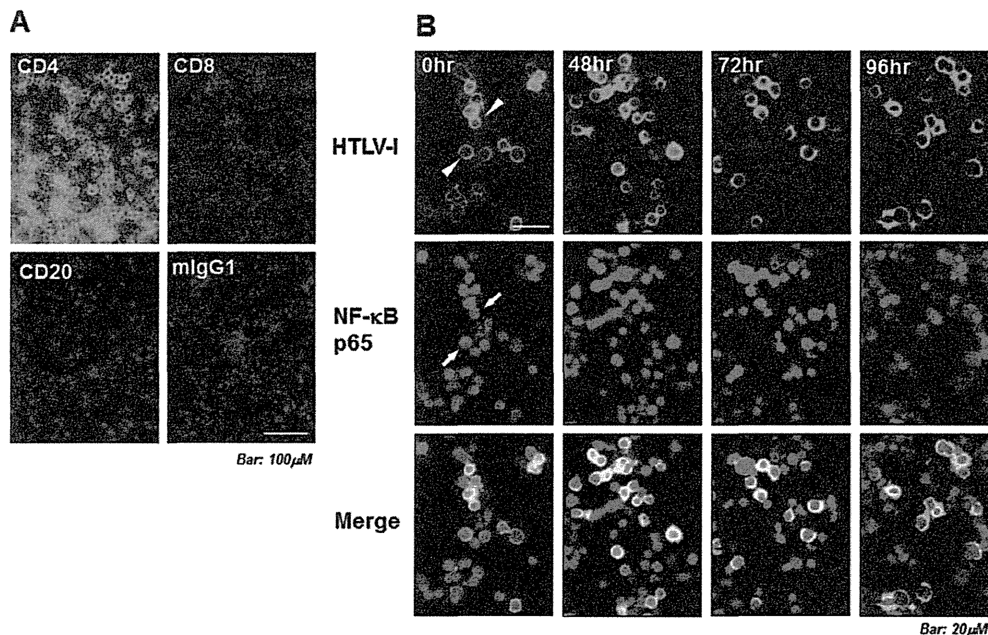


Figure 1. Characterization of the human T lymphotropic virus type I (HTLV-I)–infected HCT-5 T cell line. **A,** Phenotype of HCT-5 cells. After fixation in phosphate buffered saline containing 4% paraformaldehyde at 4°C followed by immersion in methanol at −20°C for 10 minutes, HCT-5 cells were incubated with primary antibodies (anti-CD4, anti-CD8, anti-CD20, and mouse IgG1 [mIgG1]) followed by fluorescein isothiocyanate (FITC)–conjugated secondary antibody and Hoechst 33258 (for counterstaining). **B,** Viability of HCT-5 cells. HCT-5 cells cultured for 0–96 hours in keratinocyte–serum-free medium were fixed and incubated with mouse anti–HTLV-I (p19, p28, and Gag) antibodies and rabbit anti–NF-κB p65 antibody and then incubated with FITC- and tetramethylrhodamine isothiocyanate–conjugated secondary antibodies and Hoechst 33258 (for counterstaining). **Arrowheads** indicate HTLV-I–related proteins including p19, p28, and Gag; **arrows** indicate NF-κB p65 translocation. Results are representative of 2 independent experiments with similar findings.

Cytokine detection in cocultured supernatant. A Proteome Profiler Cytokine Array system was used according to the manufacturer's instructions (R&D Systems). Briefly, after the membranes were blocked, diluted cocultured supernatant was incubated with a cocktail of biotinylated antibodies for 1 hour. The mixture of cytokines, chemokines, and antibodies was then incubated for 2 hours using this array system, 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 (HRP).

Analysis of apoptosis in cocultured lysate. A Proteome Profiler Apoptosis Antibody Array system was used to analyze apoptosis pathways, according to the manufacturer's instructions (R&D Systems). Briefly, diluted cocultured cellular extracts were incubated on membranes for 2 hours after the membranes were blocked for 1 hour. After a 2-hour incubation, a cocktail of biotinylated antibodies was added to the membranes and incubated for 1 hour. Chemiluminescent reagents were then used after incubation with streptavidin–HRP for 30 minutes.

Cytokine and chemokine enzyme-linked immunosorbent assays (ELISAs). The levels of sICAM-1, CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα, and CXCL8/IL-8 (all from R&D Systems) were measured by ELISA, according to the manufacturer's instructions. Briefly, the assigned volume of cell culture supernatant, standard, or control was added to an ELISA well and incubated for the indicated periods of time. After the wells were washed and decanted 3 times, each conjugate was added to a well and incubated for 1 hour at 4°C. After the washing process, substrate solution was added to each well and incubated for 15 minutes. After the addition of stop solution, optical density at 450 nm was measured.

In situ PCR of HTLV-I proviral DNA in cocultured SGECs. Initially, SGECs (alone or in coculture with HCT-5) were fixed in 0.5 ml Carnoy's fixative for 20 minutes at room temperature, followed by washing with 0.5 ml 70% ethanol for 15 minutes at room temperature on type I collagen–coated 12-mm^2 coverslips. After treatment with prewarmed protein kinase (1 μg/ml) at 37°C for 15 minutes and 3 washes with PBS, the SGECs were fixed with 4% PFA/PBS for 5 minutes and then were immersed in 50% formamide/2× saline–sodium citrate buffer at 4°C overnight. After being washed with deuterium-depleted water 3 times for 5 minutes each time, the cells were mixed with an amplification cocktail that consisted of a final concentration of 1× PCR buffer, 1 μg/ml forward primer (5′-CGGATACCCAGTCTACGTGT-3′), 1 μg/ml reverse primer (5′-GAGCCGATAACGCGTCC-3′) (17), 0.2 m*M* dNTP, 2.5 m*M* $MgCl_2$, 1 μ*M* Cy3–dUTP, and distilled water without DNA polymerase, and then boiled for 10 minutes.

Application of these primer sets was previously described

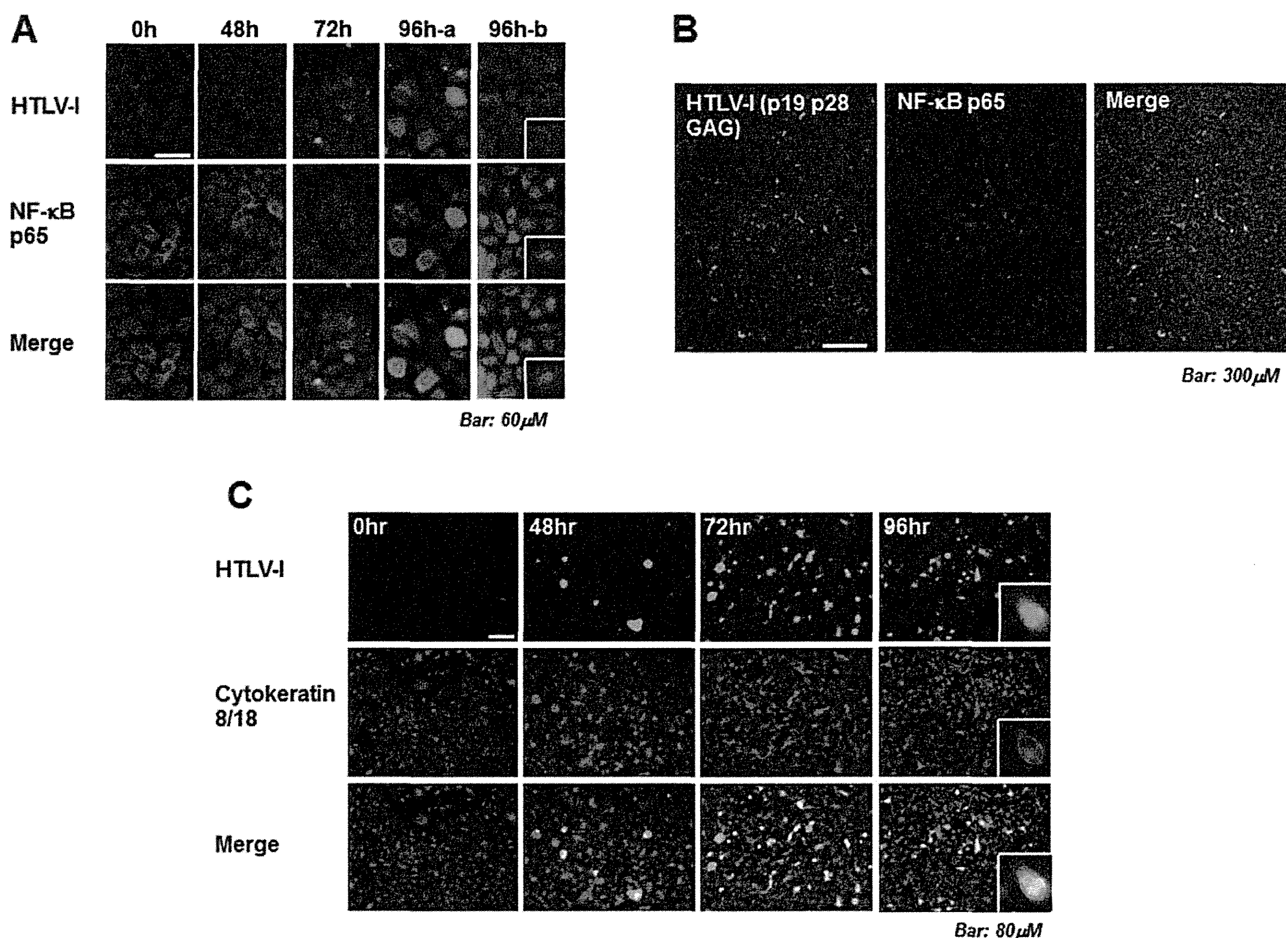


Figure 2. Detection of HTLV-I–related molecules in cocultured salivary gland epithelial cells (SGECs). **A,** Presence of HTLV-I proteins (p19, p28, and Gag), as determined by immunofluorescence analysis. SGECs cocultured for 0–96 hours were fixed in phosphate buffered saline containing 4% paraformaldehyde at 4°C followed by immersion in methanol at −20°C for 10 minutes. The SGECs were initially incubated with anti–HTLV-I antibody and NF-κB p65 followed by FITC- and tetramethylrhodamine isothiocyanate (TRITC)–conjugated secondary antibodies, respectively, and Hoechst 33258 for counterstaining. To contrast the increased expression of HTLV-I proteins without NF-κB translocation (96h-a), translocation of NF-κB is shown (96h-b). **B,** Low-magnification view of cocultured SGECs and HCT-5 cells at 96 hours, showing positive staining for HTLV-I in ~10% of SGECs. **C,** Frequency of HTLV-I–infected SGECs during 0–96-hour coculture, as determined by immunofluorescence analysis. SGECs were initially incubated with anti–HTLV-I antibody and anticytokeratin 8/18 antibody (to distinguish HTLV-I–infected SGECs from HCT-5 cells) followed by FITC- and TRITC-conjugated secondary antibodies, respectively, and Hoechst 33258 for counterstaining. In the merged view, yellow indicates HTLV-I–infected SGECs, and green indicates HCT-5 cells. Results are representative of 3 independent experiments. **Insets** in *A* and *C* show representative cells in each panel. See Figure 1 for other definitions.

in a study by Matsuoka et al, in which the positions of the forward and reverse primers were 7,358–7,377 and 7,516–7,494 of the HTLV-I pX region, respectively (17). After KAPA2G Fast DNA Polymerase complete amplification cocktail (Kapa Biosystems) was added to the SGECs and they were sealed with clear rubber covers, then the coverslips were placed in a thermocycler for in situ PCR (Hybaid). The details of the in situ PCR were as follows: each block was heated at 92°C for 3 minutes, 5 PCR cycles were performed (92°C for 1 minute, 47°C for 1 minute, and 70°C for 2 minutes), and the block was then held at 70°C for 5 minutes. The reacted coverslips were then washed 4 times with 2× saline–sodium citrate at 37°C for 15 minutes, followed by 2 washes with 0.5× saline–sodium citrate at 45°C for 15 minutes. After the coverslips were reacted with PBS once and covered with Vectashield mounting medium, SGECs were visualized using fluorochrome with a BIOREVO BZ-9000 fluorescence microscope.

Statistical analysis. Differences in ELISA results were analyzed using Student's *t*-test. *P* values less than 0.05 were considered significant.

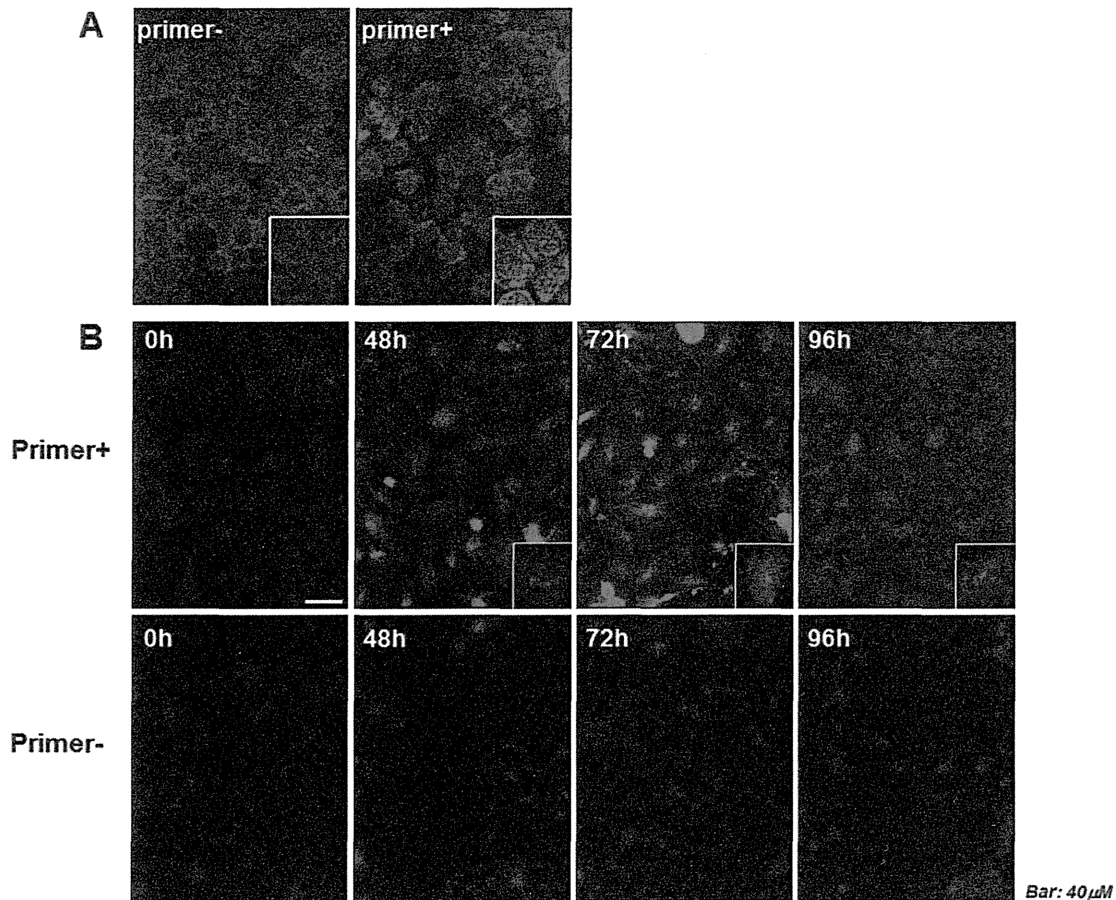


Figure 3. Detection of human T lymphotropic virus type I (HTLV-I) proviral DNA by in situ polymerase chain reaction (PCR). **A,** HTLV-I was detected in HCT-5 cells as a positive control. HCT-5 cells were treated with 1 μg/ml of protein kinase, and 5 cycles of in situ PCR were performed. Detected HTLV-I proviral DNA is shown as a granular pattern signal. **B,** Fixed salivary gland epithelial cells were treated with 1 μg/ml of protein kinase, and 5 cycles of in situ PCR were then performed in the presence or absence of primers for the HTLV-I pX region. Detected signal is indicated as the appearance of dots in the nucleus of salivary gland epithelial cells. Results are representative of 2 independent experiments with similar findings. **Insets** show representative cells in each panel.

RESULTS

Phenotype and viability of HCT-5 cells. The HCT-5 cells used for coculture with SGECs showed the CD4+ phenotype (Figure 1A), with no staining for CD8 or CD20. The HCT-5 cells cultured for 0–96 hours in keratinocyte–SFM were viable, with translocation of nuclear NF-κB into SGECs after coculture (Figure 1B).

Detection of HTLV-I–related proteins in SGECs during coculture. After coculture of SGECs and HCT-5 cells, immunofluorescence analysis showed clear signals for HTLV-I proteins p19, p28, and Gag at 72–96 hours (Figure 2A). At 96 hours, ~10% of the SGECs cocultured with HCT-5 cells showed HTLV-I–positive staining at low magnification (Figure 2B). Nuclear NF-κB p65 was also detected in 10% of the SGECs after coculture (Figures 2A and B). To distinguish HTLV-I–infected SGECs from HCT-5 cells, SGECs were stained with cytokeratin 8/18 antibodies (Figure 2C), which was reported to be one of markers for SGECs (18). In merged view (yellow signal), the mean ± SEM frequency of HTLV-I–infected SGECs was calculated as 7.8 ± 1.3%, and the remaining HCT-5 cells (green signal) were observed during coculture for 48–96 hours.

Detection of HTLV-I DNA in SGECs by in situ PCR. To clarify whether HTLV-I infected SGECs during coculture with HCT-5 cells and investigate the details, we determined HTLV-I DNA expression. As a positive control, HTLV-I proviral DNA was detected in HCT-5 cells (Figure 3A).

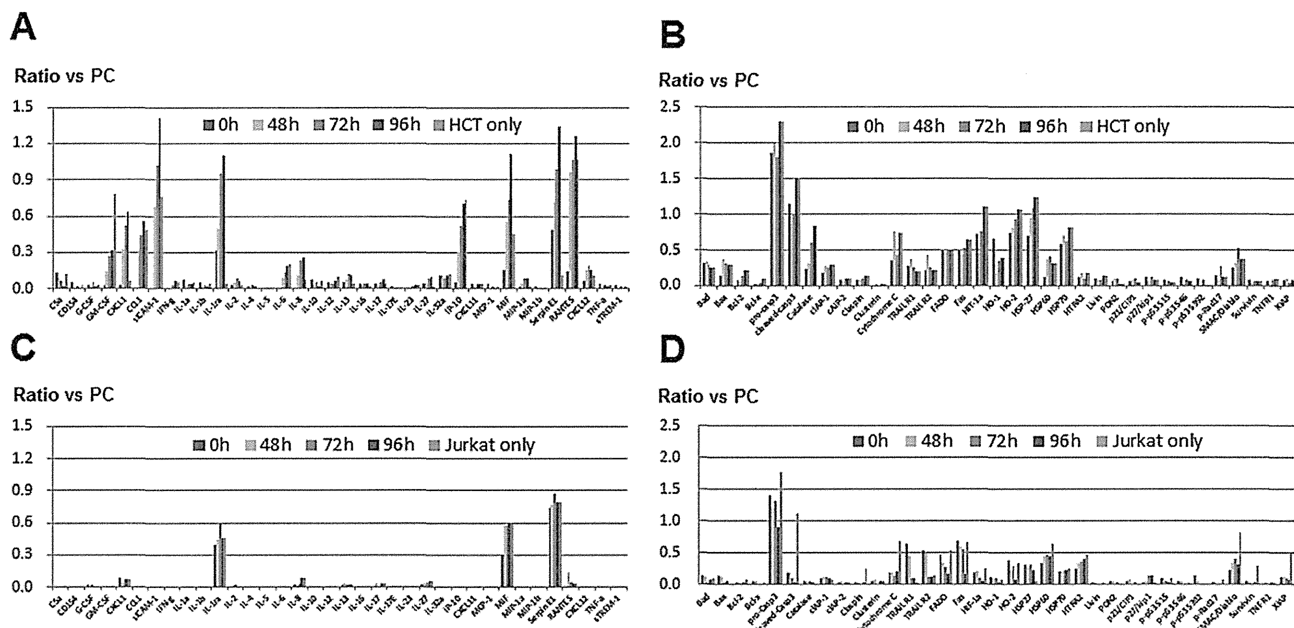


Figure 4. Expression of inflammation-related molecules and apoptosis-related molecules. **A** and **C**, Expression of inflammation-related molecules in supernatant of salivary gland epithelial cells (SGECs) cocultured with HCT-5 cells (**A**) or Jurkat cells (**C**). The semiquantitative concentrations of each molecule in culture medium (i.e., keratinocyte–serum-free medium) after coculture with HCT-5 cells or Jurkat cells is shown. "HCT-5 only" and Jurkat only" indicate the culture supernatant for HCT-5 cells and Jurkat cells, respectively. **B** and **D**, Expression of apoptosis-related molecules in lysate of SGECs cocultured with HCT-5 cells (**B**) or Jurkat cells (**D**). The semiquantitative concentration of each molecule in recovered SGEC lysate after coculture with HCT-5 cells or Jurkat cells is shown. Results are representative of 2 independent experiments with similar findings. PC = positive control.

During coculture, amplified HTLV-I DNA was observed in the nucleus of SGECs in the presence of primer at 48 hours of coculture with HCT-5 cells (Figure 3B). The strongest HTLV-I DNA signal was observed in the presence of primer at 72 hours of coculture.

Increased expression of inflammation-related molecules and apoptosis-related molecules in cocultured SGECs. As shown in Figure 4A, the expression of GM-CSF, CXCL1/GROα, CCL1, sICAM-1, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, CXCL10/IP-10, macrophage migration inhibitory factor (MIF), serpin E1, and CCR5/RANTES in cocultured HCT-5/SGEC supernatant increased in a time-dependent manner. The results of apoptosis analysis using SGEC lysate cocultured with HCT-5 are shown in Figure 4B. The responses of proapoptotic molecules including procaspase 3, cytochrome c, and Fas in the lysate were slightly increased after coculture of SGECs with HCT-5 cells. The signals for antiapoptotic molecules including Bcl-2, HO-2, Hsp27, or second mitochondria-derived activator of caspases (SMAC)/Diablo were also up-regulated after coculture.

As shown in Figures 4C and D, the expression of IL-1Ra, MIF, and serpin E1 was increased after coculture of SGECs with Jurkat cells; however, the increase was not time dependent (Figure 4C). In contrast to the results of coculture of SGECs with HCT-5, expression of other molecules including GM-CSF, CXCL1/GROα, CCL1, sICAM-1, IL-6, IL-8, and CXCL10/IP-10 was not increased following coculture of SGECs with Jurkat cells. The results of the apoptosis analysis using SGEC lysate cocultured with Jurkat cells are shown in Figure 4D. Although expression of procaspase 3 and SMAC/Diablo was similar to that observed in HCT-5/SGEC coculture, expression of cytochrome c, Fas, Bcl-2, HO-2 and Hsp27 was not up-regulated after coculture of SGECs with Jurkat cells.

The data derived from analyses using a cytokine array system and an apoptosis antibody array system were confirmed by immunofluorescence analysis and ELISA. Immunofluorescence analysis showed increased cytoplasmic expression of ICAM-1, CXCL1, RANTES, IL-8, and IP-10 (Figure 5A), with augmentation of the signals for HTLV-I p19, p28, and Gag in SGECs after

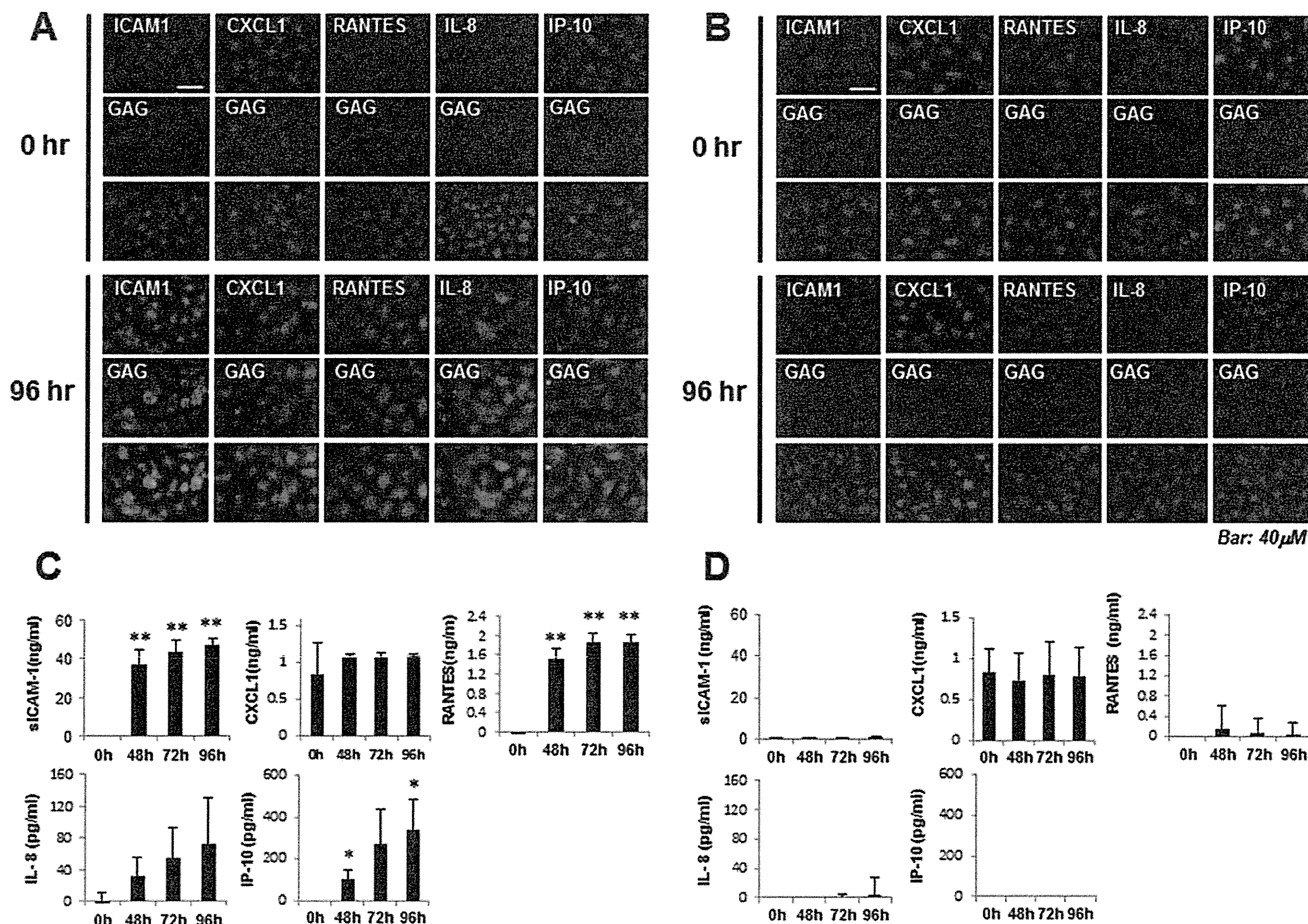


Figure 5. Increased expression of inflammation-related molecules in coculture supernatant, as confirmed by immunofluorescence analysis and enzyme-linked immunosorbent assay (ELISA). **A** and **B,** Expression of intercellular adhesion molecule 1 (ICAM-1), CXCL-1, RANTES, interleukin-8 (IL-8), and interferon-γ–inducible 10-kd protein (IP-10) in salivary gland epithelial cells (SGECs) cocultured with HCT-5 cells (**A**) or Jurkat cells (**B**), as determined by immunofluorescence analysis. The third rows in each panel show merged views. Yellow signal indicates coexpression of signal from top and middle rows. Results are representative of 2 independent experiments with similar findings. GAG = human T lymphotropic virus type I–related proteins p19, p28, and Gag. **C** and **D,** Concentrations of soluble ICAM-1 (sICAM-1), CXCL1/GROα, CCR5/RANTES, CXCL8/interleukin-8 (IL-8) and CXCL10/IP-10, in supernatant of SGECs cocultured with HCT-5 cells (**C**) or Jurket cells (**D**), as determined by ELISA. Values are the mean ± SD (n = 3 samples). * = $P < 0.05$; ** = $P < 0.01$ versus 0 hour, by Student's *t*-test.

96-hour coculture with HCT-5 cells. However, the expression of ICAM-1, CXCL1, RANTES, IL-8, and IP-10 was not increased when SGECs were cocultured with Jurkat cells (Figure 5B). Accordingly, significant increases in the expression of sICAM-1, RANTES, and IP-10 in the cocultured supernatant were confirmed by ELISA (Figure 5C). In SGEC/Jurkat cell coculture supernatant, however, no significant increase in sICAM-1, RANTES, and IP-10 expression was observed (Figure 5D). Furthermore, these molecules as well as IL-8 were scarcely detectable in SGEC/Jurkat cell coculture supernatant. The results of immunofluorescence analysis of apoptosis-related molecules also showed that the membranous expression of Fas on SGECs as well as the cytoplasmic expression of Bcl-2, cytochrome c, HO-2, and Hsp27 were up-regulated after 96-hour coculture with HCT-5 (Figure 6A). Compared with the results for HCT-5/SGEC coculture, no increase in apoptosis-related molecules was observed on SGECs cocultured with Jurkat cells (Figure 6B).

No detection of apoptosis of cocultured SGECs. We previously reported that cultured SGECs are committed to apoptosis by several stimuli (15,16). Because in the present study the expression of proapoptotic mole-

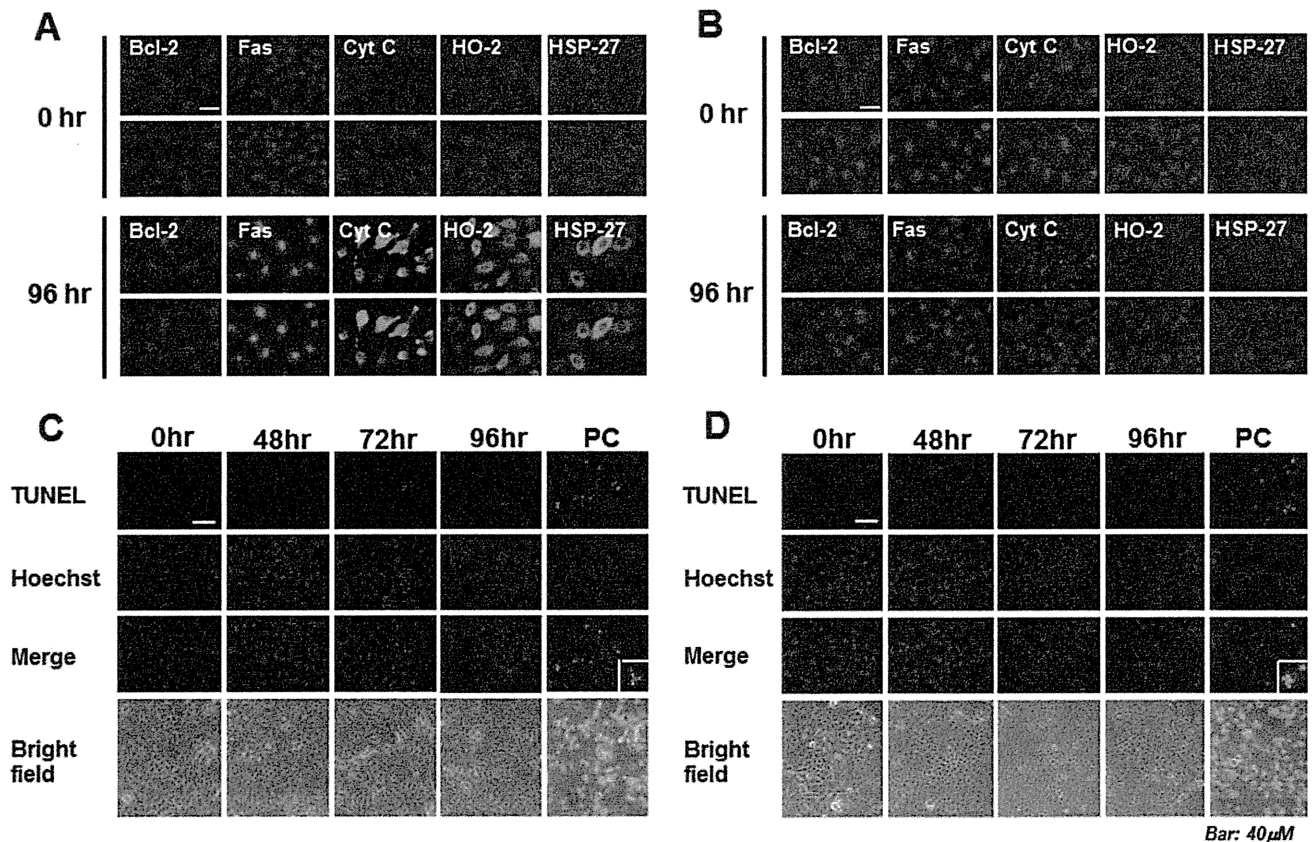


Figure 6. Expression of apoptosis-related molecules in cocultured salivary gland epithelial cells (SGECs). **A** and **B,** Expression of Bcl-2, Fas, cytochrome c (Cyt C), heme oxygenase 2 (HO-2), and Hsp27 in supernatant of SGECs cocultured with HCT-5 cells (**A**) or Jurkat cells (**B**), as determined by immunofluorescence analysis. **C** and **D,** Apoptosis of SGECs as evaluated by TUNEL staining. SGECs were cocultured with HCT-5 cells (**C**) or Jurkat cells (**D**) for 0–96 hours, followed by fixation in phosphate buffered saline containing 4% paraformaldehyde at 4°C and immersion in methanol at −20°C for 10 minutes, and were analyzed for TUNEL staining; Hoechst 33258 was used for nuclear staining. The fluorescein isothiocyanate–conjugated green signal indicates the presence of TUNEL-positive cells. As a positive control (PC), the SGECs were treated with TRAIL for 3 hours. Results are representative of 2 independent experiments with similar findings.

cules was increased by coculture with HCT-5 cells, it could be speculated that coculture with HCT-5 cells might induce apoptosis of SGECs. As we showed previously, the number of TUNEL-positive cells was clearly increased in SGECs stimulated with TRAIL (Figure 6C). In contrast, during 0–96-hour coculture, no TUNEL-positive staining was observed in SGECs cocultured with HCT-5 cells (Figure 6C). In addition, no obvious morphologic change was observed on brightfield views during coculture. Similarly, no TUNEL-positive staining was observed in SGECs during 0–96-hour coculture with Jurkat cells (Figure 6D).

DISCUSSION

With regard to the relationship between primary SS and retrovirus, Talal et al first reported that serum antibodies against human immunodeficiency virus 1 were detected in 30% of sera from patients with primary SS (19), and the presence of retroviral particles was observed in salivary gland tissue from patients with SS (20). Retroviral particles were also observed in the LSGs of patients with SS (21).

Regarding HTLV-I infection in patients with primary SS, Mariette et al reported the presence of the HTLV-I *tax* gene in the LSGs of patients with primary SS; however, the LSGs of patients with other inflammatory diseases also contained this gene, suggesting that the HTLV-I *tax* gene contributes to the development of chronic inflammatory diseases including primary SS (22,23). In addition, Green et al reported 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 study showed that HTLV-I p19 or Tax protein was expressed in 42.4% of LSG samples from 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 accumulating evidence of a 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 <10% among cocultured SGECs. The migration of HTLV-I into SGECs was suggested to induce functional alterations of SGECs, because some of the SGECs became positive for nuclear NF-κB p65, which is a transcription factor known to be activated by HTLV-I (26). Accordingly, the production of several inflammatory cytokines and chemokines increased during coculture of SGECs with HCT-5 cells in the current study. However, one or more pathways other than direct infection of SGECs by HTLV-I may be used, because a substantial population of SGECs showed no staining for HTLV-I–related proteins, HTLV-I proviral DNA, or nuclear NF-κB p65 after coculture. Autocrine or paracrine interactions of cytokines and chemokines might be involved in these processes, in which cytokines and chemokines induce the production reciprocally (27).

Alternatively, transcription factors or activators other than NF-κB p65, such as CREB/activating transcription factor and CREB-binding protein, which serves as a transcription activator, might be essential (28,29). Whether unique changes induced by HCT-5 are consequences attributable to direct infection of SGECs by HTLV-I or are an indirect effect of the molecules produced by neighboring activated cells (including HCT-5) is a crucial issue. In coculture, SGECs are spindle-shaped, and the intensity of Gag staining is not as strong as that in HCT-5 cells, suggesting that SGECs are distinguishable from HCT-5 cells. Some SGECs became double positive with Gag and inflammatory molecules in the coculture (Figure 5A). Because coculture of SGECs with the non–HTLV-I–infected T cell line Jurkat did not induce changes in the expression of functional molecules, cell-free HTLV-I virions might contribute to the changes in SGECs. Although no evidence of cell-free transmission of HTLV-I to any epithelial cells has been reported, HTLV-I virions have the potential to infect myeloid and plasmacytoid dendritic cells (DCs) (30). A previous study also showed that DC-SIGN plays an important role in cell-free HTLV-I infection of DCs (31). Further studies investigating cell-free infection of SGECs by HTLV-I virions are needed in the future.

In the current study, in addition to inflammatory cytokines and chemokines, both proapoptotic and antiapoptotic molecules were augmented in SGECs after coculture with HCT-5 cells compared with coculture with Jurkat cells. However, we should also note that the results of the apoptosis analysis might be influenced by the remaining HCT-5 cells during coculture. As shown in Figure 2C, HCT-5 cells attached to SGECs during coculture, and ~5% of HCT-5 cells still remained at 96 hours. It is possible that HCT-5 cells remained in coculture because these cells had migratory and adhesive capacity; we previously reported that CD4+ T cells derived from patients with HAM showed strong transmigrating activity (32).

The increased expression of these molecules may be induced via activation of transcription factors including NF-κB p65 or by the cytokines and chemokines produced by SGECs themselves. It has been demonstrated that the expression of both proapoptotic molecules and antiapoptotic molecules is regulated by the mechanisms described above (33,34). Increases in the expression of antiapoptotic molecules such as Bcl-2, HO-2, and Hsp27 might antagonize the apoptosis-inducing capacities of Fas and cytochrome c in SGECs, indicating that apoptosis does not occur in SGECs.

It is interesting to note that HTLV-I infection of SGECs induces the niche of SS, because the expression pattern of cytokines, chemokines, proapoptotic molecules, and antiapoptotic molecules of SGECs cocultured with HCT-5 cells in vitro resembles the pattern observed in vivo in the salivary glands of SS patients (35). However, it is debatable whether the present in vitro results truly reflect in vivo observations in patients with anti–HTLV-I antibody–positive SS. In this regard, Ohyama et al reported that HTLV-I proviral DNA was not present in either acinar cells or ductal epithelial cells in the LSGs of patients with HTLV-I antibody–positive SS but was observed in the infiltrating T lymphocytes, as demonstrated by in situ PCR (36).

It has become evident that CD4+ T cells infected by HTLV-I resemble FoxP3+ Treg cells (37). Regulatory T cells produce regulatory cytokines such as IL-10 and transforming growth factor β (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 in patients with SS.

In summary, we have shown that direct infection of human primary SGECs by HTLV-I induces the niche of the salivary glands in patients with SS. Our clinical and histologic examinations also revealed the characteristics of anti–HTLV-I antibody–positive SS patients, including the low rate of ectopic GC formation in LSGs and parotid gland destruction (7,8). Although the exact pathways used by HTLV-I in SS remain unclear, this study is the first investigation in humans showing that HTLV-I infects SGECs and thus has an impact on the induction of SS.

ACKNOWLEDGMENT

We thank Ms Rie Yamashita for providing technical assistance.

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 to be published. Dr. H. Nakamura had 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.

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

Analysis and interpretation of data. H. Nakamura, Yamamoto-Fukuda, Nakashima, Arima, T. Nakamura, Koji, 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;344:1116–9.
3. Hida A, Imaizumi M, Sera N, Akahoshi M, Soda M, Maeda R, et al. Association of human T lymphotropic virus type I with Sjögren 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 Sjögren'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-γ–induced T cell–attracting chemokines, interferon-γ–inducible 10-kd protein (CXCL10) and monokine induced by interferon-γ (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, and the European Study Group on Classification Criteria for Sjögren's Syndrome. 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, Dauphinee 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; 341:72–4.
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 Tax_1 protein stimulates tumor necrosis factor-β and immunoglobulin κ 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 transmission and 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-cell lymphotropic 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-κB and cytokines. Vitam Horm 2006;74: 283–300.
34. Liang Y, Zhou Y, Shen P. NF-κB 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.

Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2015; 25(6): 876–882
© 2015 Japan College of Rheumatology
DOI: 10.3109/14397595.2015.1034941

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Rapid decrease in salivary epidermal growth factor levels in patients with Sjögren's syndrome: A 3-year follow-up study

Naoto Azuma[1], Yoshinori Katada[2], Sachie Kitano[1], Masahiro Sekiguchi[1], Masayasu Kitano[1], Aki Nishioka[1], Naoaki Hashimoto[1,3], Kiyoshi Matsui[1], Tsuyoshi Iwasaki[1,4], and Hajime Sano[1]

[1]*Division of Rheumatology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan,* [2]*Division of Kidney, Metabolism and Immunity, Department of Internal Medicine, Sakai City Hospital, Sakai, Japan,* [3]*Hashimoto Clinic for Rheumatic Diseases, Osaka, Japan, and* [4]*Division of Pharmacotherapy, Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, Kobe, Japan*

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

Abstract

Objectives. To assess changes in salivary epidermal growth factor (EGF) levels within three years and investigate the correlation between these changes and the severity of intraoral manifestations in patients with Sjögren's syndrome (SS).
Methods. Twenty-three SS patients (14 primary SS and 9 secondary SS) and 14 controls were followed up for three years. Salivary EGF concentration was measured using an enzyme-linked immunosorbent assay, and intraoral manifestations were evaluated using a short version of the Oral Health Impact Profile (OHIP-14). Changes in salivary flow rate, EGF level, and severity of intraoral manifestations were analyzed, along with associations among them.
Results. The OHIP-14 score significantly increased and the total salivary EGF output significantly decreased after three years in the SS group (10.2 ± 8.8 vs. 12.6 ± 9.2, $p = 0.040$; 10158.4 ± 9820.9 vs. 8352.8 ± 7813.3 pg/10 min, $p = 0.032$), though the salivary flow rate did not change. The decrease in total EGF output was especially high in patients with long disease duration and poor oral health-related quality of life (OHRQoL). In patients with poor OHRQoL, the change in total EGF output significantly correlated with the OHIP-14 score ($r = -0.847$, $p = 0.008$). However, there was no correlation between the change in salivary flow rate and the OHIP-14 score.
Conclusions. The rapid decrease in salivary EGF level contributes to the progression of intraoral manifestations of SS.

Keywords

Epidermal growth factor, Intraoral manifestation, Oral mucosal involvement, Saliva, Sjögren's syndrome

History

Received 26 February 2015
Accepted 22 March 2015
Published online 28 May 2015

Introduction

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, especially the salivary and lacrimal glands. As a result of salivary gland dysfunction, most patients with SS have xerostomia caused by a reduced salivary flow rate. In addition to discomfort, xerostomia can cause various intraoral manifestations, for example, dental caries and oral mucosal involvements, such as refractory stomatitis, oral ulcer, and atrophic changes in the oral mucosa and lingual papilla. In the chronic form, these involvements can severely impair the patients' quality of life (QoL) severely [1]. The intraoral manifestations in SS patients are believed to be caused mainly by the decreased clearance in the oral cavity owing to hyposalivation. However, since saliva has several beneficial physiological effects on the environment inside the oral cavity, such as lubrication, maintenance of mucosal integrity, and antimicrobial activity [2], qualitative changes in its composition should also be considered a cause of the refractory intraoral manifestations in SS.

Epidermal growth factor (EGF), which accelerates incisor eruption and eyelid opening in newborn animals, was first isolated from mouse submandibular glands [3]. EGF is a polypeptide comprising 53 amino acids (molecular weight, 6.045 kDa) that promotes the growth of various tissues in several species [4]. In humans, EGF is produced by the salivary glands and duodenal Brunner's glands [5], and the main source of EGF in the oral cavity is the parotid glands [4,6]. However, salivary EGF has been found to be secreted not only from the parotid and submandibular glands but also from the sublingual or minor salivary gland [4,6,7]. EGF binding to the EGF receptor (EGFR) stimulates the activity of intracellular kinase cascades, producing signals that consequently alter gene regulation, which leads to cell proliferation and anti-apoptogenic survival [8,9]. Salivary EGF is considered an important cytoprotective factor against injuries, and it contributes to wound healing and maintenance of mucosal integrity in the oral cavity [10,11] and gastrointestinal tract [12]. Several studies have found that salivary EGF levels are significantly decreased in patients with intraoral inflammatory lesions, such as stomatitis aphthous [4,13] and peritonsillar abscess [4]. In addition, patients with oral mucositis induced by radiation therapy for head and neck carcinoma were also found to have markedly low salivary EGF levels [14,15]. These findings suggested that low salivary EGF levels reduce the capacity of the oral mucosa to heal after injury and maintain its physiological integrity.

Although salivary EGF levels in SS patients have not been thoroughly investigated, we previously reported that the salivary flow

Correspondence to: Naoto Azuma, MD, PhD, Division of Rheumatology, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. Tel: +81-798-45-6591. Fax: +81-798-45-6593. E-mail: naoazuuh@hyo-med.ac.jp

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

rate and EGF levels decreased with the progression of SS, and this deterioration in saliva quality as well as hyposalivation could play a role in the pathogenesis of refractory intraoral manifestations in SS patients [16]. The objective of the present study was to evaluate changes in salivary EGF levels in the course of three years in the same SS patients and to assess the association between these changes and the severity of intraoral manifestations in SS.

Materials and methods

Patients

Twenty-three patients with SS and fourteen individuals without SS serving as controls (non-SS group) who participated in our previous study [16] and were subsequently followed up for three years at the Division of Rheumatology, Hyogo College of Medicine Hospital, were enrolled in this study. Of these, 14 had primary SS and 9 had secondary SS (comorbidities: rheumatoid arthritis [RA; $n = 3$], systemic lupus erythematosus [SLE; $n = 3$], systemic sclerosis [$n = 2$], and mixed connective tissue disease [$n = 1$]). All the patients fulfilled the American-European Consensus Group classification criteria for SS [17]. No significant differences were observed in age and sex between the primary SS group (mean age, 61.4 ± 13.7 years [range, 38–83 years], 12 women and 2 men) and the secondary SS group (56.4 ± 11.0 years [38–68 years], 9 women) ($p = 0.185$ and $p = 0.502$, respectively). The non-SS group consisted of patients with RA ($n = 6$), polymyalgia rheumatica ($n = 2$), dermatomyositis ($n = 1$), bronchial asthma ($n = 1$), SLE ($n = 1$), adult-onset Still's disease ($n = 1$), relapsing polychondritis ($n = 1$), and SAPHO or synovitis, acne, pustulosis, hyperostosis, and osteitis syndrome ($n = 1$). The exclusion criteria related to factors that affect the intraoral environment or saliva secretion and salivary EGF level were as follows: current smoking; chronic alcohol use; ongoing dental treatment; recurrent oral mucositis due to conditions other than SS; treatment with anti-Parkinsonism drugs or psychiatric drugs such as antidepressants, anti-anxiety agents, and antipsychotic agents; severe diabetes mellitus; severe reflux esophagitis; history of head and neck carcinoma; previous radiation therapy of the head and neck region; and previous chemotherapy for cancer. This study was approved by the ethics committee of Hyogo College of Medicine, and all subjects provided written informed consent for participation in this study.

Saliva collection

Whole stimulated saliva was obtained in the same manner as in our previous study [16]. In particular, the subjects chewed gum (Free Zone Gum Hi-Mint®; Lotte, Tokyo, Japan) for 10 min, and expectorated saliva was collected into graduated centrifuge tubes. All samples were collected in a similar way around the same time before breakfast in the morning, with fasting, because salivary EGF concentrations show apparent changes related to food intake [4]. The final saliva volume was measured, and the samples were then centrifuged at 3000 rpm for 10 min. The supernatants were stored at −80°C until being used in the assay.

Quantification of salivary EGF

Just before the EGF measurement, the saliva was thawed and centrifuged for 10 min at 3000 rpm at 4°C. Salivary EGF levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®; R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Each sample was assayed in duplicates. EGF concentrations were measured by determining the optical density of the sample against a standard curve. Total salivary EGF output (pg/10 min) was calculated by multiplying salivary EGF concentration (pg/ml) by saliva volume (ml/10 min) [15].

Assessment of intraoral manifestations

At the time of saliva collection, subjective intraoral manifestations were assessed by means of the short Japanese version of the Oral Health Impact Profile (OHIP-14), a self-administered questionnaire, as described in our previous study [16]. The OHIP is one of the most widely used instruments to measure oral health-related QoL (OHRQoL), and its translated Japanese version (OHIP-J) has good reliability and validity [18,19]. However, since the original OHIP [20] contains 49 items, clinicians may be reluctant to use it in daily clinical practice. This led to the development of the shorter version OHIP-14, which has only 14 questions designed to measure the frequency of problems associated with the teeth, mouth, or dentures. The questions assess seven aspects: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability, and handicap. OHIP-14 has been shown to retain good reliability and validity [21]. The answers to the questions were given using a five-point scale ranging from 0 to 4 (0, never; 1, hardly ever; 2, occasionally; 3, fairly often; and 4, very often). The unweighted scores were then combined to obtain a single summary score with a possible range of 0–56, with a higher score indicating more frequent problems, that is, poorer OHRQoL. It has been demonstrated by Stewart et al. that in SS patients lower salivary flow rates are significantly associated with poorer oral health as determined using the OHIP-14 summary score [1].

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The Mann–Whitney U test, chi-square test, or Fisher's exact test was used, as appropriate, to compare differences between the SS group and the control group. The Wilcoxon signed-rank test, chi-square test, or Fisher's exact test was used, as appropriate, to evaluate changes between the results of the initial evaluation performed in our previous study [16] and the reevaluation three years later. The correlations were examined using the Spearman's rank correlation coefficient. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Comparison of the clinical data obtained at baseline and reevaluation

The characteristics of the study groups are presented in Table 1. No significant differences in age and sex were observed between the SS and the non-SS groups. The mean SS disease duration at baseline (initial evaluation) was 5.5 years.

SS group (Table 2a)

There were no significant differences in the number of patients treated with muscarinic M3 receptor agonists (pilocarpine or cevimeline), corticosteroids, or immunosuppressants (e.g., tacrolimus, methotrexate, and mycophenolate mofetil) between baseline and reevaluation. The dose of the corticosteroid did not appreciably change within three years (prednisolone (PSL): 3 − 12.5 mg/day vs. 1 − 12.5 mg/day at the starting and reevaluation points, respectively). The OHIP-14 score at reevaluation (12.6 ± 9.2) was significantly higher than that at baseline (10.2 ± 8.8) ($p = 0.040$), indicating a significant exacerbation of the OHRQoL. No significant difference was observed in the salivary flow rate at baseline (8.1 ± 5.3 ml/10 min) and reevaluation

Table 1. Clinical characteristics of the study groups.

	SS[a] ($N = 23$)	Non-SS ($N = 14$)	p value
Age (years) (range)	59.5 ± 12.7 (38–83)	59.9 ± 15.2 (33–81)	0.468
Sex (male/female, number)	2/21	4/10	0.258
Disease duration[b] (years) (range)	5.5 ± 3.9 (0.2–11; $N = 13$)	–	–
Dry eye symptoms (number [%])	20 (87)	0 (0)	< 0.0001
Xerostomia symptoms (number [%])	21 (91)	2 (14)	< 0.0001
Anti-SS-A antibody (number [%])	21 (91)	0 (0)	< 0.0001
Anti-SS-B antibody (number [%])	5 (22)	0 (0)	0.135

[a]Sjögren's syndrome.
[b]Disease duration was measured at baseline (initial evaluation).

(7.4 ± 5.1 ml/10 min) ($p = 0.149$). However, the total salivary EGF output at reevaluation (8352.8 ± 7813.3 pg/10 min) was significantly lower than that at baseline (10158.4 ± 9820.9 pg/10 min) ($p = 0.032$) (Table 2a). There were no significant differences in changes in the OHIP-14 score, salivary flow rate, and total salivary EGF output between primary SS patients (2.3 ± 6.3 ml/10 min, − 0.3 ± 3.1 ml/10 min, and −273.4 ± 2747.8 pg/10 min, respectively) and secondary SS patients (2.4 ± 5.2 ml/10 min, − 1.2 ± 3.0 ml/10 min, and −2633.4 ± 4540.4 pg/10 min, respectively) ($p = 0.479$, $p = 0.256$, and $p = 0.189$, respectively). In addition, treatment with muscarinic M3 receptor agonists, corticosteroids, or immunosuppressants did not positively affect the OHRQoL, salivary flow rate, and total salivary EGF output. No significant differences were observed in changes in the OHIP-14 score, salivary flow rate, and total salivary EGF output between muscarinic M3-receptor-agonists-treated patients (2.7 ± 6.1 ml/10 min, −0.9 ± 1.5 ml/10 min, and − 1936.2 ± 3381.8 pg/10 min, respectively) and untreated patients (1.8 ± 5.3 ml/10 min, −0.3 ± 4.6 ml/10 min, and − 1602.5 ± 3935.8 pg/10 min, respectively) ($p = 0.357$, $p = 0.336$, and $p = 0.415$, respectively), or between corticosteroids or immunosuppressants-treated patients (1.9 ± 6.6 ml/10 min, −0.6 ± 3.0 ml/10 min, and − 1009.9 ± 3082.1 pg/10 min, respectively) and untreated patients (2.6 ± 5.4 ml/10 min, −0.7 ± 3.1 ml/10 min, and −2230.0 ± 3769.7 pg/10 min, respectively) ($p = 0.385$, $p = 0.455$, and $p = 0.221$, respectively).

Non-SS group (Table 2b)

None of the patients in the non-SS group received muscarinic M3 receptor agonists, while all of them were administered corticosteroids or immunosuppressants (e.g., methotrexate and etanercept) both at baseline and reevaluation. The dose of PSL did not change substantially within three years (2 − 10 mg/day vs. 0.5 − 8 mg/day at baseline and reevaluation, respectively). No significant differences were observed in the OHIP-14 score, salivary flow rate, and total salivary EGF output at baseline(9.1 ± 6.8 ml/10 min, 16.8 ± 6.7 ml/10 min, and 13623.1 ± 9546.2 pg/10 min, respectively) and reevaluation (10.7 ± 9.4 ml/10 min, 16.7 ± 6.0 ml/10 min, and 11904.9 ± 6995.4 pg/10 min, respectively) ($p = 0.169$, $p = 0.628$, and $p = 0.184$, respectively)(Table 2b).

Changes in salivary flow rate and total EGF output according to disease duration and OHRQoL

The clinical background varied widely among the SS patients. Therefore, the SS group was subdivided into two groups depending on two clinical factors.

Disease duration

The SS group was divided into the subgroups with short and long disease duration. The cut-off level was provisionally set at 5 years at the starting point based on the mean disease duration in the entire SS group (≤5 years: short duration group [$n = 7$; mean disease duration, 2.1 ± 1.1 years]; ≥6 years: long duration group [$n = 6$; 9.3 ± 1.5 years]). In the short duration group, no significant differences were observed in the salivary flow rate and total salivary EGF output between baseline (10.5 ± 7.0 ml/10 min and 15646.9 ± 12986.2 pg/10 min, respectively) and reevaluation (9.4 ± 5.4 ml/10 min and 13187.6 ± 9902.1 pg/10 min, respectively) (Figure 1a). In contrast, although the salivary flow rate did not differ significantly at baseline (3.6 ± 1.4 ml/10 min) and reevaluation (3.3 ± 1.6 ml/10 min) in the long duration group, the total salivary EGF output at reevaluation (1640.9 ± 1774.8 pg/10 min) was significantly lower than that at baseline (3652.4 ± 4211.2 pg/10 min) ($p < 0.05$) (Figure 1b).

OHRQoL

The SS group was subdivided on the basis of the magnitude of changes in the OHIP-14 scores after three years into the

Table 2. Clinical characteristics at the initial evaluation and reevaluation three years later.

	Baseline (initial evaluation)	3 years later (reevaluation)	p value
a. SS[a] group ($N = 23$)			
Muscarinic M3 receptor agonist (number [%])	12 (52)	14 (61)	0.766
Corticosteroid or immunosuppressant (number [%])	6 (26)	8 (35)	0.749
OHIP-14[b] score (out of 56) (range)	10.2 ± 8.8 (0–39; $N = 22$)	12.6 ± 9.2 (0–32; $N = 22$)	0.040
Salivary flow rate (ml/10 min) (range)	8.1 ± 5.3 (1.0–21.4)	7.4 ± 5.1 (1.4–18.2)	0.149
Total salivary EGF[c] output (pg/10 min) (range)	10158.4 ± 9820.9 (378.3–34623.1)	8352.8 ± 7813.3 (204.7–26360.1)	0.032
b. Non-SS group ($N = 14$)			
Muscarinic M3 receptor agonist (number [%])	0 (0)	0 (0)	1
Corticosteroid or immunosuppressant (number [%])	14 (100)	14 (100)	1
OHIP-14[b] score (out of 56) (range)	9.1 ± 6.8 (0–17)	10.7 ± 9.4 (0–28)	0.169
Salivary flow rate (ml/10 min) (range)	16.8 ± 6.7 (9.7–35.3)	16.7 ± 6.0 (6.7–28.4)	0.628
Total salivary EGF[c] output (pg/10 min) (range)	13623.1 ± 9546.2 (2632.3–29996.5)	11904.9 ± 6995.4 (4625.8–31337.3)	0.184

[a]Sjögren's syndrome.
[b]short version of the Oral Health Impact Profile.
[c]epidermal growth factor.

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

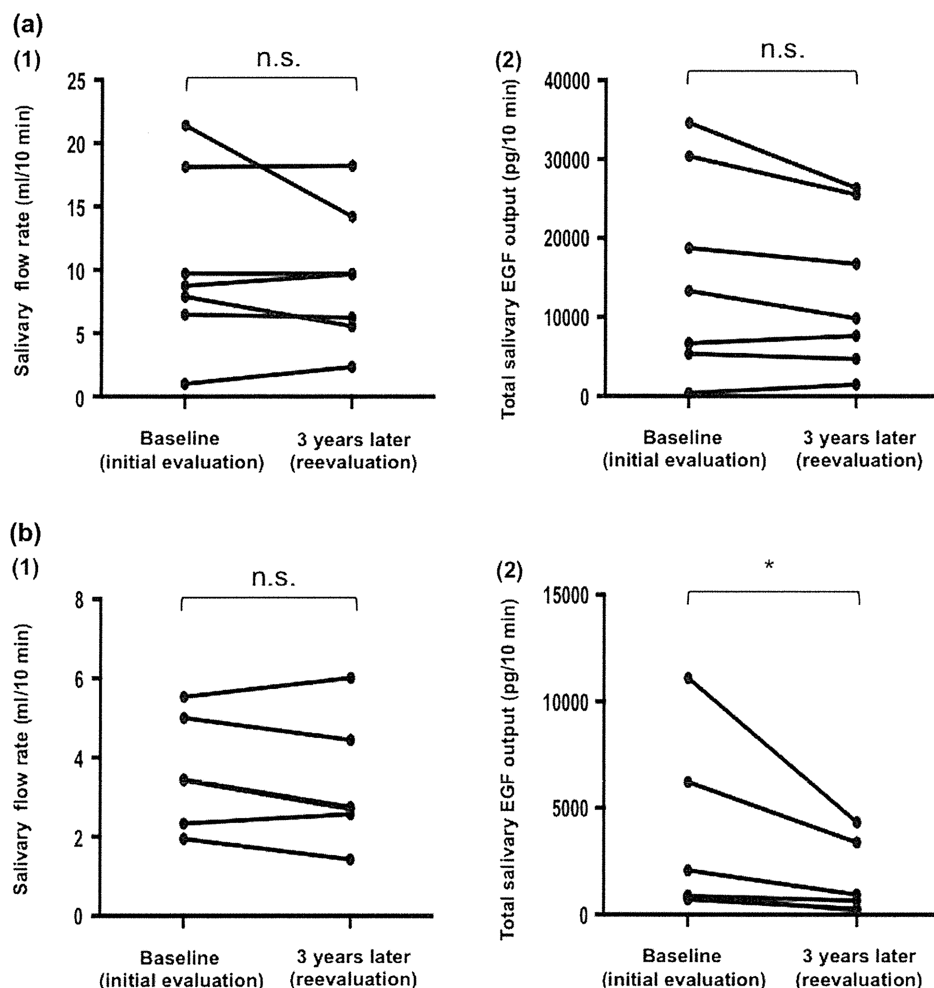


Figure 1. Changes in the salivary flow rate and total salivary EGF output according to the disease duration. (a) The group with short duration of SS (disease duration ≤ 5 years, $n = 7$). (b) The group with long duration of SS (≥ 6 years, $n = 6$). (1) Changes in the salivary flow rate. (2) Changes in the total salivary EGF output. Statistical analysis was performed by the Wilcoxon signed-rank test. $^*p < 0.05$; n.s., not significant.

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

non-exacerbation group (the score decreased or was unchanged in three years; $n = 8$) and the exacerbation group (the score increased in three years; $n = 14$). No significant difference in age was observed between the non-exacerbation (56.1 ± 14.4 years) and exacerbation (61.5 ± 12.2 years) groups ($p = 0.181$). In the non-exacerbation group, the salivary flow rate and the total salivary EGF output did not significantly differ at baseline (9.0 ± 6.3 ml/10 min and 12448.0 ± 12727.1 pg/10 min, respectively) and reevaluation point (9.0 ± 5.9 ml/10 min and 10310.9 ± 9565.9 pg/10 min, respectively) (Figure 2a). In contrast, in the exacerbation group, both the salivary flow rate and the total salivary EGF output at reevaluation point (6.8 ± 4.6 ml/10 min and 7724.2 ± 6901.4 pg/10 min, respectively) were significantly reduced compared with that at the initial value (8.0 ± 4.6 ml/10 min and 9548.6 ± 8063.6 pg/10 min, respectively) ($p < 0.05$ in both cases) (Figure 2b).

Correlation analysis

To investigate the correlations between the changes in salivary flow rate and total salivary EGF output versus the changes in OHIP-14 scores, the SS group was subdivided into groups with severe and mild intraoral manifestations using the OHIP-14 scores at baseline. The cut-off level was provisionally set at 14 points, which is equivalent to all the 14 items in the questionnaire assigned scores of 1 (≥14: severe group [$n = 7$] and ≤13: mild group [$n = 15$]) [16]. No significant differences in age and sex at baseline were observed between the severe (61.1 ± 8.6 years, 7 women) and mild (53.6 ± 14.4 years, 13 women and 2 men) groups ($p = 0.109$, $p = 1$, respectively).

In the severe group, the change ratio of total salivary EGF output was significantly and inversely correlated with the change in the OHIP-14 score ($r = -0.847$, $p = 0.008$). In contrast, there was no significant correlation between the change ratio of salivary flow rate and the change in the OHIP-14 score (Figure 3). Moreover, these three factors did not correlate in the mild group and the entire SS group.

Discussion

The following novel results were obtained in this study. (1) Although the salivary flow rate did not change in the SS patients, both the OHRQoL and total salivary EGF output decreased significantly three years after the initial evaluation. Such findings were not observed in the non-SS patients. (2) Although the salivary flow rate did not decreased in the SS patients with long disease duration, the total salivary EGF output was significantly lower at the time of reevaluation. (3) In the SS patients with reduced OHRQoL, both the salivary flow rate and total salivary EGF output significantly decreased within three years. (4) In the SS patients with short disease duration or ameliorated OHRQoL, both the salivary flow rate and total salivary EGF output did not decrease. (5) In the SS patients with severe intraoral manifestations, larger decreases in total salivary EGF output corresponded to poorer OHRQoL.

Several previous reports suggested that EGF synthesized in the salivary glands and secreted into the saliva is involved in repair mechanisms that lead to wound healing and facilitate maintenance of the integrity of the oral cavity mucosa [10,11] and demonstrated the association between intraoral inflammatory diseases

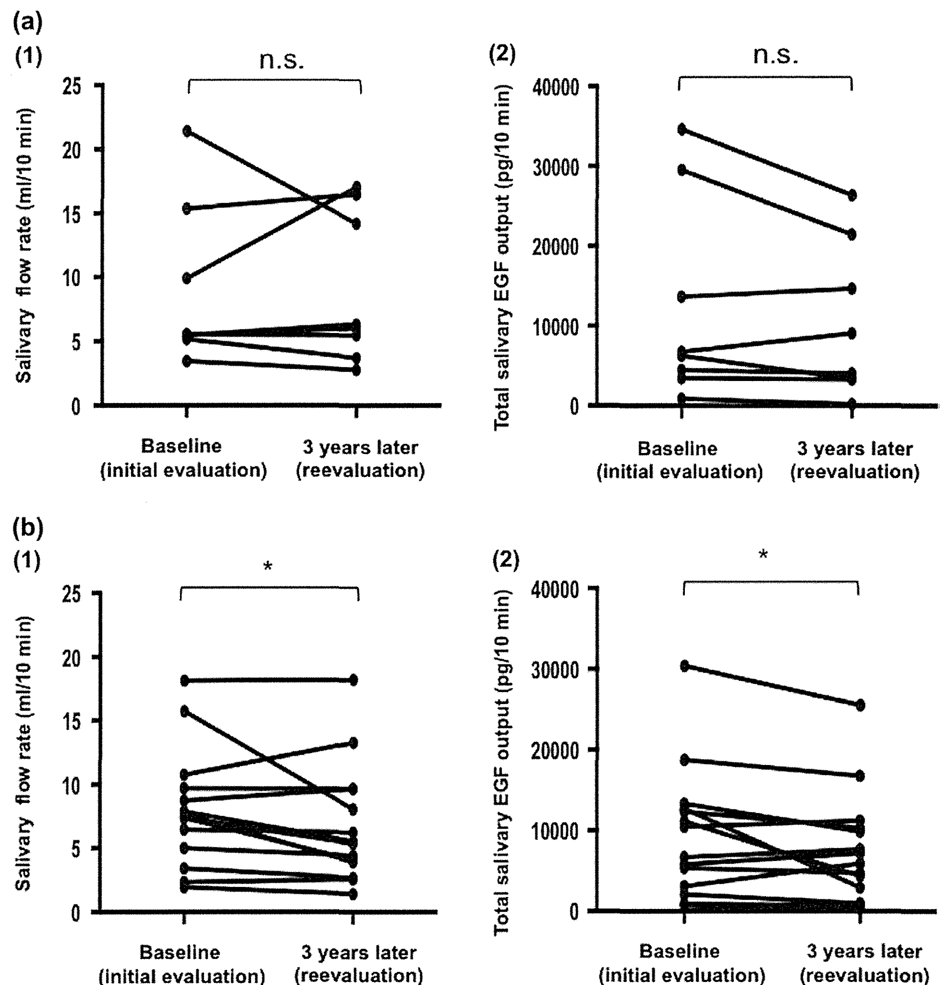


Figure 2. Changes in the salivary flow rate and total salivary EGF output according to the extent of changes in the short version of the OHIP-14 score three years after the initial evaluation. (a) The group in which the OHIP-14 score decreased or did not change (non-exacerbation group, $n = 8$). (b) The group in which the OHIP-14 score increased (exacerbation group, $n = 14$). (1) Changes in the salivary flow rate. (2) Changes in the total salivary EGF output. Statistical analysis was performed by the Wilcoxon signed-rank test. $^{*}p < 0.05$; n.s., not significant.

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

and changes in salivary EGF levels [4,13–15]. Salivary EGF concentrations were found to be significantly lower in patients with stomatitis aphthous [4,13] or peritonsillar abscess[4] and decreased further even after healing and in the absence of these lesions [4,13]. In patients with radiation-induced oral mucositis, salivary EGF levels were significantly reduced and inversely correlated with the severity of the disease [14,15]. The authors of the above reports speculated that low salivary EGF levels reduce the capacity of the oral mucosa to heal and maintain its physiologic integrity, thereby increasing susceptibility to intraoral inflammatory lesions [4,13–15]. In addition, the wound closure effect of EGF was shown in the previous study using oral epithelial cell lines [22].

Our previous report was the first to demonstrate the association between SS and salivary EGF levels [16]. The following findings of this report are important for interpretation of the present data. (1) In SS patients, the total salivary EGF output was significantly lower than in non-SS patients. Furthermore, the total salivary EGF output correlated with the salivary flow rate, decreased with time, and showed an inverse correlation with disease duration. These findings suggested that secretion of salivary EGF decreased in association with the salivary gland dysfunction induced by SS. (2) In the SS patients with short disease duration, although only the salivary flow rate was reduced, the OHRQoL was not affected. However, in patients with long disease duration, the total salivary EGF output decreased, and the OHRQoL worsened significantly. In addition, although the salivary flow rate decreased in the SS patients with mild intraoral manifestations, the total salivary EGF output did not decrease. In contrast, the total salivary EGF output decreased significantly in the patients with severe manifestations, but the decrease in the salivary flow rate was not significantly different from that in the SS patients with mild manifestations. These results indicated that an association may exist between lower levels of salivary EGF and poor OHRQoL in SS patients with long disease duration. In this regard, although few studies have investigated EGF expression in the salivary glands of SS patients, Koski et al. [23] reported that EGF expression was diminished in the labial salivary glands and concluded that, together with the reduced salivary flow, this could contribute to xerostomia and oral mucosal involvements in SS.

In the present study, we found that the salivary EGF level but not the salivary flow rate decreased significantly in the same SS patients during only three years. This decrease was especially striking in the SS patients with long disease duration and progressive exacerbation of the OHRQoL. Moreover, in the SS patients with severe intraoral manifestations at baseline, the reduction in salivary EGF levels significantly correlated with exacerbation of the OHRQoL. These findings strongly support the conclusions of our previous report and suggest that salivary EGF levels decrease rapidly within a short period of time and progressive exacerbation of the OHRQoL depends on this rapid decrease in salivary EGF levels when the SS disease duration becomes prolonged. Although the kinetics of salivary EGF has not yet been fully elucidated, Ino et al. [4] showed that salivary EGF concentration was significantly lower in younger subjects (0 – 9 years old) than in older subjects

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

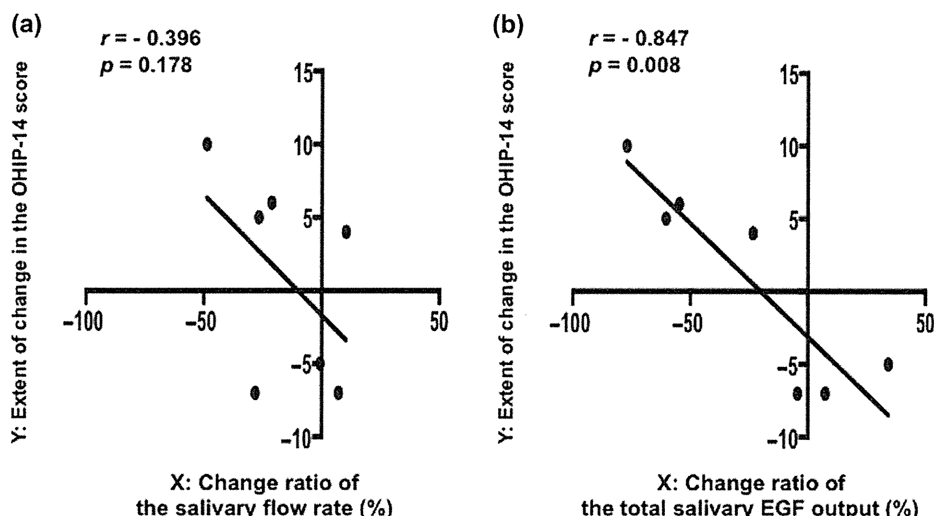


Figure 3. Correlations between the changes in the saliva and the OHRQoL in the group with severe intraoral manifestations. (a) The correlation between the change ratio of salivary flow rate and the extent of changes in the short version of the OHIP-14 score. (b) The correlation between the change ratio of total salivary EGF output and the extent of changes in the OHIP-14 score. The correlations were assessed using the Spearman's rank correlation coefficient.

(10 − 79 years old), but it was not correlated with age in the latter group. Similarly, in our previous study, age was not correlated with salivary EGF levels [16]. In addition, Nakagawa et al. [24] suggested that the progression of salivary gland destruction in SS is not necessarily associated with aging. On the basis of these findings, the rapid decrease in salivary EGF level within the three years of follow-up in the present study is likely a consequence of SS progression rather than aging.

The currently accepted treatment for xerostomia in SS consists of maintaining good oral hygiene (e.g., mouthwash), using saliva substitutes (e.g., artificial saliva), and utilizing salivary stimulation (e.g., muscarinic M3 receptor agonists). The main purpose of these components is to compensate for the reduced or inadequate supply of water via the natural saliva. Unfortunately, a substantial number of SS patients with refractory intraoral manifestations suffer from poor OHRQoL despite these treatments, especially in cases with long disease duration and declining residual salivary gland function. The findings of the present study and our previous report suggest that improving the saliva quality by EGF supplementation into oral cavity in combination with the conventional treatments may promote mucosal healing, reduce the severity of intraoral manifestations, and ameliorate the OHRQoL in SS patients. In this regard, Girdler et al. [25] investigated the effect of an EGF mouthwash on oral ulceration in patients undergoing cancer chemotherapy and noticed a slight delay in the onset and a smaller mean area of ulceration in the EGF mouthwash group compared with the placebo group. We believe that topical EGF application that can attach to wound longer than mouthwash (e.g., in the form of gel) may lead to a better result. However, the concentration and frequency of EGF administration remain to be elucidated. Moreover, an excess EGF stimulates the proliferation and differentiation of malignant cells [26]. Therefore, it should be prudent to administrate topical EGF application for intraoral manifestations.

This study has some limitations. The SS group consisted of patients treated with and without corticosteroids or immunosuppressants. In this regard, it may be desirable to examine only patients with primary SS excluding those under these medications. In addition, corticosteroids or immunosuppressants were used more frequently in the non-SS group than in the SS group. However, because the patients with non-SS group could not be diagnosed with SS or salivary gland dysfunction on the basis of clinical symptoms, physical examinations, and laboratory findings throughout the clinical course, including before the initiation of the corticosteroid or immunosuppressant therapy, we did not consider the possible influence of these medications on intraoral manifestations and salivary gland function in the present analysis.

In conclusion, salivary EGF levels in SS patients decreased rapidly in the course of three years, and the deterioration of saliva quality could contribute to the progression of the intraoral manifestations. The reliability of this result is strengthened by the fact that the present study is a follow-up reevaluation of the SS patients whom we examined in the past. We believe that the present findings may lead to the development of efficient therapies for SS.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT KAKENHI Grant Number: 22791820) and grants for intractable diseases from the Japanese Ministry of Health, Labour and Welfare.

Conflict of interest

None.

References

1. Stewart CM, Berg KM, Cha S, Reeves WH. Salivary dysfunction and quality of life in Sjögren syndrome: a critical oral-systemic connection. J Am Dent Assoc. 2008;139(3):291–9.
2. Zelles T, Purushotham KR, Macauley SP, Oxford GE, Humphreys-Beher MG. Saliva and growth factors: the fountain of youth resides in us all. J Dent Res. 1995;74(12):1826–32.
3. Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem. 1962;237:1555–62.
4. Ino M, Ushiro K, Ino C, Yamashita T, Kumazawa T. Kinetics of epidermal growth factor in saliva. Acta Otolaryngol. 1993;500:126–30.
5. Heitz PU, Kasper M, van Noorden S, Polak JM, Gregory H, Pearse AG. Immunohistochemical localisation of urogastrone to human duodenal and submandibular glands. Gut. 1978;19(5):408–13.
6. Thesleff I, Viinikka L, Saxén L, Lehtonen E, Perheentupa J. The parotid gland is the main source of human salivary epidermal growth factor. Life Sci. 1988;43(1):13–8.
7. Ino M, Ushiro K, Ino C, Yamashita T, Kumazawa T, Takahashi T. Epidermal growth factor in salivary glands –Epidermal growth factor and its receptor in human salivary glands- (in Japanese). Jibirinsho. 1992;85(5):805–14.
8. Scaltriti M, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res. 2006;12(18):5268–72.

9. Nakamura H, Kawakami A, Ide H, Koji T, Eguchi K. EGF activates PI3K-Akt and NF-κB via distinct pathways in salivary epithelial cells in Sjögren's syndrome. Rheumatol Int. 2007;28(2):127–36
10. Noguchi S, Ohba Y, Oka T. Effect of salivary epidermal growth factor on wound healing of tongue in mice. Am J Physiol. 1991; 260(4 Pt 1):E620–5.
11. Fujisawa K, Miyamoto Y, Nagayama M. Basic fibroblast growth factor and epidermal growth factor reverse impaired ulcer healing of the rabbit oral mucosa. J Oral Pathol Med. 2003;32(6):358–66.
12. Playford RJ. Peptides and gastrointestinal mucosal integrity. Gut. 1995;37(5):595–7.
13. Adişen E, Aral A, Aybay C, Gürer MA. Salivary epidermal growth factor levels in Behçet's disease and recurrent aphthous stomatitis. Dermatology. 2008;217(3):235–40.
14. Dumbrigue HB, Sandow PL, Nguyen KH, Humphreys-Beher MG. Salivary epidermal growth factor levels decrease in patients receiving radiation therapy to the head and neck. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2000;89(6):710–6.
15. Epstein JB, Gorsky M, Guglietta A, Le N, Sonis ST. The correlation between epidermal growth factor levels in saliva and the severity of oral mucositis during oropharyngeal radiation therapy. Cancer. 2000;89(11):2258–65.
16. Azuma N, Katada Y, Kitano S, Sekiguchi M, Kitano M, Nishioka A, et al. Correlation between salivary epidermal growth factor levels and refractory intraoral manifestations in patients with Sjögren's syndrome. Mod Rheumatol. 2014;24(4):626–32.
17. 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(6):554–8.
18. Ide R, Tsutsui A, Yamamoto R, Ueno K, Tsuneoka M, Mizoue T, et al. Evaluation of dental health- related quality of life -The reliability of the Japanese edition of Oral Health Impact Profile- (in Japanese). J Dent Hlth. 2002;52(1):36–42.
19. Yamazaki M, Inukai M, Baba K, John MT. Japanese version of the Oral Health Impact Profile (OHIP-J). J Oral Rehabil. 2007;34(3): 159–68.
20. Slade GD, Spencer AJ. Development and evaluation of the Oral Health Impact Profile. Community Dent Health. 1994;11(1):3–11.
21. Slade GD. Derivation and validation of a short-form oral health impact profile. Community Dent Oral Epidemiol. 1997;25(4):284–90.
22. Oudhoff MJ, Bolscher JG, Nazmi K, Kalay H, van't Hof W, Amerongen AV, Veerman EC. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. FASEB J. 2008;22(11):3805–12.
23. Koski H, Konttinen YT, Hietanen J, Tervo T, Malmström M. Epidermal growth factor, transforming growth factor-alpha, and epidermal growth factor receptor in labial salivary glands in Sjögren's syndrome. J Rheumtol. 1997;24(10): 1930–5.
24. Nakagawa Y, Kashiwazaki H, Okada K, Matsushita T, Matsuda A, Inoue N. Relationships between salivary gland destruction and aging in patients with Sjögren's syndrome (in Japanese). J J Gerodont. 2010;25(3): 307–14.
25. Girdler NM, McGurk M, Aqual S, Prince M. The effect of epidermal growth factor mouthwash on cytotoxic-induced oral ulceration. A phase I clinical trial Am J Clin Oncol. 1995;18(5):403–6.
26. Tokunaga A, Onda M, Okuda T, Teramoto T, Fujita I, Mizutani T, et al. Clinical significance of epidermal growth factor (EGF), EGF receptor, and c-erbB-2 in human gastric cancer. Cancer. 1995; 75(6 Suppl):1418–25.

Downloaded by [Hyogo College of Medicine (Hyogo Ika Daigaku)] at 00:05 16 February 2016

Effectiveness of Autologous Serum Eye Drops Combined With Punctal Plugs for the Treatment of Sjögren Syndrome–Related Dry Eye

Ying Liu, MD, Masatoshi Hirayama, MD, PhD,* Xin Cui, MD, MPH,† Samuel Connell,* Tetsuya Kawakita, MD, PhD,* and Kazuo Tsubota, MD**

Purpose: To evaluate the efficacy and safety of autologous serum (AS) eye drops combined with punctal plugs (PPs) in patients with Sjögren syndrome (SS)-related dry eye.

Methods: A retrospective clinical study was performed in patients with dry eye caused by SS. We evaluated the Schirmer test value, tear breakup time (tBUT), and fluorescein and Rose Bengal (RB) staining scores at baseline, 3 months, 6 months, 1 year, and >1 year after treatment. The dry eye indexes were also evaluated in 2 subgroups, which determined by the using of PPs, including the AS + PP group and AS only group.

Results: A total of 56 eyes of 28 patients were investigated with a mean follow-up of 42.3 ± 26.1 months. After the application of AS eye drops, the Schirmer test showed no significant changes. The tBUT (2.7 ± 1.9 seconds) was significantly improved at each time point (3.9 ± 3.1, 4.5 ± 3.1, 3.7 ± 2.5, and 5.1 ± 4.0; $P < 0.01$), fluorescein staining (4.3 ± 2.8) was significantly improved at each time point (2.2 ± 2.2, 1.9 ± 1.9, 1.8 ± 1.6, and 1.8 ± 1.8; $P < 0.01$), and RB (2.6 ± 3.0) staining was significantly improved from 6-month treatment (1.5 ± 1.9, 1.9 ± 1.9, and 1.4 ± 1.8; $P < 0.05$, 0.01, and 0.01, respectively). When combined with PPs, the tBUT and RB staining scores were found to be significantly improved in the AS + PP group compared with those of the AS only group.

Conclusions: Long-term application of AS eye drops was found to be an effective and apparently safe treatment for SS dry eye. Furthermore, PPs in combination with AS eye drops were considered to have an additive effect on SS dry eye.

Key Words: dry eye, Sjögren syndrome–related dry eye, autologous serum eye drops, long-term treatment, punctal plugs

(*Cornea* 2015;34:1214–1220)

Received for publication March 25, 2015; revision received June 2, 2015; accepted June 2, 2015. Published online ahead of print July 30, 2015.

From the *Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; and †Department of Epidemiology, Fielding School of Public Health, University of California Los Angeles, Los Angeles, CA.

Supported by Ishidsu Shun Memorial Scholarship, Japan. Statistical analysis was supported by the Center for Clinical Research, Department of Preventive Medicine and Public Health, Keio University School.

The authors have no conflicts of interest to disclose.

Reprints: Tetsuya Kawakita, MD, PhD, Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan (e-mail: kawakita@a2.keio.jp).

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

Sjögren syndrome (SS) is an autoimmune disease that leads to lacrimal gland and salivary gland dysfunction. SS is most common in women aged 40 to 60 years.[1] SS-related dry eye is a type of tear-deficient dry eye that results from autoimmune destruction of the lacrimal gland. As a refractory dry eye disease, proinflammatory cytokines in SS can result in inflammation and cumulative damage to the lacrimal gland, which leads to desiccating stress on the ocular surface.[2–4] Hence, it is important to seek effective and safe treatments for SS dry eye.

Maintaining ocular surface wetness and controlling inflammation are important considerations of SS dry eye treatment. Fundamental treatment of SS dry eye includes artificial tears and topical antiinflammatory therapy.[3,5] Punctal plugs (PPs) and oral use of exocrine gland secretagogues are also widely applied for severe SS dry eye cases.[6–8] Artificial tears, which include sodium hyaluronate, and vitamin A eye drops are commonly applied after a clinical diagnosis; however, artificial tears are not an exact substitute for natural tears, given that tears are a complex combination of water, salts, proteins, hydrocarbons, and lipids.[6,7] Topical antiinflammatory therapy, however, includes steroids, nonsteroidal, and cyclosporine eye drops. To avoid adverse effects, topical steroids can be used only in the short term; nonsteroidal antiinflammatory eye drops also have deleterious effects on the corneal epithelium.[9,10] Because of these limitations, the application of PPs is considered an effective surgical intervention.[8,11,12] Moreover, autologous serum (AS) has been prescribed as topical medication for the treatment of dry eye.[13,14] Recently, the application of AS as a topical medicine has paved the way for new treatments for refractory dry eye. Finally, the combination of PPs and AS eye drops has been proposed as an effective treatment for SS dry eye.

The components of serum are similar to those of tears; the former contains abundant growth factors and vitamins, such as the epidermal growth factor, vitamin A, and fibronectin, which promote corneal epithelial cell proliferation, maturation, and differentiation.[5,15] Since Fox et al[16] reported the application of AS for SS dry eye, the effectiveness of the treatment has been confirmed in a clinical study of 12 SS patients[5] and a case–control study of 37 eyes of patients with SS and those without SS.[13] However, the effectiveness and long-term prognosis of AS eye drops combined with PPs are still unclear.

Our study was approved by the Ethics Committee of the Keio University School of Medicine (No. 20140255). We

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

TABLE 1. Baseline Distribution of Patients' Demographic and Objective Measurements (N = 56 Eyes)

Variable	N	%
Total	56	100
Sex		
Male	6	10.7
Female	50	89.3
Age, yr	55.6 ± 13.8	
Follow-up period, mo	42.3 ± 26.1	
Schirmer test value, mm/5 min	2.7 ± 4.0	
tBUT (n = 54), s	2.7 ± 1.9	
FL score (n = 46)	4.3 ± 2.8	
RB score (n = 56)	2.6 ± 3.0	

In this study, 28 clinical patients (3 men and 25 women; 56 eyes) were administered AS eye drops. The mean patient age was 55.6 ± 13.8 years (range, 19–78 years). The mean follow-up period was 42.3 ± 26.1 months (range, 9–121 months).

retrospectively observed patients with SS dry eye who benefited from AS eye drops combined with PPs as a long-term treatment. We aimed to analyze the appearance of the ocular surface and to evaluate the efficiency and safety of the long-term use of AS eye drops and the effectiveness of long-term application of AS eye drops combined with PPs for SS dry eye treatment.

MATERIALS AND METHODS

Subjects

Medical records of patients who were under the treatment of AS eye drops between 2001 and 2011 from the Department of Ophthalmology, Keio University Hospital, Tokyo, Japan, were reviewed. A total of 56 eyes of 28 patients diagnosed with primary or secondary SS were enrolled in this study. All patients had symptoms of dry eye and a history of treatment with AS eye drops from September 2001 to November 2011 and met the diagnostic criteria of the Japanese Dry Eye research group.[17] Patients with moderate to severe dry eye underwent conventional dry eye treatment (artificial tears, vitamin A eye drops, and low concentration steroids) and/or punctal occlusion/plugs before being administered AS eye drops.

Criteria of Using PPs and AS Eye Drops

Patients were stratified by their PP status: patients in the AS + PP group were treated with PPs [Eagle Vision (TN 38133 USA)] at their first visit and then with AS eye drops, as the combination therapy. Patients in the AS group used only AS eye drops and received no PP administration.

Data Collection and Evaluation

Data from medical records, including sex and age of patients, date of the first and last visit up to medical record extraction, the frequency of AS eye drops use, and the combination use of conventional eye drops and PPs with AS eye drops were analyzed. The Schirmer test was performed at baseline (first visit) by inserting a standard filter paper strip into the temporal areas of the conjunctiva of both eyes for 5 minutes. The tear film breakup time (tBUT) was examined by instilling 2 μL of 1% fluorescein (FL) dye into the conjunctival sac and counting the tear film breakup time 3 times. The mean value was then calculated. The ocular surface was stained using 1% FL and 1% Rose Bengal (RB). FL staining scores were determined for the upper, middle, and lower areas of the cornea and rated from 0 to 9. RB staining scores were determined for the cornea, nasal, and temporal areas of the conjunctiva; each of the 3 regions were graded on a scale of 0 to 3. FL staining scores, RB staining scores, and tBUT were reviewed at baseline, 3 months, 6 months, 1 year, and more than 1 year (>1 year) of follow-up.

Protocol and Application of AS Eye Drops

AS was prepared based on a previously published protocol[5]: briefly, approximately 40 μL of blood was collected from each patient by venipuncture and centrifuged for 5 minutes at 1500 rpm. After centrifugation, the serum was carefully separated and extracted into another collection pack in a sterile manner. The serum was diluted with saline to a 20% concentration, and 5-μL aliquots were dispensed into

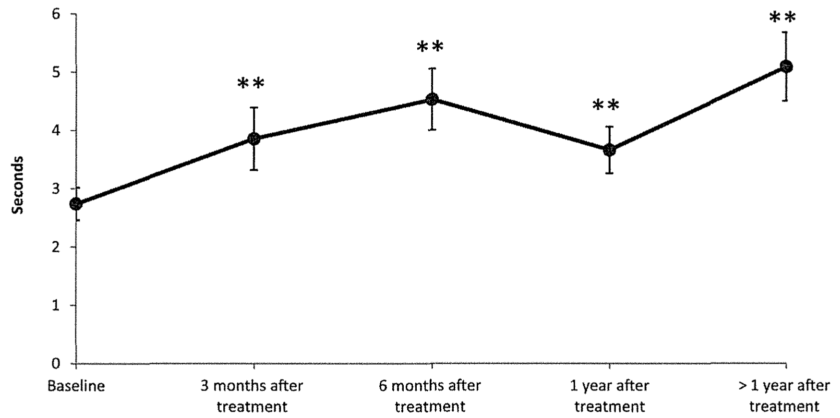
TABLE 2. Evaluation of Tear Function and Ocular Surface Vital Staining With AS Treatment

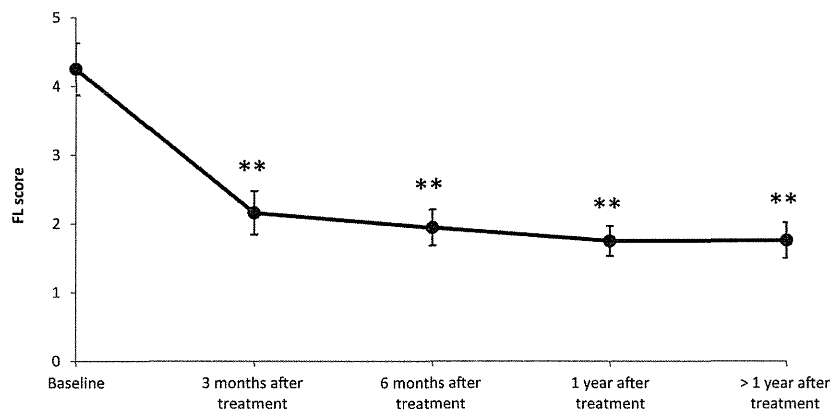
	Breakup Time, s				FL Score				RB Score			
	n	Point Estimate	95% Confidence Interval		n	Point Estimate	95% Confidence Interval		n	Point Estimate	95% Confidence Interval	
Baseline	46	2.7	2.5	3.0	46	4.3	3.9	4.6	56	2.6	2.3	3.0
3 mo	34	3.9**	2.5	3.0	50	2.2**	1.9	2.5	50	2.1	1.7	2.5
6 mo	34	4.5**	4.0	5.1	55	2.0**	3.3	4.1	52	1.5**	1.2	1.7
1 yr	38	3.7**	3.3	4.1	56	1.8**	1.7	2.2	56	1.9*	1.6	2.1
>1 yr	46	5.1**	4.5	5.7	50	1.8**	1.5	2.0	48	1.4**	1.2	1.7

*$P < 0.05$.
**$P < 0.01$.
Evaluation of tear function and ocular surface vital staining with AS treatment using a mixed model, results are shown as point estimates and 95% confidence interval. The tBUT and FL staining score significantly improved compared with those at baseline. The RB staining score significantly improved after 6 months compared with that at baseline (*$P < 0.05$ and **$P < 0.01$).

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.





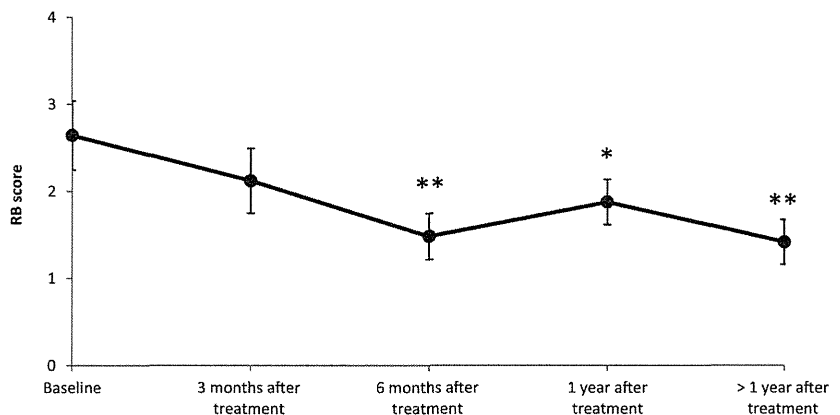


FIGURE 1. Comparison of dry eye parameters with the use of AS eye drops at each follow-up time point. A, tBUT. B and C, FL staining scores and RB staining scores of the ocular surface (* $P < 0.05$ and ** $P < 0.01$).

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

UV light–protected bottles to protect vitamin A from degradation by light. One bottle was stored in a refrigerator at 4°C for use, and all other bottles were stored in the freezer until required.

Statistical Methods

Patient demographics and baseline measurements are shown as mean ± SD or SE. We analyzed the effects of AS on tear function and ocular surface vital staining using a multilevel mixed model. FL staining scores, RB staining scores, and tBUT at each time point were compared for patients with and without PP treatment (AS + PP and AS only) using an unpaired 2-sample *t* test. All analyses were performed using SAS (9.3; SAS institute, Cary, NC).

RESULTS

Patients and Clinical Profiles

Medical records of 28 patients (3 men and 25 women) were reviewed in this study. In total, 56 eyes were analyzed. Patient age ranged from 19 to 78 years; mean age was 55.6 ± 13.8 years. All patients were treated with AS eye drops for a mean follow-up of 42.3 ± 26.1 months. Fifty eyes were (89%) followed up for more than 12 months, and 32 eyes (57%) were followed up for more than 36 months.

Although most of the patients were using conventional eye drops including vitamin A, low-concentration steroids (fluorometholone 0.1%), and intervention with PPs, the dry eye symptoms and ocular surface health did not improve before the application of AS eye drops. During our study, these treatments continued alongside the application of AS eye drops. At baseline, the mean Schirmer test value was 2.7 ± 4.0 mm and the mean tBUT was 2.7 ± 1.9 seconds, whereas mean FL and RB staining scores were 4.3 ± 2.8 and 2.6 ± 3.0, respectively (Table 1).

Changes in Dry Eye Symptoms and Ocular Surface Examination

Table 2 and Figure 1 show that the mean tBUT and FL staining scores significantly improved at 3 months, 6 months, 1 year, and >1 year compared with baseline ($P < 0.01$; Figs. 1A, B). The mean RB staining scores were significantly improved after 6 months compared with those at baseline ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively; Fig. 1C). The Schirmer test showed no significant improvement during follow-up.

Characteristics of Patients Stratified by the PP Status

There were 17 eyes in the AS only group and 12 eyes in the AS + PP group. Among patients within the AS only group, 2 were male eyes and 15 were female eyes; among patients within the AS + PP group, 12 were female eyes. Mean age of patients was 57.2 ± 9.1 years and 65.3 ± 13.3 years, respectively. In the AS + PP group, 9 eyes had both upper and lower PP occlusion at baseline, whereas 3 eyes suffered from lower PP dropping out and 3 eyes suffered from upper PP dropping out. A total of 3 eyes had only lower PP occlusion during the whole follow-up period. The retention rate at 3 months, 6 months, 1 year, and at the >1 year was 95.2%, 100%, 95.2%, and 85.7%, respectively. No complications occurred during the application of PPs. The mean follow-up period was 25.3 ± 12.0 months for the AS only group and 55.3 ± 24.5 months for the AS + PP group (Table 3).

Comparison of Dry Eye Signs Between the AS + PP Group and AS Only Group

The mean tBUT, FL staining score, and RB staining score were not significantly different between both groups at baseline. After the treatment at each time point, the mean tBUT was significantly higher in the AS + PP group at 1 year of treatment and >1 year of treatment compared with the AS only group ($P < 0.01$; Fig. 2A). The mean FL staining score was not significantly different between the 2 groups at any time point (Fig. 2B). The improvement in RB staining scores was significant in the AS + PP group compared with the AS only group after 1 year of treatment and >1 year of treatment ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2C).

Safety and Response

During our investigation period, no side effects related to AS and PP application were observed, including exacerbation of ocular surface inflammation, serious infections, punctal occlusion, epiphora, or desensitization of the corneal surface.

DISCUSSION

In this study, we observed the effectiveness of AS eye for the treatment of SS dry eye and the long-term therapeutic effect of AS combined with PPs without any side effects. Because the vitamins and growth factors in tears also exist in serum, the application of AS eye drops can supply essential nutrition to the ocular surface. Moreover, the albumin in serum has been reported to suppress apoptosis in a conjunctival cell line.[18] According to our previous research and other

TABLE 3. Characteristics of Patients Stratified by the PP Status

	Group	
Variable	**Plug− Eye Drop+**	**Plug+ Eye Drop+**
No. eyes	17	12
Sex		
Male	2	0
Female	15	12
Age, yr	57.2 ± 9.1	65.3 ± 13.3
Follow-up period, mo	25.1 ± 12.0	55.3 ± 24.5

Clinical profiles of patients with or without PPs during AS eye drop application are shown.

AS only: patients without PPs during the period of AS eye drop application.

AS + PP: patients with PPs during the period of AS eye drop application.

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.