

AD are unknown. Epidemiologic and clinical studies have revealed an association between elevated plasma homocysteine levels and increased risk of AD (Clarke et al., 1998; Miller, 1999; Seshadri et al., 2002). Homocysteine can be toxic to neurons and can increase their vulnerability to being damaged and killed by A β (Ho et al., 2001; Kruman et al., 2002), suggesting a mechanism whereby homocysteine might promote neuronal degeneration in AD. Homocysteine may not increase A β 42 production; instead it seems to impair DNA repair and reduce the threshold level of DNA damage required to trigger apoptosis (Kruman et al., 2000). Homocysteine is an intermediate in one-carbon metabolism and is produced from the amino acid methionine and homocysteine levels are normally kept low by the activities of methionine synthase, which regenerates methionine, and cystathionine β -synthase, which converts homocysteine to cysteine (Scott and Weir, 1998). Homocysteic acid (HA) is an oxidation product of homocysteine produced by brain cells and released in response to excitatory stimulation (Klanchnik et al., 1992). HA is a glutamate analogue that can activate the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor and may therefore function as an excitatory neurotransmitter in the brain (Cuenod et al., 1990). Excessive activation of NMDA receptors by HA has been implicated in the pathogenesis of epilepsy (Folbergrova et al., 2000).

HA has been shown to induce oxidative stress in neurons, which plays an important role in its neurotoxic actions (Sagara et al., 1999; Lockhart et al., 2000; Li et al., 2003). Because oxidative stress can induce the production of HA, and may also promote increased production and intracellular accumulation of A β (Gasparini et al., 1997; Paola et al., 2000), we carried out experiments aimed at determining whether intracellular A β 42 accumulation is involved in the neurotoxic actions of HA.

MATERIALS AND METHODS

Materials

Neurobasal medium, 2.5% trypsin, and B27 were obtained from Invitrogen (Carlsbad, CA) and the trypsin was diluted 10 times in phosphate-buffered saline (PBS). Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) was supplemented with 20 mM glucose, 2 mM L-glutamine, 1 mM sodium bicarbonate, and 100 U/ml gentamycin (Invitrogen). DL-Homocysteine was obtained from Wako (Tokyo, Japan), and polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA). Horseradish peroxidase (HRP)-linked F(ab')₂ fragment and enhanced chemiluminescence (ECL) system were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Fetal bovine serum (FBS), L-homocysteic acid, and all other chemicals were obtained from Sigma (St. Louis, MO).

Cell Cultures

Primary cultures of cortical neurons were prepared from embryonic Day 18–19 (E18–19) fetal Wistar or Sprague-Dawley rats. Cortical tissue fragments were incubated for 20 min in a

solution of 0.2% trypsin in calcium- and magnesium-free Hanks' balanced saline solution (HBSS), followed by incubation for 5 min in the presence of trypsin inhibitor. Cells were dissociated by trituration through the narrowed bore of a fire-polished pipette and were plated into culture dishes coated with either poly-L-lysine or polyethylenimine in DMEM medium containing 10% FBS. After cells had attached (3–6 hr post-plating) the culture medium was changed to Neurobasal medium with B27 supplements. Cultures were maintained in a humidified incubator with an atmosphere of 5% CO₂/95% air at 37°C. Experiments were carried out on cells that had been in culture for 8–12 days. Under these conditions, greater than 90% of the cells were neurons based upon immunoreactivity for microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP). Chinese hamster ovary (CHO) cell expressing human amyloid precursor protein (APP751) carrying the K670N,M671L Swedish mutation (CHO-APP_{swe}) were a generous gift from J. Shioi (Mount Sinai School of Medicine, New York).

Quantitation of Neuronal Viability

Two methods were used to quantify neuronal survival. In the first method, the mitochondrial dehydrogenase activity that cleaves 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to determine cell survival. The cortical neurons were incubated with MTT (0.5 mg/ml) for 2 hr at 37°C, and blue-colored formazan cleaved by dehydrogenase enzymes in active mitochondria was dissolved in 20% SDS/50% *N,N*-dimethylformamide (DMF) and quantified spectrophotometrically at 570 nm. The results are expressed as a percentage of the value for control cultures. Effective dose 50% (ED50) values and 95% confidence limits were determined by the method of Fieller with linear regression analysis of log-logit plot (logit conversion: $\log [p/(1 - p)]$; $p = \% \text{ neuronal viability reduction}$) using Yukms Statistical Library II v5 (Yukms Corp., Tokyo, Japan). For the second method, neuronal survival was quantified by counting viable neurons in premarked microscope fields (10 \times objective) before experimental treatment and at different time points after treatment. Viability of neurons was established by morphologic criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable, whereas neurons with fragmented neurites and a vacuolated or swollen soma were considered nonviable. Post-treatment survival values for each culture were calculated as a percentage of the number of viable neurons present before treatment.

Quantification of A β 40 and A β 42 Levels

Concentrations of A β 40 and A β 42 in culture medium were carried out by enzyme-linked immunosorbent assay (ELISA) using methods similar to those described previously (Fukamoto et al., 1999; Gasparini et al., 2004). Culture medium was collected and cleared by centrifugation (16,000 $\times g$ for 5 min at 4°C), and a 100- μ l sample of the medium was used for A β measurement. For measurement of intracellular A β , cells were solubilized in a buffer consisting of 20 mM Tris-HCl (pH 7.0), 2 mM EDTA, 0.2% SDS, 1% Triton X-100, 1% Nonidet (N)P-40, and protease inhibitors cocktail (Calbiochem). Sandwich ELISA assays were carried out to measure

TABLE I. Homocysteine, Homocysteic Acid, and Methionine Concentrations in Plasma of Alzheimer's Disease Patients and Age-Matched Control Subjects[†]

Compound	Controls (<i>n</i> = 250)	AD patients (<i>n</i> = 85)
Homocysteine (nmol/ml)	8.0 + 2.0	10.0 + 3.1*
Homocysteic acid	Not detected	Not detected
Methionine (nmol/ml)	21.2 + 6.0	13.0 + 3.0*

[†]Values are means and standard deviations. There were 70 males and 180 females in the control subject group (mean age of 77 + 8 years) and 21 males and 64 females in the Alzheimer's disease (AD) patient group (mean age of 75 + 7 years).

**P* < 0.001.

A β 40 and A β 42 according to the manufacturer's instructions (BioSource).

Human Subjects and Analyses of Plasma and Cerebrospinal Fluid Samples

Blood and cerebrospinal fluid (CSF) samples (lumbar fluid) were taken from control subjects and patients with probable AD. Information on number, gender, and average age of subjects are shown in Table I and II. All procedures were approved by the Institutional Review Board. Homocysteine and HA concentrations were measured using high-performance liquid chromatography (HPLC) with electron-capture detection using methods similar to those described previously (Grieve and Griffiths, 1992).

Statistical Analysis

All values are presented as mean \pm standard error of the mean (SEM) of at least three different experiments. Data were analyzed using one-way analysis of variance (ANOVA) between subjects, and post-hoc comparisons were made using Tukey's HSD test or Scheffé's test. In all cases, statistical significance was set at *P* < 0.05.

RESULTS

To determine whether HA affects APP processing, we measured the amounts of A β 42 in the culture medium of cultured cortical neurons that had been exposed to increasing concentrations of HA (10 nM to 100 μ M) for 12 or 24 hr. The concentration of A β 42 in the culture medium was decreased significantly in an HA concentration-dependent manner (1–100 μ M), in cultures exposed neurons that had been exposed to HA (Fig. 1A,B). In contrast, homocysteine at concentrations of 10 and 100 μ M had no significant effect on the amount of A β 42 released from the cultured neurons (data not shown). Neither HA nor homocysteine affected the amount of A β 40 released from the cultured neurons (Fig. 2).

Because HA caused a highly significant decrease in the amount of A β 42 released from neurons without affecting the amount of A β 40 released, we carried out additional analyses to determine if HA caused accumulation of A β 42 within the neurons. We measured levels of A β 42 and A β 40 in lysates of cultured neurons that had been exposed for 24 hr to increasing concentrations of HA. The amounts of intracellular A β 42 were increased signifi-

TABLE II. Homocysteine, and Homocysteic Acid Concentrations in Cerebrospinal Fluid of Alzheimer's Disease Patients and Age-Matched Control Subjects[†]

Compound	Controls (<i>n</i> = 28)	AD patients (<i>n</i> = 30)
Homocysteine (nM)	416 + 60	731 + 83*
Homocysteic acid (nM)	92 + 50	98 + 53

[†]Values are means and standard deviations. There were 12 males and 18 females in the control subject group (mean age of 66 + 6 years) and 7 males and 21 females in the Alzheimer's disease (AD) patient group (mean age of 63 + 6 years).

**P* < 0.01.

cantly by HA in a concentration-dependent manner, whereas amounts of intracellular A β 40 were not affected significantly by HA (Fig. 3). We next determined the effects of HA on the amounts of A β 40 and A β 42 retained within and released into the medium from cultured CHO cells stably overexpressing mutant human APP (Swedish mutation). These cells have been employed previously in studies of APP processing (Qin et al., 2003) and were chosen because it is known that this mutation increases the amount of A β 42 produced by cells. The amount of A β 40 released into the culture medium was decreased significantly by 10% and 98% in cells exposed to 1 and 10 μ M HA, respectively (Fig. 4). The amount of intracellular A β 40 was not affected significantly by HA. Extracellular A β 42 levels were decreased significantly and intracellular A β 42 levels increased significantly in cells exposed to HA (Fig. 4). The ability of HA to increase the accumulation of A β 42 generated from the mutant human APP strengthens the case for a role for HA in amyloid pathology in humans.

Analysis of cell viability using the MTT assay demonstrated a concentration-dependent decrease in the ability of the neurons to reduce this formazan dye when exposed to HA at concentrations of 100 and 1,000 μ M, but not at 10 μ M, after a 12-hr exposure period (Fig. 5). The latter results suggest that HA enhances intracellular accumulation of A β 42 in neurons at concentrations (<10 μ M) that are not overtly toxic to cells (Fig. 5).

Recent studies have provided evidence that A β 42 accumulates intracellularly in vulnerable neurons in AD patients (Gouras et al., 2000; Schwab and McGeer, 2000; Wirths et al., 2001; Mori et al., 2002) and in at least some mouse models of AD (Oddo et al., 2003). In addition, it has been shown that intracellular A β 1–42 can induce death in cultured neurons (Zhang et al., 2002; Pierrot et al., 2004). If accumulation of intracellular A β 1–42 plays an important role in the neurotoxic effect of HA, then inhibition of A β 42 production should increase the resistance of neurons to HA. To test this, we treated neurons with L-685,458, a compound shown previously to be a selective inhibitor of γ -secretase (Shearman et al., 2000), and then exposed them to HA. As expected, the γ -secretase inhibitor prevented the accumulation of A β 42 in the neurons exposed to HA (data not shown). Whereas HA killed 25–40% of the neurons in vehicle-treated control cultures, it killed

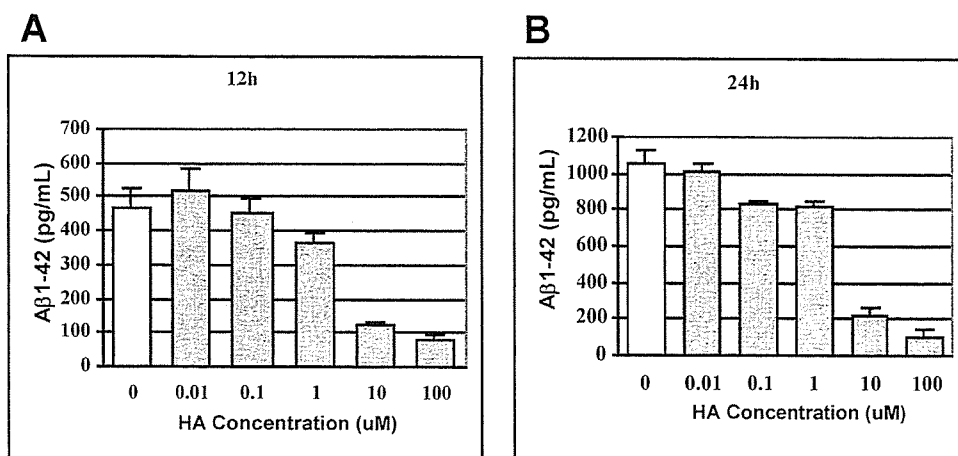


Fig. 1. Homocysteic acid (HA) decreases the amount of A β 42 released from cultured neurons. Neurons were exposed to the indicated concentrations of HA for either 12 (A) or 24 hr (B). Concentrations of A β 42 in the cultured medium were quantified by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. * $P < 0.05$, ** $P < 0.01$ compared to the value for cultures not exposed to HA.

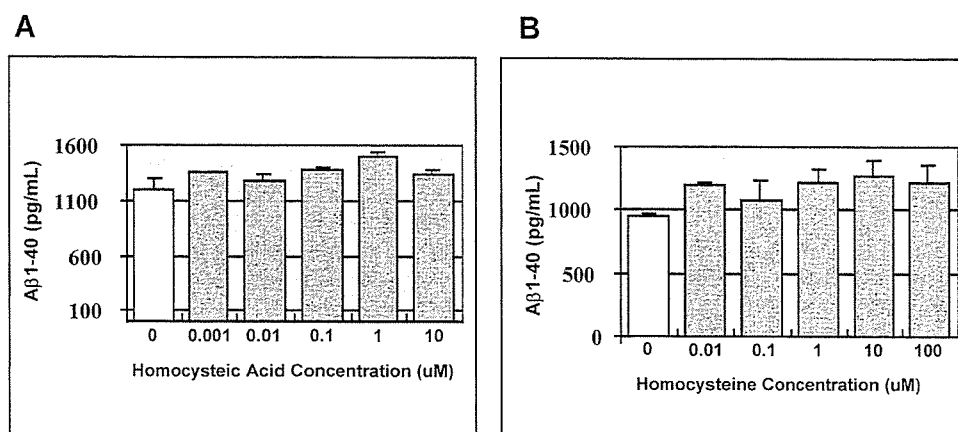


Fig. 2. Homocysteic acid (HA) does not affect the amount of A β 40 released from cultured neurons. Neurons were exposed to the indicated concentrations of either HA (A) or homocysteine (B) for 24 hr. Concentrations of A β 40 in the cultured medium were quantified by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. * $P < 0.05$ compared to the value for cultures not exposed to HA.

no neurons in cultures treated with the γ -secretase inhibitor (Fig. 6), suggesting that prevention of A β 42 production is sufficient to protect neurons against the toxicity of HA.

To test further the possibility that HA plays a role in the pathogenesis of AD, we measured concentrations of HA and homocysteine in samples of plasma and CSF from AD patients and age-matched control subjects. Consistent with previous studies (Clarke et al., 1998; Miller, 1999; Seshadri et al., 2002), homocysteine levels were elevated in plasma samples from AD patients (Table I). Consistent with a previous study (Frauscher et al., 1995), levels of HA in plasma samples from control subjects were below the limit of detection. HA was also not detectable in plasma samples

from AD patients. Methionine levels were significantly lower in plasma samples from AD patients compared to that from control subjects (Table I). Analysis of CSF revealed significantly higher levels of homocysteine in AD patient samples compared to that in samples from control subjects (Table II). HA levels in CSF exhibited considerable variability among subjects with no significant difference in samples from AD patients and control subjects (Table II).

Because HA levels were not elevated in CSF of AD patients, whereas homocysteine levels were elevated, we wondered whether elevated homocysteine levels might affect the vulnerability of neurons to being killed by HA. To this end, we determined the concentration of HA required to kill 50% of the neurons (EC₅₀) in

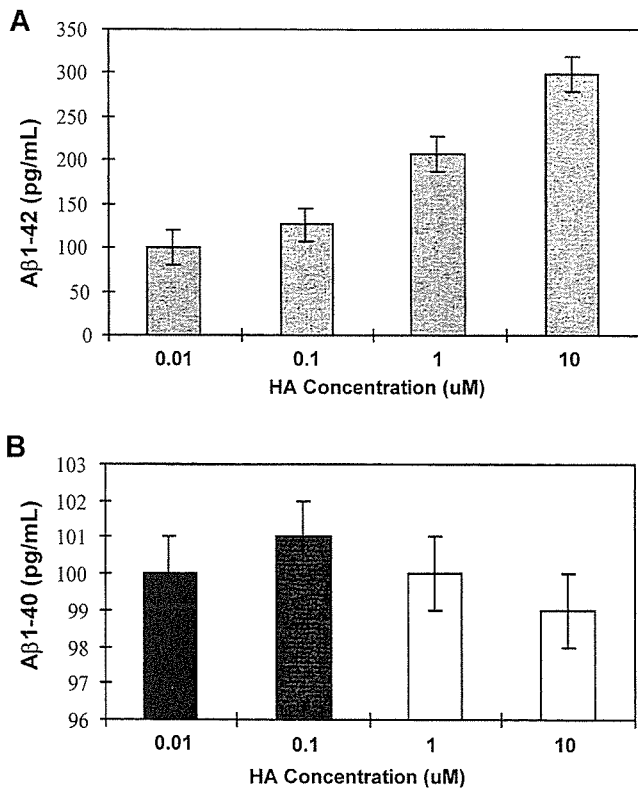


Fig. 3. Homocysteic acid (HA) increases the intracellular accumulation of A β 42 in cultured neurons. Neurons were exposed to the indicated concentrations of HA for 24 hr. Concentrations of A β 42 (A) and A β 40 (B) in the culture medium were quantified by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. * P < 0.05, ** P < 0.01 compared to the value for cultures not exposed to 0.01 μ M HA.

cortical cell cultures during a 24-hr exposure period in the absence or presence of increasing concentrations of homocysteine. The EC₅₀ values for HA were: HA alone, 175 μ M; HA plus 300 μ M homocysteine, 55 μ M; and HA plus 1 mM homocysteine, 0.6 μ M. When combined, subtoxic levels of homocysteine and HA thus can be neurotoxic.

DISCUSSION

The present findings demonstrate that exposure of neurons to HA results in the accumulation of A β 42 inside of the cells, and suggests further that the production of A β 42 contributes to the neurotoxic action of HA. The accumulation of A β 42 inside the neurons after exposure to HA was correlated their vulnerability to cell death. The ability of a γ -secretase inhibitor to attenuate the neurotoxicity of HA suggests that generation of A β 42 is a pivotal event in HA-induced neuronal death. Increasing evidence suggests that intracellular accumulation of A β 42 plays a role in the neurodegenerative process in AD. Analyses of brain tissue sections from AD patients showed that A β immunoreactivity is present in neurofibrillary tangle-bearing neurons (Hyman et al., 1989). Studies using antibodies that differentiate between

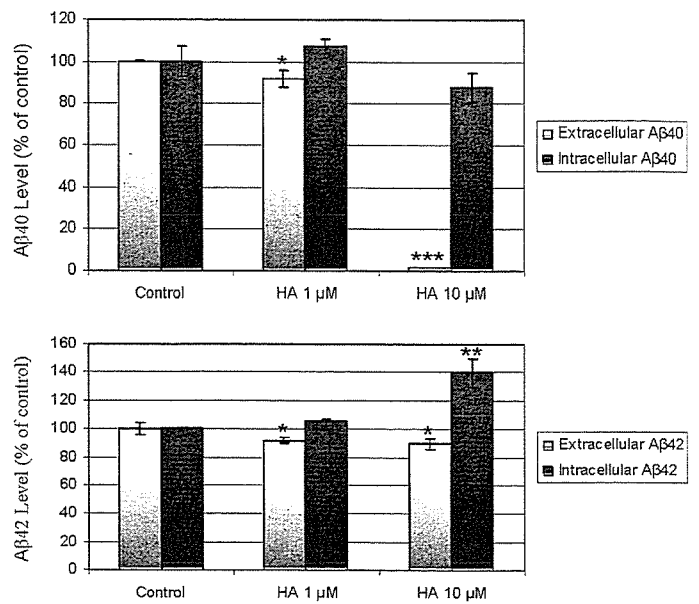


Fig. 4. Human A β 42 accumulates in Chinese hamster ovary (CHO) cells during exposure to homocysteic acid (HA). HA on the production of A β 40 and A β 42. CHO cells expressing mutant amyloid precursor protein (APP; Swedish mutation) were exposed to the indicated concentrations of HA for 24 hr. The A β 40 and A β 42 levels in cell extracts and in the conditioned medium of measured by sandwich enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM of at least four independent experiments. In control cultures, the concentrations of intracellular and extracellular A β 40 were 670 ± 48 pg/mg protein and 1130 ± 43 pg/ml, respectively; and of A β 42 were 337 ± 33 pg/mg protein and 126 ± 11 pg/ml, respectively. * P < 0.05, ** P < 0.01, and *** P < 0.001 compared to the value for control cultures.

A β 40 and A β 42 revealed that A β 42 is present in neurofibrillary tangle-bearing neurons in AD (Schwab and McGeer, 2000), and within neurons in the brains of Down syndrome patients (Mori et al., 2002). Additional analyses suggest that intraneuronal accumulation of A β 42 is an early event that may occur before synaptic dysfunction and cell degeneration (Gouras et al., 2000; Takahashi et al., 2002). Moreover, levels of A β 42 in the CSF are decreased in AD patients compared to that in control subjects (Sunderland et al., 2003), consistent with increased retention of A β 42 in neurons. We found that HA reduced release of A β 42 into the culture medium and increased the intracellular accumulation of A β 42. Our data therefore suggest that HA may play a role in the intracellular accumulation of A β 42 and consequent degeneration of neurons in AD. Consistent with this possibility, several recent studies have provided evidence that intracellular A β 42 can induce neuronal death (Kienlen-Campard et al., 2002; Zhang et al., 2002; Pierrot et al., 2004).

Although the specific mechanism whereby HA promotes intracellular accumulation of A β 42 was not established in the present study, prior information on the mechanisms by which HA and A β 42 kill neurons suggest several possibilities. HA can promote calcium influx

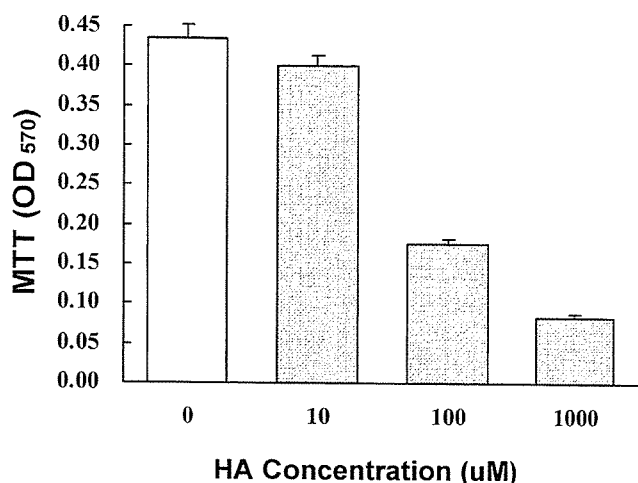


Fig. 5. Homocysteine induces neuronal death. Neurons were exposed to the indicated concentrations of homocysteic acid (HA) for 12 hr. Levels of MTT reduction in neurons were quantified. Values are the mean and SEM. $**P < 0.01$ compared to the value for cultures not exposed to HA.

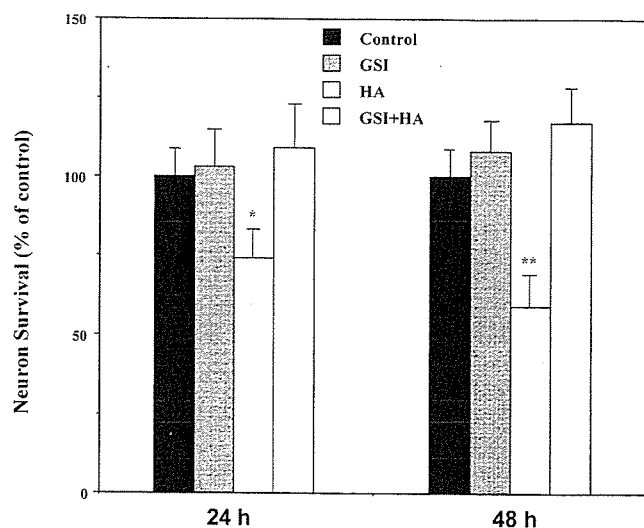


Fig. 6. A γ -secretase inhibitor protects cultured hippocampal neurons against death induced by homocysteic acid (HA). Cultures were treated with saline (Control), 100 nM of the γ -secretase inhibitor LY-411,575 (GSI), 1 μ M HA, or a combination of 100 nM GSI plus 1 μ M HA. The percentages of neurons surviving at 48 hr after treatment were determined. Values are the mean and SEM of determinations made in four separate cultures. $*P < 0.05$, $**P < 0.01$ compared to each of the other values.

into neurons by activating NMDA receptors (Kim et al., 1987) and a sustained increase in intracellular calcium levels induces intraneuronal accumulation of A β 42 (Pierrot et al., 2004). A β , in turn, can disrupt neuronal calcium homeostasis, an abnormality implicated in the pathogenesis of AD (Mattson et al., 1992; Mattson and Chan, 2003). In addition to a possible role for perturbed neuronal calcium homeostasis, oxidative stress might play a role in the adverse effects of HA on intraneuronal A β 42 accumulation and neurotoxicity. Oxidative stress is increased in neurons in AD and A β can induce membrane lipid peroxidation, which seems to be a pivotal event in synaptic dysfunction and neuronal death (Mattson, 2004b). HA induces oxidative stress and treatment of neurons with antioxidants protects them from being killed by HA (Lockhart et al., 2000). Data suggest that some antiinflammatory drugs can reduce the risk of AD, possibly by reducing A β 42 production and suppressing neuronal degeneration (Weggen et al., 2003; Yan et al., 2003). Antiinflammatory drugs reduce levels of oxidative stress through multiple mechanisms and would therefore be expected to protect neurons against the neurotoxic actions of HA.

Individuals with elevated plasma concentrations of homocysteine are at increased risk of AD (Seshadri et al., 2002). We found that subtoxic levels of homocysteine potentiate the neurotoxicity of HA, suggesting a role for HA in the AD-promoting action of homocysteine (Kruman et al., 2002; Seshadri et al., 2002). Consistent with previous findings (Miller, 1999; Seshadri et al., 2002), plasma levels of homocysteine were increased in AD patients in our study. Moreover, CSF levels of homocysteine were increased nearly twofold (Table II), suggesting the possibility that homocysteine contributes to abnormal APP processing and neuronal degeneration in

AD. HA is produced as an oxidation product of homocysteine, a process induced by hydrogen peroxide and other reactive oxygen species (Scott and Weir, 1998). The increased oxidative stress that occurs in the brain during aging, which is increased further in AD, might therefore be expected to enhance HA production in brain cells.

The effectiveness of several therapeutic agents in animal models, epidemiologic studies, and clinical trials in AD patients might involve actions that intervene in the pathway(s) by which HA damages neurons. For example, memantine is a selective open channel blocker of NMDA receptors that is beneficial in AD patients throughout the course of the disease (Ferris, 2003). Memantine can protect neurons from being damaged and killed by excitotoxins and A β (Miguel-Hidalgo et al., 2002), although it is not known if memantine decreases A β 42 production or intracellular accumulation. HA and homocysteine can increase oxidative stress by promoting calcium influx and by increasing DNA damage (Lockhart et al., 2000; Kruman et al., 2002). Increased oxidative stress contributes to the demise of neurons in AD, and antioxidants can protect neurons from being damaged and killed by A β 42, HA (Lockhart et al., 2000), and homocysteine (Kim and Pae, 1996). Oxidative stress might therefore play a role in HA-induced intracellular A β 42 accumulation, although this remains to be established. Our findings suggest that possible therapeutic approaches aimed at decreasing production of HA or blocking its neurotoxic action merit further investigation.

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A Novel Neurotrophic Agent, T-817MA [1-{3-[2-(1-Benzothiophen-5-yl) Ethoxy] Propyl}-3-azetidino] Maleate], Attenuates Amyloid- β -Induced Neurotoxicity and Promotes Neurite Outgrowth in Rat Cultured Central Nervous System Neurons

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ABSTRACT

Progressive neuronal loss in Alzheimer's disease (AD) is considered to be a consequence of the neurotoxic properties of amyloid- β peptides ($A\beta$). T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidino] maleate) was screened as a candidate therapeutic agent for the treatment of AD based on its neuroprotective potency against $A\beta$ -induced neurotoxicity and its effect of enhancing axonal regeneration in the sciatic nerve axotomy model. The neuroprotective effect of T-817MA against $A\beta(1-42)$ or oxidative stress-induced neurotoxicity was assessed using a coculture of rat cortical neurons with glia. T-817MA (0.1 and 1 μ M) was strongly protective against $A\beta(1-42)$ -induced (10 μ M for 48 h) or H_2O_2 -induced (100 μ M for 24 h) neuronal death. T-817MA suppressed the decrease of GSH

levels induced by H_2O_2 exposure (30 μ M for 4 h) in cortical neuron culture; therefore, T-817MA was likely to alleviate oxidative stress. Besides the neuroprotective effect, T-817MA (0.1 and 1 μ M) promoted neurite outgrowth in hippocampal slice cultures and reaggregation culture of rat cortical neurons. T-817MA also increased the growth-associated protein 43 content in the reaggregation culture of cortical neurons. These findings suggest that T-817MA exerts neuroprotective effect and promotes neurite outgrowth in rat primary cultured neurons. Based on these neurotrophic features, T-817MA may have a potential for disease modification and be useful for patients with neurodegenerative diseases, such as AD.

Pharmacotherapy of Alzheimer's disease (AD) is restricted to symptomatic treatment and has not been helpful in improving the deterioration of this disease (Tariot and Federoff, 2003). AD is a neurodegenerative condition that is characterized by progressive neuronal loss. There are many biological and cellular alterations in patients with AD; many aspects are involved in the pathogenesis of AD (Mattson et al., 2001). One of the most convincing hypotheses states that the conditions of AD might be a consequence of the neurotoxic properties of amyloid- β peptides ($A\beta$), although this hypothesis is still being argued (Selkoe, 1991; Naslund et al., 2000;

Rottkamp et al., 2002). $A\beta$ is considered to cause progressive synaptic degeneration and neuronal loss, thereby resulting in cognitive dysfunction and behavioral abnormalities in AD (Stepanichev et al., 2004). Extensive evidence indicates that oxidative stress may also be responsible for dysfunction or death of neuronal cells in AD (Markesbery et al., 2001; Mattson et al., 2001; Butterfield et al., 2002). The molecular mechanisms of $A\beta$ toxicity remain unclear. However, many studies support the idea that an oxidative event is critical for $A\beta$ toxicity (Rottkamp et al., 2002). For example, $A\beta$ toxicity is considered to be caused by unregulated reactive oxygen species such as hydrogen peroxide (H_2O_2) (Barnham et al., 2004). From this point of view, preventing oxidative stress may protect the remaining neurons from $A\beta$ insult during disease progression.

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ABBREVIATIONS: AD, Alzheimer's disease; $A\beta$, amyloid- β peptides; T-817MA, 1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidino] maleate; CNS, central nervous system; $A\beta(1-42)$, amyloid- $\beta(1-42)$; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MAP2, microtubule-associated protein 2; GAP-43, growth-associated protein 43; IGF-1, insulin-like growth factor-1; PBS, phosphate-buffered saline with neither Ca^{2+} nor Mg^{2+} .

Neurotrophic factors have been studied as one of the potential future therapies for AD (Tariot and Federoff, 2003). Neurotrophic factors can support the remaining neurons and protect them against disease progression in animal and cell culture models of neurodegenerative diseases (Mattson et al., 2001). In the adult nervous system, neurotrophic factors can also regulate neuronal plasticity by promoting nerve growth following injury (Gillespie, 2003) and thereby promote functional restoration (Lim et al., 2003). These features suggest that activation of neurotrophic pathways can contribute to the modification and prevention of disease progression in patients with AD.

T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidino] maleate) is a newly synthesized agent that was screened as a candidate therapeutic agent for the treatment of AD. Screening was carried out based on the neuroprotective potency against $A\beta$ -induced neurotoxicity and enhancing effect on axonal regeneration in the rat sciatic nerve axotomy model (daily treatment with T-817MA for 14 days enhanced the maximal regeneration distance of sciatic nerve axons measured using an electrophysiological analysis; Y. Nakada, unpublished observations) in the expectation of obtaining a neurotrophic agent. In the present study, to determine whether T-817MA exerts neurotrophic potency on the central nervous system (CNS), we evaluated its neuroprotective effect and neurite outgrowth promoting effect. Neuroprotective effect of T-817MA was assessed in amyloid- β (1-42)- [$A\beta$ (1-42)] or H_2O_2 -induced neuronal damages by using a coculture of rat cortical neurons with glia. The neurite outgrowth promoting effect was assessed using a hippocampal slice culture and cultured reaggregates of rat cortical neurons. The therapeutic potential of T-817MA in AD also is discussed.

Materials and Methods

Animals

Pregnant female Wistar/ST rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and kept in individual aluminum cages with laboratory bedding in an air-conditioned room on a 12-h light/dark cycle. The animals were given free access to a commercial diet (MF; Oriental Yeast Co., Ltd. Tokyo, Japan) and water. Neonatal rats were housed with their mother rat. All the experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals at Toyama Chemical Co., Ltd. (Tokyo, Japan) and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Materials

T-817MA was synthesized at Toyama Chemical Co., Ltd. The chemical structure of T-817MA is shown in Fig. 1. T-817MA was dissolved in distilled water at a concentration of 10 mM and diluted to 1, 0.1, and 0.01 μ M with Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) on the day of use. $A\beta$ (1-42): β -amyloid(1-42)

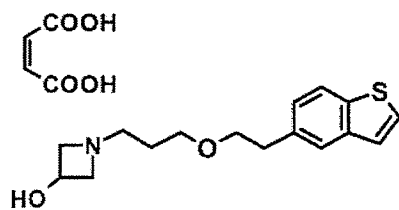


Fig. 1. Chemical structure of T-817MA.

HCl was purchased from AnaSpec Inc. (San Jose, CA). $A\beta$ (1-42) was sonicated in distilled water at a concentration of 250 μ M and then incubated at 37°C for 24 h. Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). FBS was heat-inactivated at 56°C for 30 min. Monoclonal anti-microtubule-associated protein 2 (MAP2) antibody (clone HM-2, mouse ascites fluid) and anti-gial fibrillary acidic protein were purchased from Sigma-Aldrich (St. Louis, MO). Goat polyclonal anti-growth-associated protein 43 (GAP-43) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). VECTASTAIN ABC-PO Goat IgG Kit and VECTASTAIN ABC-PO Mouse IgG Kit were purchased from Vector Laboratories (Burlingame, CA). Recombinant human insulin-like growth factor-1 (IGF-1) was purchased from PeproTech (Rocky Hill, NJ).

Neuroprotective Effect against $A\beta$ (1-42)-Induced Toxicity

Neuron/Glia Coculture. We prepared primary cortical neurons plated onto glial monolayer cultures as described previously (Pike et al., 1993). Primary cultures of cortical glial cells were prepared from 1- or 2-day-old neonatal rats. Neonatal rats were decapitated, and their whole brains were isolated. Cortices were dissected under a microscope and incubated at 37°C for 20 min with phosphate-buffered saline (PBS) devoid of Ca^{2+} and Mg^{2+} (Nissui) containing 0.25% trypsin (Invitrogen, Carlsbad, CA) and 40 units/ml DNase I (Sigma-Aldrich). Trypsinization was terminated by addition of 25% FBS. The dissociated cells were suspended in Eagle's minimum essential medium (Nissui) containing 10% FBS, 2 mM glutamine, and 25 μ g/ml gentamicin sulfate. The cells were cultured in 75-cm² flasks in a humidified CO₂ incubator with 5% CO₂/95% air at 37°C for 3 weeks to form a monolayer. The glial cells were then harvested and replated on 24-well plates at a density of 400 cells/mm² and then maintained for another 1 week. Cortical neurons were prepared from rat embryos (day 18 gestation). Cortices were dissected under the microscope and incubated with PBS containing 0.25% trypsin and 40 units/ml DNase I at 37°C for 20 min. Trypsinization was terminated by the addition of 25% FBS. Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine, and 25 μ g/ml gentamicin sulfate. They were subsequently seeded onto the cortical glial monolayer culture. Three or four days after plating cortical cells, the entire medium was replaced with DMEM containing 1% (v/v) N-2 supplement (Invitrogen), and 10 μ M cytosine arabinoside (Nacalai Tesque, Kyoto, Japan) was added to halt glial proliferation. The cells were then incubated for 7 days in this medium. $A\beta$ (1-42) was added to the coculture at a concentration of 10 μ M. T-817MA was added simultaneously with $A\beta$ (1-42) application at concentrations of 0 (control), 0.01, 0.1, and 1 μ M, and the cells were further incubated for 48 h. For the normal group, the preparations were maintained in the medium with neither T-817MA nor $A\beta$ (1-42).

Assay for the Viability of Neurons. Neuronal cell viability was quantified by measuring MAP2 immunoreactivity. This method is favorable for measuring neuronal survival in the presence of glial cells without resorting to the counting of neurons (Brooke et al., 1999). Measurement was performed using monoclonal anti-MAP2 antibody and VECTASTAIN ABC-PO mouse IgG kit in accordance with the manufacturer's manual. In brief, the cultured cells were fixed with ice-cold methanol for more than 30 min and washed with PBS three times. Following the blocking procedure with horse serum for 20 min, the cells were incubated for 30 min with anti-MAP2 antibody (1:3000 dilution). The cells were then incubated with biotinylated secondary antibody (included in the kit) for 30 min, followed by incubation with VECTASTAIN ABC Reagent for 30 min. Peroxidase activity was estimated using *o*-phenylenediamine and H₂O₂ (Maus et al., 2002). The cells were incubated with *o*-phenylenediamine solution (10 mg/ml *o*-phenylenediamine, 0.03% hydrogen peroxide, 0.05 mM citric acid, and 0.1 mM disodium hydrogen phosphate; Wako Pure Chemicals, Osaka, Japan) for 3 min. Thereafter, the reaction was terminated using 0.05 M sulfuric acid (Wako Pure

Chemicals). Each solution was diluted with 1 ml of distilled water. The absorbance of each reaction solution was measured at 490 nm. The procedures were all performed at room temperature.

Neuroprotective Effect against H₂O₂-Induced Toxicity

Assessment in Neuron/Glia Coculture. A cortical neuron/glia coculture was prepared as described above. T-817MA was added to the cocultures at concentrations of 0 (control), 0.01, 0.1, and 1 μ M, and the cells were subsequently incubated for 5 min or 24 h. H₂O₂ was then added to the coculture at a concentration of 100 μ M, and the cells were incubated for another 24 h. For the normal group, the preparations were maintained in the medium with neither T-817MA nor H₂O₂. Neuronal cell viability was quantified by measuring the MAP2 immunoreactivity as described above.

Assessment in Cortical Neuron Culture. Primary cultures of cortical neurons were prepared from rat embryos (gestational day 18). Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine, and 25 μ g/ml gentamicin sulfate. The cells were seeded onto 24-well plates precoated with poly-L-lysine (molecular weight, 30,000–70,000; Sigma-Aldrich) at a density of 1000 cells/mm². Forty-eight hours after cell seeding, cytosine arabinoside was added to the cultures (10 μ M) and removed by medium exchange after 24 h. In this condition, as shown by immunocytochemical studies using anti-MAP2 monoclonal antibody, the cultures were highly enriched in neurons, and less than 10% of the cells exhibited immunoreactivity with a rabbit antibody raised against glial fibrillary acid protein (data not shown). T-817MA was added to the cultures at concentrations of 0 (control), 0.01, 0.1, and 1 μ M. Twenty-four hours following T-817MA application, H₂O₂ was added at a concentration of 30 μ M, and the cells were incubated for another 24 h in the continuous presence of T-817MA. For the normal group, the preparations were maintained in the medium with neither T-817MA nor H₂O₂. Neuronal cell viability was quantified by measuring the MAP2 immunoreactivity as described above.

Measurement of the Intracellular GSH Content in Primary Cortical Neuron Culture. T-817MA was added to the primary culture of purified neuron at concentrations of 0.01, 0.1, and 1 μ M. Twenty-four hours following T-817MA application, H₂O₂ was added at a concentration of 30 μ M. The cells were then incubated for another 4 h in the continuous presence of T-817MA. The intracellular GSH content in the cultured neurons was measured in accordance with a previously reported method (Hiraku et al., 2002) with some modifications. In brief, the cells were washed once with ice-cold PBS, followed by the addition of 100 mM perchloric acid, and then harvested. The harvested cells were homogenized for 5 s with a microhomogenizer (Seiko Instruments, Chiba, Japan) and then centrifuged at 10,000g for 10 min at 4°C. The GSH content in the supernatant was measured using a high-pressure liquid chromatography system consisting of an L-7100 pump (Hitachi Ltd., Tokyo, Japan) and ECD-300 electrochemical detector (Eicom, Kyoto, Japan) equipped with a Symmetry C18 column (i.d., 4.6 mm \times 25 cm; Waters, Milford, MA), a WE-AU gold electrode (Eicom), and a 50-mm GS-50 gasket (Eicom). The mobile phase consisted of 4.4 mM phosphate buffer, pH 2.5, 88 mg/l 1-octanesulfonic acid, 4.4 mg/l EDTA \cdot 2Na, and 12% methanol. The measurement was carried out at room temperature at a flow rate of 0.8 ml/min. The voltage of the gold electrode was set at +600 mV against the Ag/AgCl reference electrode (RE-100; Eicom). Authentic GSH (Sigma-Aldrich) was simultaneously measured as an external standard under these conditions. Neuronal cell viability was also quantified by measuring the MAP2 immunoreactivity as described above.

Evaluation of Neurite Outgrowth Promoting Effect

Hippocampal Slice Culture. Organotypic hippocampal slice culture was prepared in accordance with the previously reported method (Stoppini et al., 1991) with some modifications. Hippocampal

slices were prepared from 7-day-old rat pups. The dorsal hippocampus was isolated and cut into transverse slices of 350- μ m thickness with a tissue chopper (Mickle Laboratory Engineering, Guilford, UK). The slices were placed onto dishes precoated with poly-L-lysine (three slices in each dish) and cultured in interface configuration with DMEM containing 12.5 mM HEPES, 1% (v/v) B-27 supplement (Invitrogen), 2 mM glutamine, and 25 μ g/ml gentamicin sulfate. T-817MA was added at concentrations of 0 (control), 0.01, 0.1, and 1 μ M at the initiation of the slice culture. The culture was then incubated at 37°C in 5% CO₂/95% air for 8 days. During the culture period, half the volume of the medium in each dish was changed every 2 or 3 days.

Reaggregation Culture of Cortical Neurons. Reaggregation culture of the cortical neurons was carried out referring to a previously reported method (Gao et al., 1992). The cortical neurons were harvested from rat embryos (embryonic day 18). Dissociated cortical cells were suspended in DMEM containing 10% FBS, 2 mM glutamine, and 25 mg/ml gentamicin sulfate and seeded onto 100-mm noncoated dishes at a density of 50,000 cells/mm² and then cultured for 4 days. As a result of this procedure, cortical neurons formed reagggregates and floated in the medium. The suspended reagggregates of neurons were collected and seeded onto six-well plates (for evaluating neurite length) or 24-well plates (for measuring contents of MAP2 and GAP-43) precoated with poly-L-lysine. The reagggregates of cells were cultured for 3 days. T-817MA was then added at concentrations of 0 (control), 0.01, 0.1, and 1 μ M, and the reagggregates of cells were cultured further for 4 days. All the cultures were incubated in the atmosphere of humidified 5% CO₂/95% air at 37°C.

Measurement of Neurite Outgrowth. Following treatment of T-817MA, cultured slices or cortical reaggregation culture was fixed with methanol. The neurites generated from slices or cell reagggregates were subsequently immunostained (Schreyer et al., 1997) with a goat polyclonal anti-GAP-43 antibody using VECTASTAIN ABC-PO Goat IgG Kit. Neurite length was defined as the distance from the edge of a slice or a reaggregate to the neurite tip. Neurite outgrowth was evaluated by measuring the length of the longest neurite in each slice or reaggregate under a microscope using a micrometer. The outgrowth was measured in three slices or three reagggregates in each dish, and their mean value was regarded as the representative value of the dish. All measurements were performed in a blinded manner. GAP-43 content was quantified by enzyme immunoassay (Schreyer et al., 1997) using VECTASTAIN ABC-PO kit with goat polyclonal anti-GAP-43 antibody.

Statistical Analysis

The results are represented as mean with S.E.M. Statistical significance of the differences between the two groups was analyzed by an analysis of variance (F-test), followed by Student's *t* test. Statistical significance of the T-817MA treatment groups from the control group was evaluated by Dunnett's test. These analyses were performed using SAS release 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). *P* < 0.05 (two-tailed) was considered to be significant.

Results

Neuroprotective Effect of T-817MA

A β (1–42)-Induced Neuronal Death. The protective effect of T-817MA on cortical neurons against A β (1–42)-induced neuronal death was investigated in the neuron/glia coculture. Typical microscope images of MAP2 immunocytochemistry are shown in Fig. 2, A–C. Many neurons that formed clusters were stained on the glial monolayer in the absence of A β (1–42) (normal). On the other hand, few neurons were detected with 10 μ M A β (1–42) treatment (control). T-817MA treatment preserved the cortical neurons in the presence of A β (1–42). To evaluate the number of surviving

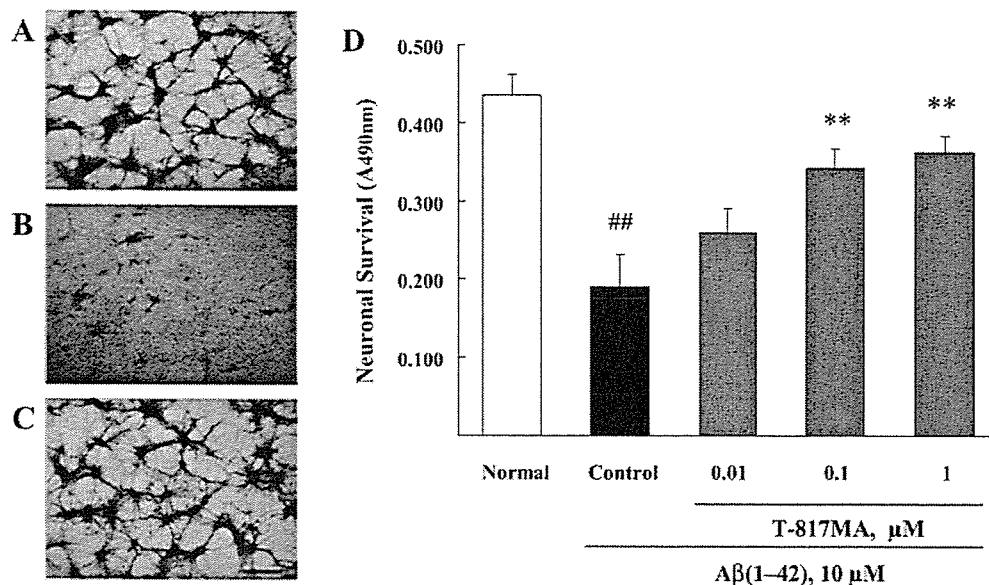


Fig. 2. Effect of T-817MA on Aβ(1-42)-induced neuronal death. Aβ(1-42)-induced neuronal death and the neuroprotective effect of T-817MA were assessed using rat neuron/glia coculture. Images of MAP2 immunocytochemistry showed typical responses: neurons with vehicle treatment (normal; A), 10 μM Aβ(1-42) treatment for 48 h (control; B), and cotreatment of 1 μM T-817MA with 10 μM Aβ(1-42) treatment for 48 h (C). Calibration bar indicates 200 μm. D, neuronal survival was estimated as MAP2 immunoreactivity using enzyme immunoassay. Results are expressed as absorbance at 490 nm. Columns and bars indicate the mean with S.E.M. ($n = 6$). **, $P < 0.01$ versus control (Dunnett's test) and ##, $P < 0.01$ versus normal (Student's t test).

neurons, MAP2 immunoreactivity, which is known to correlate the number of neurons (Brooke et al., 1999), was measured. The exposure of Aβ(1-42) significantly reduced MAP2 immunoreactivity. T-817MA significantly prevented this reduction at 0.1 and 1 μM (Fig. 2D). A peptide Aβ(42-1) did not reduce MAP2 immunoreactivity on the neuron/glia coculture (data not shown), which suggested that Aβ(1-42) specifically induced the neuronal damage.

H₂O₂-Induced Neuronal Death. Previous studies indicated that oxidative stress is supposed to contribute to Aβ-induced neuronal damages (Markesbery et al., 2001; Butterfield et al., 2002). On this basis, the effect of T-817MA on H₂O₂-induced neuronal damage was investigated in the neuron/glia coculture. The 100 μM H₂O₂ treatment greatly reduced the number of surviving neurons in the culture (Fig. 3, control). When T-817MA was pretreated for 24 h, T-817MA significantly prevented this neuronal damage at 0.1 and 1 μM (Fig. 3A). Conversely, a brief (5 min) pretreatment with T-817MA, followed by its continuous presence with H₂O₂, did not rescue the H₂O₂-treated neurons from death (Fig. 3B).

T-817MA Attenuated H₂O₂-Induced Reduction of Intracellular GSH Contents.

A similar protective effect of T-817MA was observed in the primary cortical neuron culture. H₂O₂ exposure at 30 μM for 24 h induced neuronal death (Fig. 4A, control). Twenty-four hours of pretreatment, followed by the continuous presence of T-817MA, prevented this oxidative stress-induced neuronal death at 0.1 and 1 μM (Fig. 4A). To investigate the effect of T-817MA on this oxidative stress, intracellular GSH content was measured as an index of the intracellular oxidative condition in rat cortical neurons under H₂O₂ exposure. In this experiment, primary cultures of cortical neurons were exposed to 30 μM H₂O₂ for 4 h. Viability of neurons was not altered at 4 h following 30 μM H₂O₂ exposure (Fig. 4B). On the other hand, GSH content was significantly reduced by this stress (Fig. 4C). The effect of T-817MA was examined with pretreatment for 24 h, then continuous presence for 4 h with 30 μM H₂O₂ exposure. T-817MA almost completely prevented such GSH reduction at 0.1 and 1 μM (Fig. 4C). T-817MA alone did not exert

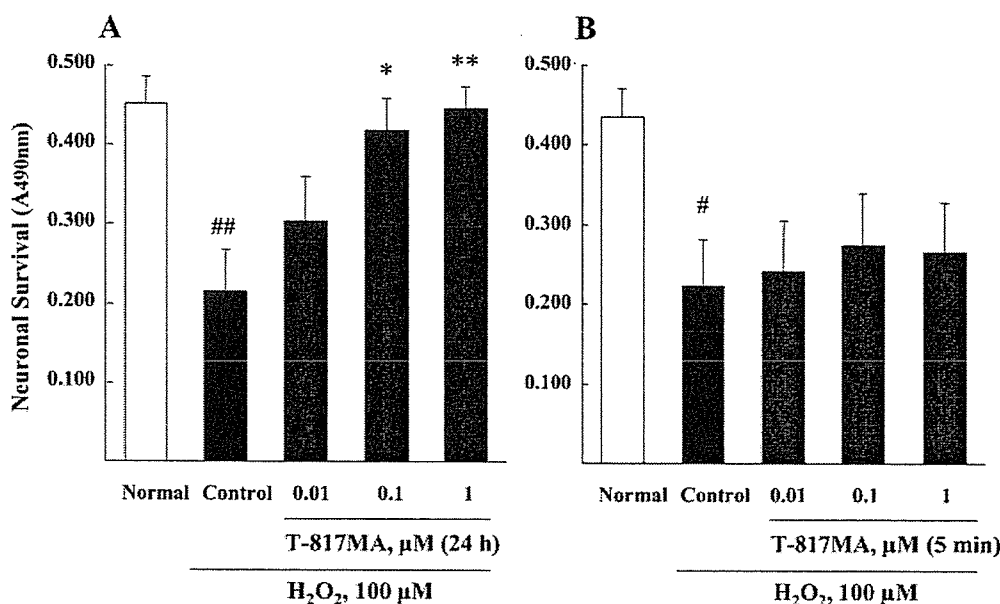


Fig. 3. Effect of T-817MA on H₂O₂-induced neuronal death. H₂O₂-induced (100 μM for 24 h) neuronal death and the neuroprotective effect of T-817MA were assessed using rat neuron/glia coculture. T-817MA was pretreated for 24 h (A) or for 5 min (B) and was continuously existed with H₂O₂. Neuronal survival was estimated as MAP2 immunoreactivity using enzyme immunoassay. Results are expressed as absorbance at 490 nm. Columns and bars indicate the mean with S.E.M. ($n = 6$). *, $P < 0.05$, **, $P < 0.01$ versus control (Dunnett's test) and #, $P < 0.05$; ##, $P < 0.01$ versus normal (Student's t test).

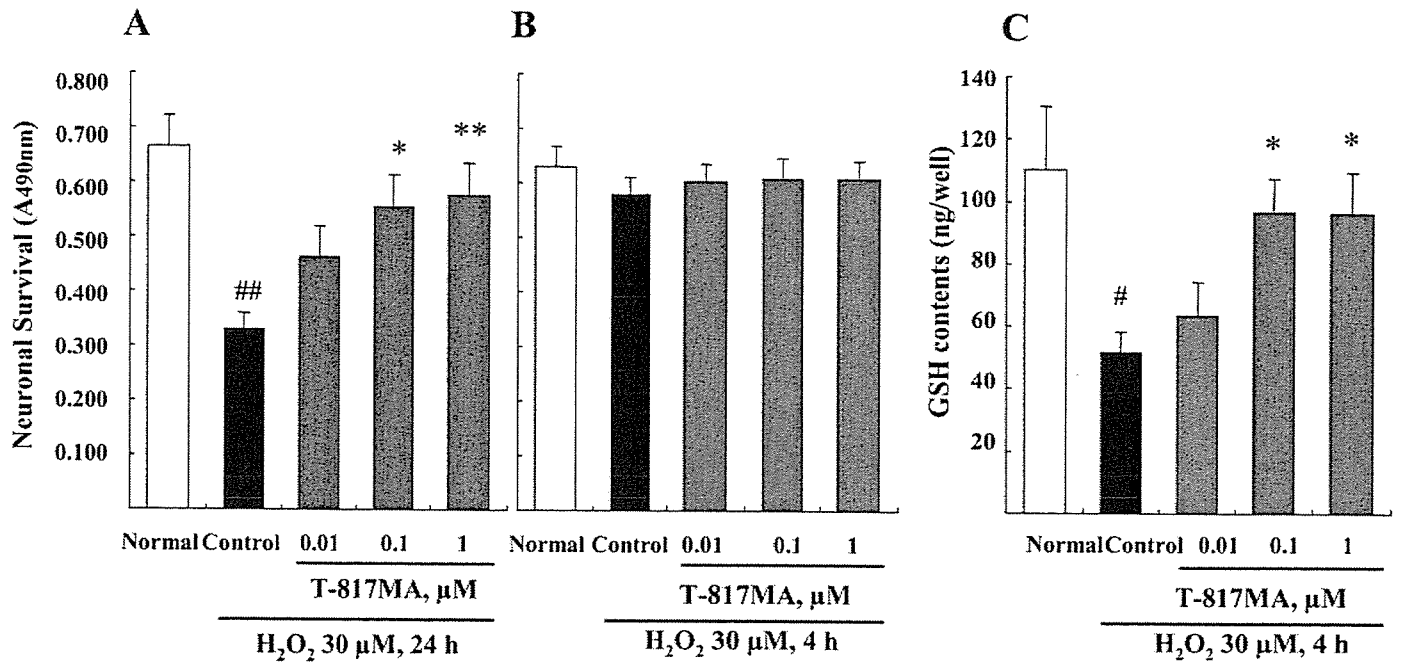


Fig. 4. Effect of T-817MA on H₂O₂-induced neuronal death and GSH reduction. Primary cultures of cortical neurons were pretreated with several concentrations of T-817MA for 24 h and then exposed to 30 μM H₂O₂ for 24 h (A) or 4 h (B) in the continuous presence of T-817MA. Neuronal survival was estimated as MAP2 immunoreactivity using enzyme immunoassay. C shows GSH contents in the culture assessed with 4-h H₂O₂ treatment, the same condition as B. Results are expressed as absorbance at 490 nm (A and B) or GSH contents (nanograms per well). Columns and bars indicate the mean with S.E.M. (*n* = 6). *, *P* < 0.05; **, *P* < 0.01 versus control (Dunnett's test) and #, *P* < 0.05; ##, *P* < 0.01 versus normal (Student's *t* test).

significant effect on the intracellular GSH content in the normal medium (data not shown).

T-817MA Promoted Neurite Outgrowth

We further investigated the neurite outgrowth promoting action of T-817MA by using two types of cultured neurons: the hippocampal slice culture and the cortical reaggregation culture. Hippocampal slices with 1 μM T-817MA treatment generated more and much longer neurites than control slices (Fig. 5, A and B). T-817MA significantly increased the neurite length at 0.1 and 1 μM (Fig. 5C).

Likewise, neurite outgrowth promotion of T-817MA was observed in the cortical reaggregation culture. Typical photomicrographs revealed that T-817MA treatment induced re-aggregates to generate longer neurites than control slices (Fig. 6, A and B). T-817MA significantly promoted neurite outgrowth at 0.1 and 1 μM (Fig. 6C). In addition to the neurite length, we also quantified the immunoreactivity of neurites for GAP-43, which is specifically located in axons. GAP-43 immunoreactivity was significantly increased by T-817MA (Fig. 6D). The effect of IGF-1, which has potential to promote neurite outgrowth in vitro (Kim et al., 1997), was

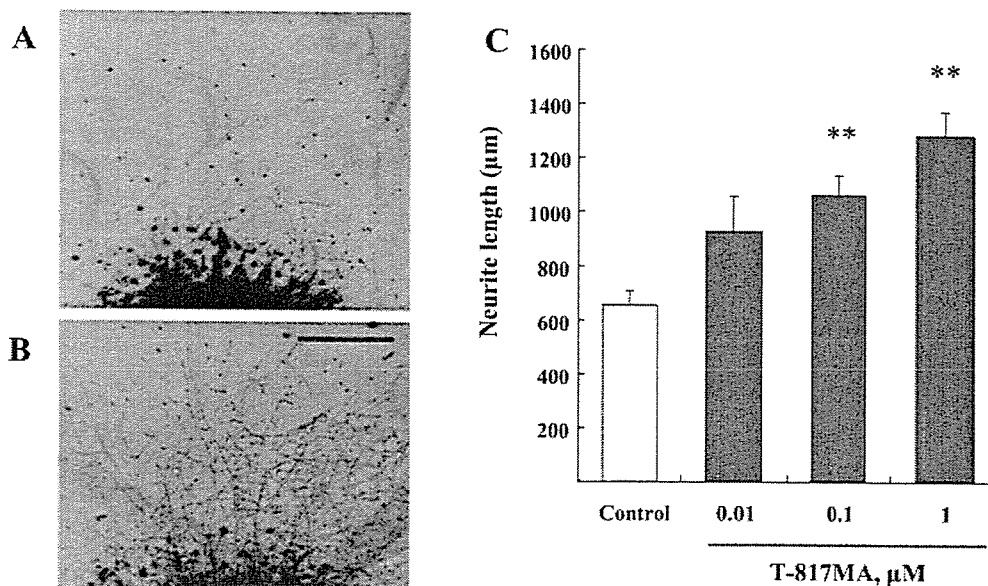


Fig. 5. Effect of T-817MA on neurite outgrowth in rat hippocampal slice cultures. Typical images of neurite response with GAP-43-immunostained neurites are shown in control (A) and 1 μM T-817MA for 8 days (B). Calibration bar, 500 μm. C, the neurite outgrowth was quantified by measuring the distance from the edge of the slice to the tip of the longest neurite (micrometers). Data are shown as mean with S.E.M. (*n* = 10). **, *P* < 0.01 versus control (Dunnett's test).

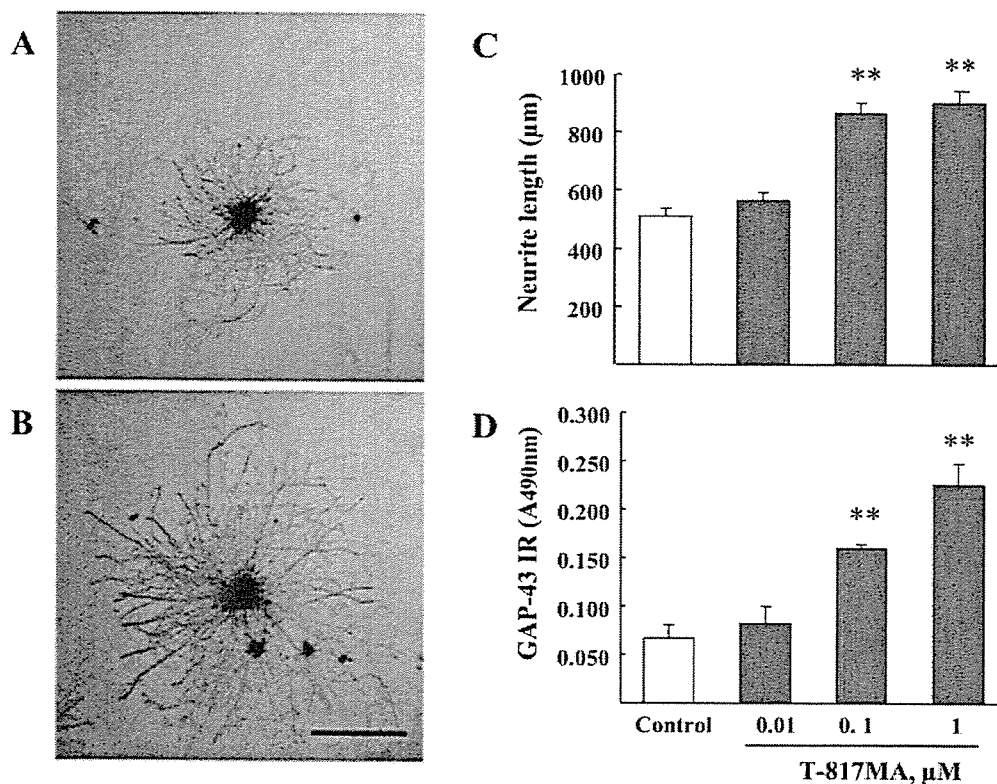


Fig. 6. Effect of T-817MA on neurite outgrowth in rat cortical reaggregation cultures. Typical images of neurite response with GAP-43-immunostained neurites are shown in control (A) and 1 μ M T-817MA for 4 days (B). Calibration bar, 500 μ m. C, the neurite outgrowth was quantified by measuring the distance from the edge of the aggregate to the tip of the longest neurite (micrometers). Quantitative analysis was performed by GAP-43 immunoreactivity (D) using enzyme immunoassay, and its results are expressed as absorbance at 490 nm. Data were shown as mean with S.E.M. (C: $n = 8$; D: $n = 4$). **, $P < 0.01$ versus control (Dunnett's test).

evaluated in this cortical reaggregation culture as a reference experiment. IGF-1 promoted neurite outgrowth; GAP-43 immunoreactivity ($A_{490 \text{ nm}}$) was 0.236 with 100 ng/ml IGF-1, which was higher than the control value ($A_{490 \text{ nm}}$, 0.158). This result indicated that the cortical reaggregation culture was useful for assessing neurite outgrowth promotion.

Discussion

AD is a neurodegenerative condition characterized by progressive neuronal loss, which may be a consequence of the neurotoxic properties of the $A\beta$ (Selkoe, 1991; Naslund et al., 2000). The current treatment with acetylcholinesterase inhibitors focuses on the activation of the remaining functional capacities (Tariot and Federoff, 2003). Although drugs focusing on neuroprotection have been actively developed recently, causal therapy for such neurodegenerative diseases is unavailable.

T-817MA was screened as a candidate therapeutic agent for AD. The present data indicate that T-817MA exerts a neuroprotective effect and promotes neurite outgrowth in rat primary cultured neurons. Considering these neurotrophic properties, T-817MA would modify or prevent pathological deterioration in AD.

In the present study, we demonstrated that T-817MA exerted a neuroprotective effect against $A\beta(1-42)$ -induced and H_2O_2 -induced neurotoxicity in the cortical neuron/glia coculture. These results indicate that T-817MA exerts a protective effect on an in vitro model of neuropathology in AD. $A\beta$ neurotoxicity is supposed to be associated with oxidative stress (Markesbery et al., 2001; Butterfield et al., 2002) and the reduction of endogenous antioxidant processes (Olivieri et al., 2001). Therefore, to understand the neuroprotective

effect of T-817MA, we focused our interest on oxidative stress-induced cell death.

T-817MA exerted its neuroprotective effect when the cells were pretreated with T-817MA for 24 h before the H_2O_2 exposure. Unlike antioxidants (Fuson et al., 1999), T-817MA was unable to protect neurons when it was applied just before oxidative stress exposure. Based on this result, it is supposed that T-817MA may exert its neuroprotective effect through the modulation of endogenous antioxidative mechanisms, rather than scavenging the reactive oxygen species. In the current study, we used a neuron/glia coculture because $A\beta$ application failed to induce clear neurotoxicity in the primary culture of the enriched cortical neurons. Previous studies indicated that coexistence of glial cells enhanced $A\beta$ -induced neurotoxicity by modifying the redox status in the neuron/glia coculture (Qin et al., 2002; Abramov et al., 2003, 2004). To demonstrate that T-817MA interacts with neurons themselves, we also investigated H_2O_2 -induced neuronal damage in a primary cortical neuron culture. In this culture set, H_2O_2 (30 μ M for 24 h) induced neuronal damage similar to that observed in the neuron/glia coculture. The neuroprotective effect of T-817MA against H_2O_2 -induced damage was also observed in the cortical neuron culture, thereby indicating that T-817MA might act on neuronal cells themselves. In this cortical neuron culture, brief exposure (4 h) of H_2O_2 did not significantly affect neuronal viability; meanwhile, this treatment reduced the GSH content in the neurons. GSH is an important intracellular antioxidant that protects the neurons against a variety of reactive oxygen species (Schulz et al., 2000). Decrease in GSH was supposed to contribute to signaling events occurring during apoptotic neuronal death (Kane et al., 1993). Disturbance of GSH homeostasis may either lead to or result from oxidative stress

in neurodegenerative disorders, and the treatments that inhibit GSH degradation may result in slowing the disease progression (Schulz et al., 2000). In our current experiment, the reduction of endogenous antioxidant processes might have preceded the neuronal damage. In this brief oxidative stress condition, T-817MA attenuated the preceding reduction of the intracellular GSH content, although T-817MA had no effect in the normal condition (data not shown). Based on these results, we assumed that T-817MA maintained the intracellular GSH content and resulted in preventing cell death under the oxidative stress condition. In addition, pretreatment of T-817MA was necessary for neuroprotection in H₂O₂-induced neuronal death in the neuron/glia coculture. These results indicate that T-817MA promotes endogenous antioxidant processes to protect the neurons from H₂O₂ stress. According to the hypothesis that A β (1–42)-induced neuronal damage in the neuron/glia coculture was mediated by reactive oxygen species generated from astrocytes (Abramov et al., 2004), promotion of the antioxidant processes in neurons might also contribute to the neuroprotective effect of T-817MA against A β (1–42)-induced neuronal damage in the neuron/glia coculture, although effects of T-817MA on glia cells cannot be excluded. Much evidence suggests that oxidative stress may be responsible for dysfunction or death of neuronal cells in AD (Markesbery et al., 2001; Mattson et al., 2001; Butterfield et al., 2002). Oxidative stress in particular has been shown to be one of the earliest changes in disease pathogenesis in AD (Nunomura et al., 2001). On these presuppositions, the antioxidative effect of T-817MA, which might be able to slow the disease progression in the early stage of AD, would be beneficial for AD treatment.

In the present study, T-817MA significantly increased neurite outgrowth. To evaluate neurite outgrowth, we used an anti-GAP-43 antibody. GAP-43 is expressed in an axon and a growth cone at high levels during periods of axon elongation (Meiri et al., 1986; Schreyer et al., 1997; Goslin et al., 1998) and was reported to be a useful tool for visualizing the sprouting of neuronal axons (McKinney et al., 1997). In the quantitative analysis with the cortical reaggregation culture, T-817MA also significantly increased GAP-43 immunoreactivity, reflecting an increase in the neurite length. Based on these results, T-817MA is believed to promote the effect of axonal outgrowth in CNS neurons.

In AD, A β deposition is considered to cause disruption of the neural network including progressive synaptic degeneration and neuronal loss, which consequently results in cognitive dysfunction and behavioral abnormalities (Stepanichev et al., 2004). Hence, reconstructing the damaged neural network is a possible therapeutic target of the disease. The neurite projection has the potential to form a target-oriented and active synapse and subsequently could reproduce functional connections (Stoppini et al., 1993; Li et al., 1994; McKinney et al., 1999). Therefore, promoting neurite outgrowth is supposed to be essential for reconstructing the damaged neural network in AD and various other neurodegenerative diseases. It is reported that some substances that possess neurite outgrowth promoting effect *in vitro* are useful in the treatment of AD (Gillespie, 2003; O'Neill et al., 2004; Tohda et al., 2004).

In the present *in vitro* investigations, T-817MA was demonstrated to have a neuroprotective effect and a neurite

outgrowth promoting effect at the same concentration range. Although the subcellular mechanisms underlying these pharmacological effects are not known, this set of features is similar to that of neurotrophins. These features are supposed to be important for the maintenance of the nervous system and for regulating certain aspects of neuronal survival. Activation of neurotrophic signaling pathways can protect neurons in animal and cell culture models of neurodegenerative diseases such as AD. Neurotrophic factors may protect neurons against age-related degeneration by modulating neurodegenerative cascades and stimulating survival-promoting mechanisms (Mattson et al., 2001). For example, brain-derived neurotrophic factor stimulates the production of various factors, such as antioxidant enzymes and antiapoptotic protein for protection against oxidative insult relevant to the pathogenesis of AD and other neurodegenerative diseases (Mattson et al., 2001). IGF-1 has a well-described neuroprotective effect against excitotoxic, metabolic, and oxidative insults in various experimental models for AD, and it promotes neurogenesis and synaptic formation throughout the brain, and IGF-1 is actively transported across the blood-brain barrier (Heck et al., 1999; Mattson et al., 2001; Wei et al., 2002; Gasparini and Xu, 2003). Therefore, IGF-1 has been indicated as a potential therapeutic target of AD (Gasparini and Xu, 2003). Despite these benefits, in general, the therapeutic application of neurotrophic factors themselves to neurodegenerative diseases is strictly limited because of their poor stability and poor CNS penetration of many of the neurotrophic factors. Considering these limitations, a neurotrophic factor-like small chemical molecule, such as T-817MA, having good CNS penetration (brain level of T-817MA was approximately 10 times higher than blood level after oral administration; A. Takagi, unpublished in-house data) may be more favorable for therapeutic use from the viewpoint of drug delivery.

In conclusion, T-817MA exerts a neuroprotective effect and promotes neurite outgrowth in rat primary cultured neurons, indicating that this compound may have a potential for disease modification and be useful for patients with neurodegenerative diseases, such as AD.

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Hydrolytic Activity of Amyloid-beta and its Inhibition with Short Peptides

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Abstract: The main component of the amyloid plaque is insoluble A β 1-42 (A β 42), which adopts a structure rich in anti-parallel β -pleated sheets. Recently, increasing awareness of A β intermediates as molten-globule states has paralleled insight into the biological activities of the A β conformer. The molten-globule state of A β 42 displays a less ordered, metastable conformation that is stabilized by the formation of fibrils. The molten-globule state of the protein has many biological properties and understanding the mechanisms of its formation is an important step in devising a therapeutic strategy for Alzheimer's disease. There have been many studies of the biological properties of A β 42 such as self-aggregation, binding to other proteins such as apolipoprotein E, cytotoxicity for neuronal cells, vasoconstriction, oxidative activity with superoxide-mediated singlet-oxygen intermediate and proteolytic activity against casein. Recent studies demonstrated that α -1 anti-chymotrypsin, a member of the serine protease inhibitor (serpin) family is also involved in the amyloid plaque. In this review, we focused on the serine protease-like activity of A β 42 for casein substrate and the effect of bio-essential metal ions for the activity and also suggest its inhibition with A β 42 derivatives; A β 15-22 (QKLVFFAE) which is a potential fragment to prevent A β self-aggregation. Consequently, we suggest that the short peptides of this kind may be of use in the therapy of Alzheimer's disease.

Keywords: Alzheimer's disease, guanidine hydrochloride, molten-globule, A β peptides, serine protease-like activity, self-aggregation, metal ions, eight-residue A β derivatives.

INTRODUCTION

Alzheimer's disease (AD) is a very common neurodegenerative disorder in senile generation and cause dementia characterized by memory loss, cognitive impairment and a variety of confusion accompanied with synaptic loss and neurotransmitter depletion [1]. Neurofibrillary tangles in the neuron and extracellular amyloid deposition as neuritic plaques are the major neuropathological features in Alzheimer's brain [2-4]. Neurofibrillary tangles consist of paired helical filaments which is composed of abnormally hyperphosphorylated tau protein and the main component of neuritic plaques is aggregated Amyloid-beta1-42(A β 42) derived from Amyloid precursor protein(APP) [5,6].

APP is a large protein and processed with proteases termed β and γ secretase to produce A β 42 and the impairment of cellular function is directly linked to the interaction of A β 42 aggregates with cellular components [7-9]. It is not certain what is the key factor to induce aggregation to A β 42 and which mechanism of aggregation process results in cell damage.

There have been many studies of the biological properties of A β 42 such as self-aggregation [10], binding to other proteins such as apolipoprotein E [11], oxidative activity with superoxide-mediated singlet oxygen intermediate and proteolytic activity against casein [12] which may contribute to cytotoxicity for neuronal cells [13,14], and vasoconstriction [15]. The frequency of the neuritic plaque appear to correlate

with the extent or severity of dementia [16,17] and the study to define the A β forms responsible for neuronal cell toxicity demonstrated the potential of fibrillar and protofibrillar species of A β but not a single conformational form [18].

The equilibrium intermediates of protein folding exist between the complete folded (native) and completely unfolded (fully denatured) states (Fig. 1). Recently, increasing awareness of A β intermediates as molten-globule states has paralleled insight into the biological activities of the A β conformer [19,20]. This findings has led to the proposal that the molten globule states of A β might be the main pathological form of A β .

In this review, we focus on the inhibition of acid-induced A β 42 aggregation with short peptides and hydrolytic activity of A β 42 for casein substrates. Prevention of A β aggregation is a potential goal in AD therapy and we suggest the possible therapeutic strategy of AD using 8-residues peptides derived from A β residues.

GUANIDINE HYDROCHLORIDE DENATURATION OF A β 42 EXPOSES THE ACID-INDUCED CRYPTIC EPITOPE

We have already demonstrated that the conformation of A β 42 varies at different pH's, and lowering the pH might generate the molten-globule state of soluble A β 42 *in vitro* [21]. Recently, we have performed unfolding experiments with acid-treated A β 42 intermediates, using guanidine hydrochloride (GdnHCl) [22]. The reactivity of monoclonal antibody, which is specific for amino acid residues 9-14 of A β 42, against acid treated-A β 42 was greatly affected by increasing concentrations of the potent denaturant GdnHCl, whereas the sample incubated at neutral pH was unaffected

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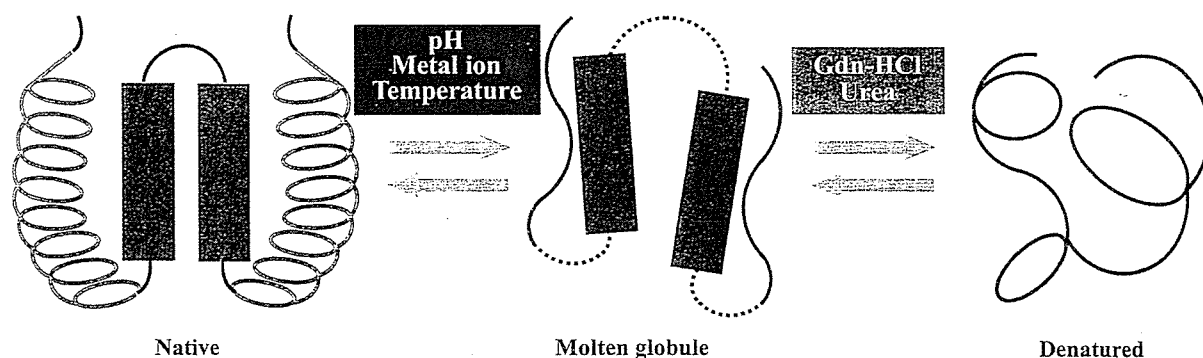


Fig. (1). The equilibrium intermediates of protein folding between native and denatured states.

Molten-globule states of A β 42 protein exist between completely folded and completely denatured states. A variety of environmental factors including pH, metal ions and temperature affect to induce the molten-globule states and Gdn-HCl and urea induce the denatured states of the protein.

and they are identical at 1.5M of GdnHCl. This finding suggests that GdnHCl induces unfolding of the sample when incubated at acid pH. It exposes amino acid residues 9-14 in A β 42, which is exposed at neutral pH and hidden at acidic pH [21,23]. In addition GdnHCl caused unfolding of partially aggregated A β 42, and this partially unfolded form may contain the folding intermediate of A β 42 that has been shown to be essential for acid-induced acquisition of resistance to proteolysis by protease K [22].

In prion studies, the two-stage denaturation of ShaPrP(90-231) induced by GdnHCl monitored by CD spectroscopy, in which the first unfolding step occurs in 1-2 M GdnHCl and the same concentration of GdnHCl exposed the cryptic epitopes in scrapie protein [24]. Guanidine hydrochloride (GdnHCl) is a commonly used protein denaturant and at high concentrations unfolds the molten globule state. However, at low concentrations it can refold acid-unfolded proteins such as apomyoglobin and cytochrome c, stabilizing their molten globule state [25].

EIGHT-RESIDUES PEPTIDES INHIBIT THE ACID-INDUCED A β 42 AGGREGATION

The A β peptide exists either mainly as a random coil/ α -helical structure or a β -sheet structure depending on pH [10], and that the kinetics of aggregation depend on which of these structures is involved, as well as on the length of the peptide examined [26,27]. Hence the adoption of a structure rich in β -sheet at acidic pH accelerates the aggregation of A β , and the abnormal A β is stabilized by intermolecular interactions with other A β monomers that have the ability to form a β -sheet conformation [28].

The compounds targeting the specific inhibition of A β aggregation may be of great importance in AD therapy. Previous reports suggested that Congo red could bind to A β however, it is not specific for A β and bind to other proteins with a high content of β -pleated sheet structure [29] and our goal in AD therapy is to identify the specific compounds to inhibit A β aggregation.

We have examined a series of partial 8-residue peptides derived from A β 42 to inhibit acid-induced aggregation of

A β 42 [22]. Previous report suggested that A β 16-22(KLVFF) fragment can bind full-length A β 40 and prevent its assembly into amyloid fibrils and that the critical amino acid residues for binding and inhibition of A β fibril formation were Lys at position 16, Leu at position 17 and Phe at position 20 [30]. N-methyl amino acids have been used to prevent the aggregation of covalently linked, parallel β -sheet dimmers [31,32] and the short peptide incorporated with N-methyl amino acids into alternative positions of a hydrophobic core sequence of A β ; NH₂-K(Me-L9V(Me-I)F(Me-A)E-CONH₂, termed A β 16-22m could be the most potent to disrupt the peptide-peptide interactions that promote A β fibrinogenesis [33].

The common feature of amyloidosis-related disorders is the abnormal folding of a natural protein into a pathologic conformer that is protease resistant [34]. We therefore investigated the ability of the short peptides to reverse the protease K susceptibility of A β aggregates in glial cells. The epitopes around residues 9-14 and 17-21 in A β 42 were dramatically affected by acidic pH [21]. The ability of partial A β fragments around A β 16-23 to inhibit A β 42 aggregation was proved by both ELISA and cell western dot blot analysis and our results indicate that peptides in the 8-residue peptides including A β 15-22, A β 16-23 and A β 17-24, which contain the central hydrophobic region of A β 42, are able to interfere with pH-induced A β 42 aggregation [22] (Fig. 2). This may be due an ability to bind to the central hydrophobic region of A β 42, including the pH-sensitive region, thereby destabilizing the interaction between A β monomers and/or oligomers necessary for fibril stability. As a result the site of protease cleavage would be exposed, and it would become sensitive to protease K. Small peptides of this kind may be useful for preventing the conformational changes leading to formation of A β intermediates in the early stages of A β aggregation.

Short fragments around A β 16-23 may be produced *in vivo* in the normal processing steps, since α -secretase is reported to act between residues 16 and 17 [35], and cathepsin D in the lysosome acted in the region of residue 21 [36,37]. Moreover, exposure to the protease responsible for insulin degradation generates the A β 17-24 fragment [38]. Hence peptides around A β 16-23 could be physiological products

and may play an important role in inhibiting self-aggregation of A β 42 *in vivo*. Studies of A β 42 aggregation kinetics have suggested that aggregation of the hydrophobic form of A β 42 can be selectively inhibited by the more soluble form, and that aggregation is driven by a hydrophobic effect of A β 42 [39].

The central region of A β 42 has been implicated in various biological functions including interaction with other proteins such as apolipoprotein E [40] and this region, comprising amino acid residues 19-25 has a very important influence on the aggregation and secondary structure of A β peptide [41]. It has been suggested that residues 10-23 may provide the structural basis of the hydrophobic behavior under physiological conditions [42]. Amino acid substitution studies indicate that the hydrophobic residues at position 17-20 are crucial for the amyloidogenic properties, with the very hydrophobic carboxy-terminal residues 29-42 corresponding to the transmembrane domain of A β 42 [30, 43]. The synthetic 5-residue peptide LPFFD, which is homologous to the central hydrophobic region 17-21 (LVFFA) of A β 42 was designed. The valine residue is replaced by proline residue to decrease the peptide's propensity to adopt a β -sheet conformation [44-46] and a aspartic acid residue was added to the C-terminus to increase solubility. This 5-residue peptide has been reported to inhibit A β fibrinogenesis *in vitro*, prevent neuronal cell death in culture and reduce A β deposition in the rat brain [47].

It has also been claimed that this peptide can reverse pre-existing A β fibrils and subsequently diminishing A β related histopathology [48]. Our previous study of the pH-induced conformational transitions of A β 42 also suggested that amino acids residues at position 9-14 and 17-21 were responsible for the changes [21]. In our present assay system, A β 16-23 inhibited A β 42 aggregation better than the 5-residue peptide LPFFD (unpublished data). We have suggested that short peptides could facilitate the formation of mixed aggregates with A β 42 that are sensitive to protease K and these findings raise the possibility that peptides around A β 16-23 could be useful for treating amyloid plaque in the AD brain. Thus, A β 15-22, A β 16-23 and A β 17-24 can inhibit A β 42 aggregation at acidic pH and these peptides may be useful in the treatment of AD.

HYDROLYTIC ACTIVITIES OF A β 42

A β has been shown recently to possess proteolytic activity against casein [49] and the molten-globule states of A β 42 at neutral pH may participate in the activity. We have studied the proteolytic activity of A β 42 for casein at neutral pH and have shown that A β 17-42 also has some activity [22]. The pH shift from 7.4 to 4.6 decreased the activity of A β 42 by about 76% and resulted in the same level of activity as A β 17-42, which was almost unaffected by acid pH. These data indicate that residues important for A β 42 activities are pH sensitive and that these must be present in the region

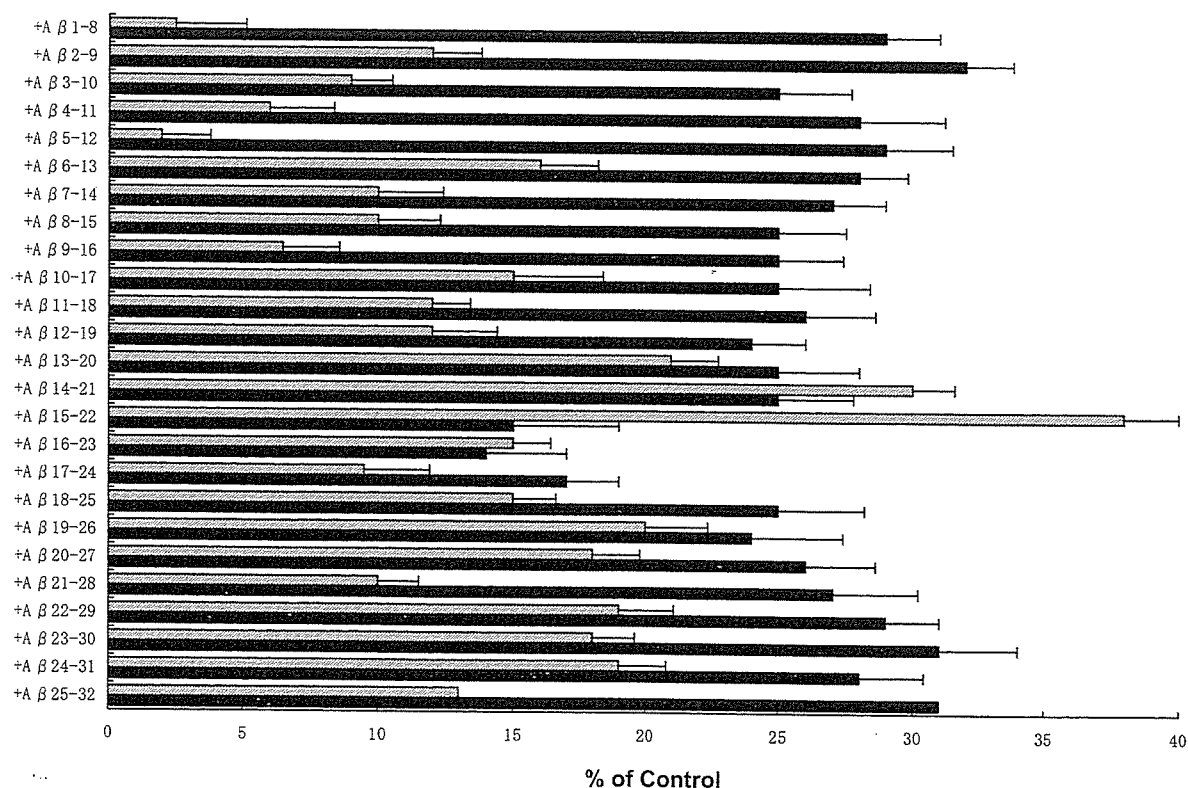


Fig. (2). Inhibition of acid-induced A β 42 aggregation and hydrolytic activity of A β 42 with short peptides.

Inhibition of acid-induced A β 42 aggregation by the short peptides was studied and the percentage of remaining protein after protease K digestion was shown as % of control (■). Inhibition of hydrolytic activity of A β 42 for casein substrate with short peptides was shown as % inhibition of control (▨).

between residues 1 and 16. We speculate that residues around 9-14 and 17-21, which are affected by pH and induce conformational changes [21], may participate in the activity.

Though A β 42 requires the first 16 residues for full activity, A β 1-16 itself has no activity. As there are no amino acid residues in A β 29-42 capable of forming the active site, the region 1-28 must contain all of the amino acid residues essential for activity. A β 1-28 corresponds to the extra-cellular domain and contains the first α -helix, and had a low level of activity. It should be noted that although NMR studies of micelle-bound A β 42 revealed the existence of an α -helix in this region [50], examination in water did not support that finding [51]. Despite the fact that the lengths of A β 1-28 and A β 17-42 are almost the same, A β 17-42 showed higher activity than A β 1-28. We suggest that the region 29-42, which contains a second α -helix and corresponds to the transmembrane domain, is essential for full activity of A β 42 and that serine 26 may contribute to the partial activity of A β 17-42.

Furthermore, residues 29-42 may be essential for stabilizing the first α -helix (residues 9-23), because these residues are reported to be essential for stabilizing the fibrils [52]. Although A β 1-16 and A β 12-28 may contribute residues crucial for A β 42 activity, both are shorter than A β 1-28 and lack the region required for stabilization. We have previously demonstrated that under neutral pH, metal ions including copper and zinc could induce conformational transition of A β 42 and amino acids residues 9-14 participate to the changes [21] and copper could also inhibit the enzymatic activity of A β 42 [22]. These results suggest that the amino acids residues 9-14, which is associated with copper, might participate to the formation of the catalytic site of A β 42 [21,22].

Chemical modification of the serine, as well as the histidine residues of A β 40, dramatically reduced proteolytic activity. However, modification of the aspartic residue has no effect. Substitution of glutamic acid by glutamine at positions 11 and 22, in the putative α -helical region, also decreased activity by 50-60%. The mutation at position 22 (E22Q) is known as the "Dutch type" and gives rise to familial early onset AD [53-55]. Such protein has potent aggregative ability and neurotoxicity in PC12 cells (rat pheochromocytoma) [56]. Our previous data suggested that the glutamic

acid at position 11 is very sensitive to acidic pH and is a key residue for preserving the conformation around region 9-21 [21] and that the serine and histidine residues participate directly in the proteolytic activity of A β 42 [22], indicating that this may be a serine protease-like activity and the glutamic acids at position 11 and 22 may be required to preserve the conformation around the catalytic site and the 29-42 region may be essential to stabilize the conformer (Fig. 3). These data are consistent with previous studies suggesting that beta-amyloid-mediated hydrolysis is Ser sensitive and His, Asp/Glu also related to the activity [49].

The central region of A β 42 has been implicated in various biological functions including interaction with other proteins such as apolipoprotein E [40] and this region, comprising amino acid residues 19-25 has a very important influence on the aggregation and secondary structure of A β peptide [41]. It has been suggested that residues 10-23 may provide the structural basis of the hydrophobic behavior under physiological conditions [42]. Amino acid substitution studies indicate that the hydrophobic residues at position 17-20 are crucial for the amyloidogenic properties, with the very hydrophobic carboxy-terminal residues 29-42 corresponding to the transmembrane domain of A β 42 [30,43]. We suggested the proteolytic activity of A β 42 and identified essential amino acid residues and further investigated effects of bioessential metal ions for the activity [22,49].

EIGHT-RESIDUES PEPTIDES INHIBIT HYDROLYTIC ACTIVITY OF A β

The α 1-antichymotrypsin that belongs to the serine protease family of inhibitors (serpins) is a major additional component of the amyloid plaque [57]. Recent reports suggest the possibility that A β 42 binds to two β -sheets of α 1-antichymotrypsin and transforms it from inhibitor to substrate [58]. The serum level of this inhibitor increases in response to the acute phase reaction of the host defense system [59]. It is mainly synthesized in the liver, although also produced by astrocytes [60], and may reduce the toxicity of the amyloid peptides in clonal cell lines as well as in cultures of primary cortical nerve cells by inhibiting their protease activity [61]. Thus, inhibition of the proteolytic activity of A β may be a useful approach for preventing cellular toxicity.

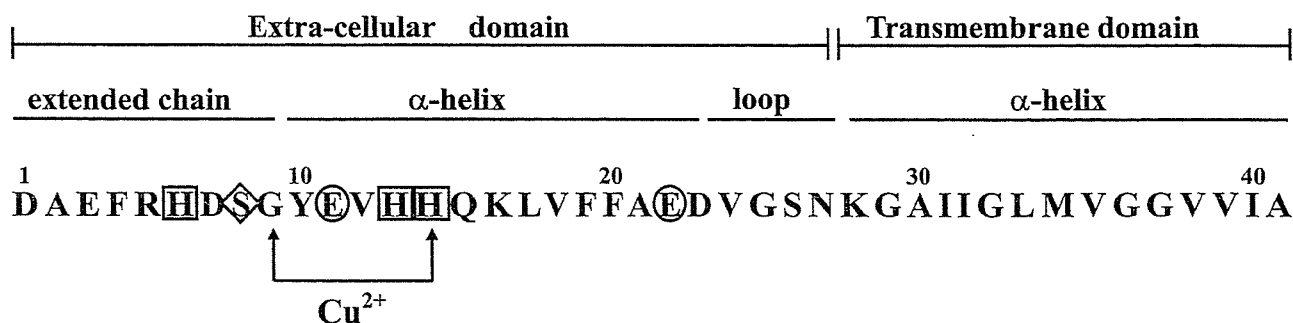


Fig. (3). Hydrolytic activity of A β 42.

Hydrolytic activity of A β 1-42 for casein substrate was tested at neutral pH.

Histidine residue at positions 6, 13 and 14 and Serine residue at a position of 8 are essential for the hydrolytic activity and glutamic acid residue at positions of 11 and 22 also participate to the activity.

We have synthesized a series of partial 8-residue peptides derived from A β 42 to study their ability to inhibit the hydrolytic activity of A β 42 at neutral pH for casein and found that short peptides of A β 14-21 and A β 15-22 are effective [22] (Fig. 2). The 5-residue peptide LPFFD, which is homologous to the central hydrophobic region 17-21 (LVFFA) of A β 42, has been reported to inhibit A β fibrinogenesis *in vitro*, prevent neuronal cell death in culture and reduce A β deposition in the rat brain [47]. Interestingly, the hydrolytic activity of A β 42 for casein at neutral pH was inhibited only by A β 14-21 and A β 15-22 [22]. The ability of partial A β peptides to inhibit the hydrolytic activity of A β 42 may be due an ability to bind to the central hydrophobic region of A β 42, including the pH-sensitive region, thereby destabilizing the interaction between A β monomers and/or oligomers necessary for fibril stability and inhibit the biological active form. We speculate that oligomerization of A β 42 may be required for full proteolytic activity and complete aggregation of A β 42 may abrogate the activity. Thus, we have suggested that short peptides A β 14-21: HQKLVFFA and A β 15-22: QKLVFFAE could be useful for the treatment of AD and A β 15-22 may be the only peptide to control both aggregation of A β 42 at acidic pH and proteolytic activity at neutral pH.

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