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PROGRESSIVE NEUROVASCULAR DISTURBANCES IN THE CEREBRAL CORTEX OF ALZHEIMER'S DISEASE-MODEL MICE: PROTECTION BY ATORVASTATIN AND PITAVASTATIN

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Abstract—Structural and functional abnormalities in the neurovascular unit (NVU) have been recently observed in Alzheimer's disease (AD). Statins, which are used clinically for reducing cholesterol levels, can also exert beneficial vascular actions. Thus, we examined the protective effects of statins on NVU disturbances in a mouse AD model. Amyloid precursor protein (APP) transgenic (Tg) mice were used as a model of AD. Atorvastatin (30 mg/kg/day, p.o.) or pitavastatin (3 mg/kg/day, p.o.) were administered from 5 to 20 months of age. Changes in the NVU, including the endothelium and basement membrane, as well as astrogliosis and matrix metalloproteinase-9 (MMP-9) activation, were assessed. There was a reduction in immunopositive staining for *N*-acetyl glucosamine oligomer (NAGO) in the endothelium and in collagen IV in the APP vehicle (APP/Ve) group, with collagen IV staining most weakest near senile plaques (SPs). There was also an increase in intensity and number of glial fibrillary acidic protein (GFAP)-positive astrocytes, particularly around the SP, where MMP-9 was more strongly labeled. Double immunofluorescent analysis showed that astrocytic endfeet had detached from the capillary endothelium in the APP/Ve group. Treatment with atorvastatin or pitavastatin ameliorated the activation of MMP-9. Overall, these data suggest that statins may have therapeutic potential for AD by protecting NVU.

Key words: Alzheimer's disease, neurovascular unit, collagen IV, astrocyte, microglia.

Alzheimer's disease (AD) is the most common form of neurodegenerative dementia in the aged population and is characterized neuropathologically by abnormal accumulation of amyloid plaques and neurofibrillary tangles (NFTs) throughout the cerebrocortical and limbic regions. Cognitive dysfunction in AD is widely associated with progressive synaptic dysfunction and neurodegeneration, initiated by the soluble form of amyloid-β peptide (Aβ) and phosphorylated tau, which is a principal component of NFTs. Transgenic (Tg) mice that overexpress mutant β-amyloid protein precursor (βAPP) in the brain have substantial Aβ deposits (Hsiao et al., 1996; Kawarabayashi et al., 2001) and exhibit memory disturbance and multiple pathological hallmarks of AD (Kurata et al., 2007).

The blood–brain barrier (BBB) exhibits unique physiological and kinetic parameters (Zlokovic et al., 1985) with a highly selective permeability compared with the heart and other peripheral organs that have a "leaky" endothelial barrier (Mann et al., 1985). Recent reports have demonstrated numerous structural and functional cerebromicrovascular abnormalities in AD subjects, including decreased microvessel density (Suter et al., 2002; Bouras et al., 2006; Kitaguchi et al., 2007). Alterations in Aβ clearance across the BBB plays a major role in brain Aβ accumulation (Zlokovic et al., 2000; Bell and Zlokovic, 2009), and decreased Aβ clearance has been shown to facilitate accumulation of Aβ in the brain and allow for the progression of AD (Bell et al., 2009).

AD patients also exhibit functional cerebrovascular alterations, including changes in cerebrovascular blood flow (CBF), which are reproduced in Tg AD mice overexpressing Aβ (Iadecola, 2004). The endothelium, neurons, and non-neuronal cells, including astrocytes, form a functional entity termed the neurovascular unit (NVU) (Wang and Shuaib, 2007; Zlokovic, 2008; Del Zoppo, 2009). Activation of matrix metalloproteinase-9 (MMP-9) induces the disruption of the NVU, which can also occur under pathological conditions, including cerebral ischemia (Lo et al., 2002; Yamashita et al., 2009) and amyotrophic lateral sclerosis (ALS) (Miyazaki et al., 2011). Altered expression of vascular specific genes is considered a key component of AD pathogenesis (Wu et al., 2005; Chow et al., 2007; Bell et al., 2009) and is related to reduced capillary density (Wu et al., 2005). Moreover, clinical studies have identified that vascular dysfunction is an early event that may contribute to the AD process (Vermeer et al., 2003; Ruitenberg et al., 2005).

Statins, which are 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are cholesterol-lowering drugs that also have effects on vascular smooth muscle cells and can reduce MMP activity (Porter and Turner, 2002). For example, atorvastatin is a strong HMG-CoA reductase inhibitor that is used clinically worldwide and has been shown to reduce brain Aβ (Petanceska et al., 2002). Pitavastatin is another potent statin that robustly reduces plasma total cholesterol (T-cho) and triglycerides (TG) (Kajinami et al., 2000; Suzuki et al., 1999). Both of these statins are lipophilic and

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; APP/At, APP atorvastatin; APP/Pi, APP pitavastatin; APP/Ve, APP vehicle; Aβ, amyloid-β peptide; BBB, blood–brain barrier; CBF, cerebrovascular blood flow; GFAP, glial fibrillary acidic protein; GFAP+, GFAP-positive; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; LEL, lycopersicon esculentum lectin; MC, methylcellulose; MMP, matrix metalloproteinases; MMP-9+, MMP-9-positive; NAGO, *N*-acetyl glucosamine oligomer; NAGO+, NAGO-positive; NVU, neurovascular unit; PBS, phosphate-buffered saline; SP, senile plaque; Tg, transgenic.

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can cross the BBB. Thus, in the present study, we examined the effects of atorvastatin and pitavastatin on the NVU and brain MMP-9 activity in an AD mouse model.

EXPERIMENTAL PROCEDURES

Animal and drug preparation

Fifty-four female hemizygous Tg mice, 3 months of age, expressing the familial AD mutant human APPK670N, M671L (line Tg2576) (B6. SJL), and 20 female non-Tg mice (B6. SJL Hybrid) were purchased from Taconic (NY, USA) and placed on a basal diet. When the mice reached 5 months of age, the non-Tg mice ($n=20$) were started on a daily dose, oral gavage, of 0.5% methylcellulose (MC) suspended in 0.1 ml water for the subsequent 5–15 months. The amyloid precursor protein (APP) mice ($n=54$) were divided into three groups: APP vehicle (APP/Ve) group ($n=17$), APP atorvastatin (APP/At) group ($n=19$), and APP pitavastatin (APP/Pi) group ($n=18$). These mice received the following treatments by oral gavage: APP/Ve group, daily oral doses of 0.5% MC only; APP/At, 0.5% MC plus atorvastatin (30 mg/kg/day); or APP/Pi, 0.5% MC plus pitavastatin (3 mg/kg/day) for the subsequent 5–15 months. Atorvastatin was provided by Pfizer Inc. (Groton, CT, USA), and pitavastatin was provided by Kowa Corporation Ltd. (Tokyo, Japan). Both statins were administered daily as a suspension with 5% MC in 0.1 ml water.

Mice in each group were sacrificed at 5, 10, 15, and 20 months of age under deep anesthesia with pentobarbital (40 mg/kg, i.p.), and the brains were removed. All experimental procedures were approved by the Animals Committee of the Graduate School of Medicine and Dentistry, Okayama University. This experiment was carried out a statement about minimizing the number of animals used and their suffering.

NAGO staining and immunohistochemistry

The dissected brains were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.6) for 8 h, and then processed and embedded in paraffin. Subsequently, 5-μm-thick sections were prepared for *N*-acetyl glucosamine oligomer (NAGO) staining and immunohistochemistry. Lycopersicon esculentum lectin (LEL) is a glycoprotein with affinity for NAGO, which is expressed by mature vascular endothelial cells (Augustin et al., 1995). For immunohistochemistry, the brain sections underwent an antigen retrieval process of heat treatment (three times) in a 500 watt microwave for 5 min in 10 mM (pH 6.0) citric acid buffer. The pretreated sections were immersed in 0.5% periodic acid to block intrinsic peroxidase activity, and then incubated with 5% normal horse serum in 50 mM phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 to block non-specific epitopes. The tissue sections were incubated overnight with primary antibodies against collagen, glial fibrillary acidic protein (GFAP), or MMP-9. LEL and primary antibody dilutions were used as follows: biotinylated LEL (1:200; Vector Laboratories, Burlingame, CA, USA), rabbit anti-collagen IV (1:100; Novotec, Lyon, France), rabbit anti-MMP-9 (1:100; Millipore, Billerica, MA, USA), and rabbit anti-GFAP (1:200; Dako, Glostrup, Denmark). Biotinylated anti-rabbit antibody was used as the secondary antibody for collagen IV, MMP-9, and GFAP immunostaining. Antibody localization was visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Brain sections were also stained without primary antibodies to confirm specificity of antibody binding (Fig. 6).

Double immunofluorescence staining

To determine spatial distribution of the NVU, sections were incubated with a mixture of NAGO (1:100) and GFAP (one: 100) antibodies. For simultaneous detection of Aβ deposits and astrogliosis, sections were incubated with a mixture of mouse anti-Aβ_{17-24} (clone 4G8, 1:1000; Signet, Dedham, MA, USA) and GFAP (1:100) antibodies overnight at 4 °C. After rinsing in PBS, the sections were incubated with appropriate Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. The tissue sections were photographed, and the images were digitized with a digital microscope camera (Olympus BX-51; Olympus Optical Corporation, Japan). Sets of fluorescent images were acquired sequentially for the red and green channels to prevent crossover of signals from green to red channels or vice versa.

Detection and analyses

The digitized images of the tissues were used for analysis. Measurements and images were made on the cerebral cortex for quantitative analysis. The numbers of GFAP-positive (GFAP+) astrocytes and MMP-9-positive (MMP-9+) neurons per 1 mm^2 area of the cerebral cortex were calculated using Adobe Photoshop software (Adobe, San Jose, CA, USA). For analyses of pixel intensity of NAGO-positive (NAGO+) endothelium, collagen IV-positive (collagen IV+) basement membrane, and GFAP+ astrocytes, the sizes of the images were kept the same under all conditions, and the images were analyzed using Scion imaging software (Scion, Frederick, MD, USA). The pixel measurement and analysis functions were used for counting the density-sliced area in the image of the positive area of the cerebral cortex. Immunohistochemical data were expressed as the percentage change compared with the 10-month-old non-Tg mouse group, which were considered to be 100%.

Western blot analysis

The removed cerebral cortex was used for Western blot analysis. Cerebral cortex samples of 20-month-old mice groups were homogenized (1000 rpm, 20 strokes) in ice-cold lysis buffer containing 50 mM Tris–HCl pH 7.2, 250 mM NaCl, 1% NP-40, and Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The lysate was centrifuged at 12,000$\times g$ for 10 min at 4 °C, and the supernatant was collected, and the protein concentration was determined by Lowry assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of total protein extract was loaded onto an 8% polyacrylamide gel, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with PBS containing 5% skimmed milk and 0.2% Tween 20, and then incubated with anti-collagen IV antibody (1:1000) overnight at 4 °C. Membranes were then washed with PBS, probed with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK), and detected using an enhanced chemiluminescent (ECL) substrate (Pierce, Rockford, IL, USA). Following ECL detection, the membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.7; 2% SDS; 0.7% β-mercaptoethanol) at 50 °C for 30 min, and then reprobed with a monoclonal anti-β-tubulin antibody (1:5000; Sigma, Tokyo, Japan) as a loading control for protein quantification. The signals were quantified using a luminoimage analyzer (LAS 1000-mini; Fuji Film, Tokyo, Japan), and quantitative densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Zymography

MMP-9 activity of the cerebral cortex of 20-month-old mice groups was examined using gelatin-zymography kit (Primary cell; Sapporo, Japan) according to the manufacturer's instructions. In brief, each sample (40 μg; the same samples used for Western blot) was loaded for electrophoresis. The gels were washed and incubated for 72 h in incubation buffer at 37 °C, and then stained with Coomassie Blue and scanned.