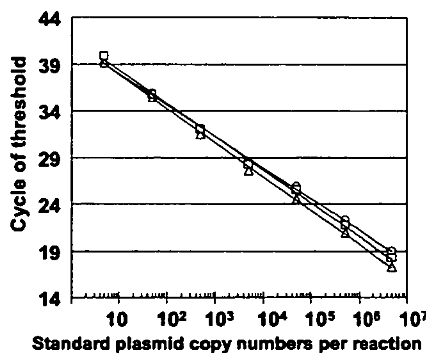

FIG. 1. Standard curves generated by multiplex real-time PCR. Serial dilutions of each viral standard ranging from 5 to 5 × 10^6 copies per reaction were used to generate the standard curves. The cycle of threshold values that corresponded to the PCR cycle number was plotted against the copy number of each viral standard. Circles, EBV DNA standard plasmid; triangles, CMV DNA standard plasmid; squares, HHV-6 standard plasmid.

inhibitor from whole-blood or plasma samples. DNA-extraction solution from either whole blood or plasma was added to serially diluted plasmid controls. The DNA solutions from both whole blood and plasma did not inhibit the amplification efficiency, indicating the absence of inhibitors in these samples. To confirm the specificity of the multiplex assay, viral DNA from standard strains was tested. None of the primer/probe sets reacted with the other viral DNA, indicating that no cross-reactivity occurred. Furthermore, the quantitative linearity, which was made using a standard plasmid, was not influenced by the presence of two other kinds of viral DNA.

The minimum detection level established with this multiplex assay was 2 copies per reaction for EBV (95% CI, 1.16 to 3.89), 2 copies for CMV (95% CI, 1.01 to 2.95), and 2 copies for HHV-6 (95% CI, 0.49 to 2.64). By contrast, the minimum detection level with the single assay was 2 copies for EBV (95% CI, 1.39 to 3.96), 2 copies for CMV (95% CI, 1.01 to 2.71), and 5 copies for HHV-6 (95% CI, 4.35 to 7.58). The multiplex assay had an overall dynamic range of 200 to 5 × 10^8 copies/ml of specimen.

DNA samples (111), which had been tested and stored previously, were evaluated with the multiplex and the single assays. Compared to the results of the single assay, the sensitivity and specificity values of the multiplex assay were 96.0 and

TABLE 2. Comparison of multiplex PCR assay and single PCR assay of previously tested DNA samples

Multiplex PCR assay	Single PCR assay (copies/ml)		Sensitivity (%)	Specificity (%)
	Positive	Negative		
EBV				
No. positive	24	0	96.0	100
No. negative	1	86		
CMV				
No. positive	18	4	94.7	95.7
No. negative	1	88		
HHV-6				
No. positive	33	4	89.2	94.6
No. negative	4	70		

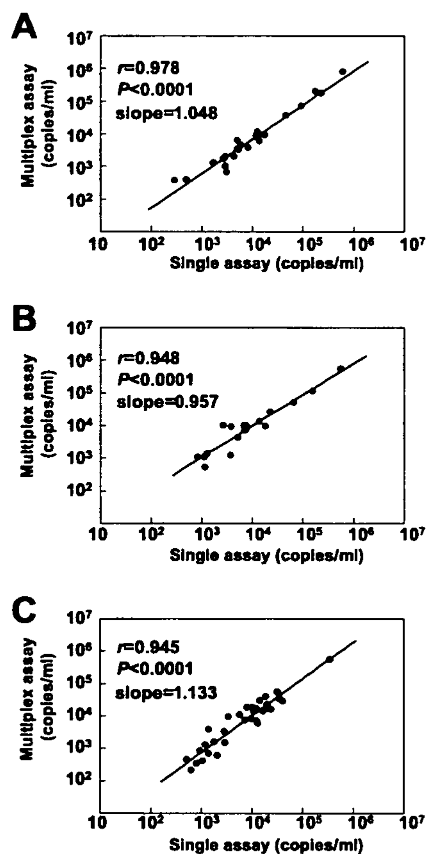

FIG. 2. Correlation of each viral DNA load determined by the multiplex and single real-time PCR assays. (A) Correlation of EBV DNA copy number (n = 24). (B) Correlation of CMV DNA copy number (n = 18). (C) Correlation of HHV-6 DNA copy number (n = 33).

100% for EBV, 94.7 and 95.7% for CMV, and 89.2 and 94.6% for HHV-6, respectively (Table 2). Some discordant results, however, were obtained between the multiplex and single assays. The viral loads of all of these discordant samples were low and around the detection limits. The viral DNA copy numbers were compared using all samples determined to be positive according to both assays. Strong correlations were detected between the viral DNA copy numbers determined by the multiplex assays and those by single assays (Fig. 2). The slopes of the correlation curves ranged from 0.957 to 1.133, indicating that no significant shifts occurred in the actual quantitative values, using the multiplex assay.

Detection of EBV, CMV, and HHV-6 DNA in whole-blood and plasma specimens from transplant recipients. Using the multiplex real-time PCR assay, we serially measured the EBV, CMV, and HHV-6 DNA levels in whole-blood and plasma specimens from 46 transplant recipients. In total, 303 paired samples (6.6 paired samples per patient) were tested. Positives were defined as any positive samples for a given patient. Because we had different numbers of samples from each patient, it was possible that the variety of sample numbers per patient would introduce some bias in the results. Using the whole-blood specimens, at least one form of viral DNA was detected in 36 of 46 recipients (78.3%), plural viral DNA forms were

TABLE 3. Detection of EBV, CMV, and HHV-6 DNA in whole blood and plasma using multiplex real-time PCR assay

Detected viral DNA	No. of samples detected from:				P value[a]
	Whole blood (n = 303)		Plasma (n = 303)		
	n	%	n	%	
EBV or CMV or HHV-6	112	37.0	37	12.2	<0.0001
EBV	72	23.8	18	5.9	<0.0001
CMV	34	11.2	16	5.3	0.0114
HHV-6	38	12.5	6	2.0	<0.0001
EBV only	48	15.8	15	5.0	
CMV only	16	5.3	13	4.3	
HHV-6 only	19	6.3	6	2.0	
EBV and CMV	10	3.3	3	1.0	
CMV and HHV-6	5	1.7	0	0	
EBV and HHV-6	11	3.6	0	0	
EBV, CMV, and HHV-6	3	1.0	0	0	
Below detection limits in all viruses	191	63.0	266	87.8	

[a] Fisher's exact test.

detected in 13 recipients (28.3%), and all three viral DNA forms were detected in 3 recipients (6.5%). Using the plasma specimens, at least one viral DNA form was detected in 18 of 46 recipients (39.1%), and plural viral DNA forms were detected in 5 recipients (10.9%).

Four patients who underwent hematopoietic stem cell transplantation developed symptomatic EBV infections and were preconditioned with antithymocyte globulin. The patients had prolonged fever, lymphadenopathy, diarrhea, or hematochezia, which could not be explained by other causes. One patient who underwent liver transplantation developed CMV hepatitis despite preemptive ganciclovir therapy. None of the patients developed HHV-6-related diseases. Each form of viral DNA was detected in both the whole-blood and plasma specimens of these five symptomatic patients, and the peaks of the viral DNA loads were concordant with the symptoms observed.

Of the 303 whole-blood samples tested, 112 were positive for at least one viral DNA form, and 29 were positive for plural viral DNA forms. Conversely, 37 of 303 plasma samples were positive for at least one viral DNA form, and only 3 samples were positive for plural viral DNA forms (Table 3). The detection rates of viral DNA in whole blood and plasma were 23.8% and 5.9% for EBV, 11.2% and 5.3% for CMV, and 12.5% and 2.0% for HHV-6, respectively. The three viral DNA forms of interest were detected more frequently in whole blood than in plasma. EBV DNA loads ranged from 220 to 8.6×10^6 copies/ml in whole blood and from 250 to 7.5×10^4 copies/ml in plasma. CMV DNA loads ranged from 220 to 3.8×10^5 copies/ml in whole blood and from 200 to 8.5×10^4 copies/ml in plasma. HHV-6 DNA loads ranged from 240 to 1.3×10^5 copies/ml in whole blood and from 290 to 1.1×10^4 copies/ml in plasma. The viral DNA copy numbers were compared using samples determined to be positive in both whole blood and plasma (Fig. 3). Weak correlations were seen between the viral loads in whole blood and those in plasma.

Comparison of EBV DNA loads between whole blood and plasma. We divided the 303 samples into two groups based on EBV infection-related symptoms, symptomatic and asymptomatic. In the symptomatic group, EBV DNA was detected in all nine whole-blood samples with high viral loads but not in two plasma samples (Fig. 4). Among the whole-blood samples, the viral loads of the symptomatic group were significantly higher than those of the asymptomatic group. However, among the plasma samples, the viral loads were similar between the two groups (Fig. 4).

Comparison of antigenemia and multiplex real-time PCR assays. The antigenemia assay was applied to 235 of 303 blood specimens that had been evaluated with the multiplex assay. CMV antigen was detected in 13 of 235 blood samples (5.5%). With the multiplex assay, CMV DNA was detected in 32 of 235 whole-blood specimens (13.6%; P = 0.002 versus antigenemia) and 14 of 235 plasma specimens (6.0%; P = 0.5 versus antigenemia). When the antigenemia assay was defined as the standard, the specificity of CMV DNA detection was higher for the plasma specimens than for the whole-blood specimens (Table 4). On the other hand, the sensitivity seemed to be higher for the whole-blood specimens, although statistical significance was not achieved, probably due to the small sample size.

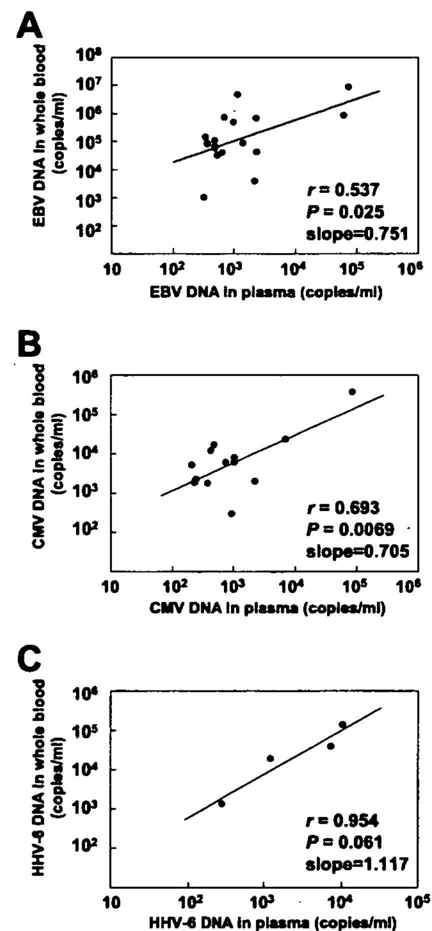

FIG. 3. Correlation of each viral DNA load between whole blood and plasma. (A) Correlation of EBV DNA copy number (n = 17). (B) Correlation of CMV DNA copy number (n = 13). (C) Correlation of HHV-6 DNA copy number (n = 4). The viral DNA copy numbers were compared using samples determined to be positive in both whole blood and plasma.

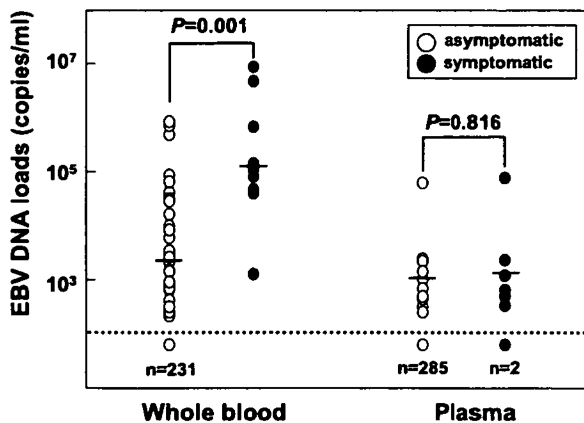

FIG. 4. Comparison of EBV DNA loads between whole-blood and plasma specimens. The dotted line indicates the detection limit in this assay. Bars show the mean copy numbers of EBV DNA. *n*, number of undetectable samples.

The 235 specimens were classified into four categories by the antigenemia values (negative, 0; low, 1 to 10; intermediate, 11 to 100; high, >100), and CMV DNA loads were compared (Table 5). Although the sample numbers for each group were small, it appeared that CMV DNA loads increased in proportion to increases in the antigenemia values. Correlations between antigenemia values and CMV DNA loads were then analyzed using the positive samples. Antigenemia values were significantly correlated with CMV DNA loads, both in whole blood ($n = 12$; $r = 0.776$; $P = 0.002$) and in plasma ($n = 9$; $r = 0.861$; $P = 0.002$).

Monitoring of EBV, CMV, and HHV-6 DNA loads in a transplant recipient. Figure 5 shows changes in the viral DNA loads in a representative case. The patient was a 4-year-old girl with severe aplastic anemia who underwent bone marrow transplantation. Two weeks after transplantation, CMV DNA was detected in her whole-blood and plasma samples (Fig. 5). Shortly after the detection of CMV DNA, the patient's blood tested positive for the CMV antigen. Although the patient had no symptoms associated with CMV infection, ganciclovir was administered preemptively. Her CMV DNA load decreased in accordance with the ganciclovir therapy. During treatment, a high level of EBV DNA was detected in her blood. In the fifth week after transplantation, she developed a fever and hematochezia. Since EBV DNA had been detected at a high level in her whole blood and plasma and other pathogens and causes that may have explained her symptoms were excluded, an EBV-related lymphoproliferative disorder was suspected. Immunosuppression was tapered, but that was not effective. Therefore, the monoclonal anti-CD20 antibody rituximab was administered. Immediately after rituximab administration, her EBV DNA load decreased in conjunction with her clinical symptoms. During the period of viral monitoring, HHV-6 DNA was undetectable in her whole blood and plasma.

DISCUSSION

Real-time PCR is a powerful tool for quantifying gene targets using fluorogenic probes and real-time laser scanning. Although multiplex real-time PCR is theoretically possible using probes with spectrally different fluorophores, the overlap of fluorophores prevents accurate quantification and limits amplification to two genes per tube (21). Only two targets could be quantified reliably in a single tube. However, recent advances in the development of real-time PCR platforms and master mix have made it possible to quantify more than three different genes in a single tube (21).

To our knowledge, this is the first study using the multiplex real-time PCR assay in the quantification of EBV, CMV, and HHV-6 DNA. Our results showed that the multiplex assay was as sensitive and specific as the single real-time PCR assay. The viral DNA loads determined by the multiplex real-time PCR assay were consistent with those described previously using other assays (6–8, 12, 26). Monospecific PCR assays require separate amplification of each target and are therefore more cost, time, and labor intensive than multiplex assays. The multiplex real-time PCR assay offers a major advantage in the field of clinical virology as it permits simultaneous amplification of several viruses in a single reaction mixture (1, 14, 21). The multiplex real-time PCR assay is particularly useful in the management of posttransplant patients, in whom frequent viral monitoring is required. The multiplex assay facilitates cost-effective diagnosis and may contribute to a decrease in the use of antiviral agents and in viral complications and hospitalizations.

One aim of the present study was to determine whether whole blood or plasma was more suitable for simultaneous virus monitoring in transplant recipients. EBV latently infects B lymphocytes. In EBV-associated lymphoproliferative disorders, EBV-infected B lymphocytes are usually found in the blood (27). Therefore, peripheral blood mononuclear cells are the best specimens to use in quantifying the EBV DNA load in transplant patients. However, whole blood, which contains mononuclear cells and is convenient to use, has been suggested as an acceptable alternative (17, 26). CMV latently infects a variety of leukocytes but predominantly cells in the monocyte/macrophage lineage. CMV quantification can be performed with acellular fractions of the blood, such as plasma and serum (7, 20, 29); however, in transplant recipients, the quantity of viral DNA is greater in leukocytes than in plasma (6, 10). HHV-6, which is closely related to CMV, infects mainly $CD4^+$ T lymphocytes and is predominantly found in latently infected monocytes/macrophages (28). Since considerable amounts of

TABLE 4. Comparison of multiplex real-time PCR assay and antigenemia assay of 235 blood specimens

Multiplex real-time PCR	Antigenemia assay: No. of positive samples	Antigenemia assay: No. of negative samples	% of sensitivity (95% CI)	% of specificity (95% CI)
Whole-blood specimens				
No. positive	12	20	92.3%[a]	91.0%[b]
No. negative	1	202	(77.8–100)	(97.2–94.8)
Plasma specimens				
No. positive	9	5	69.2%[a]	97.7%[b]
No. negative	4	217	(44.1–94.3)	(95.8–99.7)

[a] $P = 0.32$ by Fisher's exact test.
[b] $P = 0.003$ by Fisher's exact test.

TABLE 5. CMV DNA loads determined by multiplex real-time PCR assay in comparison with the antigenemia assay

Categories of antigenemia values (positive cells/ 5×10^4 cells)	Median antigenemia values (range)	No. of tested specimens	CMV DNA loads (copies/ml)			
			Whole blood		Plasma	
			No. of positive samples	Median (range)	No. of positive samples	Median (range)
0		222	20	1,420 (250–11,810)	5	280 (230–950)
1–10	2.0 (1–4)	10	9	5,040 (430–17,000)	6	620 (200–1,060)
11–100	17.5 (16–19)	2	2	12,450 (1,980–22,920)	2	4,740 (2,250–7,230)
>100	103	1	1	375,500	1	85,480

the HHV-6 genome persist in monocytes/macrophages, detection of HHV-6 DNA in whole blood may reflect both latent and active viral infection. Therefore, it has been suggested that the HHV-6 viral load in plasma is an effective indicator of active infection (16, 28).

In the present study, we compared whole-blood and plasma specimens in the simultaneous detection of EBV, CMV, and HHV-6 DNA. The detection rate for each viral DNA form was higher in the whole-blood specimens than in the plasma specimens. During the symptomatic periods, EBV DNA was found in all whole-blood specimens obtained but not in all plasma specimens. EBV DNA loads in whole blood were higher during the symptomatic period than during the asymptomatic period, whereas EBV DNA loads in plasma were similar during both periods. These results support the use of whole-blood specimens for multiplex real-time PCR assays in transplant patients, although we have insufficient data to conclude that whole blood is preferable for assaying CMV and HHV-6 loads. On the other hand, EBV, CMV, and HHV-6 latently infect blood corpuscles, and asymptomatic reactivation may occur in transplant patients. Therefore, it is necessary to determine a cutoff value that reflects clinical relevance. In this study, we could not determine the cutoff value because of the sample size and heterogeneity of the transplantations. Since the number of peripheral blood cells varies, especially after stem cell transplantation, this may influence the viral load in whole blood. A large prospective study to determine the clinical cutoff value for each virus and each transplantation type is currently under way.

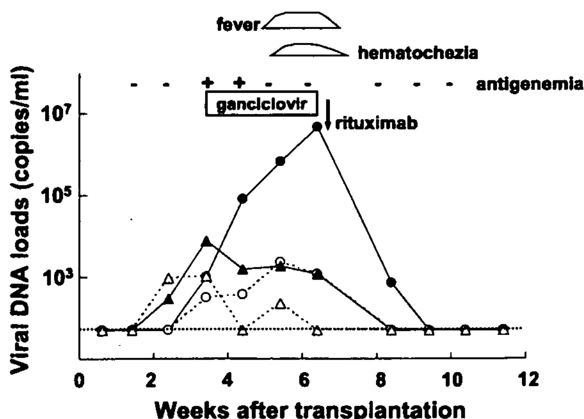

FIG. 5. Monitoring of viral DNA loads by multiplex real-time PCR in a 4-year-old girl who underwent bone marrow transplantation. Closed circles, EBV DNA copy number in whole blood; open circles, EBV DNA in plasma; closed triangles, CMV DNA in whole blood; open triangles, CMV DNA in plasma. The dotted line indicates the detection limit in this assay.

In summary, we developed a multiplex real-time PCR assay for the simultaneous detection of EBV, CMV, and HHV-6 DNA. The results of the multiplex assay were as sensitive and specific as those of the single real-time PCR assay. Compared to plasma, whole blood was more suitable for quantifying EBV DNA in transplant patients. The savings in cost, time, and labor associated with multiplex real-time PCR validate its use in the management of transplant recipients.

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ORIGINAL ARTICLE

Clinical Analysis of Risk Factors for Falls in Home-Living Stroke Patients Using Functional Evaluation Tools

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ABSTRACT. Wada N, Sohmiya M, Shimizu T, Okamoto K, Shirakura K. Clinical analysis of risk factors for falls in home-living stroke patients using functional evaluation tools. Arch Phys Med Rehabil 2007;88:1601-5.

Objectives: To identify risk factors associated with falls in home-living stroke patients and to predict falls using patient information and functional evaluation tools.

Design: Cohort study.

Setting: Community.

Participants: We recruited 101 home-living stroke patients who had hemiparesis and could walk independently with or without supporting devices. Disease duration ranged from 1 to 22 years (mean, 6.1y).

Interventions: Not applicable.

Main Outcome Measures: The score of each item of the Stroke Impairment Assessment Set (SIAS), and the FIM instrument, sex, age, duration of disease, stroke type, affected side of the body, frequency of rehabilitation, use of sedatives, and Mini-Mental State Examination score were evaluated and the occurrence of falls was observed prospectively for 12 months.

Results: Forty-five (44.6%) participants fell, 20 of whom fell repeatedly. A logistic model for predicting falls was refined until it included 4 predictors: memory score on the FIM, range of motion of the lower extremities on the SIAS, duration of disease, and affected side. The predictive value of the logistic model was 86.7%.

Conclusions: Evaluation tools were useful for predicting falls and devising preventive strategies in the high-risk group of home-living stroke patients.

Key Words: Accidental falls; Rehabilitation; Risk factors; Stroke.

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A FALL IS AMONG THE most frequent accidents among the elderly and patients with neurologic and orthopedic diseases. A fall leads to fear of falling again and sustaining serious injuries such as hip fracture and head injury.[1-4] This fear may restrict activities of daily living (ADLs) and may also result in readmission.[5]

Stroke patients have a higher risk of falls than nonstroke patients.[6] Those under acute care and rehabilitation have multiple risk factors of falls and remain in a high-risk group after discharge. Forster and Young[7] reported that 73% of stroke patients fell within 6 months of discharge from hospital. Although several studies have reported risk factors for the community-dwelling elderly,[8-11] most research on falls among people after stroke has tended to concentrate on rehabilitation hospitals and nursing homes.[12-18] Reports on falls in home-living stroke patients are scarce and involve few subjects.[19-21]

Falls in inpatient settings essentially differ from those in home settings. In inpatient settings, patients are still in the acute or subacute stage and do not yet understand their own ability to walk, so falls are caused by inattention and overconfidence. In contrast, patients in the home setting have come to understand their own ability to walk and are used to walking in this setting. The findings of studies in inpatient settings cannot therefore be applied to those of home-living patients. Consequently, the identification of group-specific risk factors is very important for preventing falls.

The aim of the present study was to identify risk factors related to falls in home-living stroke patients and to predict falls using patient information and the functional evaluation tools of the Stroke Impairment Assessment Set (SIAS), and the FIM instrument.

METHODS

Subjects were 112 stroke outpatients of our affiliated hospital, Hidakakai Hidaka Hospital. All patients had hemiparesis and were home living, and could walk independently with or without supporting devices such as a cane, orthosis of the lower extremities, and walker. All underwent a rehabilitation program involving walking, stretching, and muscle strengthening once to 3 times a week run by physical therapists and occupational therapists under the Japanese long-term care insurance system. After initial evaluation they were observed for 12 months, from January 1 to December 31, 2003. Among the 11 patients who dropped out, 3 died and 8 were readmitted due to recurrent stroke or other diseases not associated with falls. The remaining 101 stroke patients were included in the study. The study was approved by the local ethics committee at the hospital and written informed consent was obtained from all subjects and/or their families.

We evaluated the following baseline characteristics for all patients: sex, age, duration of disease, stroke type (ischemic stroke, hemorrhagic stroke, subarachnoid hemorrhage), affected side of the body (left, right), frequency of rehabilitation per week, and use of sedatives (yes, no) such as antiepileptics, psychotropic medicine, and sleeping medicine considered relevant with respect to increased fall risk. In addition, the Mini-Mental State Examination (MMSE), which has a maximum score of 30, was administered to assess cognitive function.[22]

We used the SIAS and FIM to evaluate various aspects of impairment and disability related to falls in stroke patients. The SIAS, developed for stroke outcome research in Japan,[23] has a total of 22 items, with each item scored from 0 (severely impaired) to 5 (normal) for motor function, or 0 to 3 (normal)

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Table 1: Characteristics of Stroke Patients and Comparisons Among Nonfallers and Fallers

Characteristics	Total (N 101)	Nonfallers (n 56)	Fallers (n 45)		
			Total	Occasional (n 25)	Repeat (n 20)
Sex (men/women)	62/39	35/21	27/18	15/10	12/8
Age (y)	67.2 10.0	66.9 9.2	67.5 11.1	67.4 10.7	67.6 11.9
Duration of disease (y)	6.1 4.6	5.7 4.4	6.6 4.7	6.8 4.6	6.5 5.0
Stroke type					
Ischemic	48	27	21	14	7
Hemorrhagic	49	27	22	10	12
Subarachnoid hemorrhage	4	2	2	1	1
Affected side of body (right/left)	51/50	32/24	19/26	15/10	4/16*
Frequency of rehabilitation (/wk)	1.8 0.6	1.8 0.5	1.8 0.6	1.6 0.6	2.0 0.5
Use of sedatives	29	18	11	6	5
MMSE score	24.5 4.7	24.4 4.5	24.7 4.9	24.9 5.7	24.4 3.9
Total SIAS score	46.1 11.1	46.7 11.6	45.5 10.4	45.1 9.2	46.0 11.9
Total FIM score	107.4 13.1	109.0 9.3	105.5 16.5	110.2 9.9	99.6 21.1
Motor FIM score	75.7 10.3	77.0 8.5	74.1 12.2	77.8 7.9	69.5 15.0
Cognitive FIM score	31.9 4.7	32.2 3.8	31.4 5.7	32.4 4.1	30.1 7.1

NOTE. Values are n or mean standard deviation (SD).
*Nonfallers versus repeat fallers (P .05).

for muscle tone, sensory function, range of motion (ROM), pain, trunk strength, higher cortical function, and unaffected side function. The SIAS assesses various aspects of impairment in hemiplegic patients and shows interrater reliability, predictive validity, sensitivity, and scale quality.[24] The FIM, which is used most often worldwide for evaluating the function of stroke patients,[25] has motor and cognitive scores and is subdivided into 18 subcategories, each scored on a scale of 1 (total assistance) to 7 (complete independence). Total FIM scores range from 18 to 126. It has been found to show good reliability.[26]

We defined falls as any unexpected touch of the floor by any part of a patient's body except for the soles of the feet. The information of falls was obtained from the patients or their family through interview with staff at each periodic rehabilitation. Patients were divided into 3 groups according to the frequency of falls: nonfallers, occasional fallers (patients experiencing only 1 fall during the study period), and repeat fallers (patients experiencing 2 or more falls during the study period). Table 1 summarizes the clinical characteristics of the stroke patients.

Statistical Analysis

We first carried out bivariate analyses using the t test, Mann-Whitney U test, and chi-square test to determine which variables differed significantly between the nonfallers and repeat fallers. The variables that achieved statistical significance were then included in a multivariate logistic regression. Because of the sample size and number of variables, the entry probability for logistic analysis was set at the .10 level of significance rather than the .05 level in an effort to avoid a type II error. Spearman rank-order correlation coefficients were calculated to determine which variables were related. Variables that were correlated were not placed in the same logistic regression model to avoid confounding the analysis. When 2 or more potential risk factors correlated highly or had similar P values, the factor that was clinically most important was selected for entry. The model was simplified in a stepwise fashion by removing variables with a P value greater than .05. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the risk of falling associated with independent variables. Sensitivity and specificity in predicting falls status were calculated. Sensitivity was defined as percentage of the fallers who were correctly identified. Specificity was defined as percentage of nonfallers who were correctly identified. Statistical analysis was performed using SPSS software.[a]

RESULTS

Total fallers (occasional and repeat fallers) comprised 45 (44.6%) of the 101 patients. Repeat fallers totaled 20 (19.8%) patients. The frequency of falls in repeat fallers ranged between 2 and 10 times (mean, 2.1) during the study period. Four patients sustained fractures; 1 had a humeral fracture, 1 had a

Table 2: Comparison of Nonfallers and Repeat Fallers (FIM)

Subscale and Items	Nonfallers	Repeat Fallers	P
Motor			
Self-care			
Eating	6.6 0.8	6.7 0.7	.586
Grooming	6.0 1.3	5.3 1.9	.320
Bathing	4.3 2.2	3.6 2.5	.145
Dressing upper body	5.5 1.6	4.6 2.4	.697
Dressing lower body	5.5 1.6	4.6 2.3	.382
Toileting	6.4 0.8	5.5 1.9	.086*
Sphincter control			
Bladder	6.9 0.3	6.4 1.2	.037*
Bowel	6.8 0.4	6.6 1.0	.977
Transfers			
Bed/chair	6.7 0.5	6.3 1.1	.087*
Toilet	6.4 0.5	6.0 0.9	.066*
Tub/shower	5.1 1.6	4.3 2.0	.073*
Locomotion			
Walk/chair	6.0 0.8	5.8 1.1	.496
Stairs	4.6 1.9	4.1 1.8	.270
Cognitive communication			
Comprehension	6.5 1.1	6.4 1.3	.347
Expression	6.3 1.2	6.2 1.6	.734
Social cognition			
Social interaction	6.5 1.1	6.0 1.8	.139
Problem-solving	6.1 1.1	5.6 1.8	.311
Memory	6.8 0.4	6.0 1.6	.015*

*P 0.1.

Table 3: Comparison of Nonfallers and Repeat Fallers (SIAS)

Subscale and Items	Nonfallers	Repeat Fallers	P
Motor function			
Knee-mouth	2.3 1.5	2.2 1.5	.878
Finger function	1.5 1.5	1.9 2.0	.597
Hip flexion	3.1 1.2	3.1 1.1	.779
Knee extension	2.8 1.2	2.8 1.2	.990
Foot tap	2.0 1.7	1.4 1.6	.066*
Muscle tone			
DTR: UE	1.8 0.7	2.0 0.7	.229
LE	1.7 0.7	1.8 0.7	.429
Tone: UE	1.6 0.8	1.7 0.9	.402
LE	1.8 0.9	1.9 0.9	.694
Sensory function			
Touch: UE	1.9 0.9	1.5 1.0	.124
LE	2.0 1.0	1.5 1.1	.135
Position: UE	2.0 1.1	2.0 1.1	.914
LE	2.0 1.1	1.8 1.1	.541
ROM			
Shoulder abduction	1.8 0.7	2.0 0.9	.172
Ankle dorsiflexion	1.6 0.8	2.1 0.8	.010*
Pain	2.5 0.6	2.3 1.0	.803
Trunk			
Verticality	2.7 0.5	2.7 0.6	.801
Abdominal MMT	2.2 0.7	2.2 1.0	.897
Higher cortical function			
Visuospatial	2.6 0.7	2.7 0.6	.567
Speech	2.6 0.7	2.8 0.5	.144
Unaffected side function			
Grip strength	2.2 0.6	1.9 0.7	.058*
Quadriceps MMT	2.2 0.8	2.1 0.8	.257

Abbreviations: DTR, deep tendon reflex; LE, lower extremities; MMT, manual muscle test; UE, upper extremities.
*P 0.1.

Table 5: Stepwise Logistic Models for Predicting Falls

Predictors	OR	95% CI	P
FIM			
Memory	0.252	0.093–0.679	.006
SIAS			
ROM of lower extremities	4.278	1.637–11.179	.003
Duration of disease	1.262	1.074–1.483	.005
Affected side of body (right/left)	0.076	0.013–0.444	.004

hip fracture, and 2 had a lumbar compression fracture. Most fallers fell in their home during ADLs.

No significant difference in variables between nonfallers and total fallers was recognized except for the memory score on the FIM.

Bivariate analysis revealed the following factors to differ significantly between nonfallers and repeat fallers: self-care for toileting, bladder management, transfer from bed to chair, transfer to toilet and transfer to tub or shower, as well as memory score on the FIM, foot tap test, gripping power score, and ROM of the lower extremities score of SIAS, duration of disease, and affected side (all P .10) (tables 2, 3). As shown in table 4, some variables were highly correlated and as such they could not be entered simultaneously in stepwise logistic regression analysis. The logistic model was refined until it included only 4 predictors: memory score on the FIM, ROM of the lower extremities on the SIAS, duration of disease, and affected side (table 5). The logistic model using the 4 predictors showed a high predictive value (table 6). Sensitivity of fall prediction was 81.3% and specificity was 88.1% (Hosmer-Lemeshow goodness of fit test, P .692).

DISCUSSION

This is the first study to report the ability of the SIAS and FIM to predict falls in home-living stroke patients. The fall rate in this study (44.6%) was lower than in previous studies (50% 73%)[7,19,20] aimed at home-living patients. Participants in our study continued rehabilitation during the study period, which may have reduced the risk of falls.

Comparing nonfallers and total fallers, we found no significant difference except for memory score on the FIM. Cognitive scores on the FIM reflect communicative and social cognitive function in daily life, and cognitive deficits may lead to reduced attention and thereby to an increased risk of falling.[27,28]

An important area in the research of falls is the study of repeat fallers. One fall can be a chance occurrence, but repeat falls can lead to an increased risk of injury. Therefore, we also compared the differences between nonfallers and repeat fallers, a classification of falls that has been used in several studies.[19-21]

Table 4: Spearman Correlation Coefficients Among Variables

	Toileting	Bladder Control	Bed/Chair	Toilet	Tub/Shower	Memory	Foot Tap	GP	ROM (LE)	Duration	Side
Toileting	1.000	0.277*	0.542‡	0.602‡	0.640‡	0.140	0.262*	0.182	0.042	0.293*	0.110
Bladder control		1.000	0.250*	0.265*	0.221	0.293*	0.187	0.094	0.002	0.053	0.136
Bed/chair			1.000	0.526‡	0.506‡	0.313‡	0.230	0.219	0.074	0.090	0.079
Toilet				1.000	0.519‡	0.145	0.264*	0.277*	0.059	0.024	0.119
Tub/shower					1.000	0.105	0.480‡	0.133	0.004	0.072	0.135
Memory						1.000	0.019	0.026	0.227	0.068	0.039
Foot tap							1.000	0.307	0.137	0.056	0.171
GP								1.000	0.204	0.086	0.037
ROM (LE)									1.000	0.043	0.022
Duration										1.000	0.102
Side											1.000

Abbreviations: Bed/Chair, transfer of bed, chair score in FIM; Bladder control, bladder control score in FIM; Duration, duration of disease; Foot tap, foot tap test in SIAS; GP, gripping power score in SIAS; Memory, memory score in FIM; ROM (LE), ROM of lower extremities in SIAS; Side, affected side of body; Toilet, transfer of toilet score in FIM; Toileting, self-care of toileting score in FIM; Tub/shower, transfer of tub or shower score in FIM.
*P .05; ‡P .01.

Table 6: Predictive Value of the Logistic Model

Predicted Outcome	Observed Fall (case)	Observed No Fall (case)	Predictive Value (%)
Predicted fall	13	3	81.3
No predicted fall	7	52	88.1
Overall			86.7

Aside from the FIM and SIAS scores, affected side and duration of disease were the only other variables related to fall risk in the present study. Rapport et al[29] when reporting the predictors of falls among stroke patients with an affected right hemisphere, described the causes of falls in affected left-hemisphere stroke patients to be inattention, perceptual deficits, and hemispatial neglect. Although the visuospatial function of the SIAS score was not associated with fall risk in the present study, we often find a difference in hemispatial neglect between static and dynamic situations. Several studies have shown, however, no significant relationship between the risk of fall and the affected side.[14,15]

The bivariate analysis showed that 9 category scores of the SIAS and FIM were related to fall risk: self-care for toileting, bladder management, transfer from bed to chair, transfer to toilet and transfer to tub or shower, as well as memory score on the FIM, foot tap test, gripping power score and ROM of the lower extremities score of SIAS, duration of disease, and affected side. It seems reasonable to assume that memory disturbance leads to repeat falls due to a lack of awareness of the risk. Several studies have shown the relationship between falls and cognitive deficit.[8,30,31] However, the MMSE score showed no relationship to fall risk in the present study. We suggest that because the cognitive score on the FIM was evaluated through observation of the patient's daily activities, it slightly differed from the MMSE score evaluated through examination.

The low score on self-care for toileting and bladder management on the FIM increased fall risk in this study. A possible explanation for this may be that urinary incontinence is also a plausible risk factor for falls because it is often coincident with a general decline in physical and mental functioning.[32,33] These findings indicate we must pay attention to patients who have such disorders to prevent falls.

It is interesting to note that unlike other functions, limitation in the ROM of the ankle reduced fall risk in this study. This finding suggests that immobilization of the distal portion of the affected lower extremity stabilized motion and posture.

Analysis of the total score of the FIM and scores of the motor FIM subscale and cognitive FIM subscale revealed no significant relations to falls through bivariate analysis or multivariate analysis. Whitson and Pieper[34] reported the nonlinear relationship between total FIM score and the fracture risk. In their study, patients with intermediate functional impairment had higher fracture risk than patients with mild or severe functional impairment. They thought that severely impaired patients had limited mobility and little opportunity to fracture. In our study, all the participants were living in the community and could walk independently, so their total score of the FIM was high (mean, 107.4; range, 56 124), but we found no significant relationship to falls. However, we found significant relations to falls for several individual variables including the memory score and the bladder management score, indicating that change in an individual item of the FIM and SIAS multi-item tests has little effect on the total scores. The total scores of these tests are measures of overall function, and it is therefore difficult to pinpoint which function affects fall risk. We analyzed individual items on these tests in this study in order to determine specifically what kind of impairment and disability increases the risk of falls.

Several studies have attempted to predict falls in stroke patients using balance scores among other factors.[12,21,35,36] In these studies, balance problem, arm function, ADL ability, and depressive symptoms were reported as risk factors. Our study shows that functional evaluation tests such as the SIAS and FIM are sufficiently sensitive to detect functional deficits related to falls. These evaluation tools are generally used at initial assessment and the scores can therefore enable close attention to be paid to patients with the aforementioned risk factors during rehabilitation or daily activities. The findings of the present study are also useful for designing interventions to reduce fall frequency among home-living stroke patients.

Study Limitations

Some limitations of this study should be noted. First, this model has not yet been validated in another population. To validate risk factors, their predictive abilities must be examined prospectively. Further follow-up studies are therefore needed to confirm these results. Second, there is a lack of information about the home environment and falling circumstances. In Japan, people usually take off their shoes in the house and there are many steps in a typical Japanese house. Both of these factors are likely to be closely related to falls in home-living patients, although it was difficult to estimate such a situation. To estimate these variables, a more sophisticated research method is needed.

CONCLUSIONS

Falls occurred at a rate of over 40% in home-living stroke patients. The study revealed the usefulness of clinical evaluation using the SIAS and FIM to predict repeat falls in the high-risk group of home-living stroke patients.

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High prevalence of serum autoantibodies against the amino terminal of α-enolase in Hashimoto's encephalopathy

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Abstract

Recently, we discovered autoantibodies against the amino (NH_2)-terminal of α-enolase (NAE) in patients with Hashimoto's encephalopathy (HE) (83.3%; 5/6) [Fujii, A., Yoneda, M., Ito, T., Yamamura, O., Satomi, S., Higa, H., Kimura, M., Suzuki, M., Yamashita, M., Yuasa, T., Suzuki, H., Kuriyama, M., 2005. Autoantibodies against the amino terminal of α-enolase are a useful diagnostic marker of Hashimoto's encephalopathy. J. Neuroimmunol. 162, 130–136]. We further investigated the anti-NAE autoantibodies in 25 patients who fit the diagnostic criteria for HE, based on the presence of anti-thyroid antibodies and responsiveness to immunotherapy. In this study, we demonstrated a high prevalence (68%, 17 of 25) and high specificity of anti-NAE autoantibodies in patients with HE, and clarified the clinical features of HE. This result demonstrated that anti-NAE autoantibodies, in addition to anti-thyroid autoantibodies, are emphasized as useful serological diagnostic markers of HE.
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Keywords: Hashimoto's encephalopathy; Autoantibodies; NAE; Clinical features

1. Introduction

Hashimoto's thyroiditis (HT) is the most common disorder affecting the thyroid gland. In 1966, Brain et al. reported the first case of encephalopathy associated with HT, who presented with recurrent neuropsychiatric symptoms accompanied by serum anti-thyroid antibodies in euthyroid states (Brain et al., 1966). Hashimoto's encephalopathy (HE) therefore was recognized as a nomenclature of new disease, distinct from myxoedema encephalopathy associated with hypothyroidism (Behan et al., 1988; Shaw et al., 1991; Chaudhuri and Behan, 2003; Chong et al., 2003).

Autoimmune mechanism has been proposed as an underlying pathogenesis (Brain et al., 1966; Chaudhuri and Behan, 2003; Chong et al., 2003), and immunotherapy such as steroids, immunosuppressants and/or intravenous injection of immunoglobulin (IVIg)/plasmapheresis was successfully administered (Shaw et al., 1991; Boers and Colebatch, 2001; Chaudhuri and Behan, 2003). Over 100 accumulated cases (Shaw et al., 1991; Chaudhuri and Behan, 2003; Chong et al., 2003) reported mainly by neurologists, emphasized this potentially treatable encephalopathy associated with HT in the differential diagnosis of unknown etiology of encephalopathy, and suggested the risk of under-diagnosis of HE (Ghika-Schmid et al., 1996; Maydell et al., 2002).

Several diagnostic criteria for HE have been proposed based on encephalopathy, the presence of anti-thyroid antibodies and/or responsiveness to immunotherapy including steroids (Shaw et al., 1991; Peschen-Rosin et al., 1999). Endocrinologists however argued against the terminology of HE because of the wide spectrum clinical features in patients with HE and the high prevalence of anti-thyroid antibodies in the normal population, which are usually subclinical (Sawka et al., 2002; Fatourechi, 2005).

To resolve such debates on the nomenclature and nature of HE, more specific diagnostic markers are needed (Chong et al., 2003; Fatourechi, 2005). Very recently, we discovered autoantibodies against the amino (NH_2)-terminal of α-enolase (referred to as NAE) that were highly specific in sera from a limited number of HE patients (83%, 5 of 6 with HE; 11%, 2 of 17 with HT without any neuropsychiatric features; none of controls [50 individuals] including those with other neurological or immunological conditions involving encephalopathy [25 individuals]) (Fujii et al., 2005). Thus, the anti-NAE autoantibodies are a potential tool for the diagnosis of HE and resolving the debate

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over HE described above. We further investigated the prevalence and specificity of anti-NAE autoantibodies, and clarified the clinical features in a large number of patients with HE in this study.

2. Patients and methods

2.1. Patients

In this study, we selected 25 patients with HT, who presented with encephalopathy and fit the diagnostic criteria for HE, based on the presence of anti-thyroid antibodies and responsiveness to immunotherapy such as steroids, immunosuppressants and/or IVIg/plasmapheresis. These 25 patients included our own 8 cases and 17 cases from other institutions. Neurological specialists carefully excluded other possible causes of encephalopathy including infections, other autoimmune conditions, vitamin deficiency, intoxication, cerebrovascular diseases, neoplasms and Creutzfeldt–Jakob disease and so on, and the detailed clinical information of each patient was obtained from the attending physician. The ethics committee of the University of Fukui approved this research, and written permission was obtained from each patient.

The clinical profiles of all patients were summarized in Table 1 and Fig. 1. The sex ratio of patients examined was 7:18 (male:female). The mean age was 60 years old (range: 23 to 83). All patients showed the responsiveness to steroids in variable degrees. We categorized the patients into three clinical forms such as acute encephalopathy (AE)-form, subacute psychiatric (SP)-form and others. AE was the most common clinical form (76%; 19 of 25), SP was much less frequent (16%; 4 of 25), and others were 8% (2 of 25). Four of 25 patients demonstrated recurrence (16%). Five of 25 patients had a history of HT before encephalopathy appeared (20%). All patients carried anti-thyroid antibodies (both of anti-thyroglobulin [Tg] and anti-thyroid peroxidase antibodies [TPO], 48%, 12 of 25; anti-Tg, 32%, 8 of 25; anti-TPO, 20%, 5 of 25). Most of the patients were in euthyroid states (72%, 18 of 25), except for a few patients in mild hypothyroid states treated with thyroxin (16%, 4 of 25) or transient hyperthyroid states (thyrotoxicosis) (12%, 3 of 25). All of the patients showed neuropsychiatric symptoms after recovery from dysthyroid states.

Table 1
Clinical features and anti-NAE autoantibodies in patients with Hashimoto's encephalopathy

Patient	Age/ gender	Clinical form	Anti-thyroid	Immunotherapy (response)	Anti-NAE	Neurological manifestation					Abnormal EEG	Abnormal brain MRI	Elevated CSF/ IgG protein
						C	S	C/P	I	A			
1	44, F	AE	TPO[a]	PSL (excellent)	Positive	+	+	+	Chorea	−	+	−	+
2	34, M	AE	TPO, Tg	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	−	−
3	71, F	AE[b]	TPO, Tg[a]	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	−	+
4	45, F	AE	TPO, Tg	mPSL (excellent)	Positive	+	+	−	Tremor	−	*n.d.*	−	−
5	60, F	AE	Tg	PSL (excellent)	Positive	+	−	+	−	−	+	−	+
6	78, F	AE	Tg[c]	PSL (excellent)	Positive	+	+	−	Myoclonus	−	+	−	+
7	32, F	AE (LE)	TPO,Tg	mPSL/PSL (excellent)	Positive	+	+	+	−	−	+	L	−
8	57, F	AE[b]	TPO[a]	PSL (excellent)	Positive	+	+	+	Chorea	+	+	−	−
9	78, F	AE	Tg	PSL (excellent)	Positive	+	−	+	Tremor	−	+	−	+
10	83, F	AE	TPO, Tg	mPSL/PSL (good)	Positive	+	+	−	−	−	+	−	−
11	76, M	AE	TPO, Tg[c]	PSL (good)	Positive	+	+	−	Myoclonus	−	+	−	+
12	79, F	AE	TPO, Tg[c]	PSL (good)	Positive	+	−	+	Chorea	−	+	−	−
13	36, F	AE	Tg	mPSL/PSL (good)	Positive	+	+	+	−	−	+	−	−
14	71, F	AE[b]	Tg	mPSL (fair)	Positive	+	+	+	Myoclonus	−	+	WM	*n.d.*
15	56, M	AE	Tg[d]	mPSL/PSL (good)	Negative	+	+	+	−	−	+	−	+
16	74, M	AE	Tg[d]	mPSL/PSL (good)	Negative	+	+	−	−	−	*n.d.*	−	+
17	69, M	AE (LE)	TPO	mPSL/PSL (good)	Negative	+	+	+	Myoclonus	−	+	L	−
18	58, F	AE (LE)	TPO, Tg[d]	mPSL (good)	Negative	+	+	+	Myoclonus	−	+	L	−
19	61, F	AE (LE)	TPO, Tg[d]	mPSL/PSL (fair)	Negative	+	+	+	−	−	−	L	−
20	69, F	SP	TPO[a]	PSL (good)	Positive	−	−	+	−	−	*n.d.*	−	+
21	60, M	SP	TPO, Tg	PSL (fair)	Positive	−	−	+	−	−	+	−	+
22	69, F	SP	TPO	PSL (fair) IVIg (excellent)	Positive	−	−	+	Myoclonus	−	+	−	*n.d.*
23	61, M	SP	TPO, Tg	PSL (excellent)	Negative	−	−	+	−	+	+	−	+
24	63, F	CJD[b]	Tg[a]	mPSL/DX/azathioprine (excellent)	Negative	+	+	+	Myoclonus	+	+ (PSD)	−	+
25	23, F	IVM	TPO, Tg	PSL (excellent)	Negative	−	−	−	Myoclonus	−	−	−	−

AE, acute encephalopathy form; SP, subacute psychiatric form; LE, limbic encephalopathy-like clinical feature; CJD, Creutzfeldt–Jakob disease-like clinical feature; IVM, involuntary movement dominant-form; TPO, anti-thyroid peroxidase antibodies; Tg, anti-thyroglobulin antibodies; mPSL, methylprednisolone; PSL, prednisolone; DX, dexamethasone; IVIg, intravenous administration of immunoglobulin G; C, consciousness disturbance; S, seizures; C/P, cognitive impairment/psychiatric symptoms; I, involuntary movements: A, ataxia; PSD, periodic synchronized discharge-like paroxysmal discharges; L, limbic lesions; WM, white matter lesions. *n.d.* not determined. Patients 1, 3, 8, 14, 20 and 24 were very briefly reported (Fujii et al., 2005).

[a] Known history of Hashimoto's thyroiditis before the onset of encephalopathy.

[b] Positive history of recurrence.

[c] Transient hyperthyroidism (thyrotoxicosis).

[d] Hypothyroidism treated with thyroxin.

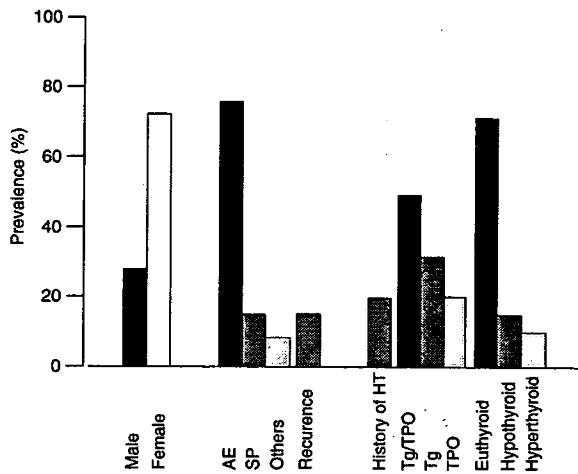

Fig. 1. Clinical profiles of patients. AE, acute encephalopathy form; SP, subacute psychiatric form; HT, Hashimoto's thyroiditis. Tg, anti-thyroglobulin antibodies; TPO, anti-thyroid peroxidase antibodies.

We examined anti-thyroid antibodies (anti-Tg and anti-TPO), steroid-responsiveness, clinical features (consciousness disturbance [C], seizures [S], cognitive impairment/psychiatric symptoms [C/P], involuntary movements [I], ataxia [A]), electroencephalogram [EEG], brain MRI and protein/immunoglobulin G in CSF, and compared these between patients with anti-NAE autoantibodies [referred to as NAE(+)] and without [NAE(−)]. Steroid-responsiveness was evaluated in three degrees: excellent, good, and fair.

2.2. SDS-PAGE and immunoblotting

Anti-NAE autoantibodies were investigated in encephalopathic patients with HT and patients with other disorders including autoimmune conditions or collagen diseases. Immunoblotting analysis of the patient's serum against the NAE expressed in human cultured cells was carried out with 12% sodium lauryl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a gel electrophoresis system (BE-220, BIO CRAFT, Tokyo, Japan), described previously (Fujii et al., 2005). The proteins on the gel were Western-blotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences, NJ) with a blotting apparatus (KS-8453, Oriental Instrument, Tokyo, Japan) at 0.3 mA/cm^2 for 8 h at 4 °C. For detection of the band specific to NAE, serum was applied to the membrane and incubated in 1% gelatin for 1 h at room temperature, then horseradish peroxidase (HRP)-conjugated anti-human goat IgG Fc (ICN Pharmateuticals, Inc., OH) was applied to the membrane as the secondary antibody, fluoresced, and developed on X-ray films (BioMax, Kodak, NY).

2.3. Statistical analysis

We used χ^2 test to assess the correlation between patients with encephalopathy with HT and anti-NAE antibodies.

3. Results

3.1. Anti-NAE autoantibodies

The clinical profiles of patients and immunological characters were summarized in Table 1 and Fig. 1. Of the 25 patients with encephalopathy with HT examined here, 68% of them were NAE(+) (17 of 25) ($p<0.001$, compared to patients with HT without encephalopathy [10%, 2 of 20], supplemented by additional data from 3 patients of HT without encephalopathy to our previous study) (Fujii et al., 2005). To our knowledge, there

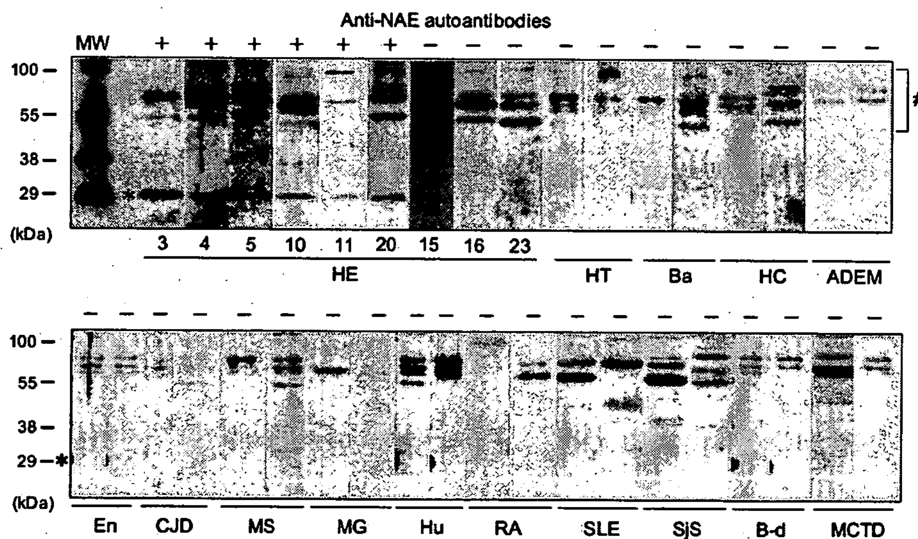

Fig. 2. Immunoblotting of the recombinant NAE with sera from patients with encephalopathy with HT, HT without encephalopathy, Basedow's disease or other neurological disorders including autoimmune conditions, or from controls. HE, Hashimoto's encephalopathy; 3–23, patients 3–23 with HE in Table 1; HT, Hashimoto's thyroiditis without encephalopathy; Ba, Basedow's disease; HC, healthy controls; ADEM, post-infectious acute disseminated encephalomyelitis; En, viral encephalitis; CJD, Creutzfeldt–Jakob disease; MS, multiple sclerosis; MG, myasthenia gravis; Hu, paraneoplastic syndrome associated with Hu-antigen; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SjS, Sjögren syndrome; B-d, Behçet disease; MCTD, mixed connective tissue disease. MW, molecular weight marker. *The position of the recombinant NAE. #Derivatives from human cultured cells for expression, which showed non-specific reactions with sera.

were no sera from 33 patients with 2 Basedow's disease, 4 postinfectious acute disseminated encephalomyelitis (ADEM), 2 viral encephalitis, 2 Creutzfeldt–Jakob disease, other autoimmune disorders or collagen diseases with neurological symptoms, including 4 multiple sclerosis, 4 myasthenia gravis, 2 paraneoplastic neurological syndrome associated with Hu-antigen, 2 rheumatoid arthritis, 3 systemic lupus erythematosus, 3 Sjögren syndrome, 3 Behçet disease, 2 mixed connective tissue disease that showed any immunological reaction to the recombinant NAE (Fig. 2). This confirmed our recent finding that anti-NAE autoantibodies were highly specific to HE.

3.2. Neurological manifestations

The neurological manifestations of patients are summarized in Table 1 and Fig. 3. There is no significant difference in the responsiveness to steroid between NAE(+) (excellent, 9; good, 5; fair 3) and NAE(−) patients (excellent, 3; good, 4; fair 1) but a bit better response in NAE(+) (Table 1 and Fig. 3). Consciousness disturbance appeared most frequently (80%, 20 of 25; 82%, 14 of 17 in NAE(+), 75%, 6 of 8 in NAE(−)). Seizures were also common (68%, 17 of 25; 64%, 11 of 17 in NAE(+), 75%, 6 of 8 in NAE(−)). Cognitive impairments/psychiatric symptoms such as memory disturbance, abnormal behaviors or hallucination occurred in 76% (19 of 25; 76%, 13 of 17 in NAE(+), 75%, 6 of 8 in NAE(−)). Involuntary movements including myoclonus/tremor and chorea were also present (52%, 13 of 25) with no difference between NAE(+) (52%; 9 of 17) and NAE(−) (50%; 4 of 8). Ataxia was much less frequent (12%, 3 of 25 in total; 5%, 1 of 17 in NAE(+); 25%, 2 of 8 in NAE(−)).

3.3. Laboratory and MRI findings

The laboratory and MRI findings of patients were summarized in Table 1 and Fig. 4. EEG abnormalities such as slow background activities and/or episodic spikes/slow waves showed very high prevalence (90%, 20 of 22) in patients examined, with a difference between NAE(+) (100%, 15 of 15) and NAE(−) (71%, 5 of 7) ($p<0.05$). By contrast, abnormalities on brain MRI were much less frequent (20%, 5 of 25), with no difference between NAE(+) (11%, 2 of 17) and NAE(−) (37%, 3 of 8). The elevated protein/immunoglobulin G in CSF was present (54%, 12 of 23), with no difference between NAE(+) (53%, 8 of 15) and NAE(−) (50%, 4 of 8).

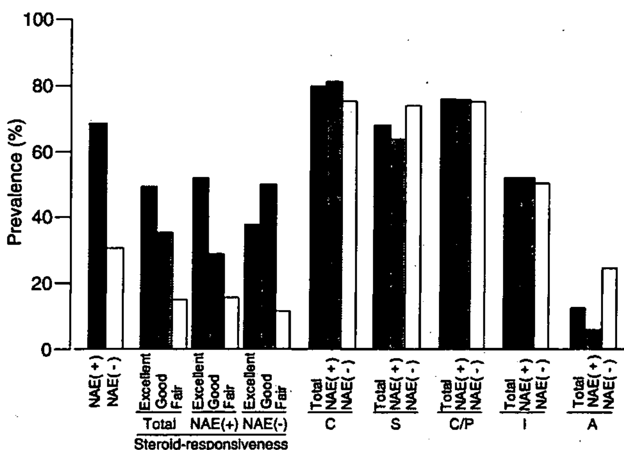

Fig. 3. Neurological manifestations compared between patients with and without anti-NAE autoantibodies. NAE(+), presence of anti-NAE autoantibodies; NAE(−), absence of anti-NAE autoantibodies. C, consciousness disturbance; S, seizures; C/P, cognitive impairment/psychiatric symptoms; I, involuntary movements: A, ataxia.

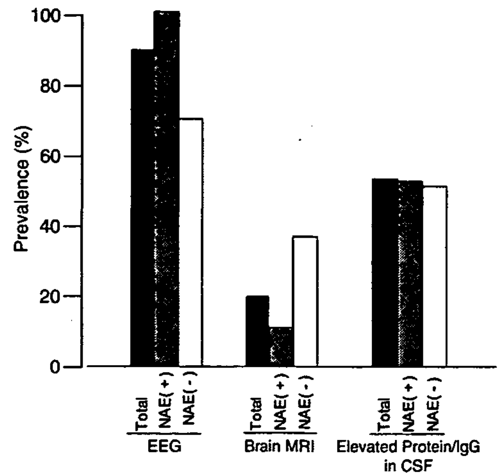

Fig. 4. Laboratory and MRI findings in patients.

4. Discussion

After Brain et al. reported the first case of encephalopathy associated with HT, there has been a debate on the nosology and nature on HE, despite the accumulation of over 100 reported cases (Behan et al., 1988; Chaudhuri and Behan, 2003; Chong et al., 2003). It is because of the wide spectrum clinical features in patients with HE, the high prevalence of anti-thyroid antibodies in the normal population (e.g. 5–10% in male, 10–25% in female in Japan), and the lack of specific diagnostic marker. HE patients presented with a variety of clinical features such as acute encephalopathy, psychosis/cognitive impairment, ataxia, recurrent acute disseminated encephalomyelitis (ADEM), involuntary movements (chorea, myoclonus or tremor) and Creutzfeldt–Jakob disease-like clinical features (Behan et al., 1988; Chaudhuri and Behan, 2003; Chong et al. 2003; Ferracci et al., 2004; Fatourechi, 2005).

In this study, the patients who presented with encephalopathy and fit the criteria for HE based on the presence of anti-thyroid antibodies and the responsiveness to immunotherapy such as steroids, immunosuppressants and/or IVIg/plasmapheresis, demonstrated a high prevalence of anti-NAE autoantibodies in their sera (68%; 17/25; $p<0.001$, compared to patients with HT without encephalopathy [10%, 2/20]). This strongly supported our previous finding of a high prevalence (83%, 5 out of 6) of anti-NAE autoantibodies in patients with HE (Fujii et al., 2005). There were no sera from patients with other disorders including autoimmune conditions or collagen diseases that showed any immunological reaction to recombinant NAE, suggesting a high specificity of anti-NAE autoantibodies to encephalopathy with HT. One third of patients examined here did not have anti-NAE

autoantibodies in their sera. NAE(−) patients can be associated with different autoantibodies because our preliminary study demonstrated other possible autoantibodies detected in NAE(−) patients (data not shown, in progress).

In the neurological features of the patients examined here, consciousness disturbance (80%), seizures (68%), cognitive impairment/psychiatric symptoms (76%) and involuntary movements (52%) were common in HE, while ataxia (12%) was much less frequent. As the clinical form, acute encephalopathy (AE) was the most common (76%). Chaudhuri and Behan investigated 18 cases of HE clinically and immunologically, and indicated that headaches (90%), seizures (67%), focal neurological deficit (67%), stupor/coma (67%), psychosis (50%) were common and ataxia (16%) and hemiparesis (16%) were rare (Chaudhuri and Behan, 2003). Although Chaudhuri and Behan emphasized myelopathy as a common clinical feature in HE with a similarity to ADEM, myelopathy was not observed in patients examined in our study. In addition, the anti-NAE autoantibodies were not detected in 4 patients with post-infectious ADEM in our study, suggesting that the clinical form of ADEM appeared relatively less frequent than Chaudhuri and Behan supposed.

In this study, relapsing was less frequent (16%) than that in Chaudhuri and Behan's study (67%). Chaudhuri and Behan carefully followed-up their patients with HE over a period of 16 years, and clarified the outcome of HE patients (Chaudhuri and Behan, 2003). The low frequency of relapse in our study seemed to depend on the short periods of the clinical courses examined (most patients were within one year). Thus, this short-term observation could have caused an underestimation of the relapse rate of HE in our study.

On laboratory/MRI findings in our study, EEG abnormalities were common (90%), compared to the low prevalence of abnormalities on brain MRI (20%). Elevation in CSF protein/IgG in our study was also a common feature (54%). Although there was no major difference in patients between NAE(+) and NAE(−) in the clinical features and laboratory/MRI findings in the present study, EEG abnormalities appeared at higher frequency and steroids tended to be more effective in NAE(+) patients.

Chaudhuri and Behan recommend immunosuppressants (e.g. azathioprine) as a potentially effective treatment besides steroids from the perspective of T-cell sensation to the antigen in HE (Chaudhuri and Behan, 2003). Although steroids have been widely administered to patients with HE in our study and others, steroid-responsiveness shows a temporally limited quality and other immunosuppressant agents are necessary to sustain long-term clinical response (Chaudhuri and Behan, 2003). Indeed, in our patient (Case 24) who showed an excellent steroid-responsiveness on the initial attack but poor response on the following attacks, this was successfully prevented from relapsing after the administration of an immunosuppressant (azathioprine) was started.

Chong et al. assumed that the combination of encephalopathy, presence of anti-thyroid antibodies and responsiveness to steroid administration seemed unlikely to be due to chance, and, however, there was no evidence of a pathogenic role for anti-thyroid antibodies in encephalopathy associated with HT (Chong et al., 2003). On the contrary, Ferracci et al. speculated that autoimmunity to neural antigens cross-reacting with thyroid antigens was the pathogenic basis of encephalopathy with HT (Ferracci et al., 2004). Additionally, the neuropathological finding of an autopsied case and brain perfusion studies in patients with HE suggested brain vasculitis, and supported the disease entity of HE (Nolte et al., 2000; Zettinig et al., 2003; Piga et al., 2004).

Sawka et al. searched for cases of encephalopathy associated with HT in Mayo Clinic from 1950 to 1996 years, and assumed that HE could be a rare autoimmune condition associated with a common autoimmune HT, in part with unknown origin (Sawka et al., 2002). In the present study, however, a large number of cases of encephalopathy associated with HT were still present, and two thirds of these patients carried anti-NAE autoantibodies even after neurological specialists had carefully excluded other possible conditions causing encephalopathy. Such discrepancy may be driven from differences between the profiles of patients treated by neurologists and compared to those treated by endocrinologists; i.e. neurologists see patients with neuropsychiatric symptoms of various causes while endocrinologists more frequently see patients with HT and less frequently encephalopathy.

Fatourechi et al. stated that a distinct clinical entity of encephalopathy associated with HT was present but the use of the term "Hashimoto's encephalopathy" was unfavorable until the pathogenesis of this condition was better defined (Fatourechi, 2005). Although the pathogenic role for anti-NAE autoantibodies in HE remained obscure, the present study demonstrated that anti-NAE autoantibodies were more specific to HE than anti-thyroid antibodies. This strongly suggests that "Hashimoto's encephalopathy" is a distinct clinical entity associated with HT although the underlying immunological condition should be better defined. Both the anti-NAE autoantibodies and anti-thyroid antibodies can be generated in the common immunological background related to HT, such as T-cell mediated antibody production (Weetman and McGregor, 1994; Stassi and De-Maria, 2002).

In conclusion, anti-NAE autoantibodies, in addition to anti-thyroid antibodies, are emphasized to as a useful serological diagnostic marker of HE, and should be included in the diagnostic criteria of this condition. Physicians must more attentively consider the possibility of HE, and test serum for anti-NAE autoantibodies as well as carefully excluding other conditions causing encephalopathy in patients with HT.

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Letter to the Editor

Limbic encephalitis with autoantibodies against the glutamate receptor epsilon 2 mimicking temporal lobe epilepsy

The N-methyl-D-aspartate-type glutamate receptor epsilon 2 (GluR ε2) channels have been implicated in synaptic plasticity associated with neural development and learning. Recently, autoantibodies against GluR ε2 were found in some patients with Rasmussen's encephalitis[1] and non-herpetic limbic encephalitis.[2] This is a first case report of non-herpetic limbic encephalitis with autoantibodies against GluR ε2 showing neither MRI signal alterations nor abnormal cerebrospinal fluid (CSF) findings.

The patient was a 20-year-old woman with no prior history of neuropsychiatric disorders. She had insomnia, palpitations, anorexia, and general fatigue for 2 months prior to admission; these symptoms worsened gradually. Five days before admission, she developed rapidly recurring complex partial seizures (CPS) characterized by a motionless stare and unresponsiveness. EEG revealed ictal discharges beginning from the left temporal area.

Her body temperature was 37.9°C. Neurological examination was normal except for potentiation of her deep tendon reflexes in four limbs. Peripheral blood leukocyte count was 9400/μL. Biochemical blood studies were normal. Routine CSF studies showed no abnormalities; protein was 23 mg/dL and cell count was 2/μL. Polymerase chain reaction did not detect herpes simplex virus-1 or -2, human herpes virus-6 or -7, cytomegalovirus, or Epstein-Barr virus in CSF. MRI at 1.5 tesla did not reveal any signal alterations in her brain. CPS was controlled by the intravenous administration of diazepam. Surprisingly, Tc-99m SPECT on day 12 showed hyperperfusion in the left temporal lobe although the epileptic seizures had already disappeared. She recovered completely around day 50 following diverse neuropsychiatric symptoms such as transient aphasia, visual hallucinations, and emotional instability. SPECT showed disappearance of hyperperfusion in the left temporal lobe. Later, IgG autoantibodies against GluR ε2 were detected in CSF. During a follow-up period of 2 years, she has not developed any epileptic seizures. IgG autoantibodies against GluR ε2 disappeared in collection of CSF 2 years after her discharge.

We initially diagnosed her as having temporal lobe epilepsy since neither MRI nor CSF studies suggested encephalitis. However, SPECT showed hyperperfusion in her left temporal lobe although she was free from epileptic seizures. Moreover, she suffered from diverse neuropsychiatric symptoms following CPS for 50 days that could not be explained solely by postictal confusion. However, a limbic encephalitis could not be diagnosed definitely because no clear evidence of encephalitis was obtained until autoantibodies against GluR ε2 were detected.

Although the patient's encephalitis may have been caused by an undetected infectious agent, we propose a different explanation. A cell-mediated immune response may have caused tissue damage during an infectious episode before admission, resulting in the production of autoantibodies against GluR ε2. Alternatively, antibodies developing in response to an infectious agent may later have acted as autoantibodies against GluR ε2 because of molecular homology.

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Exophilin4/Slp2-a Targets Glucagon Granules to the Plasma Membrane through Unique Ca^{2} -inhibitory Phospholipid-binding Activity of the C2A Domain

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Rab27a and Rab27b have recently been recognized to play versatile roles in regulating the exocytosis of secretory granules and lysosome-related organelles by using multiple effector proteins. However, the precise roles of these effector proteins in particular cell types largely remain uncharacterized, except for those in pancreatic cells and in melanocytes. Here, we showed that one of the Rab27a/b effectors, exophilin4/Slp2-a, is specifically expressed in pancreatic cells, in contrast to another effector, granuphilin, in cells. Like granuphilin toward insulin granules, exophilin4 promotes the targeting of glucagon granules to the plasma membrane. Although the interaction of granuphilin with syntaxin-1a is critical for the targeting activity, exophilin4 does this primarily through the affinity of its C2A domain toward the plasma membrane phospholipids phosphatidylserine and phosphatidylinositol-4,5-bisphosphate. Notably, the binding activity to phosphatidylserine is inhibited by a physiological range of the Ca^{2} concentration attained after secretagogue stimulation, which presents a striking contrast to the Ca^{2} -stimulatory activity of the C2A domain of synaptotagmin I. Analyses of the mutant suggested that this novel Ca^{2} -inhibitory phospholipid-binding activity not only mediates docking but also modulates the subsequent fusion of the secretory granules.

INTRODUCTION

In multicellular organisms, professional secretory cells store bioactive substances in specialized organelles, such as synaptic vesicles, secretory granules, and lysosome-related organelles, and they release them in response to specific stimuli. Although the basic exocytic protein machinery is conserved, many variations occur in the control of exocytosis that are related to the physiological role of particular cell types (Burgoyne and Morgan, 2003). It is important both biologically and medically to understand specific aspects of the exocytic mechanism, because each of the secreted materials gives a vital command to other cells to orchestrate the whole organism.

We here focused on pancreatic and cells that play a pivotal role in the maintenance of blood glucose levels. Although both cells harbor secretory granules to release peptide hormones, the two types of cells respond to opposite stimuli. The cells secrete glucagon in response to a low glucose level to prevent hypoglycemia, whereas cells secrete insulin in response to postprandial hyperglycemia to maintain a physiological range of glucose concentration. However, little is known about possible differences in the exocytic machinery between these two cell types. We previously reported a novel protein product, granuphilin, that is specifically expressed in cells but not in cells (Wang *et al.*, 1999). Subsequent analyses indicate that granuphilin functions as an effector of Rab27a (Yi *et al.*, 2002) and mediates docking of insulin granules to the plasma membrane in cells (Torii *et al.*, 2002, 2004; Gomi *et al.*, 2005). It is unknown, however, why granuphilin is not expressed in pancreatic cells if it simply executes docking of the secretory granules; nor have we identified its counterpart and its functions in cells. The identification of such a molecule may help elucidate fundamental differences in the regulatory mechanism for hormone secretion between and cells.

At the beginning of the present study, we found that Rab27a was expressed in pancreatic cells as well as in cells. Because Rab27a is thought to function through distinct effectors in a variety of regulated secretory pathways (Izumi *et al.*, 2003; Fukuda, 2005), we explored the expression of Rab27a effector proteins and discovered that exophilin4 (also called Slp2-a) was specifically expressed in pancreatic cells. In the present study, we characterized the function of exophilin4 in cultured cell lines and found that exophilin4 promotes the targeting of glucagon granules to the plasma membrane through the interaction with phospholipids via the C2A domain. Surprisingly, the binding of the C2A domain to phosphatidylserine (PS) was inhibited by the Ca^{2} concentration physiologically induced by secretagogues, which presented a marked contrast to the well-known Ca^{2} -stimulatory PS-binding activity of the synaptotagmin I-C2A domain (Südhof and Rizo, 1996; Bai and Chapman, 2004).

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Experiments using exophilin4 mutants revealed that this novel Ca^{2} -sensitive PS-binding activity plays critical roles in the docking and possibly the fusion of glucagon granules.

MATERIALS AND METHODS

DNA Construction

The cDNA clone (FLJ20163) encoding an N-terminal part of human exophilin4 was obtained from Kazusa DNA Research Institute (Kisarazu, Japan). A C-terminal part of exophilin4 cDNA was amplified from the cDNA of the HMV-II human melanoma cell line by polymerase chain reaction (PCR) and ligated to construct full-length exophilin4 cDNA. Site-directed mutagenesis of exophilin4 was performed using the following primers: 5 -GGAATTCGTTCCAGCATTTCTCCAAGA-3 and 5 -TACATATGGTTTGAACGCTGTTTTTTTACATTCGCTGC-3 for DN1, 5 -CAGCGTTCAGACCCATATGTAAAGGCC-3 , and 5 -CCCCTAGGAAACTATTGCGCTTAAATGTATTCCGATGCC-3 for DN2, and 5 -ACACTAGTAGTGCAGCAGACC-3 and 5 -CACTACTAGTGTCTGCTGCTGGCC-3 for KQ. The DN mutant was constructed by ligating the cDNA fragments containing the respective DN1 and DN2 mutations. The resulting exophilin4-C2A^{DN} has substitutions of asparagine for aspartate at amino acid positions 635, 642, and 698 of human exophilin4, whereas exophilin4-C2A^{KQ} carries substitutions of glutamine for lysine at amino acid positions 658, 659, 660, 665, and 666.

The cDNA fragments of exophilin4 encoding 579-910 (C2AB domain), 579-742 (C2A domain), and 745-910 (C2B domain) amino acids were amplified by PCR, by using a full-length exophilin4 cDNA as a template. Amplified cDNA fragments were subcloned into the pcDNA3-HA and pGEX4T-1 to express hemagglutinin (HA)-tagged and glutathione *S*-transferase (GST)-fused protein, as described previously (Yi *et al*., 2002). The cDNA fragments of N-terminal exophilin4 encoding 1-462 and 1-381 amino acids were similarly amplified and subcloned into the pcDNA3-HA and pGEX4T-1, respectively. The GST-fused cDNA constructs containing 315-673 (C2AB domain), 315-502 (C2A domain), and 487-673 (C2B domain) amino acids of granuphilin-a and that encoding the C2A domain of rat synaptotagmin I (139-267 amino acids) were described previously (Wang *et al*., 1999). A cDNA fragment encoding the C2B domain of rat synaptotagmin I (248-421 amino acids) containing glycine at 374 (Desai *et al*., 2000) was synthesized by reverse transcription-PCR, by using rat brain RNA as a template, and fused to GST. GST fusion proteins were expressed in *Escherichia coli* (BL21) and purified by affinity chromatography with glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) as described previously (Wang *et al*., 1999).

Cell Culture, Cloning, and Transfection

TC1.6 cells, kindly provided by K. Hamaguchi (Oita University, Oita, Japan), were grown in high-glucose (25 mM) DMEM supplemented with 20% fetal calf, 1% L-glutamine, 1% sodium pyruvate, and 1% minimum essential medium nonessential amino acids (Invitrogen, Carlsbad, CA). Although the TC1.6 cell line was established as a clonal cell line that specifically produces glucagon but not insulin (Hamaguchi and Leiter, 1990) from the original TC1 cell line (Powers *et al*., 1990), immunostaining analysis revealed that each cell shows variable levels of glucagon expression. We thus performed the recloning of TC1.6 cells by the limiting dilution method. Among the seven clones established, clone 6 (designated TC1.6.6) that expresses a relatively high level of glucagon was used in some immunostaining analyses to examine the protein colocalization with glucagon granules. The stable TC1.6 clones that express phogrin-enhanced green fluorescent protein (EGFP) were established as described previously using the phogrin-EGFP plasmid (Torii *et al*., 2004). Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies, Immunostaining, Immunoblotting, and Immunoprecipitation

Rabbit anti-exophilin4 antibodies (Exo4N) were raised against GST-fused N-terminal human exophilin4 protein. The rabbit anti-granuphilin antibodies (Grp-N) were described previously (Yi *et al*., 2002). Mouse anti-Rab27a and Rab3a monoclonal antibodies were purchased from BD Biosciences Transduction Laboratories (Lexington, KY). Mouse anti-Xpress monoclonal antibodies were purchased from Invitrogen. Rat anti-somatostatin and anti-HA (clone 3F10) monoclonal antibodies were purchased from Chemicon International (Temecula, CA) and Roche Diagnostics (Mannheim, Germany), respectively. Guinea pig anti-glucagon and anti-pancreatic polypeptide sera were purchased from Linco Research (St. Charles, MO). Guinea pig anti-porcine insulin serum was a gift from H. Kobayashi (Gunma University, Maebashi, Japan).

Indirect immunofluorescence analyses were performed as described previously (Wang *et al*., 1999; Torii *et al*., 2002). The mouse pancreas specimen was observed with an epifluorescence microscope (BX-50; Olympus, Tokyo, Japan) equipped with a SenSys charge-coupled device camera (Photometrics, Tucson, AZ). Cultured cells were observed with a confocal microscope LSM5 PASCAL (Carl Zeiss, Jena, Germany). Tissue extracts for immunoblotting analysis were prepared as follows. Tissue excised from C3H/He mice (0.5 g each) was homogenized using a glass-Teflon homogenizer (1000 rpm; 10 strokes) in buffer containing 10 mM HEPES, pH 7.4, 1 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride. It was then lysed on ice for 1 h in the same buffer with 0.1 M NaCl and 1% Triton X-100. After microcentrifugation at 11,000 rpm for 30 min at 4°C, the supernatant was stored at 80°C until use. Pancreatic islets were isolated by pancreatic duct injection of collagenase solution as described previously (Kasai *et al*., 2005). Immunoblotting, immunoprecipitation, and in vitro binding assay with GST fusion proteins were performed as described previously (Nagashima *et al*., 2002).

Phospholipid Binding Assays

Preparation of ^{3}H-labeled liposomes and measurement of phospholipid binding were performed according to the methods described previously (Wang *et al*., 1999; Hosaka *et al*., 2004). All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were made from 75 weight % of phosphatidylcholine (PC) and 25 weight % of PS, or 98.5 weight % of PC, and 1.5 weight % of phosphatidylinositol-4, 5-biphosphate (PIP_2), with 20 Ci of 1,2-dipalmitoyl, L-3-phosphatidyl[N-*methyl*-^{3}H]choline (GE Healthcare). Phospholipids dissolved in chloroform were dried under a stream of nitrogen gas and resuspended in 5 ml of buffer A (50 mM HEPES, pH 7.4, and 0.1 M NaCl). The mixture was shaken for 1 min and sonicated for 15 s by using a probe sonicator. Liposomes were centrifuged at 10,000 g for 10 min before use to remove aggregates. The phospholipid binding assay contained 15 g of recombinant protein bound to 10 l of glutathione beads. Beads were prewashed twice with the respective test solutions, and resuspended in 0.1 ml of buffer A containing ^{3}H-labeled liposomes (0.1 Ci/16.7 g of phospholipid) and 2 mM EGTA with or without the addition of 2.1 mM $CaCl_2$. For measuring the Ca^{2} dependence of liposome binding, the free Ca^{2} concentration was adjusted by changing the concentration of the $CaCl_2$ solution, and it was calculated using WEBMAX-C software (Patton *et al*., 2004). The mixture was incubated at room temperature for 10 min with vigorous shaking, and then it was briefly centrifuged at 2200 rpm in a tabletop centrifuge. The beads were washed three times with 1 ml of the incubation buffer, and the liposome binding was quantified by liquid scintillation counting.

Plasma Membrane Targeting and Hormone Secretion Assays

Recombinant adenoviruses bearing wild-type and mutant exophilin4 cDNAs were prepared as described previously (Yi *et al*., 2002). AxCALacZ, which expresses bacterial -galactosidase (-gal), was used as a control adenovirus. A targeting assay was performed as described previously (Torii *et al*., 2004; Izumi *et al*., 2005) with modifications. Briefly, TC1.6/phogrin-EGFP cells (clone 6) were cultured on poly-L-lysine–coated eight-well chamber slides and infected with adenoviruses encoding -gal, or wild-type or mutant HA-exophilin4. The cells were then indirectly immunostained with anti-HA antibodies to detect the exogenous exophilin4. Intrinsic EGFP and antibody staining signals were observed by confocal microscopy. A peripheral pattern of EGFP signals was quantified as follows. Cells that revealed a linear distribution along 76–100% of the whole plasma membrane were counted as 1, those along 51–75% as 0.75, those along 26–50% as 0.5, those 25% as 0.25, and those that showed no linear distribution as 0. The summed counts divided by the total number of cells examined were defined as the targeting activity. For each experiment, 60 cells were assessed unambiguously.

The effect of overexpressed exophilin4 on glucagon secretion in TC1.6 cells was examined as described previously for insulin secretion in MIN6 cells (Yi *et al*., 2002), with some modifications. Briefly, 5 10^{5} cells seeded in poly-L-lysine–coated 12-well dishes on the preceding day were infected with recombinant adenoviruses 40 h before the secretion experiment. The cells were washed once with glucose-free modified Krebs-Ringer buffer (KRB; 120 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM HEPES, pH 7.4, and 0.1% bovine serum albumin) and then incubated for 30 min in the indicated buffer in the absence or presence of high K (60 mM KCl and 65 mM NaCl). Secreted glucagon was measured using Glucagon, Human, EIA High Sensitivity kit (Peninsula Laboratories, San Carlos, CA).

RESULTS

Expression of Exophilin4

Polyclonal antibodies generated against the N terminus of exophilin4 recognized a single 130-kDa protein in the extracts of a mouse melanocyte cell line melan-a (Figure 1A), where exophilin4 is known to be expressed (Kuroda and Fukuda, 2004). The corresponding protein band was barely detected in the other tissues and cell lines examined, except for the pancreatic cell line TC1.6 (Figure 1A). Immunohistochemical analysis of mouse pancreas specimens revealed that exophilin4 is expressed only in peripheral islet

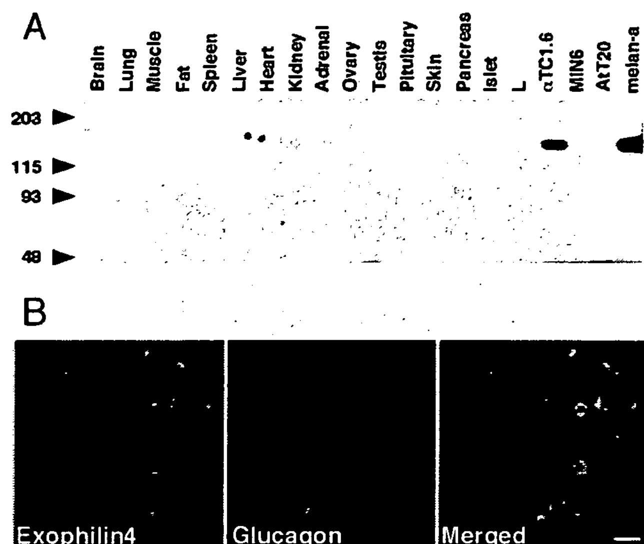

Figure 1. Tissue and cell expression of exophilin4. (A) An equal amount of protein (35 g) from tissue and cell extracts was loaded onto a polyacrylamide gel. Immunoblotting was performed using anti-exophilin4 antibodies. Numbers to the left of the panel are molecular masses in kilodaltons. (B) The pancreata of 16-wk-old male C3H/He mice were double immunostained with anti-exophilin4 and anti-glucagon antibodies. Merged fluorescent signals are also shown. Bar, 20 m.

cells but not in exocrine cells (Figure 1B). Double immunostaining analysis using antibodies against pancreatic hormones indicated that it is specifically expressed in glucagon-producing cells and pancreatic polypeptide-positive cells, but not in insulin-secreting cells or somatostatin-secreting cells (Figure 1B and Table 1). Consistent with a previous finding (Wang *et al.*, 1999), granuphilin was detected only in cells (Table 1). In contrast, Rab27a was expressed in all four types of endocrine cells but was not detected in exocrine cells (Table 1). Thus, although Rab27a is expressed in all endocrine cells in the pancreas, their effectors are differentially expressed.

Exophilin4 has a putative Rab27a binding sequence without a zinc-finger motif at the N terminus (Izumi *et al.*, 2003; Fukuda, 2005). To confirm the interaction with Rab27a, we first incubated extracts of TC1.6 cells with bacterially expressed GST-fused Rab27a protein that had been immobilized on glutathione-Sepharose beads and preloaded with either GDP or guanosine 5 -*O*-(3-thio)triphosphate (GTP S). Bound exophilin4 was then detected by immunoblotting. Exophilin4 preferentially bound to GTP S-loaded GST-Rab27a, although a weak interaction with GDP-bound Rab27a was detected (Figure 2A). To investigate whether the interaction occurs under physiological conditions, the pres-

Table 1. Summary of the distributions of exophilin4, granuphilin, and Rab27a in the pancreas

Cell type	Exophilin4	Granuphilin	Rab27a
(insulin)			
(glucagon)			
(somatostatin)			
Pancreatic polypeptide			
Exocrine			

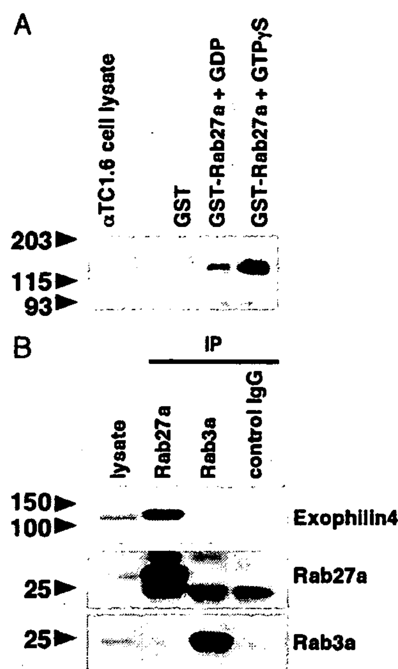

Figure 2. Complex formation between exophilin4 and Rab27a. (A) Aliquots of glutathione beads containing either GST alone or GST-fused Rab27a preloaded with GDP or GTP S (1 g of protein) were incubated with TC1.6 cell extracts (1 mg of protein) and then washed three times. Proteins that bound to the GST fusion proteins and an aliquot of the original cell lysates (20 g of protein) were analyzed by immunoblotting with anti-exophilin4 antibodies. (B) Extracts of TC1.6 cells (1 mg of protein) were incubated with anti-Rab27a, anti-Rab3a antibodies, or control mouse IgG and then with protein G-Sepharose beads. After washing the beads, immunoprecipitates (IP) and an aliquot of the original cell lysates (20 g of protein) were analyzed by immunoblotting using anti-exophilin4 and anti-Rabs antibodies. Numbers to the left of each panel are molecular masses in kilodaltons.

ence of exophilin4 was directly examined in Rab27a immunoprecipitates from TC1.6 cells. Exophilin4 was coimmunoprecipitated with endogenous Rab27a but not with Rab3a, although Rab3a was also expressed in TC1.6 cells (Yi *et al.*, 2002) and immunoprecipitated under this condition (Figure 2B). These findings are consistent with a previous finding that exophilin4 interacts with Rab27a in the B16-F1 mouse melanoma cell line (Kuroda and Fukuda, 2004).

Intracellular Localization of Exophilin4

Because Rab27a and its effector granuphilin are localized on insulin granules in pancreatic cells (Yi *et al.*, 2002), we considered that Rab27a and exophilin4 would locate on glucagon granules in cells. To confirm this, we performed an immunostaining analysis. Our anti-exophilin4 antibody, however, did not yield immunostaining signals strong enough to determine the intracellular localization of endogenous exophilin4 in TC1.6 cells. Therefore, we expressed HA-tagged exophilin4 and examined its localization by anti-HA antibodies. HA-exophilin4 was concentrated just beneath the plasma membrane and colocalized with peripheral glucagon granules (Figure 3A, a–c). Glucagon signals tended to accumulate in a more peripheral region of the transfected cells than they did in neighboring untransfected cells. The effect was specific because similarly expressed Rab27a was colocalized with glucagon in a more interior cell