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ORIGINAL ARTICLE

A Clinical Survey of Uveitis in HTLV-I Endemic Region

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ABSTRACT

Purpose: To investigate a clinical survey of uveitis in southern Kyushu of Japan, where human T-lymphotropic virus type 1 (HTLV-1) and toxoplasmosis is highly endemic.

Methods: The clinical records of patients with uveitis between 1975 and 2007 at Miyata Eye Hospital were reviewed.

Results: A total number of 1338 patients (2012 eyes), consisting of 526 men and 812 women with mean age of 50.5 years old, were analyzed. The most common clinical entity was HTLV-1 uveitis (17.1%), followed by Vogt–Koyanagi–Harada disease (9.9%), sarcoidosis (7.2%), toxoplasmosis (7.1%), Behçet's disease (4.3%) and others. Unclassified uveitis comprised 41.1% in the series. Anterior uveitis was seen in 30.8%, intermediate uveitis in 17.3%, posterior uveitis in 9.3%, and pan-uveitis in 42.6%.

Conclusions: HTLV-1 uveitis and toxoplasmosis were the major clinical entities in southern Kyushu of Japan. This relates to the high seroprevalence of the infectious agents in this region of Japan.

KEYWORDS: epidemiology; HTLV-I; intraocular inflammation; seroprevalence; uveitis

INTRODUCTION

Uveitis is an intraocular inflammatory disorder caused by a variety of infectious agents or associated with various systemic diseases, mostly autoimmune diseases. It is well known that the etiology of uveitis varies among different countries and ethnic groups depending upon prevalence of infectious agents and genetic background. Therefore, epidemiological survey of uveitis is clinically important, and many studies have been reported in many different countries and ethnic groups.[1–8]

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In Japan, Behçet's disease was the most common clinical entity of uveitis three decades ago. However, recent nationwide survey of uveitis in Japan has shown that sarcoidosis is the leading cause of uveitis, followed by Vogt–Koyanagi–Harada (VKH) disease and Behçet's disease.[8] Additionally, in the last two decades many new infectious agents were reported to be causatively related to uveitis. One example is human T-lymphotropic virus type 1 (HTLV-1), a human retrovirus known to cause adult T-cell leukemia (ATL)[9] and tropical spastic paraparesis and HTLV-1-associated myelopathy (TSP/HAM).[10,11] Ohba and his colleagues first reported that patients with HAM as well as asymptomatic carriers had various ocular manifestations including uveitis.[12] In early 1990s we reported sero-epidemiological, clinical, and virological evidences showing HTLV-1 as a causative agent to certain type of uveitis.[13] Today, it is well established that uveitis associated with

HTLV-1 (HTLV-1 uveitis) is a distinct clinical entity of uveitis. The virus is known to have unique geographic distribution and highly endemic in tropical Africa, the Caribbean basin, Latin America, Melanesia, and southwest of Japan.[14,15] HTLV-1 uveitis was previously reported to be more common in southern Kyushu island (18.4%) than in northern Kyushu island (5.4%).[16] However, clinical studies in HTLV-1 endemic area with a large number of patients with long follow up are not available.

We, therefore, conducted a clinical survey to assess the etiologies and clinical characteristics of patients with uveitis over a 32-year period in southern Kyushu island of Japan where HTLV-1 is highly endemic.

MATERIALS AND METHODS

The clinical records of patients with uveitis between June 1975 and September 2007 at Miyata Eye Hospital (Miyakonojo, Miyazaki, Japan), a referral eye center of the region, were retrospectively reviewed. Patients followed at the hospital shorter than three months were excluded from this study. The following data were entered into a computerized database system: gender, age at initial presentation, ocular symptoms and signs, etiological diagnosis, and associated systemic symptoms and diseases.

The etiological diagnoses of specific uveitis entities or associated systemic diseases were made based on medical history, extensive systemic examinations, complete ophthalmologic examinations, and laboratory tests. Complete ophthalmic examination included best corrected visual acuity, intraocular pressure, slit-lamp examination, gonioscopic examination, and indirect funduscopy in all patients. Fluorescein angiography, indocyanine green angiography, ultrasonography, electro retinography, perimetry were carried out in case of need. Additionally, in some patients where viral infection or intraocular lymphoma were suspected, aqueous humor or vitreous samples were taken and sent to the Department of Ophthalmology and Visual Science, Tokyo Medical and Dental University for polymerase chain reaction (PCR) assay. Laboratory tests performed in every patient included complete blood cell count with differential count, erythrocyte sedimentation rate, serum biochemical analysis (glucose, urea, creatinine, and bilirubin concentrations, liver enzymes and angiotensin-converting enzyme), serological tests for HTLV-1, toxoplasma, and serum fluorescent treponemal antibody absorption test. Additional tests were performed when necessary and this included chest X-ray, tuberuculin skin test, human lymphocyte antigen (HLA) typing, cerebrospinal fluid examination, and gallium-67 scintigraphy.

Standard diagnostic criteria were employed as follows. Behçet's disease was diagnosed according to the Japanese diagnostic criteria established by the Behçet's Disease Research Committee of the Ministry of Health, Labour, and Welfare of Japan[17] and sarcoidosis was diagnosed according to the Japanese criteria.[18] VKH disease was diagnosed according to the Japanese criteria reposed by Mimura[19] and all our patients met the revised international diagnostic criteria[20] and classified as complete or incomplete VKH disease. The diagnosis of HTLV-1 uveitis was made to those who were seropositivity to HTLV-1 and all other uveitic entities with defined etiology were excluded.[21] HLA-B27$^+$ acute anterior uveitis (AAU) was diagnosed in those who had AAU episode in one eye and positive to HLA-B27. Herpetic iritis was diagnosed when a patient showed anterior uveitis accompanied by herpes zoster ophthalmicus or zoster sine herpete. Herpetic iritis was also made when genomic DNA of herpes simplex or herpes zoster was detected in aqueous humor by PCR. However, when genomic DNA of cytomegalovirus was detected in patients with anterior uveitis, it was diagnosed as cytomegalovirus iritis. All systemic diseases were diagnosed according to current diagnostic criteria and the patients were also examined by the respective specialists.

RESULTS

A total number of 1338 patients (2012 eyes), consisting of 526 men (39.3%) and 812 women (60.7%) were met the inclusion criteria and analyzed in the study. The age at initial presentation ranged between 4 years and 90 years (mean ± standard deviation: 50.5±17.7). The

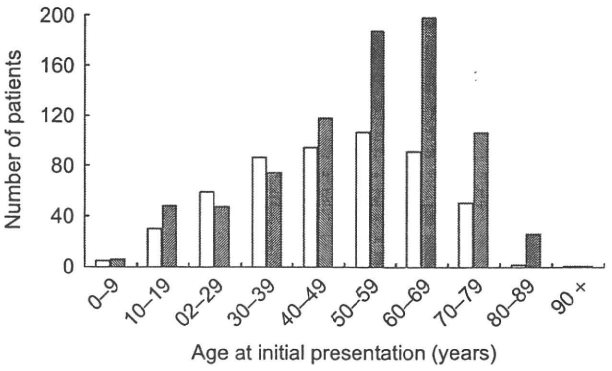

FIGURE 1 Distribution of age at initial presentation and gender of all uveitis patients. Men (open bars), women (hatched bars).

peak age of initial presentation was the fifth and sixth decades consisting of 44% of all our patients (Figure 1). During the follow-up period, 664 patients (49.6%) were unilateral involvement and 674 patients had bilateral involvement.

Etiological diagnosis

The etiological diagnosis of our patients is summarized in Table 1. The most common entity of uveitis was HTLV-1 uveitis (17.1%), followed by VKH disease (9.9%), sarcoidosis (7.2%), ocular toxoplasmosis (7.1%), Behçet's disease (4.3%), HLA-B27$^+$ AAU (1.9%), ocular toxocariasis (1.8%), Posner–Schlossman syndrome (1.3%), herpetic iritis (1.3%), and many others. Other etiological diagnoses are listed in Table 2. Infectious uveitis accounted for 31.2% of the entire study population. Of the 1338 patients, 550 patients (41.1%) were classified as etiology unknown or idiopathic uveitis despite extensive clinical and laboratory evaluations.

As for the gender and laterality in each clinical etiology, HTLV-1 uveitis and sarcoidosis were predominant in women whereas Behçet's disease and HLA-B27$^+$ AAU were predominant in men.

TABLE 1 Etiology, gender and laterality of all uveitis patients

Disease	Number of patients	(%)	Gender men/women	Laterality[a] unilateral/bilateral
HTLV-1 uveitis	229	(17.1)	65/164	141/88
VKH disease	133	(9.9)	66/67	0/133
Sarcoidosis	96	(7.2)	22/74	10/86
Ocular toxoplasmosis	95	(7.1)	43/52	82/13
Behçet's disease	58	(4.3)	41/17	12/46
HLA-B27$^+$ AAU	25	(1.9)	18/7	17/8
Ocular toxocariasis	24	(1.8)	12/12	22/2
Posner-Schlossman synd.	17	(1.3)	10/7	17/0
Herpetic iritis	17	(1.3)	4/13	17/0
Bacterial endophthalmitis	13	(1.0)	7/6	13/0
Diabetic iritis	13	(1.0)	7/6	7/6
Sclerouveitis	11	(0.8)	3/8	7/4
Acute retinal necrosis	9	(0.7)	4/5	7/2
IBD-associated uveitis	6	(0.4)	2/4	4/2
Ocular tuberculosis	5	(0.4)	0/5	0/5
Others	37	(2.8)	18/19	19/18
Unclassified uveitis	550	(41.1)	204/346	289/261
Total	1338	(100)	526/812	664/674

Abbreviations: HTLV-1 = human T-lymphotropic virus type 1; VKH = Vogt-Koyanagi-Harada; AAU = acute anterior uveitis; HLA = human leukocyte antigen; synd. = syndrome; IBD = inflammatory bowel disease.
[a]Laterality throughout clinical course.

As for the laterality, bilateral involvement was recorded in all patients with VKH disease, in 90% of sarcoidosis, and in nearly 80% of Behçet's disease. On the other hand, unilateral involvement was seen in all patients with Posner–Schlossman syndrome, herpetic iritis, and bacterial endophthalmitis, in 92% of ocular toxocariasis, in 86% of ocular toxoplasmosis, in 78% of acute retinal necrosis, and nearly 70% of HLA-B27$^+$ AAU.

The common clinical entities by age are summarized in Table 3. In childhood under 16 years old, ocular toxoplasmosis was the most common etiology followed by ocular toxocariasis. In this age group, unclassified uveitis was the most common among all age groups. Young adult (16–39 years) and middle aged (40–59 years) groups had very similar entities of uveitis: HTLV-1 uveitis, VKH disease, ocular toxoplasmosis and Behçet's disease were common diseases, and unclassified uveitis was only 36% of uveitis patients of these age groups. On the other hand, in senior age group (60 years or older), sarcoidosis and herpetic iritis became much more common than in other age groups.

The chronological changes of new patients of five major uveitis entities at our hospital are summarized in Figure 2. The new patients of HTLV-1 uveitis clearly decreased with time. However, VKH disease, sarcoidosis, ocular toxoplasmosis and Behçet's disease do not have changed much in the last 15 years.

TABLE 2 Etiological diagnosis of others

Disease	Number of patients	Gender men/women	Laterality[a] unilateral/bilateral
Sympathetic ophthalmia	4	3/1	3/1
MPPE	4	3/1	0/4
Leprosy	4	2/2	3/1
Fungal endophthalmitis	3	3/0	2/1
Syphilis	3	0/3	0/3
Uveal effusion	3	0/3	0/3
Cytomegalovirus iritis	2	2/0	2/0
Geographic chroiditis	2	2/0	1/1
Fuchs' heterochromic iridocyclitis	2	1/1	2/0
Intraocular lymphoma	2	0/2	0/2
Neuroretinitis	2	0/2	0/2
Masquerade syndrome	1	1/0	1/0
Psoriasis vulgaris	1	1/0	1/0
Cat scratch disease	1	0/1	1/0
Cytomegalovirus retinitis	1	0/1	1/0
Keratouveitis	1	0/1	1/0
Pars planitis	1	0/1	1/0

Abbreviation: MPPE = multifocal posterior pigment epitheliopathy.
[a]Laterality throughout clinical course.

TABLE 3 Most common etiological diagnosis of each generation

Age group	Disease	Number of patients	(%)
Childhood (under 16 years) (n = 56; 4.2%)	Ocular toxoplasmosis	9	(16.1)
	Ocular toxocariasis	3	(5.4)
	Herpetic iritis	2	(3.8)
	Others	7	(12.5)
	Unclassified uveitis	35	(62.5)
Young adult (16–39 years) (*n* = 298; 22.3%)	HTLV-1 uveitis	43	(14.4)
	VKH disease	40	(13.4)
	Ocular toxoplasmosis	38	(12.8)
	Behçet's disease	27	(9.1)
	Sarcoidosis	23	(7.7)
	Others	20	(6.7)
	Unclassified uveitis	107	(35.9)
Middle aged (40–59 years) (*n* = 506; 37.8%)	HTLV-1 uveitis	98	(19.4)
	VKH disease	58	(11.5)
	Ocular toxoplasmosis	36	(7.1)
	Behçet's disease	27	(5.3)
	Sarcoidosis	22	(4.3)
	Others	79	(15.6)
	Unclassified uveitis	186	(36.8)
Senior (60 years and up) (*n* = 478; 35.7%)	HTLV-1 uveitis	86	(18.0)
	Sarcoidosis	51	(10.7)
	VKH disease	35	(7.3)
	Herpetic iritis	13	(2.7)
	Ocular toxoplasmosis	12	(2.5)
	Others	59	(12.3)
	Unclassified uveitis	222	(46.4)

Abbreviation: HTLV-1 = human T-lymphotropic virus type 1; VKH = Vogt-Koyanagi-Harada disease.

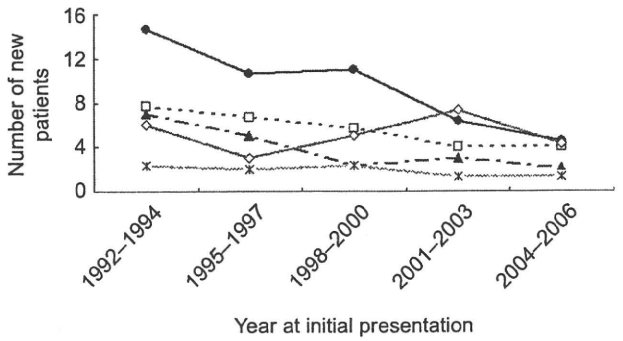

FIGURE 2 The chronological changes of new patients of five major diseases. HTLV-1 uveitis (closed circles), Vogt-Koyanagi-Harada disease (open squares), sarcoidosis (open diamonds), ocular toxoplasmosis (closed triangles) and Behçet's disease (asterisks).

Anatomical diagnosis

In the present patient population, pan-uveitis was the predominant anatomical type of uveitis (42.6%), followed by anterior uveitis (30.8%), intermediate uveitis (17.3%), and posterior uveitis (9.3%). Common entities in each group are shown in Table 4. HTLV-1 uveitis, HLA-B27$^+$ AAU, Posner–Schlossman syndrome, and herpetic iritis were most common entities in anterior uveitis. More than 60% of intermediate uveitis was HTLV-1 uveitis. On the other hand, one-third of patients in posterior uveitis were ocular toxoplasmosis. The majority of pan-uveitis consisted of systemic disorders, such as VKH disease, sarcoidosis, and Behçet's disease.

Ocular complications

Among 2012 eyes with uveitis, cataract was seen in 899 eyes (44.7%) and glaucoma in 642 eyes (31.9%) as ocular complications. Cataract surgery was undergone in 515 eyes (25.6%). Seventy eyes (3.5%) required

TABLE 4 Most common etiological diagnosis of each anatomical part of uveitis

Anatomical diagnosis	Disease	Number of patients	(%)
Anterior uveitis (*n* = 412; 30.8%)	HTLV-1 uveitis	30	(7.3)
	HLA-B27$^+$ acute anterior uveitis	25	(6.1)
	Posner-Schlossman syndrome	17	(4.1)
	Herpetic iritis	17	(4.1)
	Diabetic iritis	13	(3.2)
	Others	40	(9.7)
	Unclassified uveitis	270	(65.5)
Intermediate uveitis (*n* = 231; 17.3%)	HTLV-1 uveitis	151	(65.4)
	Sarcoidosis	12	(5.2)
	Ocular toxocariasis	4	(1.7)
	Others	8	(3.5)
	Unclassified uveitis	56	(24.2)
Posterior uveitis (*n* = 125; 9.3%)	Ocular toxoplasmosis	42	(33.6)
	Ocular toxocariasis	8	(6.4)
	Sarcoidosis	5	(4.0)
	Others	28	(22.4)
	Unclassified uveitis	42	(33.6)
Pan-uveitis (*n* = 570; 42.6%)	Vogt-Koyanagi-Harada disease	128	(22.5)
	Sarcoidosis	69	(12.1)
	Ocular toxoplasmosis	52	(9.1)
	Behçet's disease	47	(8.2)
	HTLV-1 uveitis	44	(7.7)
	Others	48	(8.4)
	Unclassified uveitis	182	(31.9)

Abbreviations: HTLV-1 = human T-lymphotropic virus type 1; HLA = human leukocyte antigen.

glaucoma surgery, consisting laser iridotomy (30 eyes), and trabeculectomy (40 eyes). Cystoid macular edema was seen in 248 eyes (12.3%), macular degeneration in 142 eyes (7.1%), and optic nerve atrophy in 79 eyes (3.9%).

Visual outcome

As for the best corrected visual acuity at the last presentation, 68.2% of eyes kept 0.8 or better, and 11.4% of eyes were worse than 0.1. Visual outcome was generally good in HLA-B27$^+$ AAU, Posner–Schlossman syndrome, VKH disease, HTLV-1 uveitis, and sarcoidosis, whereas acute retinal necrosis and Behçet's disease had much worse visual prognosis (Table 5).

Systemic complications

HTLV-1 infection can be a risk factor for HAM, ATL, and hyperthyroidism. Of the 229 cases of HTLV-1 uveitis, four patients (1.7%) developed HAM sometime after the onset of HTLV-1 uveitis. Nearly one quarter of women HTLV-1 patients (23.6%) had previous history of Graves' disease with hyperthyroidism. None of our HTLV-1 uveitis patients developed ATL in the observation periods.

Because HTLV-1 uveitis and ocular toxoplasmosis are very common in this region, we have performed serological tests in all uveitis patients after we started uveitis clinic in this hospital. The seroprevalence of HTLV-1 was 24.5% of all uveitis patients, and that of toxoplasmosis was 68.5% of the patients. The age distributions of HTLV-1 seroprevalence of all uveitis patients including HTLV-1 uveitis and of uveitis patients excluding HTLV-1 uveitis is shown in Figure 3. The HTLV-1 seroprevalence increased with the age in patients of both groups.

TABLE 5 Visual outcome of uveitis

	Final visual acuity			
Disease	0.8 ≤ eyes (%)		0.1 > eyes (%)	
HLA-B27$^+$ acute anterior uveitis	31/33	(93.9)	0/33	(0)
Posner-Schlossman syndrome	15/17	(88.3)	0/17	(0)
Vogt-Koyanagi-Harada disease	208/266	(78.2)	20/266	(7.5)
HTLV-1 uveitis	227/317	(71.6)	26/317	(8.2)
Sarcoidosis	125/182	(68.7)	8/182	(4.4)
Ocular toxoplasmosis	59/108	(54.6)	24/108	(22.2)
Herpetic iritis	9/17	(52.9)	2/17	(11.8)
Behçet's disease	51/104	(49.0)	37/104	(35.6)
Acute retinal necrosis	3/11	(27.3)	5/11	(45.5)
Unclassified uveitis	527/811	(70.5)	81/811	(10.)

Abbreviations: HLA = human leukocyte antigen; HTLV-1 = human T-lymphotropic virus type 1.

DISCUSSION

The present study showed epidemiological and clinical features of uveitis in southern Kyushu island of Japan, where HTLV-1 and toxoplasmosis are endemic. The major findings are: (1) HTLV-1 uveitis consisting of 17.1% of uveitis and ocular toxoplasmosis consisting of 7.1% are very common entities of uveitis despite the fact that these diseases are much less in other parts of Japan consisting of 1.1 % in a nation wide survey of uveitis in Japan[8]; (2) the incidence of HTLV-1 uveitis clearly decrease by time whereas other major entities such as VKH disease, sarcoidosis, ocular toxoplasmosis and Behçet's disease have not changed in the last 15 years; (3) each uveitis entities has unique features in its age distribution and gender predisposition and laterality; (4) acute retinal necrosis, Behçet's disease and ocular toxoplasmosis have poor visual prognosis whereas HLA-B27$^+$ AAU, Posner–Schlossman syndrome, VKH disease, HTLV-1 uveitis, and sarcoidosis have much better visual prognosis.

The extremely high proportion of HTLV-1 uveitis in the present study is due to a high prevalence of HTLV-1 in the southern Kyushu island.[14] The HTLV-1 seroprevalence in Miyakonojo was reported to be 11.5% in 1984.[22] Newer data are not available, but the seroprevalence in general population in Japan is known to be decreased after screening serological tests of HTLV-1 in blood donors started in 1987 in Japan because blood transfusion is one of the major routs of the viral transmission.[23] The HTLV-1 seroprevalence in uveitis patients excluding HTLV-1 uveitis in this study (7.1%) is considered to reflect the general population in this area. Conversely, the seroprevalence in all uveitis patients including HTLV-1 uveitis in this area (24.5%) was significantly higher, indicating that the viral infection is causatively related to uveitis. The

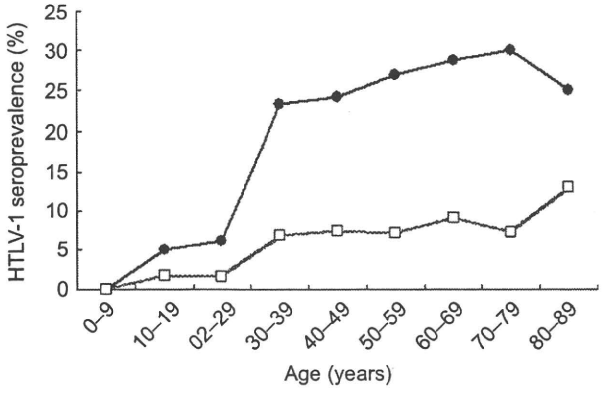

FIGURE 3 Age distribution of the seroprevalence of HTLV-1 in all uveitis patients (•) and uveitis patients excluded HTLV-1 uveitis (□).

decrease of HTLV-1 seroprevalence in general population also influences the chronological feature of the incidence of HTLV-1 uveitis; the present study for the first time demonstrated that the incidence of HTLV-1 uveitis has decreased in the last 15 years (Figure 2). Although ATL has not seen in any of our HTLV-1 uveitis patients, HAM has been found in some of the patients and Graves' disease has also been observed. We previously reported a subtype of HTLV-1 uveitis, HTLV-1 uveitis after Graves' disease.[24] The incidence of HTLV-1 uveitis after Graves' disease in the original report (25%) was very similar to that of the present study (23.6%).

The high frequency of ocular toxoplasmosis may be related to the life style and dietary habits in this region. The region is a farm country and most people living there take care of cows, chickens and horses. They also like to eat raw meat, particularly raw lever.

Sarcoidosis was 7.2% and forth most common entity of our uveitis patients. However, sarcoidosis is the most common disease consisting 13% of uveitis patients in a recent nation wide survey of uveitis in Japan.[8] The reason why sarcoidosis is relatively low in the current study is not known, but might be related to the style of our hospital. Our hospital is not a general hospital like university hospitals but an eye specialized hospital. Therefore, it is difficult to do complete systemic examinations for sarcoidosis, such as lung biopsy. The nation wide survey was carried out in 48 university hospitals in Japan and most patients with sarcoidosis were biopsy-proven by transbronchial lung biopsy.

Ocular tuberculosis and Fuchs' heterochromic iridocyclitis are very important clinical entities in some countries, but the proportion of these entities was very low in the present study, that is, ocular tuberculosis in 0.4% and Fuchs' heterochromic iridocyclitis in 0.15%. However, a nation wide survey of uveitis with over 3000 uveitis patients also reported that ocular tuberculosis was 0.7% and Fuchs' heterochromic iridocyclitis was 0.5%.[8] These data suggest that these diseases are not common diseases in Japan. Another possibility of the low prevalence of Fuchs' heterochromic iridocyclitis in Japan is that in brown-eyed patients like Japanese heterochromia is hard to identify and our diagnosis of this disease could be under-diagnosed.

As for the gender, most uveitis entities do not have gender differences though HTLV-1 uveitis, sarcoidosis, and herpetic iritis are more in women than in men, while Behçet's disease and HLA-B27$^+$ AAU are more in men than in women. HTLV-1 is known to be transmitted by infected lymphocytes in sperm and this may attributed to higher prevalence of the disease in women than in men. Despite the fact that many studies reported no gender differences in the prevalence of systemic Behçet's disease, the disease with ocular symptoms is much more in men than in women, and it is also true in this study. It is well known that sarcoidosis is common in 50–60 years old women and the trend is also seen in the present study. In fact, sarcoidosis is the second most common disease in patients older than 60 years old in this study. As for the relationship between age and uveitis entities, ocular toxoplasmosis and ocular toxocariasis are the major entities in childhood, and the proportion of unclassified uveitis (62.5%) was much higher than that of any other age groups. This is simply due to difficulties of extensive systemic examinations in children. In patients of adult age (16–59 years old), uveitis associated with VKH disease and Behçet's disease as well as two infectious diseases the pathogenic agents of which are highly endemic in this region (HTLV-1 uveitis and ocular toxoplasmosis) are the major entities. However, sarcoidosis and herpetic iritis become major entities in senior groups older than 60 years old.

As for the ocular complications and visual prognosis, cataract and glaucoma are the most common complications and many of the patients require surgical treatment for these ocular complications. However, visual prognosis is fairly good in VKH disease, sarcoidosis, and specific anterior uveitis entities like HLA-B27$^+$ AAU and Posner–Schlossman syndrome. Like in many other studies, Behçet's disease and acute retinal necrosis had very poor visual prognosis.

In conclusion, a clinical survey of uveitis in HTLV-1 endemic area in Japan disclosed that HTLV-1 uveitis is the most common uveitis entity though the incidence of the disease has decreased in the last 15 years with a decrease of HTLV-1 carriers in general population. Unlike in other parts of Japan, ocular toxoplasmosis is also very common in this region and this might be related to the life style of people living this region.

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SHORT REPORT

CXCR7 is inducible by HTLV-1 Tax and promotes growth and survival of HTLV-1-infected T cells

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Human T-lymphotropic virus type 1 (HTLV-1), the etiological agent of adult T-cell leukemia (ATL), encodes the potent transcriptional activator Tax, which is required for HTLV-1-induced immortalization of T cells. CXCR7 is an atypical chemokine receptor frequently expressed by tumor cells and known to promote cell growth and survival. We found that HTLV-1-immortalized T cells expressing Tax consistently expressed CXCR7. Induction of Tax in JPX-9 upregulated CXCR7. Wild-type Tax efficiently activated the CXCR7 promoter via a proximal NF-κB site, while a mutant Tax selectively defective in NF-κB activation did not. CCX754, a synthetic CXCR7 antagonist, inhibited cell growth and increased apoptosis of HTLV-1-immortalized T cells. Knockdown of CXCR7 by small interfering RNA also reduced cell growth. Stable expression of CXCR7 in a CXCR7-negative ATL cell line promoted cell growth and survival. Taken together, CXCR7 is inducible by Tax and may play an important role in HTLV-1-induced immortalization of T cells by promoting growth and survival of HTLV-1-infected T cells.

Key words: HTLV-1; Tax; CXCR7; NF-kB; cell growth; cell survival

Human T-lymphotropic virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL), a highly aggressive malignancy of mature $CD4^+CD25^+$ T cells.[1] *In vitro*, HTLV-1 is capable of immortalizing normal $CD4^+$ T cells.[1,2] HTLV-1 encodes the potent transcriptional activator Tax that induces the expression of viral genes and also the expression of plethora of cellular genes with the net effect of cell proliferation and activation.[3,4] Tax does not directly interact with DNA but activates various cellular transcription factors such as cAMP responsive element binding protein (CREB), nuclear factor-κB (NF-κB) and serum responsive factor (SRF).[3,4] However, ATL develops after a long latency period, usually several decades, during which tumor progression takes place through the accumulation of multiple genetic and epigenetic changes.[4] Thus, circulating ATL cells usually do not express Tax, which is also a good target for host immune surveillance,[5] and are thus considered to be mostly independent of the growth-promoting effects of Tax. Nevertheless, freshly isolated ATL cells readily express Tax upon brief *in vitro* culture.[6] Thus, Tax may still play a significant role in the pathophysiology of ATL.

Chemokines are a group of small secreted proteins that induce directed migration of hematopoietic cells and even other types of cells through interactions with chemokine receptors, a group of heptahelical G-protein-coupled receptors (GPCRs).[7] Chemokines are also known to play important roles in cancer biology.[8] For example, SDF-1/CXCL12 is known to promote metastasis, survival and proliferation of tumor cells via CXCR4. Recently, Balabanian *et al.* have reported that RDC1, an orphan GPCR closely related to the CXC chemokine receptors, is the second high-affinity receptor for CXCL12 and mediates CXCL12-induced cell migration with an efficiency similar to that of CXCR4.[9] Thus, the authors proposed RDC1 to be termed CXCR7. Subsequently, Burns *et al.* have demonstrated that not only CXCL12 but also another CXC chemokine I-TAC/CXCL11, a ligand of CXCR3, binds CXCR7, but neither CXCL12 nor CXCL11 induces calcium flux or cell migration via CXCR7.[10] Importantly, however, Burns *et al.* have also shown that CXCR7 is frequently expressed by various tumor cell lines and has a strong pro-survival effect especially under suboptimal culture conditions.[10] Furthermore, the authors have shown that, although CXCR7 mRNA is expressed in many tissues, CXCR7 protein is negative in most normal adult tissues.[10] Thus, the protein expression of CXCR7 in normal cells may be tightly regulated by a posttranscriptional mechanism(s). The expression of CXCR7 by tumor cells and its growth-promoting activity have also been demonstrated by other authors.[11–13] Furthermore, endothelial cells activated with proinflammatory cytokines as well as neovasculature associated with tumors express CXCR7.[10,11] Moreover, CXCR7 is a gene strongly upregulated in human dermal microvascular endothelial cells (DMVECs) transformed with Kaposi sarcoma-associated herpes virus (KSHV) and plays a role in focus-formation of KSHV-infected DMVECs.[14–16] CXCR7 is also a target gene of Epstein-Barr virus (EBV) transcriptional activator EBNA2 and is involved in proliferation and survival of EBV-immortalized B cells.[17] Taken together, CXCR7 is an atypical chemokine receptor not signaling in the classical manner and frequently expressed by tumor cells to contribute to their growth and survival. CXCR7 is also a common target gene of human oncogenic viruses such as KSHV and EBV.

Among human peripheral blood leukocytes, CXCR7 has been shown to be expressed on B cells, monocytes and dendritic cells.[18] On the other hand, CXCR7 is mostly negative on T cells.[10,18] In the present study, we examined the effect of HTLV-1 on CXCR7 expression in T cells. Here, we report that CXCR7 is inducible by HTLV-1 Tax and promotes growth and survival of HTLV-1-immortalized T cells.

Material and methods

Cells

HTLV-1-immortalized human T-cell lines (C8166, C91/PL, ILT8M2, MT-2, MT-4 and TCL-Kan), HTLV-1-negative human T-cell lines (CEM, MOLT-4 and Jurkat) and ATL cell lines (H582, HUT102, MT-1, KK1, KOB, SO4 and ST1) were described previously.[19,20] JPX-9 is a subline of Jurkat carrying HTLV-1 Tax under the control of the metallothionein promoter.[21] All cell lines were maintained in RPMI-1640 supplemented with

Abbreviations: ATL, adult T-cell leukemia; EBV, Epstein-Barr virus; FCS, fetal calf serum; GPCR, G-protein-coupled receptor; HTLV-1, human T-lymphotropic virus type 1; KSHV, Kaposi sarcoma-associated herpes virus; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-polymerase chain reaction.

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10% heat-inactivated fetal calf serum (FCS) and antibiotics unless otherwise described. Four ATL cell lines (KK1, KOB, SO4 and ST1) were IL-2-dependent and maintained in the presence of recombinant IL-2 at 100 U/ml.[20] Heparinized venous blood was obtained from ATL patients and healthy adult donors upon written informed consents. Peripheral blood mononuclear cells (PBMCs) were isolated by the standard density gradient centrifugation. The percentages of leukemic cells in PBMCs from ATL cells ranged from 50 to 94%. This work was approved by the ethical committees of Kinki University School of Medicine and Nagasaki University Graduate School of Medicine.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously.[22] A panel of cDNAs for normal human blood fractions was purchased from Takara Bio (Shiga, Japan; Human Blood Fractions MTC Panel, Ref. 636750). The primers used were: +5′-CTACATCTTGAACCTGGC CATTG-3′ and −5′-AGTAGGTGTCAGGCAGAGACACG-3′ for CXCR7; +5′-ATCTTCCTGCCCACCATCTACTCCATCATC-3′ and −5′-ATCCAGACGCCAACATAGACCACCTTTTCA-3′ for CXCR4; +5′-AAAAAGCGGGTCACTCTATATGCTC-3′ and −5′-CCACTGCTACCTGGTACTCTGTTGT-3′ for CD25; +5′-CGATGCCTAAATCCCAAATCGAAGCA-3′ and −5′-AATTG CTGCACTCCTTTGGGCAGTGG-3′ for CXCL11; +5′-CCCTC TGTGAGATCCGTCTTTGGCCT-3′ and −5′-TCTGATTGGAA CCTGAACCCCTGCTG-3′ for CXCL12; +5′-CCGGCGCTGCT CTCATCCCGGT-3′ and −5′-GGCCGAACATAGTCCCCCAGA G-3′ for Tax; +5′-ATCGGCTCAGCTCTACAGTTCCT-3′ and −5′-ATTCGCTTGTAGGGAACATTGGT-3′ for JPX-9 Tax; +5′-GCCAAGGTCATCCATGACAACTTTGG-3′ and −5′-GCC TGCTTCACCACCTTCTTGATGTC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification conditions were denaturation for 30 sec (5 min for the first cycle) at 94°C, annealing for 30 sec at 60°C and extension for 30 sec (5 min for the last cycle) at 72°C with 34 cycles for CXCR7, CXCR4, CXCL11, CXCL12 and CD25, 32 cycles for Tax and 26 cycles for GAPDH. Amplification products were electrophoretically separated on 2% agarose gel and stained with ethidium bromide.

Immunofluorescence staining

Monoclonal anti-human CXCR7 (clone 358426) and monoclonal anti-human CXCR4 (clone 44716) were purchased from R&D Systems (Minneapolis, MN). Control mouse IgG_{2a} and IgG_{2b} were purchased from DAKO Japan (Kyoto, Japan). Allophycocyanin (APC)-labeled goat anti-mouse IgG $F(ab')_2$ was purchased from Southern Biotechnology Associates (Birmingham, AL). FITC-labeled sheep anti-mouse IgG $F(ab')_2$ was purchased from Sigma (St. Louis, MO). For flow cytometric analysis, cells were suspended in cold PBS containing 2% FCS and 0.05% sodium azide (staining buffer) and treated with normal human AB serum for 20 min at 4°C to block Fc receptors. Cells from cell lines were stained with primary antibodies and APC-labeled secondary antibody in succession. PBMCs were stained with primary antibodies and FITC-labeled secondary antibodies in succession. PBMCs were further stained with a mixture of APC-labeled anti-CD4 (13B8.2; Beckman Coulter, Fullerton, CA) and PE-labeled anti-CD8 (DK25; DAKO Japan), or PerCP Cy5.5-labeled anti-CD19 (SJ25C1; BD Biosciences, Mountain View, CA), or a mixture of APC-labeled anti-HLA-DR (L243; BD Biosciences) and PE-labeled anti-CD14 (61D3; eBioscience, San Diego, CA), or a mixture of PE-labeled anti-CD25 (ACT-1; DAKO, Japan) and APC-labeled anti-CD4 (13B8.2; Beckman Coulter). Stained cells were immediately analyzed on FACSCalibur (BD Biosciences). Dead cells were excluded by staining with propidium iodide. For immunocytological staining, cells were adhered to poly-L-lysine-precoated Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY), fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked with 5% BSA and stained overnight with primary antibody. After washing, cells were stained with FITC-labeled secondary antibody, counterstained with TO-PRO-3 (Invitrogen, Carlsbad, CA), mounted with fluorescent mounting medium (DAKO Japan) and observed under a confocal microscopy (LSM 510 META, Carl Zeiss, Jena, Germany).

Promoter assay

By the method 5′-rapid amplification of cDNA ends, we mapped the major transcriptional start site (+1) of the human *CXCR7* gene at 10,641 bases upstream from the translation start codon (data not shown). We then amplified the promoter region from −1144 to −36 bp by PCR and cloned it into pGL3-basic luciferase reporter plasmid (Promega, Madison, WI). The serial 5′-truncation and site-directed mutagenesis were also performed using PCR amplification. The mutated sequences were as follows: CXCR7 ΔNFκB1, from G*GG*GTTTCCC to G*CC*GTTTCCC; CXCR7 ΔNFκB2, from GGAAAA*CCC*A to GGAAAA*CGG*A. The expression vectors for Tax (pHβPr.1-Tax MT2), Tax M22 (pHβPr.1-Tax M22) and control vector (pHβPr.1) were kindly provided by Dr. Masahiro Fujii (Niigata University).[23] Jurkat cells were co-transfected with 3 μg of a reporter plasmid, 1 μg of Tax, Tax M22, or control vector and 0.5 μg of pSV-β-galactosidase using DMRIE-C transfection reagent (Invitrogen). After 48 hr, cells were lysed and luciferase assays were performed using a Luciferase Assay System Kit (Promega). Luciferase activity was normalized by β-galactosidase activity that served as an internal control for transfection efficiency.

Cell growth and apoptosis assays

CCX754, a low-molecular weight synthetic CXCR7 antagonist,[10] was kindly provided by ChemoCentryx (Mountain View, CA). AMD3100 was purchased from Sigma. For growth assays, cells were suspended in RPMI-1640 supplemented with 1% FCS and cultured in the absence or presence of various concentrations of inhibitors for 72 hr using a 96-well microplate (1×10^4 cells in 200 μl/well). The level of cell growth was measured using cell counting kit-8 (WST-8; Wako Pure Chemical, Osaka, Japan). Mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provided an indication of the level of cell growth, and the absorbance at 450 nm was measured using an automated microplate reader.

For apoptosis assays, cells were suspended in RPMI-1640 supplemented with 1% FCS and cultured in the absence or presence of 5 μM of CCX754 using a 12-well plate (1×10^5 cells in 1 ml/well). After 16 hr, cells were suspended in Annexin V binding buffer (BD Biosciences), incubated with Annexin V-FITC (Wako Pure Chemical) and propidium iodide for 10 min at room temperature in the dark, and immediately analyzed with FACSCalibur.

Small interfering RNA

CXCR7 small interfering RNA (siRNA; catalog no. SI02660644) and control siRNA (catalog no. 1027281) were obtained from Qiagen (Hilden, Germany). Transfection of siRNA was performed using Amaxa Nucleofector (Amaxa, Cologne, Germany). Briefly, cells (1×10^6 cells) were suspended in 100 μl of Nucleofector Solution (Cell Line Nucleofector Kit T: VCA-1002; Amaxa) with 3 μg of siRNA. After transfection using the O-17 program, cells were resuspended in RPMI-1640 containing 1% FCS, seeded in a 96-well plate (1×10^4 cells in 200 μl/well) and cultured for 48 hr. The number of viable cells was determined with FACSCalibur by gating out cells stained with propidium iodide.

Stable transfection of CXCR7

The coding region of human CXCR7 was amplified by PCR and subcloned into pIRES2-EGFP-EF1α, which was generated by inserting the EF1α promoter into pIRES2-EGFP (Takara Bio). IL-2-dependent ATL cell line ST1[20] was stably transfected with pIRES2-EGFP-EF1α-CXCR7 or vector only. EGFP-positive cells were sorted by FACSVantage (BD Biosciences), and maintained

in medium containing geneticin. Cells were resuspended in RPMI-1640 containing 10 U/ml of recombinant IL-2 and 10% or 1% FCS, and cultured in a 96-well plate (5×10^3 cells in 200 μl/well). The number of viable cells was determined every 24 hr with FACSCalibur by gating out cells stained with propidium iodide.

Statistical analysis

Statistical significance was determined using Student's *t*-test. We considered $p < 0.05$ as statistically significant.

Results and discussion

We first examined CXCR7 expression in normal PBMCs. In contrast to CXCR4 mRNA, CXCR7 mRNA was only weakly expressed in the various fractions of normal PBMCs (Fig. 1*a*). Furthermore, activation of cells consistently downregulated CXCR7 mRNA expression. Moreover, surface CXCR7 was only detected on HLA-DR$^+$CD14$^+$ monocytes (Fig. 1*b*, upper), while all the cell fractions expressed surface CXCR4 (Fig. 1*b*, lower). Thus, normal human peripheral blood T cells were essentially negative for surface CXCR7.[18]

To examine the effect of HTLV-1 on the expression of CXCR7, we first used a panel of human T cell lines. While control T cell lines not associated with HTLV-1 hardly expressed CXCR7 mRNA, HTLV-1-immortalized human T cell lines expressing Tax mRNA strongly expressed CXCR7 mRNA (Fig. 1*c*). HTLV-1-immortalized T cell lines were also positive for surface CXCR7 (Fig. 1*d*, upper). On the other hand, all human T cell lines highly expressed CXCR4 mRNA (Fig. 1*c*) and surface CXCR4 (Fig. 1*d*, lower). It was noted that, although the signals of CXCR7 mRNA were as strong as those of CXCR4 mRNA in all HTLV-1-immortalized T cell lines (Fig. 1*c*), the surface expression of CXCR7 was generally much lower than that of CXCR4 (Fig. 1*d*). In this context, the possibility that surface CXCR7 was down-modulated by the autocrine production of any of its binding ligands, CXCL11 and/or CXCL12, was not likely, because most of these cell lines hardly expressed their mRNAs (data not shown). Therefore, compared to the mRNA expression, the protein expression and/or the surface translocation of CXCR7 might be less efficient.

We next examined the expression of CXCR7 and CXCR4 in ATL-derived cell lines. Although all ATL cell lines expressed CXCR4 mRNA at high levels, only 3 ATL cell lines (H582, MT-1 and KK1) clearly expressed CXCR7 mRNA (Fig. 2*a*). The expression of Tax mRNA was also highly variable among these ATL cell lines (Fig. 2*a*). Thus, in contrast to the case of HTLV-1-immortalized T cell lines, the expression of CXCR7 mRNA and that of Tax mRNA were not well correlated in the ATL-derived cell lines. The reason for this discrepancy appeared to be DNA-methylation, since after 5-azacytidine treatment, HUT102 and KOB became positive for CXCR7 mRNA in parallel with Tax mRNA (data not shown). We also examined the surface expression of CXCR7 and CXCR4 in these ATL cell lines. Only H582 showed surface expression of CXCR7 (Fig. 2*b*, upper). Similarly, only 3 ATL cell lines (H582, HUT102 and KOB) clearly showed surface expression of CXCR4 (Fig. 2*b*, lower). Again, the autocrine production of any of their ligands, CXCL11 and/or CXCL12, was not the likely cause of down-modulation of surface CXCR7 or CXCR4 (data not shown). We therefore analyzed intracellular CXCR7 and CXCR4 proteins by immunofluorescence staining. As shown in Figure 2*c*, both CXCR7 and CXCR4 proteins were clearly detected in the cytoplasm of KK1, albeit less strongly than in those of C91/PL and H582. Immunoblot analysis also confirmed the presence of CXCR7 protein in the cell extract of KK1 (data not shown). Similarly, SO4 and ST1 were clearly positive for CXCR4 protein in the cytoplasm (data not shown). Thus, the translocation of CXCR7 and/or CXCR4 to the cell surface may be frequently blocked in the ATL-derived cell lines.

Despite some discrepancies with the ATL cell lines, the striking parallel expression of CXCR7 mRNA and Tax mRNA in the

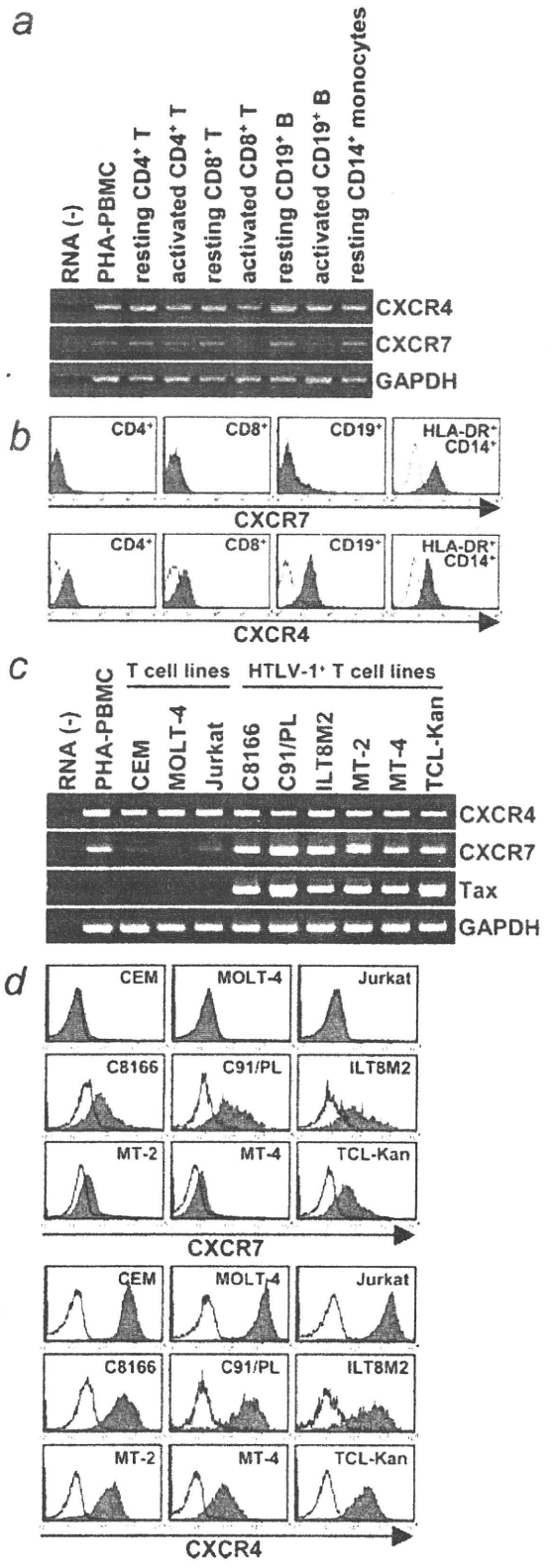

FIGURE 1 – Strong expression of CXCR7 in HTLV-1-immortalized human T cell lines. (*a*) RT-PCR analysis of the various fractions of normal PBMCs. (*b*) Flow cytometric analysis of the various fractions of normal PBMCs. (*c*) RT-PCR analysis of human T cell lines. (*d*) Flow cytometric analysis of human T cell lines. Representative results from at least 3 separate experiments are shown.

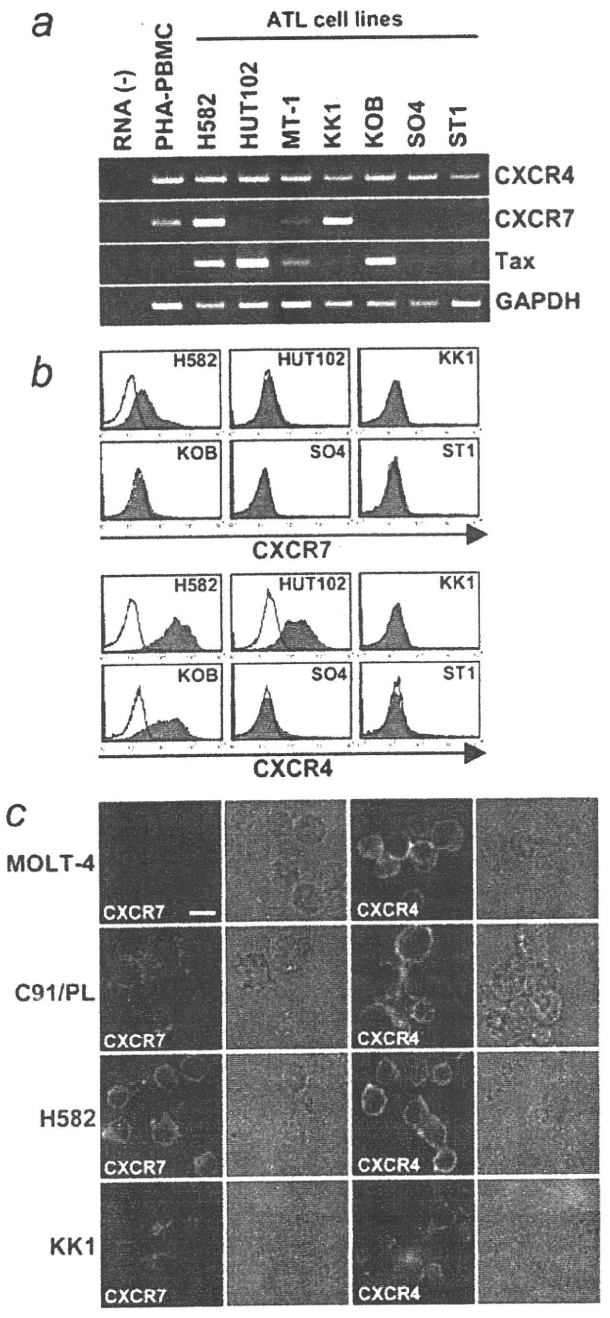

FIGURE 2 – Expression of CXCR7 in ATL cell lines. (*a*) RT-PCR analysis. (*b*) Flow cytometric analysis. (*c*) Immunofluorescence staining. The scale bar indicates 10 μm. Representative results from at least 3 separate experiments are shown.

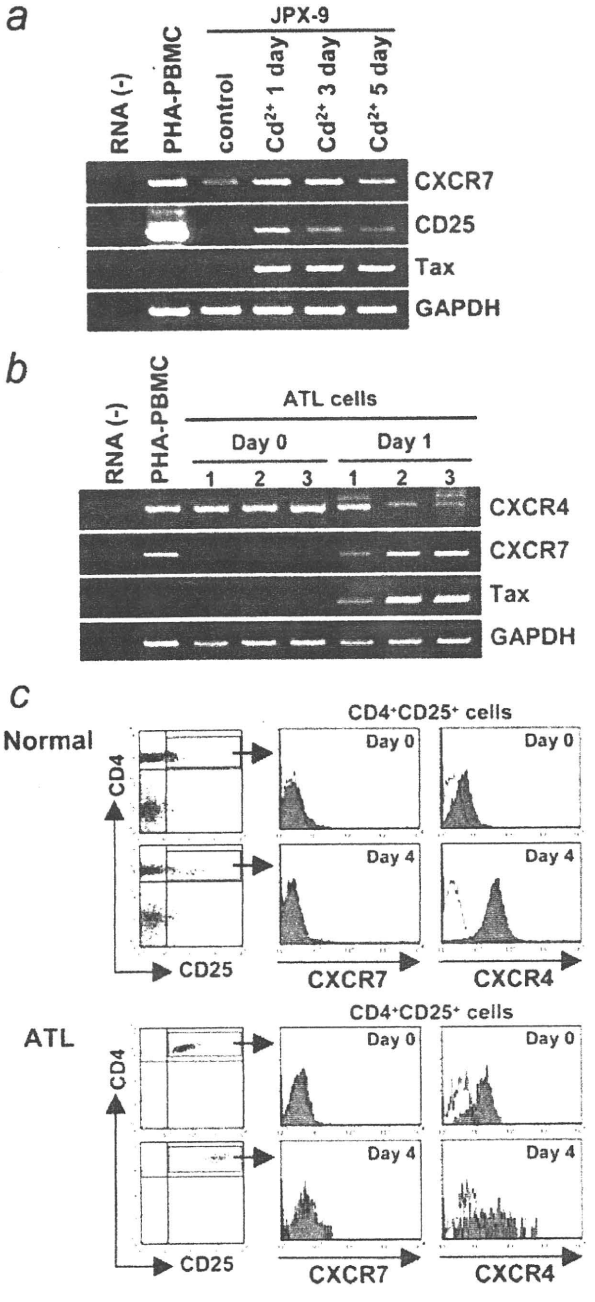

FIGURE 3 – Induction of CXCR7 in JPX-9 and primary ATL cells. (*a*) Induction of Tax mRNA and CXCR7 mRNA in JPX-9 by cadmium treatment. (*b*) Induction of Tax mRNA and CXCR7 mRNA in cultured primary ATL cells. (*c*) Flow cytometric analysis. Surface expression of CXCR7 and CXCR4 was analyzed in the $CD4^+CD25^+$ cell fraction from normal donors ($n = 3$) and ATL patients ($n = 3$). Representative results from at least 3 separate experiments are shown.

HTLV-1-immortalized T cell lines (Fig. 1*c*) strongly suggested that CXCR7 expression was inducible by Tax. To test this possibility, we first used JPX-9, a subline of Jurkat carrying the coding sequence of Tax under the control of the metallothionein promoter.[21] Upon treatment with cadmium, JPX-9 strongly expressed Tax mRNA and that of CD25, the known target gene of Tax,[3] and also upregulated CXCR7 mRNA (Fig. 3*a*). We next examined the expression of CXCR7 mRNA in primary ATL cells (Fig. 3*b*). Although circulating ATL cells freshly isolated from patients were negative for Tax mRNA, they readily expressed Tax mRNA after 1-day culture. CXCR7 mRNA was also expressed in primary ATL cells after 1-day culture in parallel with Tax mRNA. We were, however, unable to clearly detect the surface expression of CXCR7 on primary ATL cells (analyzed as $CD4^+CD25^+$ cells in PBMCs from ATL patients) even after 4 days of culture (Fig. 3*c*, lower). This might be due to inefficient translation of CXCR7 protein and/or low surface CXCR7 translocation in primary ATL cells. Taken together, these results further support the view that CXCR7 is inducible by Tax.

To directly demonstrate the transcriptional activation of the CXCR7 promoter by Tax, we co-transfected Jurkat cells with a Tax expression vector and a CXCR7 promoter-luciferase reporter

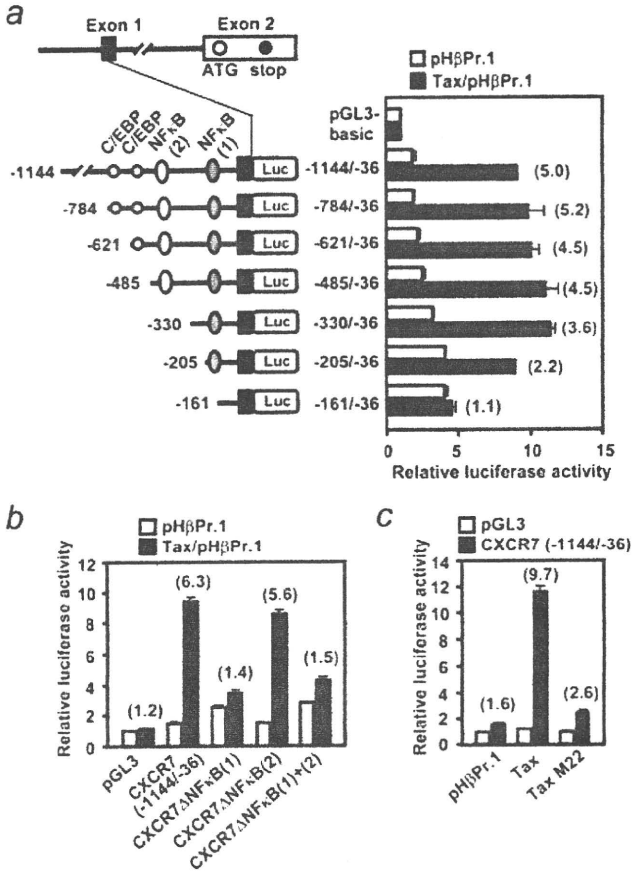

FIGURE 4 – CXCR7 promoter analysis by luciferase reporter assay. (*a*) Analysis using a serial 5′-truncation of the CXCR7 promoter. (*b*) Site-directed mutagenesis of the NF-κB sites. (*c*) Inability of Tax M22 to activate the CXCR7 promoter. Data show the means ± SD. Representative results from at least 3 separate experiments are shown.

plasmid. As shown in Figure 4*a* (right), Tax efficiently induced the luciferase reporter gene under the control of the CXCR7 promoter region from −1144 to −36 bp. Furthermore, a series of reporter plasmids with successively 5′-truncated promoter regions demonstrated that the promoter region from −205 to −161 bp contained the major Tax-responsive element. As indicated in Figure 4*a* (left), this region contained a proximal NF-κB site. To determine the importance of this and another NF-κB sites present in the CXCR7 promoter region from −1144 to −36 bp (Fig. 4*a*), we generated the reporter constructs with mutations in the proximal (#1) and/or distal (#2) NF-κB sites. Co-transfection of the reporter plasmids and the Tax-expression vector clearly demonstrated that the proximal NF-κB site was mostly responsible for the activation of the CXCR7 promoter by Tax (Fig. 4*b*). Furthermore, Tax M22, a mutant Tax selectively defective in the activation of NF-κB,[24] mostly failed to activate the CXCR7 promoter (−1144/−36; Fig. 4*c*). Collectively, Tax induces the CXCR7 gene transcription through the activation of NF-κB and mainly involving the proximal NF-κB site in the CXCR7 promoter.

Previously, Burns *et al.* have used a low molecular weight synthetic CXCR7 antagonist CCX754 to demonstrate the growth-promoting and pro-survival effects of CXCR7 on tumor cells under suboptimal low serum conditions.[10] To determine the possible role of CXCR7 on the cell growth and survival of HTLV-1-immortalized T cells, we also examined the effect of CCX754 on 2 HTLV-1-immortalized T cell lines (C91/PL and TCL-Kan) and 2 control T cell lines (CEM and MOLT-4) cultured in 1% FCS medium. As shown in Figure 5*a*, CCX754 dose-dependently suppressed the

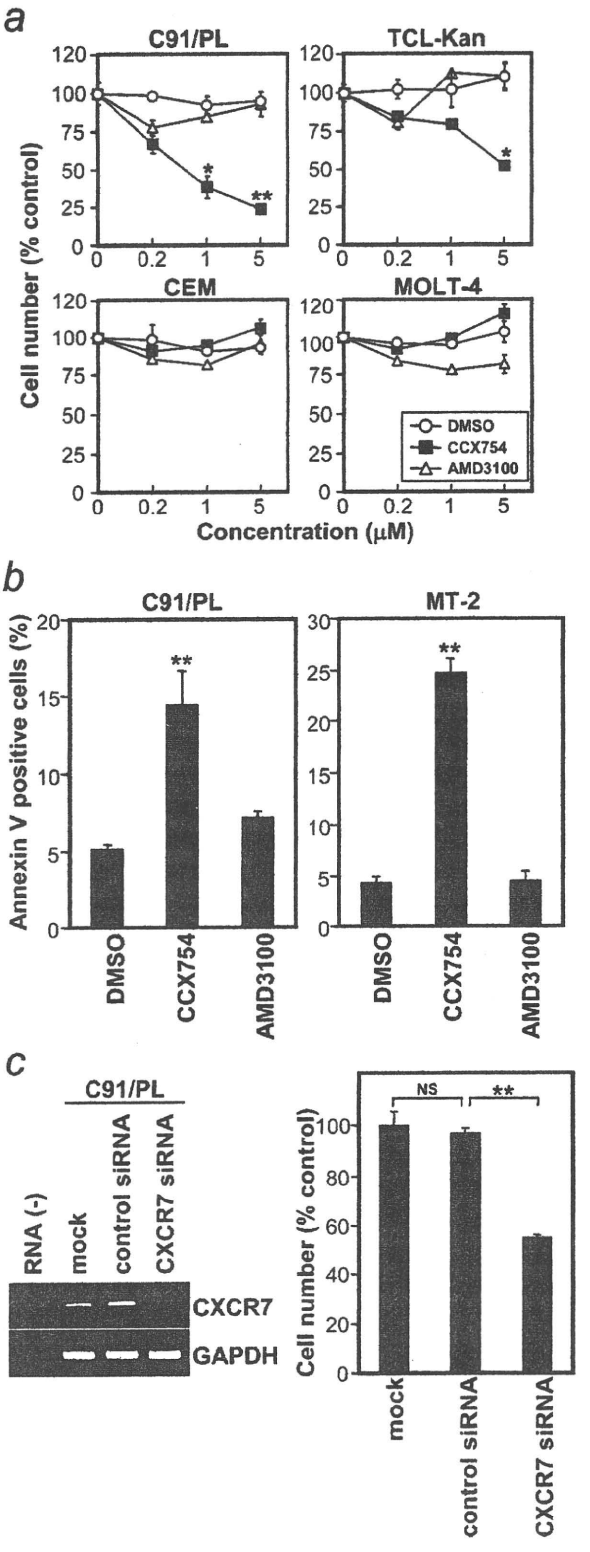

FIGURE 5 – Role of CXCR7 in cell growth and survival of HTLV-1-immortalized T cells. (*a*) Effect of CCX754 on cell growth. (*b*) Effect of CCX754 on apoptosis. (*c*) Effect of CXCR7 siRNA on cell growth. Data show the means ± SD. Representative results from at least 3 separate experiments are shown. $*p < 0.05$; $**p < 0.01$; NS, not significant.

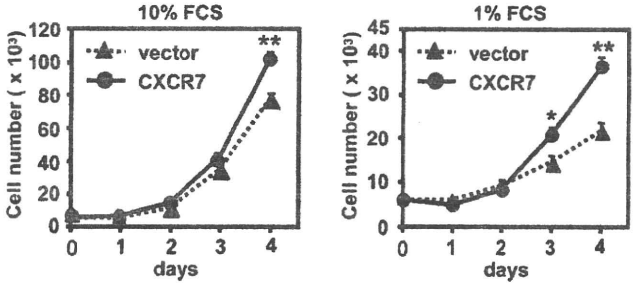

FIGURE 6 – Effect of stable CXCR7 transfection on cell growth. Stable transfectants of CXCR7 or vector only were generated using IL-2-dependent ATL cell line ST1, which was negative for CXCR7 expression. Cell growth was examined in RPMI-1640 supplemented with 10 U/ml of IL-2 and 10% FCS (left) or 1% FCS (right). Data show the means ± SD. Representative results from at least 3 separate experiments are shown. $*p < 0.01$; $**p < 0.001$.

cell growth of CXCR7-expressing HTLV-1-immortalized T cell lines but not that of control T cell lines. AMD3100, a CXCR4 antagonist,[25] had no such growth-inhibitory effect. Furthermore, the surface staining with Annexin V showed significant increases in apoptotic cells in CCX754-treated C91/PL cells and MT-2 cells (Fig. 5*b*). We also examined the effect of CXCR7 knockdown by siRNA on C91/PL cells. As shown in Figure 5*c*, CXCR7 siRNA but not control siRNA effectively reduced CXCR7 mRNA (left) and also significantly suppressed the growth of C91/PL cells cultured in 1% FCS medium (right).

To further prove the growth-promoting and pro-survival effects of CXCR7, we next generated stable CXCR7 transfectants using an IL-2-dependent ATL cell line ST1,[20] which was CXCR7-negative (Fig. 2). As shown in Figure 6, we observed a significant enhancement in cell growth of CXCR7-expressing ST1 cells both under 10% and 1% FCS culture conditions. In the former conditions, the effect of CXCR7 might be mainly on cell proliferation, while in the latter conditions where a large fraction of control vector-transfected cells went to apoptosis, the effect of CXCR7 might be more on cell survival. Therefore, CXCR7 might promote both growth and survival of HTLV-1-infected T cells.

HTLV-1 Tax is known to induce the expression of various cellular genes involved in cell proliferation and activation through interactions with the cellular transcription factors such as NF-κB, CREB and SRF.[3,4] Concerning the chemokine system, we have shown that Tax potently induces the expression of CCL22, a CCR4 ligand, which attracts $CCR4^+CD4^+$ T cells to HTLV-1-infected T cells and promotes selective transmission of HTLV-1 to $CCR4^+CD4^+$ T cells.[26] We have also shown that CCR9, the chemokine receptor mainly expressed by intestine-homing T cells,[27] is Tax-inducible and expressed by HTLV-1-immortalized T cells and primary ATL cells expressing Tax.[22] Thus, CCR9 may play a role in the gastrointestinal infiltration of HTLV-1-infected T cells and ATL cells.[22,28] In the present study, we have shown that CXCR7 is yet another target gene of Tax. We have further shown that Tax induces the expression of CXCR7 mainly through the activation of NF-κB and by involving the proximal NF-κB site in the CXCR7 promoter (Fig. 4). Although CXCR7 binds CXCL11 and CXCL12 with high affinity, its chemotactic role in response to these ligands has been currently denied.[9,10] Importantly, however, CXCR7 is frequently expressed by tumor cells and tumor-associated vascular endothelial cells, and promotes cell growth and survival apparently in a ligand-independent fashion, most notably when cells are under suboptimal culture conditions.[10–12,16] We have indeed observed growth-enhancing and pro-survival effects of CXCR7 on HTLV-1-immortalized T cells by using CCX754, a synthetic CXCR7 antagonist (Fig. 5),[10] and CXCR7 siRNA (Fig. 5) and also by generating stable CXCR7 transfectants (Fig. 6). The growth-promoting and pro-survival effects of CXCR7 are most apparent when the cells are cultured in low FCS conditions as described previously.[10] Therefore, the effect of CXCR7 may be more prominent in cell survival than in cell proliferation. It is also highly notable that the induction of CXCR7 is proven to be common in cells transformed with oncogenic viruses, namely, KSHV-infected endothelial cells,[14–16] EBV-infected B cells[17] and HTLV-1-infected T cells (the present study). Therefore, CXCR7 may have a common essential role in virus-induced cellular transformation. It remains to be seen whether CXCR7 plays a significant role in HTLV-1-infected T cells and ATL cells *in vivo* and thus can be a potential therapeutic target for HTLV-1-associated diseases. Since the protein translation as well as the surface translocation of CXCR7 seems to be tightly regulated in a cell-dependent manner,[10] it is also important to elucidate the posttranscriptional mechanisms that regulate CXCR7 expression. The molecular mechanisms of growth-promoting and pro-survival activity of CXCR7 also remain to be elucidated.

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Elevation of Serum KL-6 Glycoprotein or Surfactant Protein-D in Adult T-cell Leukemia with Distinct Pulmonary Complications

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Patients with hematological malignancies frequently suffer from lung diseases as a complication. However, it is difficult to discriminate leukemic invasion into the lung from infectious pulmonary complications. The serum level of Krebs von den Lungen-6 (KL-6), which is a mucin-like glycoprotein, is increased in more than 70% of patients with interstitial pneumonia. Surfactant protein-D (SP-D) is produced mainly in the lung by alveolar type II and bronchiolar epithelial cells and is a useful serum marker for interstitial pneumonia. We therefore measured the levels of KL-6 and SP-D in sera from 128 patients (76 males and 52 females, mean age: 59 years) with hematological malignancies, including adult T-cell leukemia (ATL). Overall, the increase in KL-6 or SP-D, above each cut-off value (500 U/ml for KL-6 and 110 ng/ml for SP-D), was detected in 11 patients (8.6%) or 10 patients (7.8%), respectively. In contrast, among 67 ATL patients, 15 patients had high serum levels of KL-6 and/or SP-D; both were elevated in 2 patients, only KL-6 was elevated in 6 patients and only SP-D was elevated in 7 patients. Thus, serum KL-6 and SP-D appear to be elevated in a mutually exclusive manner in ATL. Indeed, high serum levels of KL-6 were closely related to the stage of ATL, while the serum SP-D was elevated in ATL patients with pulmonary infection. In conclusion, the combined measurement of KL-6 and SP-D in ATL may become a useful means to discriminate leukemic pulmonary lesions from infectious pulmonary complications. ——— Krebs von den Lungen-6; surfactant protein-D; hematological malignancy; adult T-cell leukemia; interstitial pneumonia.
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Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) are sensitive markers of various interstitial pneumonias; however, the use of these markers for patients with hematological malignancies, which are frequently accompanied by lung disorders related to infections and therapies, has not been established (Kohno et al. 1999). KL-6 is a mucin-like glycoprotein that was originally discovered with a murine monoclonal antibody from a hybridoma derived from mouse splenocytes immunized with a human pulmonary adenocarcinoma cell line (Kohno et al. 1988). This KL-6 antibody recognizes a sialylated carbohydrate chain on a high molecular weight glycoprotein, later defined as a muc-1-associated epitope, implying that KL-6 is MUC1 (Stahel et al. 1994). MUC1 is predominantly membrane-bound, consisting of a cytoplasmic tail, a transmembrane domain and an extracellular domain. Shedding by a metalloproteinase appears to yield a soluble form of MUC1, KL-6, into serum and body fluids in vivo (Marcaurelle and Bertozzi 2002). Indeed, soluble, serum KL-6 has been reported to be elevated in benign lung diseases involving regenerating-pneumocytes that express high levels of MUC1 (Kohno et al. 1988). Serum KL-6 is increased in more than 70% of patients with interstitial pneumonia (Kohno 1999). Thus, soluble KL-6 has clinically applications as a sensitive and valuable marker for the diagnosis and monitoring of patients with interstitial pneumonia including idiopathic fibrosis, hypersensitive pneumonitis, collagen-related pneumonitis, and radiation pneumonitis. However, serum KL-6 is also elevated in malignant diseases derived from epithelial cells, and is elevated in 30

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to 50% of patients with adenocarcinoma of the lung, pancreas, or breast, and in 10% or less of patients with gastric, colon, or rectal cancers (Kohno et al. 1992). On the other hand, despite the expression of MUC1 and its ligand, L-selectin, in normal and malignant hematopoietic cells, the information is limited about the clinical significance of soluble KL-6 in patients with hematological malignancies (Brossart et al. 2001; Mukherjee et al. 2005). Moreover, hematological malignancies are frequently accompanied by interstitial pneumonia related to infectious agents, therapeutic drugs, and radiation and the possible relationship of KL-6 to these complications is unknown.

The protein SP-D, a member of the human collectin family, is produced mainly in the lung by alveolar type II and bronchiolar epithelial cells. The serum level of SP-D is considered to reflect damage from, or response of epithelial cells to, inflammation (Leth-Larsen et al. 2003). Accordingly, SP-D is also a useful marker for interstitial pneumonia as well as for infectious pulmonary diseases. These facts prompted us to study the serum levels of KL-6 and SP-D in patients with hematological malignancies. In particular, we focused on serum KL-6 status in adult T-cell leukemia (ATL), because ATL is characterized by a high number of invasive tumor cells and by complications due to infective interstitial pneumonias related to non-bacterial pathogens (White et al. 1995). To understand the pathological and clinical implications of serum KL-6 and SP-D, the potential correlation between their levels and those of other serum biomarkers such as lactate dehydrogenase (LD) activity, and soluble IL-2 receptor (sIL-2R) levels were examined.

Here we present the finding that serum KL-6 and SP-D are elevated in a small fraction of patients with hematological malignancies. Their elevation in serum is mutually exclusive and the measurement of KL-6 and SP-D is helpful to discriminate leukemic invasion into the lung from infectious pulmonary complications in ATL.

Patients and Materials

Serum samples from patients and healthy volunteers were collected after informed consent based on the study protocol approved the Ethics Committee of Nagasaki University Hospital (Nagasaki, Japan). A total of 363 serum samples were collected at the time of diagnosis and relapse, and stored at −30°C until use. We examined the serum levels of KL-6 and SP-D in sera from 128 patients (M : F = 76 : 52, Range: 4-84 years, Mean: 59) with hematological malignancies. The patients with hematological malignancies consisted of patients with adult T-cell leukemia (ATL; 67 patients), acute lymphoblastic leukemias (ALL; 9 patients), acute myeloid leukemia (AML; 9 patients), B-cell malignant lymphoma (ML; 14 patients), chronic lymphocytic leukemias (CLL; 12 patients), chromic myeloid leukemia (CML; 6 patients), and HTLV-1 carriers with other diseases (11 patients). All of these diseases were diagnosed based on routine clinico-pathological findings, such as morphology, immunophenotype, and cyto/molecular genetics. ATL was subclassified into 4 categories of acute, lymphoma, chronic, and smoldering subtypes according to LSG criteria (Shimoyama 1991).

Methods

KL-6 and SP-D quantification:

Serum KL-6 was quantified using the PICOLUMI KL-6 kit (Sanko Junyaku, Tokyo, Japan), an electrochemiluminescent immunoassay (ECLIA) method specific for human KL-6, according to the manufacturer's instructions. Briefly, 200 μl serum sample, pre-diluted 1 : 51 with the sample diluent, was incubated with 25 μl anti-KL-6 monoclonal antibody-coated micro magnetic beads solution for 9 min at 30°C. After the beads were washed twice with the washing solution, 200 μl ruthenium (Ru)-labeled anti-KL-6 monoclonal antibody was added to the beads and incubated for 9 min at 30°C. After the beads were washed three times with the washing solution, the beads were placed into the electrode and the photons (wavelength, 620 nm) emitted from the Ru-labeled anti-KL-6 monoclonal antibody were counted with a photo-multiplier tube. The concentration of KL-6 in the serum sample was calculated by comparing the obtained counts with photon counts obtained from calibrated KL-6 standard antigens. These ECLIA procedures were carried out with an automatic ECLIA analyzer (PICOLUMI 8220; Sanko Junyaku, Tokyo, Japan).

Serum SP-D was quantified with an SP-D EIA kit (Yamasa Co., Chiba, Japan) according to the manufacturer's instructions. Briefly, 100 μl serum sample, pre-diluted 1:11 with the sample diluent, was put into a 96-well microplate coated with an anti-SP-D monoclonal antibody. The serum sample was incubated for 18-24 h at 2-8°C. After the well was washed three times with washing solution, 100 μl monoclonal antibody-labeled peroxidase enzyme was added and incubated for 2 h at 15-30°C. After the well was washed three times with washing solution, 100 μl color development reagent including tetramethylbenzidine and hydrogen peroxide was added and incubated for 15 min at 15-30°C followed by addition of 100 μl stop solution. The absorbance of the solution in the well was measured in a microplate reader at a wavelength of 450 nm. The concentration of SP-D in the serum sample was calculated by comparing the obtained absorbance with the absorbance of calibrated SP-D standard antigens.

In addition, to evaluate the tumor behavior of ATL, soluble interleukin-2 receptor (sIL-2R) was measured according to a previously described method (Kamihira et al. 1994).

KL-6 and SP-D expression in ATL cells:

The subcellular expression of KL-6 and SP-D was examined by flow cytometry and indirect immunostaining using anti-SP-D (Yamasa, Tokyo, Japan) and anti-KL-6 (Eisai, Tokyo, Japan) antibodies and an FITC-conjugated goat anti-mouse antibody (Dako, Kyoto, Japan). The fluorescent signals were evaluated using FACSCalibur and Cellquest software (BD Biosciences Immunocytometry System, San Jose, CA) and fluorescence microscopy.

Statistical analysis

Results are expressed as mean ± standard deviation (S.D.) unless mentioned. Unpaired data were analyzed by Wilcoxon's rank sum test and Mann-Whitney *U* test, respectively. Ratio was evaluated utilizing Fisher's exact probability test. Correlation was evaluated by Spearman's correlation index. A two-tailed p value of 0.05 was considered significant.

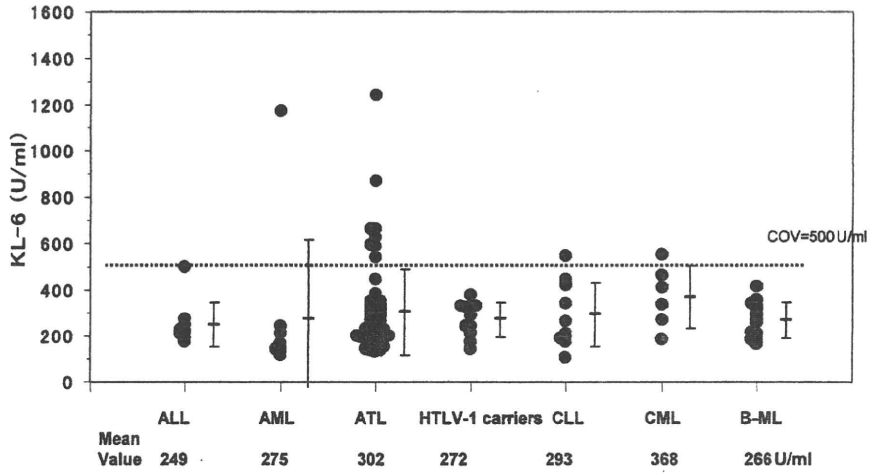

Fig. 1. Distribution plots of the serum level of KL-6 among each disease category in 128 patients with hematological malignancies. The serum level of KL-6 was higher than the cut off value of 500 U/ml (dotted line) in only 11 out of 128 patients with hematological malignancies. No statistically significant difference in the incidence or the median level among disease categories was observed.

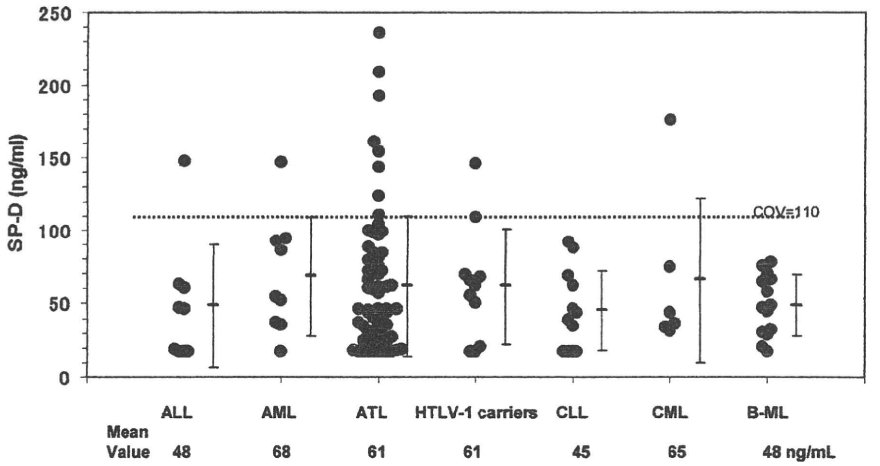

Fig. 2. Distribution plots of serum SP-D among disease categories. Serum SP-D was measured in the same 128 patients as in Fig. 1 using a cut-off point of 110 ng/ml (dotted line). The distribution of the plots is similar to that of Fig. 1.

Results

KL-6 and SP-D levels in sera from patients with hematological malignancies

Serum KL-6 and SP-D levels were measured in 128 serum samples from patients with various hematological malignancies, as shown in Figs. 1 and 2, respectively. The cut-off value was set at 500 U/ml for KL-6 and 110 ng/ml for SP-D, based on the data from healthy volunteers. Levels of KL-6 that were elevated above the cut-off value were detected in only 11 (8.9%) of 128 cases and these cases consisted of 1 (11.1%) of 9 AML, 8 (11.9%) of 67 ATL, 1 (8.3%) of 12 CLL, and 1 (16.7%) of 6 CML. No statistically significant difference in the mean value was found among the disease groups whose mean values ranged from 249 to 368 U/ml. The frequency of high serum SP-D levels in each disease group was similar to that of serum KL-6: 11% (1/9) in ALL, 11% (1/9) in AML, 12% (8/67) in ATL, 18% (2/11) in HTLV-1 carriers, 0% (0/12) in CLL, 17% (1/6) in CML, and 0% (0/14) in ML. The overall frequency is 10.2% (13/128 cases).

We next examined the potential mutual relationship between elevated levels of KL-6 and SP-D in ATL patients (Fig. 3), but found no statistically significant correlation ($r = 0.1096$, $p = 0.218$). Interestingly, the elevation in the serum levels of KL-6 or SP-D was almost completely mutually exclusive. Thus, only 2 patients showed elevated levels of both markers, while KL-6 was exclusively elevated in 11 patients and SP-D was exclusively elevated in 10 patients.

KL-6 and SP-D are elevated in a mutually exclusive manner in ATL

To determine the clinical significance of the difference in expression levels of KL-6 and SP-D and their potential use as biomarkers, further investigations were carried out using samples from ATL patients. Fifteen out of 67 ATL

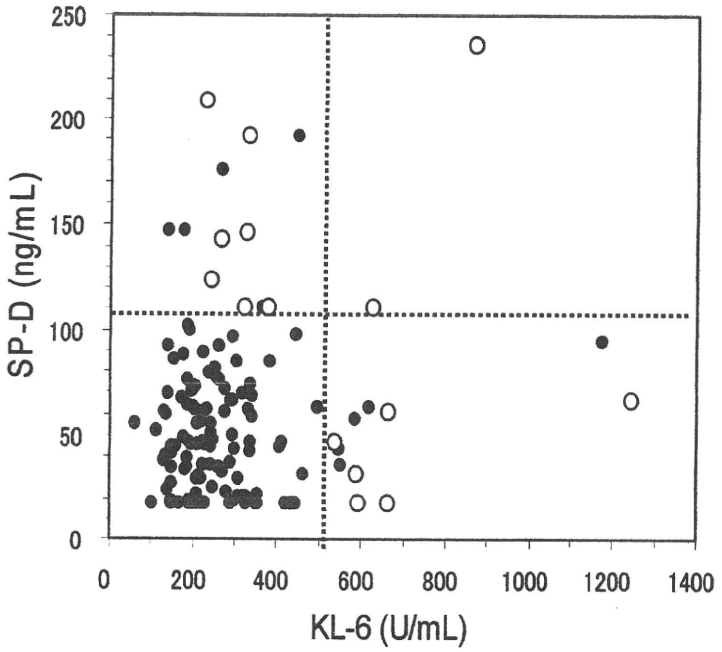

Fig. 3. Analysis of the potential relationship between serum KL-6 and SP-D levels in patients with hematological malignancies. A twin dot plot graph between KL-6 and SP-D showed no correlation between their expression levels ($y = 0.028x + 50.6$, $r = 0.1096$, $p = 0.218$). Both markers were elevated in only 2 patients. KL-6 was exclusively elevated in 11 patients, and SP-D was exclusively elevated in 10 patients. Open circles represent ATL patients with elevated levels of KL-6 or SP-D. Closed circles represent all other patients with hematological malignancies, including ATL patients.

Table 1. The clinico-pathological features of 15 patients with elevated levels of either KL-6 or SP-D.

Patient No.	Age	Gender	Subtype of ATL	KL-6 (U/mL)	SP-D (ng/mL)	Pulmonary complications	
						Tumorous	Infectious
1	76	F	Lymphoma/acute	628	110.3	±* KL-6 expressing ATL cells[1)]	–
2	56	F	Chronic	871	235.8	+ Leukemic infiltration[2)]	+
3	64	M	Chronic	1244	66.2	– KL-6 expressing ATL cells[1)]	–
4	81	M	Acute	665	17.2	+ Leukemic infiltration[2)] & PE	–
5	70	M	Lymphoma	665	60.6	±*	–
6	78	M	Lymphoma	540	46.2	±*	–
7	55	M	Lymphoma	595	17.1	±*	–
8	73	F	Smoldering	592	31.1	–	–
9	73	M	Smoldering	380	110.1	–	+
10	72	M	Lymphoma	331	192.1	–	+
11	35	M	Smoldering	327	146.4	–	+
12	40	M	Chronic	322	110.1	–	+
13	82	M	Smoldering	268	143.2	–	+
14	43	F	Acute	244	123.4	–	+
15	40	F	Acute	230	209.1	–	+

The group comprising patients 1-8 showed elevated KL-6 and was characterized by no evidence of overt pulmonary infectious complications, but had demonstrable leukemic cell invasion into the lungs (patients 2-5) and disseminated tumor cells in the body (patients 6-7). In contrast, patients (numbers 9-15) with exclusive elevation of SP-D had only infectious pulmonary complications. The cut-off values used were 500 U/ml for KL-6 and 110 ng/ml for SP-D.

[1)]KL-6-bearing ATL cells; [2)]demonstrated by BALF cytology. [3)]ATL, AML, and gastric cancer

*Clinically suspected with reticular shadow on Chest X-p,

PE, pleural effusion; CBP, chronic broncho-pneumonia; BP, broncho-pneumonia; CMV pn, cytomegalovirus pneumonia.