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An animal model of adult T-cell leukemia: humanized mice with HTLV-1– specific immunity

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INTERNAL MEDICINE

Human T-lymphotropic Virus Type-I (HTLV-I)-associated Myelopathy with Bulbar Palsy-type Amyotrophic Lateral Sclerosis-like Symptoms

Rina Ando, Noriko Nishikawa, Tomoaki Tsujii, Hirotaka Iwaki,
Hayato Yabe, Masahiro Nagai and Masahiro Nomoto

Abstract

We herein report a case of Human T-lymphotropic virus type-I (HTLV-I)-associated myelopathy with bulbar palsy-type amyotrophic lateral sclerosis-like symptoms. A 52-year-old woman developed dyslalia at approximately 40 years of age, which slowly progressed. She presented with muscular atrophy and increased tendon reflexes of the extremities as well as bulbar palsy, from which motor neuron disease was suspected. Cerebrospinal fluid (CSF) testing revealed no abnormalities except for an elevated neopterin concentration at 143.17 pmol/mL (normal ≤30 pmol/mL). Her serum and CSF anti-HTLV-I antibody titers were also high. Intravenous infusions of methylprednisolone decreased the CSF neopterin concentration to 50.33 pmol/mL. Subsequent oral prednisolone therapy was effective in alleviating the symptoms.

Key words: HTLV-I-associated myelopathy (HAM), bulbar palsy, amyotrophic lateral sclerosis (ALS), neopterin, anti-HTLV-I antibody, steroid therapy

Introduction

Human T-lymphotropic virus type-I (HTLV-I)-associated myelopathy (HAM) is a slowly progressive myelopathy caused by one of the human retroviruses, HTLV-I. The principal clinical manifestations of HAM are spastic paraplegia and pollakiuria (1). Cerebrospinal fluid (CSF) testing in HAM patients shows high levels of anti-HTLV-I antibody titer and neopterin. Immunotherapies including steroids and interferon-alpha are applied for the treatment of HAM (2). We herein report a case of HAM with bulbar palsy, muscular atrophy, and weakness of the upper limbs and trunk that required differentiation from bulbar palsy-type amyotrophic lateral sclerosis (ALS).

Case Report

A 52-year-old woman presented with the main complaint of dyslalia and gait disturbance. She had no remarkable medical history and did not smoke or drink alcohol. Her mother had suffered from dyslalia that developed in her 40s and she died at 70 years of age.

The patient developed dyslalia at approximately 40 years of age, which slowly progressed. Aspiration occurred at approximately 50 years of age. She visited our clinic due to frequent falls on level ground. She presented with muscular atrophy and increased tendon reflexes of the extremities as well as bulbar palsy. Because her deceased mother reportedly had similar symptoms, familial ALS was suspected and she was admitted to hospital for further evaluation.

On admission, the patient's height was 161 cm and body weight was 38.6 kg. Her vital signs were normal. She was alert and had normal cognitive function. The patient's facial muscle strength was slightly reduced in the upper part and moderately reduced in the lower part. She also had symptoms of cranial nerve disorder which included constant mouth opening, forced crying, dysphagia, dyslalia, a loss of gag reflex, tongue atrophy, fasciculation, and poor tongue protrusion. There were no other abnormal symptoms in the

Table.   Cases of HAM Showing ALS-like Signs in the Literature

	Matsuzaki et al.[5] (n=5)	Vernant et al.[7] (n=4)	Kuroda & Sugihara[4] (n=1)	Arimura et al.[6] (n=1)
Age at onset (years)	Mean: 52.2	49–77	57	67
Course (years)	Time to abasia: Mean:5	NR	Time to death: 4.5 years	NR
Bulbar palsy	2/5	2/4	+	+
Fasciculation in tongue	3/5	NR	+	NR
Muscular atrophy in extremities	5/5	4/4	+	+
Hyperreflexia	5/5	4/4	+	+
Babinski index	4/5	NR	+	NR
Sensory disturbance	4/5	0/4	–	+
Autonomic disturbance	4/5	1/4	+/–	+
Anti-HTLV-I antibody in CSF	5/5	NR	+	NR
Anti-HTLV-I antibody in serum	5/5	4/4	+	+
Effect of steroid therapy	Improved in 2/3 treated patients	NR	NR	NR

Abbreviations: ALS: amyotrophic lateral sclerosis, CSF: cerebrospinal fluid, HAM: HTLV-I-associated myelopathy, HTLV-I: human T-cell lymphotropic virus type-I, NR: not reported

cranial nerve system. Her extremities were spastic, and systemic muscular atrophy and fasciculation were observed. A manual muscle test revealed muscle weakness, predominantly in the proximal muscles, and was graded a 4 out of 5. The jaw reflex and tendon reflexes of the extremities were hyperactive. Babinski reflex was bilaterally positive and the patient had a spastic gait. Her autonomic symptoms included constipation and micturition frequency at approximately 8 to 10 times daily. She had no coordination disturbance or sensory disturbance.

Blood tests showed no significantly abnormal values in the patient's blood cell counts, blood biochemistry, markers of the auto-immune system, or various tumor markers. General CSF testing revealed no abnormal results. Magnetic resonance imaging scans of the brain and spinal cord revealed no abnormal findings. Nerve conduction studies also showed normal results. Needle electromyography showed acute neurogenic changes in the first dorsal interosseous muscle in the hand and anterior tibial muscle. SOD1 mutations were not identified in the genetic testing.

On admission, a presumptive diagnosis of familial ALS was made due to the patient's family history, the involvement of the upper and lower motor neurons in the brain stem (and upper and lower extremities), as well as a lack of sensory disturbance. However, the CSF neopterin concentration obtained on admission was elevated at 143.17 pmol/mL (normal concentrations ≤30 pmol/mL) (2) which was suggestive of an immune-mediated abnormality of the central nervous system. Therefore, testing for HTLV-I infection was conducted. The patient's serum and CSF anti-HTLV-I antibody titers were elevated (1:51,200 in serum and 1:128 in CSF). The HTLV-I proviral DNA load in the peripheral blood mononuclear cells (PBMCs) was high at 498 copies/10^4 cells [the mean±SD of HTLV-1 proviral load in asymptomatic HTLV-I carriers is 120±17 copies/10^4 PBMCs (3)]. We diagnosed this case as having HAM for the following reasons: 1) the titer of anti-HTLV-I antibody (1:128) in CSF

was too high as a HTLV-I carrier with the other neurological disorders and 2) the 498 copies of HTLV-I proviral load was excessively high as a HTLV-I carrier. Matsuzaki et al. reported that ALS-like HAM patients with high HTLV-I proviral loads respond well to steroid therapy (4). Therefore, steroid therapy with intravenous infusions of methylprednisolone (1,000 mg/day) for 3 days was started and the CSF neopterin concentration rapidly fell to 50.33 pmol/mL after treatment. Subsequently, therapy with oral prednisolone was initiated at a dose of 15 mg daily. Although the dyslalia still persisted, it has improved moderately. The patient's grip strength (kg) improved from 12/13 (right/left hand) to 14/18. Her micturition frequency has reduced from approximately ten times daily to six times daily. Although amelioration of the gait disturbance has not been observed, the frequency of falls has decreased. After discharge, the dose of prednisolone was tapered. At a dose of 5 mg daily, micturition frequency increased and the CSF neopterin concentration rose to 194 pmol/mL. Hence, the dose of prednisolone was increased to 10 mg daily. Thereafter, the patient's symptoms remained stable without deterioration.

Discussion

There are several reports of HAM presenting with ALS-like symptoms as was seen in our case. In one case HAM was diagnosed on the basis of autopsy results; the subject was diagnosed as having ALS in life because of bulbar palsy and involvement of the lower and upper motor neurons in the extremities without sensory disturbance or bladder and rectal dysfunction. Several studies have reported HAM patients presenting with ALS-like features (4-9); these include a patient with bulbar palsy, systemic muscular atrophy and weakness, increased tendon reflexes of the extremities, and no sensory disturbance and a patient with bulbar palsy, systemic muscular atrophy and weakness, decreased vibratory sense in the lower legs, and impaired urination.

However, an autopsy was not performed in any of these patients. Autopsy reports on ALS-like HAM patients have described infiltration of inflammatory cells and dropout and degeneration of neural cells in the dorsal column, brain stem, cerebellum, and cerebrum in addition to the main lesions in the lateral column of the thoracic spinal cord (5, 10). Another autopsy report indicated that there were no Bunina bodies, which are unique to ALS (11).

There are two possibilities of the pathogenesis of this case: 1) HAM and ALS could have been coincidental and the inflammation process of HAM modified the ALS symptoms or 2) all of the ALS-like features were caused by HTLV-1-induced inflammation in the CNS. In the present case, the patient's symptoms began with dyslalia at approximately 40 years of age and she became unable to speak at 52 years of age. However, she was able to communicate by writing and could still walk, suggesting a slower rate of progression than that of ALS. Matsuzaki et al. reported that the average interval between onset of disease and the inability to walk is 5 years in patients with ALS-like HAM with a high HTLV-I proviral load (Table), indicating a slower rate of progression compared to typical ALS (4). Steroid therapy was not effective in ALS-like HAM patients with a low HTLV-I proviral load similar to that in asymptomatic HTLV-I carriers, whereas in ALS-like HAM patients with a high HTLV-I proviral load, steroid therapy was highly effective with efficacy almost equivalent to that seen in typical HAM patients (4, 12). The improved symptoms in our patient remained stable without deterioration by oral steroid therapy. On the basis of the high HTLV-I proviral load and the favorable response to steroid therapy, in addition to a slower progression of symptoms than that of ALS, our case was considered to be consistent with HAM presenting with ALS-like features. The measurement of neopterin and HTLV-I proviral load in HAM patients may be useful in differentiating HAM with ALS-like symptoms from ALS developing in HTLV-I carriers. Even in patients who have classical symptoms of ALS, the measurement of serum anti-HTLV-I antibody is of particular significance for differentiating HAM from ALS because half of the ALS-like HAM patients who receive steroid therapy show improvement in their symptoms.

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Full-length article

Direct infection of primary salivary gland epithelial cells by HTLV-I that induces the niche of the salivary glands of Sjögren's syndrome patients

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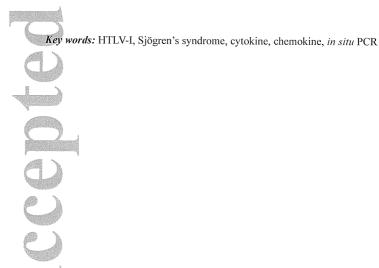Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto,

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Short title: **HTLV-I infection toward salivary gland epithelial cells**

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ABSTRACT

Objective: To explore whether Human T-cell leukemia virus type I (HTLV-I)

directly infects salivary gland epithelial cells (SGECs) and induces the niche of

Sjögren's syndrome (SS).

Methods: We determined the inflammation-related molecules profiles after the

co-culture of SGECs with HTLV-I, producing the CD4^{+} T-cell line HCT-5 or

Jurkat by antibody dot-blot array, immunofluorescence (IF) and ELISA. The

apoptosis-related molecules profile was determined by antibody dot-blot array

and IF. We investigated the presence of HTLV-I-related molecules by IF and *in*

situ PCR. The apoptosis of SGECs was evaluated by TUNEL staining.

Results: 7.8 ± 1.3 % of the SGECs were positive for HTLV-I-related proteins

after 96-h co-culture with HCT-5 cells. Nuclear expression of NF-κB p65 also

became positive in 10% of the SGECs. The presence of HTLV-I proviral DNA in

SGECs after co-culture with HCT-5 cells was detected by *in situ* PCR. A

semiquantitative analysis by dot-blot antibody array in co-cultured supernatant

with HCT-5 showed time-dependent increases of cytokines and chemokines

including sICAM-1, RANTES, and interferon γ-induced protein 10 kDa

(IP-10/CXCL10), confirmed by IF and ELISA. The expressions of pro-apoptotic

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molecules (e.g., cytochrome C and Fas) and anti-apoptotic molecules [e.g., Bcl-2,

Heme oxygenase-2 (HO-2) and HSP-27] were increased in the SGECs

co-cultured with HCT-5, showing that apoptosis of SGECs was not detected after

co-culture with HCT-5 or Jurkat.

Conclusion: HTLV-I is thought to infect SGECs and alter their cellular functions.

These changes may induce the niche of SS and contribute to the development of

SS found in anti-HTLV-I antibody-positive subjects.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) is reported to be one of the causative agents of primary Sjögren's syndrome (pSS) in endemic areas including Nagasaki City, Japan (1–3). The extremely high prevalence of SS found in patients with HTLV-I-associated myelopathy (HAM) appears to confirm a strong relationship between HTLV-I infection and SS (4–6). Our previous study also revealed the clinical characteristics of anti-HTLV-I antibody-positive SS patients, and we found that the labial salivary glands (LSGs) of such patients are not destructible compared to the LSGs of anti-HTLV-I antibody-negative SS patients (7). In addition, the low appearance of ectopic germinal center (GC) as well as the low expression of C-X-C motif chemokine 13 (CXCL13) in infiltrating mononuclear cells of LSGs were found as an immunohistological characteristic of anti-HTLV-I antibody-positive SS patients (8).

HTLV-I preferentially infects T cells, especially CD4+ T cells, and our findings described above indicated that the T-cell lineage may primarily contribute to the pathogenesis of anti-HTLV-I antibody-positive SS patients. However, the cell types other than T cells, including the human retinal pigment epithelial cell line ARPE-19 (9) and human primary fibroblast-like synovial cells

(FLS) (10) were reported to be susceptible to HTLV-I infection. In ARPE-19 cells, the expression of intercellular adhesion molecule 1 (ICAM-1) is increased by HTLV-I, and the production of granulocyte/macrophage colony-stimulating factor (GM-CSF) from FLS is induced by HTLV-I.

These observations suggested that HTLV-I may infect cell lineages other than T cells of human salivary glands and may contribute to the development of SS. In this regard, ductal epithelial cells are considered candidate cells, since varying cytokines, chemokines and apoptosis-related molecules have been shown to be expressed in these cells (1). In addition, ductal epithelial cells attract T cells into the salivary glands of SS patients through the production of an interferon-gamma (IFN-γ)-inducible 10-kDa protein (IP-10) and a monokine induced by IFN-γ (Mig) (11).

Here we investigated whether HTLV-I infects human primary salivary gland epithelial cells (SGECs) and modulates the production of functional molecules.

PATIENTS AND METHODS

Patients

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Primary SGECs from LSGs were obtained from fifteen patients with primary SS

diagnosed according to the revised criteria proposed by the American-European

Consensus Group (12). All fifteen patients were female (age: 53.2 ± 15.4) in

whom anti-HTLV-I antibody measured by a chemiluminescent enzyme

immunoassay (CLEIA) was negative.

Antibodies and reagents

Mouse anti-HTLV-I (p19, p28, and GAG) antibody (Chemicon International,

Temecula, CA, USA), mouse anti-NF-κB p65 antibody, mouse anti-cytochrome

C antibody, mouse anti-HSP-27 antibody, rabbit anti-Fas antibody and rabbit

anti-Fas antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained.

Mouse anti-heme oxygenase 2 (HO-2) antibody was purchased from OriGene

Technologies (Rockville, MD), and rabbit anti-ICAM-1 antibody, rabbit

anti-GRO/CXCL-1 antibody, anti-CCL5/RANTES antibody and rabbit

anti-IP-10/CXCL10 antibody were purchased from LifeSpan Biosciences (Seattle,

WA). Rabbit anti-IL-8 antibody was purchased from Abgend (San Diego, CA).

Secondary antibodies including donkey anti-mouse IgG conjugated with

fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated with

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tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson

ImmunoResearch Laboratories (West Grove, PA). Hoechst dye 33258 was

purchased from Sigma (St. Louis, MO). Proteome Profiler™, the human cytokine

array panel A array kit, the human apoptosis array kit, and the Quantikine®

ELISA for sICAM-1, CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα and

CXCL8/IL-8 was purchased from R&D Systems (Minneapolis, MN). Amersham

Cy3-dUTP was purchased from GE Healthcare (Buckinghamshire, UK).

Monoclonal mouse anti-human CD4, CD8, CD20cy, mouse IgG1 and

monoclonal rabbit anti-human cytokeratin 8/18 antibody were purchased from

Dako Cytomation (Glostrup, Denmark).

LSGs biopsy and cell culture

We performed a biopsy of the LSGs from each patient's lower lip under local

anesthesia and divided the samples into specimens for the diagnosis of

sialadenitis by hematoxylin staining and the culture of SGECs in a defined

keratinocyte–serum-free medium (SFM) culture medium (Invitrogen Life

Technologies, Carlsbad, CA) supplemented with hydrocortisone (Sigma) and

bovine pituitary extract (Kurabo, Osaka, Japan). All nine patients were

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compatible with the diagnosis of SS in the classification of Chisholm & Mason **(13)**.

For the co-culture of SGECs with HTLV-I-producing T cells, we co-cultured the cell line HCT-5, which is derived from cerebrospinal fluid cells of a patient with HAM **(14)** with SGECs during the designated time period in the defined keratinocyte-SFM culture medium. As a control toward HCT-5, non-HTLV-I infected T cell line Jurkat was cultured in RPMI 1640 medium with 10% fetal bovine serum. For below experiments, HCT-5 or Jurkat cells were co-cultured with SGECs at 2:1 ratio when the cells were seeded. Briefly, the SGECs were seeded on sterile cover slips for immunofluorescence. Then, HCT-5 cells were added 24 hr after SGECs stuck and grew on cover slips. For immunofluorescence, cells were stringently washed with PBS to remove remaining HCT-5 cells. Informed consent for the usage of LSGs biopsy samples was obtained from all nine patients at the commencement of the study. The study was conducted with the approval of the human ethical committee of Nagasaki University Hospital.

Immunofluorescence

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We performed the immunofluorescence studies as described **(15)**. Briefly, SGECs cultured on 12-mm^2 cover slips were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4°C, followed by immersion in methanol at −20°C for 10 min. After fixation, the SGECs were blocked in 5% normal horse serum in PBS, and then incubated in the primary antibodies for 1 h at room temperature followed by incubation with FITC-conjugated and TRITC-conjugated secondary antibodies with Hoechst dye 33258 under dark conditions. The SGECs were then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and scanned by fluorescence microscopy (BIOREVO BZ-9000, Keyence, Osaka, Japan). For the measurement of the immunofluorescence of the HCT-5 cells, fixed cells were incubated with mouse monoclonal primary antibodies as cell surface markers, followed by FITC-conjugated secondary antibody and Hoechst dye 33258. Control experiments were performed to confirm the isotype specificity of the secondary antibodies. Immunostaining of HCT-5 cells was performed in the same manner as that described above for SGECs.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

To investigate double-stranded DNA breaks of SGECs, we used TUNEL staining as described in our previous study **(16)**. After fixation, SGECs were incubated in 4% paraformaldehyde (PFA) 4°C for 15 min, followed by immersion in PBS with 0.5% Tween 20 and 0.2% bovine serum albumin using the MEBSTAIN Apoptosis kit Direct (MBL, Nagoya, Japan). The SGECs were then incubated with a 50-μL terminal deoxynucleotidyl transferase (TdT) solution at 37°C for 1 h. The FITC signal of dUTP was captured by fluorescence microscopy using the BIOREVO BZ-9000. For positive control to show induction of apoptosis, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) **(15)**.

Cytokine dot-blot array analysis for co-cultured supernatant

We used a cytokine dot-blot array system used according to the manufacturer's instructions (Proteome Profiler™, the human cytokine array panel A array kit, R&D Systems, Minneapolis, MN). Briefly, we incubated diluted co-cultured supernatant with a cocktail of biotinylated antibodies for 1 h after the membranes were blocked. The mixture of cytokines/chemokines and antibodies was then

incubated for 2 h with this array system, which was combined with an immobilized antibody on the membrane. For the detection of cytokines and chemokines, chemiluminescent reagents were used after incubation with streptavidin-horseradish peroxidase. The expressions are noted as the ratio compared with control dot-blots.

Apoptosis dot-blot array analysis for co-cultured lysate

We used an apoptosis dot-blot array system used according to the manufacturer's instructions (Proteome Profiler™, the human apoptosis array kit, R&D Systems, Minneapolis, MN). Briefly, diluted co-cultured cellular extracts were incubated on membranes for 2 h after the membranes were blocked for 1 h. After a 2-h incubation, a cocktail of biotinylated antibodies was added to the membranes and incubated for 1 h. Chemiluminescent reagents were then used after incubation with streptavidin-horseradish peroxidase for 30 min. The expressions are noted as the ratio compared with control dot-blots.

Cytokine and chemokine assay for co-cultured supernatant by ELISA

The ELISA system was used according to the manufacturer's instructions, and

the levels of sICAM-1, CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα and

CXCL8/IL-8 were measured (all from R&D Systems). Briefly, the assigned

volume of the cell culture supernatant, standard or control was added to an

ELISA well and incubated for the indicated times. After the wells were washed

and decanted three times, each conjugate was added to a well and incubated for 1

h at 4°C. After the washing process, substrate solution was added to each well

and incubated for 15 min. After the addition of stop solution, optical density at

450 nm was measured.

In situ PCR of HTLV-I proviral DNA in the co-cultured SGECs

Initially, SGECs co-cultured with or without HCT-5 were fixed in 0.5 mL

Carnoy's fixative for 20 min at room temperature, followed by washing with 0.5

mL 70% ethanol for 15 min at room temperature on type I collagen-coated

12-mm^2 cover slips. After treatment with pre-warmed protein kinase (PK) (1

μg/mL) at 37°C for 15 min and three washes with PBS, the SGECs were fixed

with 4% PFA/PBS for 5 min, then immersed in 50% formamide/2× saline sodium

citrate buffer (SSC) at 4°C overnight. After being washed with

deuterium-depleted water (DDW) three times for 5 min each time, the cells were

mixed with an amplification cocktail that consisted of a final concentration of 1×

PCR buffer, 1 μg/mL of forward primer (5'-CGGATACCCAGTCTACGTGT-3'),

1 μg/mL of reverse primer (5'-GAGCCGATAACGCGTCC-3') **(17)**, 0.2 mM

dNTP, 2.5 mM MgCl2, 1 μM Cy3-dUTP and distilled water without DNA

polymerase, and then boiled for 10 min.

The application of these primer sets had been reported by Matsuoka et al.,

in which the positions of the forward primer and reverse primer were 7358–7377

and 7516–7494 of the HTLV-I pX region, respectively **(17)**. After KAPA2G

FastDNA polymerase (Kapa Biosystems, Woburn, MA) complete amplification

cocktail was added to the SGECs and sealed with clear rubber covers, we placed

the cover slips in a thermocycler for an *in situ* PCR (Hybaid Limited, Ashford,

Middlesex, UK). The details of the *in situ* PCR reaction were as follows: each

block was heated at 92°C for 3 min, and then five cycles of PCR were performed

(92°C, 1 min; 47°C, 1 min; 70°C, 2 min), and the block was then held at 70°C for

5 min. The reacted cover slips were then washed with 2×SSC at 37°C for 15 min,

four times, followed by washing with 0.5×SSC at 45°C for 15 min, two times.

After the cover slips were reacted with PBS once and covered with Vectashield

mounting medium, we visualized the SGECs by the fluorochrome with the

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fluorescence microscope (BIOREVO BZ-9000).

Statistical analysis

Differences in the ELISA results were analyzed using Student's *t*-test. P-values <0.05 were accepted as significant.

RESULTS

The phenotype and viability of HCT-5 cells

We found that the HCT-5 cells used for co-culture with SGECs showed the CD4$^+$ phenotype **(Fig. 1A)** without staining for CD8 and CD20. The HCT-5 cells were viable with translocation of NF-κB into the nucleus in co-culture medium (i.e., the defined keratinocyte-SFM) for SGECs for 0–96 h **(Fig. 1B)**.

Detection of HTLV-I-related proteins in SGECs during co-culture

After the co-culture of SGECs and HCT-5 cells, immunofluorescence demonstrated the clear signals of HTLV-I proteins p19, p28 and GAG emerged at 72–96 h **(Fig. 2A)**. In the low-magnification view at 96h co-culture of SGECs with HCT-5 cells, approx. 10% of the SGECs showed HTLV-I-positive staining

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(Fig. 2B). Nuclear NF-κB p65 was also detected among 10% of the SGECs after co-culture **(Fig. 2A, B)**. To distinguish HTLV-I-infected SGECs from HCT-5, SGECs were stained with cytokeratin 8/18 **(Fig. 2C)** that was reported to be one of markers for SGECs **(18)**. In merged view, frequency of HTLV-I-infected SGECs was calculated as 7.8 ± 1.3 % and the remaining HCT-5 cells were observed during 48-96 h co-culture.

Detection of HTLV-I DNA in SGECs by *in situ* PCR

To investigate the details and confirm whether HTLV-I infected the SGECs during co-culture with HCT-5 cells, we determined the HTLV-I DNA expression. For a positive control, HTLV-I proviral DNA was detected in HCT-5 cells **(Fig. 3A)**. During the co-culture, amplified HTLV-I DNA was observed in the nucleus of SGECs in the presence of primer at 48 h of co-culture with HCT-5 cells **(Fig. 3B)**. The strongest HTLV-I DNA signal was observed at 72 h of co-culture in the presence of primer.

Increased expression of inflammation-related molecules and apoptosis-related molecules in the co-cultured SGECs

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The cytokine dot-blot array results for the HCT-5/SGECs co-cultured supernatant shown in **Figure 4A**. The expressions of GM-CSF, CXCL1/GROα, CCL1, sICAM-1, IL-1ra, IL-6, IL-8, CXCL10/IP-10, MIF, Serpin E1 and CCR5/RANTES were increased time-dependently after the co-culture of SGECs with HCT-5.

The results of the apoptosis dot-blot array from SGECs lysate co-cultures with HCT-5 are shown in **Figure 4B**. Pro-apoptotic molecules including pro-caspase-3, cytochrome C and Fas in the lysate showed slightly increased responses after the co-culture of SGECs with HCT-5 cells. The signals of anti-apoptotic molecules including Bcl-2, HO-2, HSP-27 or SMAC/Diablo were also up-regulated after the co-culture.

Dot-blot array results to indicate co-culture of SGECs with Jurkat were shown in Figure 4C and 4D. The expressions of IL-1ra, MIF and Serpin E1 were increased after the co-culture of SGECs with Jurkat, however, the increase was not time-dependently **(Fig. 4C)**. Different from co-culture with HCT-5, expressions of other molecules including GM-CSF, CXCL1/GROα, CCL1, sICAM-1, IL-6, IL-8 and CXCL10/IP-10 were not increased. The results of the apoptosis dot-blot array from SGECs lysate co-cultures with Jurkat are shown in **Figure 4D**.

Although expressions of pro-caspase-3 and SMAC/Diablo were similar to HCT-5/SGECs co-culture results, expressions of cytochrome C, Fas, Bcl-2, HO-2 and HSP-27 were not up-regulated after the co-culture with Jurkat.

The dot-blot data were confirmed by immunofluorescence and ELISA. As shown in **Figure 5A**, the immunofluorescence results showed the increased cytoplasmic expressions of ICAM-1, CXCL1, RANTES, IL-8 and IP-10 with augmentation of the signals for HTLV-I p19, p28 and GAG in SGECs (See spindle-shaped SGECs stained positive with both inflammatory molecules with GAG) after 96 h co-culture with HCT-5 cells. However, expressions of these molecules were not increased when SGECs were co-cultured with Jurkat **(Fig. 5B)**. Accordingly, significant increases of sICAM-1, RANTES, and IP-10 in the co-cultured supernatant compared to before the co-culture was confirmed by ELISA **(Fig. 5C)**. However, in SGECs/Jurkat co-culture supernatant, significant increase of sICAM-1, RANTES, and IP-10 was not observed **(Fig. 5D)**. Furthermore, these molecules as well as IL-8 were barely detected in SGECs/Jurkat co-culture supernatant. The immunofluorescence results of apoptosis-related molecules also showed that the membranous expression of Fas on SGECs as well as the cytoplasmic expressions of Bcl-2, cytochrome C, HO-2

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and HSP-27 were up-regulated after 96-h co-culture with HCT-5 **(Fig. 6A)**. By comparison with results from HCT-5/SGECs co-culture, no increase of apoptosis-related molecules was observed on SGECs co-cultured with Jurkat **(Fig. 6B)**.

Detection of no apoptosis of co-cultured SGECs

We have reported that cultured SGECs are committed to apoptosis by several stimuli **(15, 16)**. Since in the present study we found that the expression of pro-apoptotic molecules was increased by co-culture with HCT-5 cells, it could be speculated that the co-culture with HCT-5 cells might induce apoptosis of SGECs. As we showed previously, the SGECs stimulated with TRAIL showed clear increases of TUNEL-positive cells **(Fig. 6C, positive control)**. In contrast, during the 0 to 96 h co-culture, no TUNEL-positive staining was observed in the SGECs during co-culture with HCT-5 cells **(Fig. 6C)**. In addition, no obvious morphological change was observed on the bright field views during co-culture. Similarly, no TUNEL positive staining was observed in the SGECs during the 0 to 96 h co-culture with Jurkat **(Fig. 6D)**.

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DISCUSSION

With regard to the relationship between pSS and retrovirus, Talal et al. first reported that serum antibodies against human immunodeficiency virus (HIV)-1 were detected in 30% of sera from patients with pSS **(19)**, and the presence of retroviral particles was reported in salivary tissues from patients with SS **(20)**. The retroviral particles were also found in LSGs from patients with SS **(21)**.

Regarding HTLV-I infection in pSS, Mariette et al. reported the presence of HTLV-I *tax* gene in LSGs from pSS patients, although LSGs from patients with other inflammatory diseases also contained this gene, suggesting that HTLV-I *tax* gene contributes to the development of chronic inflammatory diseases including pSS **(22, 23)**. In addition, Green et al. showed that HTLV-I tax transgenic mice exhibited exocrinopathy involving the salivary glands, and tax protein was detected in their salivary glands and muscle specimens **(24)**.

A recent report showed that HTLV-I p19 or Tax proteins were expressed in 42.4% of LSG samples from the patients with SS, and the clinical characteristics of these SS patients (including low levels of complement and high lymphocyte counts) were identified **(25)**. Considering the above accumulating evidence of relationship between HTLV-I and SS, we speculate that HTLV-I may

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directly infect SGECs, a major cellular constituent of the salivary glands, and

change their characteristics to an inflammatory phenotype, triggering the

development of SS.

In the present study we observed for the first time that HTLV-I appears to

infect SGECs, although the expression of HTLV-I-related protein was less than

10% among co-cultured SGECs. The migration of HTLV-I into SGECs was

suggested to induce functional alterations of SGECs, since some of the SGECs

became positive for nuclear NF-κB p65, which is known as a representative

transcriptional factor activated by HTLV-I **(26)**. Accordingly, the production of

several inflammatory cytokines and chemokines was increased during the

co-culture of SGECs with HCT-5 cells in the present study. However, regarding

the above alterations of SGECs, one or more pathways other than the direct

infection of HTLV-I in SGECs may be used, since a substantial population of

SGECs after co-culture was not stained by HTLV-I related proteins, HTLV-I

proviral DNA or nuclear NF-κB p65. Autocrine or paracrine interactions of

cytokines and chemokines might be involved in these processes, in which the

cytokines and chemokines induce the production of the others **(27)**. Alternatively,

transcriptional factors or activators other than NF-κB p65, such as cyclic AMP

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response element-binding protein/activating transcription factor (CREB/ATF)

and CREB-binding protein, which serves as a transcription activator, might be

essential **(28, 29)**. Whether unique changes induced by HCT-5 are consequences

due to direct infection of HTLV-I toward SGECs or indirect effect of the

molecules, produced by neighboring activated cells including HCT-5, is a crucial

issue. In the co-culture, SGECs look like spindle-shaped and intensity of GAG

staining is not as strong as that of HCT-5 cells observed in Fig. 1B, suggesting

SGECs appear to be distinguishable from HCT-5. Some SGECs became double

positive with GAG and inflammatory molecules in the co-culture (Fig. 5A at 96

hr). Since the co-culture of SGECs with non-HTLV-I infected T cell line Jurkat

did not induce the changes of expression of functional molecules as compared

with HCT-5, cell-free HTLV-I virions might conduce the changes of SGECs.

Although no evidence of cell free transmission of HTLV-I toward any of

epithelial cells has been reported, HTLV-I virions have potential to infect

myeloid and plasmacytoid dendritic cells (DCs) **(30)**. Previous study also showed

intercellular adhesion molecule-3-grabbing nonintegrin related to DCs plays an

important role in cell-free infection of HTLV-I toward DCs **(31)**. Further studies

trying to show cell free infection of HTLV-I virions toward SGECs are needed in

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the future.

In addition to inflammatory cytokines and chemokines, pro-apoptotic molecules as well as anti-apoptotic molecules were augmented in the SGECs after co-culture with HCT-5 cells by comparison with co-culture with Jurkat in our study. However, we should also note that apoptosis dot array results might be influenced by the remaining HCT-5 cells during co-culture. As we demonstrated in Fig. 2C, HCT-5 cells stick to SGECs during co-culture and approximately 5% of HCT-5 cells still remained at 96h. The reason why HCT-5 cells remained in co-cultured is speculated that these cells had migratory and adhesive capacity as we previously reported that CD4-positive T cells derived from HAM patients showed strong transmigrating activity **(32)**.

The increase in these molecules may be induced through the activation of transcriptional factors including NF-κB p65 or the cytokines and chemokines produced by SGECs themselves. It has been demonstrated that the expressions of both pro-apoptotic molecules and anti-apoptotic molecules are regulated by the above mechanisms **(33, 34)**. Increases in the expression of anti-apoptotic molecules such as Bcl-2, HO-2 and HSP-27 might antagonize the apoptosis-inducing capacities of Fas and cytochrome C of SGECs, indicating that

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apoptosis does not occur in SGECs.

It is interesting to note that the HTLV-I infection of SGECs induces the niche of SS, since the expression pattern of cytokines, chemokines, pro-apoptotic molecules, and anti-apoptotic molecules of SGECs co-cultured with HCT-5 cells *in vitro* resembles the pattern found *in vivo* in the salivary glands of SS patients **(35)**. However, it may be disputed whether the present *in vitro* results truly reflect *in vivo* observations of patients with anti-HTLV-I antibody-positive SS. In this regard, Ohyama et al. **(36)** reported that in LSGs from patients with HTLV-I antibody-positive SS, HTLV-I proviral DNA was observed not in acinar or ductal epithelial cells of LSGs, but in the infiltrating T lymphocytes by *in situ* PCR hybridization **(36)**.

It has become evident that CD4+ T cells infected by HTLV-I resemble FoxP3+ regulatory T cells **(37)**. Regulatory T cells produce regulatory cytokines such as IL-10 and transforming growth factor (TGF)-β1 **(38),** which might affect the migration of HTLV-I into ductal epithelial cells *in vivo*. Further studies are necessary to clarify the differences and similarities of the *in vitro* role of HTLV-I infection and the *in vivo* role of HTLV-I infection observed in patients with SS.

In summary, we have shown the direct infection of HTLV-I in human

primary SGECs that induces the niche of the salivary glands of patients with SS.

In addition to the recent report from South Korea **(24)**, our clinical and

histological examinations have also revealed the characteristics of anti-HTLV-I

antibody-positive SS patients, including the low rate of ectopic germinal center

formation in LSGs and parotid gland destruction **(7, 8)**. Although we are not sure

at present about the exact pathways in SS used by HTLV-I compared with SS that

develops in anti-HTLV-I antibody-negative subjects, the present study is the first

investigation in humans showing that HTLV-I infects into SGECs, impacting on

inducing pathological condition of SS.

All authors declare no conflicts of interest in this paper.

Author contributions

All authors were involved in drafting the article or revising it critically for

important intellectual content, and all authors approved the final version for

publication. Dr. Hideki Nakamura has full access to all of the data in the study

and takes responsibility for the integrity of the data and the accuracy of the data

analysis.

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