Detection of Autoantibodies Against Hypocretin, hcrtr1, and hcrtr2 in Narcolepsy: Anti-Hcrt System Antibody in Narcolepsy

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Study Objectives: The impairment of hypocretin neurotransmission system is considered to play a major role in the pathophysiology of narcolepsy. It has been hypothesized that autoimmune abnormalities underlie the etiology of narcolepsy, based on the tight association with HLA-DRB1*1501/DQB1*0602. It remains unclear if autoantibodies against hypocretin receptors (hcrtr1 and hcrtr2) are involved in narcolepsy.

Design: We have developed a novel radioligand binding assay to address this question. Sera from 181 patients with narcolepsy, 10 patients with other hypersomnias, and 91 control subjects were used. Human [^{35}S]-Hcrt, hcrtr1, and hcrtr2 were synthesized by *in vitro* transcription/translation system. The immune complex of autoantibody and each [^{35}S]-protein were immunoprecipitated and quantified using a radioligand-binding assay.

Results: We detected autoantibodies against hypocretin in 3 patients, hcrtr1 in 1 patient, and hcrtr2 in 5 patients with narcolepsy. Positive reactions were also found against hcrtr1 in 2 and hcrtr2 in 1 control subjects. No relationships were found between these autoantibodies and HLA-DRB1*1501/DQB1*0602 haplotypes, presence of cataplexy, presence of subjective nocturnal sleep disruption, or the score on the Epworth Sleepiness Scale.

Conclusions: Although we have detected autoantibodies against the hypocretin neurotransmission system, our results do not support the hypothesis that autoantibody-mediated dysfunction in the hypocretin system underlies the pathophysiology of narcolepsy.

Keywords: Autoantibody, narcolepsy, hypocretin, orexin, hypocretin receptor, autoimmunity, sleep, radioligand assay

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INTRODUCTION

NARCOLEPSY IS A CHRONIC SLEEP DISORDER THAT AFFECTS BETWEEN 1 IN 1000 AND IN 2000 OF THE POPULATION WORLDWIDE AND APPROXIMATELY 1 in 600 of the Japanese population. This disorder is characterized by excessive daytime sleepiness and cataplexy (loss of muscle tone in response to emotional stimuli with consciousness preserved) and is frequently accompanied by other abnormal manifestations of rapid eye movement (REM) sleep, such as sleep paralysis and hypnagogic hallucinations (dream-like episodes at sleep onset). Based on several models, it has been reported that the hypocretin (Hcrt; also known as orexin) neuropeptidergic system is impaired in narcolepsy. Hcrt consists of 2 peptides (hcrt1 [also called orexin A], and hcrt2 [also called orexin B]) produced from the same precursor (preprohcrt) in neurons whose cell bodies are located in the perifornical lateral hypothalamus.[1] Canine models of autosomal recessive narcolepsy are caused by mutations of the Hcrt receptor 2 (hcrtr2) gene.[2] Murine models with the disruption of preprohcrt or Hcrt receptor gene expression exhibit narcolepsy-like behaviors.[3-5] Hcrt1 is usually undetectable in the cerebrospinal fluid (CSF) of narcoleptic patients with cataplexy.[6-9] Studies using *in situ* hybridization and immunohistochemistry have shown marked reductions in the number of Hcrt neurons in the hypothalamus of postmortem brains from patients with narcolepsy.[10,11]

Narcolepsy is generally nonfamilial and is not linked to mutations of the Hcrt system, except for 1 rare case.[10] Moreover, only 5 pairs out of 19 reported monozygotic twins are concordant for narcolepsy.[12,13] An immunological pathogenesis for narcolepsy has been suggested since the discovery of the tight association between narcolepsy and human leukocyte antigen HLA-DR2.[14] Genetic analysis has now narrowed the HLA susceptibility for narcolepsy to HLA-DQB1*0602.[15] Apart from the tight HLA association, the autoimmune hypothesis for narcolepsy is supported by the peripubertal onset of narcolepsy and the specific degeneration of Hcrt neurons in the lateral hypothalamus. The observation of gliosis-like features around Hcrt and hcrtr2 expressing neurons in the narcoleptic brain has been reported.[11,16] The Hcrt neurons appear to specifically degenerate in narcolepsy without any alteration in adjacent neurons, such as those expressing the peptide melanin-concentrating hormone.[10] Some patients with autoimmune encephalitis have shown symptoms of narcolepsy with cataplexy with reduction of CSF hcrt1 levels.[17] Immunoglobulin G in the CSF of HLA-DQB1*0602 positive narcoleptic subjects with cataplexy binds to rat hypothalamus protein extract.[18] Clinical improvement of cataplexy has been observed in narcoleptic patients after therapeutic intravenous infusion of high-dose normal-human immunoglobulin G.[19] More recently, injection of immunoglobulin obtained from patients with narcolepsy has been found to evoke behavioral arrests in mice,[20] but this remains to be confirmed. It is increasingly apparent that the blood-brain barrier does not always prevent autoantibodies shown in patients with anorexia and bulimia nervosa from reaching their targets in the hypothalamus.[21] Furthermore, hypothalamus, where Hcrt cell bodies are located, is highly vascular. Specific immunologic dysregulation against Hcrt neurotransmission might therefore be involved in the development of narcolepsy.

Recently, the screening of autoantibodies against Hcrt was con-

ducted using sera and CSF obtained from narcoleptic patients.[22,23] No autoantibodies against Hcrt could be found, suggesting the possibility for the existence of autoantibodies against other components of Hcrt neurotransmission system, such as hcrtr1 and hcrtr2. We have developed a radioligand binding assay system to detect potential autoantibodies against human Hcrt receptors. We produced recombinant human [^{35}S]-Hcrt and Hcrt receptors by *in vitro* transcription/translation and used them as antigens. Using these recombinant proteins, we investigated the presence of autoantibodies against human hcrtr1 and hcrtr2 together with Hcrt autoantibodies in sera obtained from narcoleptic patients.

MATERIALS AND METHODS

Subjects

This research was approved by the ethics committee of all collaborative institutes. Written informed consents were obtained from all participants. All patients were diagnosed clinically, in combination with the Multiple Sleep Latency Test for narcolepsy without cataplexy, at the Neuropsychiatric Research Institute (Tokyo, Japan). Diagnosis was made according to the International Classification of Sleep Disorders, second edition.[24] Blood samples and data related to sleep conditions were collected at the Tokyo Institute of Psychiatry (Tokyo, Japan) and Neuropsychiatric Research Institute (Tokyo, Japan). Control subjects were excluded if they had excessive daytime sleepiness or any signs of immunologic abnormalities based on a questionnaire obtained at the time of blood collection. All participants were Japanese except for 1 Korean and 1 American (Caucasian) narcoleptic patients. Sera from 181 patients with narcolepsy, 10 patients with other hypersomnias, and 91 healthy control subjects were examined. Five milliliters of venous blood were drawn and separated—sera were stored at -80ºC until use. The HLA typing for HLA-DRB1 and DQB1 loci were done for all the patients at NPO HLA Laboratory (Kyoto, Japan). Patients with narcolepsy consisted of 171 patients with definite cataplexy (all positive for HLA-DRB1*1501/DQB1*0602) and 10 patients without cataplexy (6 cases were HLA-DRB1*1501/DQB1*0602 positive) and were all unrelated. Patients with other hypersomnias consisted of 6 with idiopathic hypersomnias with long total sleep time and 4 cases of recurrent hypersomnia. We collected the following data to analyze potential relationships with the autoantibody index: age, sex, Epworth Sleepiness Scale[25] (ESS) at the time of blood collection, presence of nocturnal sleep disruption (subjective report), and past history of autoimmune disorders. The mean age and sex distribution are summarized in Table 1. The mean ages of patient groups were not significantly different from that of the healthy control subjects, except for the group of patients with narcolepsy without cataplexy.

Synthesis of Recombinant [^{35}S]-Hcrt, hcrtr1, and hcrtr2

The open-reading frames of Hcrt, hcrtr1, and hcrtr2 were obtained by reverse transcript-polymerase chain reaction (PCR) amplification using poly-A RNA obtained from the human hypothalamus and hippocampus (CLONTECH Laboratories, Inc., Palo Alto, CA). The first-strand cDNA was synthesized using ReverTraAce (TOYOBO, Tokyo, Japan) with random hexamers according to the manufacturer's instructions. The following primer pairs for HCRT with either an EcoR I or a Xho I site, 5'-GGAATTCatgaaccttccttccacaaaggt-3' and 5'-GGCGAGCTCtcagatcccggactgtcctcc-3' (the EcoR I and a Xho I sites are capitalized), was used for HCRT amplification. PCR was carried out using a high fidelity DNA polymerase, KOD-plus (TOYOBO, OSAKA, Japan) and Hcrt PCR product was ligated into the pET28a (+) expression vector (Novagen, Madison, WI) (HCRT/pET28a). [^{35}S]-methionine labeled Hcrt was produced using HCRT/pET28a, TNT Quick coupled Transcription/Translation System (Promega, Madison, Wisc), and [^{35}S]-methionine (Amersham Biotech, Arlington Heights, IL) according to the manufacturer's instructions. For hcrtr1 and hcrtr2, first PCR were performed using the following primer pairs, hcrtr1: 5'-atggagccctcagccaccccagg-3' and 5'-tcagggcagcactgtggtgac-3'; hcrtr2: 5'-atgtccggcaccaaattggagg-3' and 5'-ctaccagttttgaagtggtcc-3'. After the addition of 3' adenine overhangs by ExTaq (TAKARA, TOKYO, Japan), these PCR products were cloned into pGEM-T Easy vector (Promega). To add the T7 promotor, a second PCR was performed using KOD-plus DNA polymerase, cloned-vectors as templates, and the following primer pairs, hcrtr1:
5'-GGATCCTAATACGACTCACTATAGGGAGCCACCatggagccctcagccaccccagg-3' and
5'-tcagggcagcactgtggtgac-3'; hcrtr2:
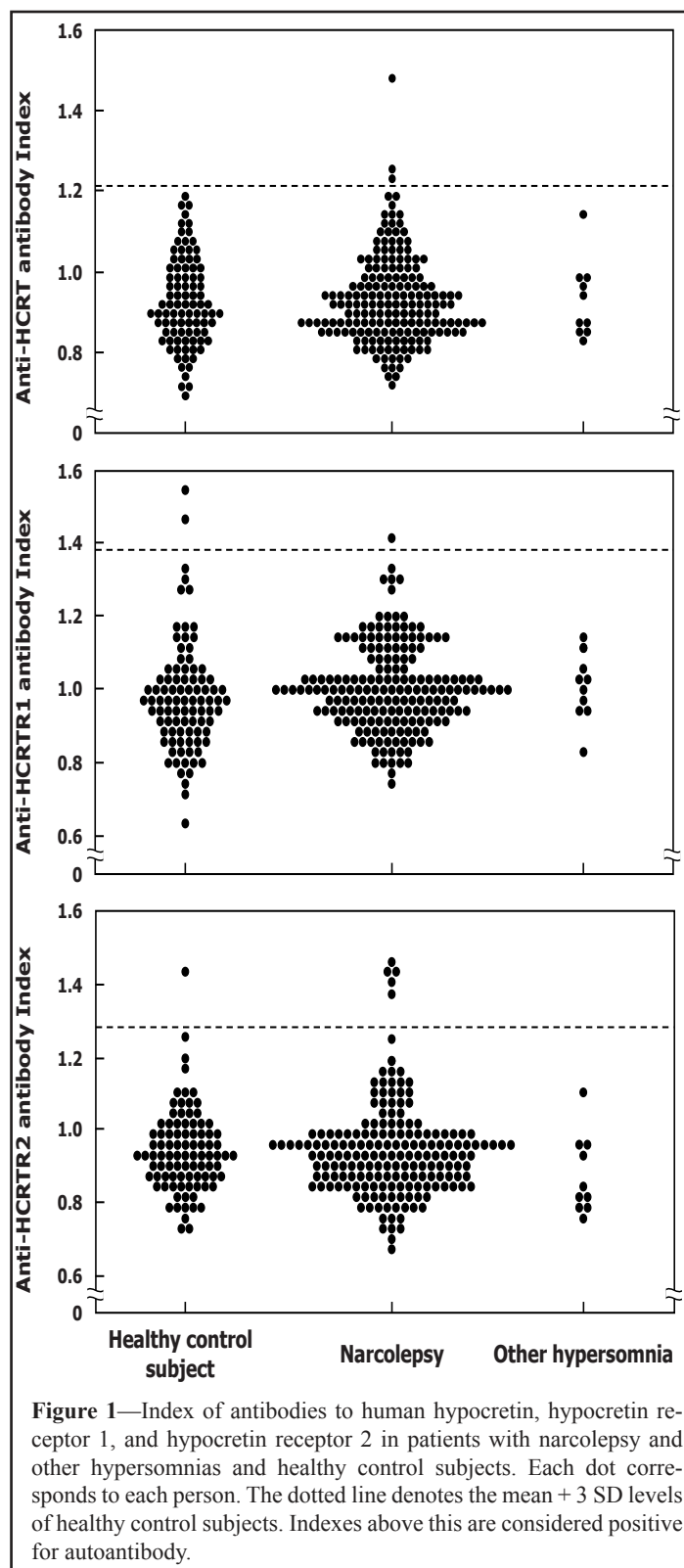5'-GGATCCTAATACGACTCACTATAGGGAGCCACCatgtccggcaccaaattggagg-3' and
5'-ctaccagttttgaagtggtcc-3' (The sequences of T7 promotor, spacer, and KOZAK translation initiation sequence are capitalized). [^{35}S]-methionine-labeled hcrtr1 and hcrtr2 were produced using second PCR products, TNT T7 Quick for PCR DNA (Promega), and [^{35}S]-methionine according to the manufacturer's instructions. Each mixture, including [^{35}S]-methionine-labeled protein, was applied to the NICK column (Amersham Biotech) to remove free [^{35}S]-methionine. SDS-PAGE analysis was carried out, and a single band corresponding to each protein (open reading frames) was found in the BAS-imaging system (data not shown). Each [^{35}S]-labeled human protein was adjusted to a 20,000 counts per minute (cpm) per 20 μL concentration by the reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% BSA, 0.1% Tween-20, and 0.1% NaN3, pH 7.4) and stored at -80ºC until use.

Table 1—Age, Sex and Autoantibodies to Hcrt Neurotransmission Systems in Hypersomnia Patients and Healthy Control Subjects.

Subjects	Number examined	Age (years mean ± SD)	Male/Female		Number of positive antibodies		
			raw number	Sex ratio	HCRT	HCRTR1	HCRTR2
Narcolepsy	181	45.2 ± 17.8	110/71	1.55	3	1	5
(With cataplexy)	(171)	(46.1 ± 18.0)	(104/67)	(1.63)	(3)	(1)	(4)
(Without cataplexy)	(10)	(27.8 ± 4.2)*+	(6/4)	(1.50)	(0)	(0)	(1)
Other hypersomnias	10	38.9 ± 18.1	2/8	0.25	0	0	0
Healthy control subject	91	42.9 ± 11.1	40/51	0.78	0	2	1

*The mean age was significantly different from healthy control subjects at p < 0.05. + The mean age was significantly different from narcoleptic patients with cataplexy at p < 0.05. HCRT: hypocretin; HCRTR1: HCRT receptor 1; HCRTR2: HCRT receptor 2

Figure 1—Index of antibodies to human hypocretin, hypocretin receptor 1, and hypocretin receptor 2 in patients with narcolepsy and other hypersomnias and healthy control subjects. Each dot corresponds to each person. The dotted line denotes the mean + 3 SD levels of healthy control subjects. Indexes above this are considered positive for autoantibody.

Radioligand Binding Assay

The detailed method for the radioligand binding assay was described previously.[26] The mixture containing 1 μL of patient serum and 20,000 cpm of each [^{35}S]-labeled human protein was incubated overnight at 4ºC (50 μL in total). The reaction mixtures containing immune complex were transferred to a 96-well filtration plate (Millipore Corp., Benford, MA). Ten microliters of 50% Protein-G Sepharose 4FF (Amersham Bioscience) were added to

each well. The plate was incubated for 45 minutes at room temperature for binding of protein-G to the immune complex and then washed 10 times with 200 μL of washing buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% Tween-20, pH 7.4) using MultiScreen vacuum manifold (Millipore). The filter was dried, and liquid scintillation counter, MicroScint 0 (PerkinElmer Life Science, Boston, MA), was added to each well. The precipitated and labeled proteins were quantified in duplicate using a Top-Count NXT apparatus (PerkinElmer Life Science). The intraassay coefficient of variation with these radioligand assays ranged from 4.5% to 5.1%, while the interassay coefficient variation ranged from 6.1% to 9.5%. In order to avoid interassay variation, the results were expressed by autoantibody index calculated as follows; (cpm of the sample serum/cpm of the pooled 91 healthy control sera). The cut-off value was calculated as the mean + 3 SD in healthy control subjects. The cut-off value with mean + 3SD is arbitrary. We selected this cut-off value based on the absorption tests in our previous experiment.[27,28] The mouse anti-Hcrt monoclonal antibodies and the rabbit polyclonal anti-Hcrt serum[29] were used as the positive standard for anti-Hcrt antibody.

Statistical Analysis

The distribution pattern was tested for 3 antibody indexes using normal probability paper. A normal distribution of antibody indexes was confirmed in hypersomnia patients and healthy control subjects. Distributions of antibody indexes among hypersomnia groups and the healthy control group were compared using the Mann-Whitney U test. The Mann-Whitney U test was also used to compare the distribution of antibody indexes among the divided groups according to the association to the following features; sex, HLA-DRB1*1501/DQB1*0602 (data available only for patients), cataplexy, and nocturnal sleep disruption. The correlations between antibody indexes and age or ESS at the time of blood sampling were examined using the Spearman correlation coefficient test. A p value less than .05 was considered as statistically significant.

RESULTS

Using a radioligand-binding assay, we examined autoantibodies against Hcrt neurotransmission systems in sera obtained from patients with narcolepsy and compared them with the sera of patients with other hypersomnias and healthy control subjects (Figure 1, Table 1). As a positive control, we used mouse monoclonal antibody and rabbit polyclonal antiserum. They showed positive reactions, and anti-Hcrt antibody indexes were 1.929 on 1:5000 dilutions and 2.155 on 1:1000 dilutions, respectively, showing the validity of our radioligand-binding assay.

There were no differences regarding the average indexes of anti-Hcrt, hcrtr1, and hcrtr2 antibody between patients with narcolepsy and healthy control subjects, patients with narcolepsy and those other hypersomnias, or patients with other hypersomnias and healthy control subjects (Figure 1, Table 1). With a cut-off values above the mean of + 3 SD of healthy control subjects (Hcrt: 1.219, hcrtr1: 1.388, hcrtr2: 1.274), positive reactions against recombinant Hcrt, hcrtr1, and hcrtr2 were found in 3, 1, and 5 patients with narcolepsy, respectively (Figure 1). As a whole, 8 patients with narcolepsy had anti-Hcrt neurotransmission system antibodies, with 1 having positive reactions against both hcrtr1 and 2. No positive reactions were detected in patients
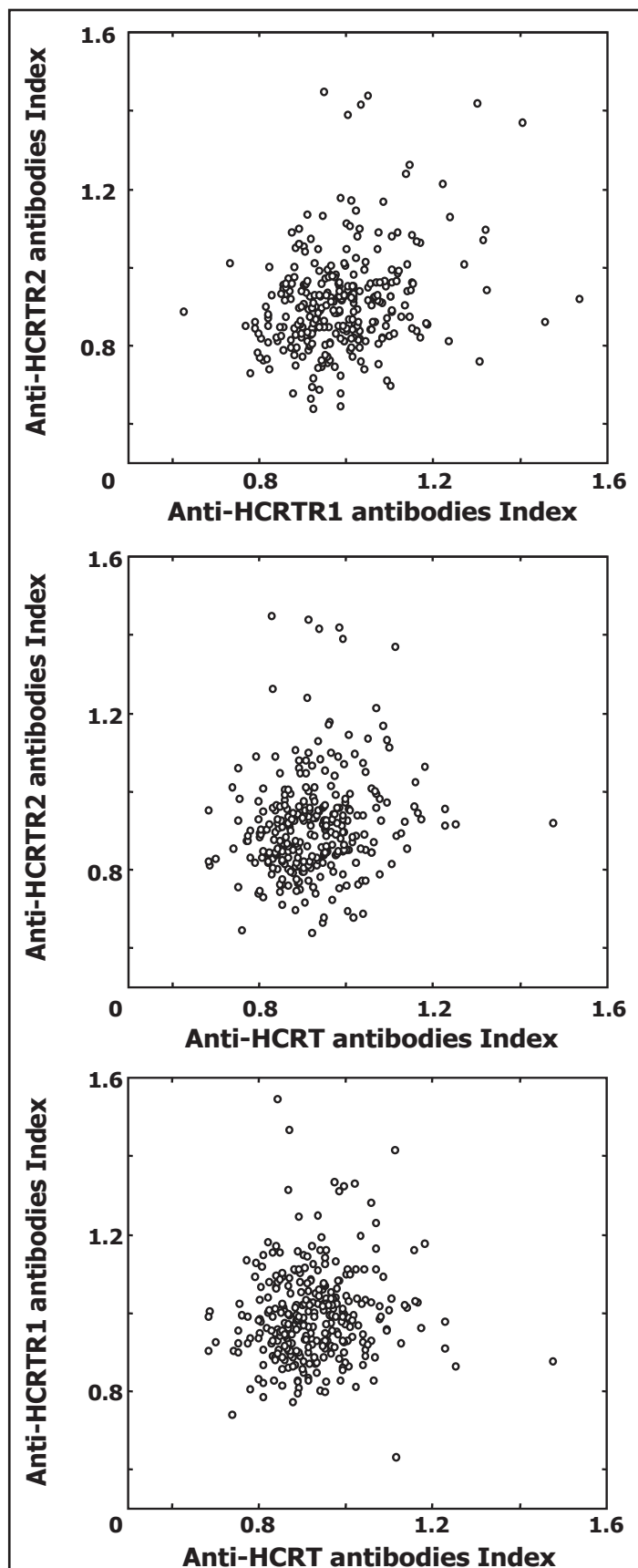
Figure 2—Comparison among 3 antibodies to human hypocretin, hypocretin receptor 1, and hypocretin receptor 2 in all subjects examined (n = 282). The Spearman correlation coefficient test was used to analyze the relationship among 3 different autoantibodies. There are no correlations among 3 antibody indexes.

with other hypersomnias, while healthy control subjects showed positive reactions against hcrtr1 (in 2 subjects) and hcrtr2 (in 1 subject).

Antibody indexes were compared with each other in order to clarify the relations among 3 different autoantibodies. No correlations were found among all subjects examined (Figure 2) or among patients with narcolepsy (data not shown).

All patients having autoantibodies against Hcrt neurotransmission systems were positive for HLA-DRB1*1501/DQB1*0602 except for 1 patient with "narcolepsy without cataplexy" who had an autoantibody against hcrtr2. The relationship between autoantibody indexes and collected clinical and demographic data were analyzed. We could not find any significant differences except for a sex difference in the mean antibody indexes. No differences in the mean antibody indexes were observed among the divided groups according to the association with HLA-DRB1*1501/DQB1*0602 (data available only for patients), presence of cataplexy, or presence of nocturnal sleep disruption, using the Mann-Whitney U test. No correlations between antibody indexes and age, or ESS, at the time of blood sampling were found using Spearman correlation coefficient test. The mean anti-Hcrt antibody index was significantly higher in women (n = 133, mean ± SD; 0.941 ± 0.113) than that in men (n = 156, mean ± SD; 0.909 ± 0.083) (p = .018). The sex differences were also noted in the subgroup of narcoleptic patients (women: n = 71, mean ± SD; 0.957 ± 0.114) (men: n = 110, mean ± SD; 0.911 ± 0.085) (p = .007).

DISCUSSION

The autoimmune hypothesis for narcolepsy has remained attractive because of the tight HLA association, peripubertal onset, specific degeneration of Hcrt neurons, and the existence of functional antibodies involving the cholinergic system in sera. In this study, we have conducted the first screening for autoantibodies against Hcrt receptors in human serum and found the existence of potential autoantibodies in some patients against Hcrt, hcrtr1, and hcrtr2 using radioligand-binding assay.

The radioligand binding assay provides several methodologic advantages compared with conventional methods used previously to investigate the autoimmune basis of narcolepsy. The recombinant proteins translated from full open reading frames are easily available as antigens in our system, compared with the conventional protein overexpression systems. It is not necessary to extract and denature the antigen for *in vitro* transcription/translation system. The Millipore MultiScreen system enables us to apply this system to high-throughput use. Repetitive washing (10 times) with high stringency detergent (1% Tween-20) can reduce background to negligible levels without any intricate operations. The radioligand binding assay also has an advantage with its ability to detect autoantibodies against conformational antigens, as compared to other methods.[30, 31] It should, however, be noted that tissue-specific posttranslational modifications of antigens are still not completely the same as natural ones.

We have shown the presence of autoantibodies in sera from both narcoleptic patients and healthy control subjects. Our observations also suggest that these autoantibodies are not likely to contribute to the pathophysiology in the majority of narcoleptic patients. They might be naturally occurring autoantibodies without pathologic function or might not cross the blood-brain barrier to cause narcolepsy. Considering the physiologic importance

of Hcrt neurotransmission system in sleep-wake regulation, it is worth discussing possible roles for these autoantibodies.

Recently, potential autoantibodies against rat hypothalamic protein using enzyme-linked immunosorbent assay have been reported in CSF obtained from HLA-DQB1*0602 positive narcoleptic patients with cataplexy.[18] On the other hand, these researchers have previously reported negative results in detecting anti-Hcrt antibodies in sera and CSF obtained from 41 narcoleptic patients, employing immunoblotting, and immunoprecipitation for Hcrt-expressing cell lines.[22] Their data have shown lower mean antibody reaction against C-terminal peptide of Hcrt in CSF from narcoleptic patients compared with that of healthy control subjects. All techniques quoted might fail to detect antibodies against various conformational antigens.

In our study, 3 patients with narcolepsy had positive reactions against [^{35}S]-recombinant Hcrt protein. These might be autoantibodies against conformational antigens, which could not be detected by other systems using linearized , partial or denatured antigens.[32] Since we used high-stringency detergent and washed the plates frequently, autoantibodies detected in the present study might have high affinities with conformational antigens. Three narcolepsy patients with positive reactions against Hcrt were all women with HLA-DRB1*1501/DQB1*0602 and cataplexy. The sex difference on the reaction of autoantibodies against Hcrt might reflect the difference in immunologic background between men and women. We need to consider the significant differences in sex ratio between the narcoleptic group and controls (110/71 = 1.55 versus 40/51= 0.78, Table 1). It is one limitation in this study and the possible reason why only anti-Hcrt antibodies did not appear in healthy control subjects.

Five patients with narcolepsy showed positive reactions against hcrtr2. One patient had autoantibodies against both hcrtr1 and hcrtr2, although no correlations among the 3 autoantibody indexes were found. The coexistence of autoantibodies against hcrtr1 and hcrtr2 suggests that this patient might produce a high level of some sorts of different autoantibodies. However, this patient (25-year-old woman with a disease duration of 13 years) showed a typical clinical course of narcolepsy without any specific complications. Therefore, these autoantibodies might not have a pathogenic role. Regarding this patient, it is worthwhile measuring the concentrations of CSF Hcrt and total immunoglobulin G in the future. We can also assert that cross-reacting antigenicity does not occur among these autoantibodies, based on the lack of correlation among the 3 autoantibodies (see Figure 2). Autoantibodies against these 3 proteins might be produced independently. One "narcolepsy without cataplexy" patient and 1 healthy control subject showed positive reactions against hcrtr2. This patient with narcolepsy without cataplexy was negative for HLA-DRB1*1501/DQB1*0602. The excessive daytime sleepiness seen in this patient could have an autoimmune etiology, but it might be driven more by environmental factors rather than HLA haplotype. Total of eight patients and 3 healthy control subjects with positive reactions to Hcrt, hcrtr1, and hcrtr2 denied the present or past history of complication from autoimmune diseases. Patients with positive reactions have narcolepsy symptoms and clinical courses similar to the majority of narcoleptic patients.

Interestingly, further inquiry revealed that 2 healthy control subjects with positive reactions against hcrtr1 had a past history of excessive daytime sleepiness in their school days. It has been speculated that traumatic events and strong stress induces disruption of the blood-brain barrier.[33,34] It has been reported that 1 type of naturally occurring antibody in the blood of control subjects has an agonist-like activity against mu-opioid receptor.[35] If anti-hcrtr1 antibodies have antagonist-like functions, the transient impairment of blood-brain barrier might have occurred in these healthy control subjects. Anti-hcrtr1 antibodies detected in 2 healthy control subjects might have some functions in sleep-wake regulation. On the other hand, Hcrt receptors are reported to be expressed in peripheral tissues.[36,37] Anti-receptor antibodies may therefore be unrelated to sleep-wake regulation.

Smith et al[20] studied immunoglobulins purified with Protein-A Sepharose in 9 narcoleptic patients and showed that all narcoleptic patients examined had functional antibodies involving the cholinergic system. In this study, we did not examine antibodies specific to the cholinergic system. Our system, however, could easily be adapted for confirmation of these and other potential antigen targets.

In conclusion, serum autoantibodies against the Hcrt and the 2 known Hcrt receptors were detected in a few narcoleptic patients. Our results showed, however, no differences in the incidence of positive numbers between narcoleptic patients and healthy control subjects and the lack of relationship between narcolepsy and these autoimmunities against Hcrt neurotransmission systems. These autoantibodies in the serum might be unrelated to the development of narcolepsy. Future analysis of CSF from patients and controls may provide further information regarding potential autoantibodies specific to the hypocretin neurotransmitter system.

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