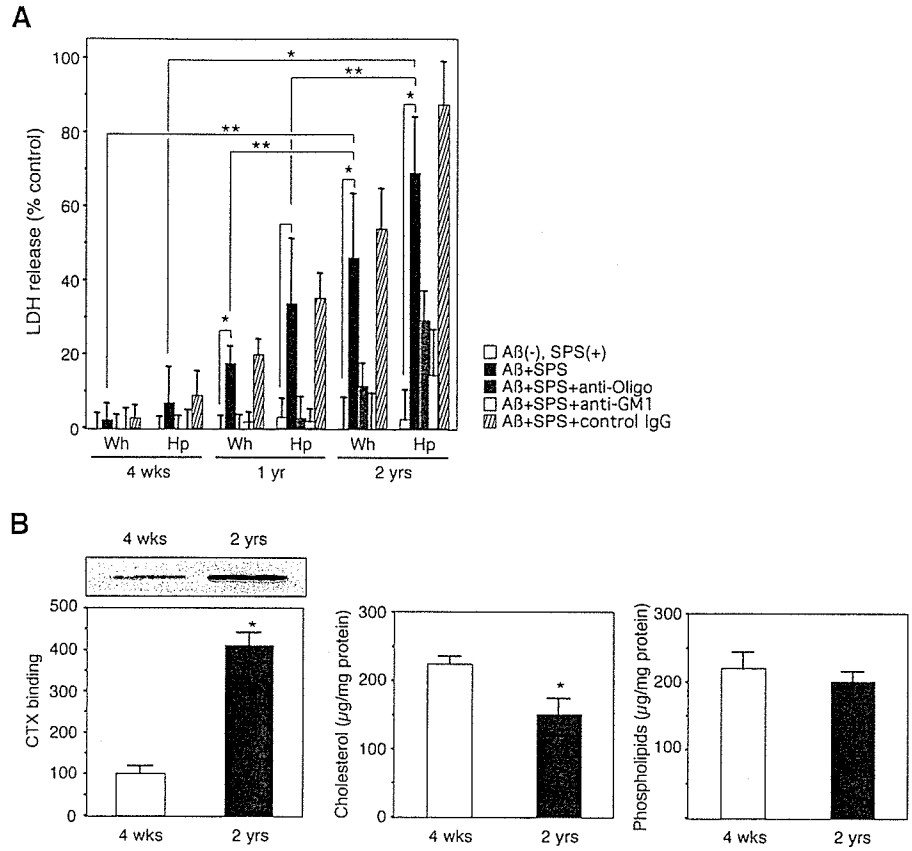

FIGURE 5. **TAβ formation from Arctic-type Aβ incubated in the presence of synaptosomes.** *A*, TAβ formation was assessed by LDH release assay of PC12N cell cultures treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been 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. *, $p < 0.0001$.

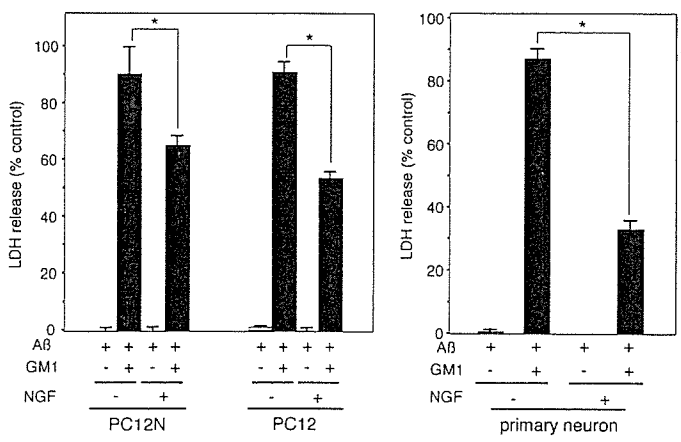

FIGURE 6. **Suppression of TAβ toxicity by the addition of exogenous NGF.** NGF-treated PC12 (PC12N), native PC12 cells, and primary neurons were treated with the incubation mixture containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 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,

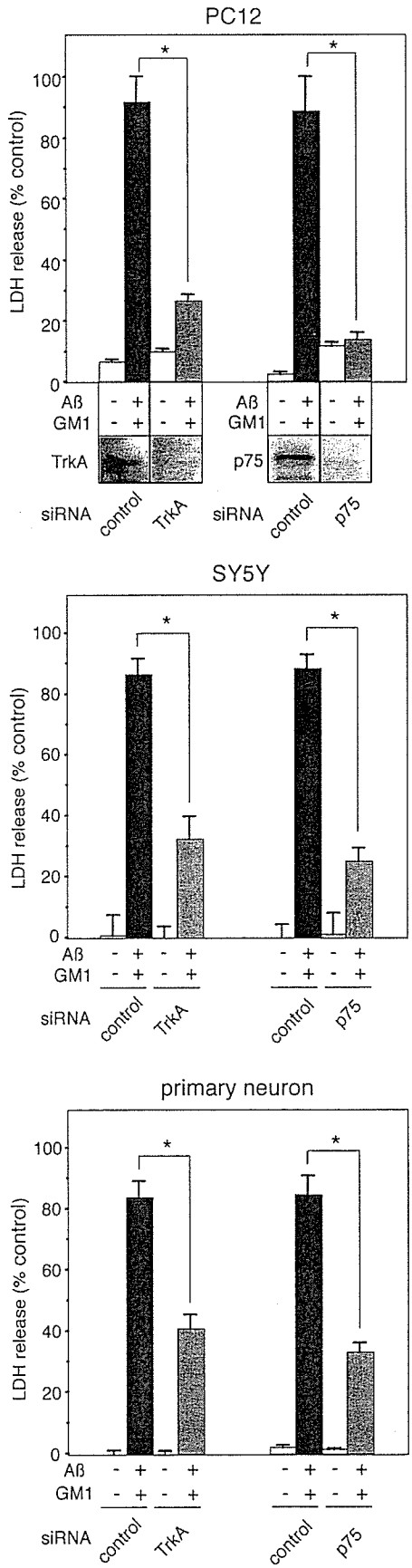

FIGURE 7. **TAβ toxicity mediated by NGF receptors.** PC12 cells, SY5Y cells, and primary neurons, which had been treated with siRNAs against TrkA or $p75^{NTR}$, were exposed to incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 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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Review

Mitochondrial membrane permeability transition and cell death

Yoshihide Tsujimoto *, Takashi Nakagawa, Shigeomi Shimizu

Osaka University Medical School, Department Medical Genetics, SORST of Japan Science and Technology Agency, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstract

Mitochondria are important organelles for energy production, Ca^{2+} homeostasis, and cell death. In recent years, the role of the mitochondria in both apoptotic and necrotic cell death has received much attention. In apoptotic and necrotic death, an increase of mitochondrial membrane permeability is considered to be one of the key events, although the detailed mechanism remains to be elucidated. The mitochondrial membrane permeability transition (MPT) is a Ca^{2+}-dependent increase in the permeability of the mitochondrial membrane that leads to loss of $\Delta\psi$, mitochondrial swelling, and rupture of the outer mitochondrial membrane. The MPT is thought to occur after the opening of a channel, which is termed the permeability transition pore (PTP) and putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin D (Cyp D: a mitochondrial peptidyl prolyl-*cis*, *trans*-isomerase), and other molecule(s). Our studies of mice lacking Cyp D have revealed that it is essential for occurrence of the MPT and that the Cyp D-dependent MPT regulates some forms of necrotic cell death, but not apoptotic death. We have also shown that two anti-apoptotic proteins, Bcl-2 and Bcl-x_L, block the MPT by directly inhibition of VDAC activity. Here we summarize a role of the MPT in cell death.

Keywords: Mitochondria; Permeability transition; Cell death

1. Introduction

Apoptosis is the best-characterized form of programmed cell death and an outline of its molecular basis is now well understood. Mammalian cells possess two major apoptotic signaling pathways, which are known as the intrinsic pathway and the extrinsic pathway [1]. Mitochondria play a crucial role in the intrinsic pathway: an increase of outer membrane permeability leads to release of proteins from the intermembrane space into the cytoplasm, including apoptogenic molecules such as cytochrome *c*, Smac/Diablo, HtrA2 (Omi), AIF, and DNaseG [1,2]. In the presence of ATP (dATP), cytochrome *c* binds to Apaf-1 and triggers its oligomerization, after which pro-caspase-9 is recruited and undergoes autoactivation. Thus, an increase in the permeability of the outer mitochondrial membrane is central to apoptosis [3,4], and membrane permeability is directly regulated by the Bcl-2 family of proteins [4,5] (see Fig. 1). However, the detailed mechanisms controlling outer mitochondrial membrane permeability during apoptosis and the exact role of Bcl–2 family members are still to be determined. The initial model used to explain the apoptotic increase of mitochondrial membrane permeability was the "mitochondrial membrane permeability transition" (MPT) [6], which has been known for some time among investigators of the mitochondria.

2. MPT

Under various conditions, such as in the presence of Ca^{2+} together with inorganic phosphate, isolated mitochondria undergo the MPT. This process is characterized by a Ca^{2+}-dependent increase in the permeability of the inner mitochondrial membrane, resulting in the loss of $\Delta\psi$, mitochondrial swelling, and rupture of the outer mitochondrial membrane [7,8] (see Fig. 1). The MPT is thought to occur after the opening of a putative channel complex, which has been termed the permeability transition pore (PTP), and consists of the voltage-dependent anion channel (VDAC: outer membrane channel), the adenine nucleotide translocator (ANT: inner membrane channel), cyclophilin D (Cyp D), and other molecule(s) [9] (see Fig. 2). The exact nature of this complex is still to be determined. A role of ANT in the MPT is supported by inhibition or activation of the MPT by bonkrekic acid and

* Corresponding author.
E-mail address: tsujimoto@gene.med.osaka-u.ac.jp (Y. Tsujimoto).

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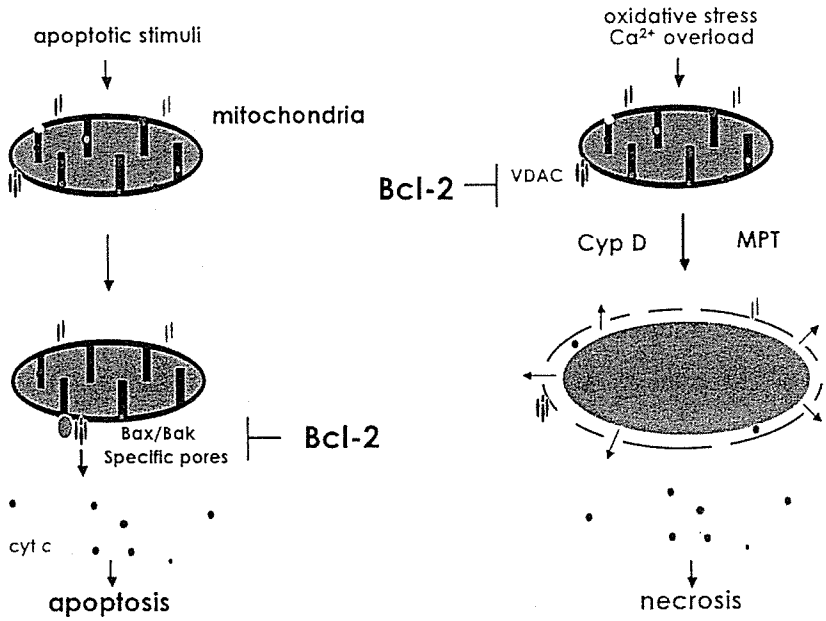

Fig. 1. Involvement of the mitochondria in apoptosis and necrosis. During apoptosis, an increase in the permeability of the outer mitochondrial membrane is crucial and is regulated by multidomain pro-apoptotic members of the Bcl-2 family (Bax and Bak), resulting in the release of several apoptogenic factors into the cytoplasm. In contrast, the Cyp D-dependent MPT (increased permeability of both the outer and inner mitochondrial membranes) is involved in necrosis induced by $Ca2^{+}$ overload and oxidative stress. Both kinds of mitochondrial membrane permeability changes are inhibited by anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x).

atractyloside, which are ligands for ANT [10]. Cyp D is a mitochondrial member of the cyclophilin family, which shows peptidyl prolyl-*cis*, *trans*-isomerase (PPIase) activity and has a crucial role in protein folding [11]. The presumed role of Cyp D in regulating the MPT is based on the observation that cyclosporin A (CsA), a specific inhibitor of the cyclophilin family, blocks the MPT [12]. Cyp D resides in the mitochondrial matrix, but associates with the inner mitochondrial membrane during the MPT. Based on the enzymatic activity of Cyp D (PPIase), it is suggested to induce a conformational change of an inner membrane channel such as ANT, leading to an increase of inner membrane permeability. In addition to the CsA-sensitive and Ca^{2+}-dependent ("regulated") MPT, the existence of a CsA-insensitive and Ca^{2+}-independent ("unregulated") MPT has also been suggested, although its mechanism and relationship to the CsA-sensitive MPT are totally unknown [13].

Some forms of apoptosis can be inhibited by CsA, suggesting a role of the CsA-sensitive MPT in this process of cell death [9,14]. The possible role of the MPT in apoptosis is also supported by the finding that apoptosis is sometimes inhibited by bonkrekic acid [10,15], although difficulty in using bonkrekic acid as a potent inhibitor of apoptosis has often been noted by

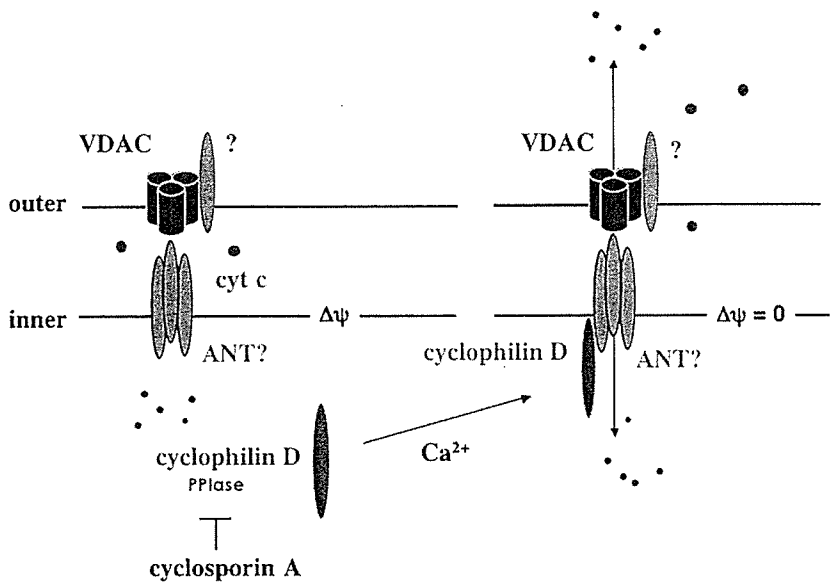

Fig. 2. Putative protein complex mediating the MPT.

many investigators. The CsA-sensitive MPT has also been implicated in the remodeling of mitochondrial cristae and mobilization of cytochrome *c* stores from the cristae during apoptosis, thus promoting the complete release of cytochrome *c* [16]. However, the overall role of the MPT in apoptosis remains controversial because there have been a number of reports that apoptosis is not inhibited by CsA [17]. Furthermore, it has been demonstrated that $\Delta\psi$ follows cytochrome *c* release in at least some types of apoptosis, suggesting that the MPT is not always the cause of cytochrome *c* release and cell death.

3. Essential players in the MPT

It has long been considered that the VDAC, ANT, and Cyp D play an essential role in the MPT, although convincing evidence was lacking until very recently.

VDAC: Experimental evidence for a direct role of the VDAC in the MPT has been provided by studies using anti-VDAC antibodies [18] (Shimizu et al., 2001). Two polyclonal anti-VDAC antibodies were obtained that recognized different epitopes of the channel, and could inhibit VDAC activity as assessed in liposomes [18]. Both of these anti-VDAC antibodies also inhibited the Ca^{2+}-induced MPT [18], supporting a crucial role for VDAC in the MPT.

ANT: The ANTs (ANT1 and 2 in mice and ANT1, 2, and 3 in humans) have also been considered important for occurrence of the MPT. It has been demonstrated that Cyp D directly interacts with ANT, although it is not known whether CsA inhibits the interaction of Cyp D and ANT [19,20]. Regarding the role of ANT in the MPT, considerable progress was made recently: it was shown that liver mitochondria from mice lacking both ANT1 and ANT2 underwent the MPT, although the threshold for Ca^{2+} was slightly increased [21], suggesting that the ANT1/2 played only a limited role, if any, in the MPT or deficiency of ANT1/2 might be compensated by other channel(s). Other channel(s) involved in the MPT might be ANT-like channels on the inner membrane, given that the MPT is modulated by ANT ligands such as bonkrekic acid or atractyloside and that the MPT is accompanied by $\Delta\psi$ loss (increased permeability of the inner mitochondrial membrane). Identification of channel(s) in the inner mitochondrial membrane, which is directly involved in the MPT and might be a target of Cyp D, would be crucial.

Cyp D: A role of Cyp D in the MPT was initially suggested because the MPT is inhibited by CsA, which inhibits the PPIase activity of cyclophilins. This has recently been confirmed by generation of Cyp D gene (*ppif*)-deficient mice [22–25]: Cyp D-deficient mitochondria isolated from mouse livers do not undergo the CsA-sensitive MPT in response to a variety of MPT inducers, including Ca^{2+}, atractyloside, and H_2O_2. Because the MPT does not occur, these mitochondria accumulate a much larger amount of Ca^{2+} than control mitochondria [22,25]. However, these Cyp D-deficient mitochondria still undergo the CsA-insensitive MPT in response to high concentrations of Ca^{2+} [22,24]. In addition, the response to reagents like ubiquinone and thiol oxidants that cause the CsA-insensitive MPT is normal in Cyp D-deficient mitochondria [24]. Thus, Cyp D is specifically involved in the CsA-sensitive MPT.

4. No role of Cyp D-dependent MPT in apoptosis

It has been controversial as to whether the MPT plays an important role in the apoptotic increase of mitochondrial membrane permeability, but recent development of Cyp D-deficient mice has finally solved this issue. Various cells isolated from Cyp D-deficient mice, such as thymocytes, MEFs, and hepatocytes, undergo apoptosis normally in response to various stimuli, including etoposide, stautosporine, and TNFα [22–25], providing the most compelling evidence that the MPT is not essential for apoptosis. These observations certainly do not exclude the possibility that some apoptosis might be mediated by the CsA-sensitive MPT, and thus may be inhibited by CsA. However, the inhibitory effect of CsA on apoptosis might need to be more carefully evaluated because CsA is normally used at relatively high concentrations that could inhibit other targets and have a secondary effect on apoptosis. It may be necessary to re-evaluate the inhibition of apoptosis by using Cyp D-deficient cells or by silencing Cyp D in cells to assess the real effect of CsA.

There have been several reports that overexpression of Cyp D protects cells against some forms of apoptosis. For example, Cyp D overexpression inhibits apoptosis induced by the over-expression of caspase-8 (but not Bax) or by exposure to arsenic trioxide [26,27]. These observations are apparently inconsistent with the findings obtained using Cyp D-deficient cells. It might be possible that apoptosis is mediated by the MPT, which is somehow affected by overexpression of Cyp D in these circumstances. However, studies of transgenic mice with myocardial expression of Cyp D have revealed that cardiac myocytes from these mice are prone to undergo mitochondrial swelling and spontaneous death [23].

5. Involvement of the MPT in necrosis

In contrast to the lack of any impact of Cyp D deficiency on apoptosis, the Cyp D-dependent MPT seems to play an important role in some forms of necrotic cell death (see Fig. 1). It has been shown that Cyp D-deficient MEFs are significantly more resistant to H_2O_2-induced necrosis [22,23], while Cyp D-deficient hepatocytes gain resistance to necrosis induced by a Ca^{2+} ionophore (A23187) or by H_2O_2 [22,23]. Interestingly, when necrosis is inhibited by Cyp D-deficiency in these cells, apoptosis does not occur as an alternate death mechanism [22], suggesting that these cells somehow block the apoptotic signaling pathway activated by H_2O_2 and Ca^{2+} overload.

6. Regulation of the MPT by Bcl-2

Although anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x_L) are known to inhibit the Bax/Bak-dependent apoptotic increase of mitochondrial membrane permeability by direct interaction with pro-apoptotic members of this family, they have also been shown to inhibit the MPT [28,29](see Fig. 1). How do these proteins act to block the MPT? Since Bax/Bak is not essential for the MPT [22], Bcl-2 (Bcl-x) might directly inhibit a component of the PTP complex. This concept is supported by the

observation that Bcl-2 (Bcl-x) can block the VDAC [28], suggesting that Bcl-2 may inhibit the MPT via VDAC blockade. It has also been shown that Bcl-2 can inhibit ANT activity [30] (Marzo et al., 1998b). However, ANT might not be a major player of the MPT [21], as described above, so Bcl-2 might inhibit other unidentified channels similar to ANT that are actually involved in the MPT. Since Bcl-2 resides mainly on the outer mitochondrial membrane, it is likely to act on the MPT by inhibiting VDAC.

7. Future studies

Studies using Cyp D-deficient mice enabled us to provide convincing evidence that the Cyp D-dependent MPT does not play a role in apoptosis. However, there are still many important questions to be answered:

(1) What is the molecular nature of the MPT pore complex? (2) What is the target of Cyp D and how does Cyp D induce the MPT? (3) What is the biological significance of the MPT? (4) What is the relationship between the Cyp D-dependent MPT and the unregulated MPT? (5) Does the unregulated MPT have a role in apoptosis or other forms of cell death? Further studies are needed to answer these important questions.

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ORIGINAL ARTICLE

Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway

K Ito[1], Y Oji[2], N Tatsumi[1], S Shimizu[3], Y Kanai[1], T Nakazawa[1], M Asada[4], T Jomgeow[1], S Aoyagi[1], Y Nakano[1], H Tamaki[4], N Sakaguchi[1], T Shirakata[4], S Nishida[5], M Kawakami[5], A Tsuboi[5], Y Oka[4], Y Tsujimoto[3] and H Sugiyama[1]

[1]Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [2]Department of Biomedical Informatics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [3]Department of Medical Genetics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; [4]Department of Molecular Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan and [5]Department of Cancer Immunotherapy, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

The *WT1* gene is overexpressed in human primary leukemia and a wide variety of solid cancers. The *WT1* gene is alternatively spliced at two sites, yielding four isoforms: 17AA(+)KTS(+), 17AA(+)KTS(−), 17AA(−)KTS(+), and 17AA(−)KTS(−). Here, we showed that 17AA(+)WT1-specific siRNA induced apoptosis in three WT1-expressing leukemia cell lines (K562, HL-60, and Kasumi-1), but not in WT1-non-expressing lymphoma cell line (Daudi). 17AA(+)WT1-specific siRNA activated caspase-3 and -9 in the intrinsic apoptosis pathway but not caspase-8 in the extrinsic one. On the other hand, 17AA(−)WT1-specific siRNA did not induce apoptosis in the three WT1-expressing cell lines. The apoptosis was associated with activation of proapoptotic Bax, which was activated upstream of the mitochondria. Constitutive expression of 17AA(+)WT1 isoforms inhibited apoptosis of K562 leukemia cells induced by apoptosis-inducing agents, etoposide and doxorubicin, through the protection of mitochondrial membrane damages, and DNA-binding zinc-finger region of 17AA(+)WT1 isoform was essential for the antiapoptotic functions. We further studied the gene(s) whose expression was altered by the expression of 17AA(+)WT1 isoforms and showed that the expression of proapoptotic Bak was decreased by the expression of 17AA(+)KTS(−)WT1 isoform. Taken together, these results indicated that 17AA(+)WT1 isoforms played antiapoptotic roles at some points upstream of the mitochondria in the intrinsic apoptosis pathway.
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Correspondence: Dr H Sugiyama, Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, 1-7, Yamada-Oka, Suita, Osaka 565-0871, Japan.
E-mail: sugiyama@sahs.med.osaka-u.ac.jp
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Introduction

The *WT1* gene was originally isolated as a tumor-suppressor gene responsible for Wilms' tumor, a neplasm of the childhood (Call *et al.*, 1990). The *WT1* gene encodes a protein with four zinc-fingers and is considered to be involved in transcriptional regulation of the genes such as PDGF-A chain (Gashler *et al.*, 1992), CSF-1 (Harrington *et al.*, 1993), IGF-II (Drummond *et al.*, 1992), IGF-IR (Werner *et al.*, 1993), and RAR-α (Goodyer *et al.*, 1995) and in RNA metabolism (Larsson *et al.*, 1995; Davies *et al.*, 1998; Niksic *et al.*, 2004). Although the *WT1* gene has been considered as a tumor-suppressor gene, the wild-type *WT1* gene is overexpressed in primary human leukemia (Inoue *et al.*, 1994) and a wide variety of solid cancers, including lung (Oji *et al.*, 2002), colon (Oji *et al.*, 2003c), esophageal (Oji *et al.*, 2004c), breast (Loeb *et al.*, 2001; Miyoshi *et al.*, 2002), thyroid (Oji *et al.*, 2003b), pancreatic ductal cancer (Oji *et al.*, 2004a), head and neck squamous cell carcinoma (HNSCC) (Oji *et al.*, 2003a), astrocytic tumors (Oji *et al.*, 2004b), and bone and soft-tissue sarcoma (Ueda *et al.*, 2003). Moreover, the following findings indicated that the wild-type *WT1* gene played oncogenic roles rather than tumor-suppressor functions in tumorigenesis of various types of cancers (Sugiyama, 2001): (a) high expression levels of *WT1* mRNA correlated with poor prognosis in leukemia (Inoue *et al.*, 1994) and breast cancer (Miyoshi *et al.*, 2002) and with high tumor stage in testicular germ-cell tumors (Harada *et al.*, 1999) and HNSCC (Oji *et al.*, 2003a), (b) growth of *WT1*-expressing leukemia and solid cancer cells was inhibited by the treatment with *WT1* antisense oligomers (Algar *et al.*, 1996; Yamagami *et al.*, 1996; Oji *et al.*, 1999, 2004a, b), (c) block of differentiation but induction of proliferation by constitutive expression of 17AA(+)KTS(+)WT1 isoform in response to granulocyte colony-stimulating factor (G-CSF) in 32D cl3 myeloid progenitor (Inoue *et al.*, 1998) and normal myeloid cells (Tsuboi *et al.*, 1999), (d) bone marrow cells with high expression level of WT1 tended to develop into leukemia in the 7,12-demethylbenz(a)anthracene

(DMVA)-induced rat leukemia (Osaka *et al.*, 1997), and (e) Lck promoter-driven 17AA(+)KTS(−)WT1 isoform-transgenic mice showed block of differentiation in T lymphoid progenitor cells (Li *et al.*, 2003).

The *WT1* gene is alternatively spliced at two sites: 17AA site, which consists of exon 5 of the *WT1* gene, and KTS site, which exists between zinc-fingers 3 and 4, and yields four isoforms: (17AA(+)KTS(+), 17AA(+)KTS(−), 17AA(−)KTS(+), and 17AA(−)KTS(−)), each of which is considered to have different functions. All of the four isoforms were expressed in primary human solid cancers, including lung cancer (Oji *et al.*, 2002), HNSCC (Oji *et al.*, 2003a), and sarcoma (Ueda *et al.*, 2003) and human primary leukemia (Siehl *et al.*, 2000). Among the four WT1 isoforms, WT1 17AA(+)KTS(+) isoform was dominantly expressed in all of the cancers examined. The results showing that constitutive expression of WT1 17AA(+)KTS(+) isoform rescued the growth inhibitory effect of WT1 antisense oligomers on cancer cells (Oji *et al.*, 1999) indicated the contribution of WT1 17AA(+)KTS(+) isoform to the growth of cancer cells (Hubinger *et al.*, 2001). As for the functions of WT1 17AA(+)KTS(−) isoform, the results showing that lck promoter-driven 17AA(+)KTS(−)WT1-transgenic mice blocked differentiation of T lymphoid progenitor cells, indicated the involvement of WT1 17AA(+)KTS(−) isoform in the tumorigenesis of lymphoid malignancy (Li *et al.*, 2003). As for 17AA(−)KTS(−) isoform, the results showing that 17AA(−)KTS(−)WT1 isoform induced G1 arrest in osteosarcoma cell lines (Englert *et al.*, 1997) and inhibited G1/S progression and accelerated differentiation in 32D cl3 murine myeloid progenitor in response to G-CSF (Loeb *et al.*, 2003) suggested tumor-suppressor roles in some tumors. As for 17AA(−)KTS(+)WT1 isoform, its functions remain unclear. These results indicate that each of the WT1 isoforms has different functions and play important roles in leukemogenesis and tumorigenesis.

Cancers are characterized by abnormal control of proliferation. The *WT1* gene is involved in the promotion of cell cycle, as it was indicated by findings that suppression of WT1 expression induced G2/M or G1 block in human leukemia K562 cells and HER2/neu-overexpressing breast cancer cells, respectively (Yamagami *et al.*, 1998; Tuna *et al.*, 2005). Moreover, 17AA(+)KTS(−)WT1 isoform increased the expression levels of antiapoptotic gene Bcl-2 in G401 rhabdoid cells (Mayo *et al.*, 1999), indicating that the *WT1* gene might be involved in suppression of apoptosis. However, there is neither a direct evidence of antiapoptotic function of the *WT1* gene nor analysis of precise mechanism of the function.

In the present study, we demonstrate that siRNA specific for 17AA(+)WT1 isoforms induces apoptosis through activation of the intrinsic apoptosis pathway in WT1-expressing leukemia cells and that this apoptosis was associated with activation of proapoptotic Bax. Furthermore, stable expression of 17AA(+)WT1 isoforms (17AA(+)KTS(+) and 17AA(+)KTS(−)) inhibited apoptosis induced by apoptosis-inducing agents, etoposide and doxorubicin, through inhibition of mitochondrial damages in leukemia cells.

Results

17AA(+)WT1-specific siRNA induces apoptosis in WT1-expressing leukemia cells

To examine the roles of 17AA(+)WT1 or 17AA(−)WT1 isoforms in protection of leukemia cells from apoptosis, 17AA(+)WT1- and 17AA(−)WT1-specific siRNA vectors were constructed. RT–PCR and Western blot analysis showed that transient expression of 17AA(+)WT1- and 17AA(−)WT1-specific siRNA vectors specifically suppressed expression of 17AA(+)WT1 and 17AA(−)WT1 isoforms, respectively, in K562 cells (Figure 1A). Three WT1-expressing leukemia cell lines (K562, HL-60, and Kasumi1) and one WT1-non-expressing lymphoma cell line (Daudi) were transfected with 17AA(+)WT1-specific siRNA, 17AA(−)WT1-specific siRNA, or control mock vector and incubated for 16 h. Annexin V-propidium iodide (PI) two-color flow cytometry showed that transient expression of 17AA(+)WT1-specific siRNA induced apoptosis in all the three WT1-expressing leukemia cell lines, but not in WT1-non-expressing lymphoma cell line (Figure 1B). On the other hand, transient expression of 17AA(−)WT1-specific siRNA did not induce apoptosis in any of the three WT1-expressing leukemia cell lines and in one WT1-non-expressing lymphoma cell line. Furthermore, the cells treated with the 17AA(+)WT1-specific siRNA were analysed for mitochondrial cytochrome *c* release and loss of mitochondrial membrane potential (MMP) by Western blot and flow cytometry, respectively. Transient expression of 17AA(+)WT1-specific siRNA induced mitochondrial cytochrome *c* release and loss of MMP in WT1-expressing K562 cells, but not in WT1-non-expressing Daudi cells (Figure 1C and D). Furthermore, 17AA(+)WT1-specific siRNA vector-transfected cells were labeled by expression of GFP protein using Gene Silencer pGSU6 shRNA Vector Kit system. As shown in Figure 1E, apoptosis was induced only in the 17AA(+)WT1-specific siRNA vector-transfected cells, but not in the cells that were not transfected with 17AA(+)WT1-specific siRNA vector. These results indicated the antiapoptotic roles of 17AA(+)WT1 isoforms in WT1-expressing leukemia cells.

Transfection of 17AA(+)WT1-specific siRNA induce apoptosis through activation of intrinsic but not extrinsic apoptosis pathway

17AA(+)WT1-specific siRNA vector was transiently expressed in K562 cells for 16 h in the presence or absence of caspase inhibitors. Annexin V-PI two-color flow cytometry showed that pan-caspase inhibitor zVAD-fmk inhibited the apoptosis, indicating that 17AA(+)WT1-specific siRNA induced apoptosis in a caspase-dependent manner (Figure 2A). Block of apoptogenic signals by a caspase-3 inhibitor Ac-DEVD-CHO, at a point where the intrinsic apoptosis pathway and the

extrinsic one meet, and of apoptogenic signals by caspase-9 inhibitor Ac-LEHD-CHO, at a point downstream of the mitochondria in the intrinsic pathway, inhibited apoptosis induced by 17AA(+)WT1-specific siRNA. On the other hand, block of apoptogenic signals by caspase-8 inhibitor Ac-IETD-CHO at a point in the extrinsic pathway did not inhibit the apoptosis.

To examine the activation of apoptosis pathway by 17AA(+)WT1-siRNA, activities of caspase-3, -8, and -9 were examined by fluorometric assay at various time points after transfection of 17AA(+)WT1-specific siRNA. Activities of caspase-3 and -9 were significantly increased in 17AA(+)WT1-specific siRNA-transfected K562 cells compared to mock vector-transfected K562 cells. However, activity of caspase-8 was not increased compared to mock vector-transfected K562 cells (Figure 2B). Moreover, to examine whether the extrinsic pathway was involved in the mitochondrial damages induced by 17AA(+)WT1-specific siRNA, mitochondrial cytochrome *c* release was analysed in the presence or absence of caspase inhibitors 16 h after the transfection of 17AA(+)WT1-specific siRNA (Figure 2C). Western blot analysis showed that block of apoptogenic signals by caspase-8 inhibitor, Ac-IETD-CHO, at a branching point from the extrinsic apoptosis pathway to the intrinsic one did not inhibit mitochondrial cytochrome *c* release, confirming that the extrinsic apoptosis pathway was not involved in mitochondrial damages induced by 17AA(+)WT1-specific siRNA.

These results indicated that 17AA(+)WT1-specific siRNA induced apoptosis through activation of the intrinsic apoptosis pathway but not through activation of the extrinsic pathway in leukemia cells, and thus that the 17AA(+)WT1 isoforms played antiapoptotic roles in the intrinsic apoptosis pathway, but not in the extrinsic one.

Transfection of 17AA(+)WT1-specific siRNA activates Bax

Since 17AA(+)WT1 isoforms were shown to play antiapoptotic roles in the intrinsic apoptosis pathway, the effect of suppression of 17AA(+)WT1 isoforms on activation of Bax, which is considered to act as a gateway for various apoptotic signals at the mitochondria, was analysed. The 17AA(+)WT1-specific siRNA vector was transiently expressed in WT1-expressing K562 leukemia cells for 16 h, and oligomerization of Bax was examined by Western blot analysis. As shown in Figure 2D, transfection of 17AA(+)WT1-specific siRNA induced dimerization and oligomerization of Bax in K562 leukemia cells. These results indicated that 17AA(+)WT1 protein acted to inhibit apoptosis at some point upstream of mitochondria in the intrinsic apoptosis pathway.

Stable expression of 17AA(+)WT1 isoforms protects leukemia cells from apoptosis induced by apoptosis-inducing agents through stabilization of mitochondrial membrane potential

To confirm that 17AA(+)*WT1* isoforms had antiapoptotic functions, K562 cell clones that stably expressed one each of four WT1 isoforms at high levels were isolated (Figure 3a). These cell clones were treated with apoptosis-inducing agents, etoposide or doxorubicin, for 24 h and analysed for apoptosis. Annexin V-PI two-color flow cytometric analysis showed that stable expression of 17AA(+)WT1 isoforms significantly inhibited etoposide-induced apoptosis in K562 cells (Figure 3b). On the other hand, stable expression of 17AA(−)WT1 isoforms (17AA(−)/KTS(+) and 17AA(−)/KTS(−)WT1 isoforms) did not inhibit etoposide-induced apoptosis. Similarly, stable expression of 17AA(+)WT1 isoforms significantly inhibited doxorubicin-induced apoptosis in K562 cells, but that of 17AA(−)WT1 isoforms did not (Figure 3c). These results confirmed the antiapoptotic functions of 17AA(+)WT1 isoforms in leukemia cells.

Since it was well known that apoptosis-inducing agents such as etoposide and doxorubicin initiated cell death primarily by triggering the mitochondrial (intrinsic) apoptosis pathway, we next examined whether or not stable expression of 17AA(+)WT1 isoforms protected mitochondrial membrane damages induced by etoposide or doxorubicin. K562 cell clones transduced with one each of four WT1 isoforms were treated with etoposide or doxorubicin for 24 h and analysed for mitochondrial cytochrome *c* release and loss of MMP by Western blot and flow cytometry, respectively. Stable expression of 17AA(+)WT1 isoforms significantly blocked etoposide-induced release of cytochrome *c* from mitochondrial membrane (Figure 4a) and inhibited loss of MMP in K562 cells (Figure 4b). On the other hand, stable expression of 17AA(−)WT1 isoforms inhibited neither mitochondrial cytochrome *c* release nor the loss of MMP induced by etoposide. Similarly, stable expression of 17AA(+)WT1 isoforms inhibited both doxorubicin-induced release of cytochrome *c* from mitochondrial membrane and loss of MMP, whereas stable expression of 17AA(−)WT1 isoforms did not (Figure 4c and d). These results indicated that stable expression of 17AA(+)WT1 isoforms stabilized mitochondrial membrane and protected leukemia cells from apoptosis induced by apoptosis-inducing agents.

17AA(+)WT1 isoforms require zinc-finger region for the antiapoptotic functions

To examine subcellular localization of four WT1 isoforms, vectors that expressed polyhistidine-tagged one each of four WT1 isoforms were constructed and stably expressed in K562 leukemia cells (Figure 5a and b). As shown in Figure 5c, it was confirmed that stable expression of polyhistidine-tagged 17AA(+)WT1 isoforms significantly inhibited etoposide-induced apoptosis but that of polyhistidine-tagged 17AA(−)WT1 isoforms did not. Then, localization of polyhistidine-tagged WT1 protein was analysed by immunocytochemistry using an anti His-tag antibody. Confocal microscopic analysis showed that all of the four polyhistidine-tagged WT1 isoforms were detected in the nucleus but not in the cytoplasm and mitochondria in K562 leukemia cells (Figure 5d). These results raised