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

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

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

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

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

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

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

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

(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

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

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).**

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

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

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

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

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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FIGURE LEGENDS

Fig. 1. Characterization of HTLV-I-infected HCT-5 T cell line.

A: After fixation in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, HCT-5 cells were reacted with primary antibodies (anti-CD4, CD8, CD20, and mouse IgG1) followed by incubation with FITC-conjugated secondary antibody with Hoechst 33258 for counterstaining. **B:** HCT-5 for 0–96 h culture in keratinocyte-SFM were fixed and incubated with mouse anti-HTLV-I (p19, p28, and GAG) antibody and rabbit anti-NF-κB p65 antibody and then reacted with FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

Fig. 2. Detection of HTLV-I-related molecules in co-cultured SGECs.

A: After the SGECs co-cultured for 0–96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, immunofluorescence was evaluated to detect the presence of HTLV-I proteins (p19, p28, and GAG). The SGECs were initially incubated with anti-HTLV-I

antibody and NF-κB p65 followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining, respectively. In contrast to increased expression of HTLV-I proteins without NF-κB translocation **(96h-a)**, the translocation of NF-κB is shown in a different view **(96h-b)**. **B:** Low-magnification view at 96 h co-culture of SGECs with HCT-5 cells. **C:** After the SGECs co-cultured for 0–96 h were fixed and immersed, immunofluorescence was evaluated to show the presence of HTLV-I proteins (p19, p28, and GAG) and SGEC marker, cytokeratin 8/18. The SGECs were initially incubated with anti-HTLV-I antibody and anti-cytokeratin 8/18 antibody followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining, respectively. HTLV-I-infected SGECs were shown as yellow staining; meanwhile HCT-5 cells were indicated as green signal in merged view. Representative results of three independent experiments are shown.

Fig. 3. Detection of HTLV-I proviral DNA by *in situ* PCR.

A: For the positive control, HCT-5 cells were used after treatment with 1 µg/mL of PK, and five cycles of *in situ* PCR were performed. **B:** The fixed SGECs were treated with 1 µg/mL of PK, and five cycles of *in situ* PCR were then performed

in the presence and absence of primers for HTLV-I pX region as reported by Matsuoka et al. **(17)**. Representative results of two independent experiments with similar findings are shown.

Fig. 4. Semiquantitative analyses of inflammation-related molecules in the supernatant and apoptosis-related molecules in lysate during co-culture.

A and C: Co-cultured supernatant was assayed with a human cytokine dot-blot array kit. Data at 0–96 h are shown as the semiquantitative concentration of each molecule in culture medium (i.e., keratinocyte SFM) for SGECs after co-culture with HCT-5 cells **(A)** or Jurkat **(C)**. "HCT-5 only" and "Jurkat only" indicate the culture supernatant for HCT-5 and Jurkat, respectively. The expressions are noted as the ratio compared with control dot-blots. Representative results of two independent experiments with similar findings are shown.

B and D: Co-cultured SGECs, HCT-5 lysate and Jurkat lysate was analyzed using a human apoptosis dot-blot array kit. Data at 0–96 h co-cultured with HCT-5 **(B)** and Jurkat **(D)** are shown as semiquantitative concentrations of each molecule in recovered SGECs lysate. "HCT-5 only" and "Jurkat only" indicate data from HCT-5 cell lysate and Jurkat lysate, respectively. The expressions are

presented as the ratio compared with control dot-blots. Representative results of two independent experiments with similar findings are shown.

Fig. 5. Confirmation of the increase in the expression of inflammation-related molecules in co-culture by immunofluorescence and ELISA.

A and B: The SGECs were co-cultured with HCT-5 **(A)** and Jurkat **(B)** for 96 h. The SGECs at 0 and 96 h were fixed in PBS containing 4% PFA at 4°C, followed by immersion in methanol at −20°C for 10 min, and then an immunofluorescence analysis was performed. The SGECs were incubated with anti-ICAM-1, CXCL-1, RANTES, IL-8 and IP-10 antibodies followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

C and D: The SGECs were co-cultured with HCT-5 **(C)** and Jurkat **(D)** for 0-96 h. Then, ELISAs were performed using the co-cultured supernatant. The concentrations of sICAM-1, CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα and CXCL8/IL-8 were detected by ELISA. Samples were collected from three independent patients, and the data shown are mean ± SD. *p<0.05 and **p<0.01

vs. 0 h (Student's *t*-test).

Fig. 6. Apoptosis of SGECs during co-culture with HCT-5 cells.

A and B: The SGECs were co-cultured with HCT-5 **(A)** and Jurkat **(B)** for 96 h. After the SGECs at 0 and 96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, an immunofluorescence analysis was performed to reveal apoptosis-related molecules. The SGECs were incubated with anti-Bcl-2, Fas, cytochrome C (Cyt C), HO-2 and HSP-27 antibodies followed by FITC-conjugated secondary antibody with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

C and D: The SGECs were co-cultured with HCT-5 **(C)** and Jurkat **(D)** for 0-96 h. The SGECs at 0–96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, then analyzed for TUNEL staining with Hoechst 33258 for nuclear staining. The FITC-conjugated green signal suggested the presence of TUNEL-positive cells. Before the TUNEL assay, observations in the bright field were also made. For the positive control (PC), the SGECs were treated with TRAIL for 3 h as reported previously **(15)**.

Representative results of two independent experiments with similar findings are

shown.

A

CD4   CD8

CD20   mIgG1

Bar: 100μM

B

HTLV-I | 0hr | 48hr | 72hr | 96hr

NF-κB p65

Merge

Bar: 20μM

Fig. 1.

A

	0h	48h	72h	96h-a	96h-b
HTLV-I					
NF-κB p65					
Merge					

Bar: 60μM

B

HTLV-I (p19 p28 GAG) | NF-κB p65 | Merge

Bar: 300μM

C

	0hr	48hr	72hr	96hr
HTLV-I				
Cytokeratin 8/18				
Merge				

Bar: 80μM

Fig. 2.

A

primer-   primer+

Fig. 3.

B

Primer+   0h   48h   72h   96h

Primer-   0h   48h   72h   96h

Bar: 40μM

A

Ratio vs PC

Legend: 0h, 48h, 72h, 96h, HCT only

B

Ratio vs PC

Legend: 0h, 48h, 72h, 96h, HCT only

C

Ratio vs PC

Legend: 0h, 48h, 72h, 96h, Jurkat only

D

Ratio vs PC

Legend: 0h, 48h, 72h, 96h, Jurkat only

Fig. 4.

Fig. 5.

A

	Bcl-2	Fas	Cyt C	HO-2	HSP-27
0 hr					
96 hr					

B

	Bcl-2	Fas	Cyt C	HO-2	HSP-27
0 hr					
96 hr					

C

	0hr	48hr	72hr	96hr	PC
TUNEL					
Hoechst					
Merge					
Bright field					

D

	0hr	48hr	72hr	96hr	PC
TUNEL					
Hoechst					
Merge					
Bright field					

Bar: 40μM

Fig. 6.