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

Hideki Nakamura[1,*], Yoshiko Takahashi[1], Tomomi Yamamoto-Fukuda[2], Yoshiro Horai[1], Yoshikazu Nakashima[1], Kazuhiko Arima[3], Tatsufumi Nakamura[4], Takehiko Koji[2], and Atsushi Kawakami[1]

[1]Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2]Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[3]Department of Public Health, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[4]Department of Human Community, Faculty of Social Welfare, Nagasaki International University, Nagasaki, Japan

* Corresponding author: Hideki Nakamura

Department of Immunology and Rheumatology, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki City, Nagasaki 852-8501, Japan.

Tel.: 81-95-819-7262, Fax: 81-958-49-7270.

E-mail: nhideki@nagasaki-u.ac.jp

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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

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