Abstract. Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by amyloid β (Aβ)-containing plaques and neurofibrillary tangles, and synaptic and neuronal loss, along with progressive cognitive impairment. Although growing evidence suggests the beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) on AD, this notion is still controversial. To evaluate the efficacy of statins for Aβ-induced cognitive impairment, we employed an Aβ injection model. Using this model, the present study demonstrated that pretreatment with fluvastatin, but not post-treatment just after Aβ exposure, prevented Aβ-induced memory impairment. We also observed that fluvastatin significantly decreased Aβ accumulation and oxidative stress after Aβ injection. Mice treated with simvastatin, but not fluvastatin, did not demonstrate the prevention of Aβ-induced memory impairment, and showed no significant decrease in oxidative stress. More importantly, fluvastatin significantly prevented the loss of neurons in the basal forebrain induced by Aβ.

Overall, the present study demonstrated that fluvastatin significantly prevented memory impairment induced by Aβ. The beneficial effects of fluvastatin might be explained by the preservation of neurons through a significant decrease in Aβ accumulation and oxidative stress. In clinical practice, the timing of the start of fluvastatin treatment might be critical in achieving a beneficial effect on cognitive function.

Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by amyloid β (Aβ)-containing plaques and neurofibrillary tangles, and synaptic and neuronal loss, along with progressive cognitive impairment. Aβ, a 38-43 amino acid peptide, is the primary component of senile plaques (1). Aβ deposited in senile plaques is considered to be primarily involved in the pathogenesis of AD, because i) familial AD (FAD) has been linked to mutations in the amyloid precursor protein (APP) (2-4), and ii) FAD-linked mutations in the amyloid precursor protein (APP) and presenilin genes (5-7) result in the increased production of Aβ42 (8,9), which is the predominant form found in senile plaques (10). Moreover, levels of total Aβ40 and Aβ42 are elevated in early dementia and levels of both peptides are strongly correlated with cognitive decline (11). Although growing evidence suggests the beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) for AD, this notion is still controversial. It was firstly reported that there is a lower prevalence of diagnosed AD in patients taking the statins, lovastatin and pravastatin, in the US (12). Another group reported that patients in the UK, receiving statins, had a lowered risk of developing cognitive impairment. Aβ, a 38-43 amino acid peptide, is the primary component of senile plaques (1). Aβ deposited in senile plaques is considered to be primarily involved in the pathogenesis of AD, because i) familial AD (FAD) has been linked to mutations in the amyloid precursor protein (APP) (2-4), and ii) FAD-linked mutations in the amyloid precursor protein (APP) and presenilin genes (5-7) result in the increased production of Aβ42 (8,9), which is the predominant form found in senile plaques (10). Moreover, levels of total Aβ40 and Aβ42 are elevated in early dementia and levels of both peptides are strongly correlated with cognitive decline (11). Although growing evidence suggests the beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) for AD, this notion is still controversial. It was firstly reported that there is a lower prevalence of diagnosed AD in patients taking the statins, lovastatin and pravastatin, in the US (12). Another group reported that patients in the UK, receiving statins, had a lowered risk of developing dementia (13). Furthermore, it was recently reported that atorvastatin produced significant positive effects on cognitive function (14,15). Consistent with clinical reports, the effect of statins on Aβ metabolism has been reported in vitro and in vivo (16-18). Simvastatin reduces Aβ levels in cell cultures and in guinea pig brain homogenate (16). Atorvastatin has been reported to activate α-secretase, and subsequently reduce the production of Aβ (17). However, other cohort studies indicated that lipid levels and the use of lipid-lowering agents do not seem to be associated with the risk of AD (19). Thus, there is an apparent discrepancy among studies regarding the effectiveness of statins for AD. In this study, we hypothesized that the timing of the start of treatment with a statin, or the kind of statin, might be critical in achieving beneficial efficacy. Thus, we employed an Aβ1-40 injection model to evaluate the effects of statins.
on memory impairment at different timings. The present study demonstrated that fluvastatin, but not simvastatin, significantly prevented memory impairment induced by Aβ through a significant decrease in Aβ accumulation and oxidative stress, and the prevention of neuronal loss.

**Materials and methods**

**Animals.** Male ddY mice (6-8 weeks old) were obtained from CLEA Japan, and housed in specific pathogen-free facilities under a standard 12/12-h light/dark cycle. All experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Osaka University School of Medicine.

**Peptide and chemicals.** Aβ1-40 was purchased from Peptide Institute. Aβ1-40 solution was prepared for each experiment as described previously (20). Briefly, 0.55 mg Aβ1-40 peptide was dissolved in 3250 μl PBS with 35% acetonitrile and 0.1% trifluoroacetic acid. To remove the remaining undissolved Aβ1-40, centrifugation was performed at 15,000 x g for 3 min before Aβ1-40 solution was aliquoted. The control peptide Aβ40-1 was also prepared in the same way as Aβ1-40. Fluvastatin was provided by Novartis Pharma AG, and simvastatin was purchased from Sigma-Aldrich.

**Aβ1-40 injection model.** To evaluate the effects of the statins on Aβ-induced cognitive impairment, we employed a mouse model produced by previously reported methods with modification (21,22). We injected Aβ1-40 into the cerebral ventricle by single injection. It has been reported that the levels of total Aβ40 and Aβ42 are elevated early in dementia, and the levels of both peptides are strongly correlated with cognitive decline (11), and that the level of Aβ was increased 80-fold compared with control (11). Thus, we calculated the quantity of Aβ to be injected. As the basal level of Aβ in the mouse brain is normally in the low nanomolar range, and in transgenic mouse models of brain amyloidosis it varies from 40 to 250 nM/kg body weight from 3 to 12 months of age (23), we determined the dose of Aβ1-40 to be injected as 200 pmol/μl. Although the quantity of Aβ injected was relatively low as compared to that in a previous report (21,22), our preliminary study revealed memory impairment induced by Aβ, as assessed by a water-finding task to evaluate spatial reference memory (data not shown).

Intracerebroventricular (i.c.v.) administration was carried out in accordance with a procedure described previously (22). Briefly, the mice were anesthetized with isoflurane gas and intraperitoneal xylazine and ketamine, Aβ1-40 (200 pmol/μl) was injected i.c.v. into the mice, aimed at 1 to 1.5 mm lateral to the midline, 0.5 mm posterior to the bregma, and 3 mm deep, using a 100 μl Hamilton syringe with a 27 gauge needle.

**Immunohistochemical detection of Aβ.** Two days after Aβ injection, the mice were sacriﬁced by transcardiac perfusion-ﬁxation with cold saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed, and postﬁxed for 12 h in the same ﬁxative. The brains were left in 15% sucrose in sodium phosphate buffer for 24 h, and 30% sucrose for 48 h at 4°C. The frozen brains were cut at 10-μm thickness with a cryostat (Leica CM3050 S, Leica Microsystems). A mouse anti-human Aβ protein monoclonal antibody (6E10; Sigma) was used at a 1:200 dilution. The tissue sections were incubated overnight at 4°C with the primary antibody. After washing in PBS three times, the tissues were incubated for 2 h at room temperature with the second antibody (goat anti-mouse antibody, Alexa488 conjugate). Images were obtained by a using fluorescence microscope (Nikon Eclipse TE300, Nikon).

**Measurement of Aβ.** The brain homogenates were analyzed by Aβ40 ELISA kit (Wako Pure Chemical Industries). To extract soluble cerebral Aβ, 150 mg of fresh frozen tissue was homogenized with a Teflon-glass homogenizer (6 strokes) in 1 ml of 70% formic acid. Homogenates were centrifuged at 100,000 x g for 1 h to remove particular material. The supernatant was neutralized with a 20-fold dilution in 1 M Tris base. After neutralization, the sample was diluted with the standard diluent in the Aβ40 ELISA kit and measured as directed in the package insert.

**Detection of superoxide anion in brain sections.** Superoxide anion was detected as described previously (25). In brief, frozen, enzymatically intact, 10-μm sections were prepared from mouse brain two days after Aβ injection and immediately incubated with dihydroethidium (DHE) (10 mol/L; Molecular Probes Inc.) in PBS for 30 min at 37°C in a light-protected humidified chamber. DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. Images were obtained using a Bio-Rad Radiance 2100 laser scanning confocal microscope (Bio-Rad Laboratories, Inc.). The intensity of the fluorescence was analyzed and quantified using ImageJ.
optical microscope. Average numbers of ChAT-positive cells in the basal forebrain were obtained by combining the data of three consecutive coronal sections.

Statistical analysis. All values are expressed as mean ± SEM. Data were statistically analyzed by Student’s t-test or ANOVA. Values of p<0.05 were considered significant.

Results

Behavioral analysis of Aβ-injected mice treated with fluvastatin before or after Aβ injection. To test the hypothesis that the timing of the start of treatment with a statin is critical to improve the memory impairment induced by Aβ injection, we initially investigated the prevention of memory impairment by statin pretreatment, in which a statin was administered from 2 weeks before Aβ injection, or post-treatment, in which a statin was administered from just after Aβ injection (Fig. 1A). In the previous report on the Aβ1-40 infusion model, Aβ accumulation was observed in the brain (21). In the present study, we observed Aβ accumulation in the cortex and hippocampus (Fig. 1B). Consistent with the previous reports, as assessed by the water-finding task, memory was significantly impaired in Aβ injection model mice. Unexpectedly, pretreatment with fluvastatin prevented the memory deficit induced by Aβ, although post-treatment with fluvastatin did not improve memory deficit (Fig. 1C). In the training trial, the number of approaches did not vary among groups, suggesting that the opportunity for the mice to learn about the apparatus was not different among the groups (data not shown). To rule out the possibility that this difference was due to the duration of fluvastatin treatment, we also treated mice with fluvastatin for 5 weeks, from just after Aβ injection. However, fluvastatin treatment for 5 weeks from just after Aβ injection did not improve Aβ-induced memory impairment (data not shown). Thus, the present study suggests that the beneficial effects of fluvastatin might be dependent on the time of starting the drug. To elucidate whether this beneficial effect of fluvastatin is due to the specific structure of fluvastatin or not, we tested another statin, simvastatin, in the model. Unexpectedly, mice treated with simvastatin did not perform the water-finding task better than control (Fig. 1D).

Molecular mechanisms of the improvement of memory impairment by fluvastatin. In light of the above, we examined why pretreatment with fluvastatin significantly improved memory impairment. Initially, we focused on the effects of fluvastatin on Aβ accumulation. When Aβ is administered into the caudate nucleus, Aβ is metabolized and eliminated from the brain relatively rapidly (23). Thus, we measured Aβ levels in brain homogenates at several time points after Aβ injection. Post-treatment with fluvastatin significantly decreased Aβ accumulation after Aβ injection (Fig. 2A). Fluvastatin also significantly reduced Aβ levels in the brain as compared with no treatment at 3 weeks after injection (Fig. 2B).

To further qualify the favorable effects of fluvastatin on Aβ accumulation, we examined whether the decrease in Aβ accumulation by fluvastatin might be due to an increase in Aβ degradation activity, since Aβ is mainly degraded by

Gel electrophoresis and Western blotting of IDE and NEP. SDS-PAGE was carried out on 4-20% Tris/glycine gradient gel (Invitrogen). The mouse brain membrane fraction was mixed with SDS sample buffer and boiled for 5 min immediately prior to electrophoresis. The samples were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). For immunoblotting, the membranes were probed with antibodies raised to insulin-degrading enzyme (IDE) (Calbiochem) or NEP (56C6; Novoceastra).

Histological analysis of choline acetyltransferase-positive cells. Two days after Aβ injection, sections of mouse brain were prepared according to the method of Aβ immunohistochemical analysis described above. The brain sections were incubated with 0.4% Triton in PBS at room temperature for 30 min. Then, immunohistochemical detection of choline acetyltransferase (ChAT) was carried out with goat anti-ChAT polyclonal antibody (1:100 dilution; Chemicon). The brain sections were incubated overnight at 4°C with the primary antibody. After washing in PBS five times, the sections were incubated for 2 h at 4°C with rabbit anti-goat IgG (1:200 dilution; Chemicon). After washing in PBS five times, the sections were incubated with goat peroxidase-anti-peroxidase (PAP) (1:400 dilution; Chemicon) for 2 h at 4°C. Then they were washed in PBS five times and incubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Wako Pure Chemical Industries) for 10 min. The tissues were reacted in 0.05% DAB and 0.01% H2O2 in TBS. Images were obtained by using a Nikon Eclipse TE300 optical microscope. Average numbers of ChAT-positive cells were quantified using the ImageJ software.
neprilysin (28) and insulin degrading enzyme (IDE) (29). An Aβ degradation assay was mainly employed to assess IDE activity (30,31) while a neprilysin assay was employed to evaluate neprilysin (27,29). Importantly, pretreatment with fluvastatin affected neither IDE activity nor neprilysin activity (Fig. 3A and B). Moreover, pretreatment with fluvastatin did not change the expression of NEP and IDE (Fig. 3C). These results indicate that the decrease in Aβ accumulation by fluvastatin was not through an increase in Aβ degradation.

We then focused on oxidative stress, since Aβ is well known to induce oxidative stress in vivo, both in the Aβ injection model (32,33) and in APP transgenic mice (34). Moreover, it has been reported that fluvastatin has potent anti-oxidative effects in various models, unlike other statins (35-38). Using DHE, an oxidative fluorescent dye, to detect superoxide in brain sections (25), the present study revealed that Aβ injection significantly induced oxidative stress in the hippocampus (Fig. 4A and B). Importantly, fluvastatin significantly reduced the oxidative stress induced by Aβ (Fig. 4A and B). These results suggest that the anti-oxidative effects of fluvastatin may have contributed to its beneficial
effects on cognitive function. Importantly, simvastatin did not reduce oxidative stress induced by Ab, unlike fluvastatin (Fig. 4A and B).

Basal forebrain cholinergic neuronal loss is a common feature of AD (39). Moreover, this phenomenon is recapitulated in APP transgenic mice (40) and the Aβ injection model (22). Different effects of fluvastatin and simvastatin were also confirmed by measurement of cholinergic neurons. Fluvastatin, but not simvastatin, significantly prevented basal forebrain cholinergic neuronal loss induced by Aβ, whereas Aβ injection induced cholinergic neuronal loss in the basal forebrain (Fig. 5A and B).

Discussion

Treatment of dementia is now becoming a social problem. Among numerous possible treatments, statins might be an attractive candidate. In a French cohort study, the Three-City Study, statins were associated with decreased risk of dementia (41). However, in the Cardiovascular Health Study, a cohort study in the US, statin therapy was not associated with a decreased risk of dementia (42). The results varied in these studies, due to the limited number of subjects. On the other hand, an early report indicated that atorvastatin produced a change in the slope of deterioration of MMSE in the treatment of mild-to-moderate AD (14). These discrepancies among clinical trials indicate the need for further studies to elucidate the role of statins in the treatment of dementia. Our present study revealed two important aspects: the timing of treatment with statins, and the selection of statin. First, we clearly demonstrated that treatment with fluvastatin before the injection of Aβ significantly prevented memory impairment induced by Aβ. Thus, the timing of administration of statins might need to be more carefully considered in clinical situations. The second question appears to be more sensitive and crucial. Our present study indicated that fluvastatin significantly improved memory impairment through a significant decrease in Aβ accumulation and oxidative stress and a significant increase in cholinergic neurons, while simvastatin did not affect memory impairment. As anti-oxidant effects of fluvastatin have been reported in patients with type 2 diabetes and hyperlipidemia as compared to simvastatin (43), the clinical comparison of anti-oxidant effects between fluvastatin and simvastatin.
simvastatin might be important to elucidate the pathological significance of oxidative stress in AD. It has been suggested that the beneficial effects of statins on AD might not be only through lipid-lowering effects, but also through pleiotropic effects. One of the important mechanisms to improve memory impairment is considered to be through antioxidative effects, although further studies are necessary.

We observed that fluvastatin significantly decreased Aβ accumulation after Aβ injection in the model. It has been reported that cholesterol-lowering drugs and cholesterol-extracting resins strongly reduce intracellular and secretory neuronal Aβ42 and Aβ40 levels in vitro, and that administration of simvastatin to guinea pigs strongly reduced cerebral Aβ levels, including that of the Aβ42 isoform (16). Another group also reported that statins inhibited the dimerization of β-secretase via both isoprenoid- and cholesterol-mediated mechanisms, and then reduced Aβ production (46). Thus, previous studies suggested that the effects of statins were through the down-regulation of Aβ production and/or secretion. In the present study, Aβ was exogenously injected and therefore, the decrease in Aβ accumulation by fluvastatin was not through the direct inhibition of Aβ production and/or secretion, but maybe through a novel action of statins on Aβ metabolism. The possibility that statins might affect Aβ degradation is not evident from the data in this study (Fig. 3). One possible explanation is related to the transport of Aβ across the microvascular endothelium (i.e. the blood-brain barrier), since the transport of Aβ is essential to control Aβ levels in the brain (47). Indeed, statins have pleiotropic action on endothelial cells through the up-regulation of eNOS transcription (48), an increase in cerebral blood flow and improvement of neurological function in mice (49). Thus, these favorable actions of statins on the endothelium might up-regulate Aβ metabolism, including the clearance of Aβ.

We also demonstrated that fluvastatin, but not simvastatin, rescued basal forebrain cholinergic neuronal loss induced by Aβ, visualized as ChAT immunoreactivity. Several studies in humans indicate that basal forebrain cholinergic pathways, especially those projecting from Meynert’s nucleus to the cortex, play a crucial role in conscious awareness and mnemonic processes (50). In AD patients, a loss of brain cholinergic functions significantly underlie the impairment of learning and memory function (52). In this study, the prevention of cholinergic neuronal loss by fluvastatin might underlie the protection against Aβ-induced memory impairment. The prevention of basal forebrain cholinergic neuronal loss by fluvastatin might be due to a decrease in Aβ accumulation and/or inhibition of oxidative stress.

Overall, the present study demonstrated that fluvastatin, but not simvastatin, significantly prevented memory impairment induced by amyloid β. The beneficial effects of fluvastatin might be explained by the prevention of cholinergic neuronal loss through a significant decrease in Aβ accumulation and oxidative stress. In clinical settings, the timing of the start of treatment and the selection of statin might be critical in achieving a beneficial effect on cognitive function.

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