Ⅲ. 研究成果の刊行に関する一覧表

別紙4

研究成果の刊行に関する一覧表

書 籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
Perry G, Mondragón-Rodríguez S, Nunomura A, ほか	Oxidative stress and balance in neurodegenerative diseases.	Dickson DW, Weller RO	Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, 2nd Ed.	Wiley-Blackwell	Hoboken, NJ	2011	10-12

雑 誌

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Ⅳ. 研究成果の刊行物・別刷

3 Oxidative Stress and Balance in Neurodegenerative Diseases

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Definition

Oxidative damage is a major feature of the cytopathology of a number of chronic neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease. The original concept of oxidative stress promoted by Denham Harmon has been used to indicate an excess of oxygen free radicals that breach oxidant defenses with consequent detriment. By this definition, detection of damage resulting from reactive oxygen species is indicative of oxidative stress [1,2]. Reactive oxygen species are a by-product of cellular oxidative metabolism and are generated in the mitochondria during oxidative phosphorylation with production of molecules with unpaired electrons such as superoxide ($O_2^{\cdot}$).

Superoxide is a short-lived molecule that is reduced by the family of superoxide dismutases (SODs) to generate hydrogen peroxide (H_2O_2). Reduction of H_2O_2, for example through the action of redox-active cations such as iron and copper, generates a hydroxyl radical (•OH), which can oxidize proteins, lipids, and nucleic acids.

Nitric oxide is another short-lived species with limited toxicity that is produced by a family of nitric oxide synthases. After interaction with superoxide, nitric oxide forms peroxynitrite (ONOO-), which is another powerful reactive species that can lead to damage of cellular macromolecules through nitration or generation of additional free radicals. Cells have evolved an elaborate array of antioxidant defenses, including SOD, glutathione reductase and catalase (Figure 3.1).

Detection of cellular oxidative damage

Cellular oxidative damage can be detected in a variety of ways. Widely used markers of oxidative damage to lipids include 4-hydroxynonenal and isoprostanes, to nucleic acids include 8-hydroxy-2'-deoxyguanosine, and to proteins include nitration and glycation [3]. Indirect evidence of cellular oxidative stress is increased expression of molecules involved in oxidant defense, such as heme oxygenases, SODs, glutathione transferases, catalase, and glucose-6-phosphate dehydrogenase. It is important to note that neurons displaying signs of oxidative stress are not necessarily succumbing to oxidative stress, but may be adapting by way of oxidant defenses. These findings suggest that neurodegenerative disorders where oxidative stress is postulated to play a role, such as Parkinson's disease and AD, are associated with mechanisms that maintain a balance between oxidative stress and adaptation to this stress, reflecting the ability of living systems to dynamically regulate their defense mechanisms in response to oxidants. Therefore, mere evidence of oxidative damage does not necessarily indicate cell death by way of oxidative stress, given that the cell may have successfully increased endogenous cellular defenses sufficiently to compensate for the increased flux of reactive oxygen responsible for the damage. It does, however, indicate that the normal balance between the production and defense reduction of oxidative stress has been challenged.

Consequences and mechanisms of cellular oxidative damage

Evidence suggests that cells that fail to compensate for oxidative stress enter apoptosis, which in turn leads to death within hours [4,5]. This is particularly germane to the discussion of degenerative diseases that have a course of years. Those cells experiencing increased oxidative damage, by their continued existence, testify to their increased compensatory response to reactive oxygen.

Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders, Second Edition. Edited by Dennis W. Dickson, Roy O. Weller.

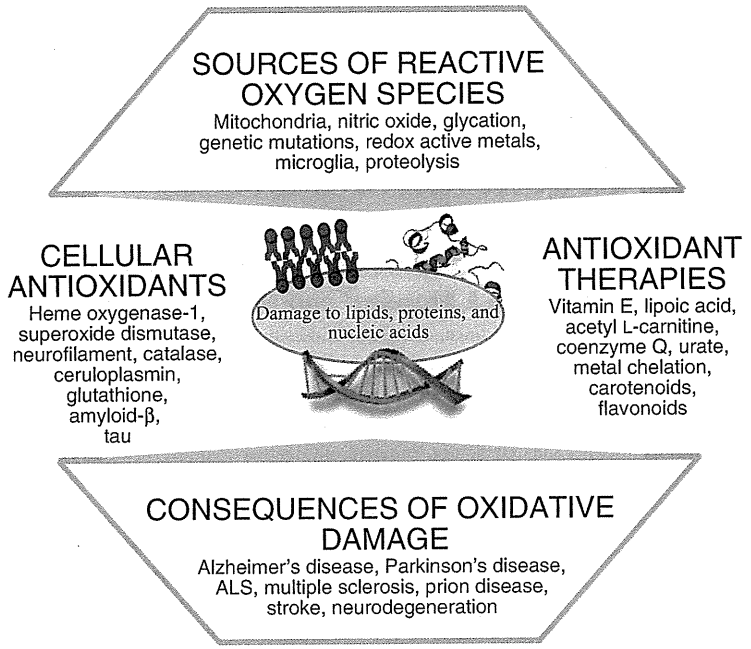

Figure 3.1 Schematic presentation of sources of products causing oxidative cellular damage influencing various central nervous system diseases. *In vivo* antioxidant and various therapeutic agents may reduce the consequences. ALS, amyotrophic lateral sclerosis.

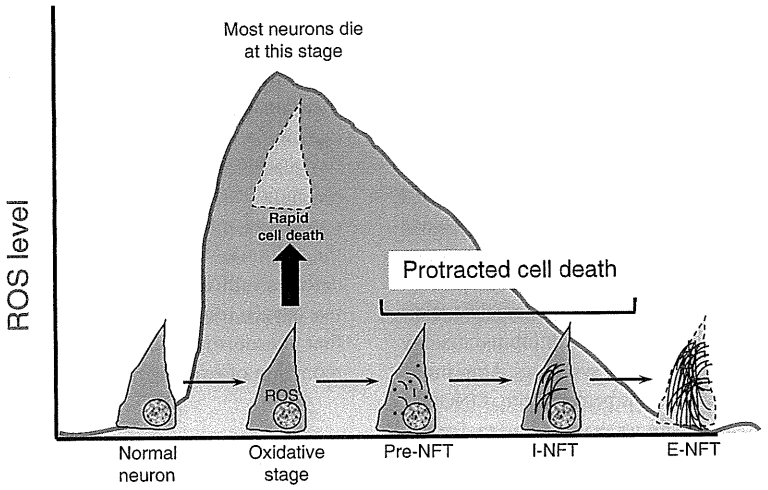

Figure 3.2 Chronology of neuronal pathology in Alzheimer's disease. Metabolic and oxidative alterations precede tau phosphorylation. The pathological lesions, neurofibrillary tangles (NFT) and senile plaques are late events. ROS, reactive oxygen species.

This is certainly the case for AD, in which oxidative damage is evident in every category of macromolecule examined, including the presence of increased sulfhydryls, induction of heme oxygenase-1, and increased expression of Cu/Zn superoxide dismutase. Even those aspects of AD thought to be most deleterious, the pathological lesions, senile plaques and neurofibrillary tangles, may be important aspects in oxidant defense [6]. Quantitative analysis of the extent of oxidative damage is actually reduced in those neurons with the most cytopathology [6-8] (Figure 3.2). This suggests that oxidative defenses extend beyond the classic antioxidant enzymes and low molecular weight reductants [9].

The distinct structural and biochemical pathological changes that are associated with and considered part of the spectrum of the disease may in fact form in response to the oxidative stress. The importance of this aspect is seen when considering that protection of critical cellular components from oxidants can be

through the incorporation of damage to less critical cellular components. At the present time, the exhaustion of cellular reductants (which are incidentally the same category of agents most often used as therapeutic antioxidants) is used as a measure of antioxidant potential; however, cellular macromolecules may share a similar function. Consistent with this view is the physiological modification of the neurofilament heavy subunit (NFH) by carbonyls [10]. Intriguingly, although NFH has a long half-life, the same extent of carbonyl modification is found throughout the normal aging process, as well as along the length of the axon. It is this slow turnover rate of NFH protein in the axon, which can take years, which may allow for oxidative protection. Therefore, NFH may be uniquely adapted as a carbonyl scavenger due to a high lysine content [10]. For example, the sequence lysine-serine-proline is repeated approximately 50 times in the sidearm portion of the molecule, a domain that is exposed on the surface of a neurofilament structure.

While more studies are required to understand the role of NFH in maintaining neuronal oxidative homeostasis, it is tempting to consider them as additional neuronal defenses important in protecting the axon from the toxic products of oxidation – reactive aldehydes.

RNA is extensively modified in AD and, while clearly damaged, the rapid turnover of RNA may also serve a protective function. With the formation of hydroxyl radicals, every macromolecule would be potentially susceptible to attack, but the most critical aspect for the cell is to reduce damage to systems, such as enzyme active sites, the compromise of which leads to cell death. While RNA alteration may lead to protein sequence anomalies [4], RNA destruction can more easily be accommodated in cellular metabolism than damage to DNA or enzyme active site destruction. The large pool of neuronal RNA may even mean that errors in protein synthesis, resulting from oxidatively modified RNA, can be corrected by the metabolic turnover of abnormal proteins. Certainly, renewal of components is a common theme in biology and, although energetically wasteful, rids the cells of the consequences of damage.

Future directions

The simple concept that oxidative damage is deleterious to cells and amenable to therapeutic increases in antioxidants may be far too simplistic. The proposed concept of homeostatic balance between oxidant stress and defenses is a possible explanation for why efforts to increase oxidative defenses by therapeutic use of antioxidants has produced, at most, moderate benefits [9]. It is imperative that the overall homeostatic system be considered before decisions are made about the short-term and long-term consequences of therapeutic antioxidants. If cells survive and function with evidence of oxidative damage, it is unlikely that the oxidant stress has damaged critical systems. Augmentation of antioxidants may only have marginal benefit or even detrimental effects by upsetting the homeostatic balance. Instead, oxidative damage should be considered both as a window to view the homeostatic compensations necessary for survival and as a means to design therapeutics to modify the more fundamental abnormalities responsible for altering oxidative balance in neurodegenerative disorders.

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YOKUKANSAN, A TRADITIONAL JAPANESE MEDICINE, AMELIORATES MEMORY DISTURBANCE AND ABNORMAL SOCIAL INTERACTION WITH ANTI-AGGREGATION EFFECT OF CEREBRAL AMYLOID β PROTEINS IN AMYLOID PRECURSOR PROTEIN TRANSGENIC MICE

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Abstract—The deposition of amyloid β protein (Aβ) is a consistent pathological hallmark of Alzheimer's disease (AD) brains. Therefore, inhibition of Aβ aggregation in the brain is an attractive therapeutic and preventive strategy in the development of disease-modifying drugs for AD. An *in vitro* study demonstrated that yokukansan (YKS), a traditional Japanese medicine, inhibited Aβ aggregation in a concentration-dependent manner. An *in vivo* study demonstrated that YKS and Uncaria hook (UH), a constituent of YKS, prevented the accumulation of cerebral Aβ. YKS also improved the memory disturbance and abnormal social interaction such as increased aggressive behavior and decreased social behavior in amyloid precursor protein transgenic mice. These results suggest that YKS is likely to be a potent and novel therapeutic agent to prevent and/or treat AD, and that this may be attributed to UH.

Key words: Alzheimer's disease, aggression, amyloid β proteins, traditional medicine, Uncaria hook, yokukansan.

Alzheimer's disease (AD), the most prevalent cause of dementia, is characterized by loss of memory and cognition in the elderly. One of the pathological characteristics of AD is the progressive deposition of insoluble amyloid β protein (Aβ) as a form of senile plaques (Wirths et al., 2004). This protein comprises peptides of approximately 39–43 amino acid residues derived from the transmembrane amyloid precursor protein (APP) (Selkoe, 2002). Aβ can exist as monomers and form a variety of different aggregate morphologies including dimers, small soluble oligomers, protofibrils, diffuse plaques, and the fibrillar deposits seen in senile plaques. Protofibrils, diffuse plaques, and fibrillar deposits seem to have a predominant β-sheet structure (Tierney et al., 1988; Barrow and Zagorski, 1991), while oligomers are believed to be more globular (Barghorn et al., 2005). Abundant evidence showing that formation of these aggregates causes primary neurodegeneration in AD has led to the amyloid hypothesis, which states that the accumulation of Aβ in the CNS is highly neurotoxic and degrades synaptic function (Selkoe, 2002; Wirths et al., 2004). Therefore, it is hypothesized that the formation, deposition, and aggregation of Aβ in the brain should be primary targets for amelioration of dementia. Currently, drugs available for dementia such as acetylcholinesterase inhibitors exert only a temporary effect on cognitive dysfunction (Millard and Broomfield, 1995; Park et al., 2000; Darreh-Shori et al., 2004), and they do not prevent or reverse the formation of Aβ deposits. Among the potentially promising strategies for developing more effective anti-dementia drugs are the inhibition of Aβ fibril formation, destabilization of aggregated Aβ, or a combination of both.

In patients with AD, not only core symptoms such as cognitive impairment, but also behavioral and psychological symptoms of dementia (BPSD) such as aggression, anxiety, and hallucinations often emerge. BPSD is a serious problem for caregivers, and because its severity and the care burden show a positive correlation, therapy for BPSD is considered to be as important as therapy for the core symptoms (Nagaratnam et al., 1998; Tanji et al., 2005). To date, anti-psychotic medicines have been used for treatment of BPSD. However, the drugs induce extrapyramidal symptoms and other adverse events, and in consequence, they decrease the quality of life and increase the difficulty of maintaining activities of daily living. Thus, new remedies without adverse effects have been sought.

Herbal remedies are used worldwide and have a long history of use to alleviate a variety of symptoms of many

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β protein; BPSD, behavioral and psychological symptoms of dementia; DLB, dementia with Lewy bodies; TBS, tris–buffered saline; Tg(+), transgenic; Tg(−), non-transgenic; UH, Uncaria hook; YKS, yokukansan.

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different conditions and diseases. Recently, clinical trials in patients with AD have also shown that some traditional Japanese medicines called kampo improved Mini-Mental State Examination scores (Iwasaki et al., 2004) and blood flow in the cerebral cortex (Maruyama et al., 2006). We have reported that several traditional herbal medicines such as kamiuntanto (*Formula lienalis angelicae compositae*) (Wang et al., 2000; Nakagawasai et al., 2004) and hachimijiogan (*Pilulae octo-medicamentorum rehmanniae*) (Iwasaki et al., 2004) ameliorated symptoms of dementia.

Yokukansan (YKS, *Pulvis depressionis hepatis*) is a traditional Japanese medicine approved by the Ministry of Health, Labour and Welfare of Japan as a remedy for neurosis, insomnia, and irritability in children. Recently, we reported that it improved such BPSD as hallucinations, agitation, and aggression in patients with Alzheimer's disease, dementia with Lewy bodies (DLB), and other forms of senile dementia (Iwasaki et al., 2005a,b). Recently, to clarify the improving effect of YKS, various basic studies have been performed (Ikarashi et al., 2009; Kawakami et al., 2009, 2010; Terawaki et al., 2010). We also previously demonstrated that Uncaria hook (UH), a constituent herb of YKS, inhibited Aβ aggregation *in vitro* (Fujiwara et al., 2006), suggesting that YKS containing UH may possess anti-aggregation activity toward Aβ, and that it may improve memory disturbance and BPSD. However, sufficient animal experiments to confirm this hypothesis have not been performed yet.

The APP transgenic [Tg(+)] mouse expressing the human form of APP695SWE is known as a model of AD. Aβ accumulates in the brain of the mice with aging (Hsiao et al., 1996; Ikarashi et al., 2004). In addition, not only cognitive dysfunction but also BPSD-like symptoms such as disinhibition, hyperactivity, and impulsive behavior have been observed in Tg(+) mice (Lalonde et al., 2003; Stackman et al., 2003; Ognibene et al., 2005; Dong et al., 2005; Adriani et al., 2006; Quinn et al., 2007). These findings suggest that the Tg(+) mouse is a valuable tool for developing new drugs for dementia and BPSD.

To clarify the hypothesis described above, in the present study, we first examined the effect of YKS on Aβ aggregation *in vitro* as well as UH. Next, the effects of YKS and UH on accumulation of Aβ in the brain and phenotypes such as memory disturbance and BPSD-like behaviors such as the increase in aggressive behavior and decrease in social behavior in the Tg(+) mice were investigated.

EXPERIMENTAL PROCEDURES

Animals

Male APP Tg(+) mice, who overexpress a 695-amino acid splice form (Swedish mutation K670N M671I) of the human amyloid β precursor protein (APP695), and non-transgenic [Tg(−)] mice were purchased from Taconic Farms Inc. (Germantown, NY, USA). Each animal was housed individually in a plastic cage (230×155×155 mm^3) and allowed free access to water and standard laboratory food in a facility with the temperature controlled at 24±1 °C and relative humidity at 55±5% and with lights on from 7:00 to 19:00 h daily until the animals were used in the experiments. Experimental protocols were approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine and complied with the procedures outlined in the Guide for the Care and Use of Laboratory Animals of Tohoku University.

Drugs and reagents

YKS is composed of seven dried medicinal herbs: *Atractylodes lancea* rhizome (4.0 g, rhizome of *Atractylodes lancea* De Candolle), Poria sclerotium (4.0 g, sclerotium of *Poria cocos* Wolf), Cnidium rhizoma (3.0 g, rhizome of *Cnidium officinale* Makino), Japanese Angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa), Bupleurum root (2.0 g, root of *Bupleurum falcatum* Linné), glycyrrhiza (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher), and UH (3.0 g, thorn of *Uncaria rhynchophylla* Miquel). The dry powdered extracts of YKS and UH were supplied by Tsumura & Co. (Tokyo, Japan).

Aβ peptides (1-40 and 1-42) and thioflavin-T were obtained from the Peptide Institute (Osaka, Japan) and Sigma (St. Louis, MO, USA), respectively. Other reagents (analytical grade) used for analysis were purchased from commercial sources.

In vitro study to evaluate effect of YKS on Aβ aggregation

Measurement of thioflavin-T to evaluate Aβ aggregation was performed using the method described by Suemoto et al. (2004) with slight modifications. Aβ (20 μM) dissolved in 50 mM potassium phosphate buffer (pH 7.4) with YKS was incubated at 37 °C for 96 h ($Aβ_{1-40}$) or 24 h ($Aβ_{1-42}$). At the end of the incubation, 3 μM thioflavin-T dissolved in 100 mM glycine buffer (pH 8.5) was added to the mixture. After incubation for 30 min at room temperature, the fluorescence of thioflavin-T bound to Aβ aggregates was measured using a microplate reader (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA, USA) with excitation at 442 nm and emission at 485 nm. The percentage inhibition was calculated by comparing the fluorescence values of test samples with those of control solutions without YKS.

In vivo study to evaluate behaviors and accumulation of Aβ

Ten-month-old Tg(+) mice were randomly divided into five groups: Tg(+) (n=10), Tg(+)+0.3% YKS (n=10), Tg(+)+1.0% YKS (n=10), Tg(+)+0.1% UH (n=10), and Tg(+)+1.0% UH (n=10). Tg(−) mice (n=10) were set as the control group. The mice in both the Tg(−) and Tg(+) groups were given normal powdered chow for 5 months from 10 to 15 months old. The mice in the Tg(+)+0.3% YKS and Tg(+)+1.0% YKS groups were given the powdered chow including 0.3% or 1.0% of YKS for 5 months. The mice in the Tg(+)+0.1% UH and Tg(+)+1.0% UH groups were given the powdered chow including 0.1% or 1.0% of UH for 5 months.

Step-through passive-avoidance tests were performed to evaluate learning ability from the age of 11 months to 14 months. Social interaction tests were performed at the age of 15 months. All behavioral tests were performed between 10:00 and 17:00 h.

After completion of behavioral tests, all mice were decapitated, and the dissected cerebral cortex was used for determination of Aβ levels.

Step-through passive-avoidance test

The apparatus (TK402D model, Neuroscience, Inc., Tokyo, Japan) for the step-through passive-avoidance test consisted of two compartments, one illuminated [100×120×100 mm^3; light at the top of compartment (27 W, 3000 lx)] and the other dark (100×170×100 mm^3). The compartments were separated by a guillotine door. During the learning stage, a mouse was placed in the illuminated safe compartment. While this compartment was lit,

the mouse stepped through the opened guillotine door into the dark compartment. The time spent in the illuminated compartment was defined as the latency period. 3 s after the mouse entered the dark compartment, a foot shock (0.01 mA, 200 V, 50 Hz ac, for 1 s) was delivered to the floor grid in the dark compartment. The mouse could escape from the shock only by stepping back into the safe illuminated compartment. Such acquisition trials during the learning stage were carried out once a day for 5 days. The mouse was judged to have learned avoidance from the foot shock when the latency period reached 300 s. Retention trials were carried out once per week for 78 days (11–14-months-old) to evaluate the retention of avoidance memory. The latency was measured for up to 300 s without delivering a foot shock. It was judged that the mouse retained the avoidance memory when it stayed in the illuminated safe compartment for 300 s.

Social interaction test

Social interaction such as aggressive behavior and social behavior in mice were evaluated by a social interaction test (File, 1980) using a square box-type open-field apparatus (50×50×40 cm^3, Neuroscience, Inc., Tokyo, Japan). A video camera was mounted vertically over the apparatus. Two mice in each group were placed together in the open-field apparatus. The interactive behaviors between the two animals were monitored by the video camera for 10 min, and the behavioral data were saved directly on a computer. Then, the total number of aggressive behaviors (tail rattling, chasing, and attacking) as the index of aggressiveness or normal social behaviors (sniffing, following, and contacting) as the index of sociability of each animal was counted by two observers blind to the treatment. The total distance traveled (cm) of each animal was analyzed as motor activity by using software (analyzing behavior system, Viewer II, Bioserve, Bonn, Germany).

Measurement of brain Aβ levels

The dissected cerebral cortex was homogenized and sonicated in Tris–buffered saline (TBS) and 70% formic acid containing 1× protease inhibitor mixtures to obtain soluble and insoluble fractions with slight modification of the method described previously (Calon et al., 2004). The homogenate was subjected to ultracentrifugation at 200,000×g at 4 °C for 20 min. The soluble supernatant was collected and frozen. To analyze the insoluble Aβ, the insoluble pellet was sonicated in 200 μl of 70% formic acid and subjected to ultracentrifugation at 300,000×g at 4 °C for 30 min to collect the soluble supernatant.

Brain $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels were measured using sandwich ELISA with a Human β Amyloid ELISA Kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according to the manufacturer's instructions. BAN50 is a monoclonal antibody raised against a synthetic peptide of human $A\beta_{1\text{-}16}$; it preferentially reacts with the N-terminal portion of human Aβ starting at Asp-1, but does not cross-react with N-terminal–truncated Aβ or with rodent-type Aβ. BA27 and BC05, which specifically recognize the C terminus of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, respectively, were conjugated with horseradish peroxidase and used as detector antibodies. The insoluble mouse brain fractions described above were neutralized and subjected to BAN50/BA27 or BAN50/BC05 ELISA. The protein concentration of the fraction was measured using a protein assay kit (Bio-Rad Lab., Hercules, CA, USA). Finally, the Aβ value was expressed as pmol per g of protein.

Data analysis

Data are expressed as mean±SEM. The date of passive-avoidance test was evaluated by analysis of variance (Kruskal-Wallis) followed by a Mann-Whitney *U* test. The data of other experiments were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn tests. The significance level in each statistical analysis was accepted at $P<0.05$.

RESULTS

Effects of YKS on Aβ aggregation *in vitro*

The effects of YKS on $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ aggregation *in vitro* are shown in Fig. 1A, B, respectively. YKS inhibited the aggregation of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in a concentration-dependent manner. Significant inhibition was observed at 10 and 100 μg/ml for $A\beta_{1\text{-}40}$ and at 100 μg/ml for $A\beta_{1\text{-}42}$.

Effects of YKS and UH on memory disturbance in Tg(+) mice

Step-through passive-avoidance tests were carried out on mice at 11–14 months of age. In the first acquisition trial of the learning stage, all mice (11 months old) in the Tg(−), Tg(+), Tg(+)+YKS, and Tg(+)+UH groups entered the dark compartment immediately after being placed in the illuminated compartment. Repeating the acquisition trial increased the latency times in all groups. All mice in all

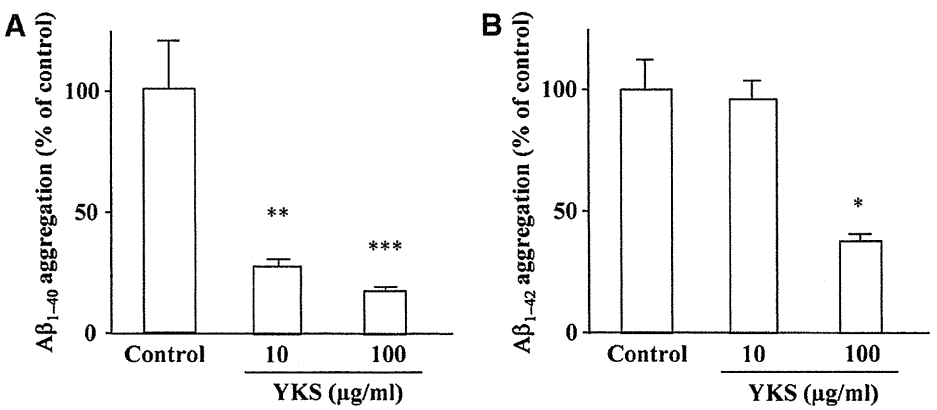

Fig. 1. Effects of YKS on $A\beta_{1\text{-}40}$ (A) or $A\beta_{1\text{-}42}$ (B) aggregation. Aβ aggregation was assessed by the thioflavin T method and expressed as the percentage of control aggregation in the absence of YKS. Values represent mean±SE from four independent experiments. Significance by Bonferroni/Dunn tests following one-way ANOVA is indicated as * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control.

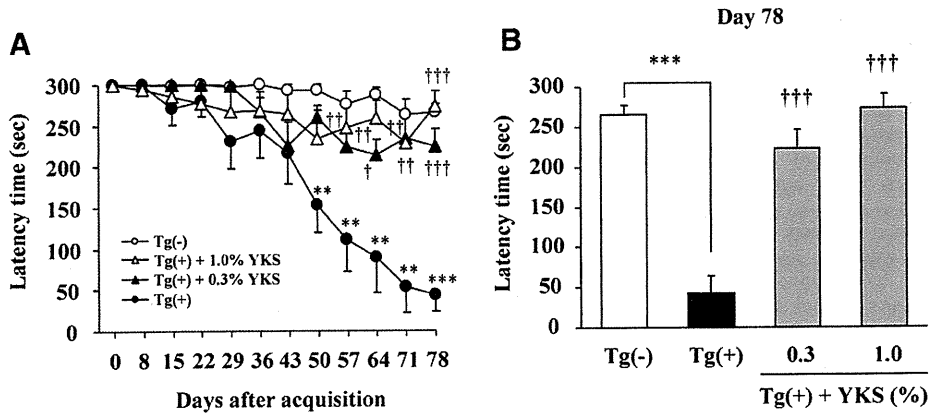

Fig. 2. Step-through latencies in the retention test of the passive-avoidance task in YKS-treated Tg mice. Changes in the latencies during retention tests for 78 d after acquisition of avoidance memory in each group are shown in (A), and the final results on day 78 are shown in (B). Values represent the means±SE (n=10 in each group). Significance by Mann-Whitney U test following analysis of variance (Kruskal-Wallis) is indicated as ** P<0.01, *** P<0.001 vs. corresponding Tg(−) control, and † P<0.05, †† P<0.01 and ††† P<0.001 vs. Tg(+) on each day.

groups acquired avoidance memory, staying in the illuminated compartment over 300 s on the fifth day. No statistically significant differences were observed in the mean latency times among all groups during the acquisition trials (data not shown).

Memory retention tests were performed once a week for 78 days after the final acquisition trial. Changes in the step-through latency in the YKS-treated groups are shown in Fig. 2A, and the results on the terminal day 78 are shown in Fig. 2B. The latency time of the Tg(+) group was significantly shorter than that of the Tg(−) group. The shorter latency was significantly prolonged by treatments with 0.3 and 1.0% YKS.

Changes in the step-through latency in UH-treated groups are shown in Fig. 3A, and then the results on the terminal day 78 are shown in Fig. 3B. The shortened latency time of the Tg(+) group was significantly prolonged by treatment with UH (0.1% and 1.0%) in a dose-dependent manner.

Effects of YKS and UH on aggressiveness and sociability in Tg(+) mice

The effects of YKS on aggressive behavior, social behavior, and motor activity are shown in Fig. 4. The aggressive behavior in the Tg(+) group increased significantly more than that in the Tg(−) group. The increase was significantly inhibited by treatment with 1.0% YKS (Fig. 4A). On the other hand, social behavior in the Tg(+) group decreased significantly more than that in the Tg(−) group. The decrease was significantly inhibited by treatment with 1.0% YKS (Fig. 4B). No significant differences of the motor activities (distance) were observed between Tg(−), Tg(+), and Tg(+)+YKS groups (Fig. 4C).

The effects of UH on aggressive behavior, social behavior, and motor activity are shown in Fig. 5. The aggressive behavior in the Tg(+) group increased significantly more than that in the Tg(−) group. The increase was

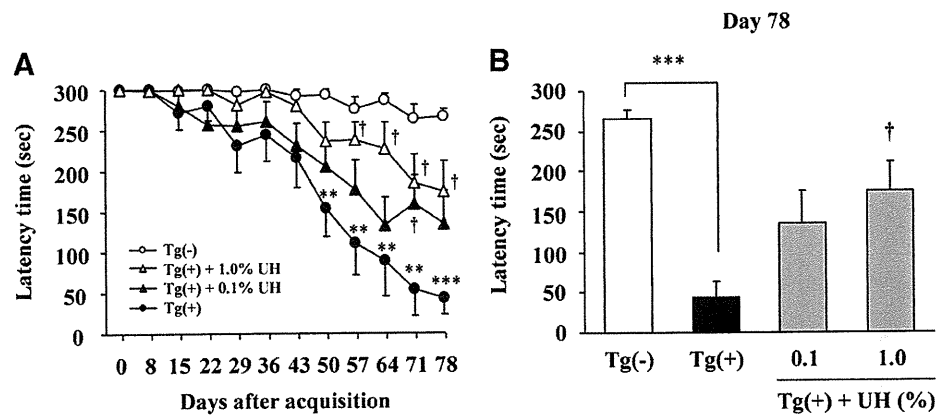

Fig. 3. Step-through latencies in the retention test of the passive-avoidance task in UH-treated Tg mice. Changes in the latencies during retention tests for 78 d after acquisition of avoidance memory in each group are shown in (A), and the final results on day 78 are shown in (B). All mice acquired avoidance memory by five repeated acquisition trials. Values represent the means±SE (n=10 in each group). Significance by Mann-Whitney U test following analysis of variance (Kruskal-Wallis) is indicated as ** P<0.01, *** P<0.001 vs. corresponding Tg(−) control, and † P<0.05 vs. Tg(+) on each day.

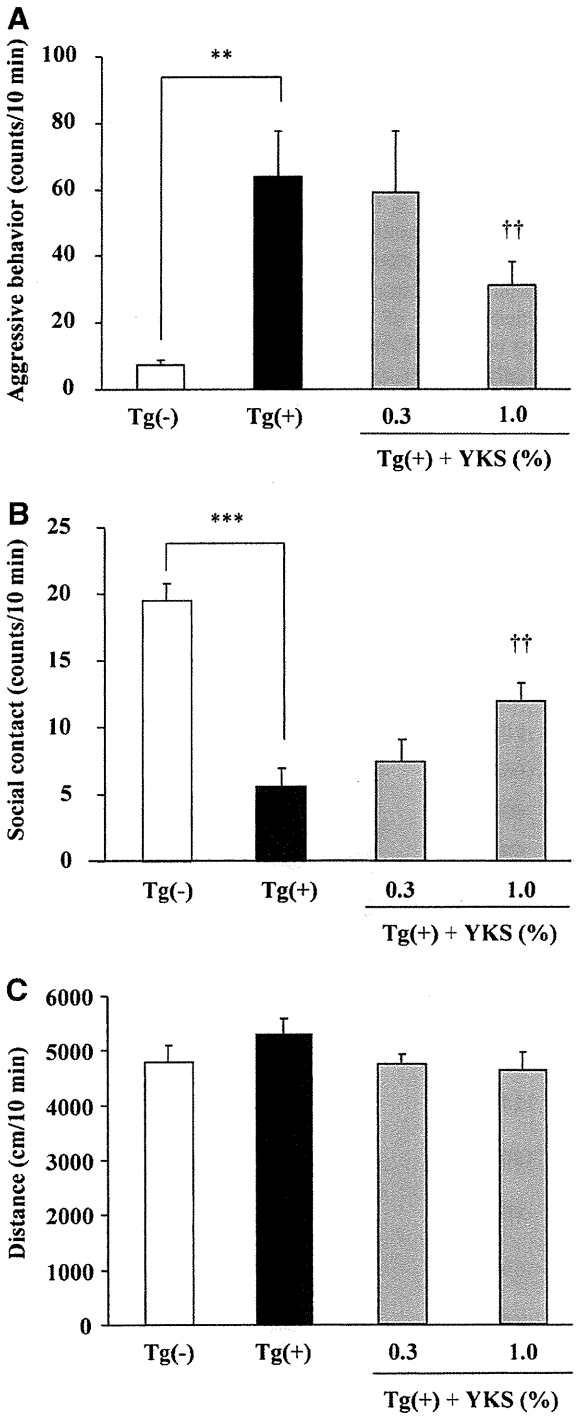

Fig. 4. Aggressive behavior (A), social contact (B), and distance as indexes of motor activity (C) of YKS-treated mice in the social interaction test. Value represents the mean±SE (n=10 in each group). Significance by Bonferroni/Dunn tests following one-way ANOVA is indicated as ** P<0.01, *** P<0.001 vs. Tg(−), and †† P<0.01 vs. Tg(+).

significantly inhibited by treatment with 1.0% UH (Fig. 5A). On the other hand, social behavior in the Tg(+) group decreased significantly more than that in the Tg(−) group. The decrease was significantly inhibited by treatment with 1.0% UH (Fig. 5B). No significant differences of the motor activities were observed between Tg(−), Tg(+), and Tg(+) + UH groups (Fig. 5C).

Effects of YKS and UH on cerebral Aβ levels in Tg(+) mice

To determine whether oral YKS or UH treatment affected the accumulation of Aβ in cerebral cortex, the cortical $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels were measured. The results are shown in Fig. 6A, B, respectively. Large amounts of both forms of Aβ were detected in the cortex of Tg(+) mice but not in Tg(−) mice. YKS or UH treatment had no significant effect on $A\beta_{1\text{-}40}$ levels in the Tg(+) mice (Fig. 6A). However, both YKS and UH inhibited $A\beta_{1\text{-}42}$ accumulation in Tg(+) mice in a dose-dependent manner (Fig. 6B).

DISCUSSION

Aβ is thought to be a causative substance of AD (Hsiao et al., 1996; Selkoe, 2002; Wirths et al., 2004). We previously demonstrated that UH (10 and 100 μg/ml) had a potent anti-aggregation effect on Aβ proteins *in vitro*, in a concentration-dependent manner (Fujiwara et al., 2006). In the present *in vitro* study, we demonstrated that YKS (100 μg/ml) significantly inhibited Aβ aggregation as shown in Fig. 1. UH is contained 14.6% in YKS, that is, 14.6 μg/ml of UH is contained in 100 μg/ml of YKS. This 14.6 μg/ml concentration is included within the range of effective concentrations (Fujiwara et al., 2006). Therefore, the anti-aggregation effect of YKS is suggested to be attributed to UH. It is important to verify whether *in vitro* results are reflected *in vivo*. However, it is difficult to compare effective dose or concentration directly between *in vitro* and *in vivo* experiments, because the experimental conditions are different between them. In the present *in vivo* study, YKS or UH were given to animals as diets containing 0.3 and 1.0% YKS, or 0.1 and 1.0% UH. The YKS intake levels in 0.3 and 1.0% YKS groups corresponded to 240 and 800 mg/kg/d when the levels were calculated from food consumption. Similarly, the UH intake levels in 0.1 and 1.0% UH groups corresponded to 80 and 800 mg/kg/d. As shown in Fig. 6B, we found that both YKS and UH dose-dependently inhibited $A\beta_{1\text{-}42}$ accumulation in the cerebral cortex of Tg(+) mice. As 800 mg/kg/d of YKS contains 117 mg/kg/d (14.6%) which is included within the range of 80 and 800 mg/kg/d of UH, we suggest the possibility that YKS (800 mg/kg/d) containing UH (117 mg/kg/d) has the anti-aggregation effect as well as the *in vitro* study. This is a first finding demonstrating that YKS and UH inhibit the accumulation of Aβ *in vivo*.

The Tg(+) mice used in the present study are known to develop disturbance of memory or cognitive function (Hsiao et al., 1996; Wegiel et al., 2001; Barnes et al., 2004; Ikarashi et al., 2004). In the present study, we evaluated the memory disturbance in Tg(+) mice using a step-through passive avoidance test. In the retention test, the latency time of the Tg(+) mice was significantly shorter

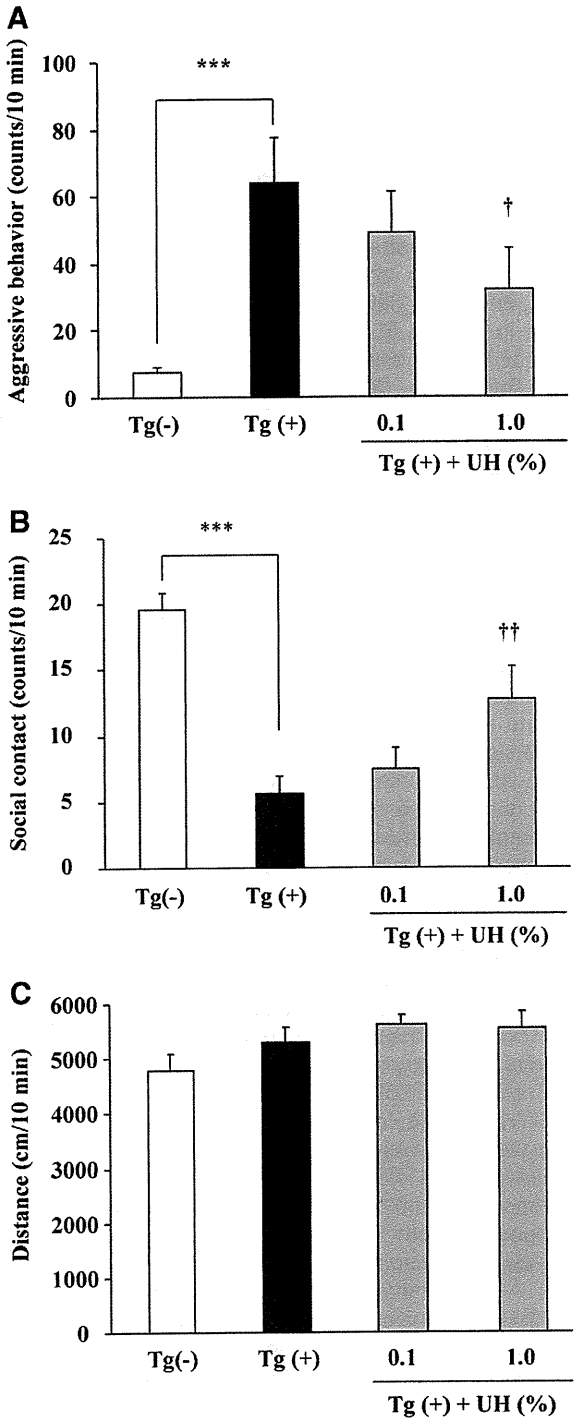

Fig. 5. Aggressive behavior (A), social contact (B), and distance as indexes of motor activity (C) of UH-treated mice in the social interaction test. Value represents the mean±SE (n=10 in each group). Significance by Bonferroni/Dunn tests following one-way ANOVA is indicated as *** $P<0.001$ vs. Tg(−), and † $P<0.05$, †† $P<0.01$ vs. Tg(+).

than that of the Tg(−) mice. The shorter latency in Tg(+) mice was significantly prolonged by treatment with YKS or UH. Though the prolongation of latency time is well-known to be affected by drug-dependent physical effects such as catalepsy and suppression of motor activity, we previously demonstrated that YKS did not induce them as haloperidol or risperidone does (Sekiguchi et al., 2009). Therefore, the ameliorative data of YKS and UH against the shorter latency in Tg(+) mice is thought to be not due to the physical disturbance, that is, these changes are selective to memory function, suggesting that YKS and UH might ameliorate the memory disturbance in Tg(+) mice.

Up till now, Tateno et al. (2008) demonstrated neuroprotective effects of YKS on Aβ-induced cytotoxicity in a primary culture of rat cortical neurons. We also recently demonstrated not only neuroprotective effects of YKS on glutamate-mediated excitotoxicity in cultured cells (Kawakami et al., 2009, 2010) but also ameliorative effects of YKS on learning and memory disturbance induced by i.c.v. injection of Aβ in mice (Sekiguchi et al., in press) and thiamine deficiency in rats (Ikarashi et al., 2009). These findings suggest that YKS has neuroprotective effects as one of the mechanisms. In addition to the mechanism, the

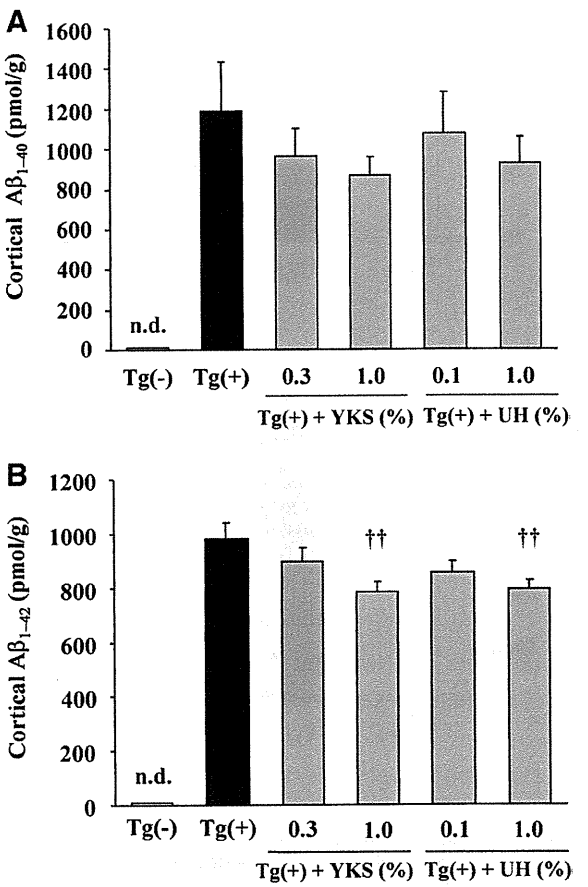

Fig. 6. Effects of YKS and UH on cortical concentrations of $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) in Tg mice. Large amounts of both forms of Aβ were detected in the cortex of Tg(+) mice but not in Tg(−) controls (n.d.). Values represent the mean±SE (n=10 in each group). Significance by Bonferroni/Dunn tests following one-way ANOVA is indicated as †† $P<0.01$ vs. Tg(+).

present results newly suggest a possibility that YKS ameliorates memory disturbance by preventing the aggregation of Aβ, which may be attributed to UH. To strongly support the participation of UH in the future, it will be necessary to confirm disappearance of the ameliorative effect of YKS by elimination of UH.

Kawarabayashi et al. (2001) demonstrated that Aβ deposit in the brain was started in the late stage (8–10 month old) though Aβ was detected biochemically in the early stage (4–5 month old). In the present study, YKS or UH was administered for 5 months from 10 to 15-month-old. In the acquisition trial for the learning at 11-month-old, no significant differences were observed in the latency times among all groups, suggesting that 11-month-old Tg(+) mice possess learning ability as well as Tg(−) control mice. However, the retention memory in the Tg(+) mice gradually decreased during 10- and 15-month-old during which Aβ deposits are facilitated. These data suggest close relation between Aβ deposit and memory disturbance. As YKS and UH ameliorated the memory disturbance in Tg(+) mice, these medicines are thought to have the preventing effect of memory disturbance.

On the other hand, in the present study, though it is true that YKS and UH statistically decreased Aβ accumulation, large amount of Aβ still existed in the brain: nevertheless, memory disturbance was ameliorated by treatment with YKS or UH. As a possible explanation for this ameliorative effect, synergistic effect of YKS including neuroprotective effect and inhibitory effect of Aβ aggregation is inferred. Furthermore, the improvement of cognition with YKS treatment was not demonstrated in clinical trials, most of which were evaluated in the comparatively short term of 4 weeks (Iwasaki et al., 2005a; Mizukami et al., 2009). To prove our hypothesis in the clinical trial, a long-term trial will be necessary in the future.

In patients with dementia, not only core symptoms but also BPSD often emerge. YKS has been reported to ameliorate BPSD such as hallucinations, agitation, and aggression in patients with AD, DLB, and other forms of senile dementia (Iwasaki et al., 2005a,b; Mizukami et al., 2009; Shinno et al., 2007, 2008). In the present study, the development of BPSD-like behaviors such as a marked increase in aggressive behavior and decrease in social behavior was observed in the Tg(+) mice, and YKS or UH ameliorated those abnormal behaviors. These effects, evaluated using social interaction tests, also are known to be influenced by changes in drug-dependent motor activity. In this test, we measured the traveling distance as an index of motor activity together with the interactive behavior, and confirmed that no significant difference in motor activity was observed among all groups. Therefore, the amelioration of aggression and sociability by YKS and UH are suggested to be a direct effect, not due to the secondary effect induced by the suppression of motor activity. The ameliorative effects by YKS of abnormal aggressiveness and sociability in Tg(+) mice are thought to support the finding in the clinical studies reporting that YKS ameliorated excitement, anger and decrease in activities of daily living in patients with AD (Iwasaki et al., 2005a).

Two mechanisms are inferred from the BPSD-ameliorating effect of YKS. One putative mechanism is that the effect may be obtained by inhibiting Aβ accumulation, which has already been discussed. However, it might be difficult to demonstrate this possibility by clinical studies because several clinical studies that evaluated YKS with a 4-week treatment period showed improvement in BPSD without amelioration of cognitive dysfunction in patients with dementia (Iwasaki et al., 2005a; Mizukami et al., 2009). This finding suggests another mechanism or possibility that YKS has a quicker improving effect on some neuronal function than the putative ameliorative effect on memory dysfunction and Aβ accumulation. Takeda et al. (2008a,b) reported that YKS attenuated the abnormal increase in cerebral glutamate release in zinc-deficient rats. Ikarashi et al. (2009) demonstrated YKS inhibited the increase in the cerebral extracellular concentration of glutamate in thiamine-deficient rats. Egashira et al. (2008) reported that YKS inhibited the 5-HT 2A receptor agonist-induced heat-twitch response by decreasing expression of 5-HT2A receptors in the prefrontal cortex. Terawaki et al. (2010) demonstrated *in vitro* that YKS and UH showed a partial agonistic effect on 5-HT1A receptors. This *in vitro* finding was also supported by *in vivo* experiment demonstrating that YKS ameliorated abnormal aggressiveness and sociability observed in para-chloroamphetamine-induced cerebral 5-HT-depletion rats, and the ameliorative effect was counteracted by co-administration of a 5-HT1A receptor antagonist, WAY-100635 (Kanno et al., 2009). Taken together, the ameliorative effects of YKS and UH on abnormal aggressive and social behaviors in Tg(+) mice may also relate to the glutamateric and serotonergic functions.

YKS has been used in Asian countries as a remedy for restlessness and agitation in children since it was developed by Xue Kai in 1555 (Iwasaki et al., 2005a). Recently, approximately over 100 million packages of YKS were sold during a year in Japan. Recent accumulated clinical studies reported not only the usefulness of YKS on BPSD but also information on the side effects. Mizukami et al. (2009) reported the appearance of hypokalaemia (two patients), gastrointestinal symptoms including vomiting/diarrhoea, nausea, epigastric distress (three patients), sedation (one patient), and leg edema (one patient) in a randomized cross-over study of YKS using 106 patients with dementia. In particular, the kampo medicines including glycyrrhiza, such as YKS, have been well-known to sometimes cause hypokalaemia (Ohtake et al., 2007; Makino et al., 2008). Therefore, the serum potassium concentration should be monitored.

CONCLUSION

In conclusion, the present study demonstrated that YKS inhibited accumulation of Aβ fibrils *in vitro* and *in vivo*. As a result, it improved not only memory deficits but also BPSD-like behaviors such as increased aggressive behavior and decreased social behavior in the APP transgenic

mice. Therefore, YKS may have potential as a therapeutic drug for patients with AD and mild cognitive impairment.

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EDITORIAL

A comprehensive strategy for dementia from primary prevention to end-stage management

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On the afternoon of 11 March 2011, as I was writing this editorial comment, the 9.0-magnitude earthquake hit the north-east part of Japan.[1] Coastal cities and towns in Miyagi Prefecture and neighbouring Iwate and Fukushima prefectures were unimaginably damaged by tsunamis. It has been estimated that over 25 000 people lost their lives or are missing. Most of the victims are reported to have drowned.[2] I was deeply heartbroken that many precious lives were cut short; I pray for them in this time of immeasurable loss. I was worried about my son because I could not reach him by phone for 5 days, but fortunately he was safe. The temperature inside our institute dropped to below freezing in the mornings and evenings because the heating system broke. During this time, I uneasily continued writing at the institute while eating supplied rice balls and being frightened by frequent aftershocks.

CURRENT PRACTICAL APPROACH TOWARD ALZHEIMER'S DISEASE

Over the past 20 years, our understanding of the molecular pathology of dementia disorders has deepened, and new diagnostic techniques and therapeutic strategies have been developed.[3] However, the number of patients with dementia has been rapidly increasing, reflecting the advent of the super-aged society, which has a strong effect on the health-care system and medical economy. Currently, 27 million people in Japan, more than 23% of the population, are 65 years or older. To cope with these demographic shifts, Tohoku University Hospital's outpatient Department of Geriatrics & Gerontology opened a memory clinic in 1991 for patients with memory loss. Many other memory clinics have subsequently opened throughout Japan. In addition, both the Japanese Psychogeriatric Society and the Japanese Society of Dementia Research have established educational programs to ensure that physicians have the expertise necessary to treat dementia patients.

From its earliest stages, dementia has a lifespan of approximately 30 years. Figure 1 outlines the life of the disease, spanning from an individual's cognitively healthy condition to the development of dementia and death, as well as important medical issues in each phase. The first two-thirds of the chart cover 20 years during which a healthy person gradually changes and develops a mild cognitive impairment and dementia. The latter third covers 10 years, over which dementia progressively worsens from mild stage to advanced stage and eventually leads to death. In patients with Alzheimer's disease, the first change in the brain is believed to be triggered by aggregation and accumulation of a small hydrophobic peptide called amyloid-β protein that is known to be toxic to neurons.[3] Toxicity may develop extremely slowly, inducing abnormal phosphorylation and polymerisation of tau protein, loss of microtubule function and eventually neuronal death. The clinical symptoms such as memory loss first become obvious when the residual ability of surviving neurons is outpaced by neuron death. For dementia

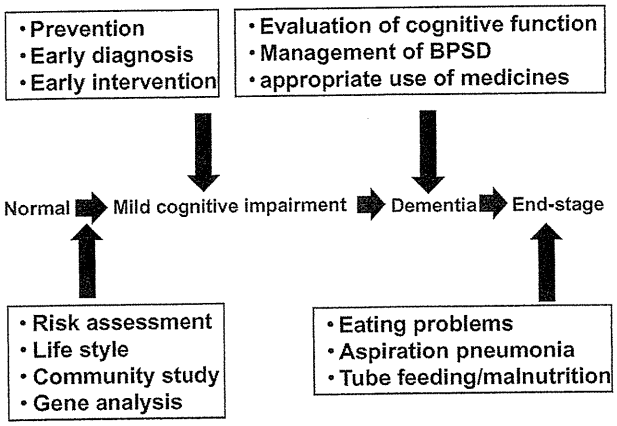

Figure 1. Several important issues at various stages of dementia are shown in a timeline beginning with the development of dementia in healthy persons and ending at the terminal stage and death. Medical assessment of dementia should be a persistent and comprehensive process involving prevention of dementia in the pre-symptomatic stage, pharmacological and non-pharmacological treatment, and treatment of eating problems, aspiration pneumonia and nutritional problem in the end-stage. In the future, the treatment framework for dementia will shift from treatment after cognitive decline begins to prevention with preemptive therapy for high-risk individuals.

in general, understanding of pathological conditions has increased along with our growing knowledge of molecular species that accumulate in the brain, such as α-synuclein in dementia with Lewy bodies and TDP-43 in frontotemporal lobar degeneration.

To fight dementia, it is essential that doctors approach treatment with a clear understanding of the entire process, from primary prevention and preemptive care to end-stage management. Since all people do not develop dementia, it is important to know the risk factors, including genetic predisposition and environmental factors, related to the development of dementia. For this purpose, a prospective cohort study with healthy community residents is valuable. The strongest genetic risk factor widely confirmed in a large-scale epidemiological study was the apolipoprotein (Apo)E4 gene. Along with biomarker development, including amyloid imaging techniques, the Alzheimer's Disease Neuroimaging Initiative has shown that amyloid-β protein may begin to accumulate in people with the apoE4 gene in their 50s, when their cognitive function is subjectively and objectively still considered to be normal.[4,5] Furthermore, several prospective cohort studies have clarified the close relationship between dementia development and mid-life lifestyle, specifically factors such as physical exercise and social engagement.[6,7] Amyloid imaging, fluorodeoxyglucose-PET, and cerebrospinal fluid biomarkers have contributed to early detection of dementia by acting as surrogate biomarkers that reflect the underlying pathological process.[8,9] In the future, standardisation and quality control of biomarkers in clinical trials of new drugs will be needed.

In 2011, new pharmacological treatments for dementia were released in Japan for the first time since 1999, when donepezil hydrochloride developed by Mr Hachiro Sugimoto of Eisai Co., Ltd. (Tokyo, Japan) was approved. Three new drugs, including two cholinesterase inhibitors and one *N*-methyl *D*-aspartate receptor antagonist, have become available for symptomatic treatment of Alzheimer's disease. Though they were released at least 10 years ago in the USA and Europe, the launch of these new drugs in Japan increased the options for the treatment of dementia. It is anticipated that general physicians will use anti-dementia drugs more frequently, but if they have little experience with diagnosing and treating dementia, doctors should introduce their patients to memory clinics to ensure that the treatment is appropriate for the diagnosis. In actual clinical practice, physicians require a large body of knowledge pertaining to management of lifestyle-related diseases,[6] diagnosis of rare dementia such as prion disease, differential diagnosis for depression and delirium, pharmacological and non-pharmacological approaches to behavioural and psychological symptoms of dementia,[10] and information on the safety/adverse effects of drug treatments, particularly with regard to older patients.[11]

DEVELOPMENT OF DISEASE-MODIFYING DRUGS AND PREEMPTIVE THERAPY

Many clinical trials of disease-modifying drugs have been suspended or unsuccessful.[12] Disease modification is a therapeutic method that aims to produce clinical benefits by stopping or delaying the process of nerve cell death and damage to nerve function. The clinical trial of active immunisation of amyloid (AN-1792) was suspended due to the serious adverse effect of autoimmune meningoencephalitis. Although a Phase II clinical trial of bapineuzumab, a passive immunisation of humanised monoclonal antibodies to amyloid, was performed in patients with mild to moderate Alzheimer's dementia for 18 months, no efficacy was observed on cognitive function or daily activities.

At present, a Phase III clinical trial comparing subject groups with apoE4 gene to those without the gene is being performed. The clinical trial of semagacestat, a γ-secretase inhibitor, was highly anticipated, but it has been discontinued because members of the active treatment group experienced a significant decrease in cognitive function and developed skin cancer; this did not occur in the placebo group. Phase III clinical trials were completed for tramiprosate, an amyloid aggregation inhibitor, and tarenflurbil, a γ-secretase modulator, but results were negative because no significant difference was found between the active treatment and placebo groups. Why did the clinical trials of these disease-modifying drugs not succeed? Many researchers think that these disease-modifying drugs might have been administered too late. Even in patients with mild Alzheimer's disease, a massive accumulation of amyloid and extensive nerve cell death may have already occurred. This raises the question of what benefits can be obtained by disease-modification – the elimination of amyloid – at this stage. To answer this question, the Alzheimer's Association and the National Institute on Aging in the USA plan to renew common conceptions of Alzheimer's disease.[13] This bold proposal involves diagnosing Alzheimer's disease when evidence shows the beginning of the disease process. For example, a positive finding of amyloid imaging or abnormal tau values in cerebrospinal fluid, even if no symptoms of Alzheimer's disease are observed, would be initial evidence of the disease process. This stage is referred to 'preclinical Alzheimer's disease'. Influenced by the Alzheimer's Prevention Initiative, led by Dr Reiman at the Banner Alzheimer's Institute (Phoenix, Arizona, USA), preemptive therapy with disease-modifying drugs in the preclinical Alzheimer's disease phase is rapidly gaining adherents.[14,15]

In Antioqiua in the northwest of Colombia, there is a significant occurrence of familial Alzheimer's disease related to the E280A presenilin (PS)–1 mutation. The E280A PS-1 mutation results in the clinical presentation of Alzheimer's disease usually when the affected individual is 48 years old. Currently, 1235 persons have undergone genetic testing, and of the people carrying the mutation, 480 have not developed the disease yet. There are plans for 1000 persons with the mutation, including some as young as 18, to eventually participate in clinical trials. According to Dr Lopera of the University of Antioquia, the study will include functional MRI, fluorodeoxyglucose-PET, Pittsburgh compound B-PET, and will sample cerebrospinal fluids in as many as subjects during the 18-month clinical trial.

The unsuccessful clinical trials of disease-modifying drugs suggest there are potentially other underlying causes of the failure such as insufficient understanding on Alzheimer's disease pathology, pitfalls in clinical diagnosis, inappropriate drug development based on the amyloid hypothesis and insufficient study design. It is intuitively understandable that developing disease-modifying drugs can no longer simply be expected to improve symptoms. Given these issues, it is important to consider how we should promote drug development for dementia. Dr Tariot of the Banner Institute has advocated that clinical trial should first be performed with healthy but high-risk subjects, such as carriers of the ApoE4 gene. If the safety of an anti-amyloid drug can be sufficiently ensured, then clinical benefits should be tested in patients who have developed Alzheimer's disease. We should redevelop research and drug development strategies for disease-modifying drugs by examining whether preemptive treatment will be the best defence. With the development of disease-modifying drugs, Alzheimer's Disease Neuroimaging Initiative will potentially take the lead in preventing Alzheimer's disease.[7]

MANAGEMENT OF DEMENTIA IN END-OF LIFE

At the 52nd annual meeting of the Japanese Geriatrics Society in Kobe City in 2010, a symposium was held that outlined the public's expectations of geriatricians. Geriatricians were expected to correctly diagnose dementia and to provide consultations concerning patients in the terminal stages. Similarly, over the next decade, the appropriate use of anti-dementia drugs, managing behavioural and psychological symptoms of dementia, and helping caregivers manage the stress resulting from their duties will be major issues relating to latter period of dementia treatment. The last phase of treatment will be care for patients in the terminal stage and deathwatch. Unlike cancer patients, dementia patients in Japan are not allowed to be treated in hospice. However, I believe that this should be changed and hospice should cover terminal stages. For this reason, there is a great need for continuous collaboration between medical service providers and caregivers. In 2009 in the *New England Journal of Medicine*, Mitchell *et al.* published the