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

Compound	Controls (n = 250)	AD patients (n = 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).
*$P < 0.001$.

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

Compound	Controls (n = 28)	AD patients (n = 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).
*$P < 0.01$.

Aβ40 and Aβ42 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 ± 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 Scheffe'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 Aβ42 in the culture medium of cultured cortical neurons that had been exposed to increasing concentrations of HA (10 nM to 100 μM) for 12 or 24 hr. The concentration of Aβ42 in the culture medium was decreased significantly in an HA concentration-dependent manner (1–100 μM), in cultures exposed neurons that had been exposed to HA (Fig. 1A,B). In contrast, homocysteine at concentrations of 10 and 100 μM had no significant effect on the amount of Aβ42 released from the cultured neurons (data not shown). Neither HA nor homocysteine affected the amount of Aβ40 released from the cultured neurons (Fig. 2).

Because HA caused a highly significant decrease in the amount of Aβ42 released from neurons without affecting the amount of Aβ40 released, we carried out additional analyses to determine if HA caused accumulation of Aβ42 within the neurons. We measured levels of Aβ42 and Aβ40 in lysates of cultured neurons that had been exposed for 24 hr to increasing concentrations of HA. The amounts of intracellular Aβ42 were increased significantly by HA in a concentration-dependent manner, whereas amounts of intracellular Aβ40 were not affected significantly by HA (Fig. 3). We next determined the effects of HA on the amounts of Aβ40 and Aβ42 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 Aβ42 produced by cells. The amount of Aβ40 released into the culture medium was decreased significantly by 10% and 98% in cells exposed to 1 and 10 μM HA, respectively (Fig. 4). The amount of intracellular Aβ40 was not affected significantly by HA. Extracellular Aβ42 levels were decreased significantly and intracellular Aβ42 levels increased significantly in cells exposed to HA (Fig. 4). The ability of HA to increase the accumulation of Aβ42 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 μM, but not at 10 μM, after a 12-hr exposure period (Fig. 5). The latter results suggest that HA enhances intracellular accumulation of Aβ42 in neurons at concentrations (<10 μM) that are not overtly toxic to cells (Fig. 5).

Recent studies have provided evidence that Aβ42 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 Aβ1–42 can induce death in cultured neurons (Zhang et al., 2002; Pierrot et al., 2004). If accumulation of intracellular Aβ1–42 plays an important role in the neurotoxic effect of HA, then inhibition of Aβ42 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 Aβ42 in the neurons exposed to HA (data not shown). Whereas HA killed 25–40% of the neurons in vehicle-treated control cultures, it killed

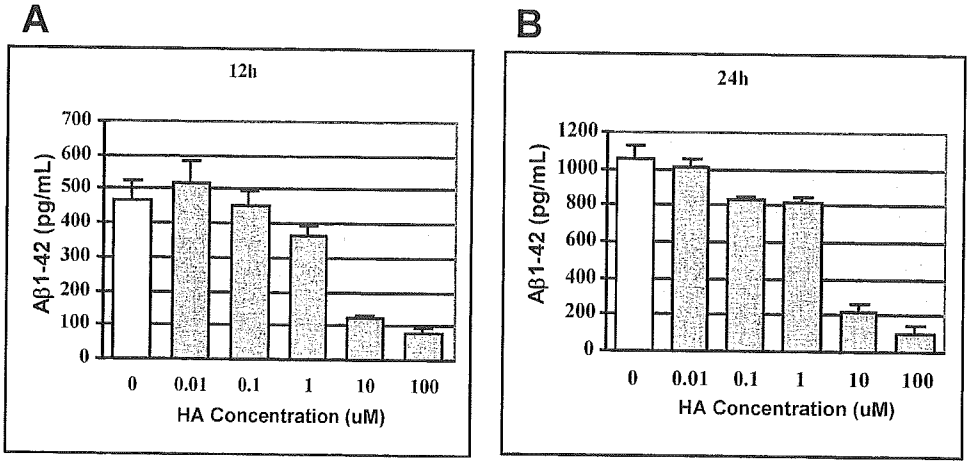

Fig. 1. Homocysteic acid (HA) decreases the amount of Aβ42 released from cultured neurons. Neurons were exposed to the indicated concentrations of HA for either 12 (**A**) or 24 hr (**B**). Concentrations of Aβ42 in the cultured medium were quantified by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the value for cultures not exposed to HA.

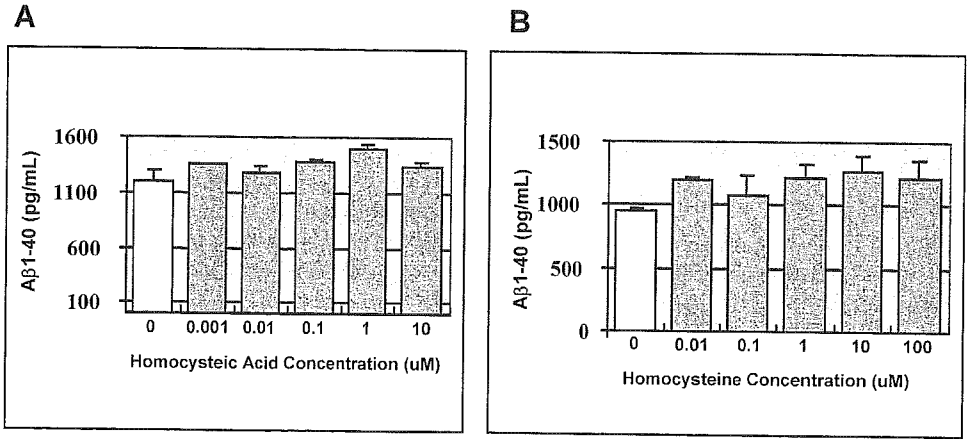

Fig. 2. Homocysteic acid (HA) does not affect the amount of Aβ40 released from cultured neurons. Neurons were exposed to the indicated concentrations of either HA (**A**) or homocysteine (**B**) for 24 hr. Concentrations of Aβ40 in the cultured medium were quantified by enzyme-linked immunosorbent assay (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 Aβ42 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

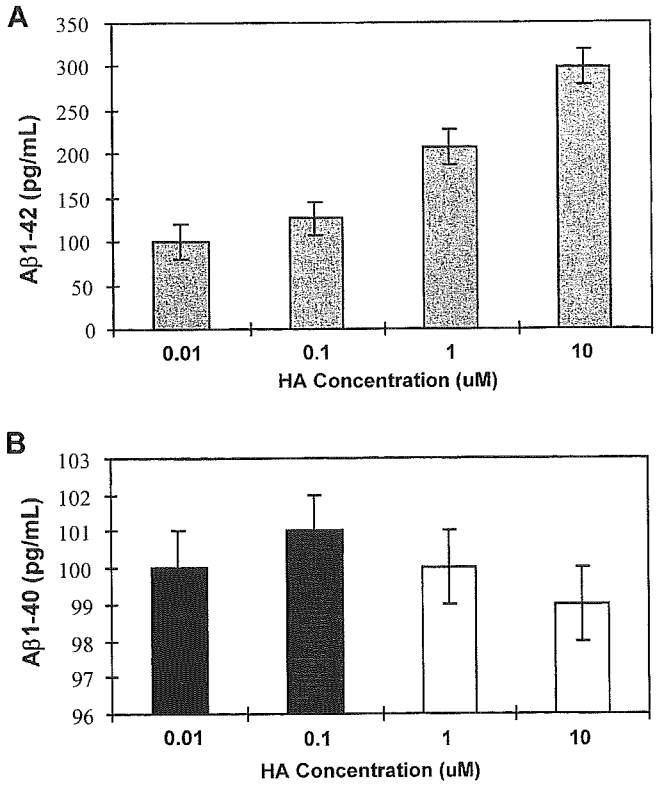

Fig. 3. Homocysteic acid (HA) increases the intracellular accumulation of Aβ42 in cultured neurons. Neurons were exposed to the indicated concentrations of HA for 24 hr. Concentrations of Aβ42 (**A**) and Aβ40 (**B**) in the culture medium were quantified by enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. $^*P < 0.05$, $^{**}P < 0.01$ compared to the value for cultures not exposed to 0.01 μM HA.

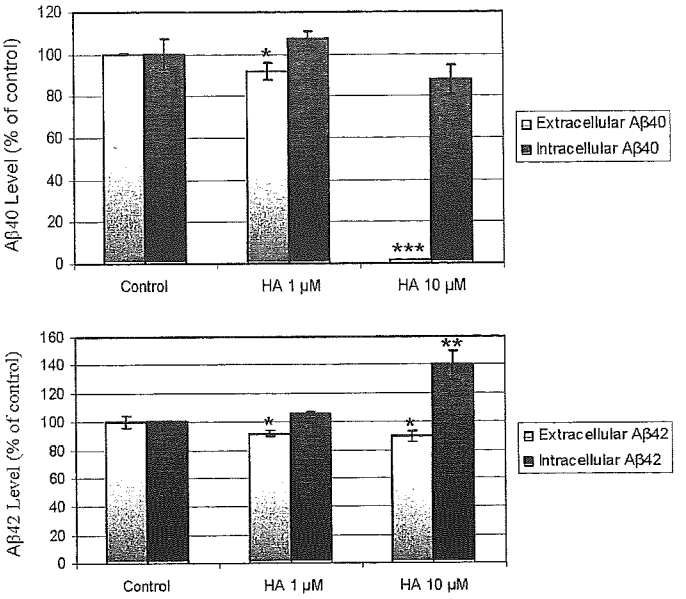

Fig. 4. Human Aβ42 accumulates in Chinese hamster ovary (CHO) cells during exposure to homocysteic acid (HA). HA on the production of Aβ40 and Aβ42. CHO cells expressing mutant amyloid precursor protein (APP; Swedish mutation) were exposed to the indicated concentrations of HA for 24 hr. The Aβ40 and Aβ42 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 Aβ40 were 670 ± 48 pg/mg protein and 1130 ± 43 pg/ml, respectively; and of Aβ42 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.

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 μM; HA plus 300 μM homocysteine, 55 μM; and HA plus 1 mM homocysteine, 0.6 μM. 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 Aβ42 inside of the cells, and suggests further that the production of Aβ42 contributes to the neurotoxic action of HA. The accumulation of Aβ42 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 Aβ42 is a pivotal event in HA-induced neuronal death. Increasing evidence suggests that intracellular accumulation of Aβ42 plays a role in the neurodegenerative process in AD. Analyses of brain tissue sections from AD patients showed that Aβ immunoreactivity is present in neurofibrillary tangle-bearing neurons (Hyman et al., 1989). Studies using antibodies that differentiate between Aβ40 and Aβ42 revealed that Aβ42 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 Aβ42 is an early event that may occur before synaptic dysfunction and cell degeneration (Gouras et al., 2000; Takahashi et al., 2002). Moreover, levels of Aβ42 in the CSF are decreased in AD patients compared to that in control subjects (Sunderland et al., 2003), consistent with increased retention of Aβ42 in neurons. We found that HA reduced release of Aβ42 into the culture medium and increased the intracellular accumulation of Aβ42. Our data therefore suggest that HA may play a role in the intracellular accumulation of Aβ42 and consequent degeneration of neurons in AD. Consistent with this possibility, several recent studies have provided evidence that intracellular Aβ42 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 Aβ42 was not established in the present study, prior information on the mechanisms by which HA and Aβ42 kill neurons suggest several possibilities. HA can promote calcium influx

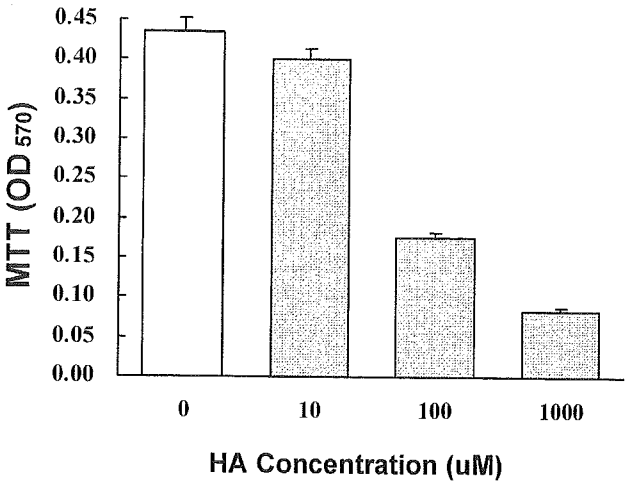

Fig. 5. Homocysteine induces neuronal death. Neurons were exposed to the indicated concentrations of homocysteic acid (HA) for 12 hr. Levels of MTT reduction in neurons were quantified. Values are the mean and SEM. $^{**}P < 0.01$ compared to the value for cultures not exposed to HA.

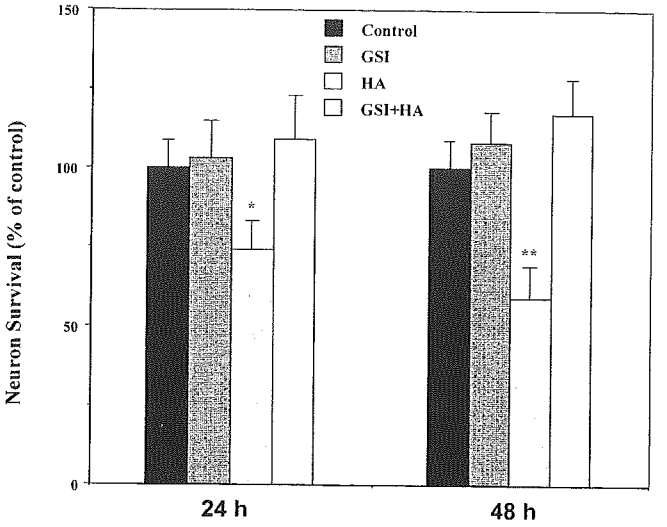

Fig. 6. A γ-secretase inhibitor protects cultured hippocampal neurons against death induced by homocysteic acid (HA). Cultures were treated with saline (Control), 100 nM of the γ-secretase inhibitor LY-411,575 (GSI), 1 μM HA, or a combination of 100 nM GSI plus 1 μM HA. The percentages of neurons surviving 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 Aβ42 (Pierrot et al., 2004). Aβ, 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 Aβ42 accumulation and neurotoxicity. Oxidative stress is increased in neurons in AD and Aβ 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 Aβ42 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 production 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 Aβ (Miguel-Hidalgo et al., 2002), although it is not known if memantine decreases Aβ42 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 Aβ42, HA (Lockhart et al., 2000), and homocysteine (Kim and Pae, 1996). Oxidative stress might therefore play a role in HA-induced intracellular Aβ42 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.

REFERENCES

Casas C, Sergeant N, Itier JM, Blanchard V, Wirths O, van der Kolk N, Vingtdeux V, van de Steeg E, Ret G, Canton T, Drobecq H, Clark A, Bonici B, Delacourte A, Benavides J, Schmitz C, Tremp G, Bayer TA, Benoit P, Pradier L. 2004. Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated Abeta42 accumulation in a novel Alzheimer transgenic model. Am J Pathol 165:1289–1300.

Clarke R, Smith AD, Jobst KA, Fefsum H, Sutton L, Ueland PM. 1998. Folate, vitamin B12, and serum total levels in confirmed Alzheimer disease. Arch Neurol 55:1449–1455.

Cuenod M, Audinat E, Do KQ, Gahwiler BH, Grandes P, Herrling P, Knopfel T, Perschak H, Streit P, Vollenweider F. 1990. Homocysteic acid as transmitter candidate in the mammalian brain and excitatory amino acids in epilepsy. Adv Exp Med Biol 268:57–63.

Ferris SH. 2003. Evaluation of memantine for the treatment of Alzheimer's disease. Expert Opin Pharmacother 4:2305–2313.

Folbergrova J, Haugvicova R, Mares P. 2000. Behavioral and metabolic changes in immature rats during seizures induced by homocysteic acid and the protective effect of NMDA and non-NMDA receptor antagonists. Exp Neurol 161:336–345.

Frauscher G, Karnaukhova E, Muehl A, Hoeger H, Lubec B. 1995. Oral administration of homocysteine leads to increased plasma triglycerides and homocysteic acid—additional mechanisms in homocysteine induced endothelial damage? Life Sci 57:813–817.

Fukamoto H, Tomita T, Matsunaga H, Ishibashi Y, Saido TC, Iwatsubo T. 1999. Primary cultures of neuronal and non-neuronal rat brain cells secrete similar proportions of amyloid beta peptides ending at Abeta40 and Abeta42. Neuroreport 10:2965–2969.

Gasparini L, Racchi M, Benussi L, Curti D, Binetti G, Bianchetti A, Trabucchi M, Govoni S. 1997. Effect of energy shortage and oxidative stress on amyloid precursor protein metabolism in COS cells. Neurosci. Lett 231:113–117.

Gasparini L, Rusconi L, Xu H, Del Soldato P, Ongini E. 2004. Modulation of beta-amyloid metabolism by non-steroidal anti-inflammatory drugs in neuronal cell cultures. J Neurochem 88:337–348.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR. 2000. Intraneuronal Abeta42 accumulation in human brain. Am J Pathol 156:15–20.

Grieve A, Griffiths R. 1992. Simultaneous measurement by HPLC of the excitatory amino acid transmitter candidates homocysteate and homocysteine sulphinate supports a predominant astrocytic localisation. Neurosci Lett 145:1–5.

Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356.

Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E, Shea TB. 2001. Homocysteine potentiates beta-amyloid neurotoxicity, role of oxidative stress. J Neurochem 78:249–253.

Hyman BT, Van Hoesen GW, Beyreuther K, Masters CL. 1989. A4 amyloid protein immunoreactivity is present in Alzheimer's disease neurofibrillary tangles. Neurosci Lett 101:352–355.

Kienlen-Campard P, Miolet S, Tasiaux B, Octave JN. 2002. Intracellular amyloid-beta 1–42, but not extracellular soluble amyloid-beta peptides, induces neuronal apoptosis. J Biol Chem 277:15666–15670.

Kim JP, Koh JY, Choi DW. 1987. L-homocysteate is a potent neurotoxin on cultured cortical neurons. Brain Res 437:103–110.

Kim WK, Pae YS. 1996. Involvement of N-methyl-D-aspartate receptor and free radical in homocysteine-mediated toxicity on rat cerebellar granule cells in culture. Neurosci Lett 216:117–120.

Klancnik JM, Cuenod M, Gahwiler BH, Jiang ZP, Do KQ. 1992. Release of endogenous amino acids, including homocyteic acid and cysteine sulphinic acid, from rat hippocampal slices evoked by electrical stimulation of Schaffer collateral-commissural fibers. Neuroscience 49:557–570.

Kruman II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L, Mattson MP. 2000. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. J Neurosci 20:6920–6926.

Kruman II, Kumaravel TS, Lohani A, Pedersen WA, Cutler RG, Kruman Y, Haughey N, Lee J, Evans M, Mattson MP. 2002. Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease J Neurosci 22:1752–1762.

LaFerla FM, Troncoso JC, Strickland DK, Kawas CH, Jay G. 1997. Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular Abeta stabilization. J Clin Invest 100:310–320.

Li J, Lin JC, Wang H, Peterson JW, Furie BC, Furie B, Booth SL, Volpe JJ, Rosenberg PA. 2003. Novel role of vitamin K in preventing oxidative injury to developing oligodendrocytes and neurons. J Neurosci 23:5816–5826.

Lockhart B, Jones C, Cuisinier D, Villain N, Peyroulan D, Lestage P. 2000. Inhibition of L-homocysteic acid and buthionine sulphoximine-mediated neurotoxicity in rat embryonic neuronal cultures with alpha-lipoic acid enantiomers. Brain Res 855:292–297.

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. 1992. beta-Amyloid peptides destablize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 12:376–389.

Mattson MP, Chan SL. 2003. Neuronal and glial calcium signaling in Alzheimer's disease. Cell Calcium 34:385–397.

Mattson MP. 2004a. Metal-catalyzed disruption of membrane protein and lipid signaling in the pathogenesis of neurodegenerative disorders. Ann N Y Acad Sci 1012:37–50.

Mattson MP. 2004b. Pathways towards and away from Alzheimer's disease. Nature 430:631–639.

Miguel-Hidalgo JJ, Alvares XA, Cacabelos R, Quack G. 2002. Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1–40). Brain Res 958:210–221.

Miller JW. 1999. Homocysteine and Alzheimer's disease. Nutr Rev 57:126–129.

Mori C, Spooner ET, Wisniewsk KE, Wisniewski TM, Yamaguch H, Saido TC, Tolan DR, Selkoe DJ, Lemere CA. 2002. Intraneuronal Abeta42 accumulation in Down syndrome brain. Amyloid 9:88–102.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39:409–421.

Paola D, Domenicotti C, Nitti M, Vitali A, Borghi R, Cottalasso D, Zaccheo D, Odetti P, Strocchi P, Marinari UM, Tabaton M, Pronzato MA. 2000. Oxidative stress induces increase in intracellular amyloid beta-protein production and selective activation of betaI and betaII PKCs in NT2 cells. Biochem Biophys Res Commun 268:642–646.

Pierrot N, Ghisdal P, Caumont AS, Octave JN. 2004. Intraneuronal amyloid-beta1–42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death. J Neurochem 88:1140–1150.

Qin W, Ho L, Pompl PN, Peng Y, Zhao Z, Xiang Z, Robakis NK, Shioi J, Suh J, Pasinetti GM. 2003. Cyclooxygenase (COX)-2 and COX-1 potentiate beta-amyloid peptide generation through mechanisms that involve gamma-secretase activity. J Biol Chem 278:50970–50977.

Sagara Y, Hendler S, Khoh-Reiter S, Gillenwater G, Carlo D, Schubert D, Chang J. 1999. Propofol hemisuccinate protects neuronal cells from oxidative injury. J Neurochem 73:2524–2530.

Schwab C, McGeer PL. 2000. Abeta42-carboxy-terminal-like immunoreactivity is associated with intracellular neurofibrillary tangles and pick bodies. Exp Neurol 161:527–534.

Scott JM, Weir DG. 1998. Folic acid, homocysteine and one-carbon metabolism: a review of the essential biochemistry. J Cardovasc Risk 5: 223–227.

Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW, Wolf PA. 2002. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 346:476–483.

Shearman MS, Beher D, Clarke EE, Lewis HD, Harrison T, Hunt P, Nadin A, Smith AL, Stevenson G, Castro JL. 2000. L-685, 458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. Biochemistry 39:8698–8704.

Su JH, Cummings BJ, Cotman CW. 1998. Plaque biogenesis in brain aging and Alzheimer's disease II. Progressive transformation and developmental sequence of dystrophic neurites. Acta Neuropathol 96:463–471.

Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, Kimmel LH, Bergeson J, Manetti GJ, Zimmermann M, Tang B, Bartko JJ, Cohen RM. 2003. Decreased beta-amyloid1–42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. JAMA 289:2094–2103.

Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK. 2002. Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am J Pathol 161:1869–1879.

Weggen S, Eriksen JL, Sagi SA, Pietrzik CU, Ozols V, Fauq A, Golde TE, Koo EH. 2003. Evidence that nonsteroidal anti-inflammatory drugs decrease amyloid beta 42 production by direct modulation of gamma-secretase activity. J Biol Chem 278:31831–31837.

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K, Bayer TA. 2001. Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. Neurosci Lett 306: 116–120.

Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, Landreth G. 2003. Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. J Neurosci 23:7504–7509.

Zhang Y, McLaughlin R, Goodyer C, LeBlanc A. 2002. Selective cytotoxicity of intracellular amyloid beta peptide1–42 through p53 and Bax in cultured primary neurons. J Cell Biol 156:519–529.

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A Novel Neurotrophic Agent, T-817MA [1-{3-[2-(1-Benzothiophen-5-yl) Ethoxy] Propyl}-3-azetidinol 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 (Aβ). T-817MA (1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidinol maleate) was screened as a candidate therapeutic agent for the treatment of AD based on its neuroprotective potency against Aβ-induced neurotoxicity and its effect of enhancing axonal regeneration in the sciatic nerve axotomy model. The neuroprotective effect of T-817MA against Aβ(1–42) or oxidative stress-induced neurotoxicity was assessed using a coculture of rat cortical neurons with glia. T-817MA (0.1 and 1 μM) was strongly protective against Aβ(1–42)-induced (10 μM for 48 h) or H_2O_2-induced (100 μM for 24 h) neuronal death. T-817MA suppressed the decrease of GSH levels induced by H_2O_2 exposure (30 μM 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 μM) 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 (Aβ), although this hypothesis is still being argued (Selkoe, 1991; Naslund et al., 2000; Rottkamp et al., 2002). Aβ 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 Aβ toxicity remain unclear. However, many studies support the idea that an oxidative event is critical for Aβ toxicity (Rottkamp et al., 2002). For example, Aβ 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 Aβ insult during disease progression.

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ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid-β peptides; T-817MA, 1-{3-[2-(1-benzothiophen-5-yl) ethoxy] propyl}-3-azetidinol maleate; CNS, central nervous system; Aβ(1–42), amyloid-β(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-azetidinol 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 Aβ-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)- [Aβ(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 μM with Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) on the day of use. Aβ(1–42): β-amyloid(1–42)·HCl was purchased from AnaSpec Inc. (San Jose, CA). Aβ(1–42) was sonicated in distilled water at a concentration of 250 μM 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-glial 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).

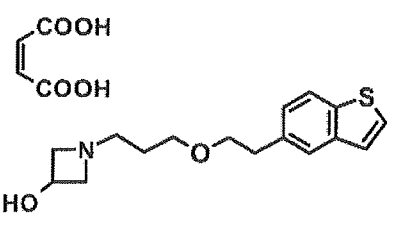

Fig. 1. Chemical structure of T-817MA.

Neuroprotective Effect against Aβ(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 μg/ml gentamicin sulfate. The cells were cultured in 75-cm^2 flasks in a humidified CO_2 incubator with 5% CO_2/95% 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/mm^2 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 μg/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 μM cytosine arabinoside (Nacalai Tesque, Kyoto, Japan) was added to halt glial proliferation. The cells were then incubated for 7 days in this medium. Aβ(1–42) was added to the coculture at a concentration of 10 μM. T-817MA was added simultaneously with Aβ(1–42) application at concentrations of 0 (control), 0.01, 0.1, and 1 μM, 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 Aβ(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 H_2O_2 (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 H_2O_2-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 μM, and the cells were subsequently incubated for 5 min or 24 h. H_2O_2 was then added to the coculture at a concentration of 100 μM, 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 H_2O_2. 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 μg/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/mm^2. Forty-eight hours after cell seeding, cytosine arabinoside was added to the cultures (10 μM) 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 μM. Twenty-four hours following T-817MA application, H_2O_2 was added at a concentration of 30 μM, 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 H_2O_2. 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 μM. Twenty-four hours following T-817MA application, H_2O_2 was added at a concentration of 30 μM. 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 10,000*g* 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 × 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 · 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-μm 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 μg/ml gentamicin sulfate. T-817MA was added at concentrations of 0 (control), 0.01, 0.1, and 1 μM at the initiation of the slice culture. The culture was then incubated at 37°C in 5% CO_2/95% 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/mm^2 and then cultured for 4 days. As a result of this procedure, cortical neurons formed reaggregates and floated in the medium. The suspended reaggregates 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 reaggregates of cells were cultured for 3 days. T-817MA was then added at concentrations of 0 (control), 0.01, 0.1, and 1 μM, and the reaggregates of cells were cultured further for 4 days. All the cultures were incubated in the atmosphere of humidified 5% CO_2/95% 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 reaggregates 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 reaggregates 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

Aβ(1–42)-Induced Neuronal Death. The protective effect of T-817MA on cortical neurons against Aβ(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 Aβ(1–42) (normal). On the other hand, few neurons were detected with 10 μM Aβ(1–42) treatment (control). T-817MA treatment preserved the cortical neurons in the presence of Aβ(1–42). To evaluate the number of surviving

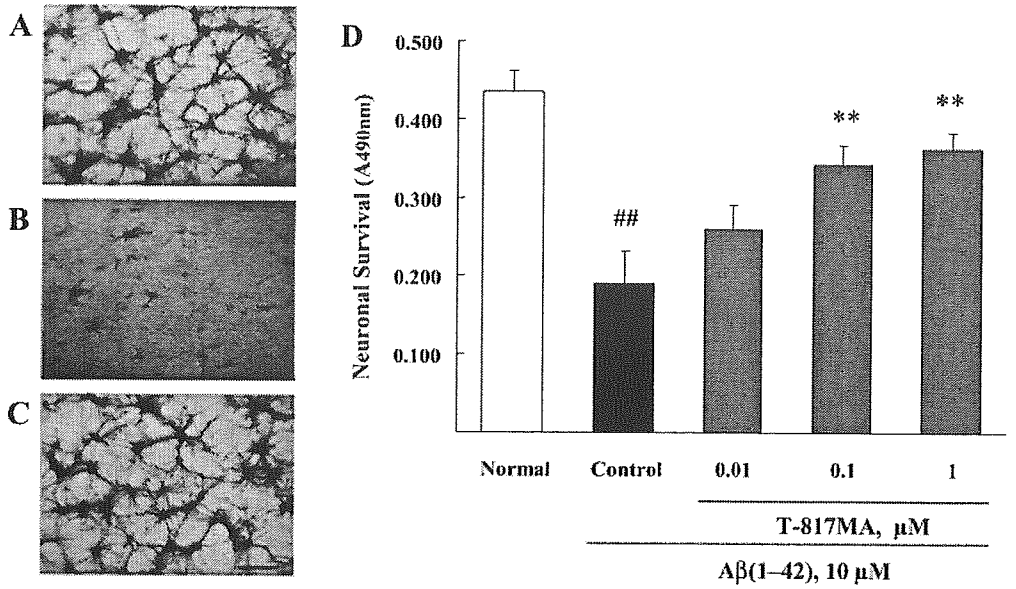

Fig. 2. Effect of T-817MA on Aβ(1–42)-induced neuronal death. Aβ(1–42)-induced neuronal death and the neuroprotective effect of T-817MA were assessed using rat neuron/glia coculture. Images of MAP2 immunocytochemistry showed typical responses: neurons with vehicle treatment (normal; A), 10 μM Aβ(1–42) 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.

H_2O_2-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 H_2O_2-induced neuronal damage was investigated in the neuron/glia coculture. The 100 μM H_2O_2 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 H_2O_2, did not rescue the H_2O_2-treated neurons from death (Fig. 3B).

T-817MA Attenuated H_2O_2-Induced Reduction of Intracellular GSH Contents. A similar protective effect of T-817MA was observed in the primary cortical neuron culture. H_2O_2 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 H_2O_2 exposure. In this experiment, primary cultures of cortical neurons were exposed to 30 μM H_2O_2 for 4 h. Viability of neurons was not altered at 4 h following 30 μM H_2O_2 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 H_2O_2 exposure. T-817MA almost completely prevented such GSH reduction at 0.1 and 1 μM (Fig. 4C). T-817MA alone did not exert

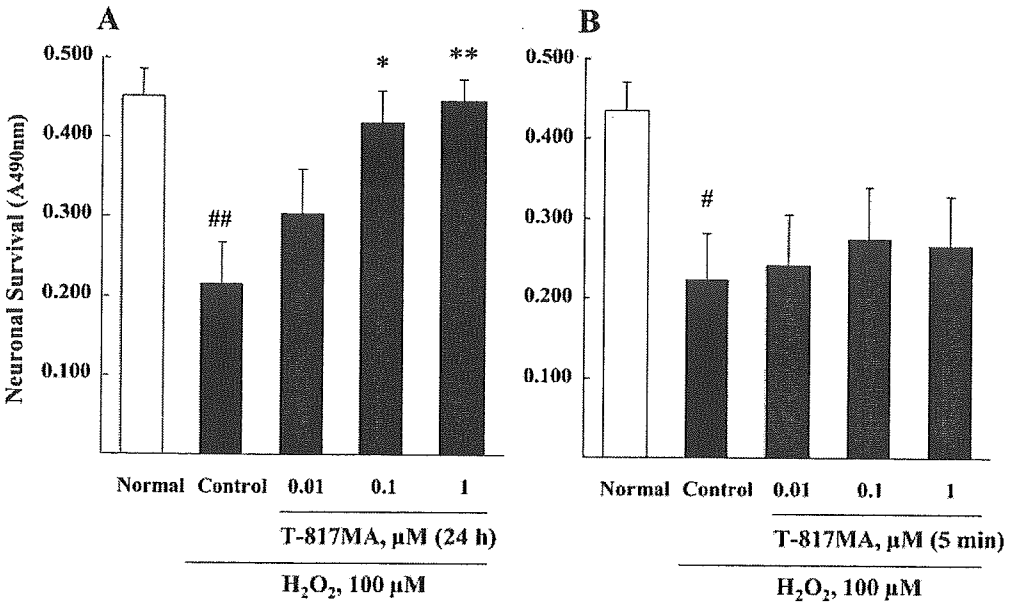

Fig. 3. Effect of T-817MA on H_2O_2-induced neuronal death. H_2O_2-induced (100 μM for 24 h) neuronal death and the neuroprotective effect of T-817MA were assessed using rat neuron/glia coculture. T-817MA was pretreated for 24 h (A) or for 5 min (B) and was continuously existed with H_2O_2. Neuronal 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).

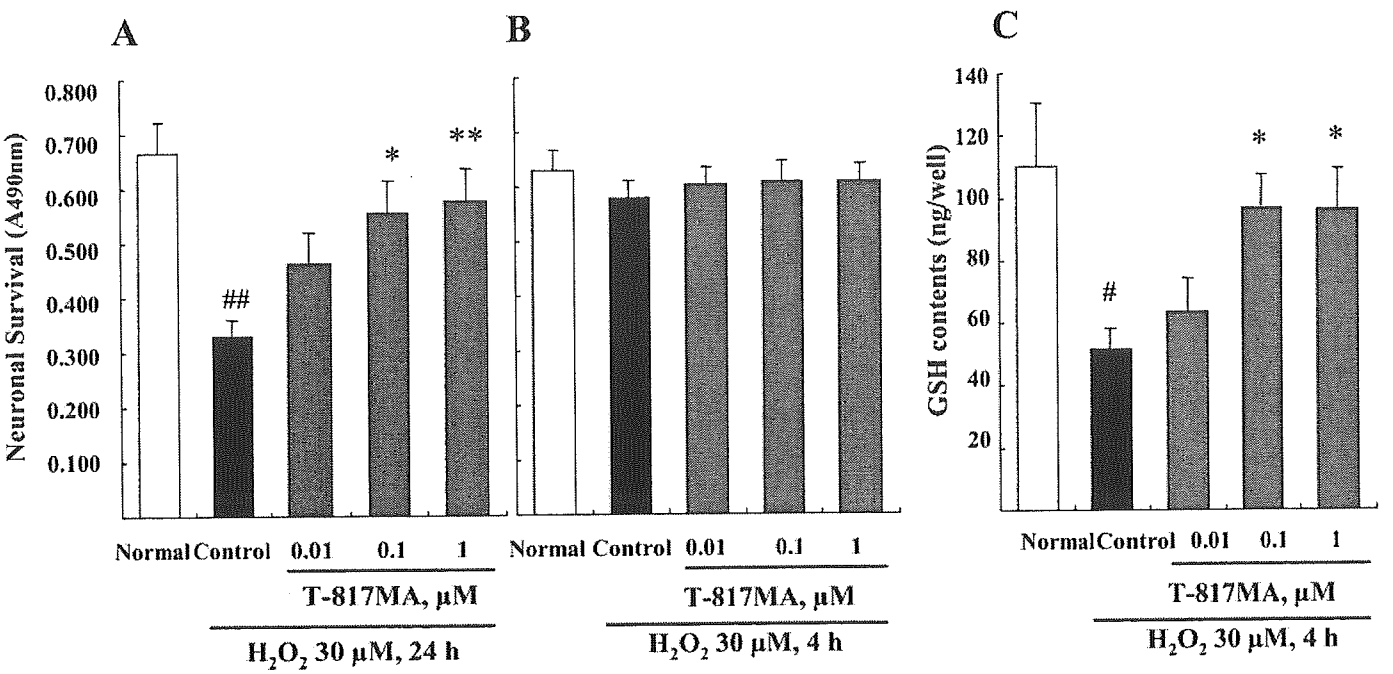

Fig. 4. Effect of T-817MA on H_2O_2-induced neuronal death and GSH reduction. Primary cultures of cortical neurons were pretreated with several concentrations of T-817MA for 24 h and then exposed to 30 μM H_2O_2 for 24 h (A) or 4 h (B) in the continuous presence of T-817MA. Neuronal survival was estimated as MAP2 immunoreactivity using enzyme immunoassay. C shows GSH contents in the culture assessed with 4-h H_2O_2 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 reaggregates 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

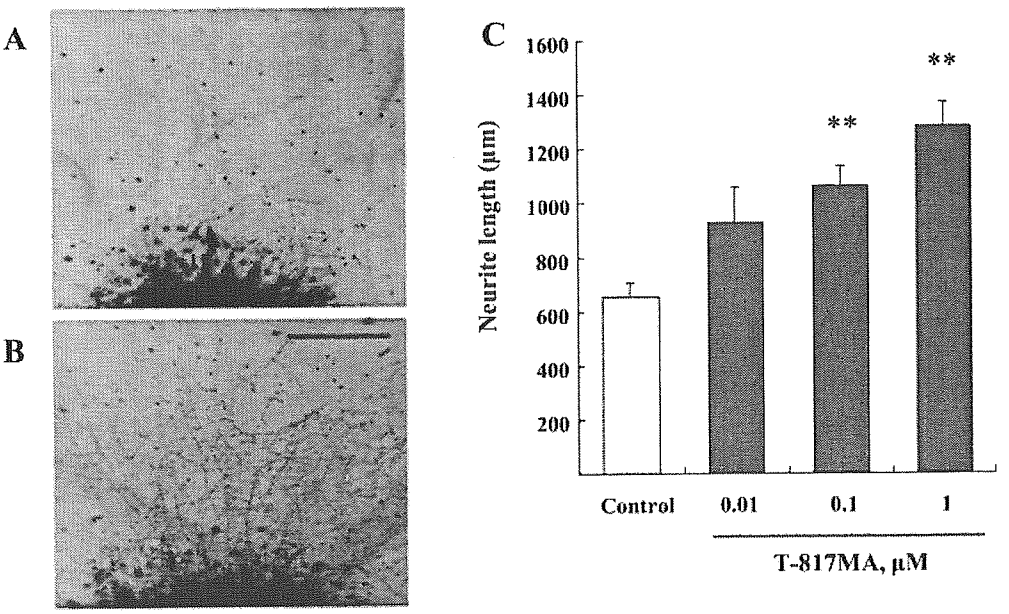

Fig. 5. Effect of T-817MA on neurite outgrowth in rat hippocampal slice cultures. Typical images of neurite response with GAP-43-immunostained neurites are shown in control (A) and 1 μM T-817MA for 8 days (B). Calibration bar, 500 μm. C, the neurite outgrowth was quantified by measuring the distance 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).

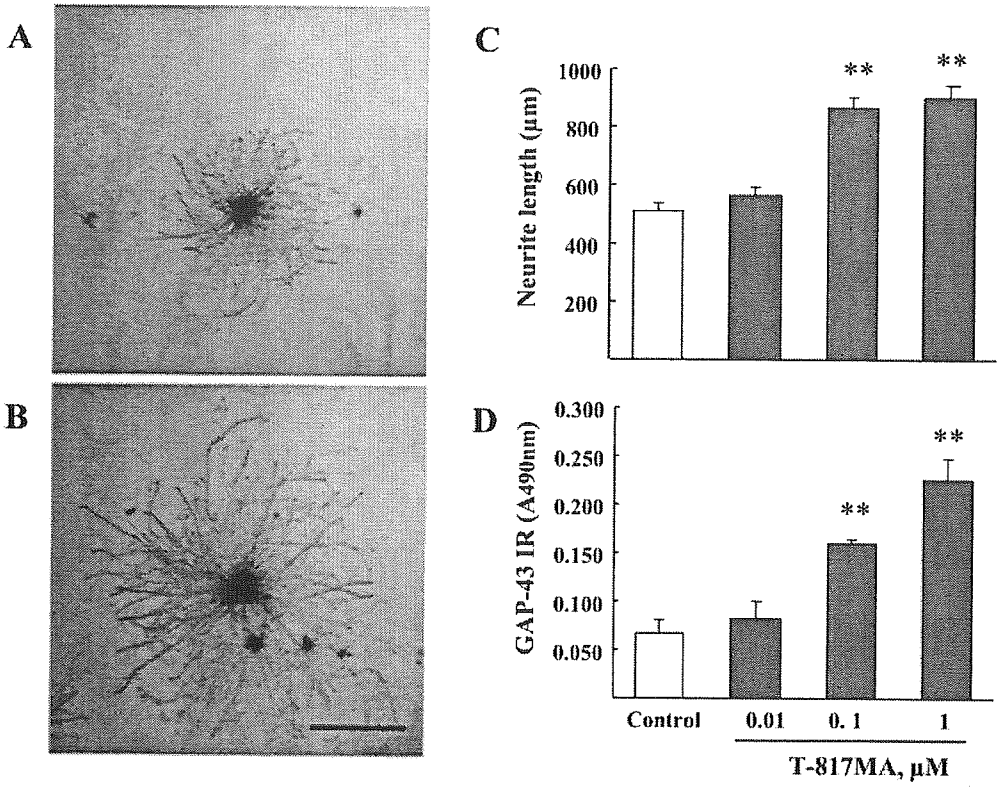

Fig. 6. Effect of T-817MA on neurite outgrowth in rat cortical reaggregation cultures. Typical images of neurite response with GAP-43-immunostained neurites are shown in control (A) and 1 μM T-817MA for 4 days (B). Calibration bar, 500 μm. C, the neurite outgrowth was quantified by measuring the distance 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 ($A_{490\ nm}$) was 0.236 with 100 ng/ml IGF-1, which was higher than the control value ($A_{490\ nm}$, 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 Aβ (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 Aβ(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. Aβ 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 Aβ application failed to induce clear neurotoxicity in the primary culture of the enriched cortical neurons. Previous studies indicated that coexistence of glial cells enhanced Aβ-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 μM 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 H_2O_2-induced neuronal death in the neuron/glia coculture. These results indicate that T-817MA promotes endogenous antioxidant processes to protect the neurons from H_2O_2 stress. According to the hypothesis that Aβ(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 Aβ(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, Aβ 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.

References

Abramov AY, Canevari L, and Duchen MR (2003) Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. *J Neurosci* **23**:5088–5095.

Abramov AY, Canevari L, and Duchen MR (2004) Beta-amyloid peptides induce mitochondrial dysfunction and oxidative stress in astrocytes and death of neurons through activation of NADPH oxidase. *J Neurosci* **24**:565–575.

Barnham KJ, Masters CL, and Bush AI (2004) Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* **3**:205–214.

Brooke SM, Bliss TM, Franklin LR, and Sapolsky RM (1999) Quantification of neuron survival in monolayer cultures using an enzyme-linked immunosorbent assay approach, rather than by cell counting. *Neurosci Lett* **267**:21–24.

Butterfield DA, Castegna A, Lauderback CM, and Drake J (2002) Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* **23**:655–664.

Fuson KS, Mark RJ, Panetta JA, and May PC (1999) Characterization of LY231617 protection against hydrogen peroxide toxicity. *J Neurochem* **72**:1154–1160.

Gao WQ, Liu XL, and Hatten ME (1992) The weaver gene encodes a nonautonomous signal for CNS neuronal differentiation. *Cell* **68**:841–854.

Gasparini L and Xu H (2003) Potential rules of insulin and IGF-1 in Alzheimer's disease. *Trends Neurosci* **26**:404–406.

Gillespie LN (2003) Regulation of axonal growth and guidance by the neurotrophin family of neurotrophic factors. *Clin Exp Pharmacol Physiol* **30**:724–733.

Goslin K, Amussen H, and Banker G (1998) Rat hippocampal neurons in low-density culture, in *Culturing Nerve Cell*, 2nd ed (Banker G and Goslin K eds) pp 339–370, The MIT Press, Cambridge.

Heck S, Lezoualc'h F, Engert S, and Behl C (1999) Insulin-like growth factor-1-mediated neuroprotection against oxidative stress is associated with activation of nuclear factor kappaB. *J Biol Chem* **274**:9828–9835.

Hiraku Y, Murata M, and Kawanishi S (2002) Determination of intracellular gluta-

thione and thiols by high performance liquid chromatography with a gold electrode at the femtomole level: comparison with a spectroscopic assay. *Biochim Biophys Acta* **1570**:47–52.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, and Bredesen DE (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Wash DC)* **262**:1274–1277.

Kim B, Leventhal PS, Saltiel AR, and Feldman EL (1997) Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires mitogen-activated protein kinase activation. *J Biol Chem* **272**:21268–21273.

Li D, Field PM, Yoshioka N, and Raisman G (1994) Axons regenerate with correct specificity in horizontal slice culture of the postnatal rat entorhino-hippocampal system. *Eur J Neurosci* **6**:1026–1037.

Lim KC, Lim ST, and Federoff HJ (2003) Neurotrophin secretory pathways and synaptic plasticity. *Neurobiol Aging* **24**:1135–1145.

Markesbery WR, Montine TJ, and Lovell MA (2001) Oxidative alterations in neurodegenerative disease, in *Pathogenesis of Neurodegenerative Disorders* (Mattson MP ed) pp 21–51, Humana Press, Totawa.

Mattson MP, Pedersen WA, and Culmsee C (2001) Cellular and molecular mechanisms underlying synaptic degeneration and neuronal death in Alzheimer's disease, in *Pathogenesis of Neurodegenerative Disorders* (Mattson MP ed) pp 113–138, Humana Press, Totawa.

Maus M, Glowinski J, and Premont J (2002) GABA is toxic for mouse striatal neurons through a transporter-mediated process. *J Neurochem* **82**:763–773.

McKinney RA, Debanne D, Gahwiler BH, and Thompson SM (1997) Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: implications for the genesis of posttraumatic epilepsy. *Nat Med* **3**:990–996.

McKinney RA, Luthi A, Bandtlow CE, Gahwiler BH, and Thompson SM (1999) Selective glutamate receptor antagonists can induce or prevent axonal sprouting in rat hippocampal slice cultures. *Proc Natl Acad Sci USA* **96**:11631–11636.

Meiri KF, Pfenninger KH, and Willard MB (1986) Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc Natl Acad Sci USA* **83**:3537–3541.

Naslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, and Buxbaum JD (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *J Am Med Assoc* **283**:1571–1577.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, et al. (2001) Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol* **60**:759–767.

Olivieri G, Baysang G, Meier F, Muller-Spahn F, Stahelin HB, Brockhaus M, and Brack C (2001) *N*-acetyl-L-cysteine protects SHSY5Y neuroblastoma cells from oxidative stress and cell cytotoxicity: effects on beta-amyloid secretion and tau phosphorylation. *J Neurochem* **76**:224–233.

O'Neill K, Chen S, and Brinton RD (2004) Impact of the selective estrogen receptor modulator, raloxifene, on neuronal survival and outgrowth following toxic insults associated with aging and Alzheimer's disease. *Exp Neurol* **185**:63–80.

Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, and Cotman CW (1993) Neurodegeneration induced by β-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci* **13**:1676–1687.

Qin L, Liu Y, Cooper C, Liu B, Wilson B, and Hong JS (2002) Microglia enhance beta-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. *J Neurochem* **83**:973–983.

Rottkamp CA, Atwood CS, Joseph JA, Nunomura A, Perry G, and Smith MA (2002) The state versus amyloid-beta: the trial of the most wanted criminal in Alzheimer disease. *Peptides* **23**:1333–1341.

Schreyer DJ, Andersen PL, Williams K, Kosatka I, and Truong TN (1997) Quantitative analysis of GAP-43 expression by neurons in microcultures using cell-ELISA. *J Neurosci Methods* **72**:137–145.

Schulz JB, Lindenau J, Seyfried J, and Dichgans J (2000) Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem* **267**:4904–4911.

Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* **6**:487–498.

Stepanichev MY, Zdobnova IM, Zarubenko II, Moiseeva YV, Lazareva NA, Onufriev MV, and Gulyaeva NV (2004) Amyloid-beta(25–35)-induced memory impairments correlate with cell loss in rat hippocampus. *Physiol Behav* **80**:647–655.

Stoppini L, Buchs PA, and Muller D (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* **37**:173–182.

Stoppini L, Buchs PA, and Muller D (1993) Lesion-induced neurite sprouting and synapse formation in hippocampal organotypic cultures. *Neuroscience* **57**:985–994.

Tariot PN and Federoff HJ (2003) Current treatment for Alzheimer disease and future prospects. *Alzheimer Dis Assoc Disord* **17 (Suppl 4)**:S105–113.

Tohda C, Matsumoto N, Zou K, Meselhy MR, and Komatsu K (2004) Abeta(25–35)-induced memory impairment, axonal atrophy and synaptic loss are ameliorated by M1, a metabolite of protopanaxadiol-type saponins. *Neuropsychopharmacology* **29**:860–868.

Wei W, Wang X, and Kusiak JW (2002) Signaling events in amyloid beta-peptide-induced neuronal death and insulin-like growth factor I protection. *J Biol Chem* **277**:17649–17656.

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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 Aβ1-42 (Aβ42), which adopts a structure rich in anti-parallel β-pleated sheets. Recently, increasing awareness of Aβ intermediates as molten-globule states has paralleled insight into the biological activities of the Aβ conformer. The molten-globule state of Aβ42 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 Aβ42 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 Aβ42 for casein substrate and the effect of bio-essential metal ions for the activity and also suggest its inhibition with Aβ42 derivatives; Aβ15-22 (QKLVFFAE) which is a potential fragment to prevent Aβ 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, Aβ peptides, serine protease-like activity, self-aggregation, metal ions, eight-residue Aβ 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 extracellulary 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(Aβ42) derived from Amyloid precursor protein(APP) [5,6].

APP is a large protein and processed with proteases termed β and γsecretase to produce Aβ42 and the impairment of cellular function is directly linked to the interaction of Aβ42 aggregates with cellular components [7-9]. It is not certain what is the key factor to induce aggregation to Aβ42 and which mechanism of aggregation process results in cell damage.

There have been many studies of the biological properties of Aβ42 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 Aβ forms responsible for neuronal cell toxicity demonstrated the potential of fibrillar and protofibrillar species of Aβ 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 Aβ intermediates as molten-globule states has paralleled insight into the biological activities of the Aβ conformer [19,20]. This findings has led to the proposal that the molten globule states of Aβ might be the main pathological form of Aβ.

In this review, we focus on the inhibition of acid-induced Aβ42 aggregation with short peptides and hydrolytic activity of Aβ 42 for casein substrates. Prevention of Aβ aggregation is a potential goal in AD therapy and we suggest the possible therapeutic strategy of AD using 8-residues peptides derived from Aβ residues.

GUANIDINE HYDROCHLORIDE DENATURATION OF Aβ42 EXPOSES THE ACID-INDUCED CRYPTIC EPITOPE

We have already demonstrated that the conformation of Aβ42 varies at different pH's, and lowering the pH might generate the molten-globule state of soluble Aβ42 *in vitro* [21]. Recently, we have performed unfolding experiments with acid-treated Aβ42 intermediates, using guanidine hydrochloride (GdnHCl) [22]. The reactivity of monoclonal antibody, which is specific for amino acid residues 9-14 of Aβ42, against acid treated-Aβ42 was greatly affected by increasing concentrations of the potent denaturant GdnHCl, whereas the sample incubated at neutral pH was unaffected

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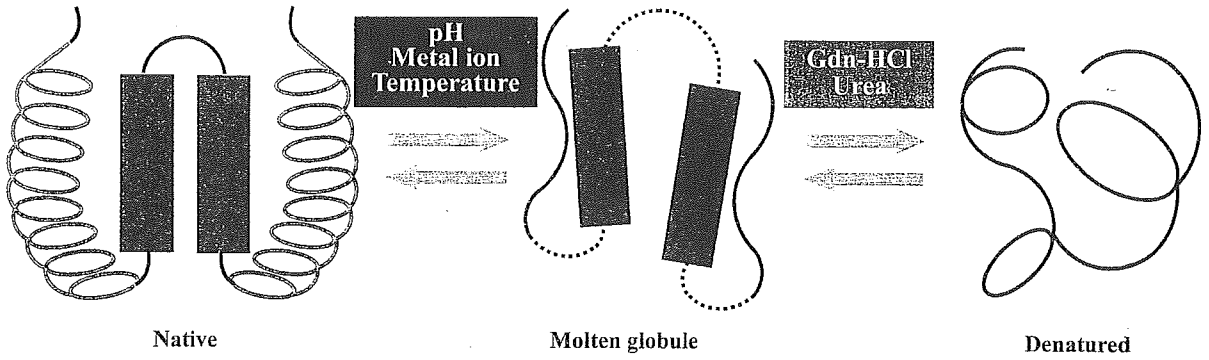

Fig. (1). The equilibrium intermediates of protein folding between native and denatured states.
Molten-globule states of Aβ42 protein exist between completely folded and completely denatured states. A variety of environmental factors including pH, metal ions and temperature affect to induce the molten-globule 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 Aβ42, which is exposed at neutral pH and hidden at acidic pH [21,23]. In addition GdnHCl caused unfolding of partially aggregated Aβ42, and this partially unfolded form may contain the folding intermediate of Aβ42 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 Aβ42 AGGREGATION

The Aβ 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 Aβ, and the abnormal Aβ is stabilized by intermolecular interactions with other Aβ monomers that have the ability to form a β-sheet conformation [28].

The compounds targeting the specific inhibition of Aβ aggregation may be of great importance in AD therapy. Previous reports suggested that Congo red could bind to Aβ however, it is not specific for Aβ 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 Aβ aggregation.

We have examined a series of partial 8-residue peptides derived from Aβ42 to inhibit acid-induced aggregation of Aβ42 [22]. Previous report suggested that Aβ 16-22(KLVFF) fragment can bind full-length Aβ40 and prevent its assembly into amyloid fibrils and that the critical amino acid residues for binding and inhibition of Aβ 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 Aβ; NH_2-K(Me-L9V(Me-l)F(Me-A)E-$CONH_2$, termed Aβ16-22m could be the most potent to disrupt the peptide-peptide interactions that promote Aβ 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 Aβ aggregates in glial cells. The epitopes around residues 9-14 and 17-21 in Aβ42 were dramatically affected by acidic pH [21]. The ability of partial Aβ fragments around Aβ16-23 to inhibit Aβ42 aggregation was proved by both ELISA and cell western dot blot analysis and our results indicate that peptides in the 8-residue peptides including Aβ15-22, Aβ16-23 and Aβ17-24, which contain the central hydrophobic region of Aβ42, are able to interfere with pH-induced Aβ42 aggregation [22] (Fig. **2**). This may be due an ability to bind to the central hydrophobic region of Aβ42, including the pH-sensitive region, thereby destabilizing the interaction between Aβ 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 Aβ intermediates in the early stages of Aβ aggregation.

Short fragments around Aβ16-23 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 Aβ17-24 fragment [38]. Hence peptides around Aβ16-23 could be physiological products

and may play an important role in inhibiting self-aggregation of Aβ42 *in vivo*. Studies of Aβ42 aggregation kinetics have suggested that aggregation of the hydrophobic form of Aβ42 can be selectively inhibited by the more soluble form, and that aggregation is driven by a hydrophobic effect of Aβ42 [39].

The central region of Aβ42 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 Aβ peptide [41]. It has been suggested that residues10-23 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 Aβ42 [30, 43]. The synthetic 5-residue peptide LPFFD, which is homologous to the central hydrophobic region 17-21 (LVFFA) of Aβ42 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 Aβ fibrinogenesis *in vitro*, prevent neuronal cell death in culture and reduce Aβ deposition in the rat brain [47].

It has also been claimed that this peptide can reverse pre-existing Aβ fibrils and subsequently diminishing Aβ related histopathology [48]. Our pervious study of the pH-induced conformational transitions of Aβ42 also suggested that amino acids residues at position 9-14 and 17-21 were responsible for the changes [21]. In our present assay system, Aβ16-23 inhibited Aβ42 aggregation better than the 5-residue peptide LPFFD (unpublished data). We have suggested that short peptides could facilitate the formation of mixed aggregates with Aβ42 that are sensitive to protease K and these findings raise the possibility that peptides around Aβ16-23 could be useful for treating amyloid plaque in the AD brain. Thus, Aβ15-22, Aβ16-23 and Aβ17-24 can inhibit Aβ42 aggregation at acidic pH and these peptides may be useful in the treatment of AD.

HYDROLYTIC ACTIVITIES OF Aβ42

Aβ has been shown recently to possess proteolytic activity against casein [49] and the molten-globule states of Aβ42 at neutral pH may participate in the activity. We have studied the proteolytic activity of Aβ42 for casein at neutral pH and have shown that Aβ17-42 also has some activity [22]. The pH shift from 7.4 to 4.6 decreased the activity of Aβ42 by about 76% and resulted in the same level of activity as Aβ17-42, which was almost unaffected by acid pH. These data indicate that residues important for Aβ42 activities are pH sensitive and that these must be present in the region

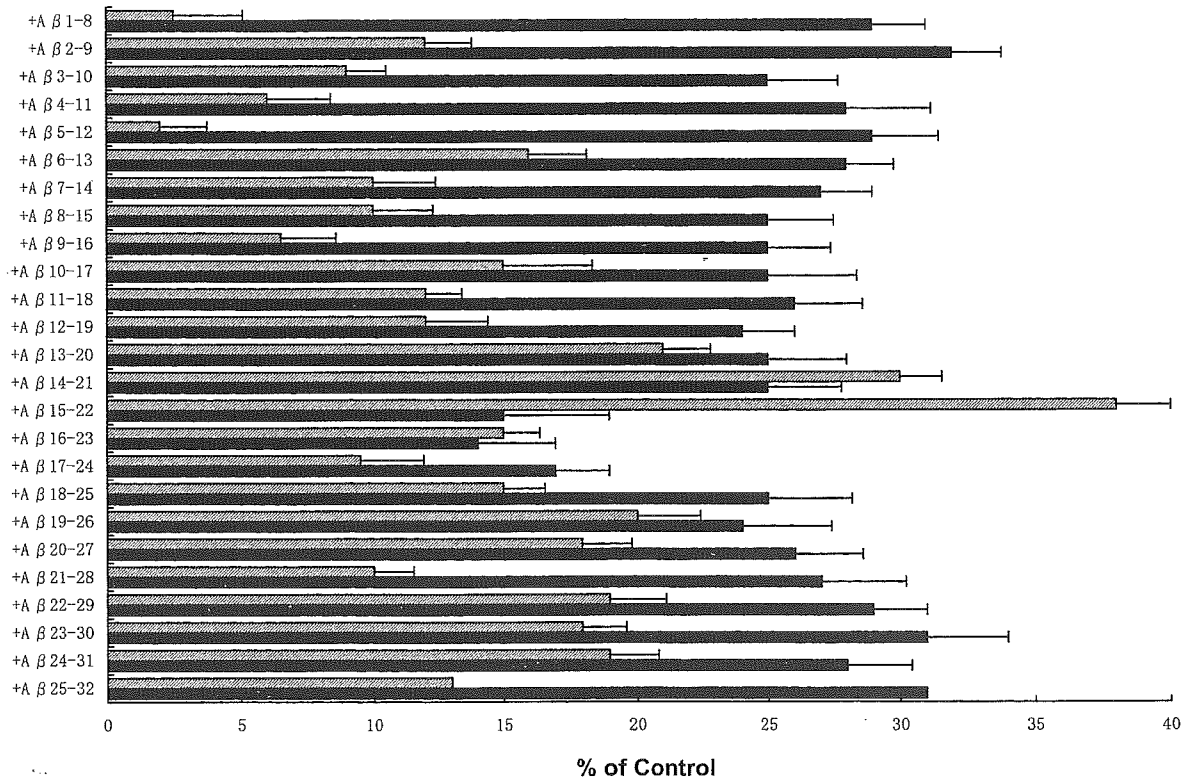

Fig. (2). Inhibition of acid-induced Aβ42 aggregation and hydrolytic activity of Aβ42 with short peptides.
Inhibition of acid-induced Aβ42 aggregation by the short peptides was studied and the percentage of remaining protein after protease K digestion was shown as % of control (■). Inhibition of hydrolytic activity of Aβ42 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 Aβ42 requires the first 16 residues for full activity, Aβ1-16 itself has no activity. As there are no amino acid residues in Aβ29-42 capable of forming the active site, the region 1-28 must contain all of the amino acid residues essential for activity. Aβ1-28 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 Aβ42 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 Aβ1-28 and Aβ17-42 are almost the same, Aβ17-42 showed higher activity than Aβ1-28. 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 Aβ42 and that serine 26 may contribute to the partial activity of Aβ17-42.

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 Aβ1-16 and Aβ12-28 may contribute residues crucial for Aβ42 activity, both are shorter than Aβ1-28 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 Aβ42 and amino acids residues 9-14 participate to the changes [21] and copper could also inhibit the enzymatic activity of Aβ42 [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 Aβ42 [21,22].

Chemical modification of the serine, as well as the histidine residues of Aβ40, 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 Aβ42 [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 Aβ42 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 Aβ 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 Aβ42 [30,43]. We suggested the proteolytic activity of Aβ42 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 Aβ

The α1-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 Aβ42 binds to two β-sheets of α1-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 Aβ may be a useful approach for preventing cellular toxicity.

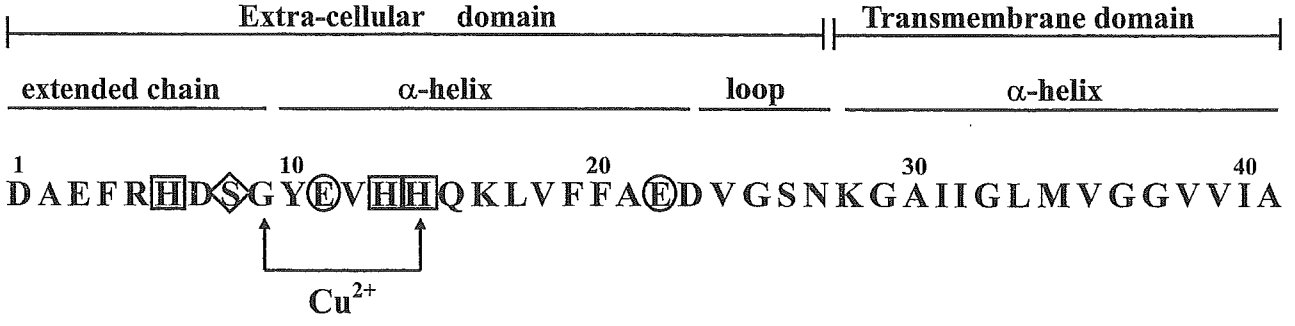

Fig. (3). Hydrolytic activity of Aβ42.

Hydrolytic activity of Aβ1-42 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 Aβ42 to study their ability to inhibit the hydrolytic activity of Aβ42 at neutral pH for casein and found that short peptides of Aβ14-21 and Aβ15-22 are effective [22] (Fig. **2**). The 5-residue peptide LPFFD, which is homologous to the central hydrophobic region 17-21 (LVFFA) of Aβ42, has been reported to inhibit Aβ fibrinogenesis *in vitro*, prevent neuronal cell death in culture and reduce Aβ deposition in the rat brain [47]. Interestingly, the hydrolytic activity of Aβ42 for casein at neutral pH was inhibited only by Aβ14-21 and Aβ15-22 [22]. The ability of partial Aβ peptides to inhibit the hydrolytic activity of Aβ42 may be due an ability to bind to the central hydrophobic region of Aβ42, including the pH-sensitive region, thereby destabilizing the interaction between Aβ monomers and/or oligomers necessary for fibril stability and inhibit the biological active form. We speculate that oligomerization of Aβ42 may be required for full proteolytic activity and complete aggregation of Aβ42 may abrogate the activity. Thus, we have suggested that short peptides Aβ14-21: HQKLVFFA and Aβ15-22: QKLVFFAE could be useful for the treatment of AD and Aβ15-22 may be the only peptide to control both aggregation of Aβ42 at acidic pH and proteolytic activity at neutral pH.

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REFERENCES

[1] Terry, R.D. *Prog. Brain Res.*, **1994**, *101*, 383.
[2] Selkoe, D.J. *Ann. Rev. Cell. Biol.*, **1994**, *10*, 373.
[3] Price, D.L.; Sisodia, S.S.; Borchelt, D.R. *Science*, **1998**, *282*, 1079.
[4] Kang, J.; Lemaire, H.G.; Unterbeck, A.; Salbaum, J. M.; Masters, C. L.; Grzeschil, K. H.; Multhaup, G.; Beyreuther, K.; Muller, H.B. *Nature*, **1987**, *325*, 733.
[5] Yankner, B. A. *Neuron*, **1996**,*16*,921.
[6] Selkoe, D. J. *Science*, **1997**, *275*, 630.
[7] Sisoida, S.S.; Koo, E.H.; Beyreuther, K.; Unterbeck, A.; Price, D.L. *Science*, **1990**, *248*, 492.
[8] Esch, F.S.; Keim, P.S.; Beattie, E.C.; Blacher, R.W.; Culwell, A.R.; Oltersdorf, T.; McClure, D.; Ward, P.J. *Science*, **1990**,*248*,1122.
[9] Golde, T.E.; Estus, S.; Younkin, L.H.; Selkoe, D.J.; Younkin, S.G. *Science*, **1992**, *255*, 728.
[10] Barrow, C.J.; Yasuda, A.; Kenny, P.T.M.; Zagorski, M.G. *J. Mol. Biol.*, **1992**, *225*, 1075.
[11] Golabek, A.A.; Soto, C.; Vogel, T.; Wisniewski, T. *J. Biol. Chem.*, **1996**, *271*, 10602.
[12] Brzyska, M.; Bacia, A.; Elbaum, D. *Eur. J. Biochem.*, **2001**, *268*, 3443.
[13] Lambert, M.P.; Barlow, A.K.; Chromy, B.A.; Edwards, C.; Freed, R.; Liosatos, M.; Morgan, T.E.; Rozovsky, I.; Trommer, B.; Viola, K.L.; Wals, P.; Zhang, C.; Finch, C.E.; Krafft, G.A.; Klein, W.L. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 6448.
[14] Roher, A.E.; Chaney, M.P.; Kuo, Y.; Webster, S.D.; Stine, W.B.; Haverkamp, L.J.; Woods, A.S.; Cotter, R.J.; Tuohy, J.M.; Krafft, G.A.; Bonnell, B.S.; Emmerling, M.R. *J. Biol. Chem.*, **1996**, *271*, 20631.
[15] Crawford, F.; Soto, C.; Suo, Z.; Fang, C.; Parker, T.; Sawar, A.; Frangione, B.; Mullan, M. *FEBS Lett.*, **1998**, *436*, 445.
[16] Selkoe, D.J. *J. Neuropathol. Exp. Neurol.*, **1994**, *53*,438.
[17] Geula, C.; Wu, C.K.; Saroff, D.; Lorenzo,A.;Yuan, M.; Yankner, B.A. *Nat. Med.*, **1998**, *4*, 827.
[18] Ward, R.V.; Jennings, K.H.; Jepras, R.; Neville, W.; Owen, D.E.; Hawkins, J.; Christie, G.; Davis, J.B.; George, A.; Karran, E.H.; Howlett, D.R. *Biochem. J.*, **2000**, *348*,137.
[19] Kelly, J.W. *Curr. Opin. Struc. Biol.*, **1998**, *8*, 101.
[20] Kirkitadze, M.D.; Condron, M.M.; Teplow, D.B. *J. Mol. Biol.*, **2001**, *312*, 1103.
[21] Matsunaga, Y.; Saito, N.; Fujii, A.; Yokotani, J.; Takakura, T.; Nishimura, T.; Esaki, H.; Yamada, T. *Biochem. J.* **2002**, *361*, 547.
[22] Matsunaga, Y.; Fujii, A.; Awasthi, A.; Yokotani, J.; Tadakazu, T.; Yamada, T. *Regul. Peptides*, **2004**, *120*, 227.
[23] Matsunaga, Y.; Zerovnik, E.; Yamada, T.; Turk, V. *Curr. Med. Chem.*, **2002**, *9*, 1717.
[24] Matsunaga,Y.; Peretz, D.; Williamson, A.; Burton, D.; Mehlhorn, I.; Groth, D.; Cohen, F.E; Prusiner, S.B.; Baldwin, M.A. *Proteins*, **2001**, 44, 110.
[25] Hagihara, Y.; Aimoto, S.; Fink, A.L.; Goto, Y. *J. Mol. Biol.*, **1993**, *231*, 180.
[26] Burdick, D.; Soreghan, B.; Kwon, M.; Kosmoski, J.; Knauer, M.; Henschen, A.; Yates, J.; Cotman, C.; Glabe, C. *J. Biol. Chem.*, **1992**, *267*, 546.
[27] Jarrett, J.T.; Berger, E.P.; Lansbury, P.T. Jr. *Biochemistry*, **1993**, *32*, 4693.
[28] Wouters, M.A.; Curmi, P.M. *Proteins*, **1995** ,*22*, 119.
[29] Lorenzo, A.; Yankner B.A. *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 12243.
[30] Tjernberg, L.O.; Lilliehook, C.; Callaway, D.J.; Naslund, J.; Hahne, S.; Thyberg, J.; Terenius, L.; Nordstedt, C. *J. Biol. Chem.*, **1997**, *272*, 12601.
[31] Chitnumsub, P.; Fiori, W.R.; Lashuel, H.A.; Diaz, H.; Kelly, J.W. *Bioorg. Med. Chem.*, **1999**, *7*, 39.
[32] Nesloney, C.L.; ,Kelly, J.W. *Bioorg. Med. Chem.*, **1996**, *4*, 739.
[33] Gordon, D.J.; Sciarretta, K.L.; Meredith, S.C. *Biochemistry*, **2001**, *40*, 8237.
[34] Nordstedt, C.; Naslund, J.; Tjernberg, L.O.; Karlstrom, A.R.; Thyberg, J.; Terenius, L. *J. Biol. Chem.*, **1994**, *269*, 30773.
[35] Miyazaki, K.; Hasegawa, M.; Funahashi, K; Umeda, M. *Nature*, **1993**, *362*, 839.
[36] Hamazaki, H. *FEBS Lett.*, **1996**, *396*, 139.
[37] McDermott, J.R.; Gibson, A.M. *Neuroreport.*, **1996**, *7*, 2163.
[38] McDermott, J, R.; Gibson, A.M. *Neurochem. Res.*, **1997**, *22*, 49.
[39] Snyder, S.W.; Ladror, U.S.; Wade, W.S.; Wang, G.T.; Barrett, L.W.; Matayoshi, E.D.; Huffaker, H.J.; Krafft, G.A.; Holzman, T.F. *Biophys. J.*, **1994**, *67*, 1216.
[40] Golabek, A.A.; Soto, C.; Vogel, T.; Wisniewski, T. *J. Biol. Chem.*, **1996**, *271*, 10602.
[41] El-agnaf, O.M.A.; Guthrie, D.J.S.; Walsh, D.M.; Irvine, B.G. *Eur. J. Biochem.*,**1998**, *256*, 560.
[42] Hilbich, C.; Kisters, W.B.; Reed, J.; Masters, C.L.; Beyreuther, K. *J. Mol. Biol.*, **1991**, *218*, 149.
[43] Hilbich, C.; Kisters, W.B.; Reed, J.; Masters, C.L.; Beyreuther, K. *J. Mol. Biol.*, **1992**, *228*, 460.
[44] Wood, S.J.; Wetzel, R.; Martin, J.D.; Hurle, M.R. *Biochemistry*, **1995**, *34*, 724.
[45] Chou, P.Y.; Fasman, G.D. *Annu. Rev. Biochem.*, **1978**, *47*, 251.
[46] Soto, C.; Castano, E.M.; Frangione, B.; Inestrosa, N.C. *J. Biol. Chem.*, **1995**, *270*, 3063.
[47] Soto, C.; Sigurdsson, E.M.; Morelli, L.; Kumar, R.A.; Castano, E.M.; Frangione, B. *Nature Med.*, **1998**, *4*, 822.
[48] Sigurdsson, E.M.; Permanne, B.; Soto, C.; Wisniewski, T.; Frangione, B. *J. Neuropathol. Exp. Neurol.*, **2000**, *59*, 11.
[49] Elbaum, D.; Brzyska, M.; Bacia, A.; Alkon, D.L. *Biochem. Biophys. Res. Commun.*, **2000**, *267*, 733.
[50] Shao, H.; Jao, S.; Ma, K; Zagorski, M.G. *J. Mol. Biol.*, **1999**, *285*, 755.
[51] Lee, J.P.; Stimson, E.R.; Ghilardi, J.R.; Mantyh, P.W.; Lu, Y-A.; Felix, A.M.; Llanos, W.; Behbin, A.; Cummings, M.; Van Criekinge, M.; Timms, W.; Maggio, J.E. *Biochemistry*, **1995**, *34*, 5191.
[52] Halverson, K.,; Fraser, P.E.; Kirschner, D.A.; Lansbury, P.T. Jr. *Biochemistry*, **1990**, *29*, 2639.
[53] Levy, E.; Carman, M.D.; Fernandez, M.I.J.; Power, M.D.; Lieberburg, I.; Van Duinen, S.G.; Bots, G.T.; luyendijk, W.; Frangione, B. *Science.*, **1990**, *248*, 1124.
[54] Wisniewski, T.; Ghiso, J.; Frangione, B. *Biochem. Biophys. Res. Commun.*, **1991**, *179*, 1247.
[55] Clements, A.; Walsh, D.M.; Williams, C.H.; Allsop, D. *Neurosci. lett.*, **1993**, *161*, 17.
[56] Murakami, K.; Irie, K.; Morimoto, A.; Ohigashi, H.; Shindo, M.; Nagao, M.; Shimizu, T.; Shirasawa, T. *Biochem. Biophys. Res. Commun.*, **2002**, *294*, 5.
[57] Abraham, C.R.; Selkoe, D.J.; Potter, H.; Price, D.L.; Cork, L.C. *Neuroscience*, **1989**, *32*, 715.

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Lib, transcriptionally induced in senile plaque-associated astrocytes, promotes glial migration through extracellular matrix

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Abstract

In an effort to identify astrocyte-derived molecules that may be intimately associated with progression of Alzheimer's disease (AD), Lib, a type I transmembrane protein belonging to leucine-rich repeat superfamily, has been identified as a distinctly inducible gene, responsive to β-amyloid as well as pro-inflammatory cytokines in astrocytes. To evaluate the roles of Lib in AD, we investigated Lib expression in AD brain. In non-AD brain, Lib mRNA has been detected in neurons but not in quiescent astrocytes. On the contrary, in AD brain, Lib mRNA is expressed in activated astrocytes associated with senile plaques, but not expressed in neurons around lesions. Lib-expressing glioma cells displayed promotion of migration ability through reconstituted extracellular matrix and recombinant Lib protein bound to constituents of extracellular matrix. These observations suggest that Lib may contribute to regulation of cell–matrix adhesion interactions with respect to astrocyte recruitment around senile plaques in AD brain.

Keywords: LRR; mRNA expression; Alzheimer's disease; Cellular migration; Extracellular matrix

Neuropathological changes in the brains from patients with Alzheimer's disease (AD) include loss of neurons, intracellular formation of neurofibrillary tangles, appearance of numerous β-amyloid (Aβ)-containing amyloid plaques, as well as reactive gliosis. Numerous reactive astrocytes observed in lesions are a common feature of an AD brain as well as in many other neurodegenerative disorders. They surround senile plaques and have morphological changes, extending processes into the lesions and producing a variety of inflammatory mediators [1–5]. These observations support the notion that the activated astrocytes in AD lesions have a significant influence on the neighboring neurons and their environment, leading to exacerbation of the disease.

In efforts to identify key molecules from astrocytes intimately involved in the disease, Lib (an LRR protein induced by β-amyloid treatment) was identified as a distinctly inducible gene, responsive to Aβ as well as to pro-inflammatory cytokines in astrocytes [6]. Lib protein is a type I transmembrane protein with an extracellular domain consisting of 15 leucine-rich repeats (LRRs) flanked by both N- and C-terminal cysteine-rich regions that form intramolecular disulfide loops, similar to the extracellular binding motifs of some adhesion proteins and receptors [7–9]. Lib is thought to play a role in inflammatory states via the LRR motif, an ideal structural framework for specific protein–protein and/or protein–matrix interactions including adhesion, target recognition or receptor–ligand binding [6–10].

In this study, the distribution of Lib mRNA expression in AD brain was evaluated to give insight into the

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