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.

Acknowledgements

The authors thank Ms. Rie Yamashita for her technical assistance. This work was supported in part by a Grant from The Ministry of Health, Labour and Welfare, Japan.

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.

Study conception and design. H. Nakamura

Salivary gland biopsy: H. Nakamura, Y. Horai, Y. Nakashima

Acquisition of data: H. Nakamura, Y. Takahashi, T. Yamamoto-Fukuda, Y. Horai

Analysis and interpretation of data: H. Nakamura, T. Yamamoto-Fukuda, K. Arima, T. Nakamura, T. Koji, A. Kawakami

REFERENCES

1. Nakamura H, Kawakami A, Eguchi K. Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjögren's syndrome. Transl Res 2006;148:281-8.

2. Terada K, Katamine S, Eguchi K, Moriuchi R, Kita M, Shimada H, et al. Prevalence of serum and salivary antibodies to HTLV-1 in Sjögren's syndrome. Lancet 1994; 22;344(8930):1116-9.

3. Hida A, Imaizumi M, Sera N, Akahoshi M, Soda M, Maeda R, et al. Association of human T lymphotropic virus type I with Sjogren syndrome. Ann Rheum Dis 2010;69:2056-7.

4. Nakamura H, Eguchi K, Nakamura T, Mizokami A, Shirabe S, Kawakami A et al. High prevalence of Sjögren's syndrome in patients with HTLV-I associated myelopathy. Ann Rheum Dis 1997;56:167-72.

5. Nakamura H, Kawakami A, Tominaga M, Hida A, Yamasaki S, Migita K, et al. Relationship between Sjögren's syndrome and human T-lymphotropic virus type I infection: follow-up study of 83 patients. J Lab Clin Med 2000;135:139-44.

6. Eguchi K, Matsuoka N, Ida H, Nakashima M, Sakai M, Sakito S et al. Primary Sjögren's syndrome with antibodies to HTLV-I: clinical and laboratory features. Ann Rheum Dis 1992;51:769-76.

7. Nakamura H, Takagi Y, Kawakami A, Ida H, Nakamura T, Nakamura T et al. HTLV-I infection results in resistance toward salivary gland destruction of Sjögren's syndrome. Clin Exp Rheumatol 2008;26:653-5.

8. Nakamura H, Kawakami A, Hayashi T, Nakamura T, Iwamoto N, Yamasaki S et al. Low prevalence of ectopic germinal centre formation in patients with HTLV-I-associated Sjogren's syndrome. Rheumatology (Oxford). 2009 ;48:854-5.

9. Liu B, Li Z, Mahesh SP, Kurup SK, Giam CZ, Nussenblatt RB. HTLV-1 infection of human retinal pigment epithelial cells and inhibition of viral infection by an antibody to ICAM-1. Invest Ophthalmol Vis Sci 2006 ;47:1510-5.

10. Sakai M, Eguchi K, Terada K, Nakashima M, Yamashita I, Ida H et al. Infection of human synovial cells by human T cell lymphotropic virus type I. Proliferation and granulocyte/macrophage colony-stimulating factor

production by synovial cells. J Clin Invest. 1993;92:1957-66.

11. Ogawa N, Ping L, Zhenjun L, Takada Y, Sugai S. Involvement of the interferon-gamma-induced T cell-attracting chemokines, interferon-gamma-inducible 10-kd protein (CXCL10) and monokine induced by interferon-gamma (CXCL9), in the salivary gland lesions of patients with Sjögren's syndrome. Arthritis Rheum 2002;46:2730-41.

12. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised Version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554-8.

13. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol 1968;21:656-60.

14. Fukushima N, Nakamura T, Nishiura Y, Ida H, Aramaki T, Eguchi K. HTLV-I production based on activation of integrin/ligand signaling in HTLV-I-infected T cell lines derived from HAM/TSP patients. Intervirology. 2008;51:123-9.

15. Nakamura H, Kawakami A, Iwamoto N, Ida H, Koji T, Eguchi K. Rapid and significant induction of TRAIL-mediated type II cells in apoptosis of primary salivary epithelial cells in primary Sjögren's syndrome. Apoptosis 2008 ;13:1322-30.

16. Nakamura H, Kawakami A, Ida H, Koji T, Eguchi K. Epidermal growth factor inhibits Fas-mediated apoptosis in salivary epithelial cells of patients with primary Sjögren's syndrome. Clin Exp Rheumatol 2007 ;25:831-7.

17. Matsuoka E, Takenouchi N, Hashimoto K, Kashio N, Moritoyo T, Higuchi I et al. Perivascular T cells are infected with HTLV-I in the spinal cord lesions with HTLV-I-associated myelopathy/tropical spastic paraparesis: double staining of immunohistochemistry and polymerase chain reaction in situ hybridization. Acta Neuropathol. 1998;96:340-6.

18.Dimitriou ID, Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN. Establishment of a convenient system for the long-term culture and study of non-neoplastic human salivary gland epithelial cells. Eur J Oral Sci. 2002;110:21-30.

19. Talal N, Dauphinée MJ, Dang H, Alexander SS, Hart DJ, Garry RF. Detection

of serum antibodies to retroviral proteins in patients with primary Sjögren's syndrome (autoimmune exocrinopathy). Arthritis Rheum. 1990;33:774-81.

20. Garry RF, Fermin CD, Hart DJ, Alexander SS, Donehower LA, Luo-Zhang H. Detection of a human intracisternal A-type retroviral particle antigenically related to HIV. Science 1990;250:1127-9.

21. Yamano S, Renard JN, Mizuno F, Narita Y, Uchida Y, Higashiyama H et al. Retrovirus in salivary glands from patients with Sjögren's syndrome. J Clin Pathol 1997;50:223-30.

22. Mariette X, Agbalika F, Daniel MT, Bisson M, Lagrange P, Brouet JC et al. Detection of human T lymphotropic virus type I tax gene in salivary gland epithelium from two patients with Sjögren's syndrome. Arthritis Rheum 1993 ;36:1423-8.

23. Mariette X, Agbalika F, Zucker-Franklin D, Clerc D, Janin A, Cherot P et al. Detection of the tax gene of HTLV-I in labial salivary glands from patients with Sjögren's syndrome and other diseases of the oral cavity. Clin Exp Rheumatol 2000;18:341-7.

24. Green JE, Hinrichs SH, Vogel J, Jay G. Exocrinopathy resembling Sjögren's syndrome in HTLV-1 tax transgenic mice. Nature 1989;341(6237):72-4.

25. Lee SJ, Lee JS, Shin MG, Tanaka Y, Park DJ, Kim TJ et al. Detection of HTLV-1 in the labial salivary glands of patients with Sjögren's syndrome: a distinct clinical subgroup? J Rheumatol 2012;39:809-15.

26. Lindholm PF, Reid RL, Brady JN. Extracellular Tax1 protein stimulates tumor necrosis factor-beta and immunoglobulin kappa light chain expression in lymphoid cells. J Virol 1992;66:1294-302.

27. Ospelt C, Neidhart M, Gay RE, Gay S. Synovial activation in rheumatoid arthritis. Front Biosci. 2004;9:2323-34.

28. Zhao LJ, Giam CZ. Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to HTLV-I 21-base-pair repeats by protein-protein interaction. Proc Natl Acad Sci U S A. 1992 ;89:7070-4.

29. Suzuki T, Fujisawa JI, Toita M, Yoshida M. The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive

element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1. Proc Natl Acad Sci U S A. 1993;90:610-4.

30. Jones KS, Petrow-Sadowski C, Huang YK, Bertolette DC, Ruscetti FW. Cell-free HTLV-1 infects dendritic cells leading to transmissionand transformation of CD4(+) T cells. Nat Med. 2008;14:429-36.

31. Jain P, Manuel SL, Khan ZK, Ahuja J, Quann K, Wigdahl B. DC-SIGN mediates cell-free infection and transmission of human T-celllymphotropic virus type 1 by dendritic cells. J Virol. 2009;83:10908-21.

32. Furuya T, Nakamura T, Shirabe S, Nishiura Y, Tsujino A, Goto H et al. Heightened transmigrating activity of CD4-positive T cells through reconstituted basement membrane in patients with human T-lymphotropic virus type I-associated myelopathy. Proc Assoc Am Physicians. 1997;109:228-36.

33. Kulms D, Schwarz T. NF-kappaB and cytokines. Vitam Horm 2006;74:283-300.

34. Liang Y, Zhou Y, Shen P. NF-kappaB and its regulation on the immune

system. Cell Mol Immunol 2004;1:343-50.

35. Delaleu N, Jonsson MV, Appel S, Jonsson R. New concepts in the pathogenesis of Sjögren's syndrome. Rheum Dis Clin North Am 2008;34:833-45

36. Ohyama Y, Nakamura S, Hara H, Shinohara M, Sasaki M, Ikebe-Hiroki A et al. Accumulation of human T lymphotropic virus type I-infected T cells in the salivary glands of patients with human T lymphotropic virus type I-associated Sjögren's syndrome. Arthritis Rheum 1998;41:1972-8.

37. Toulza F, Nosaka K, Tanaka Y, Schioppa T, Balkwill F, Taylor GP et al. Human T-lymphotropic virus type 1-induced CC chemokine ligand 22 maintains a high frequency of functional FoxP3+ regulatory T cells. J Immunol 2010 ;185:183-9.

38. Suri-Payer E, Fritzsching B. Regulatory T cells in experimental autoimmune disease. Springer Semin Immunopathol 2006;28:3-16.

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)**.