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Neurobiology

Microglia Activated with the Toll-Like Receptor 9 Ligand CpG Attenuate Oligomeric Amyloid β Neurotoxicity in *in Vitro* and *in Vivo* Models of Alzheimer's Disease

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Soluble oligomeric amyloid β (oAβ) 1-42 causes synaptic dysfunction and neuronal injury in Alzheimer's disease (AD). Although accumulation of microglia around senile plaques is a hallmark of AD pathology, the role of microglia in oAβ1-42 neurotoxicity is not fully understood. Here, we showed that oAβ but not fibrillar Aβ was neurotoxic, and microglia activated with unmethylated DNA CpG motif (CpG), a ligand for Toll-like receptor 9, attenuated oAβ1-42 neurotoxicity in primary neuron-microglia co-cultures. CpG enhanced microglial clearance of oAβ1-42 and induced higher levels of the antioxidant enzyme heme oxygenase-1 in microglia without producing neurotoxic molecules such as nitric oxide and glutamate. Among subclasses of CpGs, class B and class C activated microglia to promote neuroprotection. Moreover, intracerebroventricular administration of CpG ameliorated both the cognitive impairments induced by oAβ1-42 and the impairment of associative learning in Tg2576 mouse model of AD. We propose that CpG may be an effective therapeutic strategy for limiting oAβ1-42 neurotoxicity in AD. ***(Am J Pathol 2009, 175:2121–2132; DOI: 10.2353/ajpath.2009.090418)***

The senile plaque is a pathological hallmark of Alzheimer's disease (AD). Fibrillar amyloid β (fAβ), a major component of senile plaques, induces tau hyperphosphorylation and neuronal dystrophy.[1,2] Soluble oligomeric Aβ (oAβ) has been reported to exhibit higher neurotoxicity than fAβ. Naturally secreted oAβ inhibits hippocampal long-term potentiation and disrupts synaptic plasticity in rats *in vivo*[3]. In addition, oAβ induces neuronal reactive oxygen species (ROS) through a mechanism requiring *N*-methyl-D-aspartate receptor activation.[4] Exposure to oAβ induces rapid and massive neuronal death, while fAβ is required at higher concentrations and for longer incubations to cause neuronal dystrophy.[5]

Microglia, macrophage-like cells in the central nervous system, cluster both in and around senile plaques and have been proposed to have pivotal roles in the pathogenesis of AD. Microglia activated with Aβ may be involved in the inflammatory component of AD.[6] Both fAβ and oAβ stimulate microglial secretion of proinflammatory cytokines, chemokines, complement components, and free radicals.[7] However, microglia also perform neu-

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roprotective functions such as releasing neurotrophic factors[8] and phagocytosing and degrading Aβ.[9,10]

Toll-like receptor (TLR) ligands enhance microglial phagocytosis of Aβ. Peptidoglycan, a TLR2 ligand, and unmethylated DNA CpG motifs (CpG), a TLR9 ligand, increase Aβ phagocytosis through the G protein-coupled formyl peptide receptor-like 2.[11,12] Similarly, the TLR4 ligand lipopolysaccharide increases phagocytosis through the CD14 receptor.[13] However, microglia activated with TLR ligands also produce neurotoxic molecules such as proinflammatory cytokines, nitric oxide (NO), ROS, and peroxynitrite.[14] In particular, lipopolysaccharide-activated microglia produce a large amount of glutamate, a neurotransmitter but also potent neurotoxin.[15] Thus, factors that increase microglial clearance of oAβ without producing inflammatory mediators are candidates for the treatment of AD.

Here, we investigated the role of microglia in neurotoxicity mediated by oAβ1-42. We found that microglia activated with a low dose of CpG attenuated the neurotoxic effects of oAβ1-42 without producing other neurotoxic molecules *in vitro*. Moreover, intracerebroventricular (ICV) administration of CpG ameliorated the cognitive impairment induced by ICV injection of oAβ1-42 and the impairment of associative learning in Tg2576 mouse model of AD.

Materials and Methods

Cell Culture

The protocols for animal experiments were approved by the Animal Experiment Committee of Nagoya University. Primary neuronal cultures were prepared from the cortices of embryonic day 17 (E17) C57BL/6 mice embryos as described previously.[8] Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan) and resuspended in Nerve Culture Medium (Sumitomo Bakelite). Neurons were plated onto 12-mm-polyethyleneimine-coated glass coverslips (Asahi Techno Glass, Chiba, Japan) at a density of 5×10^4 cells/well in 24-well multidishes and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The purity of the cultures was >95% as determined by NeuN-specific immunostaining. Microglia were isolated on 14 days *in vitro* with the "shaking off" method previously described from primary mixed glial cell cultures prepared from newborn C57BL/6 mice.[16] Cultures were 97 to 100% pure as determined by Fc receptor-specific immunostaining and were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, and 0.2% glucose. Microglia were plated at a density of 7×10^4 cells/well in 8-well glass slides or at a density of 7×10^4 cells/well in 48-well multidishes. Neuron-microglia co-cultures were prepared as follows: 1×10^5 microglia in 100 μl of neuronal medium were added to neuronal cultures (5×10^4 neuronal cells) on 13 day *in vitro* in 24-well multidishes.

Preparations of Aβ Solutions

fAβ1-42 was prepared as described previously.[17] Briefly, synthetic human Aβ1-42 (Peptide Institute, Osaka, Japan) was dissolved in 0.02% ammonia solution at a concentration of 250 μmol/L, diluted to 25 μmol/L in PBS, and incubated at 37°C for 24 hours. oAβ1-42 was prepared as described previously.[18] Briefly, Aβ1-42 was dissolved to 1 mmol/L in 100% 1,1,1,3,3,3-hexafluoro-2-propanol. 1,1,1,3,3,3-Hexafluoro-2-propanol was dried by the vacuum desiccator and resuspended to 5 mmol/L in DMSO. To form oligomers, amyloid peptide was diluted to a final concentration of 100 μmol/L with Ham's F-12 and incubated at 4°C for 24 hours and then immediately added to cultures at a final concentration 5 μmol/L.

Transmission Electron Microscopy

To assess quaternary structures of Aβ, oAβ1-42 and fAβ1-42 solutions were spread on carbon-coated grids. Negative staining was performed with 3% phosphotungstic acid (pH 7.0). Proteins were then examined with a JEM-2000ExII Electron Microscope with an acceleration voltage of 160 kV.

Thioflavin T Assay

Optimum fluorescence measurements of amyloid fibrils were obtained at excitation and emission wavelength of 446 and 490 nm, respectively, with a reaction mixture containing 5 μmol/L thioflavin T (Nakalai tesque, Kyoto, Japan) and 50 mmol/L glycine-NaOH buffer (pH 8.5). Ten microliters of oAβ1-42 or fAβ1-42 solution was mixed with 100 μl of the reaction mixture, respectively.

Measurement of Heme Oxygenase-1, Interleukin-10, Matrix Metalloproteinase-9, Tumor Necrosis Factor-α, NO, and Glutamate

To measure factors produced by microglia treated with CpG and oAβ1-42, microglia were plated at a density of 7×10^4 cells/well (300 μl) in 48-well multidishes and then treated with 100 nmol/L CpG-DNA (HyCult Biotechnology, Uden, Netherlands), 100 nmol/L class A CpG (synthetic oligodeoxynucleotides (ODNs) 1585), class B CpG (ODN 1668), and class C CpG (ODN 2395). These CpG subtypes were from Alexis Biochemicals (San Diego, CA). After 3 hours of treatment with CpG, 5 μmol/L oAβ1-42 was added for 24 hours. Supernatants from microglia were assessed by ELISA kits for tumor necrosis factor-α (TNF-α) and interleukin (IL)-10 (BD Pharmingen, Franklin Lakes, NJ) and matrix metalloproteinase (MMP)-9 (R&D Systems, Minneapolis, MN). Cell extracts from microglia in extraction buffer (1% Nonidet P-40 in PBS) were measured for heme oxygenase-1 (HO-1) with an ELISA kit (Takara Bio, Mie, Japan). Measurement of NO was determined using the Griess reaction.[19] To measure glutamate, Glutamate Assay Kit colorimetric assay (Yamasa, Tokyo, Japan) was used as described previously.[20]

The mRNA expression of HO-1 was assessed by RT-PCR. Briefly, total RNA was extracted using the guanidinium thiocyanate method (RNeasy Mini Kit; Qiagen, Valencia, CA). cDNAs were generated by RT-PCR using SuperScript II (Invitrogen, Carlsbad, CA) and Ampli TaqDNA polymerase (Applied Biosystems, Branchburg, NJ) in the presence of the specific primers. HO-1 sense, 5′-CTATGTAAAGCGTCTCCA-3′; and HO-1 antisense, 5′-GTCTTTGTGTTCCTCTGTC-3′.

Measurement of ROS

To measure ROS in neuron-microglia co-cultures, we used the acetate ester form of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA-AM) probe (Invitrogen). After neuron-microglia co-cultures were treated with or without 100 nmol/L CpG for 3 hours, cells were loaded with dye by replacing media with fresh nerve culture medium containing 5 μmol/L H_2DCFDA-AM for 30 minutes. After washing, culture medium containing 5 μmol/L oAβ1-42 was added and the fluorescence of the wells was measured. Fluorescent measurements were made using a Wallac 1420 ARVOMX (PerkinElmer Japan, Yokohama, Japan).

Immunocytochemistry

Neuronal, microglial, and neuron-microglia co-cultures were fixed with 4% paraformaldehyde for 30 minutes at room temperature, then blocked with 5% normal goat serum in PBS and permeabilized with 0.3% Triton X-100. Neurons were stained with rabbit polyclonal anti-microtubule-associated protein (MAP)-2 antibody (1/500; Chemicon, Temecula, CA) and secondary antibodies conjugated to Alexa 488 (1/1000; Invitrogen). Synthetic Aβ was stained with a mouse monoclonal anti-Aβ antibody (4G8) (1/1000; Chemicon) and secondary antibodies conjugated to Alexa 568 or 647. Microglia were stained with phycoerythrin-conjugated rat anti-mouse CD11b monoclonal antibody (1/300; BD Pharmingen) before fixation. Images were analyzed with a deconvolution fluorescent microscope system (BZ-8000; Keyence, Osaka, Japan). To assess neuronal death induced by Aβ, purified neurons (5×10^4 cells/well) were plated in 24-well multidishes. A total of 5 μM oAβ1-42 or fAβ1-42 was added to the cultures on 13 days *in vitro* for 24 hours. To assess neuronal death in neuron-microglia co-cultures, 3 hours after treatment with or without TLR ligands, 5 μmol/L oAβ1-42 was added to cultures for 24 hours. Surviving neurons were identified by cytoskeletal morphology of neurons as described previously.[8] Viable neurons stained strongly with an anti-MAP-2 antibody, whereas damaged neurons stained more weakly. The number of MAP-2-positive neurons was counted in representative areas per well. More than 200 neurons were examined in each of five independent trials by a scorer blind to the experimental condition. The number of untreated viable neurons was normalized to 100%.

Western Blotting

Neuronal cultures were treated with 5 μmol/L oAβ1-42 for 24 hours. Neuron-microglia co-cultures were pretreated with CpG for 3 hours before addition of 5 μmol/L oAβ1-42 for 24 hours. The supernatants of these cultures were collected. oAβ in 10-month-old-Tg 2576 mouse brain was extracted from the soluble, extracellular-enriched fraction as described previously.[21] Hemi-forebrains were harvested in 500 μl of solution containing 50 mmol/L Tris-HCl (pH 7.6), 0.01% Nonidet P-40, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.1% SDS, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Soluble, extracellular-enriched proteins were collected from mechanically homogenized lysates following centrifugation for 5 minutes at 3000 rpm.

Collected samples were mixed with sample buffer (200 mmol/L Tris-HCl, 8% SDS, and 1% glycerol). Proteins were separated on a 5 to 20% Tris-glycine SDS-polyacrylamide gel and transferred to Hybond-P polyvinylidene difluoride membrane (GE Healthcare UK, Buckinghamshire, UK): Membranes were blocked with 1% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween20 (TBS-T). Blots were incubated in mouse anti-Aβ monoclonal antibody (6E10) (1/1000; Chemicon) diluted in 1% skim milk overnight at 4°C. Subsequently, membranes were washed in TBS-T 3 × 5 minutes and incubated with a horseradish peroxidase-conjugated anti-mouse IgG (1/5000; GE Healthcare) diluted in 1% skim milk for 1 hour. After washing in TBS-T for 1 × 15 minutes, 2 × 5 minutes, and TBS for 1 × 5 minutes, signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). The intensity of the bands was calculated by using CS Analyzer 1.0 (Atto, Tokyo, Japan).

Novel-Object Recognition Test in oAβ1-42-Induced Cognitive Impairment Mouse Model

oAβ (300 pmol/3 μl), CpG (100 nmol/L), or both oAβ and CpG were ICV injected as described previously.[22,23] The vehicle (PBS) was injected as the control. Briefly, a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long was used for these experiments. C57/BL6 mice were anesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A single injection of 3 μl of peptide or vehicle was delivered gradually over 3 minutes. The injection site was confirmed in preliminary experiments. Neither insertion of the needle nor the volume of injection had a significant influence on survival and behavioral responses or cognitive functions.

The novel-object recognition test (NORT) was performed 7 to 8 days after ICV injection of oAβ1-42 or CpG as described previously.[24,25] The experimental apparatus consisted of a plexiglas open-field box (30 × 30 × 35 high cm), with a sawdust-covered floor. The apparatus

was located in a sound-attenuated room and was illuminated with a 60 lux light source. The NORT procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box with 10 minutes of exploration in the absence of objects for 3 consecutive days (habituation session, days 4 to 6). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 minutes (day 7). The objects were constructed from a golf ball, wooden column, and wooden triangular pyramid. They were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions (day 8), the animals were placed back into the same box 24 hours after the training session, but one of the familiar objects used during training had been replaced with a novel object. The animals were then allowed to explore freely for 5 minutes, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner. A preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session over the total time exploring.

Cued and Contextual Fear-Conditioning Tests in Tg 2576 Mouse Model of AD

Cued and contextual fear conditioning tests were performed at 10 months of age according to previous report,[26] with minor modifications. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (a block plexiglas box with abundant wood tips,30 × 30 × 40 high cm) for 1 minute, then in the conditioning cage (a transparent plexiglas box, 30 × 30 × 40 high cm) for 2 minutes. For training (conditioning phase), mice were placed in the conditioning cage, then a 15-second tone (80 dB) was delivered as a conditioned stimulus. During the last 5 seconds of the tone stimulus, a foot shock of 0.6 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea). This procedure was repeated four times with 15-second intervals. Cued and contextual tests were performed 1 day after fear conditioning. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 minutes in the absence of the conditioned stimulus. For the cued test, the freezing response was measured in the neutral cage for 1 minute in the presence of a continuous-tone stimulus identical to the conditioned stimulus.

Stereotaxic injection was used for these experiments. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) before stereotaxic implantation of a microinjection cannula into the right lateral ventricle (anteroposterior, −0.3 mm, and mediolateral, +1.0 mm, from the bregma, and dorsoventral, +2.5, from the skull according to the atlas of Franklin and Paxinos).[27] CpG was dissolved in PBS at a concentration of 10 or 100 nmol/L and was injected at a volume of 3 μl for 3 minutes. Same volume of PBS was injected to vehicle mouse. A week after injection, behavioral experiment was performed.

Immunohistochemistry

Immunohistochemistry was performed on brain tissue of mice after cued and contextual fear-conditioning test. Mice were transcardially perfused with ice-cold borate-buffered 4% paraformaldehyde under deep anesthesia. The brains were rapidly removed after decapitation. Brains were then postfixed overnight in periodate lysine paraformaldehyde, equilibrated in phosphate buffered 20% sucrose for 48 hours, and embedded into Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C overnight. Coronal brain sections (20 μm) were cut with a cryostat. The sections were permeabilized with 1% Triton X-100 after blocking with 10% normal goat serum for 30 minutes. The cell nucleus was stained with Hoechst 33342 (1 μg/ml; Invitrogen). Aβ was stained with mouse monoclonal anti-Aβ antibody (4G8) (1/1000; Chemicon) and secondary antibodies conjugated to Alexa 488. Microglia were stained with a rat anti-mouse CD11b monoclonal antibody (1/1000; AbD Serotec, Oxford, UK) and secondary antibodies conjugated to Alexa 568. Images were collected and analyzed with a deconvolution fluorescent microscope system. Aβ load in immunostained tissue sections were quantified using BZ-analyzer (Keyence) as reported previously.[28] Seven sections were analyzed per animal. Total Aβ burden was quantified for the cortex and for the hippocampus on coronal plane sections stained with the monoclonal antibody 4G8. The cortical area was dorsomedial from the cingulate cortex and extended ventrolaterally to the rhinal fissure within the right hemisphere. Test areas (640 μm × 480 μm) were randomly selected. Total Aβ burden was calculated as the percentage of test area occupied by Aβ. Hippocampal measurements (600 × 600 μm) were performed similarly to the cortical analysis.

Statistical Analysis

Statistical significance of the biochemical experiments and the behavioral data were assessed with one-way analysis of variance, followed by post hoc Tukey test or Newman-Keuls test using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).

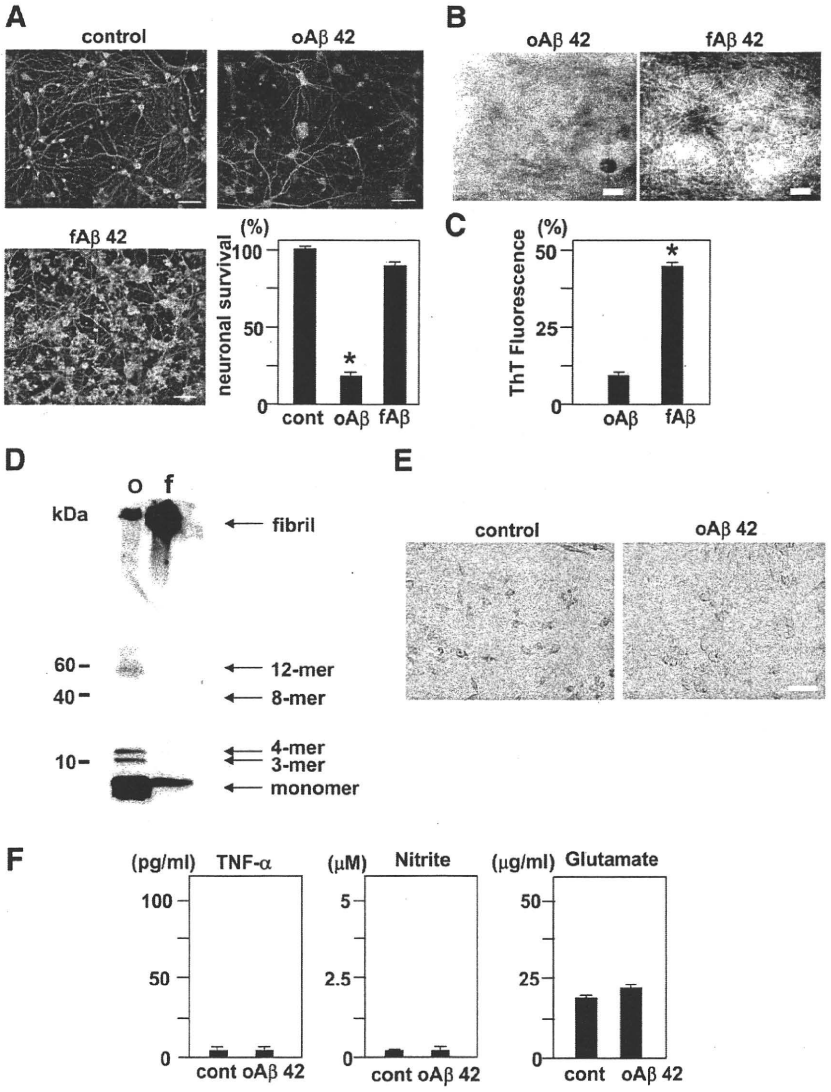

Figure 1. Neurotoxicity and morphologies of oAβ1-42 and fAβ1-42, and microglial response to oAβ1-42. **A:** The evaluation of neurotoxicity induced by oAβ1-42 and fAβ1-42. Neuronal cultures were treated with 5 μmol/L oAβ1-42 or fAβ1-42 for 24 hours. Neurons were stained with an anti-MAP-2 antibody (green). Aβ was stained with a mouse anti-amyloid β protein monoclonal antibody (4G8) (red). oAβ1-42 exhibited more striking neurotoxicity than fAβ1-42. Scale bar, 50 μm. Neuronal survival rate in oAβ1-42 treatment significantly decreased. $^*P < 0.05$ as compared with the rate in control cultures. Each column indicates the mean ± SEM ($n = 5$). **B:** Images of oAβ1-42 (**left**) and fAβ1-42 (**right**) collected with an electron microscope. Scale bar, 100 nm. **C:** Thioflavin T assay for oAβ1-42 and fAβ1-42. $^*P < 0.05$ as compared with the value of oAβ1-42. **D:** Western blot analysis of oAβ1-42 and fAβ1-42. oAβ1-42 (o) contained monomers, small oligomeric trimers (3-mer) and tetramers (4-mer), and the larger oligomers (octamers (8-mer) and dodecamers (12-mer)), whereas fAβ1-42 (f) contained monomers and fibrils. **E:** Microglial cultures were treated with or without 5 μmol/L oAβ1-42 for 24 hours. In a phase contrast, oAβ1-42 induced microglial adhesion. Scale bar, 50 μm. **F:** The measurement of TNF-α (**left**), nitrite (**middle**), and glutamate (**right**) produced by microglia activated with oAβ1-42. Microglial cultures were treated with 5 μmol/L oAβ1-42 for 24 hours. Each column indicates the mean ± SEM ($n = 7$).

Results

Neurotoxicity of oAβ1-42

First, we investigated the toxic effects of oAβ and fAβ on primary cortical neurons. Administration of 5 μmol/L oAβ1-42 to cortical cultures on DIV 13 for 24 hours resulted in significant neuronal death. The network of MAP-2-positive dendrites collapsed and neuronal survival decreased to 20% (Figure 1A). In contrast, administration of fAβ1-42 did not induce neuronal cell death, although Aβ deposition was observed on dendrites (Figure 1A). Thus, oAβ1-42 exhibits a more potent neurotoxicity than fAβ1-42. Both oAβ1-40 and fAβ1-40 did not induce neuronal cell death (Supplemental Figure S1, see *http://ajp.amjpathol.org*). We evaluated the morphology of oAβ1-42 and fAβ1-42 by transmission electron microscopy. We observed fine spherical particles of oAβ1-42 and fibril formation by fAβ1-42 (Figure 1B). The fluorescence of Thioflavin T, a marker for amyloid fibril formation, was associated with fAβ1-42 (Figure 1C). Western blotting with an antibody directed against Aβ (6E10) revealed that a solution of oAβ1-42 contained monomers, trimers (3-mer), tetramers (4-mer), and larger oligomers (octamers (8-mer) and dodecamers (12-mer)). In contrast, a solution of fAβ1-42 contained monomers and fibrils, but not oligomers (Figure 1D).

In primary microglial culture, administration of 5 μmol/L oAβ1-42 for 24 hours induced microglial adhesion (Figure 1E), but did not induce the production of neurotoxic mediators such as TNF-α, glutamate, or nitrite, a stable breakdown product of NO (Figure 1F).

Microglia Activated with CpG Attenuate the Neurotoxicity Induced by oAβ1-42

To define the role of microglia in the neurotoxicity of oAβ1-42, we evaluated neuronal survival in neuron-microglia co-cultures. Neurons stained with anti-MAP-2 antibody exhibited no detectable morphological abnormalities and possessed intact cell bodies and dendrites, and

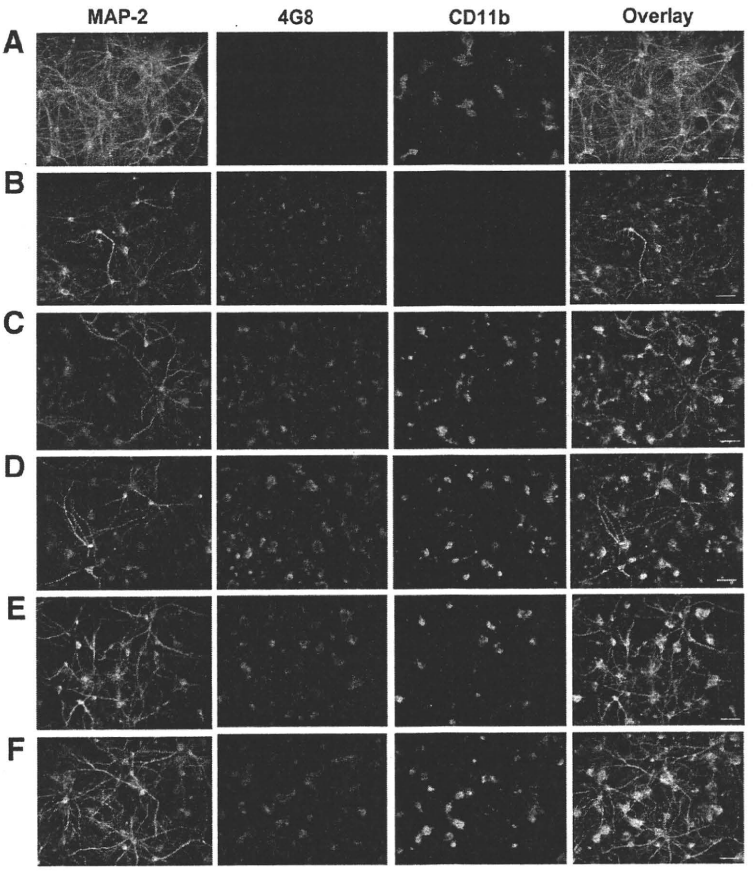

Figure 2. Protective effect of microglia activated with CpG against oAβ1-42 neurotoxicity. **A:** Representative deconvolution fluorescent images of control neuron-microglia co-cultures (1:2 of neuron: microglia). **B:** Neuronal cultures treated with 5 μmol/L oAβ1-42. **C:** Neuron-microglia co-cultures (1:2 neuron to microglia) treated with oAβ1-42. **D:** Neuron-microglia co-cultures stimulated with 1 nmol/L CpG and oAβ1-42 (**E**) or with 10 nmol/L CpG and oAβ1-42 (**F**) or with 100 nmol/L CpG and oAβ1-42. After 3 hours of treatment with CpG, cultures were treated with oAβ1-42 for 24 hours. Neurons were stained with an anti-MAP-2 antibody (green). Aβ was stained with 4G8 (red) and microglia were stained with a phycoerythrin-conjugated anti-CD11b antibody (blue). Scale bar, 50 μm. **G:** Neuronal survival rate was quantified as the percentage of intact neurons following treatment relative to control wells. The viability of untreated neurons (control) was normalized to 100%. $^*P < 0.05$ as compared with the survival rate of neuron-microglia co-cultures treated with oAβ1-42. Each column indicates the mean ± SEM ($n = 7$).

microglia stained with anti-CD11b antibody were also intact in unstimulated co-cultures (Figure 2A). After treatment of neuronal cultures with 5 μmol/L oAβ1-42 for 24 hours, the neuronal cells were severely damaged, and the survival rate decreased to 18% (Figure 2, B and G). Similarly, the neuronal survival rate was not improved in neuron-microglia co-cultures treated with oAβ1-42 (Figure 2, C and G), which implies that unstimulated microglia have not protective effect against oAβ1-42 neurotoxicity. Administration of CpG to neuronal cultures or neuron microglia co-cultures induced no toxic change (Supplemental Figure S2A, see *http://ajp.amjpathol.org*). After 3 hours of treatment with 1 nmol/L, 10 nmol/L, or 100 nmol/L CpG, 5 μmol/L oAβ1-42 was added to neuron-microglia co-cultures for 24 hours. The neuroprotective effect was not evident in culture with 1 nmol/L CpG (Figure 2, D and G). However, microglia treated with 10 or 100 nmol/L CpG prevented neuronal cell death, and the neuronal survival rate was significantly improved reaching 53 and 62%, respectively (Figure 2, E–G). In neuronal cultures, CpG did not attenuate the neurotoxicity induced by oAβ1-42 (Figure 2G). Moreover, 100 nmol/L CpG attenuated oAβ1-42-induced neurotoxicity for 48 hours, whereas other TLR ligands such as peptidoglycan and lipopolysaccharide did not (Supplemental Figure S2B, see *http://ajp.amjpathol.org*). We conclude from these findings that CpG-activated microglia have neuroprotective effect against oAβ1-42 neurotoxicity *in vitro*.

CpG-Activated Microglia Increase the Clearance of oAβ1-42, Produce the Antioxidant Enzyme HO-1 and Aβ-Degrading Enzyme MMP-9, and Release Fewer Neurotoxic Molecules

To elucidate the mechanisms of neuroprotection by microglia activated with CpG, we examined whether CpG increased the clearance of oAβ1-42. Western blot analysis revealed that there was no significant difference between the amount of oAβ1-42 present in the supernatants of neuronal cultures and in neuron-microglia co-cultures without CpG administration. (Figure 3A). However, CpG dose-dependently decreased the amount of oAβ1-42 in neuron-microglia co-cultures, especially treatment with 100 nmol/L CpG significantly decreased the amount of 3-, 4-, 8-, and 12-mer of oAβ1-42 (Figure 3, B and C). Moreover, we examined the effect of CpG on Aβ uptake by microglia alone at 1 and 24 hours time points. We found that CpG significantly enhanced microglial uptake of oAβ at both 1 and 24 hours (Supplemental Figure S3A, see *http://ajp.amjpathol.org*).

Because oxidative stress is a major component of oAβ1-42 neurotoxicity, we examined whether microglia activated with CpG express the antioxidant enzyme HO-1. CpG-activated microglia produced HO-1 in a dose-dependent manner. A total of 10 and 100 nmol/L CpG significantly increased the production of HO-1. The

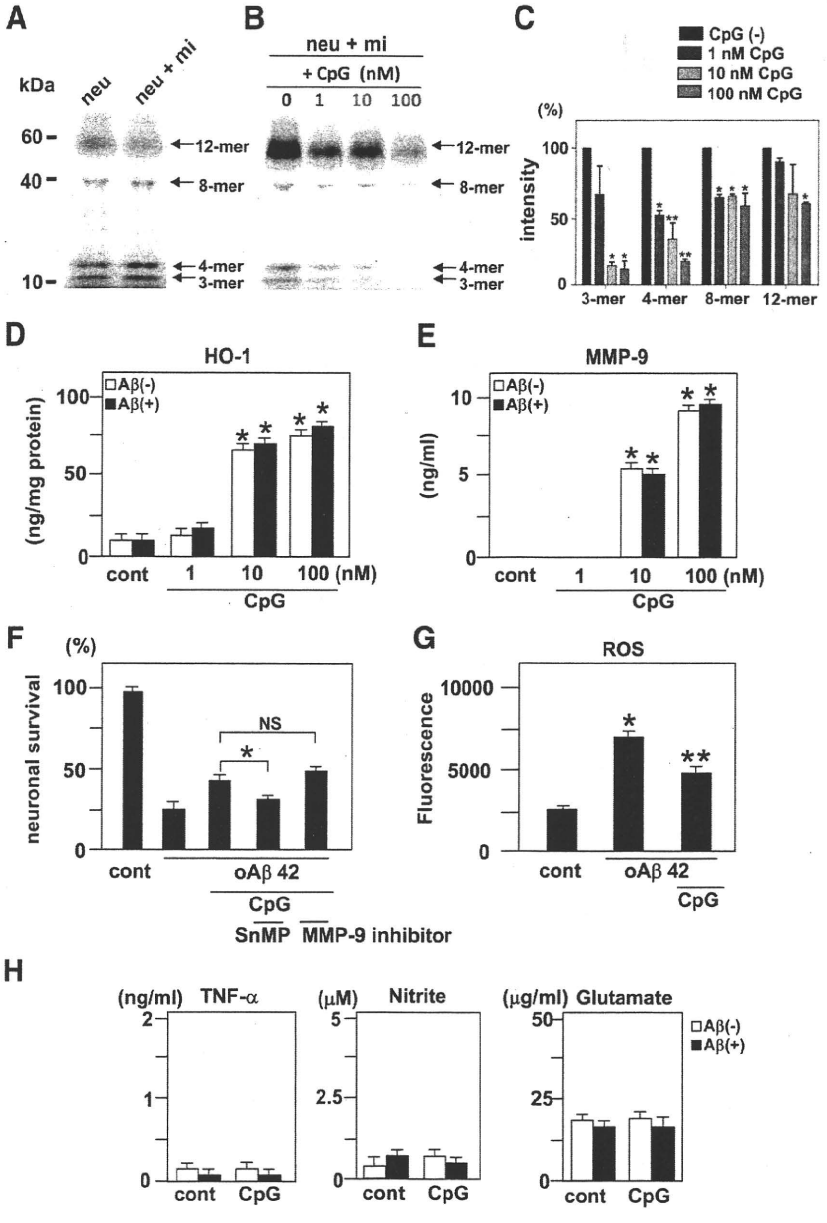

Figure 3. Clearance of oAβ1-42 and the production of HO-1, MMP-9, and neurotoxic molecules by microglia activated with CpG. **A:** Western blot analysis of oAβ1-42 in neuronal cultures (neu) and neuron-microglia co-cultures (neu + mi). Twenty-four hours after addition of 5 μmol/L oAβ1-42, oAβ1-42 present in the supernatants of these cultures was detected by Western blotting. **B:** Western blot analysis of oAβ1-42 in neuron-microglia co-cultures with CpG treatment. Neuron-microglia co-cultures (neu + mi) were treated with 5 μmol/L oAβ1-42 for 24 hours following 3 hours of treatment with 1, 10, or 100 nmol/L CpG. Microglia activated with CpG dose-dependently reduced the amount of oAβ1-42 in the supernatants. **C:** Semiquantification of oAβ1-42 in **B** by densitometric analysis. The amount of oAβ1-42 in neuron-microglial co-cultures without CpG (black) was normalized to 100%. oAβ1-42 in co-cultures treated with 1 nmol/L CpG (blue), 10 nmol/L CpG (green), or 100 nmol/L CpG (red) was calculated. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with the intensity of oAβ1-42 in neuron-microglia co-cultures without CpG. Each column indicates the mean ± SEM ($n = 6$). The production of HO-1 (**D**) and MMP-9 (**E**) by microglia activated with CpG in the absence or presence of oAβ1-42. After 3 hours of treatment with CpG, microglial cultures were treated with or without oAβ1-42 for 24 hours. $^{*}P < 0.05$ as compared with untreated controls. Each column indicates the mean ± SEM ($n = 3$–5). **F:** The effect of HO-1 and MMP-9 on oAβ1-42 neurotoxicity. Neuron-microgila co-cultures were treated with 100 nmol/L CpG in the presence of 10 μmol/L tin-mesoporphyrin (SnMP) IX, a specific HO-1 inhibitor, or 50 nmol/L MMP-9 inhibitor for 3 hours, and then oAβ1-42 was added to the cultures for 24 hours. Tin-mesoporphyrin IX, but not the MMP-9 inhibitor, decreased neuronal survival rate. $^{*}P < 0.05$ as compared with CpG-treated cultures without inhibitors. Each column indicates the mean ± SEM ($n = 6$–9). **G:** The suppressive effect of CpG on ROS production by oAβ in the neuron microglia co-cultures. After neuron-microglia co-cultures were treated with or without 100 nmol/L CpG for 3 hours, cells were loaded with fresh nerve culture medium containing 5 μmol/L H_2DCFDA-AM for 30 minutes. After washing, culture medium containing 5 μmol/L oAβ1-42 was added and the increment of the fluorescence was calculated at 5 minutes. $^{*}P < 0.05$ as compared with untreated controls. $^{**}P < 0.05$ as compared with co-culture cells treated with oAβ1-42. Each column indicates the mean ± SEM ($n = 4$). **H:** The measurement of TNF-α (**left**), nitrite (**middle**), and glutamate (**right**) produced by microglia activated with 100 nmol/L CpG with or without oAβ1-42. After 3 hours treatment with CpG, microglial cultures were treated with or without oAβ1-42 for 24 hours. $^{*}P < 0.05$ as compared with untreated microglia. Each column indicates the mean ± SEM ($n = 7$).

production levels were not influenced by exposure to oAβ1-42 (Figure 3D). Since the anti-inflammatory cytokine IL-10 induces HO-1 expression by macrophages,[29] we also examined and confirmed that IL-10 induced HO-1 mRNA expression by microglia (Supplemental Figure S3B, see *http://ajp.amjpathol.org*). Although CpG induced IL-10 in microglia, the expression was suppressed by oAβ1-42 treatment (Supplemental Figure S3C, see *http://ajp.amjpathol.org*).

MMP-9 is also thought to play a neuroprotective role in AD because it degrades both oAβ and fAβ. A total of 10 and 100 nmol/L CpG significantly induced MMP-9 production in microglia with or without treatment of oAβ1-42 (Figure 3E). To determine whether HO-1 and MMP-9 contribute to the neuroprotective effects of CpG-activated microglia, we applied the specific HO-1 inhibitor tin-mesoporphyrin IX (Frontier Scientific, Logan, UT) and MMP-9 inhibitor (Merck, Darmstadt, Germany). The neuroprotective effect of CpG was abolished by treatment with 10 μmol/L tin-mesoporphyrin IX (Figure 3E). However, inhibition of MMP-9 with an MMP-9 inhibitor at 50 nmol/L did not influence the neuroprotective effect of CpG-activated microglia (Figure 3F). These results imply that HO-1 rather than MMP-9 may contribute to the

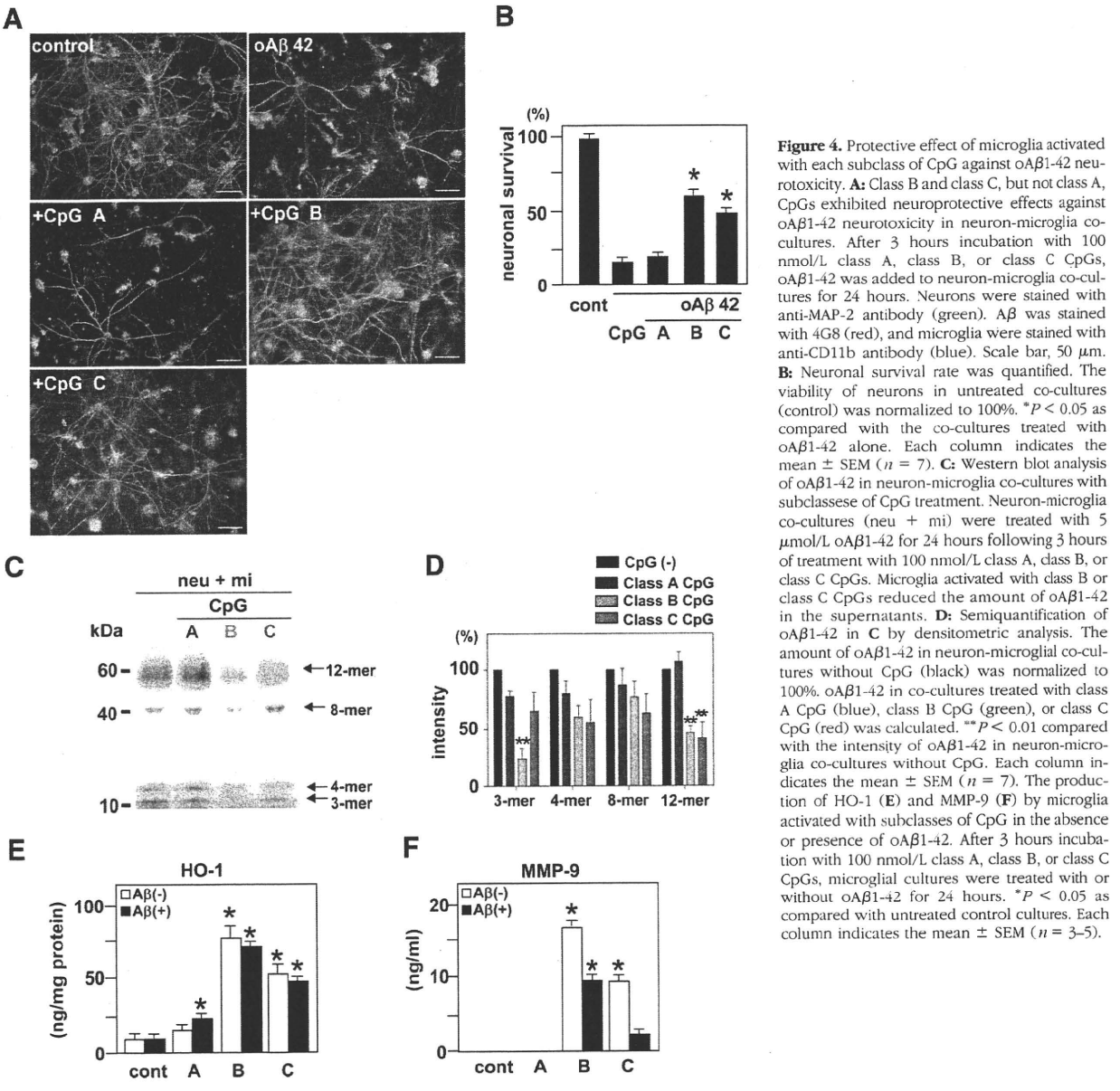

Figure 4. Protective effect of microglia activated with each subclass of CpG against oAβ1-42 neurotoxicity. **A:** Class B and class C, but not class A, CpGs exhibited neuroprotective effects against oAβ1-42 neurotoxicity in neuron-microglia co-cultures. After 3 hours incubation with 100 nmol/L class A, class B, or class C CpGs, oAβ1-42 was added to neuron-microglia co-cultures for 24 hours. Neurons were stained with anti-MAP-2 antibody (green). Aβ was stained with 4G8 (red), and microglia were stained with anti-CD11b antibody (blue). Scale bar, 50 μm. **B:** Neuronal survival rate was quantified. The viability of neurons in untreated co-cultures (control) was normalized to 100%. $^{*}P < 0.05$ as compared with the co-cultures treated with oAβ1-42 alone. Each column indicates the mean ± SEM ($n = 7$). **C:** Western blot analysis of oAβ1-42 in neuron-microglia co-cultures with subclassese of CpG treatment. Neuron-microglia co-cultures (neu + mi) were treated with 5 μmol/L oAβ1-42 for 24 hours following 3 hours of treatment with 100 nmol/L class A, class B, or class C CpGs. Microglia activated with class B or class C CpGs reduced the amount of oAβ1-42 in the supernatants. **D:** Semiquantification of oAβ1-42 in **C** by densitometric analysis. The amount of oAβ1-42 in neuron-microglial co-cultures without CpG (black) was normalized to 100%. oAβ1-42 in co-cultures treated with class A CpG (blue), class B CpG (green), or class C CpG (red) was calculated. $^{**}P < 0.01$ compared with the intensity of oAβ1-42 in neuron-microglia co-cultures without CpG. Each column indicates the mean ± SEM ($n = 7$). The production of HO-1 (**E**) and MMP-9 (**F**) by microglia activated with subclasses of CpG in the absence or presence of oAβ1-42. After 3 hours incubation with 100 nmol/L class A, class B, or class C CpGs, microglial cultures were treated with or without oAβ1-42 for 24 hours. $^{*}P < 0.05$ as compared with untreated control cultures. Each column indicates the mean ± SEM ($n = 3$–5).

neuroprotection of CpG. Furthermore, treatment of oAβ significantly increased ROS production in the neuron microglia co-cultures. Pretreatment of 100 nmol/L CpG significantly suppressed ROS production by oAβ (Figure 3G).

Although TLR4 ligand lipopolysaccharide generally increases microglial production of neurotoxic molecules including TNF-α, nitrite and glutamate; 100 nmol/L CpG did not induce these toxic molecules in microglia (Figure 3H).

Microglia Activated with Class B and C, but not Class A CpG, Attenuate oAβ1-42 Neurotoxicity

CpG ODNs are divided into three classes by their ability to induce IFN-α expression in plasmacytoid dendritic cells (class A) and to promote survival, activation, and maturation of B cells and plasmacytoid dendritic cell (class B) or both (class C)[30]. We examined which class of CpG induces the neuroprotective effects of microglia. Class A CpG neither activated microglia nor induced neuroprotective effects against oAβ1-42 toxicity, whereas both class B and C CpGs activated microglia and significantly increased neuronal survival, to 58 and 49% following oAβ1-42 treatment, respectively (Figures 4, A and B). Western blot analysis revealed that class B CpG significantly decreased the amount of trimers and dodecamers of oAβ1-42 present in the supernatants of neuron-microglia co-cultures, and class C CpG significantly decreased dodecamers of oAβ1-42, whereas oAβ1-42 did not decrease by the administration of class A CpG (Figure 4, C and D). In addition, microglia activated with class B and C CpGs expressed HO-1 in both the absence or presence of oAβ1-42, whereas treatment with class A CpG only slightly increased HO-1 expression in the pres-

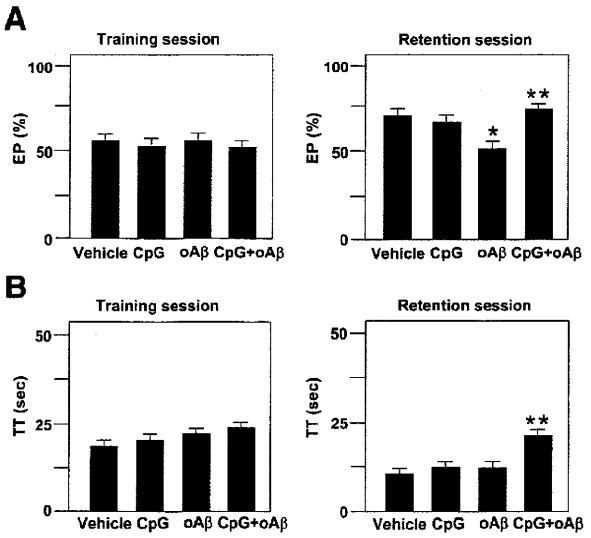

Figure 5. Effect of ICV injection of CpG on oAβ1-42-induced cognitive impairment in the NORT. **A:** Exploratory preference (EP) and total exploring time (TT) (**B**) in the training session (**left**) and the retention session (**right**). NORT was performed 7 to 8 days after ICV injection of oAβ1-42. Each column indicates the mean ± SEM (n = 6–7). $^{*}P < 0.05$ as compared with controls. $^{**}P < 0.05$ as compared with oAβ1-42-injected mice.

ence of oAβ1-42 (Figure 4E). Microglia activated with class B and C CpGs also produced MMP-9 (Figures 4F).

ICV Injection of CpG Ameliorates oAβ-Induced Impairment of Recognition Memory in the NORT

To examine the role of CpG-activated microglia in cognitive dysfunction induced by oAβ1-42, we investigated the effect of *in vivo* administration of CpG on the impairment of recognition memory in the NORT after ICV injection of oAβ1-42. Mice injected with oAβ1-42 displayed significantly reduced exploratory preference for the novel object in the retention session ($F_{(3,21)} = 3.68$, $P < 0.05$; Figure 5A), although total exploration time in the training and retention sessions was unaffected. The result implies that oAβ1-42 induces the impairment of recognition memory. Simultaneous injection of CpG with oAβ1-42 significantly improved both exploratory preference ($F_{(3,21)} = 3.68$, $P < 0.05$; Figure 5A) and total exploration time ($F_{(3,21)} = 4.41$, $P < 0.05$; Figure 5B) in the retention session, although exploratory preference and total exploration time were unaffected in the training session.

ICV Injection of CPG Ameliorates the Impairment of Associative Learning in the Cued and Contextual Fear-Conditioning Tests in Tg2576 Mice

Next, we examined the effect of CpG on the cognitive function of Tg2576 mouse model of AD. We evaluated associative learning at the age of 10 months in a conditioned fear learning test. In the preconditioning phase (training), the mice hardly showed any freezing response. There were no differences in basal levels of freezing response between the groups (data not shown). In the contextual learning test, wild-type mice showed a marked contextual freezing response 24 hours after fear conditioning (Figure 6A). However, vehicle-injected Tg2576 mice exhibited less of a freezing response in the contextual tests (Figure 6A), indicating an impairment of associative learning. The CpG (10 or 100 nmol/L)-injected Tg2576 mice were indistinguishable from wild-type mice, and the CpG treatment dose-dependently and significantly reversed the contextual freezing response as compared with vehicle-injected Tg2576 mice ($F_{(3,35)} = 9.54$, $P < 0.05$; Figure 6A). In the cued (tone) learning test, although there was no significant difference in the cued freezing response at 24 hours after fear conditioning between wild-type and vehicle-injected Tg2576 mice, both injection of 10 and 100 nmol/L CpG showed a tendency to reverse the cued freezing response (Figure 6B). No alterations of nociceptive response were found in any of the mutant mice: there was no difference in the minimal current required to elicit flinching/running, jumping, or vocalization among the mice (data not shown). Then, we examined whether CpG decreased Aβ deposits in the cortex (Figure 6C) and the hippocampus (Figure 6D) of Tg 2576 mice. No Aβ deposits were seen in wild-type mice. Although Aβ deposits (green) were abundant in the cortex and the hippocampus of vehicle-injected Tg2576 mice, ICV injection of CpG significantly decreased Aβ deposits in both areas. Microglia (red) clustered around Aβ deposits (Figure 6, C and D). CpG decreased Aβ load in a significant, dose-dependent manner in both areas (Figure 6E). We examined oAβ in the soluble, extracellular-enriched fractions of the hemi-forebrains of mice and detected 12-mer oAβ in vehicle-injected Tg2576 mice by Western blotting. The 12-mer oAβ was strikingly and significantly decreased in 100 nmol/L CpG-injected Tg2576 mice (Figure 6, F and G).

Discussion

Recent studies have proposed that oAβ1-42 contributes to the neurotoxicity associated with AD. AD begins with subtle alterations of hippocampal synaptic efficacy before frank neuronal degeneration, and this synaptic dysfunction is caused by diffusible oAβ.[31] Disruption of hippocampal long-term potentiation and synaptic plasticity by oAβ1-42 appears to involve Ca^{2+} signaling,[32] oxidative stress mediated by an *N*-methyl-D-aspartate receptor,[4,33] and protein phosphatase 1.[34] In addition, oAβ interferes with insulin receptor function in hippocampal neurons and inhibits the activation of specific kinases required for long-term potentiation.[35] In the present study, we have confirmed that oAβ1-42 exhibits more potent neurotoxicity than fAβ1-42 in murine cortical cultures.[5,36] Therefore, decreasing or preventing formation of oAβ1-42 is a potential therapeutic strategy against AD.

The precise role of microglia in oAβ1-42 toxicity remains unclear. Microglia stimulated with Aβ are reported to release proinflammatory cytokines via the nuclear factor κB[37,38] and contribute to the pathogenesis of AD. However, in our experimental conditions, oAβ1-42 neither

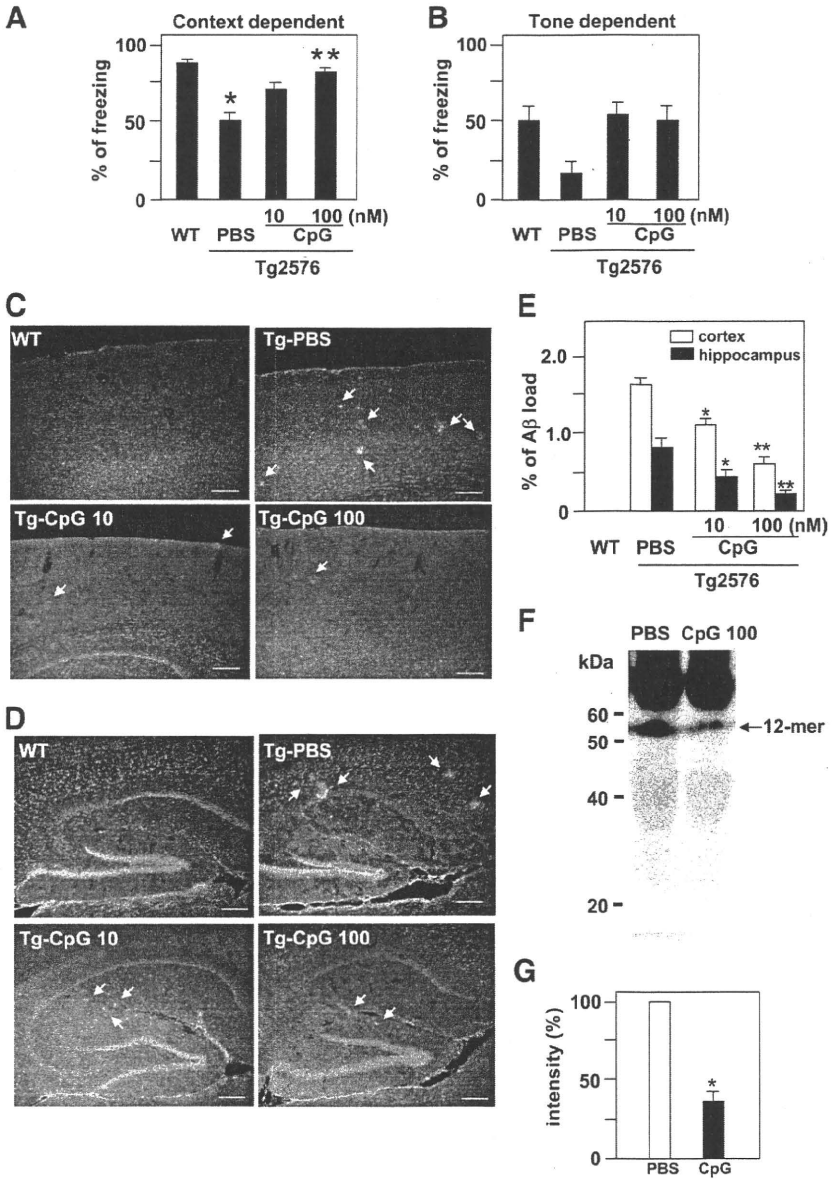

Figure 6. Effect of ICV injection of CpG on associative learning in the cued and contextual conditioning tests and Aβ clearance in Tg2576 mice. The retention session was performed 24 hours after the training. Context-dependent (**A**) and tone-dependent (**B**) freezing times were measured at the age of 10 months. Each column indicates the mean ± SEM (wild-type (WT) mice, $n = 14$; vehicle-treated (PBS) Tg2576 mice, $n = 9$; CpG (10 nmol/L)-treated Tg2576, $n = 8$; CpG (100 nmol/L)-treated Tg2576, $n = 8$). $^{*}P < 0.05$ compared with wild type. $^{**}P < 0.05$ as compared with vehicle-treated Tg2576 mice. **C:** Aβ deposits in the cortex. Aβ was stained with 4G8 (green), microglia stained with anti-CD11b antibody (red), and cell nucleus stained with Hoechst 33342 (blue). **White arrows** indicate Aβ deposits surrounding by microglia. Scale bar, 200 μm. **D:** Aβ deposits in the hippocampus. Scale bar, 200 μm. **E:** Quantification of Aβ deposits in **C** and **D**. The ratio between the Aβ deposits area and the total area of the analyzed region was multiplied by 100. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with vehicle-treated Tg2576 mice. Each column indicates the mean ± SEM ($n = 3$). **F:** Western blot analysis of oAβ extracted from the hemi-forebrains of Tg 2576 mice. A 12-mer oAβ was detected in vehicle-treated Tg2576 mice and decreased in 100 nmol/L CpG-injected Tg2576 mice. **G:** Semi-quantification of 12-mer oAβ by densitometric analysis. The amount of 12-mer oAβ1-42 in vehicle-treated Tg2576 mice was normalized to 100%. $^{*}P < 0.05$ as compared with vehicle-treated Tg2576 mice. Each column indicates the mean ± SEM ($n = 4$).

activated microglia nor induced the release of neurotoxic proinflammatory cytokines, NO, or glutamate. These results suggest that oAβ1-42 is not acting to trigger of microglial neurotoxicity.

TLR signaling pathways contribute to phagocytosis of Aβ. TLR2 acts as an endogenous receptor for the clearance of Aβ by bone marrow-derived microglia.[9] Interestingly, a TLR4 mutation exacerbates Aβ burden in mouse models of AD.[39] Thus, we investigated whether microglia activated with TLR ligands exert neuroprotective effects against oAβ1-42 toxicity. Consequently, we found that TLR9 ligand CpG enhanced microglial neuroprotection. Furthermore, CpG exerted the neuroprotective effect of BV-2 microglial cell line against oAβ toxicity (data not shown). TLR9, which detects single-stranded DNA containing unmethylated CpG, is located in intracellular endosomal-lysosomal compartment. We confirmed that microglia expressed TLR9 at a higher level, whereas astrocytes and neuronal cells expressed it at a lower level (data not shown). Thus, CpG mainly acts on microglia in the central nervous system. TLR7 and TLR8 are closely associated with TLR9. They are also located in endosomal-lysosomal compartment and detect single-stranded RNA. The ligands for TLR7 and TLR8 may also have some roles in microglial neuroprotection.

Western blot analysis revealed that microglia activated with CpG reduced the amount of oAβ present in the supernatant of treated cultures. Moreover, CpG was a potent inducer of antioxidant enzyme HO-1. The up-regulation of HO-1 in microglia by CpG treatment may lead to neuroprotection via suppression of ROS production by oAβ. HO-1, a member of the heat-shock protein family, is a microsomal enzyme that oxidatively cleaves heme to produce biliverdin, carbon monoxide, and iron.[40] Aβ binds to heme to promote a functional heme deficiency, mitochondrial dysfunction, and neurotoxicity.[41] Amyloid

precursor protein also binds to HO, and oxidative neurotoxicity is markedly enhanced in cerebral cortical cultures from amyloid precursor protein Swedish mutant transgenic mice.[42]

HO-1 is reported to be induced by the anti-inflammatory cytokine IL-10.[29] Although CpG induced IL-10 in microglia in the absence of oAβ, IL-10 production was inhibited in the presence of oAβ. Therefore, HO-1 may be induced by a discrete mechanism independent from IL-10 in AD.

MMP-9, a protease that degrades Aβ, is expressed at higher levels in the brains of AD patients and may play an important role in amyloid clearance by degrading both oAβ and fAβ.[10] MMP-9 expression is increased by serum amyloid A through formyl peptide receptor-like-1.[43] Although CpG stimulation induced MMP-9 in microglia, inhibiting MMP-9 pharmacologically did not affect neuroprotection by microglia. Thus, MMP-9 may not mainly contribute to the neuroprotection provided by CpG-activated microglia.

In the present study, CpG induced fewer neurotoxic molecules such as TNF-α, NO, and glutamate in microglia, whereas previous studies have reported that CpG-activated microglia produce TNF-α, IL-12, and NO[44] and induce neuronal damage.[45] The discrepancies between these studies and our experiments may be a consequence of differences in the concentrations of TLR ligands used. Higher concentration (10 μmol/L) of CpG have been used for microglial activation in previous reports, whereas here we have used lower concentrations (1 to 100 nmol/L) of CpG.

In addition, we observed that the neuroprotective effect differs among CpG ODN classes. The responses of microglia to the different classes of CpG have not been fully understood. Here, we showed for the first time that class A CpG did not activate microglia, whereas class B and C CpGs induced neuroprotection by microglia that was mediated by clearance of oAβ and induction of HO-1. Three major classes of CpG ODN are structurally distinct. The structures of class A CpG include poly-G motifs at the 5′ and/or 3′ ends that are capable of forming very stable but complex higher-ordered structures and a central phosphodiester region containing one or more CpG motifs in a self-complementary palindrome. Class B CpG has a completely phosphorothioate backbone and does not form typically higher-ordered structures. Class C CpG has a phosphorothioate backbone, and 3′ palindrome forms duplex.[46] These distinct structures of CpG ODN may reflect different microglial responses.

Finally, we examined the effect of CpG on oAβ1-42 neurotoxicity in two different *in vivo* studies. The ICV administration of Aβ25-35 is reported to cause cognitive impairment in a NORT. Oxidative stress contributes to the onset of this cognitive dysfunction.[23] We found that injection of oAβ1-42 also induced cognitive impairment as assessed by NORT. Surprisingly, one-time ICV injection of CpG improved both the cognitive impairment by oAβ1-42 in NORT. The impairment of associative learning in Tg 2576 mouse model of AD was also effectively suppressed by ICV injection of CpG. We also confirmed that CpG treatment decreased Aβ deposits and oAβ in Tg 2576 mice. Our results concur with the recent study that a total of 14 i.p. injection of CpG into Tg 2576 mice beginning at the age of 6 weeks, and once a month, ameliorates AD-related pathology,[28] Aβ plaques are reported to form extraordinarily quickly, over 24 hours. Within 1 to 2 days of a new plaque's appearance, microglia are activated and recruited to the site.[47] ICV injection of CpG may directly induce microglial activation via TLR9 and enhance microglial rapid uptake of oAβ through fluid-phase macropinocytosis as reported recently.[48] CpG may also enhance microglial phagocytosis of fAβ through formyl peptide receptor-like 2.[11] Such mechanisms of Aβ clearance by CpG can decrease Aβ plaque formation in Tg 2576 mice.

Recently, the therapeutic potential of CpG has generated great interest.[46] CpG offers a potent adjuvant activity that elicits a more effective immune response to infectious agents or tumors. A previous report[49] demonstrated that CpG strongly inhibits the effector phase of inflammatory arthritis. In addition, CpG can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury.[50] Our findings suggest that CpG, especially class B and C, may also be effective therapeutic agents against oAβ1-42 neurotoxicity in AD.

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Can an individualized and comprehensive care strategy improve urinary incontinence (UI) among nursing home residents?

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ABSTRACT

Urinary incontinence (UI) is one of the most common and distressing conditions among nursing home residents. Although scheduled care is usually provided for them, incontinence care should be individualized regarding going to the toilet, changing diapers, and taking food and water. We have developed an individualized and comprehensive care strategy to address the problem. We conducted an intervention study that involved training chiefs of staffs, who in turn trained other staffs, and encouraging residents. A total of 153 elderly subjects selected from 1290 residents in 17 nursing homes were eligible to receive our individualized and comprehensive care. The goals of the care strategy were (i) to complete meal intake; (ii) to take fluids up to 1500 ml/day; (iii) to urinate in a toilet; (iv) to spend over 6 h out of bed; and (v) to reduce time spent in wet diapers. We explained the aims of our strategy to the chiefs of staff of each nursing home and instructed them to encourage residents to take an active part in our individualized and comprehensive care strategy for 12 weeks. For 3 days before and after that period, we assessed the changes in fluid volume intake, time spent in wet diapers, size of diaper pads, and urination habits. The result was that fluid volume intake significantly increased ($p < 0.001$) while time spent in wet diapers decreased ($p < 0.001$). The number of residents wearing diapers decreased as did the size of pads during the day ($p = 0.0017$). The proportion of residents using diapers at night was reduced and those using toilets at night increased ($p = 0.007$). This study suggests that such an individualized and comprehensive care strategy can offer a measurable improvement in UI care.

1. Introduction

Urinary incontinence (UI) is one of the most common and distressing conditions affecting nursing home residents and their caregivers on staff (Ouslander et al., 1982, 1993; Ouslander and Fowler, 1985; Mizoguchi et al., 1995; Toba et al., 1996; Schnelle et al., 1998; Fader et al., 2003). It has an adverse impact on physical health, psychosocial status, and the costs of health care (Ouslander et al., 1982, 1993; Ouslander and Fowler, 1985). Ouslander et al. (1982) examined the characteristics of residents with UI in nursing home settings, and indicated that improved care will provide a better quality of life for these patients as the staff's knowledge of incontinence increases.

Several studies have suggested that individualized incontinence care was able to reduce the rate of UI among nursing home residents (Gotoh et al., 2001; Mori, 2001; Ouslander et al., 2001; Toba, 2002; Fader et al., 2003). In fact, some clinical trials have demonstrated that prompted voiding, a typical individualized incontinence care method, and other similar behavioral interventions can significantly reduce the frequency and volume of UI among nursing home residents (Ouslander et al., 1993; Schnelle et al., 1998; Mori, 2001; Fader et al., 2003). However, most such residents still receive inadequately individualized or scheduled toileting assistance (Ouslander and Fowler, 1985; Mori, 2001; Toba, 2002; Fader et al., 2003; Schnelle et al., 2004). In addition, there has been much debate on what kind of individualized care

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could best address UI problems. Some previous studies have reported that inconsistency in applying care principles caused nursing home care variations each night during the study observation period (Ouslander and Fowler, 1985; Schnelle et al., 1998, 2004, 2006).

In Japan, scheduled incontinence care, called as "scheduled toileting," have become common in most nursing homes. The residents go to the toilet as a group and have to line up in front of it. Most residents are compelled to urinate and defecate on commode chairs or in diapers due to lack of toilets or of the care staff required to accompany them. A previous study reported that Japanese nursing home residents remained in wet diapers for an hour or more on average due to scheduled toileting (Toba et al., 1996). In addition to scheduled toileting, meals are also tightly scheduled. The residents usually take their meals together in large dining halls at strictly scheduled hours (usually 8:00, 12:00, and 18:00). Nursing home staffs provide physical assistance to those who cannot eat or drink without help. Because often only one staff is available to take care of many residents, the quality of physical assistance suffers. As for drinks, roasted green tea, Houji-cha, is usually delivered to the residents before each meal. Some nursing homes provide some snacks at 10:00 and 15:00. However, the variety of available beverages and snacks is limited. Additionally, most nursing home residents need some help from staff to maintain a sitting position due to their weak physical condition. However, such assistance is insufficient, leaving the residents bedridden in most nursing homes.

To significantly improve the knowledge of incontinence among nursing home residents and develop an individualized care strategy, Takahashi et al. (2004) surveyed UI problems in nursing homes in Fukushima Prefecture, Japan and conducted a preliminary intervention study. They measured residents' fluid intake volume, frequency of diaper changes, and time spent in wet diapers. As a result, they confirmed that residents' fluid intake was unstable and low, as other similar studies had reported (Ouslander and Fowler, 1985; Schnelle et al., 2004, 2006; Simmons and Schnelle, 2006). Consequently, they suggested that creative solutions were needed to comprehensively improve residents' quality of life across the board, including the issue of UI. Additionally, they obtained a preliminary result of an improvement in UI rates by educating nursing home caregivers about UI.

We hypothesized that an improvement of UI could best be achieved by a system of individualized and comprehensive care that focused on providing adequate fluids and meals, encouraging patients to use toilets and reducing the size of their diaper pads. This approach would differ significantly from the usual UI care in which diapers would be changed only at scheduled times. The aim of this study was to investigate whether such a strategy was in fact able to increase the intake of fluids and food, and to reduce the proportion of diaper users and the size of their diaper pads, thus leading to an enhanced quality of life.

2. Methods

2.1. Study setting

2.1.1. Nursing homes

The present study was conducted at 17 nursing homes: 9 welfare institutions for the elderly (Kaigo Rojin Fukushi Shisetsu); 6 health care facilities for the aged (Kaigo Rojin Hoken Shisetsu); 1 hospital; and 1 institution for dementia patients (Group Home).

2.1.2. Nursing home staffs

The ratio of nursing home residents to caretakers on staff was approximately 1.5 among nursing homes in this study. Since the corresponding ratio of the Japanese standard is 3.0, the nursing homes in this study were relatively adequate regarding care staffs.

2.2. Study subjects

A total of 153 elderly subjects selected from 1290 residents in 17 nursing homes were eligible to participate. Eligibility criteria were the ability (1) to maintain a sitting position, and (2) to express their wish to defecate. Of those subjects, 31 were ineligible to receive our individualized and comprehensive care, due to declining co-morbidity or to having been discharged from their facilities. Thus, we obtained complete data from 122 residents, with informed consent obtained from either the subject or his/her representative, that is, spouse, son, or daughter.

2.3. Education program for care staff

One care staff was selected from each nursing home as the head care staff who participated in a training program for the individualized and comprehensive care strategy and, after finishing the program, educated other care staffs in turn.

First, all chiefs on staff were informed of the current excretion status of all residents in their facility based on our investigation of the actual circumstances among all 1290 residents prior to launching the current study (unpublished data). Second, they were informed of the volume of current intakes of food and water, and the time spent in wet diapers among our 153 subjects. Our data indicated that both food and water intake volumes of residents were inadequate. Third, they learned the rationale for our hypothesis that UI problems among residents improved after participation in our care strategy. The protocol for that strategy can be summarized as follows: (1) to encourage complete meal intake; (2) to increase fluid intake up to 1500 ml/day; (3) to encourage urination in a toilet; (4) to encourage time spent out of bed to exceed 6 h; and (5) to reduce time spent in wet diapers (Table 1).

In addition to the above five goals, we asked care staffs to choose diapers with smaller pads to improve skin condition and lower costs. Larger wet pads have been shown to not only adversely affect larger areas of skin than smaller ones, but also to force nursing home residents and their families to bear both the higher cost and extra expense of disposal.

2.4. Details of our strategy

2.4.1. To encourage complete meal intake

Care staffs provided patients with strong physical assistance at meal times, and encouraged them to finish their meals if they appeared reluctant to do so. The care staffs recorded each resident's meal intake volume in check sheets to assess the rate of meals completely consumed.

2.4.2. To increase fluid intake up to 1500 ml/day

Care staffs delivered beverages other than before each meal, e.g., in the early morning, 10:00, 15:00, and before sleep. In addition to roasted green tea, Houji-cha, Japanese green tea which elderly residents preferred was delivered. Some care staffs were specially assigned to engage in tea delivery, as they were able to deliver tea anytime. Nursing homes prepared various kinds of beverages other than green tea including coffee, black tea, cocoa, milk, orange juice, apple juice, etc. If the residents preferred some beverages other than green tea, the staffs served them. Care staffs patiently assisted the residents in drinking the beverages and

Table 1
Concepts of each protocol for individualized and comprehensive care strategy.

Summary of protocol	Concept
(1) To encourage complete meal intake Since nursing homes manage nutritional states of their residents, incomplete meal intakes lead to insufficient nutrition (Sugiyama, 2006). Malnutrition is associated with the risk of poor health status. If energy and protein are appropriately supplied to malnourished elderly persons, their physical functions are improved (Akner and Cederholm, 2001).	
(2) To increase fluid intake up to 1500 ml/day Adults usually require 2400 ml water a day: 1200 ml from fluids; 900 ml from solids; and 300 ml from oxidation in the body (Ono, 2000). Our goal for fluids was approximately from 1200 to 1500 ml.	
(3) To encourage urination in a toilet Ms. Tanaka, a co-author, hypothesized that sitting on a toilet facilitates evacuation aided by gravity. In fact, when one sits on the toilet to defecate, the anorectal angle becomes obtuse so as to allow easy defecation (Konishi and Kanazawa, 1989). In addition, nursing home residents probably experience a higher quality of life when using toilets rather than diapers.	
(4) To encourage time spent out of bed to over 6 h Ms. Tanaka also hypothesized that nursing home residents, rather than remaining in bed, retain better control of their blood pressure and sense of balance when out of bed, and using their autonomous nervous system and preserving their trunk muscular strength. Time spent out of bed can also sharpen their appetite.	
(5) To reduce time spent in wet diapers Urinary incontinence can lead to physical complications such as skin irritation and urinary tract infection (Ouslander et al., 1982; Ouslander and Fowler, 1985; Schnelle et al., 1998, 2004, 2006; Fader et al., 2003). To minimize skin irritation caused by wet diaper pads, minimum-sized pads that can fully absorb a one-time volume of urination are recommended.	

encouraged them to finish as much of their drink as they could manage. If the residents seemed to feel tired, they drank a cup of tea from time to time. Water intake volume was also recorded in residents' check sheets.

2.4.3. To encourage urination in a toilet

Care staffs provided individual assistance for resident toileting in this study. They assessed whether residents could maintain their sitting position unaided or would need assistance, and provided encouragement to use the toilet or commode chair. They actually accompanied residents to the toilet, prompted them to void, and asked them to stay seated for at least 15 min as necessary when attempting to urinate or defecate.

2.4.4. To encourage time spent out of bed to over 6 h

Care staffs tried to elevate bedridden residents, and encouraged ambulatory residents to spend their time socializing with others in nursing home living rooms. The care staffs attempted to keep usually bedridden residents sitting up longer than before this study. Staff also urged the ambulatory residents to frequent the living rooms for a longer time than before, and they arranged for residents to spend quality time in those living rooms by, for example, playing music CDs and chatting with one another frequently.

2.4.5. To reduce time spent in wet diapers

Care staffs conscientiously checked out the condition of diapers every 2 h. They carefully recorded the condition of diapers (dry or wet) on the check sheets when residents wet their diapers, and furnished them with fresh ones as soon as possible. Hours spent in wet diapers were calculated by subtracting the total time spent in dry diapers from 24 h. In addition, the care staffs were trained to determine when they started to use smaller instead of larger pads by assessing the weight of absorbed urine and comparing them with dry ones.

2.5. Assessment

Twice before and after a 12-week exposure to our individualized and comprehensive care strategy, the residents were assessed by Care level (Grade 1–5; with Grade 5 denoting the most severe state), by Independency level of the elderly with dementia (Grade I, II, III, IV, and M; with Grade M denoting the most severe state), and by Independency level of the elderly (J1, J2, A1, A2, B1, B2, C1, C2; with C2 denoting the most dependent state) using the criteria of the Japanese Ministry of Health, Labor and Welfare.

2.6. Statistical analysis

We used a paired *t*-test to compare 3-day mean water intakes and hours spent in wet diapers before and after the 12-week intensive care strategy. The Wilcoxon signed rank sum test was used to compare the size of the diaper pad before and after the 12-week period. We ranked 24 combination patterns among pants, diapers, and pads from rank 1 denoting cloth pants without pads to rank 24 denoting cloth diapers with two large-size pads; two kinds of pants and two kinds of diapers (i.e., 4 choices) with or without pads [6 choices: none, small, medium, large, extra large (LL) size pads, and two large-size pads]. We used SPSS 12.0J for Windows (SPSS Japan Inc., Tokyo) for statistical analysis.

3. Results

The mean age of the 122 nursing home residents participating in this study was 85.2 years (81.2 among males and 85.9 among females). Females comprised 85.2% of the study subjects. Fifty-seven percent of all subjects were in care level Grade 4. As to the

Table 2
Characteristics of 122 nursing home residents participating in this study.

Factors	Categories n (%)
Sex	
Men	18 (14.8)
Women	104 (85.2)
Care level[a]	
No	1 (0.8)
Grade 1 (mild)	0 (0)
Grade 2	5 (4.1)
Grade 3	28 (23)
Grade 4	70 (57.4)
Grade 5 (severe)	18 (14.8)
Independence level of elderly with dementia[b]	
Grade I (mild)	2 (1.7)
Grade II	18 (14.9)
Grade III	59 (48.8)
Grade IV (severe)	42 (34.7)
Independence level of elderly[c]	
J1 (independent)	0 (0)
J2	0 (0)
A1	7 (5.8)
A2	15 (12.4)
B1	28 (23.1)
B2	52 (43)
C1	7 (5.8)
C2 (dependent)	12 (9.9)

[a] Judged by the extent of time required for care giving. Elderly persons requiring a longer time are ranked at a more severe level. This judgement is used for payment of the Long-term Care Insurance managed by the Japanese Government.

[b] This classification aims to enable co-medical staffs to objectively and quickly determine the requirements of care giving for the elderly diagnosed with dementia by a physician so as to provide appropriate care.

[c] This classification served to spread the idea of being bedridden (Netakiri). The aim is for co-medical staffs to objectively and quickly determine the care giving requirements for the elderly with a disability in order to provide appropriate care.

Table 3
Difference in fluid intake and time spent in wet diapers before changing (wet time) before and after intervention by our comprehensive care strategy.

	Before	After	Difference, CI	p^a
Fluid intake volume (ml/day)	881.1 ± 263.8	1146.4 ± 365.2	265.3 (213.5–317.2)	<0.001
Wet time (h/day)	13.9 ± 5.5	12.2 ± 5.9	−1.7 (−2.41 to −0.92)	<0.001

Note: Mean ± S.D. calculated from 3-day assessments were used to obtain difference before and after intervention.
[a] Paired *t*-test was used to evaluate difference before and after intervention.

independency level of the elderly, those categorized with dementia Grade III accounted for 48.8% of the study subjects, and those judged to be at the B1 to C2 independency level accounted for 81.8% (Table 2). The distribution of those three indices did not change after our 12-week intensive care strategy (data not shown).

The mean water intake volume of nursing home residents significantly increased after our intervention from 881.1 ± 263.8) (±S.D.) ml/day to 1146.4 ± 365.2 ml/day (mean difference of 265.3, 95% Confidence interval (CI) 213.5–317.2; $p < 0.001$) (Table 3). The mean time that residents spent before changing from wet diapers to fresh ones decreased from 13.9 ± 5.5 h to 12.2 ± 5.9 h/day (mean difference −1.7, CI = −2.41 to −0.92; $p < 0.001$).

After intervention, 34 residents (27.9%) improved their daytime UI care, 68 were unchanged, and 20 became worse ($p = 0.017$; Table 4). Among all residents, 17 who were wearing cloth pants with pads changed to smaller pads after the intervention. Two residents who had used training pants also changed to smaller pads. Five residents used no diapers or pads after the intervention as did as aged persons without dementia. No statistically significant improvement in nocturnal UI was observed.

The method of urination during daytime did not significantly change before and after intervention (14 residents improved, 5 worsened, and 103 were unchanged, Table 5). That method showed a statistically significant improvement during nighttime ($p = 0.007$; Table 6); 23 subjects (18.9%) improved, 8 worsened, and 91 were unchanged after the intervention; among those 23, 2 changed from using commode chairs to using toilets, while among residents who wore diapers, 10 changed to commode chairs and 8 to toilets.

4. Discussion

We confirmed that nursing home residents had a low volume of water intake (in average 881 ml/day), as other similar studies had

Table 4
Changing types of pants or diapers and the size of pads during daytime.

Before	After																						
	Cloth pants						Training pants						Diapers						Cloth diapers						
	None	S	M	L	LL	Two L	None	S	M	L	LL	Two L	None	S	M	L	LL	Two L	None	S	M	L	LL	Two L	Total
Cloth pants																									
None	1	2	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
S	1	9	6	1	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	20
M	1	6	28	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34
L	0	2	6	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Two L	0	1	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Training pants																									
None	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
S	1	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
M	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
L	2	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Two L	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Diaper																									
None	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2
M	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	3
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	4
LL	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Two L	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Cloth diaper																									
None	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	6
S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M	0	1	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2
LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Two L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	21	41	6	1	3	0	9	12	8	0	0	0	3	3	3	0	0	4	0	0	2	0	0	122

Note: Cloth pants are the most desirable and cloth diapers the least desirable option. Among similar types of pants or diapers, eliminating use of pads is the most desirable and use of two L pads is the least desirable option. "Two L" denotes using two large-size pads. A small-size pad can absorb less than 150 ml, a medium-size 150–449 ml, a large-size pad 450–999 ml, a LL-size pad 1000 ml or more.
Improved: 34 residents (under the diagonal).
Worsened: 20 residents (over the diagonal).
Unchanged: 68 residents (on the diagonal).

Table 5
Change in method of daytime urination.

	After				
	Toilet	Commode chair	Urinary chamber pot	Diaper	Total
Before					
Toilet	81	1	0	4	86
Commode chair	0	4	0	0	4
Urinary chamber pot	1	0	2	0	3
Diaper	7	4	2	16	29
Total	89	9	4	20	122

Improved: 14 residents (under the diagonal).
Worsened: 5 residents (over the diagonal).
Unchanged: 103 residents (on the diagonal).

Table 6
Change in method of nighttime urination.

	After					
	Toilet	Commode chair	Urinary chamber pot	Diaper	Cont. ind. catheter	Total
Before						
Toilet	7	1	0	3	0	11
Commode chair	2	8	1	1	0	12
Urinary chamber pot	0	0	0	1	0	1
Diaper	8	10	3	76	1	98
Total	17	19	4	81	1	122

Improved: 23 residents (under the diagonal).
Worsened: 8 residents (over the diagonal).
Unchanged: 91 residents (on the diagonal).

reported (Ouslander and Fowler, 1985; Schnelle et al., 2004, 2006; Simmons and Schnelle, 2006). Our study was the first in Japan to quantitatively clarify the level of water intake among nursing home residents. Following our individualized and comprehensive care strategy, we observed an increased volume of water intake and a reduction in the time residents wore wet diapers after urination. This strategy resulted in the following improvements in UI care: approximately a quarter of residents switched from diapers to pants or from larger to smaller pads during the day; and about one-fifth improved their method of urination from diapers to toilets at night.

Since we found that nursing home residents consumed insufficient water, we aimed to increase their water intake. However, an increase of water intake without any restriction may not necessarily improve UI care. A previous study has reported that many nursing homes advocated encouraging fluids to an "adequate level," often over 2000 ml/day in the United States (Ouslander and Fowler, 1985). An adequate fluid level and/or fluid restriction in the evening is usually encouraged for bladder training (Ouslander and Fowler, 1985). In other words, an adequate fluid intake reduces the risk of urinary tract infection and maintains normal bladder function. However, such adequate levels are often impractical and can worsen UI in patients with unstable bladders, though they may prove worthwhile for some patients with indwelling catheters (Ouslander and Fowler, 1985).

Although we tried to encourage a fluid intake volume of at least 1500 ml, the mean volume was only 1146.4 ml in the assessment conducted after the 12-week period. That volume proved insufficient, even when nursing home staff would deliberately increase residents' fluid intake; it would have been even lower, had the staff not tried to increase it. Previous studies have reported that medical records kept by nursing home staffs reflected an overestimate of residents' food and fluid intakes (Schnelle et al., 2004, 2006; Simmons and Schnelle, 2006). Prior to the introduction of our current strategy, nursing home staffs were not aware of the importance of accurately monitoring fluid volume, even though they encouraged residents to drink often. Moreover, staffs were seldom trained to accurately measure the volume of food intake. As a result, they were often slipshod in recording the volume, causing inaccurate estimations to be made. One reason for such inaccuracies may be the lack of an adequate staff who would carefully observe and record residents' intake volumes (Schnelle et al., 2004).

We observed a decrease in the amount of time residents were left unattended before their wet diapers were changed. Since UI can lead to physical complications such as skin irritation and urinary tract infection (Ouslander et al., 1982; Ouslander and Fowler, 1985; Schnelle et al., 1998, 2004, 2006; Fader et al., 2003), it is desirable that time spent in a wet diaper be kept to a minimum. According to a previous study using a micturition-monitoring device, the mean wet time was over 1 h for every UI episode if the nursing home staff failed to encourage prompt voiding (Toba et al., 1996). Another study showed that in most nursing homes the mean time spent in a wet diaper before changing was approximately 200 min (Schnelle et al., 1998).

As a result of our strategy, we observed improvements in incontinence care among one-fourth of residents such as changing from diapers to pants or from larger to smaller pads. Such improvements, however, occurred in daytime rather than at night, one possible reason being an insufficiency of nighttime nursing home staffs. In that situation, residents' pads were changed less often. To reduce the frequency of pad changing, staffs may have preferred larger pads but been unable to substitute them for smaller ones.

To improve the quality of care for nursing home residents, more and better educational programs on individualized care and the quality control of care are needed. Several studies have suggested that individualized incontinence care was able to improve UI (Gotoh et al., 2001; Mori, 2001; Ouslander et al., 2001; Toba, 2002; Fader et al., 2003; Takahashi et al., 2004). However, almost all nursing homes in our study had no prior strategy of individual incontinence care before this study, since the staffs were unaware that such care could improve a resident's incontinence. If, as in our study, nursing home staffs are educated and encouraged to practice intensive and individualized incontinence care including precise ongoing assessments of their residents' incontinence, some residents currently wearing diapers may no longer need them. In short, inappropriate care for the elderly who wear diapers may be simply due to a lack of sufficient education and training regarding UI. In addition, adequate management systems and auditing technologies are also needed (Schnelle et al., 2004). However, attempting to educate or motivate staffs to provide unrealistic levels of care may be even more ineffective and counterproductive (Schnelle et al., 2004).

Although a preintervention assessment showed that more residents wore diapers at night than in the day, only nighttime incontinence care had improved after the intervention period. One reason may be the unnecessary use of diapers for residents who had improved nighttime control. Although nursing home staffs should individualize nighttime incontinence care to minimize disrupting the sleep of other residents (Ouslander et al., 1993, 2001; Schnelle et al., 1998; Fader et al., 2003), they often indiscriminately used diapers with pads large enough to absorb an entire night's volume of urine. Nighttime staffs are often too few to conduct painstaking incontinence care such as prompt voiding (Gotoh et al., 2001; Fader et al., 2003).

There are several limitations to the current study. First, since this is not a randomized controlled trial, several unavoidable factors could have biased the results. One such factor is measurement bias.

Because some of the data were collected by those who actually performed the individualized and comprehensive care strategy, they might have overestimated the effect of their intervention. The second limitation is in the procedure for checking wetness. We defined diapers or pads as wet when trained staffs felt them to be heavier than identical dry diapers or pads. The third limitation concerns the necessity of working with diapers and pads made by different manufacturers, which could lead to variations in evaluating wetness depending on the brands of diapers or pads available. The fourth limitation is the difficulty of obtaining accurate information on the frequency of incontinent episodes and other medical information, e.g., present and past illness, and diagnosis by physician, from nursing staff or nursing home records. In the nursing home records we examined, there were no specific evaluations documented by urologists similar to those in other studies (Ouslander et al., 1982; Ouslander and Fowler, 1985; Mori et al., 1999; Gotoh et al., 2001; Toba, 2002; Okamura et al., 2003a,b).

In conclusion, this study suggests that our individualized and comprehensive care strategy of educating nursing home staffs to encourage residents to raise their food and water intake levels and to reduce the time they spend in wet diapers may be useful in improving UI.

Conflict of interest

None.

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Mechanism Underlying Apolipoprotein E (ApoE) Isoform-dependent Lipid Efflux From Neural Cells in Culture

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We determined the molecular mechanisms underlying apolipoprotein E (ApoE)-isoform-dependent lipid efflux from neurons and ApoE-deficient astrocytes in culture. The ability of ApoE3 to induce lipid efflux was 2.5- to 3.9-fold greater than ApoE4. To explore the contributions of the amino- and carboxyl-terminal tertiary structure domains of ApoE to cellular lipid efflux, each domain was studied separately. The amino-terminal fragment of ApoE3 (22-kDa-ApoE3) induced lipid efflux greater than 22-kDa-ApoE4, whereas the common carboxyl-terminal fragment of ApoE induced very low levels of lipid efflux. Addition of segments of the carboxyl-terminal domain to 22-kDa-ApoE3 additively induced lipid efflux in a length-dependent manner; in contrast, this effect did not occur with ApoE4. This observation, coupled with the fact that introduction of the E255A mutation (which disrupts domain–domain interaction) into ApoE4 increases lipid efflux, indicates that interaction between the amino- and carboxyl-terminal domains in ApoE4 reduces the ability of this isoform to mediate lipid efflux from neural cells. Dimeric 22-kDa or intact ApoE3 induced higher lipid efflux than monomeric 22-kDa or intact ApoE3, respectively, indicating that dimerization of ApoE3 enhances the ability to release lipids. The adenosine triphosphate–binding cassette protein A1 (ABCA1) is involved in ApoE-induced lipid efflux. In conclusion, there are two major factors, intramolecular domain interaction and intermolecular dimerization, that cause ApoE-isoform-dependent lipid efflux from neural cells in culture.

Key words: Alzheimer's disease; apolipoprotein E; high-density lipoprotein (HDL); neurons; astrocyte

The lipoprotein found in the central nervous system (CNS) is the high-density lipoprotein (HDL), and apolipoprotein E (ApoE) is one of the major apolipoproteins regulating lipid transport in CNS (Roheim et al., 1979; Pitas et al., 1987b; Weisgraber et al., 1994). Astrocytes and microglia synthesize and secrete ApoE (Boyles et al., 1985; Nakai et al., 1996), which interacts with adenosine triphosphate (ATP)-binding cassette protein A1 (ABCA1) (Krimbou et al., 2004) to remove cholesterol from cells and generate HDL particles in the cerebrospinal fluid and cultured media (Pitas et al., 1987a; Borghini et al., 1995; LaDu et al., 1998).

ApoE-inducible lipid efflux is ApoE-isoform dependent (Michikawa et al., 2000; Gong et al., 2002; Xu et al., 2004), and ApoE3 generates a similar number of HDL particles to but with a smaller number of ApoE molecules than ApoE4 (Gong et al., 2002). HDL synthesis mediated by ApoE contributes to cholesterol release from the cell membrane. On the other hand, HDL associated with ApoE is taken up by cells via ApoE receptors and the cholesterol in HDL is used for maintaining cholesterol homeostasis in CNS neurons. Thus, this isoform-specific action of ApoE to remove cholesterol and

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