The possible role of IL-5 in the pathogenesis of lupus has not been extensively evaluated. Several studies suggested that abnormally high levels of IL-5 may have a role in the abnormal expansion of auto-reactive B-1 cells and the subsequent suppression of autoimmune disease [33]. Additionally, elevated IL-5 mRNA has been reported in cutaneous lupus erythematosus [34]. The authors of those studies suggested that IL-5, produced by the Th2 subset of CD4+ T cells may contribute to the development of autoimmunity in lupus. However, the potential relevance of these results to NPSLE is unclear.

FGF-basic is a potent angiogenic factor whose activity is involved in endothelial cell and fibroblast survival, proliferation, migration, and tube formation, together with VEGF [35]. An increased FGF-basic level was reported in lupus and tended to be correlated with disease activity [36].

There has been no thorough comparison of the CSF of NPSLE, MS and NMO patients, to the best of our knowledge. Here, we found that NPSLE patients showed significantly higher levels of cytokines, chemokines and growth factors compared to MS and NMO patients. Among these factors, IL-17, IL-15, IL-9, IL-5, IL-12 p70, IL-7, FGF-basic, eotaxin, IL-13, GM-CSF and VEGF had not been observed in the CSF of NPSLE patients. In the present examination, we were able to detect cytokines, chemokines and growth factors with the Bio-plex assay, even if they were present in very small amounts. Since almost all of the above markers had already been shown elsewhere to be increased in MS or NMO, it would be difficult to distinguish MS or NMO from NPSLE using the above markers. We therefore devised a numerical prediction scoring system that clearly separated the NPSLE patients from the other patients. We identified six "minimum predictive markers"—IL-17, IL-2, IFN-γ, IL-5, FGF-basic and IL-15—by a weighted-voting algorithm that showed the highest MCC and predicted the test set with 94.59% accuracy.

These predictive markers seem not to be correlated with each other; however they certainly involve Th1, Th2 and Th17 cells. This may indicate that these six markers contribute more strongly to induce both pro-inflammatory and antibody-mediated neuronal and glial cells' destruction in NPSLE compared to MS and NMO, and we suspect that they are essential for the pathogenesis of NPSLE.

Focusing on other markers is also important. We found that the correlations of pairs of cytokines/chemokines/growth factors showed different patterns in NPSLE, MS and NMO. The correlation coefficients of the cytokines/chemokines were the most significant: PDGF-BB, MIP-1a, MIP-1b and RANTES in NPSLE, IL-1b and IL-1ra in MS, and IL-12(p70) and VEGF in NMO (Suppl. Fig. 2). Examinations of each single marker may be meaningless, because each marker operates together with one or more other markers in a rather complex system. These results indicate that the downstream pathway of Th1/Th2/Th17-related mediators differs among NPSLE, MS and NMO.

Various markers are known to be involved in the pathology of NPSLE; however, no predictive markers for detecting NPSLE were reported prior to the present study. Here, we were able to identify six minimum predictive markers (IL-17, IL-2, IFN-γ, IL-5, FGF-basic and IL-15), by using a weighted-voting algorithm with the highest MCC that could predict NPSLE from other autoimmune neurological

diseases with 94.59% accuracy. We examined the correlations of cytokines/chemokines/growth factors in NPSLE, MS and NMO, and we found that many cytokines/chemokines/growth factors were operating together in NPSLE compared to MS and NMO. The determination of various types of CSF cytokine profiles may contribute to the diagnosis of NPSLE and may help elucidate the mechanisms underlying this disease.

Conflict of interest

The authors have no conflicts of interest to report.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2015.01.010.

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Direct Infection of Primary Salivary Gland Epithelial Cells by Human T Lymphotropic Virus Type I in Patients With Sjögren's Syndrome

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Objective. To investigate whether human T lymphotropic virus type I (HTLV-I) directly infects salivary gland epithelial cells (SGECs) and induces the niche of the salivary glands in patients with Sjögren's syndrome (SS).

Methods. SGECs were cultured with the HTLV-I–producing CD4+ T cell line HCT-5 or with Jurkat cells. Antibody arrays, immunofluorescence analysis, and enzyme-linked immunosorbent assay (ELISA) were used to determine the profiles of inflammation-related molecules, and the profiles of apoptosis-related molecules were determined by antibody array and immunofluorescence analysis. The presence of HTLV-I–related molecules was assessed by immunofluorescence analysis and in situ polymerase chain reaction. Apoptosis of SGECs was evaluated by TUNEL staining.

Results. Among the SGECs, 7.8 ± 1.3% (mean ± SD) were positive for HTLV-I–related proteins after 96-hour coculture with HCT-5 cells. Nuclear NF-κB p65 was also detected in 10% of the SGECs. The presence of HTLV-I proviral DNA in SGECs after coculture with HCT-5 cells was detected by in situ polymerase chain reaction. After coculture of SGECs with HCT-5, the expression of cytokines and chemokines, including soluble intercellular adhesion molecule 1, RANTES, and interferon γ–induced protein 10 kd (IP-10/CXCL10) was increased in a time-dependent manner. The expression of proapoptotic molecules (e.g., cytochrome c and Fas) and antiapoptotic molecules (e.g., Bcl-2, Heme oxygenase 2, and Hsp27) was increased in the SGECs cocultured with HCT-5, showing that apoptosis of SGECs was not detected after coculture with HCT-5 or Jurkat cells.

Conclusion. HTLV-I is thought to infect SGECs and alter their cellular functions. These changes may induce the niche of SS and contribute to the development of SS in anti–HTLV-I antibody–positive individuals.

Human T lymphotropic virus type I (HTLV-I) has been reported to be involved in the pathogenesis of primary Sjögren's syndrome (SS) in endemic areas, including Nagasaki City, Japan (1–3). The extremely high prevalence of SS among patients with HTLV-I–associated myelopathy (HAM) appears to confirm a strong relationship between HTLV-I infection and SS (4–6). A previous study by our group also revealed the clinical characteristics of anti–HTLV-I antibody–positive SS patients and showed that the labial salivary glands (LSGs) of such patients are not destructible compared with the LSGs of anti–HTLV-I antibody–negative patients with SS (7). In addition, the low prevalence of ectopic germinal centers (GCs) as well as the low expression of CXCL13 in infiltrating mononuclear cells in LSGs were shown to be immunohistologic characteristics of anti–HTLV-I antibody–positive patients with SS (8).

HTLV-I preferentially infects T cells, especially CD4+ T cells, and the observations described above indicate that the T cell lineage may primarily contribute

to the pathogenesis of anti–HTLV-I antibody–positive SS. However, cell types other than T cells, including the human retinal pigment epithelial cell line ARPE-19 (9), and human primary fibroblast-like synoviocytes (FLS) (10) were reported to be susceptible to HTLV-I infection. In ARPE-19 cells, the expression of intercellular adhesion molecule 1 (ICAM-1) is increased by HTLV-I, and the production of granulocyte–macrophage colony-stimulating factor (GM-CSF) from FLS is induced by HTLV-I.

These observations suggested that HTLV-I may infect cell lineages other than T cells in human salivary glands and may contribute to the development of SS. In this regard, ductal epithelial cells are considered candidate cells, because various cytokines, chemokines, and apoptosis-related molecules have been shown to be expressed in these cells (1). In addition, ductal epithelial cells attract T cells into the salivary glands of patients with SS through production of interferon-γ (IFNγ)–inducible 10-kd protein (IP-10; CXCL10) and monokine induced by IFNγ (CXCL9) (11).

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

PATIENTS AND METHODS

Patients. Primary SGECs were obtained from the LSGs of 15 female patients with primary SS (mean ± SD age 53.2 ± 15.4 years). In all patients, SS was diagnosed according to the revised criteria proposed by the American–European Consensus Group (12), and anti–HTLV-I antibodies were absent, as measured by chemiluminescent enzyme immunoassay.

Antibodies and reagents. Mouse anti–HTLV-I antibodies (p19, p38, and Gag) were obtained from Chemicon, and mouse anti–NF-κB p65 antibody, mouse anti–cytochrome c antibody, mouse anti-Hsp27 antibody, and rabbit anti-Fas antibody were obtained from Santa Cruz Biotechnology. Mouse anti–heme oxygenase 2 (anti–HO-2) antibody was purchased from OriGene, and rabbit anti–ICAM-1 antibody, rabbit anti–growth-related oncogene (anti-GRO)/CXCL1 antibody, anti-CCL5/RANTES antibody, and rabbit anti–IP-10/CXCL10 antibody were purchased from LifeSpan Biosciences. Rabbit anti–interleukin-8 (anti–IL-8) antibody was purchased from ABgene. Secondary antibodies, including fluorescein isothiocyanate (FITC)–conjugated donkey anti-mouse IgG and tetramethylrhodamine isothiocyanate (TRITC)–conjugated donkey anti-rabbit IgG, were purchased from Jackson ImmunoResearch. Hoechst 33258 was purchased from Sigma. A Proteome Profiler Human Cytokine Array Kit, Panel A, and a Quantikine ELISA kit for soluble ICAM-1 (sICAM-1), CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα, and CXCL8/IL-8 were purchased from R&D Systems. Cy3–dUTP was purchased from GE Healthcare. Monoclonal mouse anti-human CD4, anti-human CD8, anti-human CD20cy, mouse

IgG1, and monoclonal rabbit anti-human cytokeratin 8/18 antibodies were purchased from Dako.

LSG biopsy and cell culture. Each patient underwent a lower lip salivary gland biopsy under local anesthesia. Some of the specimens were stained with hematoxylin to diagnose sialadenitis, and some were used for culture of SGECs in a defined keratinocyte–serum-free medium (SFM) (Invitrogen Life Technologies) supplemented with hydrocortisone (Sigma) and bovine pituitary extract (Kurabo). In all 15 patients, the diagnosis of SS was compatible with the Chisholm and Mason scale for histologic grading of LSG biopsy tissue (13).

For the coculture of SGECs with HTLV-I–producing T cells, HCT-5 cells (which are derived from the cerebrospinal fluid cells of patients with HAM [14]), were cultured with SGECs for the designated period of time in defined keratinocyte–SFM culture medium. As a control toward HCT-5, the non–HTLV-I–infected T cell line Jurkat was cultured in RPMI 1640 medium with 10% fetal bovine serum. For the experiments described below, HCT-5 or Jurkat cells were cocultured (2:1) with SGECs at the time when the cells were seeded. Briefly, the SGECs were seeded onto sterile coverslips for immunofluorescence analysis. Next, HCT-5 cells were added 24 hours after the SGECs attached to and grew on the coverslips. For immunofluorescence analysis, the cells were stringently washed with phosphate buffered saline (PBS) to remove any remaining HCT-5 cells. Informed consent for the use of LSG biopsy samples was obtained from all 9 patients at the commencement of the study. The study was conducted with the approval of the human ethics committee at Nagasaki University Hospital.

Immunofluorescence analysis. Immunofluorescence analyses were performed as previously described (15). Briefly, SGECs cultured on 12-mm² coverslips were fixed in PBS containing 4% paraformaldehyde (PFA) at 4°C, followed by immersion in methanol at −20°C for 10 minutes. After fixation, the SGECs were blocked in 5% normal horse serum in PBS and then incubated with the primary antibodies for 1 hour at room temperature, followed by incubation with FITC-conjugated and TRITC-conjugated secondary antibodies and Hoechst 33258, in the dark. The SGECs were then mounted in Vectashield mounting medium (Vector) and scanned with a fluorescence microscope (BIOREVO BZ-9000; Keyence). To measure the immunofluorescence of the HCT-5 cells, fixed cells were incubated with mouse primary monoclonal antibodies as cell surface markers, followed by FITC-conjugated secondary antibody and Hoechst 33258. Control experiments were performed to confirm the isotype specificity of the secondary antibodies. Immunostaining of HCT-5 cells was performed in the same manner as that described above for SGECs.

TUNEL staining. To investigate DNA double-strand breaks in SGECs, TUNEL staining was performed as described in a previous study by our group (16). After fixation, SGECs were incubated in 4% PFA at 4°C for 15 minutes, followed by immersion in PBS with 0.5% Tween 20 and 0.2% bovine serum albumin, using a MEBSTAIN Apoptosis Kit Direct (MBL). The SGECs were then incubated with a 50-μl terminal deoxynucleotidyl transferase solution at 37°C for 1 hour. The dUTP signal as detected by FITC was captured using a BIOREVO BZ-9000 fluorescence microscope (Keyence). TRAIL was used as a positive control to show induction of apoptosis (15).
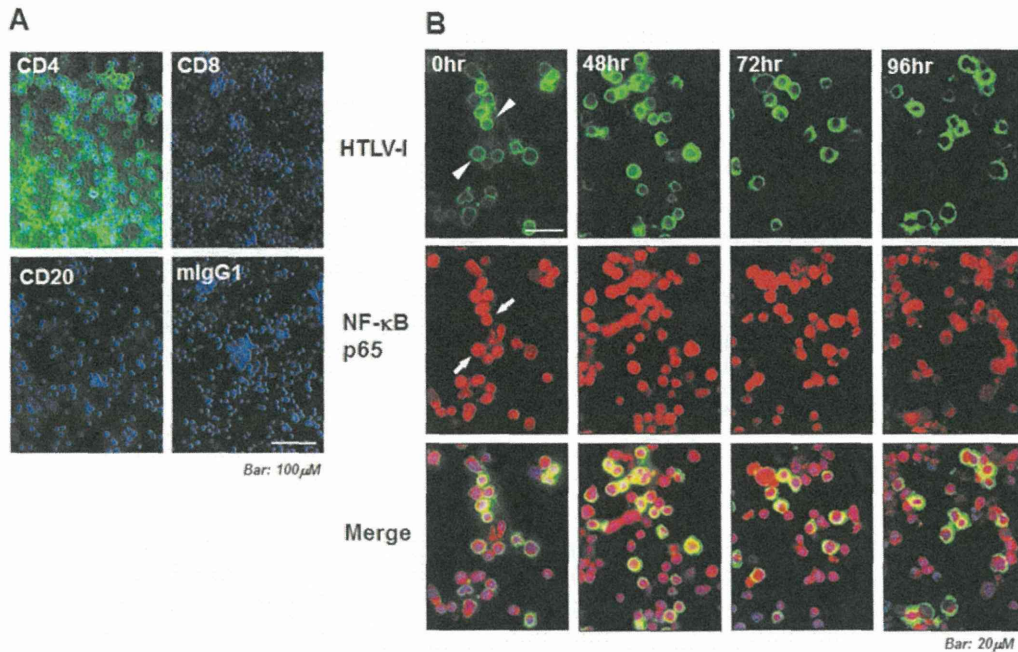
Figure 1. Characterization of the human T lymphotropic virus type I (HTLV-I)–infected HCT-5 T cell line. **A,** Phenotype of HCT-5 cells. After fixation in phosphate buffered saline containing 4% paraformaldehyde at 4°C followed by immersion in methanol at −20°C for 10 minutes, HCT-5 cells were incubated 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 vol-

ume 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-mm² 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 mM dNTP, 2.5 mM MgCl₂, 1 μM Cy3–dUTP, and distilled water without DNA polymerase, and then boiled for 10 minutes.
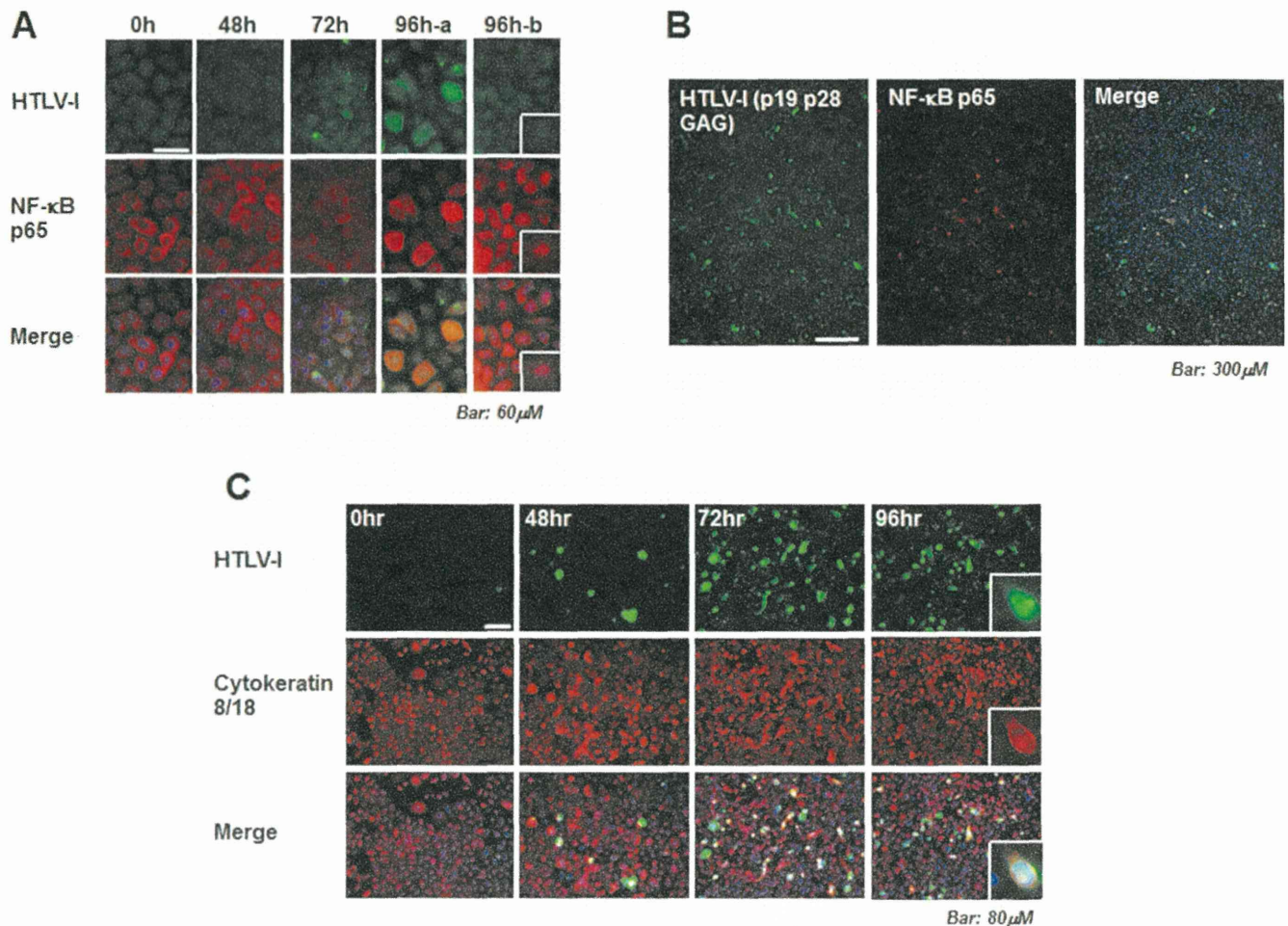
Application of these primer sets was previously described

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.
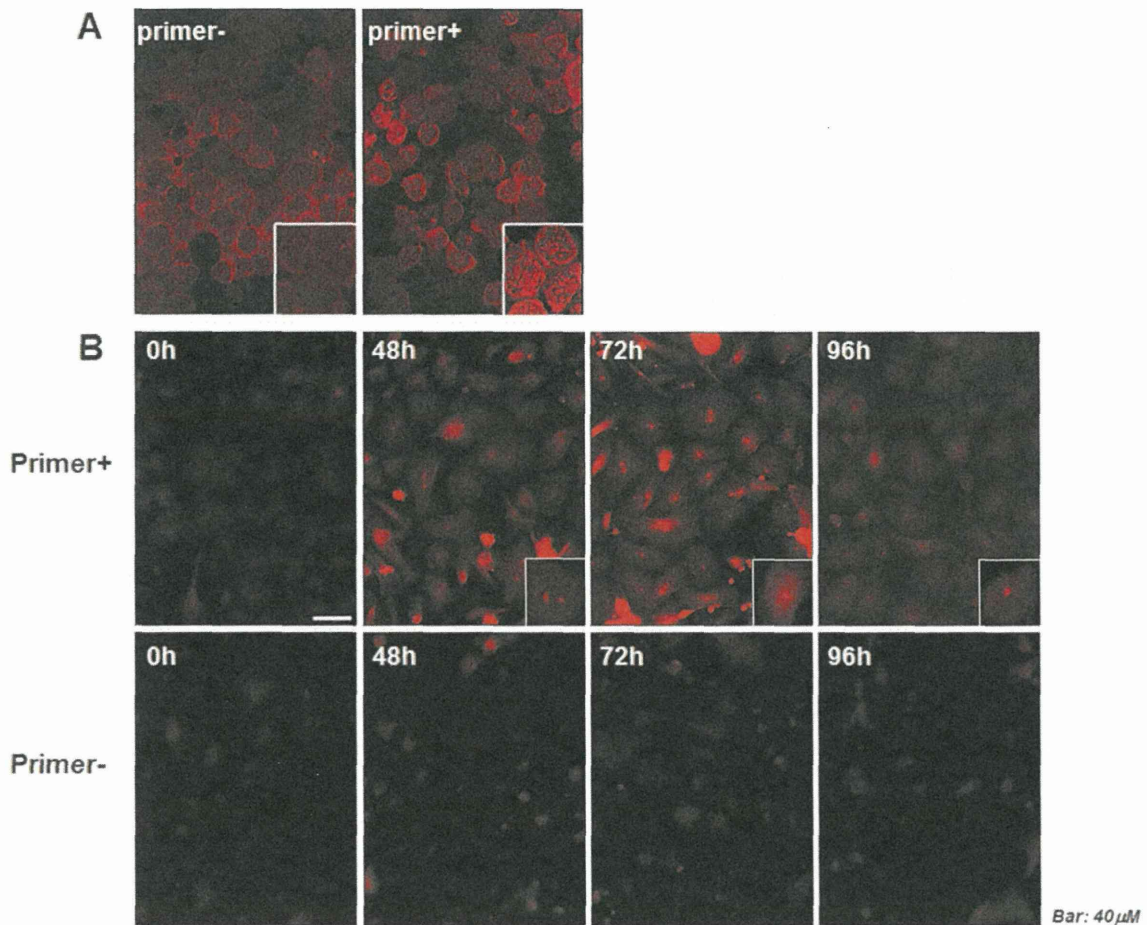
Figure 3. Detection of human T lymphotropic virus type I (HTLV-I) proviral DNA by in situ polymerase chain reaction (PCR). **A,** HTLV-I was detected in HCT-5 cells as a positive control. HCT-5 cells were treated with 1 μg/ml of protein kinase, and 5 cycles of in situ PCR were performed. Detected HTLV-I 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).
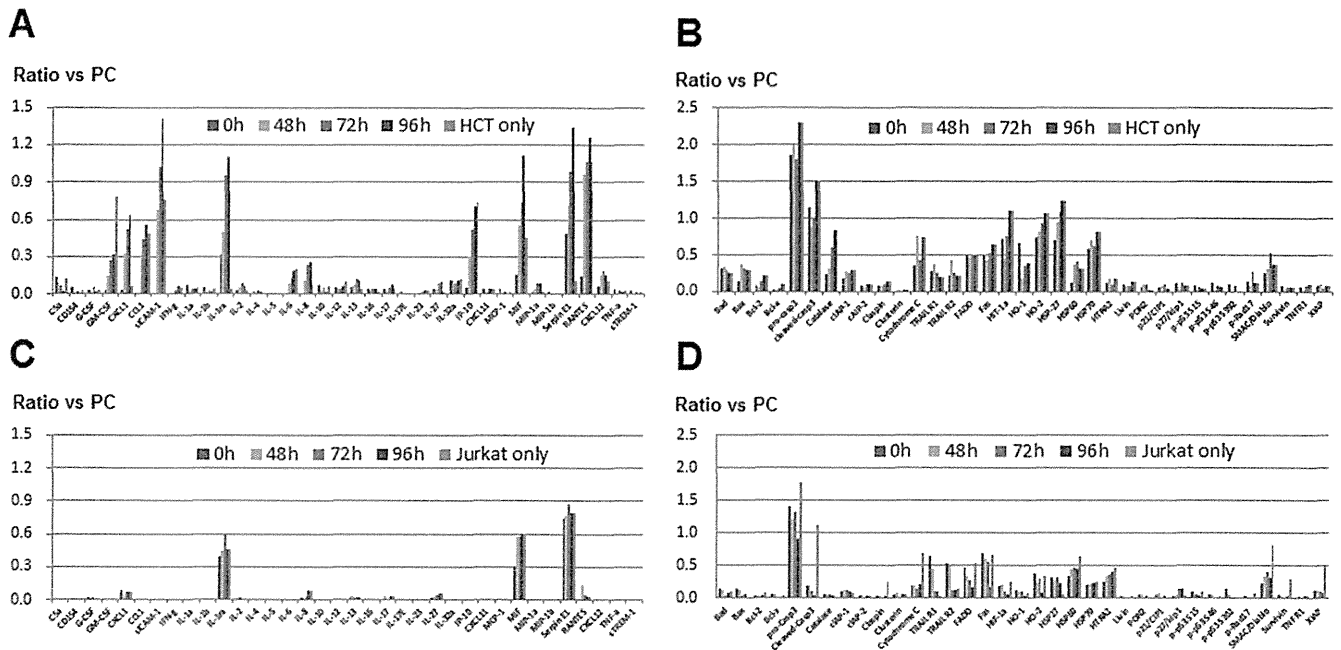
Figure 4. Expression of inflammation-related molecules and apoptosis-related molecules. **A** and **C,** Expression of inflammation-related molecules in supernatant of salivary gland epithelial cells (SGECs) cocultured with HCT-5 cells (A) or Jurkat cells (C). The semiquantitative concentrations of each 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
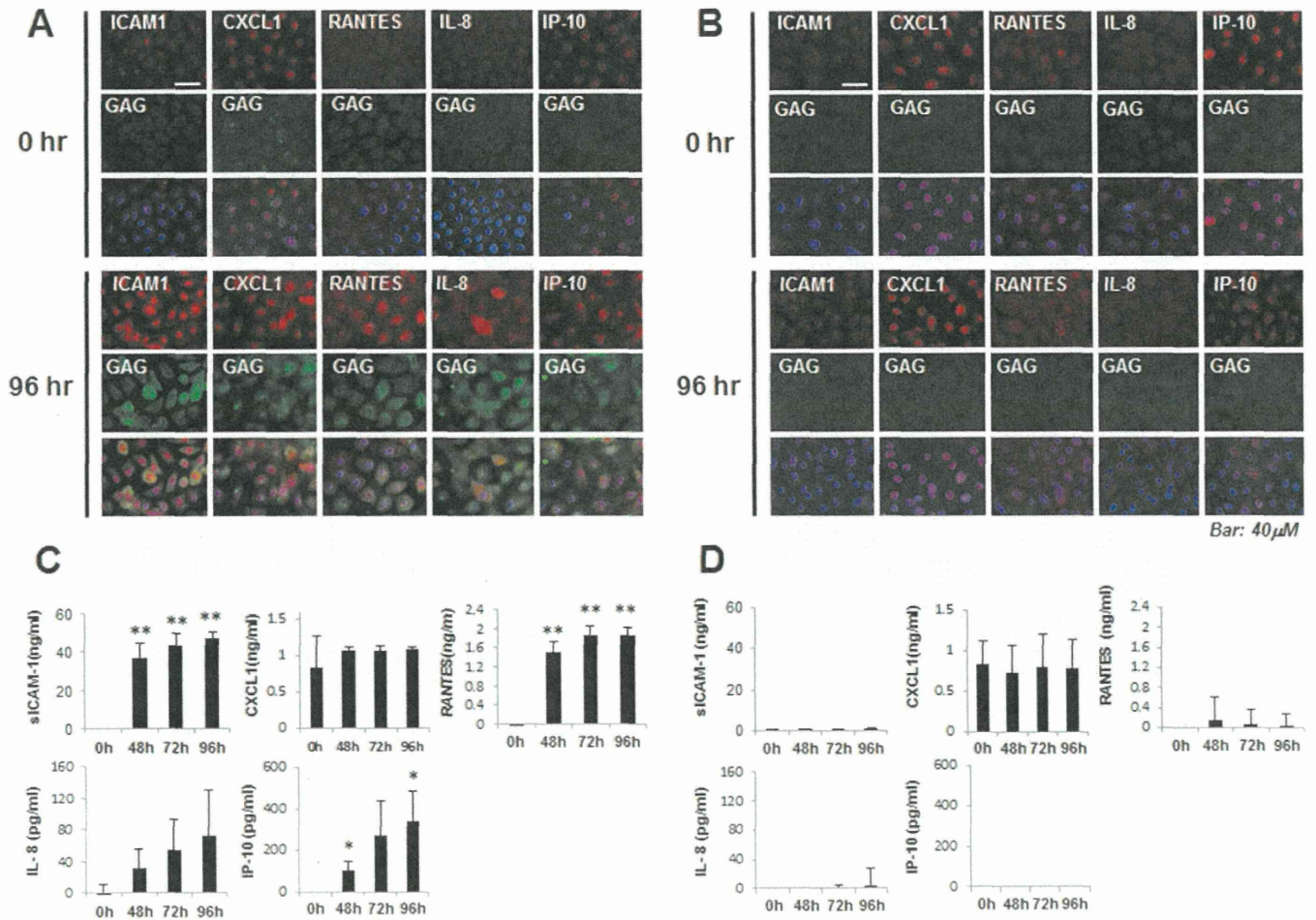
Figure 5. Increased expression of inflammation-related molecules in coculture supernatant, as confirmed by immunofluorescence analysis and enzyme-linked immunosorbent assay (ELISA). **A** and **B**, Expression of intercellular adhesion molecule 1 (ICAM-1), CXCL-1, RANTES, interleukin-8 (IL-8), and interferon-γ–inducible 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-
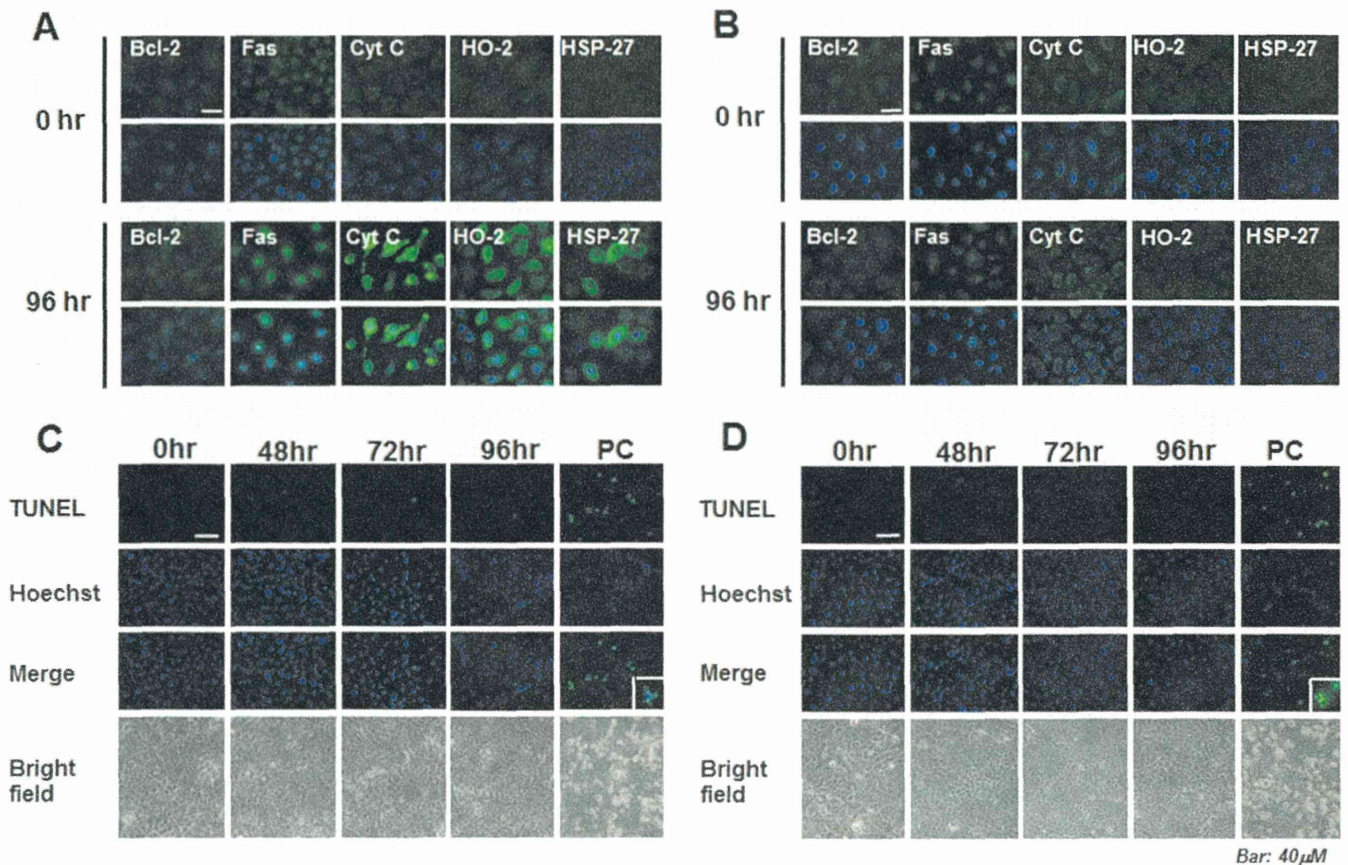
Figure 6. Expression of apoptosis-related molecules in cocultured salivary gland epithelial cells (SGECs). **A** and **B,** Expression of Bcl-2, Fas, cytochrome c (Cyt C), heme oxygenase 2 (HO-2), and Hsp27 in supernatant of SGECs cocultured with HCT-5 cells (**A**) or Jurkat cells (**B**), as determined 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.

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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.

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Editorial

IL-6 targeting compared to TNF targeting in rheumatoid arthritis: studies of olokizumab, sarilumab and sirukumab

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The combination of synthetic disease-modifying anti-rheumatic drugs (sDMARDs) such as methotrexate (MTX) and biologic DMARDs (bDMARDs) targeting inflammatory cytokines such as tumour necrosis factor (TNF) has enabled markedly efficient control of disease activity in patients with rheumatoid arthritis (RA) with inadequate response to MTX (MTX-IR).[1–7] Although TNF inhibitors have offered pivotal strategies for rheumatologists in daily practice and 20–50% of RA patients treated with TNF inhibitors achieve clinical remission within 6 months, the remaining patients still have active disease and progressive disability. IL-6 is also a pleiotropic cytokine with diverse activities and plays a central role in the pathogenesis of RA by contributing to T cell activation, B cell activation, synoviocyte stimulation, endothelial activation, osteoclast maturation and production of acute-phase proteins. Serum levels of IL-6 and soluble IL-6 receptor (IL-6R) are elevated and correlate with disease activity in RA patients and so blocking IL-6/IL-6R has been considered beneficial for the treatment of RA. In accordance with this, accumulated evidence has shown the clinical efficacy as well as the adequate safety of tocilizumab, a humanised anti-IL-6R monoclonal antibody (mAb), as monotherapy or in combination with sDMARDs such as MTX in patients who are sDMARD naive and have an inadequate response to TNF inhibitors (TNF-IR).[8–13] Tocilizumab was, therefore, approved as a first-line bDMARD in patients responding insufficiently to MTX or other sDMARDs in Japan and Europe. Also, in the 2013 EULAR recommendations for the management of RA,

tocilizumab was listed as a first-line TNF inhibitor in patients with sDMARD-IR.[14] The successful treatment of RA by tocilizumab has encouraged the development of novel bDMARDs targeting IL-6 or IL-6R. In addition to tocilizumab, the phase II clinical trials of olokizumab, sarilumab and sirukumab, three new bDMARDs targeting IL-6, are reported.

Olokizumab is a humanised anti-IL-6 mAb. Genovese et al[15] report the findings of a 12-week phase IIb study to assess the safety and efficacy of subcutaneous olokizumab in RA patients with moderate-to-severe disease activity despite TNF inhibitors. A total of 221 patients were randomised to one of nine treatment arms receiving placebo or olokizumab (60, 120 or 240 mg) every 4 weeks (q4w) or every 2 weeks (q2w), or 8 mg/kg tocilizumab q4w. All patients received background MTX. Treatment with olokizumab met the primary endpoint (change from baseline in DAS28 C-reactive protein (CRP)) as compared to placebo at week 12 at all olokizumab doses tested (60 mg, p=0.0001; 120 mg and 240 mg olokizumab, p<0.0001). Olokizumab at various doses demonstrated similar efficacy to tocilizumab across multiple endpoints. The greatest improvement in DAS28-CRP scores was observed in the olokizumab 240 mg q2w group. In addition, pharmacokinetic modelling demonstrated a shallow dose–exposure response relationship in terms of the percentage of patients with DAS28<2.6. Olokizumab was also superior to placebo according to American College of Rheumatology (ACR) responses. Most treatment emergent adverse events (TEAEs) were comparable between the olokizumab and tocilizumab treatment groups, the incidence of serious TEAEs (SAEs) was similar between treatment groups, and no serious SAEs were reported by more than one patient. There was one recorded SAE of increased blood triglycerides in the tocilizumab group.

Sarilumab is a human anti-IL-6Ra mAb. The results of a 12-week phase II study to assess the safety and efficacy of subcutaneous sarilumab are reported by Huizinga

et al.[16] A total of 306 patients with active RA despite MTX were randomised to one of six treatment arms receiving placebo or sarilumab (100 mg q2w, 150 mg q2w, 100 mg qw, 200 mg q2w or 150 mg qw for 12 weeks) with background MTX. The proportion of patients achieving the primary endpoint, an ACR20 response at week 12, compared to placebo was significantly higher for sarilumab 150 mg qw (72.0% vs 46.2%, multiplicity adjusted p=0.0203) and higher ACR20 responses were also attained with 150 mg q2w (67%; unadjusted (nominal) p=0.0363) and 200 mg q2w (65%; unadjusted p=0.0426) versus placebo. Infections were the most common TEAEs, although no serious infections were reported. At week 12, mean total cholesterol was higher in the four highest dose groups; the increase from baseline was 9.4%, 10%, 16.4% and 21.1%, respectively, in the 150 mg q2w, 100 mg qw, 200 mg q2w and 150 mg qw groups, compared to 4.9% in the placebo group.

Sirukumab is a human anti-IL-6 mAb. Smolen et al[17] report the findings of two parts of a phase II study to assess the safety and efficacy of subcutaneous sirukumab in patients with active RA despite MTX. In part A, the proof of concept study, 36 patients were randomised to placebo or sirukumab 100 mg q2w through week 10, with crossover treatment during weeks 12–22. In part B (dose finding), 151 patients were randomised to sirukumab (100 mg q2w, 100 mg q4w, 50 mg q4w or 25 mg q4w) through week 24, or placebo through week 10 with crossover to sirukumab 100 mg q2w. The primary endpoint (ACR50 at week 12 in part B) was achieved only with sirukumab 100 mg q2w (26.7% vs 3.3% with placebo; p=0.026). Greater improvements in the mean DAS28-CRP score at week 12 were observed with sirukumab 100 mg q2w versus placebo in parts A (2.1 vs 0.6, p<0.001) and B (2.2 vs 1.1; p<0.001). Through week 12 in parts A and B, the incidence of TEAEs was similar among the sirukumab and placebo groups. There were no reports of opportunistic infections, tuberculosis or gastrointestinal perforations. Changes in laboratory values, including neutropenia, liver transaminases and total cholesterol, were consistent with reports for tocilizumab.

Promising findings in a phase IIb study using clazakizumab, a humanised anti-IL-6 mAb, for RA patients have also been previously reported.[18 19] The combination of MTX and clazakizumab (80, 160 and 320 mg intravenously at day 1 and week 8) was associated with rapid and

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Editorial

significant improvements in disease activity as measured by ACR20 and DAS28 in 127 RA patients with MTX-IR within 12 or 16 weeks after treatment.

The ACR20 response rates achieved with tocilizumab, olokizumab, sarilumab and sirukumab were significantly higher than with placebo and were generally consistent except for olokizumab in RA patients with MTX-IR, although the background characteristics of enrolled patients differed among the studies.[8–10 15–17] Also, improvements in DAS28 were comparable between tocilizumab and olokizumab in TNF-IR patients.[15] In general, clinical efficacy as well as safety profiles, as shown below, appear similar among the five mAbs (tocilizumab, olokizumab, sarilumab, sirukumab and clazakizumab), making it difficult to differentiate between them compared to tocilizumab. In fact, UCB has out-licensed olokizumab to R-Pharma after its phase II trial. Anti-IL-6R mAbs indiscriminately affect both the membrane form and the soluble form of the receptor, but these results suggest that anti-IL-6 mAbs could inhibit IL-6 from binding to soluble receptor or membrane receptor, which results in a similar efficacy profile among three anti-IL-6 mAbs and two anti-IL-6R mAbs. On the other hand, Nishimoto *et al* reported that serum levels of IL-6 in Castleman disease were lower than those in RA, while there was no difference in soluble IL-6R levels between the two conditions. However, the increase in IL-6 levels after tocilizumab therapy was much greater in Castleman disease than in RA.[20] Thus, the pathological relevance of the difference between serum IL-6 and soluble/membrane IL-6R remains unclear; it is also difficult to interpret the difference between ligand inhibition and receptor inhibition for the treatment of RA.

Several clinical and functional assessments indicate that switching to tocilizumab is successful in patients with TNF-IR.[11 12] As described, olokizumab resulted in significant improvement in DAS28 as compared to placebo at week 12 in RA patients with TNF-IR.[15] Furthermore, we reported that tocilizumab was a good treatment option for improving signs and symptoms and inhibiting progression of joint damage in 45 RA patients with structural as well as clinical TNF-IR in the REACTION study.[13] Thus, bDMARDs targeting IL-6 were initially recommended as second-line therapy for patients with TNF-IR.[21] However, recent clinical research such as the ADACTA study has changed the ranking of tocilizumab. In this study, comparison of tocilizumab and adalimumab monotherapy for RA patients with MTX-IR revealed that

tocilizumab monotherapy was superior to adalimumab for reducing disease activity in RA, that safety was comparable between both therapies, and that their adverse events (AEs) were consistent with previous findings.[22] Tocilizumab is, therefore, ranked as a first-line bDMARD, similarly to TNF inhibitors in patients with MTX-IR.[14]

Furthermore, tocilizumab appears to have several advantages: (i) tocilizumab monotherapy is significantly superior to MTX, in contrast to monotherapy with TNF inhibitors[23–26]; (ii) tocilizumab is highly effective for systemic juvenile idiopathic arthritis characterised by spiking fever, evanescent skin rash, lymphadenopathy, hepatosplenomegaly and serositis in addition to arthritis[27 28]; and (iii) tocilizumab ameliorates the amyloidosis secondary to RA because it normalises serum levels of amyloid A.[29 30] These promising results in tocilizumab studies have encouraged the development in other bDMARDs targeting IL-6. On the other hand, TNF inhibitors are superior to tocilizumab for the treatment of ankylosing spondylitis and inflammatory bowel diseases, indicating that differential use of TNF inhibitors and IL-6 inhibitors could be another theme to be addressed. Furthermore, although good radiological results with tocilizumab have been documented in multiple reports, studies comparing IL-6 inhibitors with TNF inhibitors should be carried out in order to clarify their similar effects on structural damage.

Soon five bDMARDs will be available for targeting IL-6, which will raise questions as to when and how these agents should be employed. Although more treatment options may be better for patients, several crucial points remain unclear from a clinical point of view. For instance, if patients fail to respond to an anti-IL-6R mAb, might they respond to another anti-IL-6 or anti-IL-6R mAb as happens with TNF inhibitors? Are there any grounds for considering switching between IL-6 inhibitors or, as described above, switching from anti-IL-6R to anti-IL-6 or vice versa? Does switching between IL-6 inhibitors improve their efficacy? Further studies are warranted to establish whether there are important differences among the five IL-6 inhibitors, and to determine which inhibitor should be chosen for a particular patient from a clinical standpoint as regards clinical response and/or structural damage, AEs, and efficacy in patients with TNF-IR.

The safety profiles of olokizumab, sarilumab and sirukumab are similar to each other and to that of tocilizumab as

determined in clinical trials, post-marketing surveillance and clinical practice.[31 32] Commonly reported AEs with IL-6 inhibitors include gastrointestinal disorders, upper and lower respiratory tract and urinary tract infections, and nervous system disorders, similar to those found for olokizumab, sarilumab and sirukumab and to AEs observed for tocilizumab and multiple bDMARDs targeting TNF. However, there were no reports of opportunistic infections, tuberculosis or gastrointestinal perforations in patients with diverticulitis, possibly because of the careful inclusion criteria for each trial (eg, patients with diverticulum were not included in the trials of olokizumab, sarilumab and sirukumab). Nonetheless, safety data from daily practical clinics should be collected. Common laboratory changes were primarily neutropenia and elevated liver function tests and serum lipids with excessive levels of total cholesterol, although the exact clinical consequences and mechanisms remain to be clarified. However, because these trials were too short and too small for strong conclusions on safety and there were no negative findings, safety should be determined with multiple long-term extension studies and nation-wide registries in clinical practice.

Taken together, the safety and efficacy profiles in clinical trials of olokizumab, sarilumab and sirukumab are similar and are consistent with those observed in RA patients treated with tocilizumab. Furthermore, the clinical efficacy of these IL-6 inhibitors is similar to that of TNF inhibitors in patients with MTX-IR and TNF-IR. Screening of biomarkers or genetics in each RA patient, for instance, baseline serum levels of TNF and/or soluble IL-6R, may help to predict the efficacy of each drug and to select patients for cytokine-oriented targeted therapies.[33] However, better strategies are warranted for selecting and identifying appropriate patients earlier once bDMARDs targeting IL-6 are launched in the near future. We also need to determine whether there are important differences between the many IL-6 inhibitors and which are suitable for particular patients, otherwise companies may waste time and money in development.

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Prevalence of Hepatitis B Virus Infection in Patients with Rheumatic Diseases in Tohoku Area: A Retrospective Multicenter Survey

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Hepatitis B virus (HBV) reactivation has been increasingly recognized in patients receiving chemotherapy and immunosuppressive therapy; however, the prevalence of HBV infection and rate of HBV screening in patients with rheumatic diseases remains unclear. In this study, we aimed to assess the prevalence of HBV infection and fulminant HBV hepatitis in patients with rheumatic diseases. We also investigated the rate of HBV screening before immunosuppressive therapy in patients with rheumatic diseases. A retrospective questionnaire survey was conducted in the North-east area (Tohoku) of Japan. Questionnaires, comprising 6 questions, were sent to 318 rheumatologists in May 2010, and responses were gathered until June 2011. In total, 71 rheumatologists (22.3%) responded to the survey. We enrolled 7,650 patients with rheumatoid arthritis (RA) and 1,031 patients with systemic lupus erythematosus (SLE). When limited to institutes at which almost all (≥ 90%) patients were tested for HBV serology, 1.1% (40/3,580) patients with RA and 0.3% (3/1,128) patients with SLE were positive for hepatitis B surface antigen (HBsAg), and 25.2% (177/703) patients with RA and 13.7% (34/248) patients with SLE were positive for hepatitis B core antibody (HBcAb).

About one-third of rheumatologists did not check HBsAg and more than half did not check hepatitis B surface antibody (HBsAb) or HBcAb at all before therapy. Fulminant HBV hepatitis was observed in 1 RA patient who was current HBV carrier. In conclusion, the prevalence of HBV infection is high in patients with RA and SLE. HBV screening before immunosuppressive therapy should be strictly performed.

Introduction

After the widespread use of rituximab, an anti-CD20 monoclonal antibody, hepatitis B virus (HBV) reactivation has been increasingly recognized in patients receiving chemotherapy and immunosuppressive therapy (Oketani et al. 2012). Some studies have shown that HBV reactivation occurs not only in 'current HBV carriers', who are positive for hepatitis B surface antigen (HBsAg), but also in 'resolved HBV carriers', who are negative for HBsAg but positive for hepatitis B surface antibody (HBsAb) and/or hepatitis B core antibody (HBcAb) (Oketani et al. 2012). Particularly, HBV reactivation in resolved carriers may often cause a type of fulminant hepatitis, termed as *de novo* HBV hepatitis, with an extremely high mortality rate (Umemura et al. 2008).

Based on these results, Centers for Disease Control (CDC) recommended screening for HBV serology before chemotherapy and immunosuppressive therapy (Weinbaum et al. 2008). The American College of Rheumatology (ACR) also recommends HBV screening before immunosuppressive therapy (Singh et al. 2012), and recently, the Japanese College of Rheumatology (JCR) proposed an algorithm for HBV screening (Harigai et al. 2014). According to this algorithm, all patients should be screened for HBsAg before immunosuppressive therapy. In addition, those who are negative for HBsAg should be tested for HBsAb and HBcAb. HBV DNA quantification by real-time polymerase chain reaction (RT-PCR) should be performed in resolved HBV carriers. When HBV DNA becomes positive during and after therapy, prophylactic nucleoside analogs such as entecavir should be administered (Harigai et al. 2014). However, evidence to support validity of this algorithm to prevent severe hepatitis is not sufficient and especially needs to clarify cost-benefit relations.

HBV is endemic in Japan, and approximately 20% Japanese individuals are infected with HBV (Kiyosawa et al. 1994). Therefore, HBV screening before treatment should be more strictly performed in Japan than in other non-endemic countries. However, only few studies have reported the prevalence of HBV infection and rate of HBV screening in Japanese patients with rheumatic diseases (Urata et al. 2011; Mori 2011; Watanabe et al. 2013)

In this study, we assessed the prevalence of HBV infection and fulminant HBV hepatitis in patients with rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In addition, we investigated the rate of HBV screening before immunosuppressive therapy in patients with rheumatic diseases.

Methods

A retrospective questionnaire survey was conducted in the North-east area (Tohoku) of Japan. Questionnaires were sent to 318 rheumatologists in May 2010, and we waited for the response until June 2011. Following are the 6 questions listed in the questionnaire: (1) How many patients with RA have you treated? (2) How prevalent is HBV infection in patients with RA? (3) How many patients with SLE have you treated? (4) How prevalent is HBV infection in patients with SLE? (5) Do you examine serological HBV markers before treatment in patients with rheumatic diseases? (6) Have you ever experienced patients with fulminant HBV hepatitis? In this simplified questionnaire survey, we did not check a detail on sex, age, and hepatitis enzymes. We checked each HBV serological marker independently. Therefore, we did not check HBsAg positivity in HBcAb-positive patients.

Diagnoses of RA and SLE were based on RA classification criteria and SLE classification criteria (Arnett et al. 1988; Hochberg 1997; Aletaha et al. 2010). HBV serological tests were performed before starting immunosuppressive therapy at each institute. Immunosuppressive therapy was defined as the use of biologics, immunosuppressive disease-modifying antirheumatic drugs (DMARDs) including methotrexate (MTX), tacrolimus, leflunomide, mizoribine, corticosteroids, and other immunosuppressive agents (Harigai et al. 2014). The study protocol was approved by the ethics committees of Tohoku University Graduate School of Medicine.

Results

Overall response rate

Of the 318 rheumatologists, 71 (22.3%) responded to the questionnaire. Although we waited for the response until June 2011, all the answers obtained were before the Great East-Japan Earthquake (March 11, 2011).

Prevalence of HBV infection in patients with RA

In total, 7,650 patients with RA were enrolled. 0.7% (50/7,650) patients with RA were considered to be current HBV carriers, and 25.6% (214/837) were positive for HBcAb (Table 1). When the patient cohort was limited to institutes at which HBV serology was examined for almost all patients (≥ 90%), 1.1% (40/3,580) patients with RA were current HBV carriers, and 25.2% (177/703) were positive for HBcAb. Among patients receiving biologics, 0.3% (3/1,128) patients were positive for HBsAg, indicating that

Table 1. Positivity rate for each HBV serological marker in patients with RA.

		HBsAg	HBsAb	HBcAb
All patients	Total	50/7,650 (0.7%)	245/1,295 (18.9%)	214/837 (25.6%)
	at institutes ≥ 90% patients were examined	40/3,580 (1.1%)	169/1,011 (16.7%)	177/703 (25.2%)
Patients with biologics	Total	3/1,634 (0.2%)	68/512 (13.3%)	64/274 (23.4%)
	at institutes ≥ 90% patients were examined	3/1,128 (0.3%)	49/391 (12.5%)	57/199 (28.6%)

Table 2. Positivity rate for each HBV serological marker in patients with SLE.

	HBsAg	HBsAb	HBcAb
Total	3/1,031 (0.3%)	26/284 (9.2%)	38/267 (14.2%)
at institutes ≥ 90% patients were examined	3/704 (0.4%)	25/248 (10.1%)	34/248 (13.7%)

Table 3. Screening rate for each HBV marker before starting immunosuppressive therapy in 71 rheumatologists.

	HBsAg	HBsAb	HBcAb	HBV DNA
All patients	18 (25%)	2 (3%)	4 (6%)	0 (0%)
Not all patients	30 (42%)	24 (34%)	29 (41%)	21 (30%)
None	23 (32%)	45 (63%)	38 (54%)	50 (70%)
Total	71	71	71	71

biologics tended to be avoided in current HBV carriers. In contrast, biologics were prescribed for resolved HBV carriers at a similar rate to patients without HBV infection.

Prevalence of HBV infection in patients with SLE

Among 1,031 patients with SLE, 3 patients (0.3%) were positive for HBsAg (Table 2). When limited to institutes at which HBV serology was examined for almost all patients (≥ 90%), 0.4% (3/704) patients were positive for HBsAg and 13.7% (34/248) showed positive results for HBcAb, indicating that the prevalence of HBV infection in patients with SLE was lower than that in patients with RA (p = 0.0002, Chi-square test).

Rate of HBV screening before immunosuppressive therapy

The rate of screening for HBV serological markers before initiating treatment is summarized in Table 3. 71 rheumatologists answered it with respect to each HBV serological marker. HBsAg was examined for all patients by a relatively high number of clinicians (18/71, 25%); however, approximately one-third clinicians (23/71, 32%) did not check HBsAg at all and more than half of the clinicians did not check HBsAb or HBcAb at all.

Fulminant HBV hepatitis

Among all answers, fulminant HBV hepatitis was reported in 1 patient with RA. This patient was 70's female and current HBV carrier (HBsAg-positive, and hepatitis Be antigen-negative), but was treated by MTX 8 mg/week and prednisolone (PSL) 5 mg/day without nucleoside analogs in general physician's clinic. HBV DNA quantification was not performed in this clinic. The patient was admitted to a nearest university hospital and treated with plasma exchange and entecavir, but died of fulminant HBV hepatitis confirmed by HBV DNA quantification and autopsy.

Discussion

This retrospective multicenter questionnaire survey conducted in the North-east area of Japan demonstrated that approximately 1% patients with RA were current HBV carriers and more than 25% were considered to be resolved carriers. Previous reports in Japan showed similar results estimating that 25% (60/239) patients in Kumamoto and 31.5% (135/428) in Aomori were infected with HBV (Urata et al. 2011; Mori 2011), indicating that more than one-fourth patients with RA may be infected with HBV in Japan. However, the number of patients enrolled in this study is much larger than previous reports. To our knowledge, this is the largest study regarding prevalence of HBV infection in RA patients in Japan. Our data suggest that HBV screening and appropriate management of HBV should be strictly performed when initiating immunosuppressive therapy. Prevalence of HBV infection was significantly lower (16.5%; 41/248) in patients with SLE than that in patients with RA (Watanabe et al. 2013). It has been reported that older adults had a higher frequency of HBV

infection that younger adults in Japan (Tanaka et al. 2011). Therefore, one reason to explain this may be the different ages of onset of RA and SLE. However, because high-dose corticosteroids and intensive immunosuppressive therapy, such as cyclophosphamide, are often required to treat patients with SLE, we should not overlook the risk of HBV reactivation, even in patients with SLE.

This retrospective survey is the first report about the rate of HBV screening before immunosuppressive therapy in Japan and also showed that the rate was low in routine clinical practice. However, previous reports from oncologists showed similar results (Tran et al. 2010; Day et al. 2011a, b; Hwang et al. 2012; Zurawska et al. 2012; Lee et al. 2012). For example, Hwang et al. (2012) reported that among 10,729 patients who received chemotherapy, only 1,787 (16.7%) underwent screening for HBsAg or HBcAb. As for rheumatologists, Stine et al. (2010) conducted a nationwide questionnaire survey about HBV screening in 1,000 ACR members. Responses obtained (153/1,000, 15.3%) were highly variable and more than half of the members did not check HBsAb or HBcAb prior to initiating therapy. The authors concluded that it is necessary to improve education among rheumatologists regarding the risks of HBV reactivation in patients in whom immunosuppressive therapy needs to be started.

Recent advances in treatment have changed the therapeutic goal of patients with RA and other rheumatic diseases (Harigai et al. 2014). Biologics, high-dose MTX, and corticosteroids are being increasingly used to induce remission or low disease activity. Among them, the use of biologics, such as tumor necrosis factor-α inhibitors, may cause HBV reactivation (Urata et al. 2011). However, MTX and corticosteroids also have the potential to induce HBV reactivation and de novo hepatitis (Urata et al. 2011; Harigai et al. 2014). The use of biologics is now contraindicated in current HBV carriers (Harigai et al. 2014); however, some rheumatologists prescribe them for current HBV carriers in this study (Table 1). Recently, with an increasing interest in HBV reactivation in Japan, the rate of HBV screening may continue to increase. Nevertheless, a new framework to improve the assessment of the risk of HBV reactivation and fulminant HBV hepatitis by Japanese rheumatologists needs to be developed.

Although fulminant HBV hepatitis was observed in 1 patient who was current carrier, fulminant hepatitis due to HBV reactivation in resolved carriers was not reported in this retrospective study. However, this does not mean that fulminant HBV hepatitis does not occur in resolved carriers because the rate of HBV screening was low. Therefore, we are now conducting a multicenter prospective study to investigate the rate of HBV reactivation in patients with rheumatic diseases under immunosuppressive therapy.

In conclusion, this retrospective questionnaire study demonstrated that approximately 20% or more patients with rheumatic diseases were infected with HBV and the rate of HBV screening before immunosuppressive therapy among

rheumatologists was low in routine clinical practice. Although the incidence of fulminant HBV hepatitis was low, we rheumatologists should improve our consciousness regarding the risk of HBV reactivation and management of HBV infection.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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