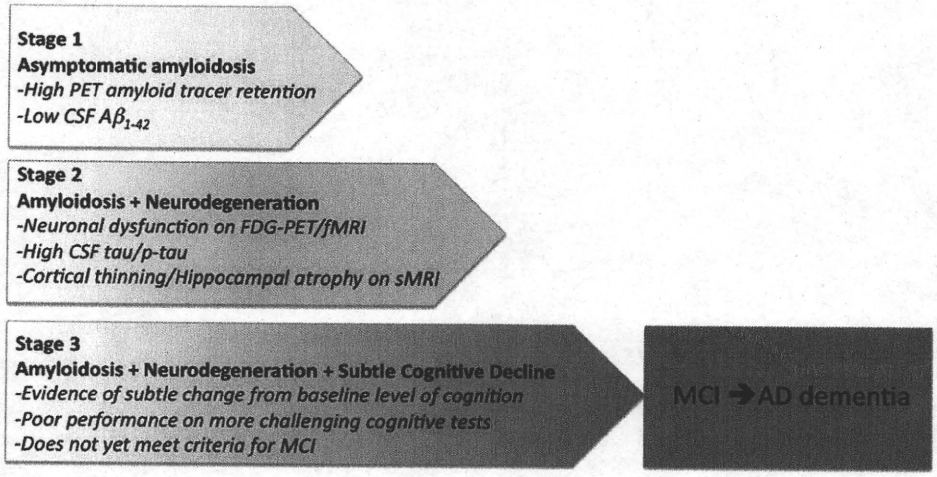

Fig. 5. Graphic representation of the proposed staging framework for preclinical AD. Note that some individuals will not progress beyond Stage 1 or Stage 2. Individuals in Stage 3 are postulated to be more likely to progress to MCI and AD dementia. Abbreviations: AD, Alzheimer's disease; Ab, amyloid beta; PET, position emission tomography; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose, fMRI, functional magnetic resonance imaging, sMRI, structural magnetic resonance imaging.

will likely require further modification as new findings emerge, and that the feasibility of delineating these stages in recruiting clinical research cohorts remains unclear. It may be easiest to recruit individuals on the basis of Aβ positivity and perform post hoc analyses to determine the predictive value of specific combinations of biomarker abnormalities. These proposed research criteria are intended to facilitate the standardized collection of new data to better define the spectrum of preclinical AD, and to elucidate the endophenotype of individuals who are most likely to progress toward AD-C.

9.1. Stage 1: The stage of asymptomatic cerebral amyloidosis

These individuals have biomarker evidence of Aβ accumulation with elevated tracer retention on PET amyloid imaging and/or low $A\beta_{42}$ in CSF assay, but no detectable evidence of additional brain alterations suggestive of neurodegeneration or subtle cognitive and/or behavioral symptomatology. The standards for determining "amyloid-positivity" are still evolving (refer to the next section). Although recent work suggests there may be a CSF $A\beta_{42}$ cutoff value that is predictive of progression from MCI to AD dementia [66], it is unknown whether a similar threshold will be optimal in prediction of decline in individuals with normal or near normal cognition. Similarly, using PET imaging techniques, it remains unknown whether a summary numeric threshold within an aggregate cortical region or within specific anatomic region will provide the most useful predictive value. Recent data suggest that although CSF $A\beta_{42}$ is strongly inversely correlated with quantitative PET amyloid imaging measures (distribution value ratio or standardized uptake value), there are some individuals who demonstrate decreased CSF $A\beta_{42}$ and who would not be considered amyloid positive on PET scans [67]. It remains unclear whether this finding reflects different thresholds used across these techniques or if decreased CSF $A\beta_{42}$ is an earlier marker of accumulation. In addition, there may be genetic effects that are specific to CSF or PET markers of Aβ.

As mentioned previously, we note that the currently available CSF and PET imaging biomarkers of Aβ primarily provide evidence of amyloid accumulation and deposition of fibrillar forms of amyloid. Although limited, current data suggest that soluble or oligomeric forms of Aβ are likely in equilibrium with plaques, which may serve as reservoirs, but it remains unknown whether there is an identifiable preplaque stage in which only soluble forms of Aβ are present. Because laboratory data increasingly suggest that oligomeric forms of amyloid may be critical in the pathological cascade, there is ongoing work to develop CSF and plasma assays for oligomeric forms of Aβ. There are also emerging data from genetic-risk cohorts that suggest early synaptic changes may be present before evidence of amyloid accumulation using currently available amyloid markers. Thus, it may be possible in the future to detect a stage of disease that precedes stage 1.

9.2. Stage 2: Amyloid positivity + evidence of synaptic dysfunction and/or early neurodegeneration

These individuals have evidence of amyloid positivity and presence of one or more markers of "downstream" AD-P-related neuronal injury. The current markers of neuronal injury with the greatest validation are: (1) elevated CSF tau or phospho-tau, (2) hypometabolism in an AD-like pattern (i.e., posterior cingulate, precuneus, and/or temporoparietal cortices) on FDG-PET, and (3) cortical thinning/gray matter loss in a specific anatomic distribution (i.e., lateral

and medial parietal, posterior cingulate, and lateral temporal cortices) and/or hippocampal atrophy on volumetric MRI. Future markers may also include fMRI measures of default network connectivity. Although previous studies have demonstrated that, on average, amyloid-positive individuals demonstrate significantly greater abnormalities on these markers as compared with amyloid-negative individuals, there is significant interindividual variability. We hypothesize that amyloid-positive individuals with evidence of early neurodegeneration may be farther down the trajectory (i.e., in later stages of preclinical AD). It remains unclear whether it will be feasible to detect differences among these other biomarkers of AD-P, but there is some evidence that early synaptic dysfunction, as assessed by functional imaging techniques such as FDG-PET and fMRI, may be detectable before volumetric loss.

9.3. Stage 3: Amyloid positivity + evidence of neurodegeneration + subtle cognitive decline

We postulate that individuals with biomarker evidence of amyloid accumulation, early neurodegeneration, and evidence of subtle cognitive decline are in the last stage of preclinical AD, and are approaching the border zone with the proposed clinical criteria for MCI. These individuals may demonstrate evidence of decline from their own baseline (particularly if proxies of cognitive reserve are taken into consideration), even if they still perform within the "normal" range on standard cognitive measures. There is emerging evidence that more sensitive cognitive measures, particularly with challenging episodic memory measures, may detect very subtle cognitive impairment in amyloid-positive individuals. It remains unclear whether self-complaint of memory decline or other subtle neurobehavioral changes will be a useful predictor of progression, but it is possible that the combination of biomarkers and subjective assessment of subtle change will prove to be useful.

10. Need for additional study

We propose a general framework with biomarker criteria for the study of the preclinical phase of AD; however, more work is needed to clarify the optimal CSF assays, PET or MRI analytic techniques, and in particular, the specific thresholds needed to meet these criteria. There are significant challenges in implementing standardized biomarker "cut-off" values across centers, studies, and countries. Work to standardize and validate both fluid-based and imaging biomarker thresholds is ongoing in multiple academic and pharmaceutical industry laboratories, as well as in several multicenter initiatives. These criteria will need to be validated in large multicenter natural history studies, or as provisional criteria for the planning of preventative clinical trials. For instance, it will be important to establish the test–retest and cross-center reliability of biomarker measurements, further characterize the sequence of biomarker changes, and the extent to which these biomarkers predict subsequent clinical decline or clinical benefit. In particular, there is an important need to evaluate methods for determining "amyloid-positivity" because it remains unclear whether there is a biologically relevant continuum of Aβ accumulation, or whether there is a clear threshold or "cut-off" value that could be defined on the basis of predictive value for subsequent clinical decline, as has been suggested in several CSF studies [28,66]. It also remains unknown whether these thresholds should be adjusted for age or genotype. After these thresholds are established, it may be most feasible to select research cohorts for large studies solely on the basis of "amyloid-positivity" on CSF or PET amyloid imaging, and to use additional biomarker and cognitive measures for post hoc analyses to determine additional predictive value.

Although recent advances in biomarkers have revolutionized our ability to detect evidence of early AD-P there is still a need for novel biomarker development. In particular, although the current biomarkers provide evidence of Aβ deposition, an *in vivo* marker of oligomeric forms of Aβ would be of great value. Imaging markers of intraneuronal pathology, including specific markers of specific forms of tau/tangles and alpha-synuclein, are also needed. In addition, more sensitive imaging biomarkers that can detect early synaptic dysfunction and functional and structural disconnection, such as fMRI and diffusion tensor imaging, may one day prove to be useful to track early response to amyloid-lowering therapies. Finally, we may be able to use the currently available biomarkers as a new "gold standard" to re-evaluate simple blood and urine markers that were discarded on the basis of excessive overlap between clinically normal and AD patients. The significant proportion of clinically normal individuals who are "amyloid-positive" on both CSF and PET imaging may have confounded previous studies attempting to differentiate "normal" controls from patients with AD.

Similarly, additional work is required to identify and validate neuropsychological and neurobehavioral measures to detect the earliest clinical manifestations of AD. We need to develop sensitive measures in multiple cognitive and behavioral domains that will reveal evidence of early synaptic dysfunction in neural networks vulnerable to AD pathology. We also need to develop measures of very early functional changes in other domains, including social interaction, mood, psychomotor aspects of function, and decision making. These measures would allow us to link better the pathological processes to the emergence of clinical symptoms, and may be particularly useful to monitor response to potential disease-modifying therapies in these very early stages.

The proposed criteria apply primarily to individuals at risk by virtue of advanced age because inclusion criteria for trials in autosomal dominant mutation carriers and homozygous *APOE* ε4 carriers will be likely defined primarily on genetic status. Trials in genetic-risk populations might use these criteria to stage individuals within the preclinical phase of AD. In genetic-risk cohorts, it may even be possible to detect an even

earlier stage of presymptomatic AD, before the point when there is already detectable cerebral amyloidosis. Several FDG-PET and fMRI studies have suggested that evidence of synaptic dysfunction may be present in young and middle-aged *APOE* ε4 carriers (see Fig. 3), and there may be other biological alterations that are present before significant deposition of fibrillar forms of amyloid that would be preferentially responsive to presymptomatic intervention.

The emerging concept of preclinical AD and the role of biomarkers in the detection and tracking in this stage of the disease have important implications for the development of effective treatments. Therapies for preclinical AD would be intended to postpone, reduce the risk of, or completely prevent the clinical stages of the disorder. As recently noted, the use of clinical endpoints in clinical trials of such treatments would require large numbers of healthy volunteers, large amounts of money, and many years of study. Researchers have raised the possibility of evaluating biomarker endpoints for these treatments in cognitively normal people at increased risk for AD because these studies might be performed more rapidly than otherwise possible. Subjects enrolled in these studies could include individuals with autosomal dominant mutation carriers (with essentially a 100% chance of developing clinical AD) or those at increased risk of developing sporadic AD (e.g., *APOE* ε4 carriers or subjects with biomarker evidence of preclinical AD pathology). The use of biomarkers rather than clinical outcomes could accelerate progress in these trials; however, regulatory agencies must be assured that a given biomarker is "reasonably likely" to predict a clinically meaningful outcome before they would grant approval for treatments tested in trials using biomarkers as surrogate endpoints. Research strategies have been proposed to provide this evidence by embedding the most promising biomarkers in preclinical AD trials of people at the highest imminent risk of clinical onset to establish a link between a biomarker effect and the onset of clinical symptoms of AD. We envision the time when the scientific means and accelerated regulatory approval pathway support multiple preclinical AD trials using biomarkers to identify subjects and provide shorter term outcomes, such that demonstrably effective treatments to ward off the clinical stages of AD are found as quickly as possible. There are several burgeoning efforts to design and conduct clinical trials in both genetic at-risk and amyloid-positive older individuals, including the Dominantly Inherited Alzheimer Network (study of familial AD), the Alzheimer Prevention Initiative, and Anti-Amyloid Treatment in Asymptomatic AD (A4) trial being considered by the Alzheimer's Disease Cooperative Study.

Finally, the ethical and practical implications surrounding the issues of future implementation of making a "diagnosis" of AD at a preclinical stage need to be studied, should the postulates put forth previously prove to be correct. Although at this point our recommendations are *strictly for research purposes only*, the public controversy surrounding the identification of asymptomatic individuals with evidence of AD-P raised several important points that the field must consider. In particular, the poignant question of "why would anyone want to know they have AD a decade before they might develop symptoms, if there is nothing they can do about it?" should be carefully considered well before any results from research is translated into clinical practice. First, there may be important reasons, including social and financial planning, why some individuals would want to know their likelihood of developing AD dementia within the next decade, even in the absence of an available disease-modifying therapy. It is our hope, however, that the advances in preclinical detection of AD-P will enable earlier, more effective treatment, just as nearly all of therapeutic gains in cancer, cardiovascular disease, osteoporosis, and diabetes involve treatment before significant clinical symptoms are present. It is entirely possible that promising drugs, particularly amyloid-modifying agents, will fail to affect the clinical course of AD at the stage of dementia or even MCI, when the neurodegenerative process is well entrenched, but may be efficacious at the earliest stages of the AD-P, before the onset of symptoms.

The definitive studies to determine whether the majority of asymptomatic individuals with evidence of AD-P are indeed destined to develop AD dementia, to elucidate the biomarker and/or cognitive endophenotype that is most predictive of cognitive decline, and to determine whether intervention with potential disease-modifying therapies in the preclinical stages of AD will prevent dementia are likely to take more than a decade to fully accomplish. Thus, we must move quickly to test the postulates put forth previously, and adjust our models and study designs as new data become available. Because potential biologically active treatments may be associated with small but significant risk of adverse side effects, we will need to determine whether we can predict the emergence of cognitive symptoms with sufficient certainty to appropriately weigh the risk/benefit ratios to begin treatment in asymptomatic individuals. It is clear that many questions remain to be answered, and that there may be additional factors which will influence the probability of developing clinical AD. However, the considerable progress made over the past two decades now enables a strategic path forward to test these hypotheses, move the field toward earlier intervention, and ultimately, toward the prevention of AD dementia.

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Japanese Alzheimer's Disease Neuroimaging Initiative: Present status and future

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Abstract Japanese Alzheimer's Disease Neuroimaging Initiative (J-ADNI) was launched in 2008, aiming at conducting a longitudinal workup of a standardized neuroimaging, biomarker and clinico-psychological surveys. The research protocol was designed to maximize compatibility with that of US-ADNI, including structural magnetic resonance imaging analysis for the evaluation of brain atrophy, fluorodeoxyglucose and amyloid positron emission tomography, cerebrospinal fluid sampling, *APOE* genotyping, together with a set of clinical and psychometric tests that were prepared to achieve the highest compatibility to those used in the United States. Japanese ADNI has recruited ~357 participants (142 amnestic mild cognitive impairment, ~134 normal aged and 72 mild Alzheimer's disease (AD), as of April 15, 2010). World-wide ADNI activities will establish the rigorous quantitative descriptions of the natural course of AD in its very early stages. The data, as well as the methodologies and infrastructures, will facilitate the clinical trials of disease-modifying therapies for AD using surrogate biomarkers.

Keywords: ADNI; Neuroimaging; Biomarker; Alzheimer's disease; Disease-modifying therapy

Currently, there is a compelling need to establish novel treatments for Alzheimer's disease (AD), and to demonstrate, as well as track, the efficacy of potential treatments in clinical trials, especially those for disease-modifying drugs that target the pathophysiological mechanism of AD. At this time, clinical trials of AD are conducted in a stage of the disease that is considered late in the trajectory of the pathological process. In addition, clinical studies require large numbers of participants with AD because the statistical power of our currently available measures, that is, clinico-neuropsychological scales, is low because of the large fluctuation in data. Thus, biomarkers, including neuroimaging and body fluid chemistry, hold great promise that would assist in many of these challenges.

To identify such biomarkers, Alzheimer's Disease Neuroimaging Initiative (ADNI) was launched in the United States in 2005. US-ADNI already completed the recruitment and is continuing the longitudinal follow-up of ~800 participants including mild cognitive impairment (MCI) as the major target population, a major proportion of which represents the very early stage of AD.

We started discussions about the need for Japanese version of ADNI in 2006 for several reasons. First, there was an urgent need to meet with the requirements for global clinical trials of disease-modifying drugs for AD that were about to start in Japan, although we had little experience in nationwide or global-level clinical studies on AD, despite the relatively high activities of neurologists, psychiatrists, and geriatricians who had been involved in the clinical studies of dementia. Second, we did not have sufficient infrastructures, such as clinical study coordination center like Alzheimer's Disease Cooperative Study or imaging data repository like Laboratory of Neuro Imaging, that are required for clinical studies or trials of AD. Third, we realized that we would be able to improve the Japanese AD clinical sciences to an international level by conducting rigorous and comprehensive clinical study like ADNI, in collaboration with international experts in this field.

In this way, we submitted proposals for Japanese ADNI (J-ADNI) to the two major governmental funding agencies, that is, Ministry of health, labor and welfare (MHLW), and

URL of J-ADNI: http://www.j-adni.org/.

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New Energy and Industrial Technology Development Organization (NEDO; a foundation of Ministry of economy, technology, and industry), and got funded in 2007. Seven domestic pharmas (Astellas, Eisai, Daiichi-Sankyo, Dainippon-Sumitomo, Shionogi, Takeda, Tanabe-Mitsubishi) and four international pharmas (Bristol-Myers Squibb, Eli-Lilly, Merck-Banyu, Pfizer) also decided to contribute one-third of the total budget; the total costs for J-ADNI amounts to ~500 million yen/year.

We designed the research protocol to maximize the compatibility with that of US-ADNI, including structural magnetic resonance imaging (MRI) analysis, fluorodeoxyglucose (FDG) and amyloid positron emission tomography (PET), cerebrospinal fluid sampling, *APOE* genotyping, combined with a set of clinical and psychometric tests that were prepared to achieve the highest compatibility to those used in US-ADNI. We are going to recruit 300 individuals with amnestic MCI (using logical memory cut off based on education), 150 early AD and 150 cognitively normal individuals by the end of 2010, following them up until 2013 (Fig. 1).

The organization of J-ADNI is shown in Fig. 2. In total, 38 clinical sites participated in J-ADNI.

The clinical core is headed by Takashi Asada (Tsukuba University, Psychiatry) and Hiroyuki Arai (Tohoku University, Gerontology) and is responsible for the registration and clinical evaluation of the participants. The clinical core closely collaborates with the neuropsychology core led by Morihiro Sugishita (Niigata Rehabilitation University). During the preparation stage, Sugishita corrected the Japanese translation as well as the configuration of several major clinical and neuropsychological batteries, including ADAS-COG, MMSE, and Clinical Dementia Rating, to maximize the harmonization between English and Japanese versions. Currently, the compatibility of the test batteries is being demonstrated through the analysis of the baseline data of J-ADNI.

Hiroshi Matsuda (Saitama Medical University, MRI core PI), in collaboration with Fumio Yamashita (National Center for Neurology and Psychiatry) and other core members, has established an algorithm to achieve the standardization of MRI scans among clinical sites using different MRI equipments from various vendors, based on 3D-MPRAGE scan protocol using ADNI phantom. They also have created programs for the correction and calibration of signal equity or distortion of the images, which enabled the rigorous volumetric analysis.

Kengo Ito (National Institute for Longevity Sciences, PET core PI) and Michio Senda (Institute of Biomedical Research and Innovation, PET quality control PI) also have established the standardized protocol for PET imaging in J-ADNI, in collaboration with Kenji Ishii (Tokyo Metropolitan Institute for Gerontology, amyloid PET PI). Twenty-eight sites are conducting FDG-PET, so far covering ~71% of participants (253 cases). Amyloid PET core has established a standardized protocol for ^{11}C-PiB PET using dynamic scan data acquisition (in addition to late-phase images), as well as that for ^{11}C-BF-227, the latter being developed by Kudo and colleagues in Japan. ^{11}C-PiB PET is being conducted in 15 sites and ^{11}C-BF-227 is used in two sites.

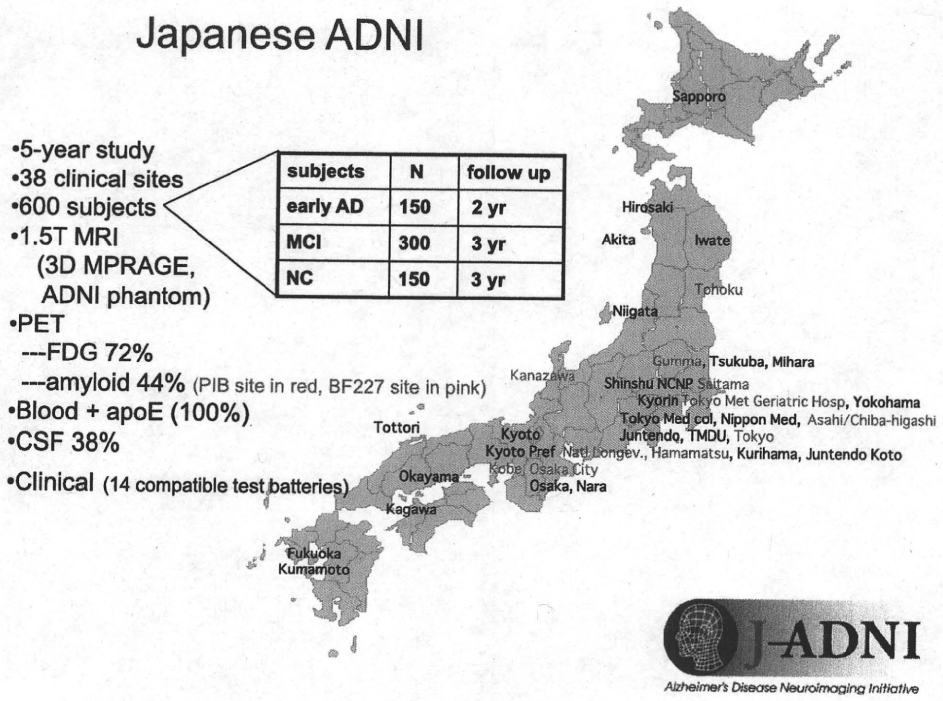

Fig. 1. Overview of J-ADNI.

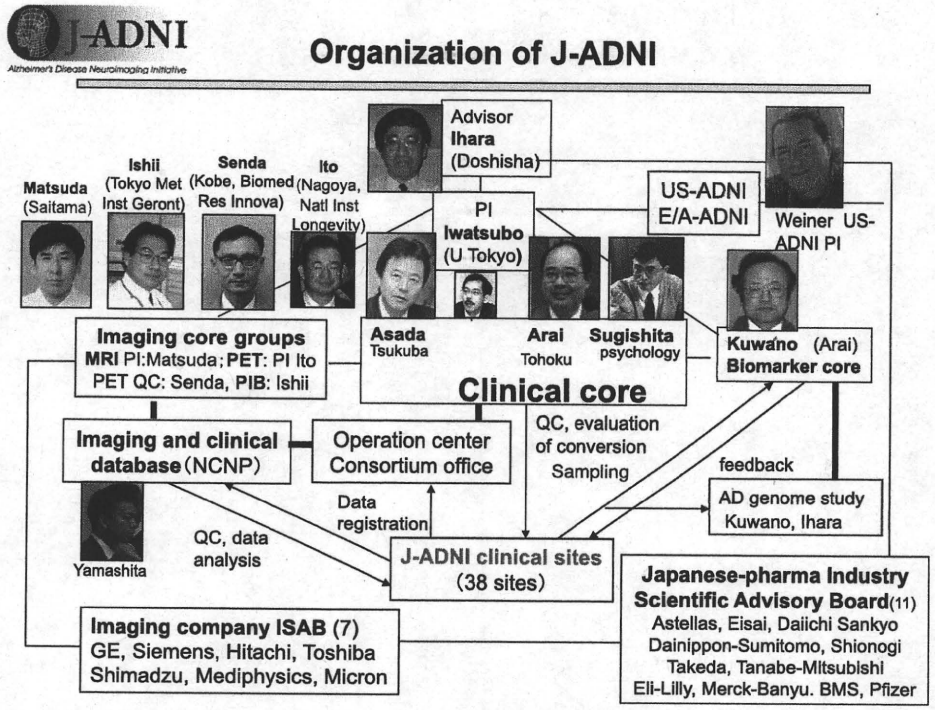

Fig. 2. Organization of J-ADNI.

Currently ~44% of total participants (157 cases) have undergone amyloid PET scan.

Biomarker core is led by Ryozo Kuwano as PI (Niigata University), with the assistance of Hiroyuki Arai as co-PI. They established the J-ADNI biosample repository in Niigata, based on the nationwide collection network of biofluid samples with the assistance of SRL company. Blood samples were collected from all participants upon every visit. So far, 139 participants (~39% of total) had lumber tap and donated cerebrospinal fluid samples. *APOE* genotype also is characterized at the Niigata site.

Until now, 38 clinical sites have screened 483 individuals and enrolled 357 participants who met with the inclusion criteria (151 amnestic MCI, 134 cognitively normal aged, and 72 early AD, as of April 15, 2010). The overall exclusion rate upon screening was 21.0% (8.8% in CN, 27.8% in MCI and 25.0% in AD), which was lower than that in US-ADNI. Currently longitudinal follow-up examination is underway with a relatively low drop-out rate (~6.5%/year).

Use of highly compatible protocols between J-ADNI and US-ADNI will enable us to establish the rigorous quantitative descriptions of the natural course of AD in its very early stages. The data, as well as the methodologies and infrastructures, will facilitate clinical trials of disease-modifying therapies for AD using surrogate biomarkers, enabling the application of effective therapies to AD/MCI patients, and eventually the prevention of AD.

Acknowledgments

The author thanks all the participants, research members, funding agencies (NEDO, MHLW) and J-ADNI ISAB member companies for their efforts and willingness to foster the J-ADNI research.

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Invited Review for the 90th Anniversary

Geriatric Medicine, Japanese Alzheimer's Disease Neuroimaging Initiative and Biomarker Development

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Due to a change in disease spectrum in aged countries, the primary role of geriatricians should be directed to an appropriate management and prevention of 1) cognitive decline and dementia, 2) swallowing and aspiration pneumonia and 3) falls and fractures. Management of dementia constitutes a central part in the practice of geriatric medicine in order to support independence of life in elderly people. The current paradigm of cognitive function-based testing for the diagnosis and treatment of Alzheimer's disease (AD) is going to drastically shift to a biomarker-based test approach, a shift that will correspond to the emergence of disease-modifying drugs. In addition, a new molecular imaging technique that visualizes neuronal protein deposits or pathological features has been developed in Japan and the U.S.A. Based on these achievements, the Alzheimer's Disease Neuroimaging Initiative (ADNI) was proposed and initiated in 2005. The ADNI is a long-term observational study being conducted in the U.S.A., Europe, Australia, and Japan using identical protocols. The objectives of ADNI are: 1) to establish methodology which will allow standard values related to long-term changes in imaging data, such as MRI and PET, in patients with AD and mild cognitive impairment and normal elderly persons; 2) to obtain clinical indices, psychological test data, and blood/cerebrospinal fluid biomarkers to demonstrate the validity of image-based surrogate markers; and 3) to establish optimum methods to monitor the therapeutic effects of disease-modifying drugs for AD. Patient enrollment in the Japanese ADNI has begun in July 2008. Imaging of AD pathology not only acts as a reliable biomarker with which to assay curative drug development by novel pharmaceutical companies, but it also helps health promotion toward AD prevention.

Keywords: geriatric medicine; Alzheimer's disease; Amyloid β-peptide; Biomarker; Amyloid imaging; ADNI
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Geriatrician's role and proposal of "Geriatric Triangle"

Geriatric medicine is an independent internal medicine division that is specialized for management of medical problems of elderly people. Despite a fact that elderly people appear healthy, a variable latent organ dysfunction may be present due to a limited residual capacity. A condition referred to as geriatric syndrome is a complex and multi-organ disease especially suffered by elderly people. The geriatric syndrome consists of more than 50 medical conditions such as dementia, depression, delirium, pneumonia, urinary incontinence, osteoporosis and fractures as well as malnutrition, sarcopenia, skin ulceration and renal failure. Importantly, these clinical conditions often occur in combination rather than separately. As illustrated in Fig.1, most important functions which support independence of life in later years are: 1) Thinking and judgments; 2) Eating and swallowing; and 3) Standing and walking. Loss of these basic functions alone or in combination will directly lead to devastating health implications and reduced quality of life. Disturbance of cognitive ability manifests as dementia. Impairment of ordered oropharyngeal functions causes a disturbed swallowing or dysphasia followed by development of aspiration pneumonia. Failure of standing and walking results in repeated falls and fractures. — all being hardly present before the age of 65 but highly prevalent over the age of 75. Moreover, these problems not merely occur in separate occasions but they also are inter-related each other. For example, people with advanced dementia are likely to develop eating problems and aspiration (Nakagawa et al. 1997; Wada et al. 2001; Mitchell et al.

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2009). Repeated episodes of pneumonia will develop disturbed nutrition and dehydration which leads to sarcopenia with an increased risk of falls and fractures (Lang et al. 2010). A long-term bedridden state due to hip or vertebral fractures will result in worsening of dementia (Muir et al. 2009). Conversely, demented patients who were treated by anti-psychotic drugs are associated with an increased risk of falls and fractures (Horikawa et al. 2005). A long-term bedridden state due to hip or vertebral fractures are prone to develop esophageal regurgitation and aspiration (Matsui et al. 2002). Drugs that up-regulate brain dopaminergic function are occasionally beneficial to prevent aspiration pneumonia in the elderly (Yamaya et al. 2001). Here, we propose to term such a closely-related condition as "geriatric triangle" as shown in Fig.1. Patients diagnosed as having geriatric triangle are likely to be placed on a long-term care facility due to reduced quality of life (Sasaki 2008). Therefore, the primary role of geriatricians should be directed to an appropriate management and prevention of geriatric triangle. Moreover, every single geriatrician should be capable of managing the geriatric triangle beyond a scope of each organ specialist (Sasaki 2008). Hence, primary targets of geriatric medicine may include assessment and treatment of 1) cognitive decline and dementia, 2) swallowing and aspiration pneumonia and 3) falls and fractures. On the other hand, it is unlikely as a primary role of geriatrician only to manage elderly people with diseases which are spanning entire stages of life. Such diseases, for example, hypertension and diabetes mellitus, can be taken care of by each organ specialist. Due to a change in disease spectrum in aged countries, it should be emphasized that geriatric medicine has become a separate and independent practice division from other organ-specialized fields of internal medicine.

Current scientific approach toward understanding of Alzheimer's disease (AD) pathogenesis

Alzheimer's disease (AD) deprives sufferers of variable life-supporting functions that are necessary for independence in the later years of life. Development of AD leads to parting from society. Care-taking families sacrifice their quality of life and their mental and physical burdens are immeasurable. Loss of personality due to alteration of brain function while physical appearance remains the same is horrible and miserable. As an essential domain of geriatric triangle as described in Fig. 1, prevalence of dementia (the number of people with the disease at any one time) doubles for every 5-year age group beyond the age 65. Briefly, dementia hardly develops prior to age 60. However, according to data from Ministry of Health, Labor and Welfare in Japan, the prevalence of dementia is estimated to be 1.5% for age 65-69, 3.6% for age 70-74, 7.1% for age 75-79, 14.6% for age 80-84, 27.3% beyond age 85 (http://www.mhlw.go.jp/english/index.html). The elderly population aged 65 or older is now approximately 22% of the whole population in Japan. Therefore, it is likely that dementia becomes quite common over the age of 65. According to recently conducted community survey, AD is a leading cause of dementia among elderly Japanese population (Yamada et al. 2001; Wada-Isoe et al. 2009). The rapid increase in the number of AD patients can be a consequence of a rapid increase in human life span. In Japan, an average life span in 1947 was 50.6 years for men and 53.9 years for women. Surprisingly, that was 79.3 years for men and 86.1 years for women in 2008. It is possible that AD is only encountered when the nation reaches a sufficiently aged society. Furthermore, AD is a major factor in increasing national medical expense. It is a universal desire to find a way to control AD. The U.S.A. calls the rapid increase in

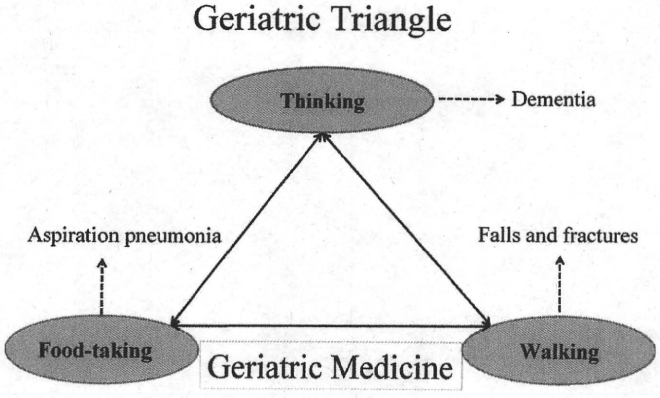

Fig. 1. Proposed concept of geriatric triangle.
At least three physical and mental functions are needed to support independence of life in elderly people. They are 1) Food-taking ability, 2) Standing and walking, and 3) Thinking and judgments. Loss of these basic functions alone or in combination will lead to devastating health implications and reduced quality of life through a vicious circle. Here, we propose to term such a vicious circle as "geriatric triangle". Geriatric triangle constitutes a major part of geriatric syndrome. Therefore, each geriatrician should be capable of managing the geriatric triangle beyond a scope of each organ specialist.

AD patients and concomitant pressure on federal budget a "National Crisis" which illustrates the seriousness of the problem (A National Alzheimer's Strategic Plan, 2009).

Understanding of pathogenesis of AD has markedly progressed in the last 3 decades. Pathological changes of AD occur gradually initially in cognitively normal people with dementia representing the end stage of many years of accumulation of amyloid β-peptide (Aβ). Aβ was first sequenced from meningeal blood vessels of AD brains (Glenner&Wang 1984). A year later, the same peptide was discovered as the primary components of senile plaques (Masters et al. 1985). Shortly after these earlier findings, cloning of the gene encoding amyloid β-peptide precursor protein (APP) and its localization to chromosome 21coupled with the recognition that Down's syndrome (trisomy 21) leads invariably to AD neuropathology set a initial hypothesis that Aβ is a primary driving force in the pathogenesis of AD. The other neuropathological features that are characteristic of AD include neurofibrillary changes and neuron death. Spatial distribution of senile plaques differs from that of neurofibrillary changes (Arriagada et al. 1992a; Arriagada et al. 1992b). A major building block of neurofibrillary changes was shown to be abnormally phosphorylated tau (Lee et al. 1991). According to the amyloid hypothesis, cortical Aβ accumulation causes all of the disease process associated with AD including microglial and astroglial activation, synaptic injury, oxidative injury followed by abnormal tau phosphorylation and eventually loss of neurons and dementia (Hardy and Selkoe 2002). The amyloid hypothesis also tells us that control of amyloid deposition would achieve success to control AD. There have been several conceptually important observations that strongly support the amyloid hypothesis. First, we occasionally see Aβ-positive but tau-negative brains from cognitively normal elderly people in autopsy samples, suggesting that Aβ deposition predates tau deposition (Arai et al. 1990). This time framework was further evidenced by the observation that Aβ-positive senile plaques occur at age 30's, whereas tau-positive neurofibrillary changes are seen only after the age of 40 in the brains afflicted with Down's syndrome (Mann et al. 1989). Thirdly, genetic mutations causing autosomal dominant familial AD were discovered in the APP gene clustering at or very near the sites that are normally cleaved by proteases called β or γ-secretases (Goate et al. 1991). These mutations enhance proteolytic processing of APP to generate amyloidogenic Aβ (Citron et al. 1992). Other AD-causing mutations in PS-1 and PS-2 gene also enhance generation of amyloidogenic Aβ by changing proteolytic processing of APP (Scheuner et al. 1996). Finally, a distinct Aβ species ending at amino acid 42 (Aβ42) is highly amyloidogenic, and there was a uniform pattern of Aβ42 deposition as an initial event of pathology either in non-demented, AD or Down's syndrome patients (Iwatsubo et al. 1994). As illustrated in Fig. 2, we can use a hypothetical assumption to think about the progression of AD. Namely, assuming that memory loss became noticeable at the age 70 followed by progression of multiple cognitive decline and behavioral problems at the age of 75. The patient was eventually diagnosed as suffering AD. In such an instance, we can assume that accumulation of cerebral Aβ may have started at around 50 years of age followed by intracellular accumulation of tau in the form of neurofibrillary changes as well as neuron death may have started at approximately 60-65 years of age. Therefore, it should be emphasized that there is an approximately 20-year time lag between the initiation of amyloid protein deposition and onset of the earliest clinical manifestations of dementia in AD. During this lag-period, individuals are cognitively normal but they are not aware of what changes are taking place in their brains. We assume that such individuals would ultimately develop AD if he or she lived long enough. Furthermore, a prodromal stage of AD often referred to as mild cognitive impairment (Petersen et al. 2009) is characterized by onset of mildest cognitive symptoms despite a massive neuron loss in vulnerable cortical areas (Gómez-Isla et al. 1997). Hence, there is an extremely high need for development of methods that simply and reliably detect amyloid and tau deposits. One such approach is a recently developed molecular imaging technique called "amyloid imaging".

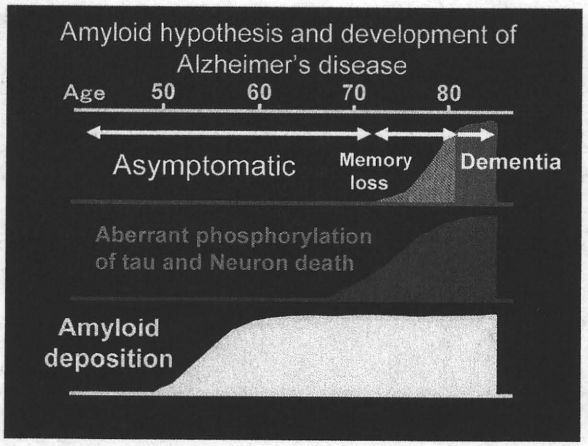

Fig. 2. Hypothetical scheme of progression of AD from amyloid deposition to development of dementia.
It is noteworthy that brain amyloid continues to be accumulating towards the onset of AD during which subjects are not aware of what changes are taking place in their brains. When subjects are first symptomatic, abundant neurofibrillary changes and a massive neuron death have already begun in vulnerable brain regions such as hippocampal or entorhinal cortex. Original description was made by Yasuo Ihara.

A paradigm shift in the diagnosis and treatment of AD

Fig. 3 illustrates a superimposition of the diagnostic and treatment framework in the context of the hypothetical amyloid cascade described above. AD has so far been diag-

nosed clinically only by demonstrating "cognitive decline" which has progressed to a stage that is sufficient enough to disturb independent social or occupational life. It is likely that cognitive decline is associated with a massive neuron death that exceeds so-called "cognitive reserve capacity" (Stern 2009). In addition to cognitive testing, two other diagnostic techniques including magnetic resonance imaging (MRI) and fluorodeoxyglucose (FDG)-PET are currently in common use to demonstrate a mass of dead nerve cells directly or indirectly. Symptomatic drugs such as donepezil hydrochloride and memantine hydrochloride are best considered at this point. However, a dramatic improvement of memory function cannot be expected since disturbance of episodic memory is based upon a massive loss of hippocampal and entorhinal cortical neurons. Accordingly, if we assume that AD represents chronic effects of a long-standing imbalance between Aβ production and Aβ clearance and this imbalance causes all existing events in the downstream of Aβ, a special attention should be directly paid to amyloid and tau depositions in the development of preventive strategies. If we are successful in developing diagnostic methodologies to detect amyloid or tau deposition before a massive neuron death occurs, such approaches will make a great contribution to developing a disease-modifying or curative treatment that directly targets amyloid and also tau. A paradigm of cognitive function-based testing for the diagnosis and treatment of AD is going to drastically shift to a biomarker-based test approach in accordance with the emergence of disease-modifying drugs. Hope for prevention of AD would be potentially carried out. As mentioned later, the Alzheimer's Disease Neuroimaging Initiatives (ADNI) will change paradigm of diagnostic and treatment of AD drastically with biomarkers as a bridging role in the paradigm shift.

Biomarkers with a bridging role in the paradigm shift

In general, biomarkers of AD are defined as indicators of specific features that characterize AD in vivo. Either biochemical or imaging biomarkers are expected to provide potentially diverse purposes as summarized elsewhere (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association & NIAWG 1998; Frank et al. 2003; Shaw et al. 2009). First, biomarkers will support preonset diagnosis. As demonstrated in Fig. 2 and 3, AD pathology has already started with abundant amyloid pathology even though individuals are otherwise normal and are still independent in their daily living activities,. This stage can be an ideal therapeutic time point in which disease-modifying or curative drugs should be indicated before neurodegenerative cascade is triggered. Such biomarkers will enable us to move from disease modification to prevention of AD. Second purpose is evaluation of disease severity. Currently, severity or clinical stage of AD is evaluated by neuropsychological testing. However, neuropsychological test results are likely to vary due to the patient's physical condition on the day of the test and experience of the examiners. In a study involving 192 AD patients performed by Jack et al., the annual change in ADAS-Cog score in mild to moderate AD was 4.25 ± 7.2 (mean ± S.D.) points, while the yearly change in hippocampal volume on MRI in the same patients was −234 ± 144 (mean ± S.D.) mm^3 (Jack et al. 2003; Petersen et al. 2005). The SD, representing variation of the values, of the hippo-

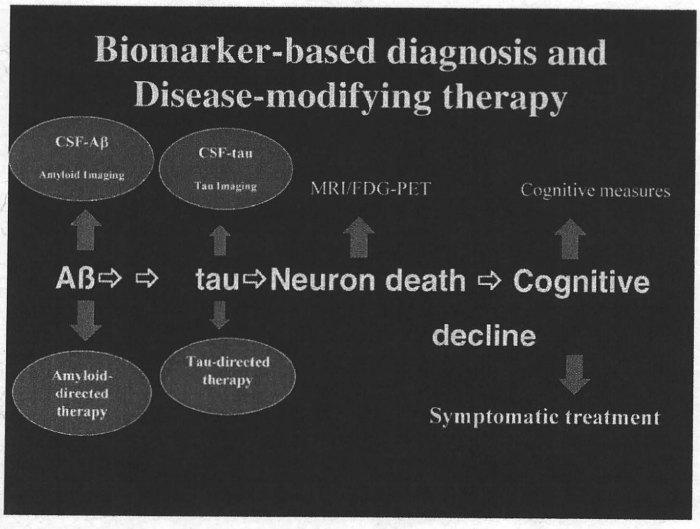

Fig. 3. Strategies for new diagnostic and therapeutic approaches for AD are presented based on amyloid hypothesis.
This figure illustrates a superimposition of the diagnostic and treatment framework in the context of the hypothetical amyloid cascade as described in Fig. 2. In the hypothesis, amyloid is located upstream probably due to a causative agent of AD. Therefore, amyloid imaging is quite attracting because this technology will facilitate both detection and intervention that targets amyloid. If tau imaging would also be possible, tau-targeting therapy might be considered.

campal index was only 0.6 times the mean, while that of ADAS-Cog was 1.7 times. Since image processing is a uniform mechanical task, variation of the imaging biomarker should be small. Sensitive biomarkers which reliably and objectively reflect changes in lesions, even though the effect size is small, are expected to be used analogously to commonly used laboratory test indices for evaluation of the disease severity in clinical practice such as C-reactive protein in inflammatory diseases, serum transaminase levels in liver diseases as well as serum creatinine kinase levels in muscular diseases. Thirdly, we need biomarkers that support evaluation of therapeutic effects. Several classes of amyloid-reducing drugs such as γ-secretase inhibitors (De Strooper et al. 2010) and amyloid immunization therapy (Tabira 2010) might become available in the near future. For the development of these therapeutic drugs, development of methodology to objectively access "decrease or removal of amyloid" is necessary. For example, when the brain amyloid level is reduced by a novel treatment, the biomarker levels are expected to return closer to normal range. Ideal biomarkers may also provide important information regarding the timing of treatment initiation, discontinuation and changing of drug treatment. However, it may be unlikely that a single biomarker meets all conditions described above, and it may be more realistic to prepare a combination or panel of several different biomarkers.

Since therapy is likely to be most effective at or before symptom onset, early or pre-symptomatic detection of AD is highly desirable before neurodegeneration becomes obvious. Thus, there is a great need for blood and CSF biomarkers that substantially aid tracking disease progression of AD and eventually promoting prevention strategy. As reviewed elsewhere (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association & NIAWG 1998; Frank et al. 2003), ideal AD biomarkers should detect a fundamental feature of AD neuropathology, be validated in autopsy confirmed cases, have a diagnostic sensitivity > 80% for detecting AD and a specificity of > 80% for distinguishing AD from other dementias. Moreover, assays using AD biomarkers should be reliable, reproducible, non-invasive, simple to perform and inexpensive. Further, validation of AD biomarkers requires confirmation by at least 2 independent studies from qualified investigators published in peer-reviewed journals. Tau and Aβ are major components of the two neuropathological hallmarks of AD (tangles and plaques respectively), and they are the most intensively studied candidate AD biomarkers where they are best studied in cerebrospinal fluid (CSF) using extensively characterized ELISAs (Arai et al. 1995; Arai et al. 1997; Arai et al. 1998; Tomita et al. 2007). A recent examination of > 100 subjects with autopsy-confirmed diagnoses reached a conclusion that elevated CSF tau levels are associated with the presence of AD pathology and CSF Aβ42 levels are decreased in AD (Clark et al. 2003). Currently, it is widely accepted that biomarkers of brain amyloid burden are reductions in CSF Aβ42 and increased amyloid PET tracer retention (Fagan et al. 2006; Jack et al. 2010). As shown in Fig. 2, after a lag period, which varies from patient to patient, neuronal dysfunction and neurodegeneration become the dominant pathological processes. Biomarkers of neuronal injury and neurodegeneration are increased CSF tau and structural MRI measures of cerebral atrophy (Arai et al. 1995). Neurodegeneration is accompanied by synaptic dysfunction, which is indicated by decreased FDG-PET (Jack et al. 2010).

Development and clinical applications of amyloid imaging

Amyloid imaging is currently considered to be the most promising candidate biomarker since it meets many possible conditions of an ideal biomarker as described above. The most difficult hurdle for clinical application of this technology is to find a probe with following excellent characteristics: 1) it should selectively bind to Aβ aggregates with β-sheet-structure; 2) it should readily penetrate the blood-brain barrier (BBB) while being rapidly cleared off from the brain in the absence of the target; 3) the labeled form should not lose the characteristics of the mother compound. In our experience, enhancing one of several necessary characteristics causes loss in another, requiring extensive adjustment.

Although brain Aβ deposits are still well beyond the resolution of conventional neuroimaging techniques such as MRI, the density of these deposits in the brain tissue can be visualized through specific radiotracer and positron emission tomography (PET). The first compound to emerge as an amyloid-imaging agent was Chrysamine-G (Klunk et al. 1995). This compound shows similar binding characteristics to Congo-red, but unfortunately, due to its limited BBB permeability, there was no use as a clinical PET tracer. A marked progression in the development of amyloid-imaging tracers was made by the development of 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl) amino]-2-naphthyl}ethylidene) malononitrile ([^{18}F]FDDNP) (Agdeppa et al. 2001). This compound is highly lipophilic and can easily cross BBB, and has been used in human PET studies (Shoghi-Jadid et al. 2002; Small et al. 2006; Barrio et al. 2008). However, this agent has some limitations in its practical use due to its low signal-to-background ratio (Tolboom et al. 2009). Currently, the most successful amyloid-binding agent is a thioflavin-T derivative, N-methyl-[^{11}C] 2-(4′-methylaminophenyl)-6-hydroxybenzothiazol ([^{11}C]PIB) which has been shown to possess a high affinity for Aβ fibrils (Klunk et al. 2003; Mathis et al. 2003; Klunk et al. 2004). An autoradiographic study using AD brain sections revealed that [^{11}C]PIB, in addition to binding to the classical fibrillar Aβ plaques, also binds to a range of Aβ containing lesions including diffuse plaques and cerebrovascular amyloid angiopathy (Lockhart et al. 2007). In vitro binding studies indicated that PIB preferentially binds to Aβ1-42 fibrils with high affinity (Klunk et al. 2003) with a negligible binding to α-synuclein and tau (Lockhart et al. 2007; Fodero-

Tavoletti et al. 2007). The [^{11}C]PIB retention in the neocortical areas is correlated with the Aβ plaque load (Bacskai et al. 2007; Ikonomovic et al. 2008) with an inverse relation to CSF Aβ42 levels (Fagan et al. 2006). The frequency of cognitively normal individuals with positive PIB binding rose in an age-dependent manner from 0% at ages 45-49 years to 30.3% at ages 80-89 years. (Rowe et al. 2007; Morris et al. 2010). Further, CSF tau and phospho-tau$_{181}$ increased with cortical PIB binding in cognitively normal individuals (Fagan et al. 2009). However, there is currently no evidence of how frequently PIB-positive normal individuals will convert to develop dementia or how long is the interval between the detection of significant Aβ burdens and the onset of dementia. Longitudinal amyloid imaging studies are needed to demonstrate the reality of amyloid hypothesis via looking at relation between amyloid deposition and temporal AD progression.

Benzoxazole derivatives are also promising alternatives as amyloid-imaging probes (Okamura et al. 2004). A PET study using the ^{11}C-labeled benzoxazole derivative 2-(2-[2-dimethylaminothiazol-5-yl]ethenyl)-6-(2-[fluoro]ethoxy) benzoxazole (BF-227) demonstrated significantly higher retention of this tracer in cerebral cortices of AD patients compared to the majority of healthy elderly subjects (Kudo et al. 2007). The retention of this tracer in cerebral cortices of mild cognitive impairment patients was intermediate between AD and healthy normal subjects (Waragai et al. 2009; Furukawa et al. 2010). A voxel-by-voxel analysis demonstrated a higher retention of [^{11}C]BF-227 in the posterior association cortex of AD patients. The pattern of this distribution corresponds well with the distribution of neuritic plaques in postmortem AD brains (Okamura et al. 2009). These findings suggest [^{11}C]BF-227 may be distinct from [^{11}C]PIB in detecting different populations of amyloid deposits. In addition, glucose metabolism demonstrated by FDG-PET was negatively correlated with that of BF-227, suggesting that extracellular amyloid surrounds synapses and impairs neuronal function (Furukawa et al. 2010). In my personal view, a highly expected value of amyloid imaging may be its capability to monitor treatment effects in PIB or BF-227 positive normal individuals who have received amyloid-reducing therapies (Rinne et al. 2010). The [^{11}C]-labeled form has a short half-life (20.4 minutes) and its synthesis requires a facility capable of radioisotope synthesis using a cyclotron, whereas the [^{18}F] -labeled form has a longer half-life (109.7 minutes), which may be amenable for delivery to various sites. Therefore, the [^{18}F]-labeled compounds, for example, [^{18}F]AV-45 will probably be a standardized agent for future clinical uses (Personal communication from Skovronsky D).

Future prospects of the Japanese ADNI

Development of curative molecular targeting therapy for AD has rapidly progressed centering mainly in work done by U.S. pharmaceutical companies. Clinical trials of symptomatic treatments currently on the market could be completed within about 6 months, but planned disease-modifying drugs to delay progression of AD may require trial durations of at least one year or longer to confirm sufficient drug effect. Development of a surrogate biomarker which reflects the pathology of the disease and monitors its progression may be desperately needed for conducting long-term clinical trials. Based on this consideration, an observational clinical study called "The Alzheimer's Disease Neuroimaging Initiative (ADNI)", was proposed and initiated in the U.S.A. in 2005 (Mueller et al. 2005; http://www.adni-info.org/ ; http://www.loni.ucla.edu/ADNI/). ADNI is a non-randomized long-term observational study undertaken in the U.S.A., Europe, Australia, and Japan using an identical protocol in each participant nation. Japanese ADNI (J-ADNI) is planning to follow 300 patients with MCI for 3 years, 150 patients with early AD for 2 years, and the other 150 normal subjects for 3 years in a cooperative study of a total of 38 facilities nationwide with sufficient experience in the management of dementia (http://www.j-adni.org/). The principle investigator is Professor Takeshi Iwatsubo at University of Tokyo. The study objectives are: 1) to establish methodology that will determine standard values related to long-term changes in image data, such as MRI and PET, in AD and MCI patients and normal elderly persons; 2) to simultaneously collect clinical indices, psychological tests, and blood/cerebrospinal fluid biomarkers to demonstrate the validity of image surrogate markers, and 3) to establish the optimum method to monitor therapeutic effects of curative drugs (disease-modifying drugs) for AD, for which analyses of the following observation items are prioritized: 1) Rate of conversion from MCI to AD, 2) rates of whole brain and hippocampus volume changes via MRI, 3) rates of change in blood and cerebrospinal fluid biomarkers, and 4) rate of change in glucose metabolism on FDG-PET. In addition, baseline amyloid PET scans are given to subjects who agreed it in J-ADNI. We hope that J-ADNI project promotes long-delayed improvements of Japanese infrastructure of medical care system for dementia. It is inadvisable for Japanese medical society to ignore that in the U.S.A. a paradigm shift in AD from 'cognitive measures-based to biomarker-based' has begun after deliberation and discussion on subjects such as clinical trial efficiency and cost reduction. Many different curative drugs are under development by pharmaceutical manufacturers, and global clinical trials of these new drugs are ongoing.

In J-ADNI, firstly, several of Japanese version of the cognitive test batteries were revised by Sugishita M. et al. to normalize the relative difficulty and to enhance maximum compatibility of the test with World Wide ADNI and later for global clinical trials of new drugs. The first patient was successfully enrolled at the National Center of Neurology and Psychiatry in July 2008. More than 330 patients have already been enrolled as of March 10, 2010. The consent rate to FDG-PET, amyloid PET, and sampling of cerebrospinal fluid was obtained from 80, 44, and 40% of the participants, respectively. We will attempt to increase the

number of patients enrolled and the rate of consent to biomarker sampling, aiming at a great success of J-ADNI and World Wide ADNI together.

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Homocysteine, Another Risk Factor for Alzheimer Disease, Impairs Apolipoprotein E3 Function*[S]

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Apolipoprotein E (apoE) ε4 and hyperhomocysteinemia are risk factors for Alzheimer disease (AD). The dimerization of apoE3 by disulfide bonds between cysteine residues enhances apoE3 function to generate HDL. Because homocysteine (Hcy) harbors a thiol group, we examined whether Hcy interferes with the dimerization of apoE3 and thereby impairs apoE3 function. We found that Hcy inhibits the dimerization of apoE3 and reduces apoE3-mediated HDL generation to a level similar to that by apoE4, whereas Hcy does not affect apoE4 function. Western blot analysis of cerebrospinal fluid showed that the ratio of apoE3 dimers was significantly lower in the samples from the patients with hyperhomocysteinemia than in those that from control subjects. Hyperhomocysteinemia induced by subcutaneous injection of Hcy to apoE3 knock-in mice decreased the level of the apoE3 dimer in the brain homogenate. Because apoE-HDL plays a role in amyloid β-protein clearance, these results suggest that two different risk factors, apoE4 and hyperhomocysteinemia, may share a common mechanism that accelerates the pathogenesis of AD in terms of reduced HDL generation.

It has been shown that the possession of the apolipoprotein E (apoE) ε4 allele is a major risk factor for Alzheimer disease (AD)[2] (1). In the central nervous system, apoE is one of the major lipid acceptors to remove cholesterol from cells and generate HDL particles. Previous studies have shown that apoE isoforms do not affect apoE binding to ABCA1, that apoE-mediated ABCA1-dependent cholesterol efflux is not affected by apoE isoforms in fibroblasts (2), and that there is no apoE-isoform-dependence on apoE-mediated lipid efflux from mouse astrocytes (3). Other lines of evidence have shown that apoE induces lipid efflux from macrophages and neural cells in an isoform-dependent manner; apoE3 induces a greater lipid efflux than apoE4 (4–9). It has been shown that two major factors cause this apoE-isoform-dependent generation of HDL. Namely, intramolecular domain interaction occurring in apoE4 attenuates apoE4 ability to generate HDL and intermolecular dimerization by disulfide bonds between cysteines in apoE3 enhances apoE3 ability to generate HDL in neural cells (10).

Recent studies have shown other functions of apoE as well, including an intracellular function of apoE (11, 12) and a function of apoE in clearance and degradation of Aβ. It has been demonstrated that apoE isoforms differentially regulate Aβ clearance from the brain (13), and that an increased level of lipidated apoE, namely, apoE-HDL, stimulates Aβ degradation (14). These lines of evidence suggest that the lower ability of apoE4 than apoE3 to generate HDL would result in an enhanced Aβ deposition in the brain owing to the lower Aβ degradation/clearance from the brain. Similarly, apoE-isoform-dependent HDL generation results in a lower HDL-cholesterol level in serum in those who possess apoE ε4 allele, which is a risk factor for atherosclerosis (15) and cerebral infarction (16).

In light of these findings, it is interesting to note that similar to the apoE ε4 allele, hyperhomocysteinemia is a risk factor not only for atherosclerosis (17), cerebral infarction (18), and vascular dementia (19), but also for AD (20–23), and that homocysteine (Hcy) level has been reported to increase in the cerebrospinal fluid (CSF) of patients with AD compared with that of control subjects (24). Hcy is generated from the metabolism of methionine, the sulfur-containing amino acid. Previous studies have shown that Hcy generates oxidative stress, leading to cell damage (25), impairs blood-brain barrier function (26), and increases brain Aβ levels (27, 28). However, the molecular mechanism underlying hyperhomocysteinemia-mediated AD development has not yet been fully understood. Because Hcy is a molecule harboring a thiol, it is possible that the thiol of Hcy associates with the thiol of cysteine residues in apoE3, and this disulfide bonds interferes with apoE3 dimerization. Because the dimerization of apoE3 enhances its ability to generate HDL (7, 10), Hcy bound to cysteine residues of apoE3 deteriorates apoE3 HDL generation. In this study, we found that Hcy interferes with apoE3 dimerization by forming disulfide bonds with cysteine residues of apoE3 and impairs apoE3 ability to generate HDL to a level similar to that of apoE4. This is also the case

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, Table 1, and Figs. 1–3.

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[2] The abbreviations used are: AD, Alzheimer disease; DTT, dithiothreitol; PC, phosphatidylcholine; CSF, cerebrospinal fluid; Hcy, homocysteine.

for human CSF obtained from patients with hyperhomocysteinemia and for brain of apoE3 knock-in mice subcutaneously injected with Hcy. These results suggest that two different risk factors for AD, namely, apoE4 and hyperhomocysteinemia, may share a common mechanism; that is, apoE4 has a lower ability to generate HDL than apoE3 and the Hcy modification of apoE3 impairs the ability of apoE3 to generate HDL to a level similar to that of apoE4.

EXPERIMENTAL PROCEDURES

Animals—ApoE knock-out mice (B6.129P2-*Apoe*^{tm1Unc}/J) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice expressing human apoE3 in place of mouse apoE (apoE3 knock-in mice) (29) were kindly provided by the Mitsubishi Kagaku Institute of Life Sciences.

Cell Culture—All experiments were performed in compliance with existing laws and institutional guidelines. Highly astrocyte- and neuron-rich cultures were prepared in accordance with a method described previously (30). The astrocyte-rich cultures were maintained in DMEM containing 10% FBS until use.

ApoE Preparation and Hcy Treatment—Five hundred micrograms of apoE3 or apoE4, purchased from Wako (Osaka, Japan), was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 6 M urea to obtain 1 ml of an apoE-containing solution, dialyzed against PBS overnight at 4 °C, and stocked in aliquots at −80 °C as described previously (10). Hcy was dissolved in 10 mM Tris-HCl buffer (pH 8.0) to make a stock solution at a concentration of 100 mM, and the Hcy stock solution was divided into aliquots and kept at −80 °C until use. For Hcy treatment, 7.5 μl of 100 mM Hcy was added to 500 μl of an apoE-containing solution. The apoE solutions with or without Hcy were incubated overnight at room temperature. The samples were then dialyzed against PBS overnight at 4 °C. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce) and used for experiments to determine lipid efflux. For dithiothreitol (DTT) treatment, 5 mM DTT was incubated with an apoE stock solution overnight at room temperature. The samples were then dialyzed against PBS overnight at 4 °C. The protein concentration of each sample was determined using a BCA protein assay kit and used for experiments to determine lipid efflux.

Determination of Levels of Cholesterol and Phosphatidylcholine (PC) Released from Astrocytes Labeled with [^{14}C]Acetate—Astrocytes plated in 12-well dishes were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin solution for 72 h. The cultures were then treated with 37 kBq/ml [^{14}C]acetate (Moravek Biochemicals, Inc., Brea, CA) for 48 h. The astrocytes were washed in DMEM twice and treated with apoE in DMEM. The culture medium was quickly removed, and the astrocytes were dried at room temperature, and the levels of cholesterol and PC released were determined as described previously (7). The levels of [^{14}C]cholesterol and [^{14}C]PC efflux were calculated using the following formula: % efflux = media × 100/(media + cell).

Reverse-phase High Performance Liquid Chromatography and Mass Spectrometry—A synthetic peptide, LGADMEDVCGR or LGADMEDVC(Hcy)GR, or recombinant apoE3 was dissolved in PBS, to a concentration of 1 mM or 15 μM, respectively. Synthetic peptide LGADMEDVCGR or ApoE3 was mixed with or without 10-fold molar concentration of Hcy at 4 °C for 1 day using a vortex mixer. ApoE3 incubated with or without Hcy was digested with trypsin (1 μg/ml; Trypsin Gold, Promega) at an enzyme/substrate ratio of 1:100 (w/w) at 37 °C overnight. Incubated synthetic peptides or apoE3 tryptic peptides were separated by reverse-phase HPLC (model 1100 Series; Agilent Technology, Waldbronn, Germany) on a C18 column (2 × 30 mm; Cadenza CD-C18, Imtakts, Kyoto, Japan) with a linear gradient of 0–64% acetonitrile in 0.1% TFA for 64 min at a flow rate of 0.2 ml/min. The fractionated peptides were subjected to mass spectrometry (AXIMA-CFR, Shimadzu, Kyoto, Japan). Mass spectrometric analysis was performed by MALDI-TOF MS. Samples were prepared by mixing with α-cyano-4-hydroxycinnamic acid as a matrix.

Sampling of Human Plasma and CSF—Human plasma and CSF were obtained from patients in Fukushimura Hospital (Toyohashi, Japan). The plasma and CSF were frozen immediately in liquid nitrogen at lumbar tap and then stored at −80 °C until use. Experiments using human CSF were performed after obtaining informed consent from the patients' guardians for diagnosis and research for biochemical, molecular biological, and genomic analysis.

Determination of Levels of Hcy in Plasma and CSF—The Hcy concentrations in human plasma and CSF were determined by HPLC as demonstrated previously (24). The apoE genotype was also determined and the samples from apoEε3/3 patients were used for this study.

Western Blot Analysis—For the determination of apoE3 dimers, the conditioned media or human CSF were dissolved in a sampling buffer consisting of 100 mM Tris-HCl (pH 7.4), 10% glycerol, 4% SDS, and 0.01% bromphenol blue and analyzed by 12.5% Tris/Tricine SDS-PAGE under nonreducing conditions. Blots were probed for 4 h at room temperature with a goat anti-apoE polyclonal antibody, AB947 (1: 2,000; Chemicon, Temecula, CA). Band detection was carried out using an ECL kit (GE Healthcare). The signals corresponding to apoE of each sample in the immunoblot membrane were quantified by densitometry with NIH ImageJ software, with varying concentrations of recombinant apoE protein (Wako, Tokyo, Japan) as standards. Standard signals were demonstrated to be linear in the range of apoE protein amounts from 0 to 2 μg per lane. The apoE concentrations in the conditioned culture media within this range were used for analysis.

Chemically Induced Hyperhomocysteinemia—Mice expressing human apoE3 in place of mouse apoE (apoE3 knock-in mice) (29) at 40–42 weeks of age were subcutaneously injected with Hcy. PBS (100 μl) containing Hcy at a concentration of 13 μmol/μl (0.6 μmol/g body weight) was injected into the mice twice a day (in the morning and evening) for 6 days. For control mice, 100 μl of PBS was injected. In the morning of the seventh day, the animals were deeply anesthetized with isofluorane. Through an incision of the skin covering the occipital bone and the cervical dorsum, the atlanto-occipital membrane was exposed and incised under an operating microscope. The animals were perfused transcardially with PBS, and the brains were removed. Peripheral blood (0.5–1.0 ml) was collected from the

caudal vena cava immediately before the perfusion. For the preparation of brain samples, the brain hemispheres from a PBS- or Hcy-injected apoE3 mouse were homogenized in 800 μl of PBS containing a protease inhibitor mixture (Roche Applied Science, Mannheim, Germany), and then centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatant was then used for Western blot analysis.

Statistical Analyses—StatView computer software (Windows) was used for statistical analysis. The statistical significance of differences between samples was evaluated by multiple pairwise comparisons among the sets of data using analysis of variance and the Bonferroni *t* test.

RESULTS

We examined the cholesterol and PC efflux from cultured astrocytes induced by apoE3, apoE3 pretreated with Hcy, and apoE4 24 h after the commencement of treatment of apoEs at various concentrations (Fig. 1*A*). The levels of cholesterol and PC efflux induced by apoE3 were higher than those induced by apoE3 preincubated with Hcy or apoE4 at 0.1, 0.3, and 1.0 μM. Because our previous study showed that the dimer formation of apoE3 enhances apoE3 ability to release lipids (6, 10), we determined the levels of cholesterol and PC efflux and also the assembly state of apoE3 and apoE4. The levels of cholesterol and PC efflux induced by apoE3 were significantly higher than those induced by apoE4 and apoE3 pretreated with Hcy 24 h after the commencement of treatment (Fig. 1*B*). A reduced level of lipid efflux was accompanied by a reduced level of apoE3 dimers in apoE3 samples pretreated with Hcy (Fig. 1*C*, *asterisks*). ApoE4 does not form dimers owing to a lack of cysteine. In these experiments, we confirmed that Hcy at concentrations used in our study was not toxic (see supplemental Fig. 1).

These results suggest the possibility that Hcy inhibits the dimer formation of apoE3 and this may be responsible for the reduced level of lipid efflux induced by apoE3 pretreated with Hcy, because our previous studies showed that apoE serves as a lipid acceptor in an isoform-dependent manner; apoE3 induces greater HDL generation than apoE4 (6, 7, 9). It is possible to assume that the thiol in Hcy can form disulfide bonds with the thiol of cysteine residues in apoE3. To examine whether the inhibition of thiol-disulfide bonds in apoE3 dimers affects apoE3-mediated cholesterol efflux, apoE3 or apoE4 was preincubated with a thiol-reducing agent, DTT, and then dialyzed against PBS and used for the experiment to determine cholesterol efflux. The levels of cholesterol released by apoE3 were significantly greater than those released by apoE3 pretreated with Hcy or DTT at 24 h after the commencement of treatment (Fig. 1*D*). The level of cholesterol efflux induced by apoE4 was significantly lower than that induced by apoE3, and Hcy or DTT pretreatment did not affect apoE4-induced cholesterol release (Fig. 1*D*). A reduced level of lipid efflux was accompanied by a reduced level of apoE3 dimers in apoE3 samples pretreated with Hcy or DTT (Fig. 1*D*). The effects of Hcy and DTT on apoE3 dimer formation are shown in Fig. 1*E*. The levels of apoE3 dimers (Fig. 1*E*, *asterisks*) in apoE3 samples pretreated with Hcy or DTT decreased in a Hcy- or DTT-dose-dependent manner (Fig. 1*E*). These lines of evidence suggest that the lower level of HDL generated by Hcy-bound apoE3 than by intact

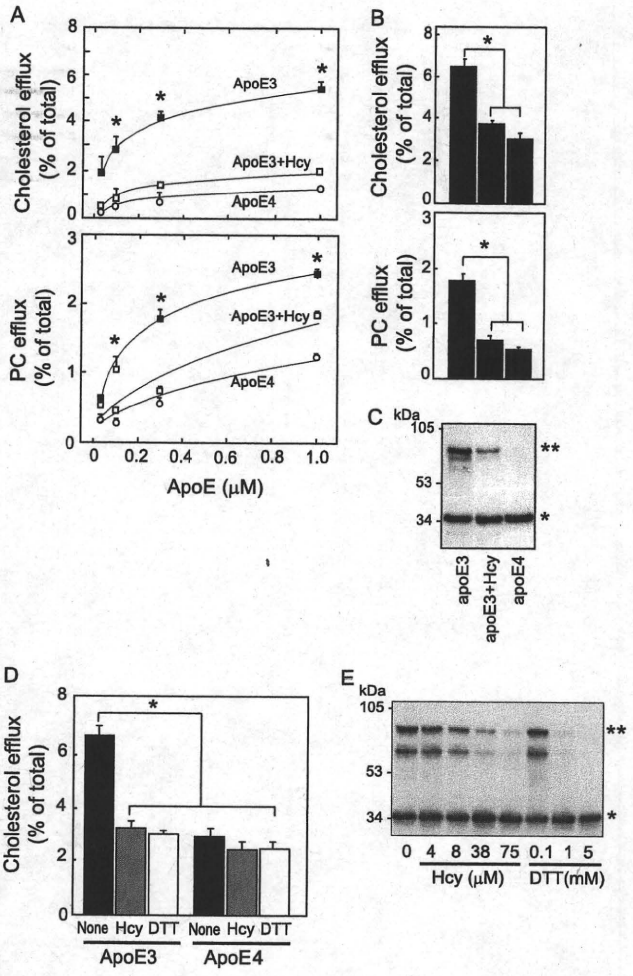

FIGURE 1. **Hcy impairs apoE3 function to generate HDL in cultured astrocytes.** *A*, the cultured astrocytes labeled with [^{14}C]acetate were exposed to apoE3 (*black squares*), apoE3+Hcy (*red squares*), and apoE4 (*yellow circles*) at 0.05, 0.01, 0.3, and 1.0 μM for 24 h. The lipids released into the media, and the lipids retained in the cells were determined. Data are means ± S.E. of four samples. *, $p < 0.001$ *versus* apoE3+Hcy and apoE4 at each dose point. The basal levels of cholesterol and PC efflux in the absence of apoEs are 1.0 ± 0.1 (%) and 0.4 ± 0.1 (%), respectively. Three independent experiments showed similar results. *B*, the percentages of released cholesterol and PC levels with respect to the total levels were calculated. Data are means ± S.E. of four samples. *, $p < 0.001$. Three independent experiments showed similar results. *C*, Western blot analysis of the samples of apoE3, apoE3+Hcy, and apoE4 was performed under nonreducing conditions. *D*, each culture was exposed to apoE3, apoE3+Hcy, apoE3+DTT, apoE4, apoE4+Hcy, and apoE4+DTT at an apoE concentration of 0.3 μM for 24 h. The percentages of released cholesterol levels with respect to the total levels were calculated. Data are means ± S.E. of four samples. *, $p < 0.001$. Three independent experiments showed similar results. *E*, Hcy at various concentrations of 4, 8, 38, and 75 μM and DTT at 0.1, 1, and 5 mM were added to the apoE3 solution, and the apoE3 solution was incubated for 24 h at 4 °C. Each solution was then dialyzed using a cassette dialyzer in PBS for 15 h at 4 °C. The apoE3-containing solutions were then analyzed by Western blot analysis under nonreducing conditions using the anti-apoE antibody (AB947). * and **, apoE3 monomers and dimers, respectively.

apoE3 results in an earlier Aβ deposition and inferior synaptic plasticity, causing earlier AD development.

We next determined whether these are also the cases for neurons. We have examined the cholesterol efflux from cultured neurons induced by apoE3, apoE3 pretreated with Hcy, apoE4, and apoE4 pretreated with Hcy at varying hours after