Table 3 Distribution of age at onset of SLE, PM/DM, and SSc in fiscal 2007: percentile, mean, most susceptible age

Diseases	Sex	Number	Percentile (%)					Mean	SD	Most susceptible age[a] (years)
			10	25	50	75	90			
SLE	Male	2,042	15	23	35	54	68	38.5	19.3	15–44
	Female	16,995	16	22	32	43	54	33.7	14.5	20–39
PM/DM	Male	1,581	30	42	53	62	70	50.8	16.9	45–64
	Female	4,167	28	40	51	60	69	49.2	16.0	40–64
SSc	Male	1,018	35	46	55	64	70	54.0	14.1	50–69
	Female	7,875	32	42	51	59	67	50.2	13.7	40–59

Patients with unknown age at onset were excluded

SLE systemic lupus erythematosus, *PM/DM* polymyositis/dermatomyositis, *SSc* systemic sclerosis, *SD* standard deviation

[a] We determined the most susceptible age as the minimum range that includes the peak onset age and 50 % of onsets

32 years for men and women, respectively. For PM/DM and SSc, this was in the 50s for both sexes. Mean age at onset was similar to these 50th percentiles in all three diseases. We determined the most susceptible age as the minimum range that included peak occurrence and 50 % of onsets. This showed that men and women are most susceptible to SLE over the age range of 15–44 and 20–39 years, respectively. For PM/DM, this was 45–64 and 40–64, and for SSc, 50–69 and 40–59 years, for men and women, respectively.

Discussion

Here we report in detail present age and distribution of age at onset in patients with SLE, PM/DM, and SSc in Japan. Because these diseases are relatively rare, to produce an epidemiologically effective study, it is necessary to accumulate a large number of patients using nationwide surveys. We used data from a large number of such patients in Japan, which were especially informative regarding age of onset distribution. These data represent very important epidemiological information that has not been reported in any previous studies in Japan, with one exception that analyzed older data [3].

There are several reports describing age, occurrence frequency in men and women, and age of onset of SLE [4–6], PM/DM [7, 8], and SSc [9–11] in Western populations. In Caucasians, peak incidence of SLE occurs between ages 15 and 45 years, with a female-to-male ratio of 6–10:1 [4–6]. The pattern of occurrence of idiopathic inflammatory myopathy was bimodal, with a small childhood peak between 10 and 15 years and adult peak between 45 and 60 years [7, 8], with a female-to-male incidence ratio of 2.5:1 [7]. Age at onset of SSc is most commonly in the range of 45–65 years. As with the other two diseases, SSc is also predominant in women, with a female-to-male ratio of 4–6:1 [9–11]. The report presented here shows that the Japanese population seems similar to Western populations in the factors assessed here. However, age at PM/DM onset did not show a childhood peak. The reason for this may be that alternative financial support for medical treatment for children is provided by local government and therefore some of them are not registered in the national database. It means that a childhood peak may exist in Japanese, but our observation on the national database could not detect it.

There are some limitations to this study. One issue is data representativeness. The data we used, however, are derived from less than half of all registered patients, which depended on the electronic data entry rate. As the reason for the low entry rate is that some prefectures did not enter electronic data at a high enough rate due to financial or clerical problems, it is unlikely that the data entry rate differs between sexes and at different ages. In the observation of the characteristics of sex, age, and age of onset, we can expect that data is representative of the entire registered patient population. Other issues would be possible bias due to the use of data from the registration system, such as accuracy of disease diagnosis and coverage of all patients. Accuracy of data contained within this registration system is likely to be good, because specialist committees were organized in each prefectural government to check diagnoses according to standard criteria ordained by the government. Most patients are expected to be diagnosed and registered in this system, but clearly, we cannot be sure of the rate of omissions. Thus, calculations of prevalence rates from these data may be an underestimation. The prevalence of childhood patients may be also underestimated, as stated above. The last issue is the observation of incidence. We can distinguish newly registered cases, but as some of those may be recurrences or reregistration of patients who have moved (because when a patient changes address across prefectures, the registration may be renewed), not all the apparently newly registered cases are new incidence cases. Therefore, we did not show the incidence here.

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In conclusion, gender distribution, present age, and age at onset of recent SLE, PM/DM, and SSc patients in Japan was surveyed nationwide for fiscal 2007. Our findings provide new information on the natural history of disease development in Japan, which, despite ethnic and other differences, appears similar to that familiar in Western populations.

Acknowledgments This study was supported by grants from Health and Labour Science Research of the Ministry of Health, Labour and Welfare, Japan: Research Committee on Epidemiology of Intractable Diseases and Research Committee on Autoimmune Diseases.

Conflict of interest None.

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ORIGINAL ARTICLE

TLR3-mediated apoptosis and activation of phosphorylated Akt in the salivary gland epithelial cells of primary Sjögren's syndrome patients

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Abstract This study aimed at ascertain whether innate immunity is involved in the apoptosis of primary cultured salivary gland epithelial cells (SGECs) in primary Sjögren's syndrome (pSS). Induction of apoptosis of SGECs was performed using a TLR3 ligand, poly (I:C). Activation of phosphorylated-Akt (pAkt) and cleaved-caspase 3 was determined by Western blotting or immunofluorescence. Expression of TLR2 and TLR3 with pAkt was observed in cultured SGECs after 24-h stimulation with each ligand. Compared with stimulation with the peptidoglycan or lipopolysaccharide, that with poly (I:C) induced significant nuclear fragmentation, as determined by Hoechst staining ($p = 0.0098$). Apoptosis was confirmed by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining of SGECs from pSS patients and a normal subject. A significant increase in TUNEL-positive cells was observed by the addition of a PI3K inhibitor, LY294002. Poly (I:C) phosphorylated stress-activated protein kinase/Jun-terminal kinase and p44/42 MAP kinase as well as Akt. Furthermore, poly (I:C)-induced caspase 3 cleavage in SGECs was also inhibited by LY294002. Similar results were obtained using SGECs obtained from a normal subject. The results demonstrated for the first time that TLR3 induces the apoptotic cell death of SGECs via the PI3K-Akt signaling pathway.

Keywords Sjögren's syndrome · TLR3 · Akt · MAP kinase · Caspase3

Abbreviations

IFN	Interferon
IRF3	Interferon (IFN) regulatory factor 3
FITC	Fluorescein isothiocyanate
LPS	Lipopolysaccharide
LSG	Labial salivary gland
MAP	Mitogen-activated protein
PBS	Phosphate-buffered saline
PGN	Peptidoglycan
PI3K	Phosphatidylinositol 3-kinase
pSS	Primary Sjögren's syndrome
SGECs	Salivary gland epithelial cells
TRIF	TIR domain-containing adaptor-inducing IFNβ
TRITC	Tetramethyl rhodamine isothiocyanate
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling

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Introduction

Toll-like receptors (TLRs) are known as intermediation receptors involved in innate immunity [1]. Some TLRs are signaling adaptor molecules that are stimulated by bacterial or viral nucleic acid sequences [2, 3]. We previously reported the expression of TLR2, 3, and 4 in labial salivary glands (LSGs) obtained from patients with primary Sjögren's syndrome (pSS) [4] showing sicca symptoms due to salivary gland destruction [5, 6]. These three types of TLRs

have been shown to be expressed in a human salivary gland (HSG) cell line, as well as in LSGs from pSS patients in vitro [4]. Additionally, in vitro stimulation of an immortalized human salivary gland cell line, HSG cells, with TLR ligands did not induce Akt phosphorylation but rather the phosphorylation of mitogen-activated protein kinases (MAPKs) [4]. However, no detailed kinetic analyses of apoptotic signals and Akt activation in cultured primary salivary gland epithelial cells (SGECs) of pSS patients have been conducted to date. In our series of studies, apoptotic sensitivity to pro-apoptotic signaling in SGECs differed from that in HSGs [7, 8]. For instance, a significant difference in sensitivity to anti-Fas antibody was observed between these cell types. Although HSGs showed sensitivity to a single stimulation with anti-Fas antibody, cultured SGECs required stimulation with both anti-Fas antibody and phosphoinositide-3-kinase (PI3K) inhibitor to induce apoptosis [7]. Since recent studies have shown that TLRs can induce apoptosis in certain types of cells such as human breast tumor cells [9, 10], it is reasonable to speculate that SGECs and HSGs may respond differently to TLR ligands. Thus, findings obtained thus far with SGECs appear to be more relevant to the clinical setting than those of series using HSGs. In the present study, we investigated TLR-mediated cell death and the expression of relevant anti-apoptotic molecules in the SGECs of pSS patients.

Materials and methods

Patients

This study contained three female patients with pSS (age: 62.7 ± 4.7). Diagnosis of pSS was determined by the revised criteria proposed by the American-European Consensus Group [11, 12]. SGECs obtained from a 59-year-old female who showed sicca symptoms without a diagnosis of pSS were used as the normal control. Labial salivary gland (LSG) biopsies were performed after informed consent was obtained from all participants. The study was conducted in accordance with the human experimental guidelines of our institution.

Antibodies and reagents

Anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal goat anti-TLR2, 3, and 4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) and donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Hoechst dye 33258 was purchased from Sigma (St. Louis, MO, USA). The selective PI3K inhibitor LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Peptidoglycan (PGN) from Staphylococcus aureus and poly (I:C) were purchased from InvivoGen (San Diego, CA, USA) and lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma (St. Louis, MO, USA).

Culture of primary salivary epithelial cells

The method used for culturing SGECs from pSS patients has been described in our previous reports [7, 8]. Briefly, minor salivary gland tissue was excised and cultured in a defined keratinocyte-SFM culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with hydrocortisone (Sigma) and bovine pituitary extract (Kurabo, Osaka, Japan). For immunofluorescence studies, the SGECs were cultured on 12-mm^2 cover slips that were prospectively coated with a Type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Immunofluorescence

The SGECs on 12-mm^2 cover slips were incubated for 10 min in PBS containing 4 % paraformaldehyde at 4 °C, and the cells were subsequently immersed in methanol at −20 °C for 10 min. After the reaction was blocked in 5 % normal horse serum in PBS, the SGECs were incubated in the primary antibodies for 1 h at room temperature. After the cells were washed three times in PBS, the SGECs were incubated with FITC-labeled and TRITC-labeled secondary antibodies in medium supplemented with Hoechst dye 33258 under dark conditions. After incubation with the secondary antibodies, the SGECs were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and were scanned by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany). Control experiments were performed to confirm the isotype specificity of the secondary antibodies.

Induction of apoptosis

After 12 h of growth-supplement starvation, the primary cultured SGECs were treated with poly (I:C) (final concentration: 25 μg/ml) for 24 h.

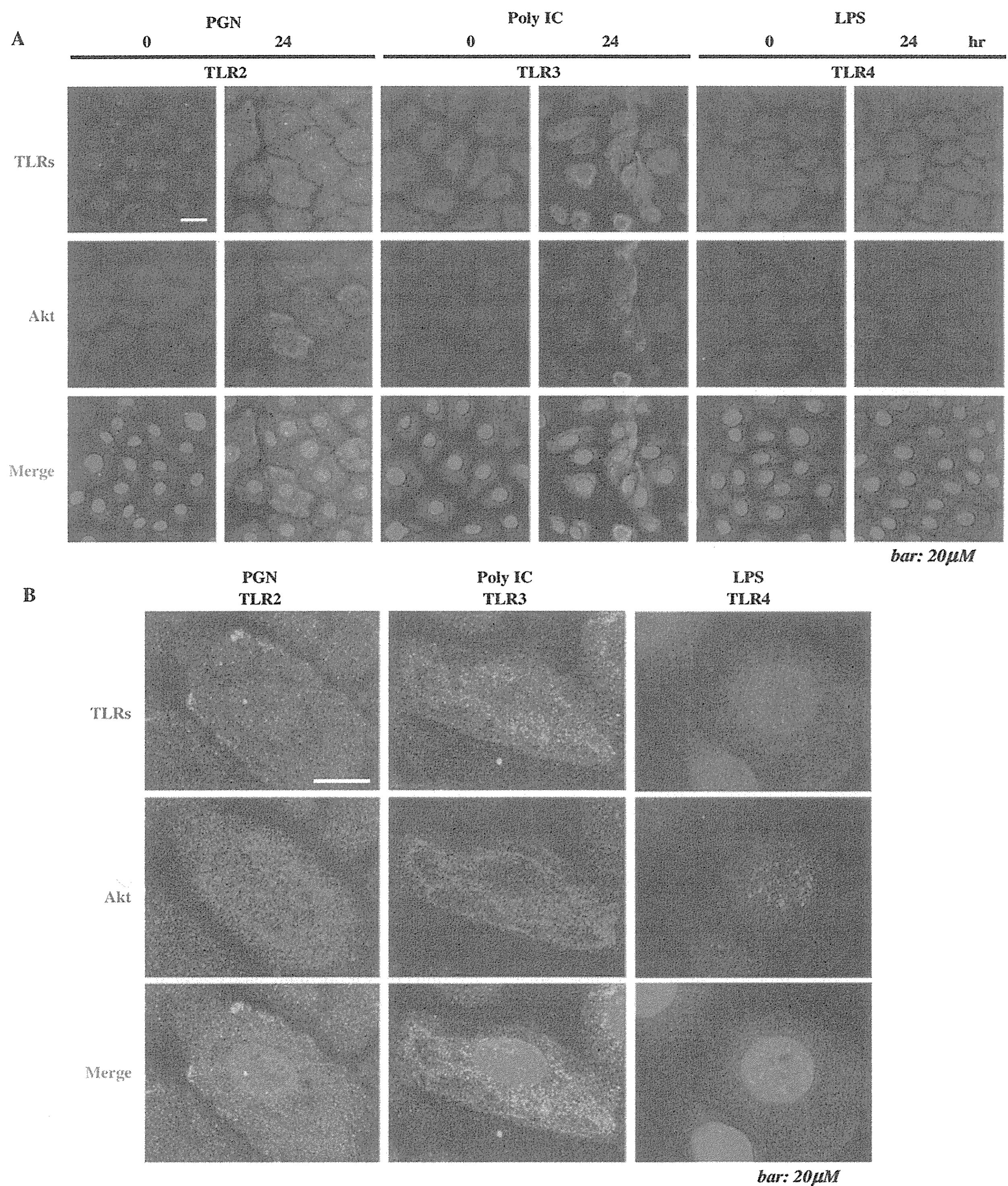

Fig. 1 Expression of TLRs in the presence of their ligands in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 10 μg/ml of PGN, 25 μg/ml of poly (I:C), or 1 μg/ml of LPS for 24 h (low magnification; **a**). The SGECs were double-labeled using goat anti-TLR2, 3, or 4 antibody with FITC-conjugated secondary antibody (*green*) and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (*red*). The status of the nucleus was observed by Hoechst staining (*blue*). **b** A higher-magnification view after 24-h stimulation with each ligand. Shown are the representative results of three independent experiments (*bar*, 20 μM)

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

For the detection of apoptosis, TUNEL staining was employed to demonstrate double-stranded DNA breaks, as shown in our previous study [13]. Later, the SGECs were fixed in 4 % PFA 4 °C for 15 min followed by immersion in PBS with 0.5 % Tween 20 and 0.2 % bovine serum albumin using the MEBSTAIN Apoptosis kit Direct (MBL, Nagoya, Japan). The SGECs were incubated with a 50 µl terminal deoxynucleotidyl transferase (TdT) solution at 37 °C for 1 h. The stained SGECs were captured by confocal microscopy and analyzed by WinROOF software (Mitani Corporation, Fukui, Japan) [14].

Western blot analysis

The method used for Western blot analysis has also been described in our previous reports [8]. Briefly, the SGECs were lysed and the protein concentrations were determined, and identical amounts of protein were subjected to 12.5 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After the proteins were transferred to a polyvinylidene fluoride filter, blocking for 1 h using 5 % nonfat dried milk in Tris-buffered saline containing 0.1 % Tween 20 was performed, after which the cells were incubated at 4 °C overnight with anti-cleaved caspase 3 rabbit monoclonal antibody, phosphorylated-Akt S^{473}, phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase rabbit polyclonal antibodies. After incubation with a 1:1,000 dilution of donkey anti-rabbit IgG, coupled with horseradish peroxidase, detection with an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA) was performed. For statistical analysis, the Student's t test was used ($p < 0.05$; considered as statistically significant).

Results

Expression of TLRs and phosphorylated Akt in primary SGECs with TLR ligand stimulation

We initially examined the expression of three types of TLR in primary cultured SGECs stimulated by TLR ligands (Fig. 1a). Although TLR2 and TLR3 were detected in the cell membrane or cytoplasm in the presence of PGN and poly (I:C), no TLR4 signal was detected (Fig. 1b). Phosphorylated Akt was also detected in the presence of PGN and poly (I:C).

Frequency of nuclear fragmentation under the presence of TLR ligands in primary SGEC from pSS patients and a normal subject

Nuclear fragmentation was detected using Hoechst staining in pSS patients. When 100 cells in three different fields

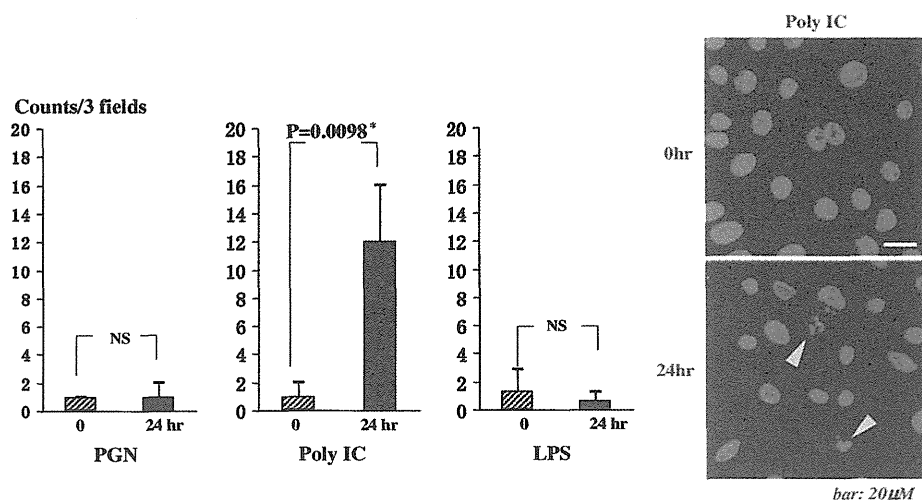

Fig. 2 Nuclear fragmentation induced by poly (I:C) in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 10 µg/ml of PGN, 25 µg/ml of poly (I:C), or 1 µg/ml of LPS for 24 h. Then, to quantify nuclear fragmentation identified by Hoechst staining, 100 cells of interest were counted in three different fields. In the *left panel*, the average number of cell deaths observed among poly (I:C)-stimulated cells was statistically compared with that of PGN or LPS-stimulated cells, as evaluated by unpaired Student's t test ($p < 0.05$; statistically significant). *NS* not significant. Shown are the representative results of two independent experiments. The *right panel* shows representative nuclear fragmentation (*arrowheads*) induced by poly (I:C)

were counted to quantify the fragmented cells, poly (I:C) stimulation induced a statistically significant amount of fragmentation (p value = 0.0098, determined by Student's t test, $p < 0.05$; statistically significant) compared with that induced by PGN or LPS (Fig. 2, left panel). For the normal subject, poly (I:C) stimulation also induced significant nuclear fragmentation (p value = 0.0023). A representative fragmentation in a pSS patient was observed (Fig. 2, right panel).

Detection of poly (I:C)-induced apoptosis by TUNEL assay

TUNEL staining was employed to determine whether the fragmentation determined by Hoechst staining was due to cell death. Twenty-four hours after stimulation with poly (I:C) in SGECs from pSS patients, nuclear fragmentation was detected by bright-field and Hoechst staining. The Hoechst-positive cells were merged, as shown by TUNEL

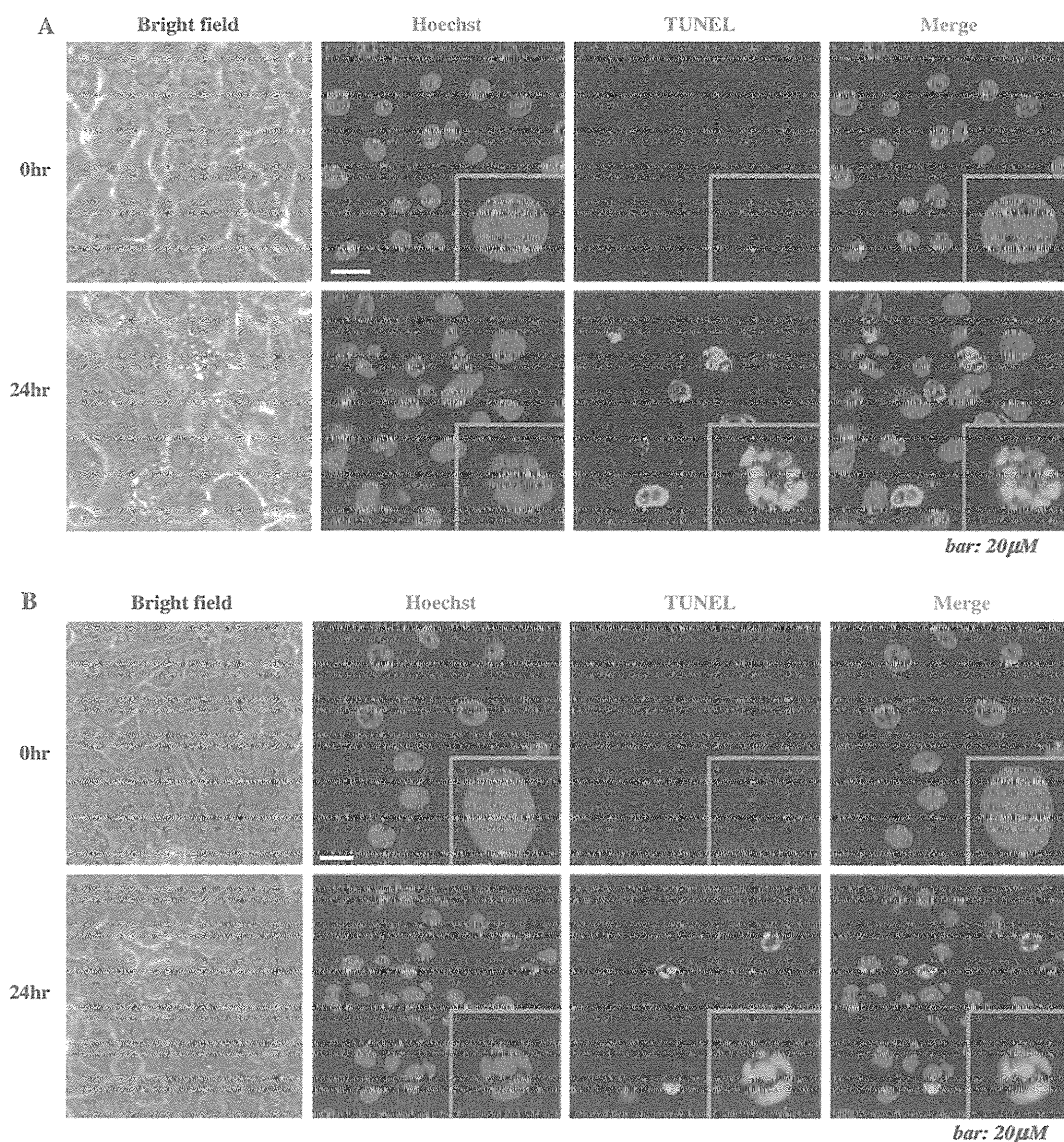

Fig. 3 Detection of double-stranded DNA breakage by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h. To confirm apoptosis as a double-stranded DNA break at the site of nuclear fragmentation, we employed TUNEL-staining coupled with bright-field view. **a**, **b** The results from a pSS patient and a normal subject, respectively. The inset shows representative staining for each panel. The merged view shows that nuclear fragmentation corresponded to apoptosis (*bar*, 20 μM). Shown in **a** are the representative results of two independent experiments with pSS patients

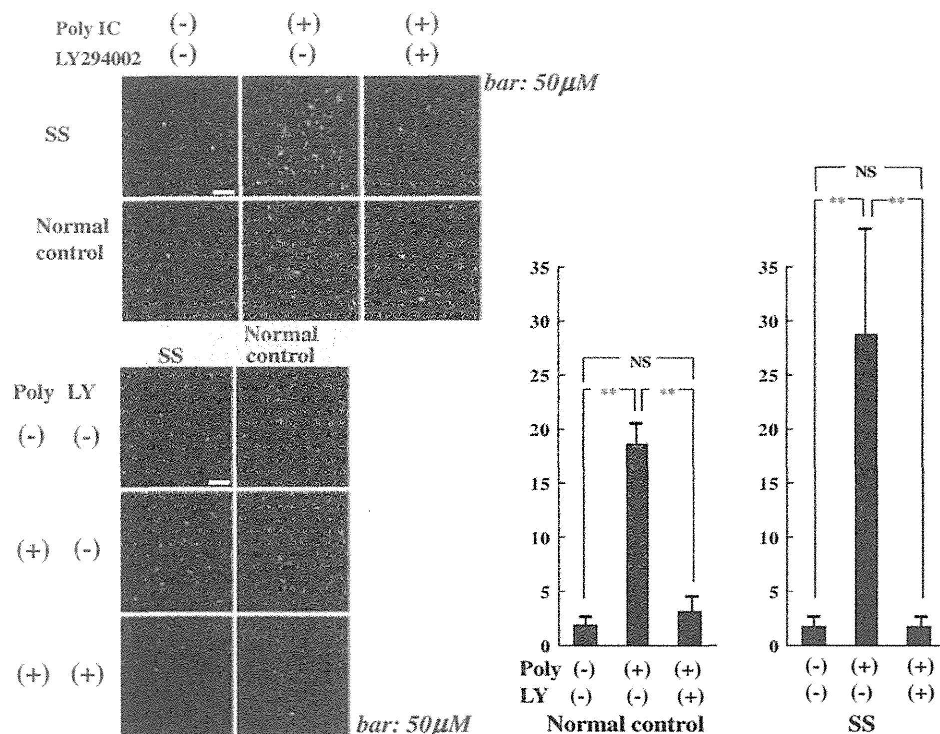

Fig. 4 Quantification of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive apoptotic cells in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h. The TUNEL staining image from a pSS patient and a normal subject (*upper panel*) was merged by Hoechst staining (merged view; *bar*, 50 μM). Then, poly (I:C)-induced apoptosis with or without 50 μM of the PI3K inhibitor, LY294002, was detected by TUNEL staining (green) in pSS patients and a normal control, followed by quantification with WinROOF software (pink signal) (*lower panel*). The calculated areas of the captured signals were statistically compared using unpaired Student's *t* test ($p < 0.05$; statistically significant) (*right panel*). *NS* not significant. Shown are the representative results of two independent experiments

staining (Fig. 3a). Furthermore, poly (I:C)-induced TUNEL-positive cells had merged, as determined by Hoechst staining in the SGECs from one normal subject (Fig. 3b). Poly (I:C)-induced apoptosis detected by TUNEL staining (Fig. 4, upper panel) in pSS patients and the normal control was quantified by converting the TUNEL-positive signal (green) into a pink signal, as observed with an image analyzer (Fig. 4, lower panel); significant acceleration of poly (I:C)-induced apoptosis was seen, as was subsequent inhibition by the addition of a PI3K inhibitor, LY294002, in both groups (Fig. 4, right panel). There was also significant difference of poly (I:C)-induced apoptosis between in both groups ($p < 0.01$).

Akt phosphorylation at the poly (I:C)-induced apoptotic site

To determine whether the phosphorylation of Akt is associated with the poly (I:C)-induced cell death of SGECs, immunostaining of phosphorylated Akt and TLR3 was performed at the site of nuclear fragmentation determined by Hoechst staining. In the SGECs from pSS patients, clear expression of TLR3 and phosphorylated Akt was observed in concert with nuclear fragmentation (Fig. 5, left panel). In the normal subject, poly (I:C)-induced expression of TLR3 and phosphorylated Akt was observed at the site of nuclear fragmentation (Fig. 5, right panel). In the normal subject, poly IC-induced expression of TLR3 and phosphorylated Akt was also observed in cells lacking nuclear fragmentation (Fig. 5, lower panel), which was similar to the co-expression of TLR3 and phosphorylated Akt found in patients with pSS described in Fig. 1b.

Poly (I:C)-induced MAP kinase cleavage of caspase 3 and reversal of effect by PI3K inhibitor

Poly (I:C)-induced expression of MAP kinases including phosphorylated stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated p38 MAP kinase, and phosphorylated p44/42 MAP kinase was performed. Phosphorylation of SAPK/JNK and p44/42 MAP kinase

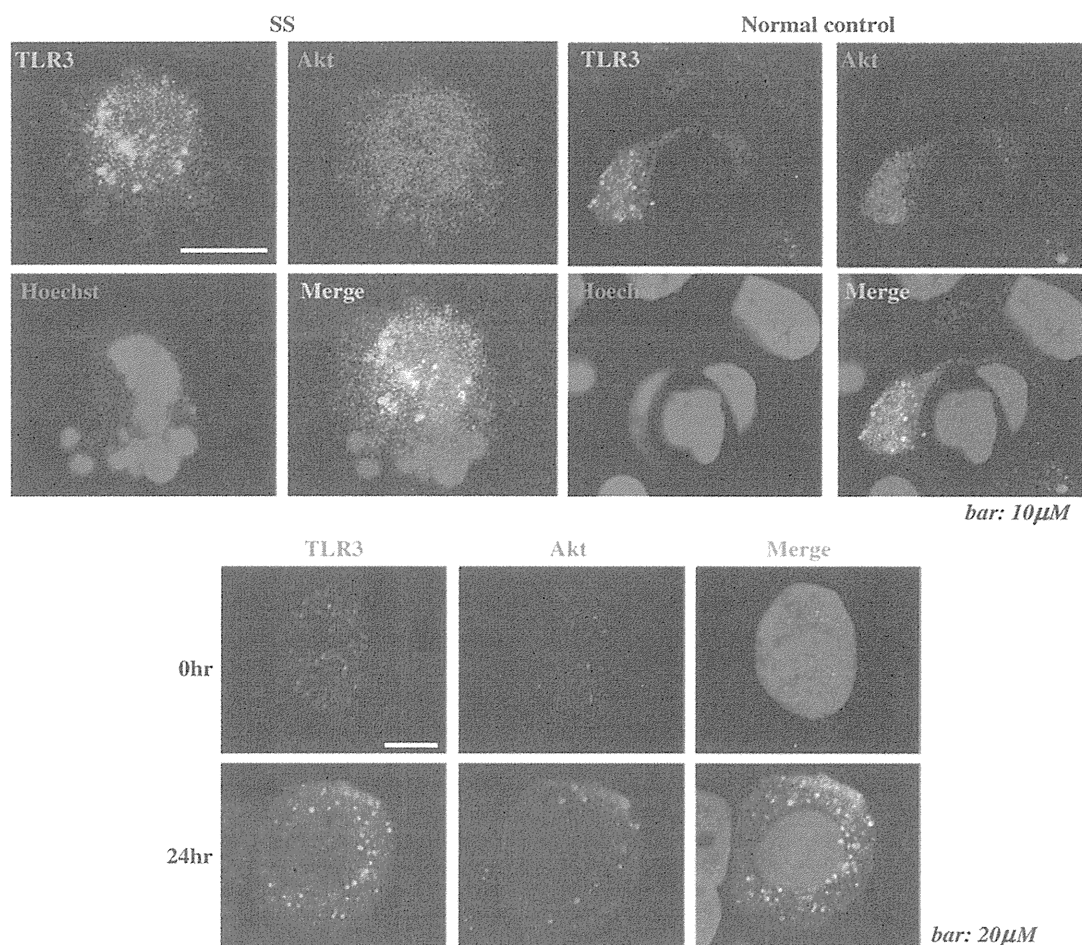

Fig. 5 Co-expression of TLR3 and phosphorylated Akt in apoptotic cells in primary cultured salivary gland epithelial cells (SGECs). After 12 h of starvation of growth supplement, primary cultured SGECs were treated with 25 μg/ml of poly (I:C) for 24 h with or without 50 μM of the PI3K inhibitor LY294002. Nuclear fragmentation detected by Hoechst staining from a pSS patient (*left panel*) and a normal subject (*right panel*) was observed, and the results of Hoechst staining were merged with double-labeled samples using goat anti-TLR3 antibody with FITC-conjugated secondary antibody (*green*), and rabbit anti-phosphorylated Akt antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (*red*). (*Bar* 10 μM). Shown are the representative results of two independent experiments. The *lower panel* shows the expression of TLR3 along with phosphorylated Akt in primary cultured SGECs treated with 25 μg/ml poly (I:C) for 24 h in a normal subject. (*Bar* 20 μM). Shown are the representative results of two independent experiments with pSS patients

was observed, although phosphorylation of p38 was not found. In addition, poly (I:C)-induced signal of phosphorylated SAPK/JNK and p44/42 MAP kinase in pSS was stronger than that in a normal subject. Slight phosphorylation of Akt induced by poly (I:C) was also found. Poly (I:C)-induced cleavage of caspase 3 was examined by Western blot analysis (Fig. 6). Poly (I:C) stimulation clearly revealed cleavage of caspase 3 in the pSS-SGEC lysate by Western blotting, and this result was also obtained in the case of the lysate from the normal subject. Furthermore, cleavage of caspase 3 induced by poly (I:C) was reversed by the addition of LY294002.

Discussion

In this study, TLR-induced apoptosis was clearly observed in the SGECs of pSS patients, as well as in a normal subject. Hsu et al. [15] initially demonstrated that TLRs had the potential to induce MyD88-independent apoptosis in the presence of the protein kinase PKR. Liew et al. [16] reported that TLR2, TLR3, and TLR4 could induce caspase-dependent or -independent apoptosis, in which MyD88-dependent and TIR domain-containing adaptor-inducing IFNβ (TRIF)-dependent pathways were initiated. In addition, Khvalevsky et al. [10] reported that TLR3

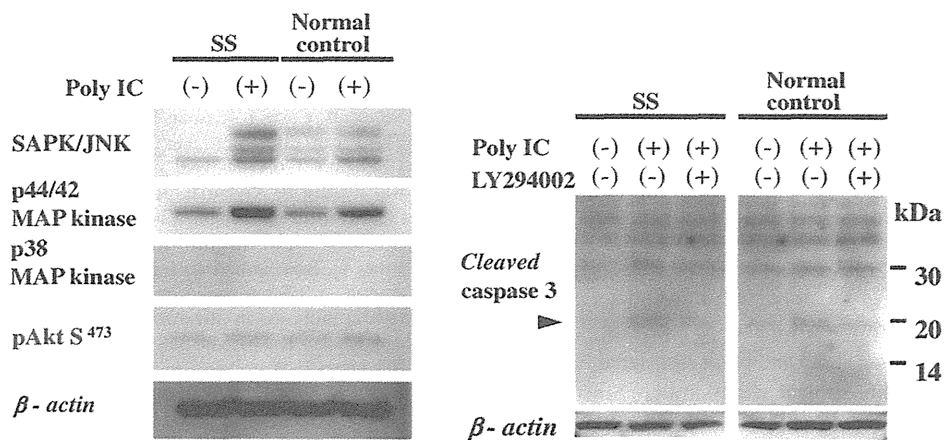

Fig. 6 Detection of poly (I:C)-induced mitogen-activated protein (MAP) kinases and cleavage of caspase 3 in the presence of PI3K inhibitor in primary cultured salivary gland epithelial cells (SGECs). Primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 h. Then, poly (I:C)-induced expression of Akt and mitogen-activated protein kinases including phosphorylated-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), phosphorylated-p38 MAP kinase, and phosphorylated-p44/42 MAP kinase was determined by Western blotting (Fig. 6 *left panel*). With or without 50 μM of the PI3K inhibitor LY294002, primary cultured SGECs from a pSS patient or a normal subject were treated with 25 μg/ml of poly (I:C) for 24 h. Then, poly (I:C)-induced cleavage of caspase 3 was determined by Western blotting (Fig. 6 *right panel*). As a control, β-actin was used. Shown are the representative results of two independent experiments with pSS patients

signaling also induced apoptosis in specific cell lines. Usually, in TLR3 signaling, TRIF is recruited after ligand binding, followed by the activation of NF-κB [17] and interferon (IFN) regulatory factor 3 (IRF3). However, some cell lines showed no induction of NF-κB or IRF3; instead, TLR3-dependent cell death was induced in these cell lines. However, in the report [10] by Khvalevsky and co-workers, higher levels of poly (I:C)-induced apoptosis were observed in a colon adenocarcinoma cell line, HepG2, then in a hepatoma cell line, Huh7, or in a human embryonic kidney cell line, HEK293, which suggested that sensitivity to poly (I:C) might be cell-species specific. One explanation for such a difference in apoptotic sensitivity was suggested by Meylan et al. [18], who previously noted that RIP1 activity was required in TLR3 signaling, which indicated that in some cell species, the ability of RIP1 to induce caspase activation was impaired.

With regard to the involvement of Akt in TLR3 signaling, Sarkar et al. [19] revealed that the PI3K-Akt pathway was crucial for TLR3-mediated double-strand RNA-induced genes such as ISG56. Sarkar and colleagues demonstrated that TLR3 downstream of IRF3 was not fully phosphorylated when recruitment of PI3 kinase to TLR3 was blocked, suggesting an essential role for the PI3K-Akt pathway in the TLR3-mediated innate response. Inhibition of PIK3 by a specific inhibitor, LY294002, was followed by clearly impaired TLR3-mediated signaling. In our experiment, phosphorylation of Akt was accompanied by poly (I:C)-induced apoptosis of SGECs. It remains unclear why the phosphorylation of Akt was correlated with the apoptotic process; however, the downstream signal following the adaptation of TRIF to TLR3 might be involved in the phosphorylation of Akt, coupled with RIP1 activation, which is known to lead to the cleavage of caspase.

Proapoptotic signals in pSS have been reported in previous studies of Fas/Fas ligands, i.e., granzyme/perforin cytotoxic granules generated by CD8+ cytotoxic T lymphocytes [20–24]. As we have reported recently, in cultured SGECs, the Fas/Fas ligand system is well understood [7, 8], and the Fas signal accompanying PI3K inhibition is known to have the potential to induce apoptosis. Furthermore, we recently revealed a rapid induction of apoptosis by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the SGECs of pSS [8]. As regards TLR3-mediated cell death in patients with pSS, Manoussakis et al. [25] recently reported that detachment-induced apoptosis was observed in poly (I:C)-treated SGECs from patients with SS. However, the innate immunity-related induction of apoptosis and the anti-apoptotic system in pSS has not yet been fully elucidated. Although no direct association with SS was observed, Numata et al. [26] more recently demonstrated TLR3-mediated apoptosis of human bronchial epithelial cells. Their study clearly showed that insulin-dependent PI3K-Akt signaling inhibited TLR3-mediated cell death. Thus, their results might help elucidate the role of the PI3K-Akt pathway as an anti-apoptotic process in TLR3-mediated cell death. Here, another possibility except TLR3 to induce apoptosis can be considered because TLR3

expression was observed after 24-h stimulation with poly (I:C). Since poly (I:C) also strongly induced melanoma differentiation-associated gene-5 or retinoic acid-induced protein I [27], these mechanisms should be concerned in the apoptotic process of SGEC in SS. The present study suggests a new mechanism to account for salivary gland cell death. The detailed relationship between PI3K-Akt signaling and molecules downstream of the ligation with TLR3 remains supported by the evidence, since PI3K-Akt appears to act as an inducer of the poly (I:C)-induced apoptotic cell death of SGECs. However, we should note that poly (I:C)-induced expression of TLR3 and phosphorylated Akt in pSS patients and a normal subject were similar. Since the difference was found in poly (I:C)-induced MAP kinases and apoptosis, the phenomenon might explain difference of sensitivity toward pro-apoptotic signal in both groups. Phosphorylation of MAP kinases induced by poly (I:C) is different due to cell species or time course. For example, phosphorylation of p38 is found in corneal fibroblasts at 60-min stimulation with poly (I:C) [28].

In summary, we focused on TLR3-induced apoptosis and the associated phosphorylation of Akt in pSS. These findings may provide novel insights into the apoptotic and anti-apoptotic systems found in the labial salivary glands in pSS. However, the precise signals downstream of TLR-3 have yet to be determined. Downstream signal analysis and related investigations will be necessary to elucidate TLR3-mediated apoptosis of SGECs in pSS.

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ORIGINAL ARTICLE

Correlation between salivary epidermal growth factor levels and refractory intraoral manifestations in patients with Sjögren's syndrome

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Abstract

Objective. To assess changes in salivary epidermal growth factor (EGF) levels and the correlation between these levels and the severity of intraoral manifestations in Sjögren's syndrome (SS).
Methods. Forty SS patients and 23 controls were enrolled. 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). The associations among salivary flow rate, EGF levels and the severity of intraoral manifestations were analyzed.
Results. The total salivary EGF output was significantly decreased in the SS patients compared with the controls (9237.6 ± 8447.0 vs. 13296.9 ± 7907.1 pg/10 min, respectively, $p = 0.033$). In the SS patients, total EGF output and salivary flow rate showed a strong positive correlation ($r_s = 0.824$, $p = 0.0005$), while total EGF output and disease duration showed a negative correlation ($r_s = -0.484$, $p = 0.008$). Further, total EGF output was significantly correlated with the OHIP-14 score ($r_s = -0.721$, $p = 0.012$).
Conclusions. The salivary flow rate and EGF levels are decreased in SS, and this deterioration in saliva quality causes refractory intraoral manifestations. Our findings have provided new therapeutic targets for SS.

Keywords

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

History

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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, related to a reduced salivary flow rate. In addition to the discomfort due to xerostomia, dry mouth can cause various intraoral manifestations, that is dental caries and oral mucosal involvements, such as refractory stomatitis, oral ulcer and atrophic changes in the oral mucosa and lingual papilla, and because of complexities caused by these involvements and the chronicity of SS, patients' quality of life (QOL) can be impaired severely [1]. The intraoral manifestations in SS patients are believed to be caused mainly by a decrease in the clearance in the oral cavity owing to hyposalivation. However, considering that saliva has several beneficial physiological effects on the environment inside the oral cavity, such as lubrication and maintenance of mucosal integrity and antimicrobial activity [2], qualitative changes in sialochemistry 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 new-born 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 are the parotid glands [4,6]. The distribution EGF concentration in parotid gland saliva, submandibular saliva and whole saliva is in the ratio 6:1:4 [4]. 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]. 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 [8,9] and gastrointestinal tract [10–13]. Additionally, previous studies in animal [14] and human models [15] suggest that topical EGF significantly enhances the healing of skin wounds. Therefore, many skin-care cosmetics containing EGF have been produced recently.

Although the detailed mechanisms by which EGF secretion into saliva is controlled are not yet known, studies have found that salivary EGF levels were significantly decreased in patients with intraoral inflammatory lesions, such as stomatitis aphthosa [4,16] and peritonsillar abscess [4]. Patients with oral mucositis induced by radiation therapy for head and neck carcinoma also were found to have markedly low salivary EGF levels [17,18]. These findings suggested that low salivary EGF levels reduce the capacity of the oral mucosa to heal after injury and maintain its physiologic integrity.

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To the best of our knowledge, no study conducted thus far has measured salivary EGF levels in SS patients. The objective of this study then was to evaluate changes in salivary EGF levels in SS patients and to assess the association between salivary EGF levels and the severity of intraoral manifestations in SS.

Materials and methods

Patients

Forty patients with SS, followed up at Division of Rheumatology, Hyogo College of Medicine Hospital, participated in this study. Of these, 27 had primary SS and 13 had secondary SS (comorbidities: rheumatoid arthritis (RA; $n = 4$), systemic lupus erythematosus (SLE; $n = 3$), systemic sclerosis ($n = 3$), CREST syndrome ($n = 1$), dermatomyositis (DM; $n = 1$) and mixed connective tissue disease ($n = 1$)). All patients fulfilled the American–European Consensus Group classification criteria for SS [19]. No significant difference was observed in age and sex between the primary SS group (mean age, 55.0 ± 13.9 years (range, 29–81 years), 24 women and 3 men) and secondary SS group (mean age 56.0 ± 12.0 years (range, 34–77 years), 13 women) ($p = 0.416$ and $p = 0.538$, respectively). Twenty-three individuals without SS, including healthy individuals ($n = 3$) and those with RA ($n = 7$), polymyalgia rheumatica ($n = 4$), DM ($n = 2$), bronchial asthma ($n = 2$), SLE ($n = 1$), adult-onset Still's disease ($n = 1$), relapsing polychondritis ($n = 1$), SAPHO syndrome ($n = 1$) and eosinophilia ($n = 1$) were recruited as controls (non-SS group). The exclusion criteria, which are related to factors that affect the intraoral environment or saliva secretion and salivary EGF, 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, antianxiety drugs and antipsychotic drugs; severe diabetes mellitus; severe reflux esophagitis; past history of head and neck carcinoma; previous radiation therapy to the head and neck region; and patients who had 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 the study.

Saliva collection

Whole stimulated saliva was collected after the subjects chewed gum (Free Zone Gum Hi-Mint®; Lotte, Tokyo, Japan) for 10 min and expectorated into graduated centrifuge tubes. All samples were similarly collected before breakfast around the same time 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 − 20°C until the assay.

Quantification of salivary EGF

Just before EGF measurement, the saliva was defrosted and centrifuged for 10 min at a temperature of 4°C and a speed of 3000 rpm. Salivary EGF levels were measured using a commercial enzyme-linked immunosorbent assay kit (Quantikine®; R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Each sample was assayed in duplicate. 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) [18].

Quantification of intraoral manifestations

At the time of saliva collection, subjective intraoral manifestations were assessed by means of a short Japanese version of the Oral Health Impact Profile (OHIP-14), which is a self-administered questionnaire. The OHIP is one of the most widely used instruments to measure oral health-related QOL (OHRQoL). Since the original OHIP [20] is 49-item measure, clinicians may not be inclined to use it in daily clinical practice. Therefore, the short OHIP-14, which has only 14 questions and has good reliability and validity, was developed as a modified version of the OHIP [21]. In addition, the OHIP was translated from English to Japanese, and this Japanese version of the OHIP (OHIP-J) reportedly has good reliability and translated validity [22,23]. Although the OHIP has been generally used for assessing OHRQoL in the elderly, Ide et al. proved that the OHIP-J is suitable for assessing OHRQoL in young and middle-aged adults as well [24]. Moreover, Stewart et al. showed that in SS patients, lower salivary flow rates were significantly associated with poorer oral health as determined using the OHIP-14 summary score [1].

The OHIP-14 consists of 14 questions designed to measure the frequency of problems associated with the teeth, mouth or dentures. The questions have seven aspects: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap. Using a five-point scale ranging from 0 to 4 (0, never; 1, hardly ever; 2, occasionally; 3, fairly often and 4, very often), participants rated how frequently they had experienced each item addressed in the 14 questions. The unweighted ratings for the 14 questions were then summed, and a single summary score with a possible range of 0 to 56 was calculated on the basis of the combined scores. Higher scores indicated more frequent problems, that is poorer OHRQoL.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The Mann–Whitney *U* test, chi-square test or Fisher's exact test were used as appropriate, to compare differences between the SS group and the non-SS control group. The correlations between various factors were examined using Spearman's rank correlation coefficient. A value of $p < 0.05$ was considered statistically significant.

Results

Patient characteristics in the study using salivary sample and the OHIP-14 questionnaire

The characteristics of the study groups are presented in Table 1. No significant difference in age and sex was observed between the groups. The mean disease duration of SS was 5.6 years. Twenty-one patients in the SS group received therapy with muscarinic M3 receptor agonists (pilocarpine or cevimeline), while 13 patients from this group and 17 patients from the non-SS group were administered corticosteroid or immunosuppressant (e.g. azathioprine, cyclosporine, tacrolimus, methotrexate and etanercept). The salivary flow rate in the SS group (7.8 ± 4.4 ml/10 min) was significantly lower than that in the non-SS group (16.9 ± 5.9 ml/10 min) ($p < 0.0001$). The OHIP-14 score in the SS group (11.3 ± 9.4) was significantly higher than that in the non-SS group (7.1 ± 7.6) ($p = 0.037$). Thus, the OHRQoL of SS patients was poor compared with that of the non-SS patients.

Comparison of salivary EGF levels between SS and non-SS patients

The salivary EGF concentration in the SS group (1109.4 ± 852.3 pg/ml) was significantly higher than that in the non-SS group

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Table 1. Clinical characteristics of the study groups.

	SS (N = 40)	Non-SS (N = 23)	p value
Age (years) (range)	55.4 ± 13.2 (29–81)	56.1 ± 17.4 (31–82)	0.425
Sex (male/female, number)	3/37	5/18	0.129
Disease duration (years) (range)	5.6 ± 3.7 (0.2–12; N = 24)	–	–
Dry eye symptoms (number (%))	34 (85)	1 (4)	< 0.0001
Xerostomia symptoms (number (%))	35 (88)	3 (13)	< 0.0001
Anti-SS-A antibody (number (%))	37 (93)	0 (0)	< 0.0001
Anti-SS-B antibody (number (%))	11 (28)	0 (0)	0.005
Muscarinic M3 receptor agonist (number (%))	21 (53)	0 (0)	< 0.0001
Corticosteroid or immunosuppressant (number (%))	13 (33)	17 (74)	0.004
Salivary flow rate (ml/10 min) (range)	7.8 ± 4.4 (1.0–21.4)	16.9 ± 5.9 (8.9–35.3)	< 0.0001
OHIP-14 score (out of 56) (range)	11.3 ± 9.4 (0–39; N = 35)	7.1 ± 7.6 (0–25)	0.037
Salivary EGF concentration (pg/ml) (range)	1109.4 ± 852.3 (60.9–3852.5)	778.5 ± 371.9 (271.7–1699.7)	0.041
Total salivary EGF output (pg/10 min) (range)	9237.6 ± 8447.0 (356.5–34623.1)	13296.9 ± 7907.1 (2632.3–29996.5)	0.033

(778.5 ± 371.9 pg/ml) (p = 0.041), whereas the total salivary EGF output in the SS group (9237.6 ± 8447.0 pg/10 min) was significantly lower than that in the non-SS group (13296.9 ± 7907.1 pg/10 min) (p = 0.033) (Table 1). No significant difference was observed in the salivary flow rate, salivary EGF concentration or total salivary EGF output between the primary SS group (7.7 ± 4.0 ml/10 min, 1195.4 ± 938.7 pg/ml and 9521.7 ± 8222.1 pg/10 min, respectively) and secondary SS group (8.0 ± 5.5 ml/10 min, 930.6 ± 632.4 pg/ml and 8647.4 ± 9212.0 pg/10 min, respectively) (p = 0.420, p = 0.182 and p = 0.382, respectively). Because the clinical background varied widely among the SS patients, this group was divided into two groups depending on two clinical factors.

First, the SS group was divided into the long duration and the short duration groups by disease duration. The cut-off level was provisionally set at 5.6 years based on the mean disease duration of entire SS group (≥ 5.6 years: long-duration group (mean disease duration, 9.2 ± 1.8 years), < 5.6 years: short duration group (2.6 ± 1.3 years)). The mean age in the long-duration SS group (63.9 ± 5.9 years) was significantly higher than that in the short duration SS group (53.2 ± 13.0 years) ($p < 0.01$). The OHIP-14 score in the long-duration SS group (13.9 ± 10.8) was significantly higher than that in the non-SS group (7.1 ± 7.6) ($p < 0.05$), but the score did not differ significantly between the short-duration SS group and the non-SS group. With regard to the salivary flow rate, the rate was significantly lower in the long-duration SS group (4.7 ± 2.4 ml/10 min) than the short-duration SS group (9.1 ± 5.7 ml/10 min) and the non-SS group (16.9 ± 5.9 ml/10 min) ($p < 0.05$ and $p < 0.0001$, respectively). The rate in the short-duration SS group was also significantly lower than that in the non-SS group ($p < 0.001$) (Table 2a). Further, salivary EGF concentration in the long-duration SS group (759.0 ± 646.5 pg/ml) was significantly lower than that in the short-duration SS group (1513.4 ± 1058.2 pg/ml) ($p < 0.05$), and total salivary EGF output in this group (4087.2 ± 4356.7 pg/10 min) was also significantly lower than the short-duration SS group (13881.3 ± 10480.2 pg/10 min) and the non-SS group (13296.9 ± 7907.1 pg/10 min) ($p < 0.01$ and $p < 0.001$, respectively). On the other hand, the EGF concentration in the short-duration SS group was significantly higher than that in the non-SS group ($p < 0.01$), but no significant difference was found in the total EGF output in this group compared with the non-SS group (Figure 1a).

Second, the SS group was divided on the basis of the OHIP-14 score into the severe intraoral manifestations group and the mild group. When one point out of four was given on all 14 questions, the total OHIP-14 score was 14. Therefore, the cut-off level was provisionally set at 14 points (≥ 14: severe group, ≤ 13: mild group). The mean age in the severe SS group (61.5 ± 10.4 years) was significantly higher than that in the mild SS group (52.7 ± 14.0 years) ($p < 0.05$). In the severe SS group, disease duration was longer and salivary flow rate was less than those in the mild SS group, but

Table 2. Clinical characteristics of the SS group and non-SS group.

	Long SS duration (≥ 5.6 y) (N = 11)	Short SS duration (< 5.6 y) (N = 13)	Non-SS (N = 23)
a Classification of the SS group by disease duration			
Disease duration (years) (range)	9.2 ± 1.8* (7–12)	2.6 ± 1.3 (0.2–5)	–
Age (years) (range)	63.9 ± 5.9** (57–77)	53.2 ± 13.0 (37–81)	56.1 ± 17.4 (31–82)
OHIP-14 score (out of 56) (range)	13.9 ± 10.8† (2–31; N = 11)	8.6 ± 6.6 (0–21; N = 11)	7.1 ± 7.6 (0–25)
Salivary flow rate (ml/10 min) (range)	4.7 ± 2.4***††† (2.0–8.6)	9.1 ± 5.7†† (1.0–21.4)	16.9 ± 5.9 (8.9–35.3)

*$p < 0.0001$ versus the short SS duration (< 5.6 y) group.
**$p < 0.01$ versus the short SS duration (< 5.6 y) group.
***$p < 0.05$ versus the short SS duration (< 5.6 y) group.
†$p < 0.05$ versus the non-SS group.
††$p < 0.001$ versus the non-SS group.
†††$p < 0.0001$ versus the non-SS group.

	Severe SS (≥ 14) (N = 14)	Mild SS (≤ 13) (N = 21)	Non-SS (N = 23)
b Classification of the SS group by oral health-related QOL (OHIP-14 score)			
Age (years) (range)	61.5 ± 10.4* (47–79)	52.7 ± 14.0 (31–81)	56.1 ± 17.4 (31–82)
Disease duration (years) (range)	7.2 ± 3.7 (2.7–12; N = 9)	4.9 ± 3.6 (0.2–10; N = 13)	–
Salivary flow rate (ml/10 min) (range)	6.9 ± 4.2† (2.0–15.4)	9.3 ± 4.4† (3.5–21.4)	16.9 ± 5.9 (8.9–35.3)

*$p < 0.05$ versus the mild SS (≤ 13) group.
†p < 0.0001 versus the non-SS group.

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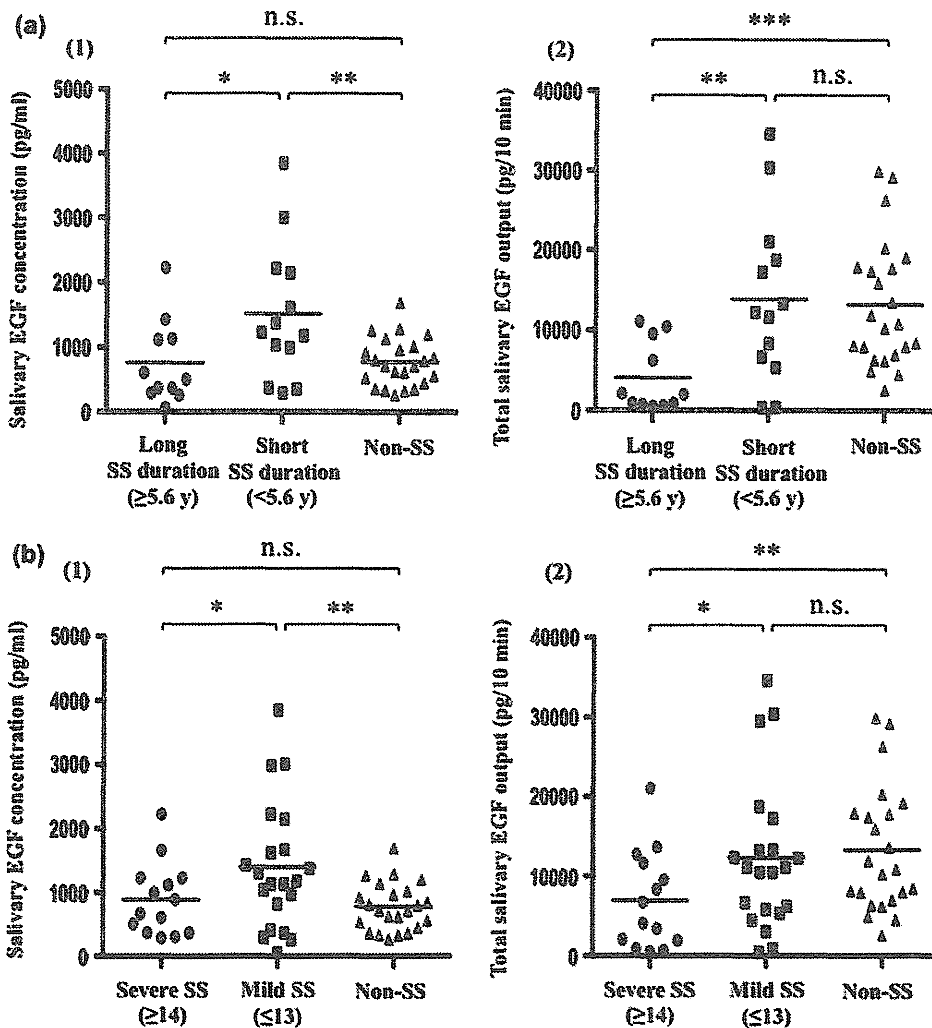

Figure 1. Salivary EGF levels of the SS and non-SS groups. (**a**) The SS group was divided into the long-duration group and short-duration group depending on disease duration, and salivary EGF levels were compared among these groups and the non-SS group. (1) Salivary EGF concentration. (2) Total salivary EGF output. (**b**) The SS group was divided into the severe and mild groups depending on the severity of intraoral manifestations determined using the OHIP-14 score, and the salivary EGF levels were compared among these groups and the non-SS group. (1) Salivary EGF concentration. (2) Total salivary EGF output. Statistical differences were assessed using the Mann–Whitney U test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant.

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neither showed a significant difference (Table 2b). Salivary EGF concentration in the severe SS group (888.9 ± 564.5 pg/ml) was significantly lower than that in the mild SS group (1392.5 ± 991.5 pg/ml) ($p < 0.05$), and the total salivary EGF output in this group (6965.8 ± 6161.1 pg/10 min) was significantly lower than that in the mild SS group (12275.7 ± 9420.0 pg/10 min) and the non-SS group (13296.9 ± 7907.1 pg/10 min) ($p < 0.05$ and $p < 0.01$, respectively). In contrast, although the EGF concentration in the mild SS group was significantly higher than that in the non-SS group (778.5 ± 371.9 pg/ml) ($p < 0.01$), the total EGF output did not differ significantly between the mild SS group and the non-SS group (Figure 1b).

Correlation analysis

The correlation between salivary flow rate, salivary EGF levels, OHIP-14 score and disease duration was assessed in the SS group.

The correlation between salivary flow rate and salivary EGF levels was evaluated in 13 patients with SS excluding those under medical treatment that might affect salivary flow rate (e.g. muscarinic M3 receptor agonist, corticosteroids and immunosuppressants). Salivary flow rate was found to be significantly correlated with salivary EGF concentration and total salivary EGF output ($r_s = 0.566$, $p = 0.023$ and $r_s = 0.824$, $p = 0.0005$, respectively) (Figure 2a).

In the entire SS group, including patients for whom disease duration could be confirmed and those under medical treatment ($n = 24$), disease duration was found to be significantly and inversely correlated with salivary flow rate, salivary EGF concentration and total salivary EGF output ($r_s = -0.512$, $p = 0.005$, $r_s = -0.389$, $p = 0.030$ and $r_s = -0.484$, $p = 0.008$, respectively) (Figure 2b). The same analysis with only six patients excluding those under the abovementioned medical treatment showed that although disease duration was not significantly correlated with salivary flow rate, the salivary EGF concentration and total salivary EGF output, the correlation between disease duration and each saliva-associated factor tended to show an inverse relationship ($r_s = -0.657$, $p = 0.088$; $r_s = -0.771$, $p = 0.051$ and $r_s = -0.657$, $p = 0.088$, respectively).

The same analysis was also conducted in 10 patients with SS excluding those under the abovementioned medical treatment to test the correlation between the OHIP-14 score and each saliva-associated factor. The OHIP-14 score was significantly and inversely correlated with salivary flow rate, salivary EGF concentration and total salivary EGF output ($r_s = -0.661$, $p = 0.022$; $r_s = -0.697$, $p = 0.015$ and $r_s = -0.721$, $p = 0.012$, respectively) (Figure 2c).

Discussion

Several novel findings were demonstrated in this study: (1) Total salivary EGF output in SS patients was significantly lower than that in non-SS patients. (2) Salivary EGF concentration and total

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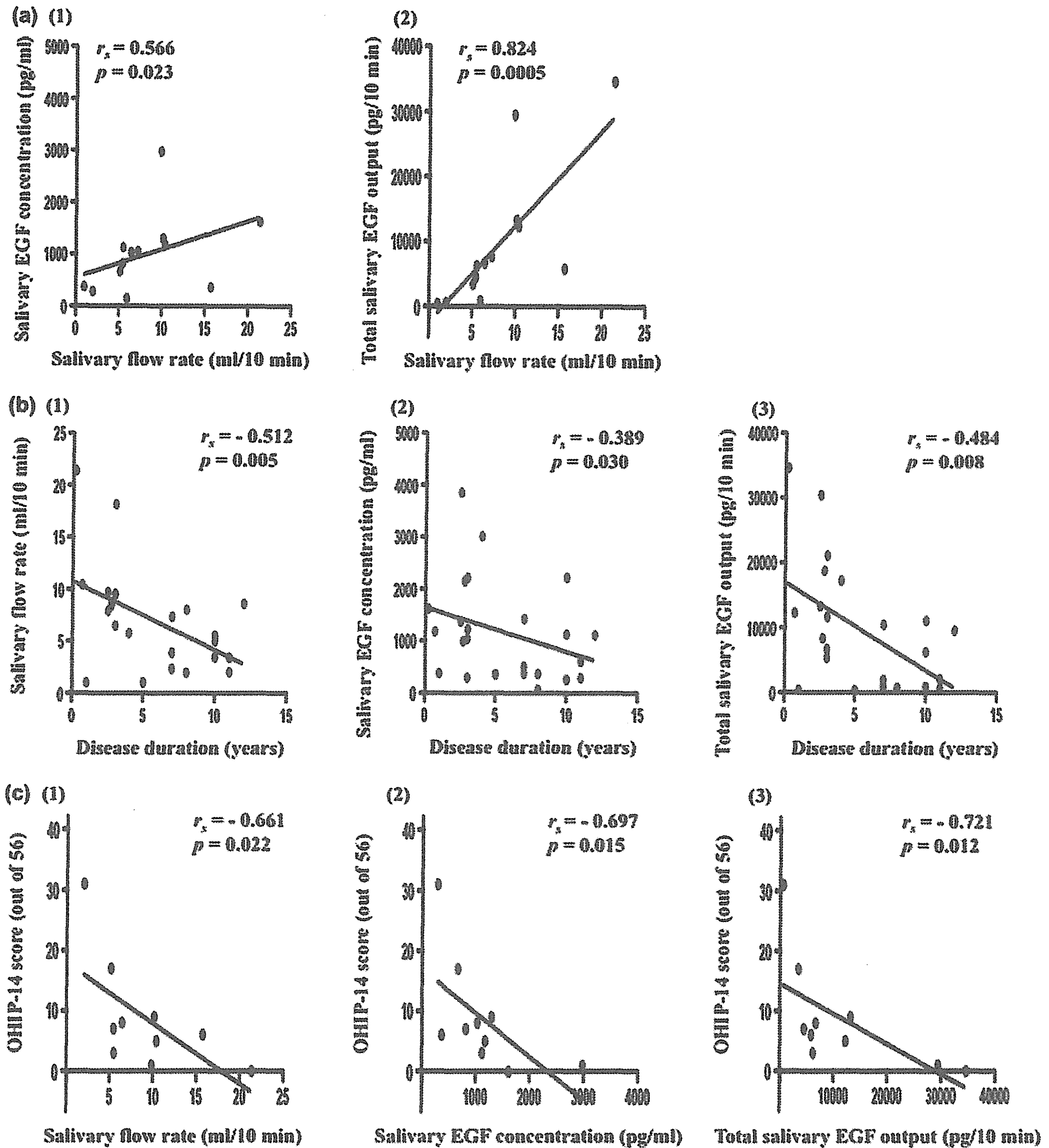

Figure 2. Correlations between each factor in the SS group. (a) Correlation of salivary flow rate with salivary EGF concentration (1) and total salivary EGF output (2). (b) Correlation of disease duration with salivary flow rate (1), salivary EGF concentration (2) and total salivary EGF output (3). (c) Correlation of OHIP-14 score with salivary flow rate (1), salivary EGF concentration (2) and total salivary EGF output (3). Correlations were assessed using Spearman's rank correlation coefficient.

output were correlated with salivary flow rate in the SS patients. (3) Further, in the SS patients, salivary EGF concentration and total output as well as salivary flow rate were inversely correlated with disease duration and decreased with time. (4) In the SS patients, the lower the salivary EGF concentration, total output and salivary flow rate became, the poorer the OHRQoL was. (5) In SS patients with long disease duration and severe intraoral manifestations, both salivary EGF concentration and total output were significantly decreased. (6) In SS patients with short disease duration and mild intraoral manifestations, although the salivary flow rate was low, both salivary EGF concentration and total output did not decrease. In the present study, the use of corticosteroid or immunosuppressants was more frequent in the non-SS group than in the SS group. Because the non-SS group patients could not be diagnosed with SS or salivary gland dysfunction on the basis of their clinical symptoms, physical findings and laboratory findings through the clinical course, including before the start of corticosteroid or immunosuppressant therapy, we did not consider the influence of these medications on intraoral manifestations and salivary gland function in the non-SS group patients during the analysis in our study.

Hutson et al. [25] showed that wound healing of the skin was enhanced by licking, that is transfer of saliva to the wound. Subsequent reports suggested that EGF synthesized in salivary glands

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and secreted into the saliva is involved in wound healing inside and outside the oral cavity. In animal models, oral wound healing was delayed significantly after removal of the submandibular glands, which are the major source of salivary EGF in rodents, and oral administration or topical application of EGF was found to restore the rate of wound healing [8,9]. Fujisawa et al. [9] reported that topical EGF application promoted proliferation of fibroblasts and keratinocytes and accelerated healing of gingival ulcers. These findings suggest that salivary EGF is involved in repair mechanisms that lead to wound healing and maintenance of the integrity of the mucosa of the oral cavity.

Although the kinetics of salivary EGF is not yet known, Ino et al. [4] showed the following: (1) salivary EGF concentration was significantly lower in the young group (0–9 years old) than the old group (10–79 years old) but was not correlated with age in the latter. Similarly, in the non-SS group in our study, age was not correlated with salivary EGF concentration and total salivary EGF output ($r_s = 0.175$, $p = 0.212$; and $r_s = 0.086$, $p = 0.348$, respectively). (2) This parameter did not differ significantly between male and female subjects. (3) Salivary EGF concentration showed an apparent diurnal rhythm related to meal consumption, that is it was the highest in the morning, when the subjects had fasted, and decreased once meals were consumed; it increased again during fasting. The proposed underlying reason was that salivary EGF was produced and secreted constantly and showed a low concentration because of dilution with the increased amount of saliva stimulated by meal consumption. Therefore, we collected saliva samples before breakfast, when the subjects had fasted, and we ensured that all samples were collected around the same time in the morning, when the salivary EGF concentration was considered the most stable and unaffected by meals.

Several studies have demonstrated the association between intraoral inflammatory diseases and changes in salivary EGF levels. Salivary EGF concentrations were found to be significantly low in patients with stomatitis aphthosa [4,16] or peritonsillar abscess [4] and decreased even after healing and in the absence of these lesions [4,16]. In patients with radiation-induced oral mucositis, salivary EGF levels were significantly decreased and were inversely correlated with the severity of oral mucositis [17,18]. Every author has speculated that low salivary EGF levels reduce the capacity of the oral mucosa to heal after injury and maintain physiologic integrity, thereby increasing susceptibility to intraoral inflammatory lesions [4,16–18]. In SS, a number of patients frequently develop refractory intraoral inflammatory lesions, such as oral mucositis and glossitis.

In the SS patients in the present study, the total salivary EGF output was significantly lower than that in the non-SS patients, and the salivary EGF concentration and total output were correlated with the salivary flow rate, decreased with time and showed an inverse correlation with disease duration. These findings suggested that the secretion of salivary EGF decreased in association with the salivary gland dysfunction induced by SS. Few previous reports have investigated EGF expression in the salivary glands of SS patients: Koski et al. [26] reported that EGF expression was diminished in the labial salivary glands of SS patients and concluded that the continuous lymphocytic inflammation in SS distributed not only salivary flow but also EGF production by salivary gland. They also concluded that diminished salivary flow and EGF for export could contribute to xerostomia and oral mucosal involvements in SS. SS and radiation therapy to head and neck are representative causes of histological damage to the salivary glands, resulting in impaired saliva secretion and changes in saliva composition. In patients undergoing radiation therapy to the head and neck region, saliva volume [18,27], salivary EGF concentration [17] and total salivary EGF output [18] are markedly decreased in the first week of therapy and remain reduced throughout radiation therapy. However, the kinetics of salivary EGF levels in the SS patients in this study was different from that in patients with radiation-induced oral mucositis. In the early phase of SS, although the salivary flow rate reduced, total salivary EGF output did not decrease. Therefore, salivary EGF concentration increased because of saliva enrichment. When the SS disease duration became prolonged, in addition to the progression of the decrease in the salivary flow rate, the total salivary EGF output decreased as well. Further, the salivary EGF concentration also decreased. These findings demonstrated that in SS, although the secretion of saliva decreases from the early stage of SS, the secretion of EGF begins to decrease several years after salivary secretion reduction. These differences in the kinetics of salivary EGF output between SS and radiation injury were considered to depend on differences in the rate at which salivary gland destruction progresses between these conditions. In the early phase of SS, when only the salivary flow rate was reduced, the OHRQoL was not impaired. Subsequently, in the late phase, when the salivary EGF concentration and total output decreased, OHRQoL worsened significantly. Moreover, in the group that had mild intraoral manifestations, although the salivary flow rate decreased, the salivary EGF concentration and total output did not decrease. In the group with severe manifestations, the salivary EGF concentration and total output decreased significantly, but the decrease in the salivary flow rate was not significantly different compared with the mild group. These findings suggest that lower levels of salivary EGF may be associated with poor OHRQoL in SS patients, and they support the findings of previous reports that low levels of salivary EGF may be associated with reduced healing of the oral mucosa after injury.

Kelly et al. [28] showed that the concentration and total production of salivary EGF was lower in patients with RA than in controls (patients with musculoskeletal disorders other than RA or primary sicca syndrome). In the present study, many patients with RA and other rheumatic diseases were included in the non-SS group as controls. If these patients had not been included, the decrease in the salivary EGF levels in the SS patients may have been more remarkable than that in the non-SS patients. Kelly et al.'s report [28] also showed that the total production of salivary EGF was reduced even further in patients with RA plus sicca syndrome and primary sicca syndrome than those with just RA. It seemed that the patients were diagnosed as sicca syndrome only by the results of Schirmer's test. Therefore, it was not correctly certain whether the patients were SS. However, their results correspond well with our findings.

The findings of the present study and previous reports suggest that topical application of EGF may promote mucosal healing and reduce the severity of oral mucosal manifestations in SS patients. In previous studies using oral epithelial cell lines, the cell migration response [29] and wound-closure effect [30] of EGF were shown. In human, one study examined the effect of EGF mouthwash application in patients undergoing cancer chemotherapy [31]. Although the rate of healing of established ulcers in patients who received EGF mouthwash and placebo did not differ, a slight delay in the onset and a smaller mean area of ulceration were noted with EGF application. The investigators concluded that the EGF mouthwash did not accelerate oral mucosal wound healing, but it may have the potential to protect the oral epithelium from cytotoxic damage and reduce the overall severity of cytotoxic damage [31]. Patients develop oral mucosal manifestations rapidly in a few days after the initiation of chemotherapy [31]. In SS, the progression of oral mucosal manifestations is not rapid compared with that as a consequence of chemotherapy. Considering this pathological mechanism in SS-associated mucositis, we expect that topical EGF application, for example a mouthwash, will be more effective for SS patients with reduced salivary EGF levels than for patients undergoing chemotherapy. In addition, the EGF

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concentration was found to be decreased in the tear fluid of SS patients [32,33]. Tsubota et al. [34] reported that corneal epithelial damages decreased significantly after the initiation of the treatment with autologous serum eye drops containing EGF, vitamin A and transforming growth factor-β. These results strongly indicate the efficacy of topical EGF application in the treatment of oral mucosal manifestations in SS patients.

In conclusion, the decrease in salivary flow rate and salivary EGF levels that appears with the progression of SS and indicates lower intraoral clearance by hyposalivation and deterioration of saliva quality could play a role in the pathogenesis of refractory intraoral manifestations in SS patients. Our findings provide new specific targets for therapeutic intervention.

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Conflict of interest

None.

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Angiotensin II Type 1 Receptor Antagonist Attenuates Lacrimal Gland, Lung, and Liver Fibrosis in a Murine Model of Chronic Graft-Versus-Host Disease

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Abstract

Chronic graft-versus-host disease (cGVHD), a serious complication following allogeneic HSCT (hematopoietic stem cell transplantation), is characterized by systemic fibrosis. The tissue renin-angiotensin system (RAS) is involved in the fibrotic pathogenesis, and an angiotensin II type 1 receptor (AT1R) antagonist can attenuate fibrosis. Tissue RAS is present in the lacrimal gland, lung, and liver, and is known to be involved in the fibrotic pathogenesis of the lung and liver. This study aimed to determine whether RAS is involved in fibrotic pathogenesis in the lacrimal gland and to assess the effect of an AT1R antagonist on preventing lacrimal gland, lung, and liver fibrosis in cGVHD model mice. We used the B10.D2→BALB/c (H-2^d) MHC-compatible, multiple minor histocompatibility antigen-mismatched model, which reflects clinical and pathological symptoms of human cGVHD. First, we examined the localization and expression of RAS components in the lacrimal glands using immunohistochemistry and quantitative real-time polymerase chain reaction (PCR). Next, we administered an AT1R antagonist (valsartan; 10 mg/kg) or angiotensin II type 2 receptor (AT2R) antagonist (PD123319; 10 mg/kg) intraperitoneally into cGVHD model mice and assessed the fibrotic change in the lacrimal gland, lung, and liver. We demonstrated that fibroblasts expressed angiotensin II, AT1R, and AT2R, and that the mRNA expression of angiotensinogen was greater in the lacrimal glands of cGVHD model mice than in controls generated by syngeneic-HSCT. The inhibition experiment revealed that fibrosis of the lacrimal gland, lung, and liver was suppressed in mice treated with the AT1R antagonist, but not the AT2R antagonist. We conclude that RAS is involved in fibrotic pathogenesis in the lacrimal gland and that AT1R antagonist has a therapeutic effect on lacrimal gland, lung, and liver fibrosis in cGVHD model mice. Our findings point to AT1R antagonist as a possible target for therapeutic intervention in cGVHD.

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Introduction

Chronic graft-versus-host disease (cGVHD), a multisystem chronic allo-immune and auto-immune disorder, is a serious and potentially life-threatening long-term complication of allogeneic HSCT (hematopoietic stem cell transplantation) [1].

Clinical manifestations of cGVHD include inflammation and fibrosis [2].

We previously reported that cGVHD is frequently related to dry eye, with 50% of patients developing or experiencing worsened pre-existing dry eye after HSCT [3], and that the lacrimal glands of cGVHD patients show marked fibrosis and inflammatory cell infiltration around medium-sized ducts in the interlobular areas [4]. Chronic pulmonary dysfunction occurs in 20% to 50% of cGVHD patients, depending on the donor source and the time interval after HSCT. The chronic lung injury is subdivided into two types: obstructive lung disease (OLD) and restrictive lung disease (RLD). In each type, collagen deposition and fibrosis development are observed either in the peribronchiolar space (OLD) or interstitial space (RLD) [5]. Evidence of cholestasis is present in approximately 80% of patients with cGVHD. In the histopathology of liver cGVHD, ductopenia, portal fibrosis, and chronic cholestasis reflect chronicity [6]. However, the pathophysiology of cGVHD is not completely understood, and an effective therapy for it has not been established.

Only a few animal models have been developed to examine cGVHD. Among them, the B10.D2→BALB/c (H-2^d) MHC-compatible, multiple minor histocompatibility antigen-mismatched mouse shows several characteristics resembling human cGVHD. Its features include skin fibrosis with increased collagen deposition [7,8], pulmonary fibrosis [7], inflammation and destruction of the salivary and lacrimal glands [9], and hepatic