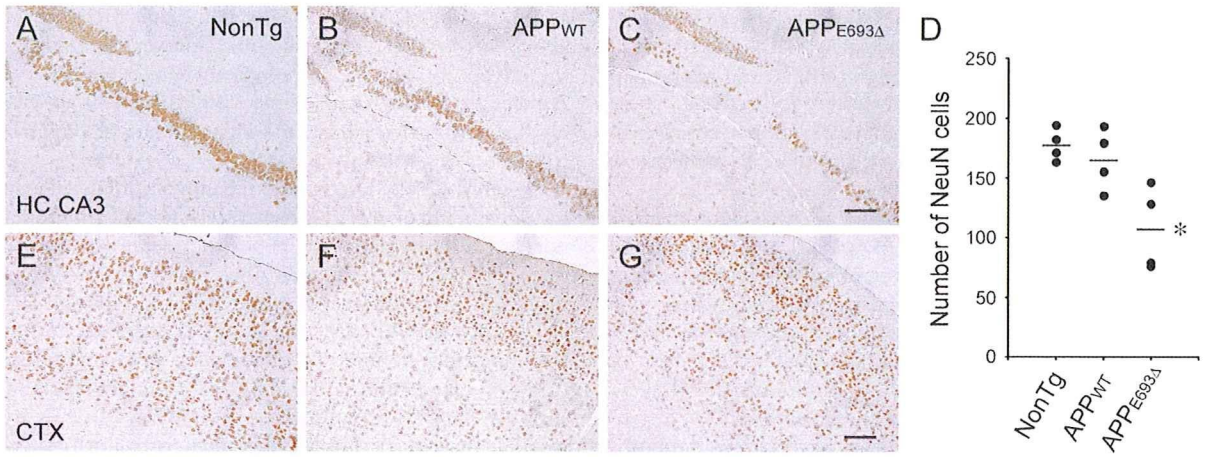

Figure 8. Neuronal loss in $APP_{E693\Delta}$-Tg mice. ***A–C, E–G***, Brain sections of Tg mice were stained with an antibody to NeuN, a marker of mature neurons. Compared with non-Tg littermates (***A***, ***E***) and APP_{WT}-Tg mice (***B***, ***F***), $APP_{E693\Delta}$-Tg mice (***C***, ***G***) exhibited significant decrease in NeuN-positive cells in the hippocampal CA3 region, but no decrease in cerebral cortex at 24 months; hippocampal CA3 region (***A–C***) and cerebral cortex (***E–G***). No significant difference was observed between non-Tg littermates and APP_{WT}-Tg mice at 24 months. ***D***, NeuN-positive cells in the pyramidal cell layer of the hippocampal CA3 region were counted within 900 μm from its end toward the dentate gyrus in the photographs. $^*p = 0.0044$ versus NonTg; $p = 0.0121$ versus APP_{WT}-Tg ($n = 4$). CTX, Cerebral cortex; HC, hippocampus. Scale bars, 100 μm.

8*C*,*G*) exhibited a significant decrease in NeuN-positive cells in the hippocampal CA3 region at 24 months (Fig. 8*D*). No apparent decrease in NeuN-positive cells was observed in the cerebral cortex at this age. The APP_{WT}-Tg mice did not exhibit significant neuronal loss compared with the non-Tg littermates. These findings suggest that Aβ oligomers triggered the pathological cascades leading to neuronal death, in which neuronal loss occurred a long period of time after the start of intraneuronal accumulation of Aβ oligomers at 8 months. The hippocampal CA3 region appears to be particularly vulnerable to the toxic effects of Aβ oligomers.

These findings together suggest that Aβ oligomers, which are localized within neurons in $APP_{E693\Delta}$-Tg mice, significantly contribute not only to synaptic alteration but also to other features of AD pathology *in vivo*.

Discussion

In the present study, we generated novel APP-Tg mice expressing the E693Δ mutation to test *in vivo* the ability of Aβ oligomers to produce the synaptic, cognitive, and neuropathological features of AD. On immunohistochemical examination, $APP_{E693\Delta}$-Tg mice exhibited age-dependent accumulation of Aβ oligomers within neurons in the cerebral cortex and hippocampus from 8 months, but no amyloid plaques even at 24 months. Biochemical analysis confirmed the accumulation of Aβ dimers and possibly trimers in their brains. This is consistent with our previous findings that mutant Aβ E22Δ peptides neither formed amyloid fibrils *in vitro* nor were deposited in amyloid plaques in AD patient brains, and instead formed abundant oligomers *in vitro* (Tomiyama et al., 2008) and accumulated in oligomeric forms within transfected cells (Nishitsuji et al., 2009). The enhanced formation of Aβ oligomers and the lack of amyloid plaques in our $APP_{E693\Delta}$-Tg mice indicate that this mouse model is suitable for study of the contribution of Aβ oligomers to the pathogenesis of AD.

We initially tested in our $APP_{E693\Delta}$-Tg mice whether the consensus that Aβ oligomers cause early synaptic pathology in AD is correct. Synaptic and cognitive functions were examined by *in vivo* electrophysiology and behavioral tests using the Morris water maze at 8 months. By this age, Aβ oligomers had begun to accumulate within neurons in the hippocampus and cerebral cortex of the $APP_{E693\Delta}$-Tg mice. Although their basal synaptic transmission was not affected at this age, short-term and long-term synaptic plasticity (PPF and LTP, respectively) and spatial reference memory were significantly impaired. Our control APP_{WT}-Tg mice also exhibited weaker but significant impairment of synaptic plasticity at 8 months, despite little accumulation of Aβ oligomers. One possible explanation for these findings is that *in vivo* electrophysiology is a very sensitive assay, and that therefore even trace amounts of Aβ oligomers in the APP_{WT}-Tg mice could be found to cause synaptic dysfunction. Such small amounts of Aβ oligomers may not be detected by immunohistochemistry or Western blotting, and may be insufficient to cause cognitive impairment in water maze tasks which may be less sensitive than other behavioral tasks. Another possibility is that elevated secretion of Aβ from neurons may lead to synaptic depression (Kamenetz et al., 2003; Ting et al., 2007), in which Aβ monomer, as well as oligomers, may act as a negative regulator of synaptic transmission. Alternatively, overexpression of APP in neurons may itself cause synaptic dysfunction, independent of Aβ production. We also examined whether loss of synapses occurred in our $APP_{E693\Delta}$-Tg mice. Immunohistochemistry for the presynaptic marker synaptophysin demonstrated that synaptic density in the hippocampus of these mice decreased in age-dependent fashion from 8 months, in parallel with accumulation of Aβ oligomers. Thus, Aβ oligomers were confirmed to cause early synaptic pathology in the absence of amyloid plaques in our $APP_{E693\Delta}$-Tg mice.

The next and main objective of the present study was to clarify whether Aβ oligomers contribute *in vivo* to features of AD pathology other than synaptic alteration. Although numerous lines of APP-Tg mice have been shown to exhibit abnormal tau phosphorylation in the brain, this was detected only after amyloid plaque formation and within the dystrophic neurites surrounding plaques (Duyckaerts et al., 2008). We examined our $APP_{E693\Delta}$-Tg mice for tau pathology using immunohistochemistry. In the absence of amyloid plaques, the mice displayed abnormal tau phosphorylation in the hippocampus and cerebral cortex from 8 months, in parallel with intraneuronal accumulation of

Aβ oligomers. Tau hyperphosphorylation was detected in hippocampal mossy fibers and the cingulum, which connect granule cells of the dentate gyrus and the hippocampal CA3 region and the cingulate gyrus and entorhinal cortex, respectively. These neural connections have been thought to play important roles in learning, memory, and consciousness, and to be altered early in AD (Arendt, 2004; Villain et al., 2008).

Clustering of activated astrocytes and microglia around amyloid plaques is another feature of AD pathology thought to be involved in neurodegeneration via cytokine and chemokine release from these cells. Activation of astrocytes and/or microglia has been observed in many APP-Tg mice, though again this was only after amyloid plaque formation and was in the vicinity of plaques (Duyckaerts et al., 2008). On immunohistochemical examination, the $APP_{E693\Delta}$-Tg mice exhibited activation of microglia from 12 months and activation of astrocytes from 18 months in the cerebral cortex and hippocampus. Although we did not confirm the presence of extracellular Aβ oligomers in $APP_{E693\Delta}$-Tg mouse brain, the observed glial activation and recruitment may be caused by overflow of extracellular diffusible Aβ oligomers from neurons (Oddo et al., 2006). Alternatively, glial cells might have been activated and recruited by cytokines or chemokines released from glial cells which neighbored and were in contact with aberrant neurons containing Aβ oligomers.

The most striking feature of AD pathology is neuronal loss. In APP-Tg mice, the occurrence of neuronal loss has in several lines been reported only after intense development of amyloid plaques (Duyckaerts et al., 2008). On immunohistochemical examination, we found that our $APP_{E693\Delta}$-Tg mice, despite their lack of amyloid plaques, exhibited significant neuronal loss in the hippocampal CA3 region at 24 months. To our knowledge, this is the first report that neuronal loss was induced by Aβ oligomers alone *in vivo* in the absence of amyloid plaques. No neuronal loss was observed at younger ages or in other brain regions, suggesting that Aβ oligomer-induced neuronal death requires long exposure of cells to Aβ oligomers *in vivo* and occurs in cells vulnerable to the toxic effects of Aβ oligomers. This is consistent with our observation that, in patients with the E693Δ mutation, mild atrophy began to occur initially in the hippocampus several years after the onset of AD (H. Shimada, S. Ataka, T. Tomiyama, J. Takeuchi, H. Takechi, H. Mori, and T. Miki, unpublished observations).

Our findings imply that intracellular Aβ plays important roles in synaptic alteration and subsequent neuropathology. A similar relationship between intracellular Aβ and synaptic pathology has been reported in other Tg mice. In the triple transgenic 3xTg-AD mice, synaptic and cognitive dysfunction were found to be correlated with the accumulation of intraneuronal Aβ in the hippocampus (4 months) before amyloid plaque formation (12 months) (Oddo et al., 2003; Billings et al., 2005). In arcAβ mice, cognitive dysfunction was observed at 6 months, after the intraneuronal accumulation of Aβ (3 months) but before amyloid plaque formation (7 months) (Knobloch et al., 2007). Furthermore, in AD and Tg2576 mouse brains, morphological alterations of synapses occurred in association with intraneuronal accumulation of Aβ (Takahashi et al., 2002), and some of the intraneuronal Aβ formed oligomers (Takahashi et al., 2004). We also previously observed that in AD brains, synaptophysin was decreased around neurons containing Aβ oligomers (Ishibashi et al., 2006). Many other studies of patients with AD (Gouras et al., 2000; Fernández-Vizarra et al., 2004) and Down syndrome (Mori et al., 2002) and of APP-Tg mice (Casas et al., 2004; Lord et al., 2006; Oakley et al., 2006) have demonstrated that intraneuronal accumulation of Aβ is an early pathological change observed before amyloid plaque formation. However, we cannot exclude the possibility that the pathological changes we observed were induced by extracellular soluble Aβ oligomers. It appears possible that both extracellular and intracellular Aβ oligomers contribute to the pathology of AD.

Notably, Aβ oligomers predominantly accumulated in insoluble fractions, particularly the FA-extracted fraction, in $APP_{E693\Delta}$-Tg mice. This finding appears inconsistent with the prevalent view that Aβ oligomers are soluble. However, detergent-insoluble features of intracellular Aβ have also been demonstrated in 3xTg-AD mice (Billings et al., 2005). It may be that the soluble fraction of intracellular Aβ oligomers is in an equilibrium with the insoluble fraction, and that the E22Δ mutation in Aβ tends to shift the equilibrium toward the insoluble fraction. It is unclear whether these insoluble Aβ oligomers form fibrils within cells. While very weak thioflavin S staining was observed in neurons in the $APP_{E693\Delta}$-Tg mice, this staining may have reflected its binding to Aβ oligomers rather than Aβ fibrils. Alternatively, intraneuronal thioflavin S staining may reflect the presence of fibrillar aggregates of hyperphosphorylated tau. In our previous study, Aβ was found to localize largely at endosomes, and to a lesser extent in the ER, Golgi, lysosomes, and autophagosomes in cultured cells (Nishitsuji et al., 2009). In AD and Tg2576 mouse brains, Aβ was found to accumulate in multivesicular bodies, which are a type of endosome (Takahashi et al., 2002). Thus, endosomes may be the main site of accumulation of insoluble Aβ. A study to determine the subcellular localization of Aβ oligomers in our $APP_{E693\Delta}$-Tg mice is ongoing.

In summary, we found that Aβ oligomers caused not only synaptic alteration but also abnormal tau phosphorylation, microglial activation, astrocyte activation, and neuronal loss *in vivo* in the absence of amyloid plaques. Our findings provide a new insight into the pathogenesis of AD, that amyloid plaque formation is not an absolute requirement for the onset and development of AD. Instead, Aβ oligomers play pivotal roles throughout the progression of AD. It is true that we have not yet succeeded in forming neurofibrillary tangles in these mice and thus need to refine this model. Nevertheless, our Tg mice are, at present, the only animal model of Aβ oligomer-induced pathology avoiding the effects of amyloid plaques even at old ages, and are thus a valuable means of investigation of the pathological and physiological roles of Aβ oligomers and for evaluation of strategies for treatment of AD that specifically target Aβ oligomers.

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Short communication

DCP-LA neutralizes mutant amyloid β peptide-induced impairment of long-term potentiation and spatial learning

Tetsu Nagata[a], Takemi Tominaga[b], Hiroshi Mori[b], Takahiro Yaguchi[a], Tomoyuki Nishizaki[a,*]

[a] Division of Bioinformation, Department of Physiology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
[b] Department of Neuroscience, Osaka City University, Graduate School of Medicine, Osaka, Japan

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ABSTRACT

Long-term potentiation (LTP) was monitored from the CA1 region of the intact rat hippocampus by delivering high frequency stimulation (HFS) to the Schaffer collateral commissural pathway. Intraventricular injection with mutant amyloid β_{1-42} peptide lacking glutamate-22 ($A\beta_{1-42}E22\Delta$), favoring oligomerization, 10 min prior to HFS, inhibited expression of LTP, with the potency more than wild-type amyloid β_{1-42} peptide. Intraperitoneal injection with the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) 70 min prior to HFS neutralized mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition. In the water maze test, continuous intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide for 14 days prolonged the acquisition latency as compared with that for control, with the potency similar to wild-type $A\beta_{1-42}$ peptide, and intraperitoneal injection with DCP-LA shortened the prolonged latency to control levels. The results of the present study indicate that DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced impairment of LTP and spatial learning.

1. Introduction

Accumulating studies have pointed to the implication of amyloid β (Aβ) peptide in the pathogenesis of Alzheimer disease. Lines of evidence have suggested that soluble Aβ oligomers, but not Aβ fibrils, play a critical role in synaptic and cognitive disorders for Alzheimer disease [1,3,7,13]. Indeed, Aβ oligomers decrease synaptic density [8,14], inhibit long-term potentiation (LTP), a cellular model of learning and memory, in the hippocampus [9,17,18], and impair memory function [2,10]. We have found a novel amyloid precursor protein mutation (E693Δ) to produce variant $A\beta_{1-42}$ lacking glutamate-22 ($A\beta_{1-42}E22\Delta$) in Japanese pedigrees showing Alzheimer-type dementia and Alzheimer disease [16]. Notably, $A\beta_{1-42}E22\Delta$ peptide, favoring oligomerization, inhibited expression of the *in vivo* hippocampal LTP [16].

In our earlier study, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a newly synthesized linoleic acid derivative, stimulated presynaptic glutamate release by enhancing activity of presynaptic α7 nicotinic acetylcholine (ACh) receptors through direct and selective activation of protein kinase C-ε (PKC-ε), thereby inducing an 'LTP-like' long-lasting facilitation of the *in vitro* and the *in vivo* hippocampal synaptic transmission [6,15,20]. In addition, DCP-LA ameliorated disorder of spatial learning and memory induced by intraperitoneal injection with scopolamine or continuous intraventricular injection with $A\beta_{1-40}$ peptide for rats [12] and improves age-related learning impairment for accelerated-senescence-prone mice 8 (SAMP8) [19].

The present study examined the effect of DCP-LA on LTP inhibition and spatial learning disorders induced by $A\beta_{1-42}E22\Delta$ peptide. The results show that DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced impairment of LTP and spatial learning.

2. Materials and methods

2.1. Animal care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Aβ peptides

Wild-type $A\beta_{1-42}$ peptide was purchased from American Peptide Company (Sunnyvale, CA, USA) and $A\beta_{1-42}E22\Delta$ peptide was synthesized based upon the results of mass spectrometry (American Peptide Company).

2.3. LTP monitoring

Under urethane (1.5 g/kg, intraperitoneal injection) anesthesia, field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region of the hippocampus of Wistar rats (male, 6w) by electrically stimulating the Schaffer collateral (0.033 Hz, 0.1 ms in duration). The parameters for high frequency stimulation (HFS) to induce LTP were four trains with an inter-train interval of 200 ms and each train consisted of ten 30-s 200-Hz pulses. Phosphate-buffered saline (PBS)

* Corresponding author. Tel.: +81 798 45 6397; fax: +81 798 45 6649.
E-mail address: tomoyuki@hyo-med.ac.jp (T. Nishizaki).

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or DCP-LA (1 mg/kg weight) was intraperitoneally injected 70 min before HFS and subsequently, PBS (2 μl), wild-type $A\beta_{1-42}$ peptide (10 ng in 2 μl PBS), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng in 2 μl PBS) was injected in the right-sided lateral ventricle for 30 s using a Hamilton microsyringe 10 min before HFS.

2.4. Water maze test

PBS, wild-type $A\beta_{1-42}$ peptide (10 ng/day), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng/day) was continuously injected in the right-sided lateral ventricle of Wistar rats (male, 6w) for 14 days using an osmotic pressure pump, and then PBS or DCP-LA (1 mg/kg weight) was intraperitoneally injected 30 min prior to water maze task.

A circular plastic water tank with 180 cm in diameter and 45 cm in deep was used for a water maze test. The entire inside of the pool was painted black, and the pool was filled up to 25 cm from the bottom with muddy water containing India ink at 22 °C. A platform (11 cm in diameter) painted black was placed into water, the top sinking 1 cm below water surface. The pool was put in a test room, where there were several marks those rats are able to see from the pool. The position of the marks remained unchanged throughout testing. A platform was located in the constant position, i.e., in the middle of one quadrant, equidistant from the center and edge of the pool. Rats facing the wall of the pool were placed into water at one of 5 positions selected at random, and time from start to escape onto the platform (acquisition latency) was measured. When succeeded, rats were allowed to stay on the platform for 10 s. Rats received the task for consecutive 16 days and the acquisition latency (time form the start to arrival onto the plate) was measured.

2.5. Statistical analysis

Statistical analysis was carried out using Fisher's Protected Least Significant Difference (PLSD) test.

3. Results

3.1. DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition

We monitored fEPSPs from the CA1 region of the intact rat hippocampus by electrically stimulating the Schaffer collateral commissural pathway. For control, HFS enhanced fEPSP slopes to 170–250% of basal levels, being evident at 120 min after HFS (LTP) (Fig. 1A and B). Intraventricular injection with wild-type $A\beta_{1-42}$ peptide inhibited expression of LTP ($P<0.001$ as compared with control LTP, Fisher's PLSD test), and DCP-LA (1 mg/kg, i.p.) significantly recovered the LTP inhibition to a level similar to control LTP ($P<0.0001$ as compared with LTP for wild-type $A\beta_{1-42}$ peptide treatment, Fisher's PLSD test) (Fig. 1A). More marked inhibition of LTP was obtained with mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng) ($P<0.0001$ as compared with LTP for wild-type $A\beta_{1-42}$ peptide treatment, Fisher's PLSD test), and DCP-LA (1 mg/kg, i.p.) significantly improved the LTP disruption ($P<0.0001$ as compared with LTP for mutant $A\beta_{1-42}E22\Delta$ peptide treatment, Fisher's PLSD test); conversely, DCP-LA enhanced LTP more than control LTP (Fig. 1B).

3.2. DCP-LA ameliorates mutant $A\beta_{1-42}E22\Delta$ peptide-induced spatial learning impairment

In the water maze test, intraventricular injection with wild-type $A\beta_{1-42}$ peptide prolonged the acquisition latency (Fig. 2A). A similar effect was obtained with mutant $A\beta_{1-42}E22\Delta$ peptide, with no significant difference in the latency between wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide (Fig. 2B). Intraperitoneal injection with DCP-LA (1 mg/kg, i.p.) significantly shortened the prolonged latency for both the treatment with wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide (each $P<0.001$ as compared with the latency for wild-type $A\beta_{1-42}$ or mutant $A\beta_{1-42}E22\Delta$ peptide treatment without DCP-LA, Fisher's PLSD test), reaching the levels for control (Fig. 2A and B). This indicates that DCP-LA ameliorates spatial learning disorders induced not only by wild-type $A\beta_{1-42}$ peptide but mutant $A\beta_{1-42}E22\Delta$ peptide.

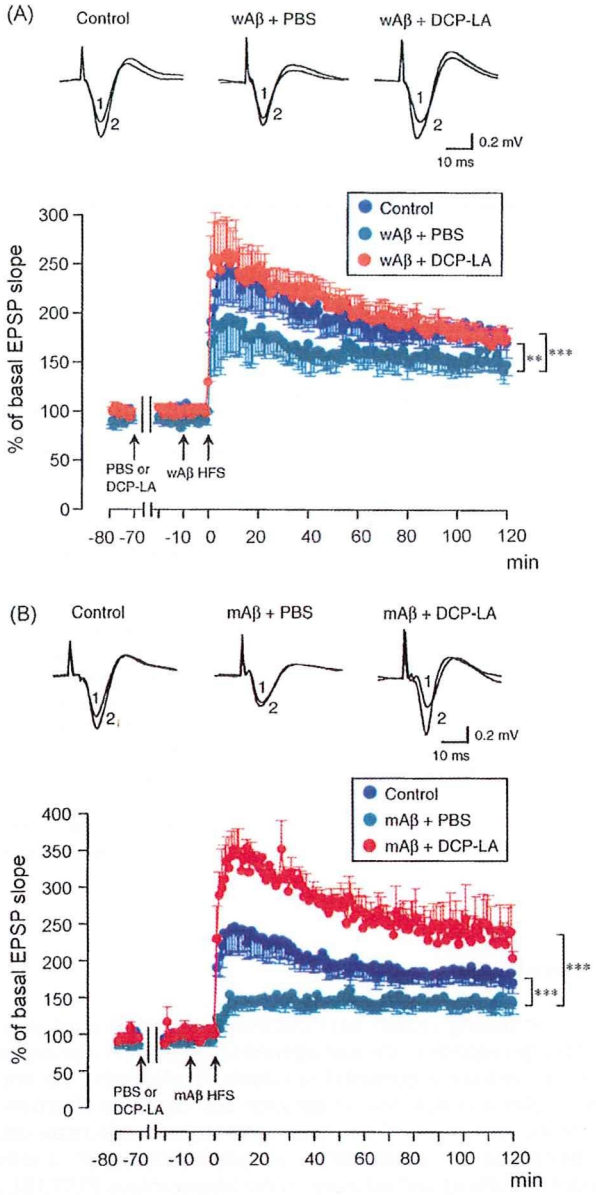

Fig. 1. Effect of DCP-LA on LTP inhibition induced by wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide. fEPSPs were monitored from the CA1 region of the intact rat hippocampus. DCP-LA (1 mg/kg) or PBS was intraperitoneally injected 70 min prior to HFS, to induce LTP, followed by intraventricular injection with wild-type $A\beta_{1-42}$ peptide (wAβ) (10 ng) (A) or mutant $A\beta_{1-42}E22\Delta$ peptide (mAβ) (10 ng) (B) 10 min prior to HFS. fEPSPs recorded at 0 (1) and 120 min (2) are superimposed. Control, intraventricular injection with PBS and intraperitoneal injection with PBS; wAβ + PBS, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with PBS; wAβ + DCP-LA, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with DCP-LA; mAβ + PBS, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with PBS; mAβ + DCP-LA, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with DCP-LA. In the graphs, each point represents the mean (±SEM) percentage of basal fEPSP slope (0 min) ($n=5$). $^{**}P<0.001$; $^{***}P<0.0001$, Fisher's PLSD test.

4. Discussion

One might think that the effects of the linoleic acid derivative DCP-LA on synaptic transmission are similar to the effects of nicotine, an agonist of nicotinic ACh receptors including α7 receptors. DCP-LA, however, is not an agonist of nicotinic ACh receptors, distinct from nicotine, and therefore, DCP-LA does not

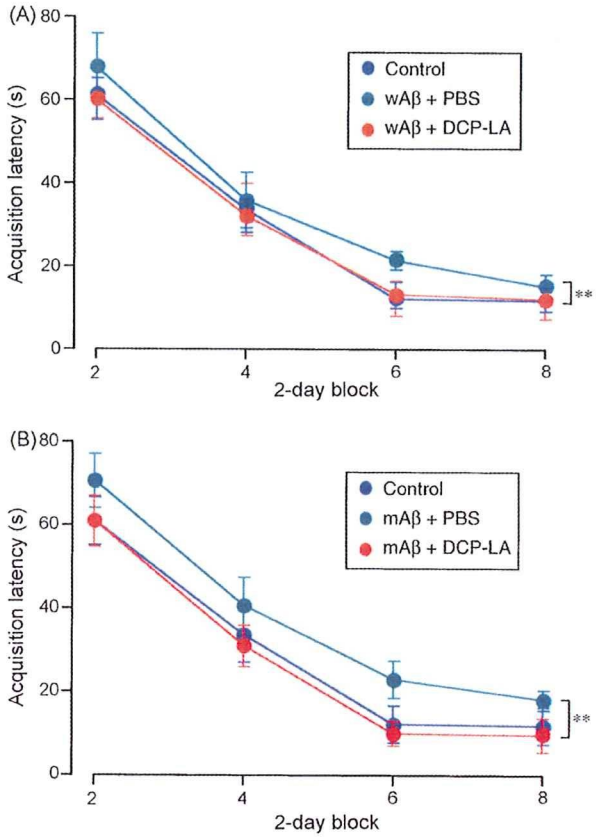

Fig. 2. Effect of DCP-LA on spatial learning impairment induced by wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide. PBS, wild-type $A\beta_{1-42}$ peptide (10 ng/day) (A), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng/day) (B) was continuously injected in the ventricle for 14 days, and then PBS or DCP-LA (1 mg/kg weight) was intraperitoneally injected 30 min prior to water maze task. Water maze task was performed two trials per day and the acquisition latency was measured. Control, intraventricular injection with PBS and intraperitoneal injection with PBS; wAβ + PBS, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with PBS; wAβ + DCP-LA, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with DCP-LA; mAβ + PBS, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with PBS; mAβ + DCP-LA, intraventricular injection with mutant $A\beta_{1-42}$ E22Δ peptide and intraperitoneal injection with DCP-LA. In the graphs, each point represents the mean (±SEM) acquisition latency from consecutive 2 days ($n = 10$). $^{**}P < 0.001$, Fisher's PLSD test.

affect the receptor inactivation. DCP-LA serves as a potentiator of nicotinic ACh receptor responses under the control of PKC [6,15,20]. With regard to the presynaptic action, DCP-LA directly and selectively activated PKC-ε, that is enriched in presynaptic terminals, thereby enhancing activity of presynaptic α7 ACh receptors, causing an increase in presynaptic glutamate release responsible for long-lasting facilitation of hippocampal synaptic transmission [6,15,20]. With regard to the postsynaptic action, DCP-LA stimulated exocytosis of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits, GluR1 and GluR2, by activating Ca^{2+}/calmodulin-dependent protein kinase II through protein phosphatase-1 inhibition in rat hippocampal slices [5], which also participates in DCP-LA-induced facilitation of hippocampal synaptic transmission. The long-lasting facilitation of hippocampal synaptic transmission would account for the beneficial action of DCP-LA on learning and memory deficits induced by scopolamine and $A\beta_{1-40}$ peptide or in association with aging [12,19].

We have confirmed that mutant $A\beta_{1-42}E22\Delta$ peptide tends to favor soluble oligomers rather than fibrils [16]. Much attractive finding was that a considerably low dose of mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng) is capable of inhibiting LTP. Like mutant $A\beta_{1-42}E22\Delta$ peptide, wild-type $A\beta_{1-42}$ peptide still inhibited LTP, but to a lesser extent. These results may support the note that Aβ oligomers, but not Aβ fibrils, are a critical factor for Alzheimer-type dementia. Soluble Aβ oligomers, accordingly, could be a target of Alzheimer disease [4]. In the present study, DCP-LA recovered LTP inhibition induced by wild-type $A\beta_{1-42}$ peptide. Surprisingly, DCP-LA abolished mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition and furthermore, enhanced the LTP greater than control LTP. The underlying mechanism, however, is presently unknown. A study shows that docosahexaenoic acid, a *cis*-unsaturated free fatty acid, suppresses τ phosphorylation induced by Aβ oligomers [11]. This may account for the protective effect of the linoleic acid derivative DCP-LA against synaptotoxicity induced by Aβ oligomers.

In the water maze test, intraventricular injection with a low dose (total 140 ng) of mutant $A\beta_{1-42}E22\Delta$ peptide prolonged the acquisition latency as compared with that for control, with the potency similar to wild-type $A\beta_{1-42}$ peptide, indicating that soluble wild-type $A\beta_{1-42}$ or mutant $A\beta_{1-42}E22\Delta$ peptide might cause Alzheimer-type dementia. DCP-LA shortened each prolonged latency to control levels. DCP-LA, thus, appears to exert its beneficial action on spatial learning disorders induced by mutant $A\beta_{1-42}E22\Delta$ oligomers as well as wild-type $A\beta_{1-42}$ peptide bearing synaptotoxicity.

In conclusion, the results of the present study show that mutant $A\beta_{1-42}E22\Delta$ peptide inhibits LTP, with the potency more than wild-type $A\beta_{1-42}$ peptide, and impairs spatial learning. DCP-LA neutralizes the LTP inhibition and improves spatial learning disorders. This represents that DCP-LA could be developed as a promising drug for treatment of Alzheimer-type dementia and Alzheimer disease.

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Oligomeric Amyloid β-Protein as a Therapeutic Target in Alzheimer's Disease: Its Significance Based on its Distinct Localization and the Occurrence of a Familial Variant Form

Kazuhiro Ito, Ken-Ichi Ishibashi, Takami Tomiyama, Tomohiro Umeda, Kei-Ichi Yamamoto, Erika Kitajima, Toshiki Idomoto, Toshie Nagatomo, and Hiroshi Mori*

Departments of Neuroscience, Osaka City University Graduate School of Medicine, Osaka, Japan

Abstract: Oligomer Aβ is the term utilized for multimeric but non-fibrillar forms of amyloid β-protein (Aβ). The most prominent property of oligomer Aβ is considered to be its solubility and structure. Here, we examined the histochemical localization of oligomer Aβ in AD brains. At present, little information is available on the structure and function of cerebral oligomer Aβ. We therefore studied the molecular localization of oligomer Aβ using a newly generated polyclonal mouse antisera against a variant Aβ with a deletion mutation of the 22nd glutamate that we found recently in a patient with familial Alzheimer's disease. Intracellular as well as extracellular oligomer Aβ are herein discussed to define their structure and pathological roles in disease.

Keywords: Alzheimer disease, amyloid, Aβ, oligomer, synapse, recessive hereditary.

INTRODUCTION

Oligomer Aβ was first documented as a detergent-stable soluble Aβ species [1] and has been determined to induce synaptic malfunction [2]. This finding may, in part, explain the synaptic alteration that occurs in Alzheimer's disease (AD) [3] as it has been proposed that an early event in AD involves synaptic failure [4]. In light of this, oligomer Aβ is currently believed to be closely related with the disease, and may be an important factor in mild cognitive impairment (MCI). A requisite neuropathological hallmark of AD is the presence of amyloid plaques. Such plaques contain Aβ primarily as a fibrillar structure, and are argued to induce cellular dysfunction that can lead to toxicity and the formation of dystrophic neurites [5, 6]. As oligomer Aβ has not been extensively compared with other non-filamentous Aβ species, like ADDL (Aβ-derived diffusible ligands: [7]) and Aβ*56 [8], our knowledge of its function and role remains limited. Following up on the discovery of the London mutation, APP(V717I), that demonstrated the involvement of the *APP* gene in AD [9], other genetic studies have confirmed that many missense mutations in APP are associated with familial AD and cerebral amyloid angiopathy (CAA). In general, these pathogenic mutations have been shown to influence Aβ metabolism, impacting its production, as well as it aggregation by various means. Our recent discovery of a novel APP mutation, (E693Δ), that produces a variant Aβ that lacks glutamate 22 (E22Δ), in a Japanese pedigree with early-onset familial AD extends this and provides support for a role of oligomer Aβ in AD [10]. Whereas the secretion of total Aβ was markedly reduced by this mutation, the variant Aβ proved to be more resistant to proteolytic degradation and, notably, showed the unique aggregation property of enhanced oligomerization but no fibrillization [10]. The fact that variant Aβ inhibited hippocampal long-term potentiation more potently than the wild-type peptide (normal Aβ) in rodents, supports the central involvement of oligomer Aβ in AD [10].

In this study, we examined oligomer Aβ for its localization in AD brain and its association with plaque Aβ.

MATERIALS AND METHODS

Antisera

Anti-Aβ (referred to as β001) was used in the current study as the primary amyloid antibody, as previously described [11]. According to a conventional protocol for mouse immunization [12], anti-oligomer Aβ was newly prepared in three mice immunized with synthetic Aβ peptide (Aβ1-42d) to comprise the full amino acid sequence of Aβ from aspartate (the first residue) to alanine (the 42nd residue) but lacking glutamate (the 22nd amino acid), to represent the E693Δ mutation [10]. For absorption experiments, synthetic wild-type Aβ1-42 peptide was incubated to allow formation of fibrillar structures at room temperature for three hours. The resultant supernatant was obtained as oligomer Aβ antisera after sedimentation at 15,000 rpm for 20 min to adsorb residual trace amounts of activity for recognition of fibrillar Aβ.

Neuropathological Analysis

Paraffin-embedded tissues from the temporal cortex of AD brain were used for analysis. Sections (5 μm thick) were examined immunohistochemically using anti-oligomer antibody. Subsequently, sections were incubated for 45 min in biotinylated secondary antibody (horse anti-mouse IgG; Vector Laboratories, Burlingame, CA, USA) and ABC solution

*Address correspondence to this author at the Department of Neuroscience, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan; Tel: 81-6-6645-3920; Fax: 81-6-6645-3922; E-mail: mori@med.osaka-cu.ac.jp

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for 1 h (Vectastain ABC Elite kit; Vector). The reaction product was visualized with 0.05% diaminobenzidine/0.01% hydrogen peroxide solution for 10 min. The specimens were also observed in detail using a confocal laser microscope (Axiovert 100; Zeiss, Wetzlar, Germany). Each figure was obtained as the average image of four scans [13].

Immunospecificity was confirmed with use of antigen peptide in the adsorption experiment. In addition, other controls were performed without the primary or secondary antibody to confirm the specificity of immunoreaction.

RESULTS AND DISCUSSION

Preparation and Characterization of Anti-Oligomer Aβ Antisera

Aβ1-42d peptide was injected four times each in three mice for immunization. After the third booster immunization, three serum samples were obtained from three mice and tested in a dot-blot test on membrane with the synthetic Aβ1-42d peptide. The mutant Aβ1-42d peptide was recognized by conventional anti-Aβ antibody (β001) (lane a in Fig. (**1**)) and by three other serum samples as well (lanes b-d in Fig. (**1**)). Since this Aβ1-42d peptide solution included oligomer Aβ and monomer Aβ, we next examined if the sera obtained exclusively bound oligomer Aβ or bound both this oligomer and monomer Aβ. Anti-Aβ antibody (β001) immunoprecipitated monomer Aβ as well as dimer, trimer, and tetramer Aβ (a in Fig. (**2**)) while all three sera predominantly immunoprecipitated Aβ1-42d peptide in oligomer rather than monomer Aβ form (b-d in Fig. (**2**)), indicating higher specific binding to oligomer Aβ than monomer Aβ. These sera were not specific to Aβ1-42d peptide in oligomer Aβ and recognized wild-type Aβ (unpublished observation).

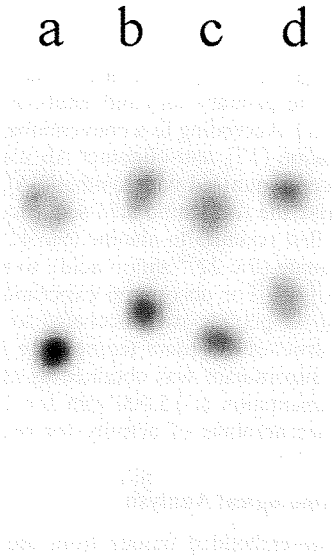

Fig. (1). Dot blot assay with anti-oligomer Aβ antibody.
The synthetic mutant Aβ1-42d peptide including the E22Δ mutation was dotted onto a PVDF membrane at protein contents of 1ng and 0.5ng from the top. Bovine serum albumin was dotted onto the bottom. The antibodies used were (a) β001, (b) #1 mouse serum, (c) #2 mouse serum, and (d) #3 mouse serum.

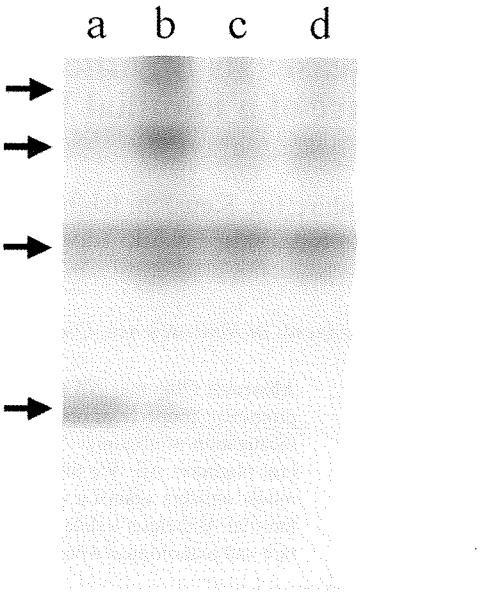

Fig. (2). Oligomer Aβ with deletion mutation.
The variant Aβ peptide was incubated to enable oligomer formation but not fibril formation. The resultant variant Aβ oligomer was incubated with (a) β001, (b) #1 mouse serum, (c) #2 mouse serum, and (d) #3 mouse serum for immunoprecipitation together with protein A-agarose beads. After spin centrifugation, the resultant sediments were washed with RIPA buffer followed by gel electrophoresis and elecroblotting on PVDF membrane. The membrane was blocked with 10% calf serum and then incubated with anti-Aβ antibody (β001). Arrows represent tetramer, trimer, dimer, and monomer from the top.

Neuropathology

We used one of these three oligomer Aβ sera to stain AD brain sections to determine the cerebral localization of oligomer Aβ. As expected, we observed no distinct well-known forms of pathology such as Aβ plaques with anti-oligomer Aβ antiserum (Fig. **3**). Careful observation revealed weak but clearly increased immunoreactivity in neuronal perikarya in brain sections whose adjacent sections exhibited the presence of senile plaques with anti-Aβ antibody (β001) in association with which spherical amyloid burden with or without cores of variable sizes was observed (Fig. (**4**)). Moreover, immunoreactivity appeared to be associated with unidentifiable dot-like cellular organelles. These immunoreactions were completely abolished when antisera against oligomer Aβ were adsorbed with synthetic oligomer Aβ peptide (Fig. (**3b**)). Oligomer Aβ thus differed from fibrillar Aβ in senile plaques. Oligomer Aβ was detected as dot or dot-like deposits. These findings indicated that oligomer Aβ is not strongly associated with the Aβ plaque. The localization of oligomer Aβ for the most part differs from that of fibrillar Aβ.

Intracellular Oligomer Aβ

Intracellular oligomer Aβ was previously documented in biochemical experiments [14] and by microscopic examination [15]. Here, we extend such observations for oligomer Aβ with confocal laser microscopy. As shown in (Fig. **5**), dot signals representing oligomer Aβ were observed in the peri-

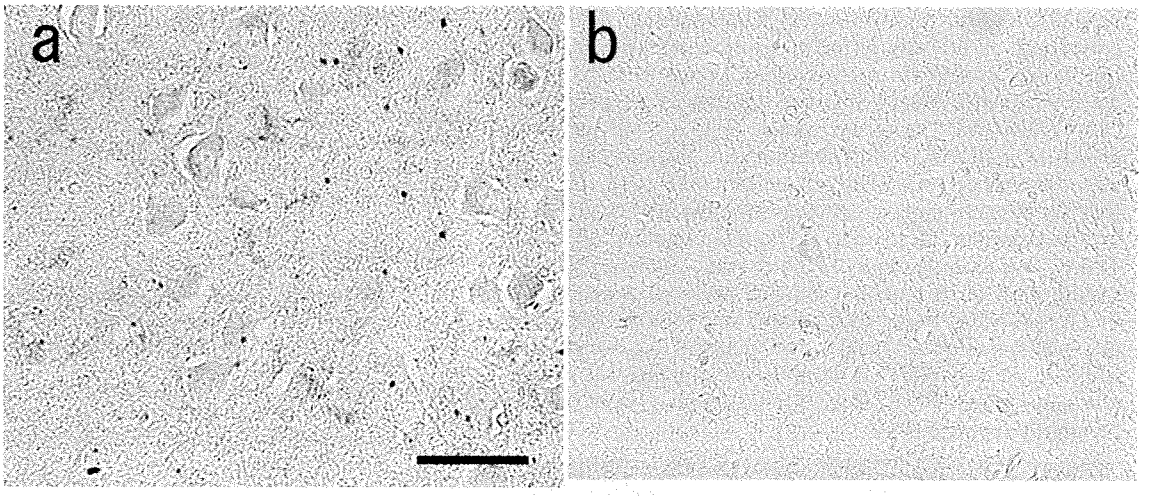

Fig. (3). Immunohistochemstry of oligomer Aβ in AD brain sections.
Temporal lobe sections were stained **(a)** with anti-oligomer Aβ antibody and **(b)** anti-oligomer Aβ antibody after adsorption by synthetic oligomer Aβ peptide. Bar represents 50 μm.

karya of hippocampal pyramidal neurons of AD brain. However, the relationship between these dots and cell organelles remains unclear.

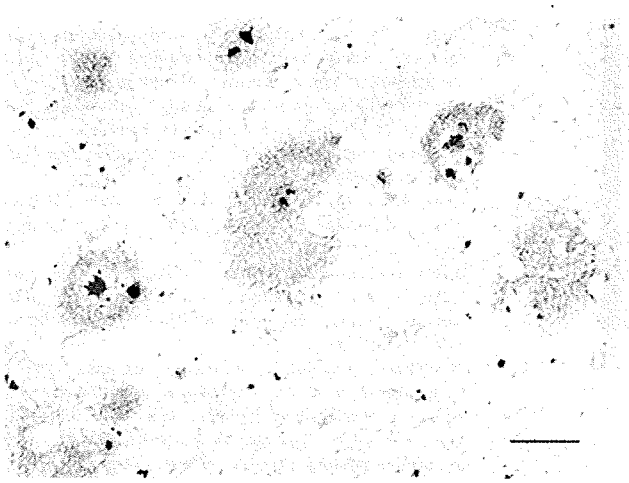

Fig. (4). Immunohistochemstry of fibrillar Aβ in AD brain section. A temporal lobe section from AD brain was pretreated with formic acid and stained with conventional anti-Aβ antibody. Bar represents 50 mm.

In addition to the perikaryal occurrence of oligomer Aβ, we observed oligomer Aβ in blood vessels (Fig. **6**). Taking into consideration that oligomer Aβ is different from fibrillar Aβ, it was interesting to see the relation between fibrillar Aβ and oligomer Aβin vascular Aβ. It is currently unclear in AD brains whether such cellular oligomer Aβ plays a role in deterioration of synaptic function as does exogenous oligomer Aβ, since little evidence was provided for cerebral oligomerized Aβ. It is certainly important to characterize and compare *in vitro* and *in vivo* oligomer Aβ.

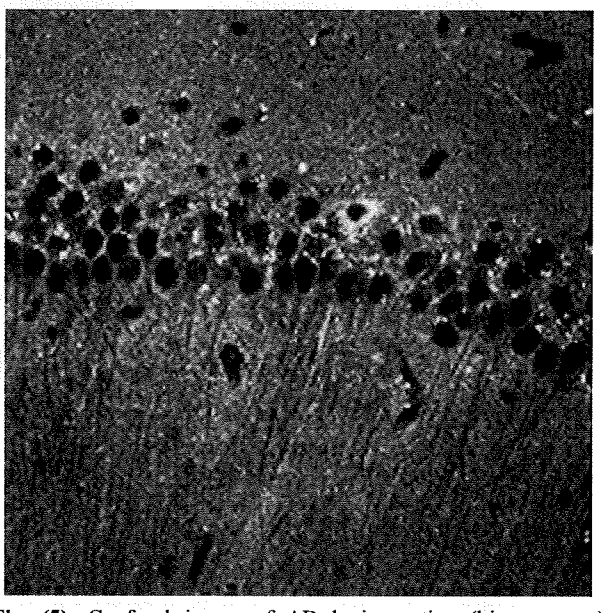

Fig. (5). Confocal image of AD brain section (hippocampus) stained with anti-oligomer Aβ antibody.

Pathological Oligomerization of Aβ

The biological function of oligomer Aβ was first discussed by Selkoe and colleagues, who derived and examined it from culture media secreted by cells transfected with APP [2]. They also noted that oligomer Aβ existed in cells. They observed that exogenous oligomer Aβ disturbed LTP function in synapses. They are referred to as low-n oligomer Aβ and it remains inconclusive whether or not they are different from ADDLs or Aβ*56. We observed an oligomeric profile similar to that of low-n oligomer [1, 15], but cannot exclude the presence of other oligomer species since we do not know the relationships among these three oligomers. Although it is known that oligomer Aβ is a normal product of APP metabo-

lism [2], it is not clear how and where nascent Aβ is oligomerized, particularly as pathological or harmful oligomer Aβ species in neurons. Furthermore, it remains to be elucidated whether or not all oligomer Aβ species are pathologically equivalent. The recent variant oligomer Aβ has been shown to induce LTP deterioration in a manner similar to oligomer Aβ derived from cell culture studies [2, 10, 16]. Indeed, variant oligomer Aβ containing the E693Δ mutation exhibited greater effects than did normal oligomer Aβ. More directly, we recently observed that variant oligomer Aβ containing the E693Δ mutation induced neuronal degeneration in a hippocampal tissue slice culture likely via the induction of synaptic malfunction rather than neuronal toxicity [17]. In addition, we consider that intracellular oligomer Aβ in hippocampus observed here (Fig. (**5**)) has a an important role, as previously reported [13].

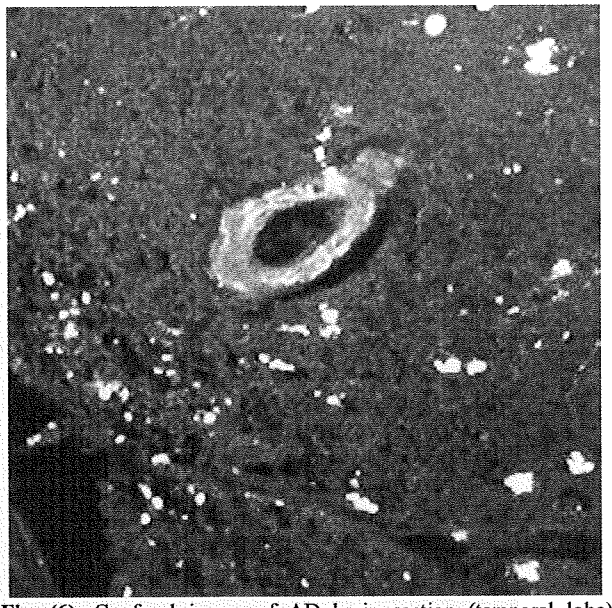

Fig. (6). Confocal image of AD brain section (temporal lobe) stained with anti-oligomer Aβ antibody.

Significance of Oligomer Aβ

The present observation of intracellular accumulation of oligomer Aβ appears to be in good agreement with our previous finding of decreased secretion of oligomer Aβ with E693Δ mutation in transfection experiments [10]. Oligomerization of Aβ, particularly with the E693Δ mutation, is likely to suggest another role. As described before [10], we observed patients only in homozygotes, but not heterozygotes of the mutation allele. This indicates that the E693Δ mutation represents the first autosomal recessive inheritance linked to the disease on human genetics. At least in these pedigrees, the disease onset may be linked with the gene dosage of *APP* to include oligomer Aβ. Thus, more information is needed on the structure and cellular metabolism of oligomer Aβ to clarify its mode of hereditary transmission. Oligomer Aβ like a dimer form may transform into fibrillar Aβ or serve as a seed for further complex [18]. We believe that our recent finding (Aβ E22Δ) provides a critical and seminal clue to aid understand the structural and pathological function of oligomer Aβ.

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Molecular Pathogenesis of Genetic and Inherited Diseases

The E693Δ Mutation in Amyloid Precursor Protein Increases Intracellular Accumulation of Amyloid β Oligomers and Causes Endoplasmic Reticulum Stress-Induced Apoptosis in Cultured Cells

Kazuchika Nishitsuji,* Takami Tomiyama,* Kenichi Ishibashi,* Kazuhiro Ito,* Rie Teraoka,* Mary P. Lambert,† William L. Klein,† and Hiroshi Mori*

From the Department of Neuroscience, Osaka City University Graduate School of Medicine, Osaka, Japan; and the Department of Neurobiology and Physiology,† Northwestern University, Evanston, Illinois*

The E693Δ mutation within the amyloid precursor protein (APP) has been suggested to cause dementia via the enhanced formation of synaptotoxic amyloid β (Aβ) oligomers. However, this mutation markedly decreases Aβ secretion, implying the existence of an additional mechanism of neuronal dysfunction that is independent of extracellular Aβ. We therefore examined the effects of this mutation on both APP processing to produce Aβ as well as subcellular localization and accumulation of Aβ in transfected HEK293 and COS-7 cells. Both β- and γ-cleavage of mutant APP increased, indicating a lack of inhibition in Aβ production. Instead, this mutation promoted Aβ accumulation within cells, including the endoplasmic reticulum (ER), Golgi apparatus, early and late endosomes, lysosomes, and autophagosomes, all of which have been proposed as intracellular sites of Aβ generation and/or degradation, suggesting impairment of APP/Aβ trafficking. Notably, the intracellular mutant Aβ was found to predominantly form oligomers. Concomitant with this accumulation, the ER stress markers Grp78 and phosphorylated eIF2α were both strongly induced. Furthermore, the activation of caspase-4 and -3 as well as DNA fragmentation were detected in these cells. These results suggest that mutant Aβ induces alteration of Aβ trafficking and subsequent ER stress-induced apoptosis via enhancement of its intracellular oligomerization. Our findings suggest that Aβ oligomers exhibit toxicity in the extracellular space and within the cells themselves. *(Am J Pathol 2009, 174:957–969; DOI: 10.2353/ajpath.2009.080480)*

Soluble oligomers of amyloid β (Aβ) peptide are believed to cause synaptic and cognitive dysfunction in the early stages of Alzheimer's disease (AD).[1,2] Natural low-n Aβ oligomers, such as dimers and trimers, have been shown to inhibit hippocampal long-term potentiation (LTP)[3,4] and memory[4,5] when injected into rat cerebral ventricle. Synthetic and natural larger-size Aβ oligomers, such as 12-mers termed Aβ-derived diffusible ligands[6,7] and Aβ*56,[8] have also been demonstrated to inhibit LTP in rat hippocampal slices[6] and disrupt memory when administered into rat cerebral ventricle.[8] Both low-n oligomers and Aβ-derived diffusible ligands have been shown to induce loss of synapses when applied exogenously in hippocampal slices and neurons.[9,10] In addition to direct evidence for the synaptotoxicity of Aβ oligomers, many correlative studies between soluble Aβ and synaptic and cognitive dysfunction have been reported.[7,11–16] Taken together, these findings have established the so-called oligomer hypothesis that AD begins with synaptic dysfunction caused by diffusible, extracellular Aβ oligomers.

Nevertheless, it is still unclear whether this mechanism is actually responsible for AD in humans. We previously identified an amyloid precursor protein (APP) mutation, E693Δ, in Japanese pedigrees exhibiting AD and Alzheimer's-like dementia.[17] This mutation is located within the Aβ sequence and produces variant Aβ lacking glutamate-22 (E22Δ). Aggregation studies using syn-

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Address reprint requests to Takami Tomiyama, Ph.D., Department of Neuroscience, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. E-mail: tomi@med.osaka-cu.ac.jp.

thetic peptides demonstrated that the mutant Aβ exhibited a unique property of enhanced oligomerization but no fibrillization. Amyloid imaging of patient's brains using Pittsburgh compound-B revealed few amyloid plaques. In line with the oligomer hypothesis, this mutant peptide inhibited hippocampal LTP more potently than wild-type peptide when injected into rat cerebral ventricle. In addition, this mutant peptide induced loss of synapses more potently than wild-type peptide in mouse hippocampal slices.[12] These findings suggest that the E693Δ mutation causes dementia by enhanced formation of synaptotoxic Aβ oligomers, which may provide genetic validation in humans for the oligomer hypothesis.

However, this mutation caused a marked reduction in Aβ40 and Aβ42 secretion from transfected cells,[17] a finding that appears incompatible with a pathological mutation. This observation led us to speculate that the E693Δ mutation may disturb neuronal function not only by forming extracellular Aβ oligomers but also by an additional, intracellular mechanism independent of extracellular Aβ. To test this possibility, we examined the effects of this mutation on APP processing to produce Aβ and on subcellular localization and accumulation of Aβ in transfected cells. The E693Δ mutation exhibited no inhibitory effects on β- and γ-cleavage of the mutant APP, and instead enhanced them. This mutation thus increased Aβ accumulation within cells. Immunocytochemical analyses suggested that the E693Δ mutation affects Aβ trafficking and induces endoplasmic reticulum (ER) stress-mediated apoptosis probably via enhancement of Aβ oligomerization. Such toxic effects of intracellular Aβ are probably not restricted to the E693Δ mutation and appear instead to be a common mechanism by which Aβ oligomers cause neuronal dysfunction.

Materials and Methods

Antibodies

Monoclonal antibodies specific to Aβ42 (11C)[19] and to Aβ oligomers (NU-1)[20] and polyclonal antibodies to the N-terminal region of Aβ (β001)[19] and to the C-terminal region of APP (C40)[21] were prepared in our laboratories. A monoclonal antibody, 6E10, to residues 3 to 8 of Aβ (Signet Laboratories, Inc., Dedham, MA), a polyclonal antibody to actin (Sigma-Aldrich, Inc., St Louis, MO), and polyclonal antibodies to organelle markers were purchased, including anti-calnexin antibody (Stressgen Bioreagents Corp., Ann Arbor, MI) for ER, anti-furin antibody (Affinity Bioreagents, Golden, CO) for Golgi apparatus, anti-early endosome antigen-1 (EEA1) antibody (Upstate, Lake Placid, NY) for early endosomes, anti-mannose 6 phosphate receptor (M6PR) antibody (Abcam, Inc., Cambridge, MA) for late endosomes, anti-lysosome-associated membrane protein-2 (LAMP-2) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for lysosomes, and anti-microtubule-associated protein-1 light chain 3 (LC3) antibody (MBL, Nagoya, Japan) for autophagosomes. A polyclonal antibody to Grp78 (BiP) was obtained from Stressgen Bioreagents Corp., and polyclonal antibodies to the eukaryotic initiation factor 2α subunit (eIF2α) and to phosphorylated eIF2α were from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal antibodies to caspase-4 (4B9; MBL) and to cleaved caspase-3 (5A1, Cell Signaling Technology) were also purchased.

APP and C99 Constructs

Wild-type human APP_{695} (APP_{WT}) cDNA was amplified by polymerase chain reaction (PCR) from pooled human cDNA, and cloned into a pCI mammalian expression vector (Promega Corp., Madison, WI) at the *Nhe*I and *Not*I sites. Mutant APP cDNAs with the E693Δ and Swedish (K670N/M671L) mutations ($APP_{E693\Delta}$ and APP_{SW}, respectively) were prepared by site-directed mutagenesis and cloned into pCI vector at the same sites. Wild-type and mutant C99 cDNAs were amplified by PCR from these APP constructs. To express C99 on cellular membranes, we prepared a PCR primer overlapping the APP leader sequence (corresponding to the first 17 amino acids of APP) and the N-terminal region of C99. The APP leader sequence-C99 fusion cDNAs were cloned into pCI vector at the *Nhe*I and *Not*I sites. To prepare molecular size markers in Western blotting, C59 and C50 cDNAs were also amplified by PCR from the APP_{WT} construct using PCR primers containing the start codon (ATG), and cloned into pCI vector at the same sites.

Western Blotting to Measure β-Cleavage Products

HEK293 cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs using the Lipofectamine Plus reagent (Invitrogen Corp., Carlsbad, CA). The cells were cultured overnight in OPTI-MEM I (Gibco BRL, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), and media were replaced every day with serum-free OPTI-MEM I containing 1 μmol/L γ-secretase inhibitor L-685,458 (Peptide Institute, Osaka, Japan). Three days after transfection, the conditioned media were harvested and subjected to Aβ enzyme-linked immunosorbent assay (BioSource International, Inc., Camarillo, CA) to confirm that L-685,458 sufficiently inhibited γ-secretase activity. The cells were washed with phosphate-buffered saline (PBS), harvested using a cell scraper, and homogenized by sonication in 1% Triton X-100/Tris-buffered saline (100 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl) containing protease inhibitor cocktail P8340 (Sigma). After agitation at 4°C for 1 hour, the cell homogenates were centrifuged at 1000 × *g* for 10 minutes at 4°C to remove cell debris and insoluble materials. The supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% NuPage Bis-Tris gels (Invitrogen), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). APP and its products C-terminal fragment (CTF)α and CTFβ were probed with C40 followed by horseradish peroxidase-labeled anti-rabbit antibody (Bio-Rad Laboratories, Inc., Hercules, CA) and the chemiluminescent substrate ECL Plus

(Amersham, GE Health care, Buckinghamshire, UK). Signals were visualized and quantified using a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Western Blotting to Measure γ-Cleavage Products

HEK293 cells were transfected with $C99_{WT}$ and $C99_{E693\Delta}$ constructs. The cells were cultured overnight in 10% FCS/OPTI-MEM I, and the media were replaced with serum-free OPTI-MEM I. Three days after transfection, the cells were harvested and homogenized as described above. After centrifugation at 1000 × *g* for 10 minutes at 4°C, the supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. For molecular size markers, SDS-PAGE samples were also prepared from C59 and C50 transfectants and loaded on the gels. C99 and its product APP intracellular domain (AICD) were probed with C40 and quantified as described above.

Immunoprecipitation/Western Blotting of Intracellular Aβ

HEK293 cells were transfected with APP_{WT}, $APP_{E693\Delta}$, APP_{SW}, and $APP_{SW/E693\Delta}$ constructs. The cells were cultured overnight in 10% FCS/OPTI-MEM I, and media were replaced with serum-free OPTI-MEM I. Two and three days after transfection, the cells were harvested and homogenized as described above. The cell homogenates were centrifuged at 14,000 × *g* for 15 minutes at 4°C. Aliquots of the supernatants were subjected to SDS-PAGE followed by Western blotting with C40 for quantification of APP levels. The remaining portions of the supernatants were combined into one tube to combine cell extracts from five culture dishes (10 cm diameter). APP and CTFs in the samples were precleared by immunodepletion with C40 and protein A Sepharose (Pharmacia, Piscataway, NJ) at 4°C overnight. Aβ in the samples was then immunoprecipitated with 6E10 and protein A Sepharose at 4°C overnight. The precipitates were washed three times with 1% Triton X-100/Tris-buffered saline, once with Tris-buffered saline, and boiled for 5 minutes in SDS sample buffer to elute Aβ. The eluates were subjected to SDS-PAGE with 12% Bis-Tris gels, and transferred to PVDF membranes. The membranes were boiled in PBS for 10 minutes to enhance signals, and Aβ was probed with β001 and visualized as described above.

Immunocytochemistry

COS-7 cells grown on poly-L-lysine-coated coverslips were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs, as described above. The cells were cultured overnight in 10% FCS/OPTI-MEM I, and the media were replaced with serum-free OPTI-MEM I. Two days after transfection, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 30 minutes and permeabilized with 1% saponin in PBS for 10 minutes. In the experiment on endocytic inhibition, transfected cells were treated with 25 μg/ml of the clathrin-dependent endocytosis inhibitor chlorpromazine (Sigma) and 25 μg/ml of the clathrin-independent endocytosis inhibitor nystatin (Sigma) for 15 minutes at 37°C on day 2, and then fixed. After washing with PBS, the cells were incubated with blocking buffer containing 20% calf serum in PBS overnight at 4°C. The cells were then incubated with the primary antibodies followed by rhodamine (TRITC)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The primary antibodies included 11C (1:5), NU-1 (1:1000), β001 (1:5000), anti-calnexin antibody (1:300), anti-furin antibody (1:500), anti-EEA1 antibody (1:500), anti-M6PR antibody (1:300), anti-LAMP2 antibody (1:500), anti-LC3 antibody (1:200), anti-Grp78 antibody (1:1000), and anti-phosphorylated eIF2α antibody (1:500). Antibodies were diluted with 10% calf serum in PBS. The stained specimens were mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and examined under a LSM 510 confocal laser microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

To compare intracellular Aβ oligomerization between APP_{WT} and $APP_{E693\Delta}$, 10 β001-positive cells were randomly selected from each specimen, and relative fluorescence intensities in regions of interest were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). The ratio of oligomers (NU-1-positive staining) to total Aβ (β001-positive staining) in each cell was calculated from the relative fluorescence intensities with NU-1 and β001.

Western Blotting of eIF2α, Phosphorylated eIF2α, and Caspase-4

COS-7 cells grown in culture dishes (10 cm diameter) were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs, as described above. The cells were cultured overnight in 10% FCS/OPTI-MEM I, and media were replaced with serum-free OPTI-MEM I. In some experiments, cells were cultured in serum-free OPTI-MEM I containing 1 μmol/L γ-secretase inhibitor L-685,458. Two days after transfection, cells were harvested and homogenized by sonication in 1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/Tris-buffered saline containing protease inhibitor cocktail at 4°C. After centrifugation at 1000 × *g* for 10 minutes at 4°C, the supernatants were subjected to SDS-PAGE and transferred to PVDF membranes. The eIF2α, phosphorylated eIF2α, and caspase-4 were probed with corresponding antibodies. The protein contents of cell lysates were normalized to actin.

Caspase-3 Assay

Activation of caspase-3 was assessed by Western blotting to detect cleaved caspase-3 fragments and enzyme assay to measure caspase-3 activity. COS-7 cells grown in 96-well culture plates (5000 cells/100 μl/well) were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs. The

cells were cultured overnight in 10% FCS/OPTI-MEM I, and media were replaced with serum-free OPTI-MEM I. Two days after transfection, staurosporine (Sigma) was added to some wells of mock transfection at a concentration of 1 μmol/L and incubated for 4 hours at 37°C to make positive control for apoptosis. For Western blotting, culture media were removed from four wells of each transfectant and SDS sample buffer was directly added to the wells (50 μl/well) to lyse cells. Cell lysates from these wells were combined into one tube, homogenized by sonication, and boiled. The samples were subjected to SDS-PAGE, transferred to PVDF membranes, and probed with an antibody to cleaved caspase-3. The protein contents of cell lysates were normalized to actin. In enzyme assay, we used the Caspase-Glo 3/7 assay kit (Promega). The luminogenic caspase-3/7 substrate/luciferase mixture was added to another four wells (100 μl/well) of each transfectant and incubated for 1 hour at room temperature, which resulted in cell lysis, caspase cleavage of the substrate, and generation of luminescent signal produced by luciferase. Cell lysates were transferred to white-walled 96-well plates and luminescence was measured using a Wallac 1420 ARVO SX multilabel counter (Wallac Oy, Turku, Finland). Values were normalized to the number of cells, which was determined by counting cells grown in the other two wells of culture plates.

Terminal dUTP Nick-End Labeling (TUNEL) Assay

COS-7 cells grown on coverslips were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs, as described above. After fixation as described above, the cells were washed twice with PBS and incubated with 50 μl of TUNEL label mix containing TUNEL enzyme (both from Roche Diagnostic GmbH, Mannheim, Germany) for 60 minutes at 37°C. Subsequently the cells were washed three times and stained with NU-1, as described above. The specimens were examined under a confocal microscope. Five fields were randomly selected, and NU-1-positive and TUNEL-/NU-1-positive cells were counted. The experiment was repeated three times, and the mean ratio of TUNEL-/NU-1-positive cells to NU-1-positive cells (~300 cells in each experiment) was calculated.

Results

Effects of the E693Δ Mutation on β-Cleavage of APP

We previously showed that the E693Δ mutation markedly reduced both Aβ40 and Aβ42 secretion from transfected HEK293 cells.[17] This reduction may reflect low efficiency of β- and/or γ-cleavage of the mutant APP. To address this question, we studied the effects of this mutation on APP processing to produce Aβ. The β-cleavage of APP was examined by measuring the levels of CTFβ, a β-cleavage product, in HEK293 cells transfected with APP_{WT} and $APP_{E693\Delta}$ constructs. To prevent further cleavage of newly generated CTFβ, the cells were cultured in the presence of 1 μmol/L L-685,458, a γ-secretase inhibitor. The levels of Aβ in conditioned media were decreased to levels similar those with mock transfection, indicating that this inhibitor sufficiently inhibited γ-secretase at the concentration used (data not shown). APP and its product CTFs in cell lysates were analyzed by Western blotting with C40, a polyclonal antibody to the C-terminal region of APP. CTFα/APP and CTFβ/APP ratios were both increased by the presence of the E693Δ mutation (Figure 1, A and B). The CTFβ/CTFα ratio was higher in the mutant APP than wild-type APP. Thus, this mutation increased cleavages of the ectodomain of APP, particularly at the β-cleavage site.

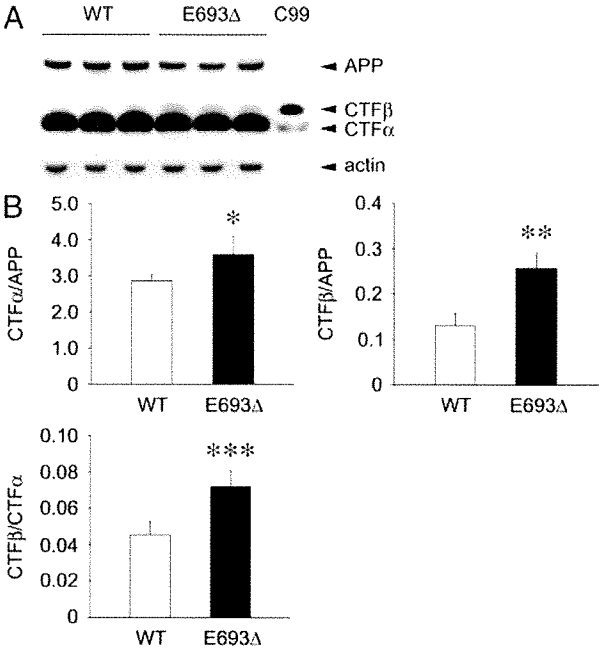

Figure 1. Increased β-cleavage of APP in the presence of E693Δ mutation. HEK293 cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs and cultured for 3 days in the presence of 1 μmol/L γ-secretase inhibitor L-685,458. **A:** Cell lysates were subjected to Western blotting with C40, a polyclonal antibody to the C-terminal region of APP. A sample prepared from cells transfected with C99 construct was also loaded on the gels as a molecular size marker. CTFα in the C99 lane was probably generated from transfected C99 and/or endogenous APP by α-secretase. **B:** Signals for APP, CTFα, and CTFβ were quantified using a LAS-3000 luminescent image analyzer, and CTFα/APP, CTFβ/APP, and CTFβ/CTFα ratios were calculated. The columns and bars represent the means ± SD for five transfectants. $^{*}P = 0.0145$, $^{**}P = 0.0002$, and $^{***}P = 0.0007$ versus wild-type (WT) by unpaired Student's *t*-test. The E693Δ mutation increased both α- and β-cleavage of APP, particularly β-cleavage.

Effects of the E693Δ Mutation on γ-Cleavage of APP

γ-Cleavage of APP was evaluated by measuring the levels of AICD, a γ-cleavage product. For this purpose, we used C99 (equivalent to CTFβ) constructs to avoid effects of β-cleavage. C99 and its product AICD in cell lysates were analyzed by Western blotting with C40. For molecular size markers, samples were also prepared from C59

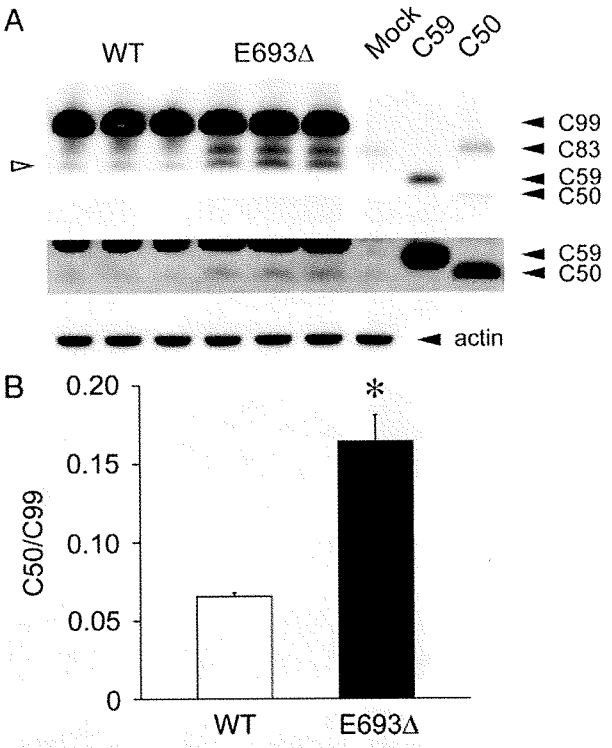

Figure 2. Increased γ-cleavage of C99 in the presence of E693Δ mutation. HEK293 cells were transfected with $C99_{WT}$ and $C99_{E693\Delta}$ constructs and cultured for 3 days. **A:** Cell lysates were subjected to Western blotting with C40. For molecular size markers, samples prepared from cells transfected with C59 and C50 constructs were also loaded on the gels. C83, equivalent to CTFα, was probably generated from transfected C99 and/or endogenous APP. **Open arrowhead** indicates an unidentified fragment of C99. **B:** Signals for C99 and C50 were quantified using a LAS-3000, and C50/C99 ratios were calculated. The columns and bars represent the means ± SD for three transfectants. $^*P = 0.011$ versus wild-type (WT) by unpaired Student's *t*-test. The E693Δ mutation increased γ-cleavage of C99. Taken together with the results in Figure 1, this mutation was shown to increase Aβ production.

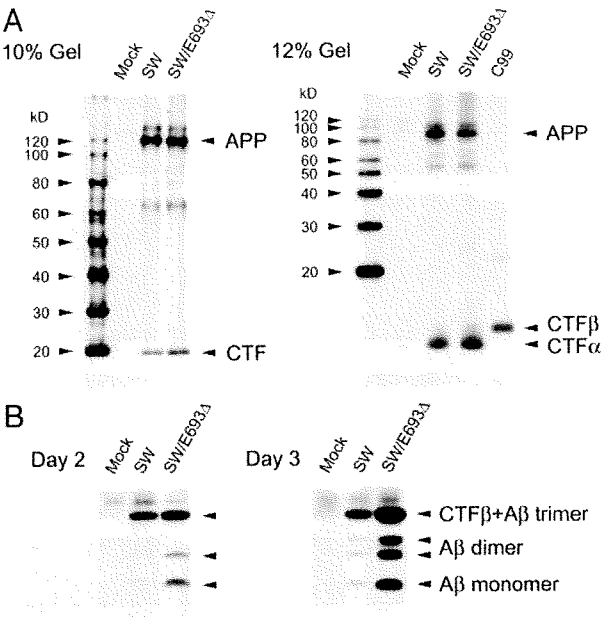

Figure 3. Increased intracellular accumulation of the mutant Aβ. HEK293 cells were transfected with APP_{SW} and $APPSW/_{E693\Delta}$ constructs and cultured for 2 and 3 days. **A:** Cell lysates were subjected to Western blotting with C40. Levels of expression of APP were confirmed to be nearly identical among different transfectants. No significant increase or appearance of APP-related fragments other than CTFs was observed in $APPSW/_{E693\Delta}$-transfected cells compared with APP_{SW}-transfected cells. **B:** Cell lysates from five dishes were combined, and intracellular Aβ was immunoprecipitated with 6E10, an anti-Aβ monoclonal antibody. The eluates from the immunoprecipitates were subjected to Western blotting with β001, a polyclonal antibody to the N-terminal region of Aβ. E695Δ mutation increased intracellular accumulation of Aβ. Notably, the intracellular Aβ appeared to form SDS-stable low-n oligomers, primarily dimers and possibly trimers.

and C50 transfectants and loaded on the gels. C59 and C50 are thought to be generated from CTFβ by function of γ-secretase at the γ40- and ε-cleavage sites, respectively.[22] Again, the C50/C99 ratio was increased by the presence of the mutation (Figure 2, A and B), indicating that this mutation enhanced the γ-cleavage of APP. We could not detect signals corresponding to C59 in this assay. Taken together, these findings showed that the E693Δ mutation did not inhibit Aβ production, and instead increased both β- and γ-cleavage of APP.

Effects of the E693Δ Mutation on Intracellular Aβ Accumulation

Despite the enhanced processing of the mutant APP to produce Aβ, extracellular Aβ40 and Aβ42 levels were lower in $APP_{E693\Delta}$-transfected cells than APP_{WT}-transfected cells,[17] suggesting that this mutation causes increased intracellular accumulation or accelerated degradation of Aβ in the extracellular space. The latter possibility is unlikely because the mutant Aβ was shown to be more resistant to proteolytic degradation.[17] We therefore examined the levels of intracellular Aβ in HEK293 cells transfected with APP_{WT}, $APP_{E693\Delta}$, APP_{SW}, and $APP_{SW/E693\Delta}$ constructs. Levels of expression of APP were confirmed to be nearly identical among different transfectants (Figure 3A). After preclearing of APP and CTFs from cell lysates with C40, Aβ was immunoprecipitated with the anti-Aβ monoclonal antibody 6E10, and detected by Western blotting with β001, a polyclonal antibody to the N-terminal region of Aβ. Compared with APP_{SW} and $APP_{SW/F693\Delta}$, the mutant Aβ was shown to accumulate more abundantly than wild-type Aβ (Figure 3B). Similar results were obtained with APP_{WT} and $APP_{E693\Delta}$, although the amounts of intracellular Aβ were much lower (data not shown). Notably, the intracellular Aβ appeared to form SDS-stable low-n oligomers, primarily dimers. It is possible that Aβ trimers also accumulated, although we could not distinguish Aβ trimers from CTFβ in this assay, in which CTFβ could not be completely precleared with C40 and could be co-immunoprecipitated with 6E10 and stained with β001. The results obtained suggest that the reduction in Aβ secretion caused by the E693Δ mutation is attributable to increased intracellular accumulation of Aβ.

Subcellular Localization and Accumulation of Aβ

We next examined the subcellular localization of Aβ by immunocytochemistry to identify intracellular sites of Aβ accumulation. We used COS-7 cells for this, because the cell bodies of COS-7 cells are larger than those of

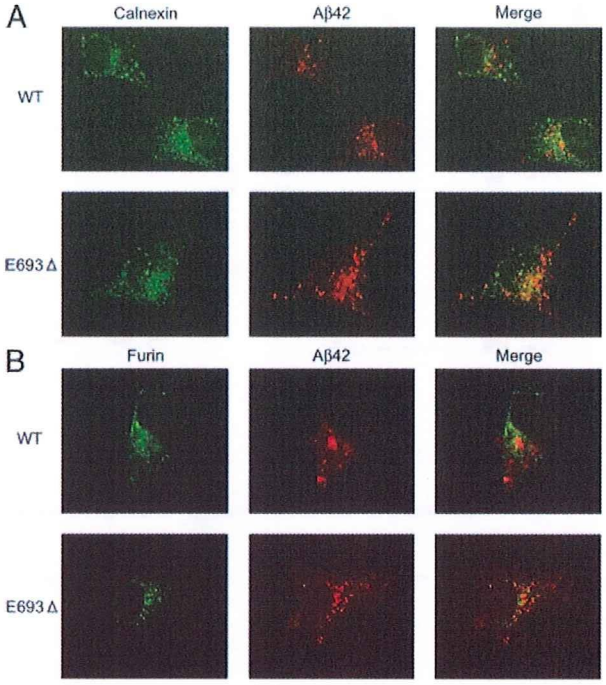

Figure 4. Increased accumulation of the mutant Aβ in the secretory pathway. COS-7 cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs and cultured for 2 days. The cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 1% saponin in PBS. After blocking with 20% calf serum in PBS, the cells were stained with the anti-Aβ42 monoclonal antibody 11C (red) in combination with an anti-calnexin antibody for ER (green) (**A**) or anti-furin antibody for Golgi apparatus (green) (**B**). The mutant Aβ was shown to accumulate in ER and Golgi apparatus more abundantly than wild-type (WT) Aβ.

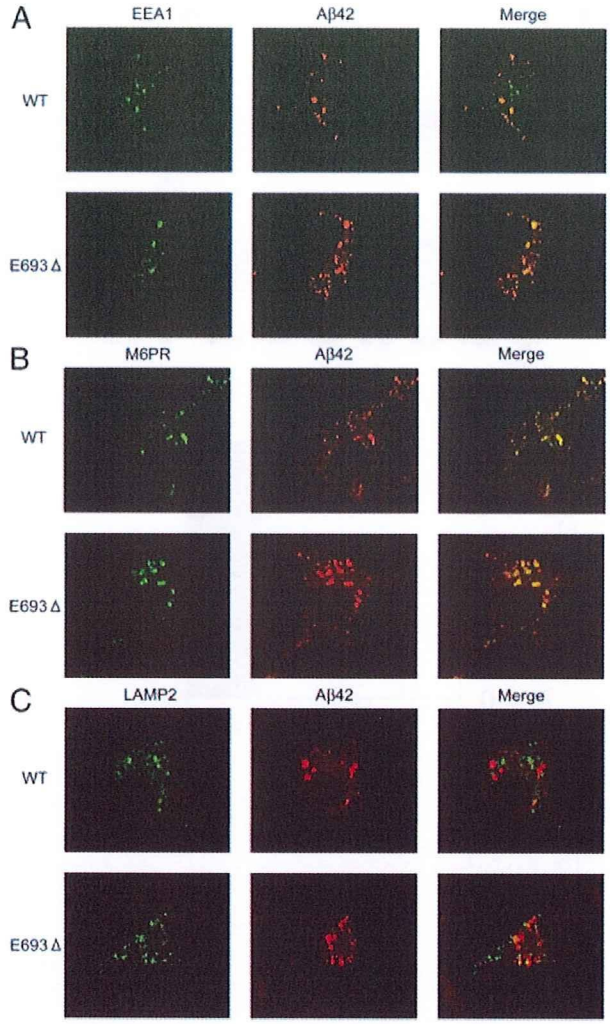

Figure 5. Increased accumulation of the mutant Aβ in the endocytic pathway. COS-7 cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs and cultured for 2 days. The cells were fixed, permeabilized, and blocked as described in Figure 4, and then stained with 11C (red) in combination with an anti-EEA1 antibody for early endosomes (green) (**A**), anti-M6PR antibody for late endosomes (green) (**B**), or anti-LAMP2 antibody for lysosomes (green) (**C**). The mutant Aβ was shown to accumulate in early and late endosomes and lysosomes more abundantly than wild-type (WT) Aβ.

HEK293 cells, making this cell line more suitable for examination of localization of Aβ. Cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs and stained with the anti-Aβ42 monoclonal antibody 11C in combination with polyclonal antibodies to organelles, including ER (calnexin), Golgi apparatus (Furin), early endosomes (EEA1), late endosomes (M6PR), lysosomes (LAMP-2), and autophagosomes (LC3), all of which have been suggested to be intracellular sites of Aβ generation and/or degradation.[23–28] No difference in level of APP expression was observed between APP_{WT}- and $APP_{E693\Delta}$-transfected cells on Western blotting (data not shown). In both APP_{WT}- and $APP_{E693\Delta}$-transfected cells, Aβ immunoreactivities were detected in all organelles tested, with preferential localization in late endosomes. Consistent with our immunoprecipitation/Western blotting results, the mutant Aβ was found to accumulate more abundantly than wild-type Aβ within cells.

Aβ Accumulation in the Secretory Pathway

Higher accumulation of the mutant Aβ was observed in ER (Figure 4A) and the Golgi apparatus (Figure 4B). These organelles are involved in control of protein folding, modifications, and sorting in the secretory pathway. Despite the increased accumulation of the mutant Aβ in ER and Golgi apparatus, its secretion from cells was markedly reduced,[17] implying impairment of APP/Aβ trafficking in this pathway. Reduced trafficking of APP to the plasma membrane has also been suggested in another APP mutation, the Arctic (E693G) mutation, which increased intracellular Aβ levels in transfected cells.[29]

Aβ Accumulation in the Endocytic Pathway

Higher accumulation of the mutant Aβ was also observed in early (Figure 5A) and late endosomes (Figure 5B) and lysosomes (Figure 5C). Of all organelles we tested, Aβ accumulation was most prominent in late endosomes. Enhanced accumulation of the mutant Aβ in endosomes/lysosomes suggests impaired sorting of Aβ in endosomal vesicles to lysosomes, which may have been caused by insufficient degradation of the mutant Aβ in lysosomes. Alternatively, endocytosis of the mutant

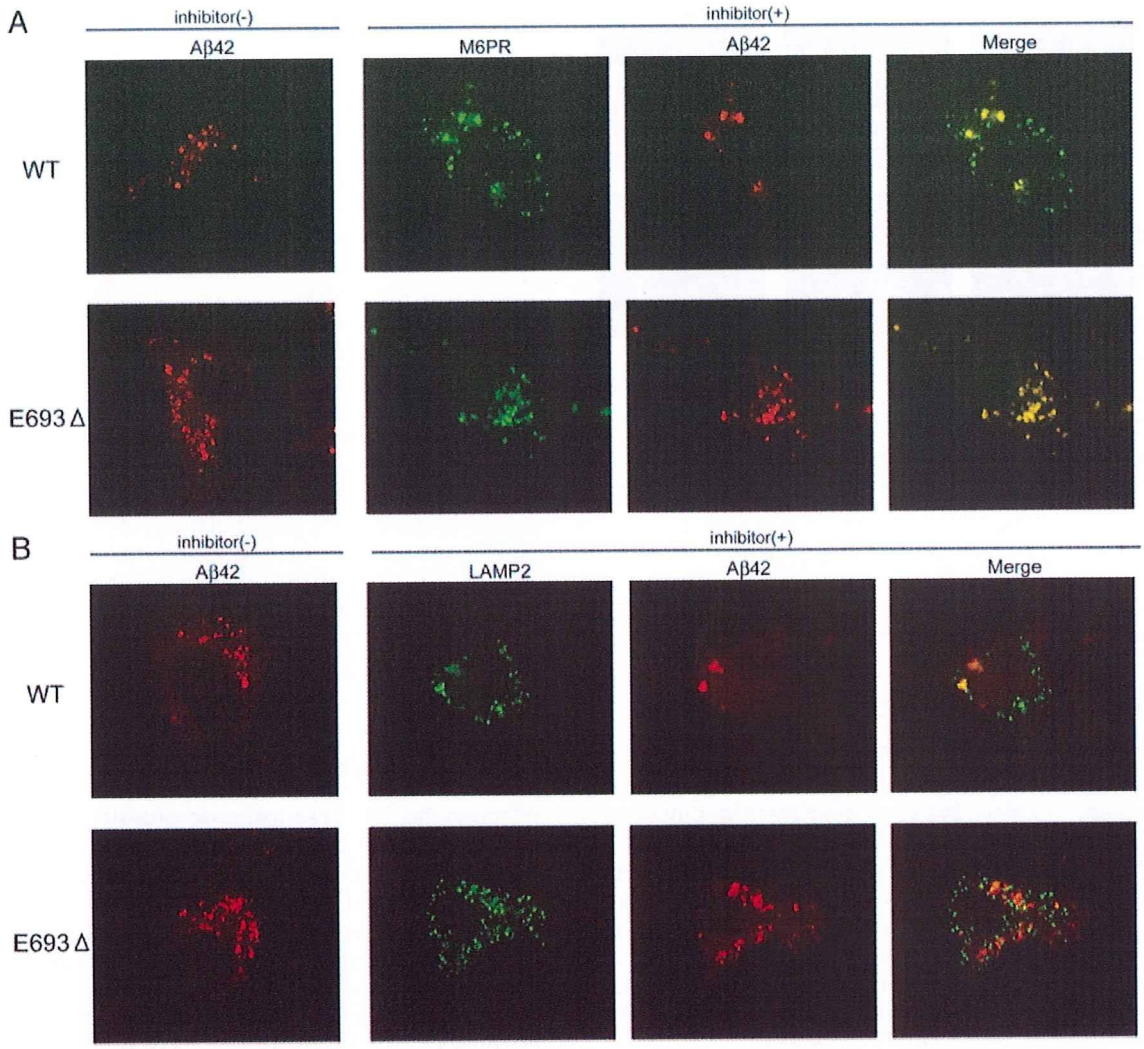

Figure 6. Effects of endocytic inhibition on endosomal/lysosomal accumulation of the mutant Aβ. COS-7 cells were transfected with APP_{WT} and $APP_{E693\Delta}$ constructs and cultured for 2 days. On day 2, the cells were treated with 25 μg/ml of the clathrin-dependent endocytosis inhibitor chlorpromazine and 25 μg/ml of the clathrin-independent endocytosis inhibitor nystatin for 15 minutes at 37°C. Soon after this treatment, the cells were fixed, permeabilized, and blocked as described in Figure 4, and then stained with 11C (red) in combination with an anti-M6PR antibody (green) (**A**) or anti-LAMP2 antibody (green) (**B**). The endosomal/lysosomal accumulation of wild-type (WT) Aβ was markedly attenuated by this treatment, whereas that of the mutant Aβ was not significantly affected.

APP may be increased, as suggested in the Arctic mutation,[29] which would result in increased Aβ production in these vesicles. To test the latter possibility, endocytosis was halted by treating cells with the endocytosis inhibitors chlorpromazine and nystatin. Endosomal/lysosomal accumulation of wild-type Aβ was markedly attenuated by this treatment, whereas that of mutant Aβ was not significantly affected (Figure 6, A and B). This result suggests that the mutant Aβ accumulated in these vesicles have been primarily generated via pathways other than endocytosis, such as the secretory and autophagic pathways, and transported into endosomal/lysosomal vesicles beyond their capacity to dispose it. However, we cannot exclude the possibility that the difference in effect of treatment between APP_{WT}- and $APP_{E693\Delta}$-transfected cells may have just reflected the difference in amount of Aβ accumulated intracellularly and that the time of treatment we used (15 minutes) was not enough to clear the mutant Aβ from these vesicles.

Aβ Accumulation in the Autophagic Pathway

Impairment of APP/Aβ trafficking and abnormal accumulation of Aβ in organelles may elicit the induction of autophagy, by which aged and dysfunctioning organelles are transported to late endosomes and lysosomes to be degraded. In support of this speculation, autophagosomes were much more strongly induced in $APP_{E693\Delta}$-transfected cells (Figure 7). In addition, higher immunoreactivity of the mutant Aβ was observed in these vesicles. Such an activation of the autophagic pathway should provide a certain amount of Aβ to endosomes/lysosomes, which may account for the steady accumulation of Aβ in endosomes/lyso-