lower glucose. However our measurement of the glutamate in the medium may not precisely reflect the concentration of extracellular glutamate in the slice. Using glutamate oxidase attached to a micro-probe, we reported the increase of extracellular glutamate from the CA3 dendritic region 10 min after the initiation of glucose deprivation (Takata et al., 1995) when the ATP levels in the slice are well preserved (Takata and Okada, 1995). While these results indicate that mild extracellular glutamate release triggering the induction of lactate-supported PS may occur during hypoxia with lower glucose levels, more detailed investigations will be required to substantiate this observation.

In summary, our present experiments confirm our previous conclusions that lactate cannot be utilized initially as a primary substrate to maintain synaptic activity instead of glucose in the adult hippocampal neurons, especially the CA3 pyramidal neurons and the granular neurons of dentate gyrus. It still remained to be proved that whether the CA1 pyramidal neurons show the similar characteristic with the paradigm we used here in current experiment. However, the significant point overlooked so far by other investigators is that lactate can maintain energy levels in hippocampal slices, but fails to be a complete alternative to glucose for the maintenance of synaptic function in immediate prepared slices (see Section 1, Yamane et al., 2000) and at physiological temperature (35–37 °C). Moreover, conditions of glucose shortage, such as hypoxia with reduced glucose or glucose deprivation, triggers a shift in substrate utilization from the adult form (glucose alone) to the immature form (lactate as well as glucose, Wada et al., 1997). We also previously demonstrated that pyruvate and ß-hydroxybutyrate as well as lactate could not sustain the synaptic potential despite the fact that energy levels of the slices were well-maintained in the adult neuron (Kanatani et al., 1995; Wada et al., 1997). However, immature neurons can maintain both synaptic activity and energy levels using these substrates. Studying this dynamic shift in energy metabolism from the adult to the immature form in adult neurons will have a great impact on the understanding of the pathophysiology of neurons injured under post-ischemic or degenerative conditions.

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ORIGINAL ARTICLE

Evaluation of a computerized test system to screen for mild cognitive impairment

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Abstract

Background: Mild cognitive impairment (MCI) refers to the clinical condition between normal aging and Alzheimer's disease (AD) and has a high probability of developing into AD. Early detection of MCI is important because early detection and appropriate follow-up treatment can prevent the disease from progressing. Therefore, MCI is an important candidate for screening and possible intervention.

Methods: We have developed a computerized screening test system to identify cognitive decline. This system consists of six tests (age and year-of-birth validity test, three-word memory test, time orientation test, first modified delayed-recall test, visual working memory test and second modified delayed-recall test). The scores obtained from three groups (MCI patients, AD patients and healthy control subjects) were analyzed to evaluate the sensitivity and specificity required for the screening of MCI.

Results: The system was well accepted by the patients. All of the test procedures were completed within 5 min. Significant group differences in all test results were found. The system has sensitivity and specificity values of 82% and 87%, respectively, when used as a screen for MCI.

Conclusion: The system is useful for the screening of cognitive disorders.

INTRODUCTION

The population of aged persons is increasing along with the extension of the average span of human life. The number of aged persons over 65 years of age is now over 24 million, which accounted for 19% of all Japanese people in 2003. The prevalence rate of dementia was reported to be 7.5% of all people over 65 years of age.[1] This means that the number of persons with dementia is estimated to have reached more than two million in Japan. Therefore, preventing an increase in the number of persons who suffer from cognitive disorders is regarded as an urgent task. Nowadays, a lot of attention is focused on mild cognitive impairment (MCI).[2] MCI refers to the clinical condition between normal aging and Alzheimer's disease (AD) and has a high probability of developing into AD.[3] Early detection of MCI is important because early detection and appropriate follow-up treatment can prevent the disease from progressing.[4] Therefore, MCI is an important candidate for screening and possible intervention.[5,6] In the case of mass screening, the necessary requirements which a test method must fulfill are speed, objectiveness and unbiased results even if the examiner changes. Using a computerized cognitive test system yields some useful advantages; for example, it can provide quick, objective and precise results based on the same standard.[7] We have developed a computerized screening test system to identify cognitive decline. In this paper, we present a description of the system and the results obtained in our study.

SUBJECTS AND METHODS

Subjects

Fifty-one outpatients in the memory disorder and dementia clinics at Tottori University Hospital were enrolled for the study. They received neuropsychological tests as well as a neuroimaging examination and other medical checks. The diagnosis of dementia was made according to the *Diagnostic and Statistical Manual of Mental Disorders*, revised third edition (DSM-III-R)[8] criteria, and the diagnosis of AD was based on the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA)[9] criteria. The diagnosis of MCI was made according to the five criteria for amnestic mild cognitive impairment, which are: (i) subjective memory complaints; (ii) impaired memory function for age and education level; (iii) preserved general cognitive function; (vi) intact activities of daily living; and (v) absence of dementia, as previously reported.[3] Finally, 29 patients (eight men and 21 women; mean age of 78.1 ± 5.2 years) were diagnosed with senile dementia of the Alzheimer type (SDAT) and 22 patients (eight men and 14 women; mean age of 72.0 ± 9.6 years) were diagnosed with MCI. We recruited healthy control subjects from among the people who attended the Health Promotion event and from the patients' spouses who visited the hospital. Fifty-five persons (11 men and 44 women; mean age of 72.6 ± 7.3 years) accepted our invitation to participate in the study.

Computer system description

The computer program was developed with Microsoft Visual Basic 6.0 and runs under the Windows operating system on any IBM PC-compatible computer. The computer must have an audio output device because audio instructions are provided along with written instructions. The system has been designed for users who are mainly aged people. Most aged people can hardly operate a computer by using a keyboard and mouse. Therefore, we adopted a touch screen display as an input device so that aged people can operate the system easily. All interactions can be performed by touching the icon shown on the display without using a keyboard and mouse. Throughout the whole process, users are guided not only by text prompts, but also by voice instructions. In this study, we used a Hitachi PC1NH5 laptop computer (Hitachi, Tokyo, Japan) and a Totoku CV511PJ touch screen display (Totoku, Tokyo, Japan).

Details of test procedure

The system presents six tests as follows:

1. Age and year-of-birth validity test

The purpose of this test is to examine the subject's ability to recall his or her age and year of birth. The system will ask the subject for his or her age and year of birth, and the subject is then required to respond by touching the corresponding icons on the screen display. The system awards one point for the correct response.

2. Three-word memory test

The purpose of this test is to assess the immediate memory of the subject. The system will voice three words (for example, apple, swallow and car) and will immediately ask the subject what the words were. Then the system displays nine choice icons and requires the subject to select the three correct icons. The system awards a maximum of three points, one for each correct icon chosen. Before this test ends, the system informs the subject that the following modified delayed-recall test will query the subject for the three words again.

3. Time orientation test

For this test, the system presents four screens in turn, and asks the subject what year, month, date and day it is, respectively. The system awards a maximum of four points, one for each correct response.

4. First modified delayed-recall test

The purpose of this test is to assess the short-term memory of the subject. The system asks the subject to recall the same words voiced in Test 2. Nine choice icons are displayed as in Test 2, but the arrangement is different. The system awards a maximum of three points, one point for each correct answer.

5. Visual working memory test

The purpose of this test is to examine the visual working memory of the subject. Two types of figures are

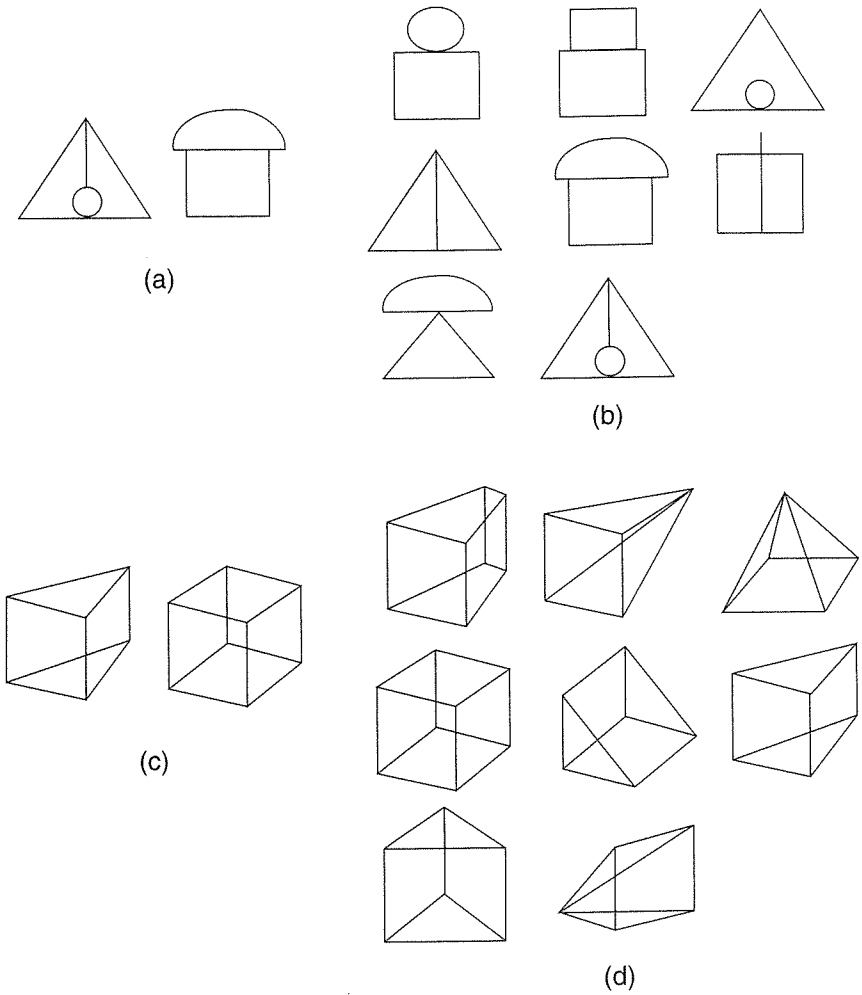

Figure 1 Figures used in the visual working memory test. (a) 2-D target stimuli; (b) 2-D figure choices; (c) 3-D target stimuli; and (d) 3-D figure choices.

used for this test: two-dimensional (2-D) and three-dimensional (3-D). Figure 1 shows the target figures and choices for this test. First, two 2-D target figures are presented for 10 s, after which the system displays eight possible choices of 2-D figures. The subject is then required to select the two previously displayed figures from among the eight choices. One point is awarded for each correct answer, for a maximum of two points. This process is then repeated with 3-D figures.

6. Second modified delayed-recall test

This test also assesses the short-term memory of the subject and is similar to the first delayed-recall test (Test 4, explained above). A maximum of three points are awarded, one for each correct response. For this test, the arrangement of the choice icons is different from that in Test 2 and Test 4.

All tests are usually completed within 5 min and the maximum number of points that can be awarded is 18 points.

Statistical analysis

Variations between the groups were first analyzed for significance by the Kruskal–Wallis test and then pairs of groups were directly compared using the Mann–Whitney test. Moreover, the differences between the scores obtained from three tests (three-word memory test; first and second modified delayed-recall tests) for each group were examined by the Friedman non-parametric test. These three statistical procedures were performed using statistical software (SPSS version 11 for Windows; SPSS Inc., Chicago, IL, USA). Receiver operating characteristic (ROC) analysis was performed to determine optimal cut-off points for the total score correspond-

Table 1 Demographic characteristics of samples and the results of individual tests for all three groups

	Control	MCI	SDAT
Sample size	55	22	29
Age (years)	72.6 ± 7.3	72 ± 9.6	78.1 ± 5.2
Sex (M/F)	11/44	8/14	8/21
Age and year-of-birth validity test	1 ± 0.00	0.89 ± 0.32	0.66 ± 0.48
Three-word memory test	2.96 ± 0.20	2.83 ± 0.38	2.66 ± 0.61
Time orientation test	3.86 ± 0.35	3.83 ± 0.38	1.69 ± 1.31
First delayed-recall test	2.94 ± 0.24	2.67 ± 0.49	1.76 ± 1.15
Visual working memory test for 2-D figures	1.88 ± 0.33	1.83 ± 0.38	0.97 ± 0.78
Visual working memory test for 3-D figures	1.80 ± 0.40	1.44 ± 0.51	1.31 ± 0.66
Second delayed-recall test	2.86 ± 0.35	2.44 ± 0.51	1.38 ± 0.98
Total score	17.31 ± 0.93	15.94 ± 1.26	10.41 ± 4.06

Results are expressed as: mean ± standard deviation. MCI, mild cognitive impairment; SDAT, senile dementia of the Alzheimer type.

Table 2 Results of the Kruskal–Wallis test showing significant group differences for all tests

	χ^2-value
Age and year-of-birth validity test	20.2**
Three-word memory test	9.39*
Time orientation test	59.3**
First delayed-recall test	35.4**
Visual working memory test for 2-D figures	37.3**
Visual working memory test for 3-D figures	15.7**
Second delayed-recall test	49.3**
Total score	65.0**

*$P < 0.01$, **$P < 0.001$.

ing to the screening between MCI and control subjects.

RESULTS

All subjects could understand how to interact with the computer and could complete the tests by themselves. Table 1 presents the demographic characteristics of samples and the results of individual tests from the three groups. At the group level, the SDAT group performed worse than the other two groups in each of the six tests, and the control group performed best in each of the six tasks. The results of the Kruskal–Wallis test are shown in Table 2 and significant group differences can be found for all tests. The results of the Mann–Whitney test in which the differences for all pairs of groups were compared are shown in Table 3. Significant differences between the control group and the SDAT group were found for all tests. Significant differences between the control group and the MCI group were found for the age and year-of-birth validity test, the visual working memory test for 3-D figures, the second delayed-recall test and the total score. Significant differences between the MCI group and SDAT group were found for the time orientation test, the visual working memory test for 2-D figures, the second delayed-recall test and the total score. The results of the Friedman nonparametric test are shown in Table 4. Significant differences were found in the SDAT and MCI groups at 99.9% level ($P < 0.001$) and 95% level ($P < 0.05$), respectively, and no significant differences were found in the control group. ROC analysis to identify the control and MCI yielded the best sensitivity and specificity values of 82% and 87%, respectively, with a cut-off point of 16.

DISCUSSION

The mini-mental state examination (MMSE)[10] is a widely recognized tool used for the detection of cognitive impairment, and in addition appears to be quite useful for examining patients with an increased risk of dementia (e.g. MCI patients).[11] When we developed the screening system, we referred to the tests used in the MMSE. However, these were originally designed for a method based on face-to-face interviews, so it was difficult to adopt all the tests for the computerized procedure. We paid attention to the tests of memory and the tests of visual working memory which are sensitive neuropsychological measures to detect early cognitive decline. Finally, we assembled the six tests explained above to form the computerized test system.

The mean value of the total score for all tests decreases in this order: control group, MCI group and SDAT group. In the validity test for age and year of

Table 3 Results of the Mann–Whitney test comparing the differences in all pairs of groups

	Control and MCI	Control and SDAT	MCI and SDAT
Age and year-of-birth validity test	*	**	NS
Three-word memory test	NS	**	NS
Time orientation test	NS	**	**
First delayed-recall test	NS	**	NS
Visual working memory test for 2-D figures	NS	**	**
Visual working memory test for 3-D figures	**	**	NS
Second delayed-recall test	**	**	**
Total score	**	**	**

*$P < 0.01$, **$P < 0.001$. MCI, mild cognitive impairment; NS, not significant; SDAT, senile dementia of the Alzheimer type.

Table 4 Results of the Friedman non-parametric test analyzing the differences in the scores from three tests for each group

	χ^2-value
Control	7
MCI	8.22*
SDAT	32.1**

*$P < 0.05$, **$P < 0.001$. MCI, mild cognitive impairment; SDAT, senile dementia of the Alzheimer type.

birth, all control subjects gave the correct answer. However, some MCI patients and one-third of the SDAT patients gave the wrong answer. A person who is unable to give his/her correct age and year of birth can be suspected to have MCI or SDAT. A combination of the three-word memory test and the subsequent delayed-recall test has been proposed as a useful test to screen for dementia.[12] In this study, there were no significant differences between the three-word memory test and the two subsequent modified delayed-recall tests in the control group. However, it is statistically significant that the score for the latter test was worse than that of the earlier test in the MCI and SDAT groups. This result is especially obvious in the SDAT group. It also suggests that these tests are useful for identifying cognitive decline. Visual working memory is also an important factor in the diagnosis of dementia.[13] In this study, we used two types of figures as target stimuli for the visual working memory test: 2-D and 3-D figures. In the case of 2-D figures, there were no statistical differences between the control and MCI groups. However, in the case of 3-D figures, statistical differences were found between the control group and MCI group. As for the MCI patients, they performed as well as the healthy control subjects in the visual working memory test for 2-D figures, but their performance was significantly impaired in the 3-D visual working memory test compared to the control subjects. In contrast, the visual working memory of the SDAT group for 2-D and 3-D figures was significantly impaired compared to the control group. Therefore, the visual working memory test also seems to be a useful indicator in identifying cognitive decline.

Conventional neuropsychological tests, such as the MMSE, involve dialogs between the interviewer and subject. However, it is difficult for a computer to understand what a subject is saying. Therefore, the system can only be designed to recognize a response when the user touches the choice button shown on the computer screen. Showing the answer choices is like giving a small hint and it does not require users to generate answers freely. In the delayed-recall test, presenting cues during the elicit retrieval phase improves the specificity of distinguishing individuals with dementia from unimpaired elderly people.[14] People who are able to answer the question with the help of a hint seem to be able to retain their cognitive ability and they usually get a high score. However, people who obtain a low test score even when hints are given are strongly suspected to be in cognitive decline. The selection of one answer from a range of choices seems to be adequate for the assessment of memory impairment and the method is easily adopted as a computerized procedure. However, computers are unsuitable for the assessment of dialogic ability, such as fluency, and this is a disadvantage of using computers.

We were able to identify MCI patients with an accuracy of 82% in this study, which is similar to the results presented by Darby *et al.*[15] Some other studies using biochemical examinations[16] and medical imaging equipment[17] have distinguished normal aged persons from those with dementia with high sensitivity

and a specificity of over 90%. However, such examinations are invasive or expensive. Therefore, we think that neuropsychological tests like ours are better for mass screening at the present time.

As conventional tests are based on a face-to-face interview, we often feel awkward when patients are unable to answer the questions correctly. The patients also seem to be embarrassed. In such cases, even though they are in the presence of a doctor, it seems that the patients' pride is hurt by showing their cognitive decline. But in the case of a computerized screening system, they do not feel uncomfortable when they cannot answer the question correctly. There appear to be some advantages and disadvantages in the methods of assessment using human interviewers and using computers. Human interviewers can respond flexibly according to the condition of the patient. However, their prescribed treatment is likely to be biased according to their experience or knowledge, and there is a risk that the assessment criteria could vary from tester to tester.[18] In contrast, the computerized testing system is good at extracting the required information based on the same standards without the above-mentioned problems. Standardization is required for screening tests to be consistent in assessment and for their widespread use. It has been pointed out that a useful dementia screening system should be fast, simple and accurate.[19] Our computerized test system can provide quick, objective and precise results based on the same standard. Although computerized testing cannot replace a human interviewer in all cases, this system is useful for the screening of cognitive disorders.

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Genetic Analysis of Familial Alzheimer's Disease in a Japanese Population

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Alzheimer's disease (AD) is one of the most common neurodegenerative disorders in the elderly population. The pathological hallmarks (amyloid plaque, neurofibrillary tangle and neuronal cell loss) have been well characterized. The causal genes for early-onset familial Alzheimer's disease (FAD) are the presenilin 1 (PS-1) gene on chromosome 14 [1], the presenilin 2 (PS-2) gene on chromosome 1 [2] and the amyloid precursor protein (APP) gene on chromosome 21 [3]. In addition, apolipoprotein E (APOE) allele 4 (ε4), located on chromosome 19, is a well-established genetic risk factor for sporadic AD [4]. Among these genes, mutations in PS-1 seem to be the most common genetic factor underlying the development of early-onset FAD. To date, over 100 missense mutations for PS-1, 8 mutations for PS-2 and 16 mutations for APP are cited in an online database (AD mutation database; http://molgen-www.uia.ac.be/ADMutations/). Several PS-1 mutations and only an APP mutation (V717I) were previously described in the Japanese population (table 1) [2, 5–19]. Eighteen missense mutations in the PS-1 gene were reported in the Japanese familial AD (FAD) pedigrees. No pathogenic mutation of the PS-2 gene has been identified in the Japanese population. In this chapter, we report the results of our most recent studies of these three genes in FAD and sporadic AD patients in a Japanese population.

Subjects and Methods

Patient Samples

Twenty-two Japanese patients were selected from 5 early-onset (<65 years old) FAD patients (mean age of onset: 58.2 years), 7 late-onset (>65 years old) FAD patients

Table 1. APP, PS-1 and PS-2 gene mutations in Japanese FAD and sporadic AD

	Exon	Mutation	Reference
PS-1	5	V96F	5
		E123K	6
	6	H163R[1]	5, 7, 8, 9
	7	E184D	10
		G209R	11
		I213T	5
		G217D	12
		F237I[2]	13
	8	A260V	2, 14
		S266G	15
	8	R269H	9
		E273A	9
		E280A	8
		A285V	2
	9	S290C	16
	11	G384A	9
		N405S	17
	12	A431V	18
APP	17	V717I	19

[1]This mutation was reported in early-onset FAD and early-onset sporadic AD.

[2]This mutation was reported in early-onset FAD with spastic paraparesis.

(mean age of onset: 70.3 years old), and 10 early-onset sporadic AD patients (mean age of onset: 55.4 years). All patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorder Association (NINCDS-ADRDA) criteria for probable and possible AD [20]. These diagnoses were assisted by MRI or CT imaging studies. We defined patients as having FAD if at least 2 members were affected in a family and the difference in age of onset was less than 20 years. Genomic DNA was extracted from peripheral leukocytes using the standard phenol-chloroform method and subjected to PCR amplifications.

Primers and PCR Amplification

Intronic primers were generated to amplify exons 16, 17 and 18 of the APP gene, exons 3–12 of the PS-1 and PS-2 genes. The primer sequences are provided in table 2. In brief, 50–100 ng DNA was amplified using PCR in each 15 μl reaction mixture using 1 mmol of specific primers and 0.8 units of Taq DNA polymerase (TaKaRa, Tokyo, Japan) in supplied 1 × PCR buffer for 35 cycles of 30 s at 94°C for denaturing, 30 s at 58°C for annealing, and 40 s at 72°C for extension.

Table 2. PCR primers (5′→3′)

APP		Size, bp
APP Ex16-F	TAGAAAGAAGTTTTGGGTAGGCTTT	260
APP Ex16-R	AGAGTTAATAGGTCATTTGGCAAGACA	
APP Ex17-F	AATGAAATTCTTCTAATTGCGTTT	258
APP Ex17-R	TTCTCTCATAGTCTTAATTCCCACTT	
APP Ex18-F	TTACTGCTTCTCCATGTTCACC	249
APP Ex18-R	TTCTATAAATGGACACCGATGG	
PS-1		
PS1Ex-3F	TCTTTCCCTTTTCAGAACCTCA	222
PS1-Ex3R	CAGAGGTGAGGGGAGATGATAA	
PS1-Ex4F	TCATAGTGACGGGTCTGTTGTT	423
PS1-Ex4R	CTTCAAGGTGATGATGACATGC	
PS1-Ex5F	TTCTGTGTTGGAGGTGGTAATG	287
PS1-Ex5R	CTGTGACAAGAATACCCAACCA	
PS1-Ex6F	AGTCTGGGCGACAAAGTGAG	237
PS1-Ex6R	TGATAGCTACACAGCACAAAGG	
PS1-Ex7F	GTTTGGGAGCCATCACATTATT	373
PS1-Ex7R	AGATGAGGAAAGAAAACACTCCA	
PS1-Ex8F	CCTTCGTTAATTCCTCCCTACC	248
PS1-Ex8R	CATGTGCTTCAGTTCCGATAAA	
PS1-Ex9F	GGAGAAATGATGGCTTGTTGTT	266
PS1-Ex9R	GGAGTCTATGACCAAAGAAAGACG	
PS1-Ex10F	CAATGACAGCTAGTTACTGTTTCCA	346
PS1-Ex10R	TTCATTTTATTCTCAAAAAGGTTGA	
PS1-Ex11F	ATTCATTGTGGGGTTGAGTAGG	294
PS1-Ex11R	GAACTGCCTTAAAGGGACTGTG	
PS1-Ex12F	CTTGTGATTGAGTTTTGCCTGA	294
PS1-Ex12R	TGTCCTCCCCAGATTTTGTTAT	

PS-2		Size, bp
PS2-Ex3F	GTCTCACAGGAAAGTGGAACAAG	284
PS2-Ex3R	GAATGTCTGGTTTTCATCTGTAAGG	
PS2-Ex4F	TGGAAAGCAACATTCAAACTTC	456
PS2-Ex4R	TTCTTCATCCCTGCTCTTTACC	
PS2-Ex5F	TAGCAGGTCCAGAATCACTCAAG	318
PS2-Ex5R	GTTTTTCTAAAGGCGGCTGTTT	
PS2-Ex6F	GCTTGGGTATCAGTCTCAGGAT	258
PS2-Ex6R	GAGCTCGATGGTCATCTTTCC	
PS2-Ex7F	GAATGGTGGTAAACTGCTAGGC	407
PS2-Ex7R	CTCTGCAGAAAGGGATGCAAG	
PS2-Ex8F	GCTACAGGGCAGGCTCTTCT	206
PS2-Ex8R	ACCCCGAGTCAGGCAGAG	
PS2-Ex9F	GCTTTCTGGGACGCAGACT	311
PS2-Ex9R	TGGTTTTCAACGGACCTTTC	
PS2-Ex10F	ACCCCTTCTTGGAGCTTTGT	282
PS2-Ex10R	GAGCCTCCACCCTCTGTCTC	
PS2-Ex11F	GGGCCAGAGTTTCTCTTCTTTT	281
PS2-Ex11R	CCCTAGGGATCCTGAGACCTG	
PS2-Ex12F	CCCAGGGACTAGACCATGACT	290
PS2-Ex12R	AACTGCATCCAATGAAAATTCC	

Table 3. Identified mutation and polymorphism

	Exon (PCR region)	Polymorphism	Amino acid	NCBI SNP cluster ID
APP	Exon 16	2032 G/A	D678N	–
	Exon 18	IVS17-10 T/C	intron	–
PS-2	Exon 3	69 T/C	A23A	rs11405
		129 C/T	N43N	rs6759
	Exon 4	IVS3-42 G/A	intron	rs1295644[1]
		IVS3-29 T/C	intron	rs1295643
		260 T/C	H87H	rs1046240[1]
	Exon 5	IVS5+30 G/C	intron	rs2236910
	Exon 8	861 C/T	P287P	–
	Exon 9	IVS8-24 G/A	intron	rs2802267
	Exon 11	IVS11+24 G/A	intron	rs2855562

[1]These are linked polymorphisms in our samples.

Single-Strand Conformation Polymorphism Analysis and Sequence Analysis

PCR products of AD samples for screening were subjected to single-strand conformation polymorphism (SSCP) analysis. One microliter of PCR product was denatured in formamide-containing buffer at 95°C for 8 min, quickly chilled on ice, and electrophoresed on a 12% polyacrylamide gel with 10% glycerol at 4°C for 24 h at 200 V. DNA bands were visualized using silver staining. The mobility-shifted band was directly cut from the gel using a freshly prepared razor blade. The eluted band was re-amplified under identical PCR conditions for 45 cycles. The purified PCR product derived from the extra band was subjected to direct sequencing using a Big Dye cycle sequence kit (Amersham Bioscience Japan, Tokyo, Japan) and the ALF automated luminescent sequencer (Applied Biosystems Japan, Tokyo, Japan). APOE genotyping was carried out according to standard procedures [22].

Results

In PCR-SSCP analysis, 2 extra conformers in the APP gene (exon 16, exon 18), none in the PS-1 gene and 9 in the PS-2 gene were observed. Table 3 shows the identified mutation and polymorphisms identified by sequence analysis. No missense mutations in the PS-1 and PS-2 gene were detected in any samples. Except for the IVS17-10 T/C polymorphism of the APP gene and the 861 C/T (P287P) polymorphism of the PS-2 gene, the identified polymorphisms were previously reported in the NCBI SNP database (the APP gene; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId = 35, the PS-1 gene; http://www.ncbi.nlm.

nih.gov/SNP/snp_ref.cgi?locusId = 5663 and the PS-2 gene; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId = 5664). We identified an entirely novel APP gene mutation (2032 G/A D678N [APP770 numbering]) in an early-onset FAD pedigree containing 3 affected members with a mean age of onset of 59.7 years and APOE genotype ε3/ε3.

Discussion

In the present study, we systematically conducted mutation screening of the PS-1, PS-2 and APP genes in samples from patients diagnosed with varied forms of AD. No pathogenic mutations of the PS-1 or PS-2 genes were identified. In the APP gene, we identified a novel mutation (D678N) in an early-onset FAD pedigree. This mutation is equivalent to an amino acid substitution of Asp at position 7 of amyloid-β (Aβ) (Asp7-Aβ) with Asn (Asn7-Aβ). We hypothesize that Asn7-Aβ derived from the D678N mutant APP has altered fibrillogenic and/or catabolic properties that increase accumulation of protein and/or the neurotoxic potential of Aβ, eventually leading to AD. In vitro studies will be necessary to characterize the pathogenic impact of the D678 mutation on fibrillogenesis and/or secretase activity.

We identified 2 novel SNPs in the APP gene and 9 SNPs (including 1 novel SNP) in the PS-2 gene. The IVS17-10 T/C polymorphism of the APP gene, identified from an EOSAD patient (age of onset: 59 years), is close to a splicing acceptor site and may raise a possibility to influence splicing efficiency. The genetic case-control study of this polymorphism is currently under way. While 861 C/T (P287P) polymorphism of the PS-2 gene was found in an FAD pedigree of variable age of onset (age 63–75), we have not obtained sufficient segregation data regarding this mutation. Furthermore, since 861 C/T (P287P) is a silent polymorphism, it is unclear whether this polymorphism (or a linked mutation) of the PS-2 gene contributes to the development of this FAD pedigree.

Genetic linkage studies have demonstrated multiple susceptible loci for FAD [22–26]. Additional studies are required to identify as many candidate genes as possible to elucidate the pathomechanisms of AD and improve our strategies for treatment and prevention of AD.

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Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients

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Objectives – To investigate a possible implication of inflammatory processes in the development of dementia in cerebrovascular disease. *Patients and methods* – We examined the levels of interleukin-6 (IL-6) in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) ($n = 26$), ischemic cerebrovascular disease without dementia (CVD) ($n = 11$), vascular dementia (VD) ($n = 11$), and other neurological disorders ($n = 21$) using sensitive enzyme-linked immunosorbent assay. *Results* – The CSF concentrations of IL-6 were significantly elevated in patients with VD compared with those of patients with AD or CVD. *Conclusion* – The CSF IL-6 levels are increased in patients with VD, suggesting that inflammatory mechanisms may be involved in the development of cognitive decline in some patients with cerebrovascular disease. CSF IL-6 may be a biological marker for dementia in cerebrovascular disease.

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Key words: vascular dementia; Alzheimer's disease; cerebrovascular disease; interleukin-6; cytokines; tau protein; cerebrospinal fluid; biological marker

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Vascular dementia (VD) is a common cause of dementia in Japan (1). However, the mechanisms of clinical cognitive deterioration in patients with cerebral ischemia are not completely understood. Several recent studies have provided insight into the possible role of inflammatory processes in the development of brain ischemia and multi-infarct cognitive impairment, as demonstrated by the accumulation of inflammatory cells and mediators in the ischemic brain (2–5). Vila et al. (6) reported that interleukin-6 (IL-6) participates in the acute-phase response that follows cerebral ischemia and that an association exists between high levels of IL-6 and early neurological worsening. A case–control genetic study reported a positive association of the -174 G/C IL-6 gene polymorphism and the risk of multi-infarct dementia (7). These data led the hypothesis that inflammatory mechanisms play a crucial role in the pathogenesis of the development of dementia in cerebrovascular disease. Cerebrospinal fluid (CSF) levels of IL-6 are elevated in central nervous system (CNS) infections and non-infectious CNS inflammatory diseases, indicating that levels of IL-6 in the CSF reflect the inflammatory processes (8–10). Little has been reported about the IL-6 levels in the CSF of patients with VD. Previous studies reported that these levels did not differ from those of controls (11–13). However, some patients with VD in the previous study investigated by Yamada et al. (13) showed high CSF concentrations of IL-6. In order to clarify the association of inflammatory mechanisms with VD, we examined the CSF levels of IL-6 in patients with VD as well as in patients with Alzheimer's disease (AD) and ischemic cerebrovascular disease without dementia (CVD).

Subjects and methods

The subjects were 26 patients with AD (mean age ± SD 66.8 ± 8.2 years), 11 patients with CVD (70.0 ± 6.2 years), 11 patients with VD (74.5 ± 4.5 years), and 21 patients with other neurological disease (OND) (68.4 ± 5.8 years). Assessments of these patients included a carefully examined medical history, physiological examination, drug inventory, neurological examination, comprehensive cognitive evaluation with the use of the Functional Assessment Staging of Alzheimer's disease (FAST staging), the Mini-Mental State Examination (MMSE), neuroimaging assessments of CT scan or MRI and single photon emission computed tomography of the head, and routine laboratory tests, such as blood analysis and biological

examination. Patients who satisfied the Diagnostic and Statistical Manual of Mental Disorders, third edition-revised (DSM-III-R) (14) and the diagnostic criteria of the National Institute of Neurological and Communicative Disorders Association (NINCDS-ADRDA) (15) and those scoring 4 points or less on Hachinski's ischemic score (16) were diagnosed as having AD. Patients who satisfied the DSM-III-R and the ADDTC criteria for ischemic VD (17) and those scoring 7 points or more on Hachinski's ischemic score were diagnosed as having VD. All the patients with VD showed stepwise deterioration of cognitive function and one or more infarcts outside the cerebellum detected by neuroimaging. The CVD group was defined as patients who had a history of stroke episode and with CT scan or MRI findings of infarcts without dementia. OND patients consisted of seven patients with Parkinson's disease (PD), four patients with amyotrophic lateral sclerosis (ALS), four patients with spinocerebellar degeneration, two patients with peripheral neuropathy, two patients with tension-type headache, one patient with myopathy and one patient with essential tremor. OND patients did not show any cognitive impairment. After informed consent from patients or their families, CSF was collected by lumbar puncture. CSF samples were stored at −80°C until assay. Collections of CSF from the patients with CVD or VD were performed during the chronic phase of the diseases when the progression of neurological deterioration was no longer observed. CSF IL-6 levels were determined in duplicate, using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Inc, Minneapolis, MN, USA). CSF total tau protein levels were measured using ELISA kit (Innogenetics, Gent, Belgium). Statistical significance was analyzed by one-way ANOVA, followed by *post hoc* tests. Correlation was analyzed by Spearman rank correlation test.

Results

As shown in Fig. 1, the concentrations of IL-6 in the CSF of VD patients were 5.67 ± 1.7 pg/ml (mean ± SE); those of patients with AD were 2.53 ± 0.87 pg/ml; those of patients with CVD were 2.15 ± 0.38 pg/ml; and those of patients with OND were 3.15 ± 0.67 pg/ml. Significantly elevated levels of IL-6 were found in the CSF of patients with VD compared with those in the CSF of patients with AD, CVD, and OND. There was no significant difference in CSF IL-6 levels between AD patients and CVD and OND patients. There were not a correlation between CSF IL-6 levels and

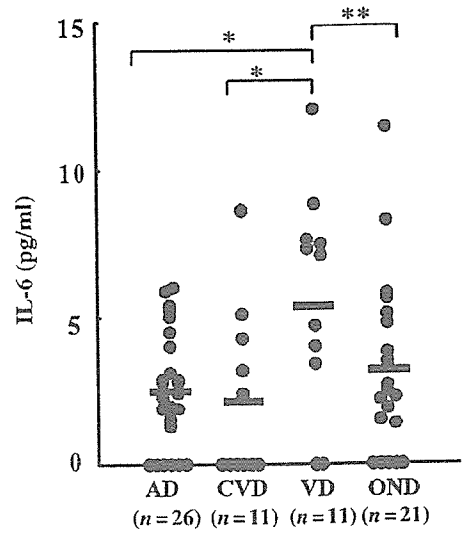

Figure 1. Interleukin-6 levels in the cerebrospinal fluid of the patients with Alzheimer's disease, ischemic cerebrovascular disease without dementia, vascular dementia, and other neurological disease. The horizontal bar indicates the mean level. Statistical differences were calculated using one-way ANOVA followed by a *post hoc* test; $*P < 0.01$, $**P < 0.05$.

Table 1 Total tau levels in cerebrospinal fluid

Disease	Mean ± SE (pg/ml)
AD	236.0 ± 17.2
CVD	115.0 ± 39.3
VD	116.8 ± 28.1
OND	126.4 ± 16.4

AD, Alzheimer's disease; CVD, cerebrovascular disease without dementia; VD, vascular dementia; OND, other neurological disorders.

MMSE scores in VD patients (data not shown). The levels of total tau protein in CSF were shown in Table 1. Significantly elevated levels of tau protein were found in the CSF of patients with AD compared with those in the CSF of patients with VD ($P < 0.01$). There were not significant differences between CSF tau levels in VD and those in CVD or OND.

Discussion

Interleukin-6 was described initially by Hirano (18) as a B cell differentiation factor usually derived from T cells and it can also be produced by astrocytes and microglia in the CNS (9, 19, 20). CSF levels of IL-6 were examined in patients with infectious or non-infectious inflammatory diseases of the CNS. CSF levels of IL-6 were also examined in patients with neurodegenerative disorders. In particular, controversial results ranging from no changed (21, 22), to increased (23) or decreased

(13) levels of IL-6 in the CSF have been reported in AD patients. Differences in sample size, selection of patients with AD and control subjects, or experimental procedures may account for these varying results. Our results show that there are no significant differences in the CSF levels of IL-6 between patients with AD and patients with CVD or OND. The OND group included patients with PD and ALS, in which higher levels of CSF IL-6 have been reported (23, 24). Indeed, the patients with the two highest levels in OND group in our study were a PD patient and an ALS patient, but CSF IL-6 levels were not altered in other patients with ALS or PD. We conclude that the levels of IL-6 are not altered in patients with AD, and that CSF IL-6 may not be a biological marker for the diagnosis of AD. On the contrary, we obtained significantly higher CSF levels of IL-6 in patients with VD, but not in patients with CVD who did not have dementia. However, previous reports indicated that CSF levels of IL-6 in VD patients were not significantly elevated (11, 12). It has been reported that patients with VD are heterogeneous and diagnosis criteria for VD are not interchangeable (25). Selection of patients may account for these differences. Not all the patients with VD showed higher levels of IL-6 in CSF in this study, but we used the DSM-III-R and ADTTC criteria for diagnosis of VD and employed probable cases in this study and these cases also showed significant lower levels of total tau protein in CSF compared with AD patients, suggesting that clinical diagnosis of VD patients was sufficient to segregate AD patients from VD cases. Recent reports indicated inflammatory process might be involved in cerebrovascular disease. Higher baseline levels of CSF IL-6 were shown to be related to early neurologic worsening in ischemic stroke, independent of the initial size topography or mechanism of ischemic infarction (6, 26). A genetic association of IL-6 polymorphism with multi-infarct dementia (7) and activation of the micoglia in Binswanger's disease, a form of VD has been shown (3). The increased intrathecal production of granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that stimulates microglial cell growth and has inflammatory properties, has been found in patients with VD (4). Taken together with our result, the inflammatory activations in the CNS might be associated with some part of VD patients and measurement of CSF IL-6 might provide a clue to differential diagnosis of dementia. Our study is a cross-sectional design, further studies using a longitudinal design with large samples are necessary to support these results.

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Soluble Aβ homeostasis in AD and DS: impairment of anti-amyloidogenic protection by lipoproteins

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Abstract

In order to assess whether lipoproteins are physiologically able to balance and modulate the sAβ homeostasis in vivo, soluble Aβ levels in lipoprotein-depleted plasma were measured as a function of age in normal controls, Alzheimer's disease (AD) patients, and Down's syndrome (DS) cases. The reshaping of sAβ homeostasis, in particular the sAβ42-lipoprotein interaction, takes place over normal-60's, whereas mild AD patients appear to have impaired this anti-amyloidogenic mechanism resulting in a significant increase of lipoprotein-free sAβ42. Similar loss of function takes place in Down's syndrome patients. Lipoprotein-free sAβ remains significantly elevated from the pre-symptomatic through the symptomatic stages of the disease, and declines with the progression of the AD-like pathology. The dissociation of sAβ from lipoprotein-particles also occurs in brain parenchyma and the presence of soluble dimeric lipoprotein-free Aβ prior to its parenchymal deposition in AD brains would support the hypothesis that functionally declined lipoproteins may be major determinants in the production of metabolic conditions leading to higher levels of sAβ species and cerebral amyloidosis.

Keywords: Alzheimer's disease; Down's syndrome; sAβ homeostasis; Lipoprotein-free Aβ; Aβ dimer

1. Introduction

Amyloid β (Aβ) is the major constituent of the fibrils deposited in senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) and Down's syndrome (DS). This peptide, originally thought not to be derived by normal processing of its precursor APP [30], is now known to be a normal soluble component (sAβ) of biological fluids [8,28,29,40] and brain parenchyma [24,33]. It has been reported that an increased amount of sAβ precedes the appearance of Aβ deposits in AD and DS brains [35] and accumulates with age [6], suggesting that sAβ species represent the direct precursors of the deposited fibrils. Ninety percent or more of the sAβ found in normal human plasma is associated with lipoprotein particles [20], which had been shown to maintain and stabilize the peptide solubility in vitro [19,21] and modulate its catabolism [8,20]. A recent study suggests that brain levels of total extractable Aβ becomes elevated very early in the disease process and correlates with measurable decline in cognitive functions [23], pointing to the in vivo sAβ homeostasis as a potential therapeutic target.

It is well known that aging is the most prevailing risk factor for sporadic AD patients. In vivo studies have shown that Aβ neurotoxicity is closely related to the aging brain via unknown age-related factors [7], perhaps reflecting metabolic alterations. Our previous experiments demonstrated that a functional decline for the physiologic

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