based on distinct clinical and pathologic features. If we use the information of the skin manifestation of all the patients, PM and DM could be separated. Although the skin information of some proportion of patients is not well described in the database, further consideration may solve the problem in further study.

In conclusion, we provide estimates of the recent prevalence of PM/DM, and the incidence of the disease in Japan for the first time at the nationwide population level. As prevalence has been showing an increasing trend recently, a continuing concern must be to maintain monitoring of these epidemiologic features.

Funding

This study was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan, for Research Committee on Autoimmune Diseases.

Conflict of interest

H. Kohsaka has served as a consultant to Chugai Pharmaceutical and has received research grants from Eisai Pharmaceutical and Takeda Pharamaceutical. All other authors have declared no conflicts of interest.

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

Mod Rheumatol, 2015; 25(1): 85–89
© 2014 Japan College of Rheumatology
DOI: 10.3109/14397595.2014.900843

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ORIGINAL ARTICLE

Serum interferon-α is a useful biomarker in patients with anti-melanoma differentiation-associated gene 5 (MDA5) antibody-positive dermatomyositis

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Abstract

Objective. We have tried to clarify the clinical importance of the measurement of serum type-I interferon (IFN) in patients with anti-melanoma differentiation-associated gene 5 Ab (MDA5 Ab)-positive dermatomyositis (DM).

Methods. We studied 30 patients with DM: 10 were anti-MDA5 Ab-positive and 20 were anti-MDA5 Ab-negative. At each patient's initial visit, serum IFN-α, IFN-β, interleukin 18 (IL-18), ferritin, and the titer of anti-MDA5 Ab were measured using enzyme-linked immunosorbent assays (ELISAs). The associations between the IFNs and with the other variables were examined.

Results. Rapidly progressive interstitial lung disease (RPILD) was confirmed in 10 patients, most of whom were complicated in the anti-MDA5 Ab-positive DM patients. The presence of clinically amyopathic dermatomyositis (CADM) as well as the serum concentrations of IFN-α and ferritin was significantly higher in the anti-MDA5 Ab-positive DM patients. Serum concentration of IL-18 did not differ between anti-MDA5 Ab-positive and anti-MDA5 Ab-negative groups; however, a positive correlation was found between IFN-α and IL-18 in the anti-MDA5 Ab-positive DM patients ($r = 0.8139$, $p = 0.0146$).

Conclusion. Serum IFN-α can be used as a useful biomarker in patients with anti-MDA5 Ab-positive DM, which may reflect the presence of RPILD.

Keywords

IFN-α, anti-MDA5 antibody, CADM, RPILD, Ferritin

History

Received 15 November 2013
Accepted 25 February 2014
Published online 8 April 2014

Introduction

Dermatomyositis (DM) is a group of chronic inflammatory disorders commonly involved in skeletal muscles and skin. DM is often associated with interstitial lung diseases (ILDs) and is known to cause significant organ damage, adversely affecting the prognosis of ILD patients [1,2]. Clinically amyopathic dermatomyositis (CADM) is a subgroup of DM characterized by various skin manifestations and none-to-mild muscle symptoms [3]. CADM is often complicated with rapidly progressive interstitial lung disease (RPILD), which can be treatment resistant and life threatening [3]. Therefore, the presence of RPILD is of significant importance in ILD found in DM patients, and the identification of biomarkers to identify CADM complicated with RPILD is desirable.

Regarding the clinical diagnosis of CADM complicated with RPILD, anti-CADM 140-kDa polypeptide Abs (anti-CADM-140 Abs) were found in sera from patients with CADM complicated with RPILD [4], and more recently, an RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) was identified as a major autoantigen which is targeted by anti-CADM140 Abs [5]. MDA5 is a member of the Rig-I-like receptors (RLR) family that recognizes double-stranded RNA (dsRNA) within the cytosolic compartment and induces the production of inflammatory cytokines and cell surface molecules involved in the antiviral response [6]. In this regard, Sun et al. recently reported that interferon-alpha (IFN-α) in serum is high in CADM patients complicated with ILD; however, they did not investigate the presence of anti-MDA5 Ab or RPILD [7].

One of the characteristics of anti-MDA5 Ab-positive CADM patients is hyperferritinemia [8,9]. These patients often express high serum interleukin 18 (IL-18) [10]. It is possible that the

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production of anti-MDA5 Ab is an epiphenomenon during virus infection that is associated with the onset of CADM and RPILD. Considering that dsRNA virus-mediated innate immune responses might be activated in anti-MDA5 Ab-positive CADM patients, type I IFNs could be crucial biomarkers reflecting organ damage and serologic disease activity.

The present study was undertaken to clarify the clinical importance of the measurement of serum type I IFNs in patients with anti-MDA5 Ab-positive DM. Our present data suggest that serum IFN-α could be used as a biomarker that reflects the disease activity in patients with anti-MDA5 Ab-positive DM complicated by RPILD.

Patients and methods

Patients

The study population consisted of 30 patients (22 females and 8 males) with DM (CADM or classical DM) who had been admitted to our hospital (Nagasaki University Hospital and Kurume University Hospital) between May 2009 and October 2012. The diagnosis of classical DM was based on Bohan and Peter criteria [11,12]. The diagnosis of CADM was based on Sontheimer criteria [13]. The diagnosis of classical DM or CADM was carefully evaluated by Japan College of Rheumatology-certified rheumatologists (M.T., H.N., H.I., T.O., and A.K.) using the patients' medical records. The diagnosis of ILD was based on the results of chest X-ray and high-resolution computed tomography, reported by Japanese board-certified radiologists. RPILD was defined as progressive dyspnea and hypoxemia, and a worsening of interstitial change on chest radiography within 1 month from the onset of respiratory symptoms [4]. Patients gave their informed consent to be subjected to the protocol, which was approved by the Institutional Review Board of Nagasaki University.

Blood samples

Blood specimens were obtained from all patients in this study at their first visit for serological analysis. Blood samples were stored at − 20°C until use, and they were used to measure anti-MDA5 Ab, IFN-α, IFN-β, ferritin, and IL-18. The cut-off value of anti-MDA5 Ab was defined as 8.0 U/mL. An ELISA system using recombinant MDA5 as an antigen source was performed as described [5]. All anti-MDA5 Ab-positive sera samples were positive with anti-CADM140 Abs confirmed using immunoprecipitation. The measurement of IFN-α and IFN-β was performed with the VeriKine™ Human IFN-α ELISA Kit (Product #41100) and the VeriKine™ Human IFN-β ELISA Kit (Product #41410), respectively, following the manufacturer's instructions. The measurement of ferritin was performed with the AssayMax Human Ferritin ELISA Kit (Assaypro, Catalog No. EF 2003-1) following the manufacturer's instructions. The measurement of IL-18 was performed with the Human IL-18 ELISA Kit (Code No. 7620, Medical & Biological Laboratories Co., Nagoya, Japan) following the manufacturer's instructions. In addition to DM patients, serum IFN-α, IFN-β, ferritin, and IL-18 were measured in 36 healthy controls.

Statistical analyses

In order to identify and evaluate differences in variables between the anti-MDA5 Ab-positive DM patient group and the anti-MDA5 Ab-negative DM patient group, we used the Mann–Whitney U-test or the χ^2 test (Fisher's exact probability test when appropriate). Correlations between two serum markers were calculated using the Spearman's rank correlation test. Values of p less than 0.05 were considered significant.

Results

Table 1 summarizes the patients' data. Ten of the thirty patients were positive for anti- MDA5 Ab. In the anti-MDA5 Ab-positive group, CADM was identified in eight patients and classical DM was diagnosed in the other two patients. In the group of 20 patients negative for anti-MDA5 Ab, classical DM was diagnosed in 17 patients, and CADM was diagnosed in the other three patients, indicating the significant prevalence of CADM in the anti-MDA5 Ab-positive group ($p = 0.0006$). Accordingly, ILD, especially RPILD, was preferentially distributed in the anti-MDA5 Ab-positive group ($p = 0.0038$ in ILD, $p < 0.0001$ in RPILD). In fact, all 10 cases of the anti-MDA5 Ab-positive patients were complicated by RPILD. Significantly high levels of anti-MDA5 Ab titer ($p < 0.0001$), IFN-α ($p = 0.0003$) and ferritin ($p = 0.0003$) were found in the anti-MDA5 Ab-positive patients compared to those of the anti-MDA5 Ab-negative patients (Figure 1). In contrast, no significant difference was observed between the two groups in terms of IFN-β or IL-18 (Figure 1). Then we examined the differences of cytokine levels between the anti-MDA5Ab-positive DM and anti-MDA5-negative DM patients complicated with ILD. The result is that IFN-α and ferritin levels were significantly elevated in anti-MDA5 Ab-positive patients than those in anti-MDA5 Ab-negative with ILD (Table 2). Three anti-MDA5 Ab-positive patients died during the observation period. We examined the differences of cytokine levels between the alive and the deceased patients; however, the differences were not statistically significant (Table 3).

We further examined the correlations of the serum concentrations of IFN-α with the other markers in anti-MDA5-positive DM patients (Figure 2). Serum concentration of IL-18 did not differ between anti-MDA5 Ab-positive and anti-MDA5 Ab-negative groups (Figure 1); however, a positive correlation was found

Table 1. Comparison of clinical and serologic manifestations between patients with anti-MDA5 Ab and without anti-MDA5 Ab.

	Anti-MDA5 Ab		
Variable	Positive ($n = 10$)	Negative ($n = 20$)	p value
Age, years (SD)	58.9 (12.38)	44.8 (19.85)	0.0444
Female, n (%)	9 (90%)	13 (62.5%)	0.1512
CADM, n (%)	8 (80%)	3 (15%)	0.0006
ILD, n (%)	10 (100%)	9 (45%)	0.0038
RPILD, n (%)	10 (100%)	1 (5%)	<0.0001
Anti-MDA5 Ab (U/mL), median (range)	154.423 (16.946–1448.155)	1.255 (0.765–4.782)	<0.0001
Death, n (%)	3 (30%)	0 (0%)	0.0111

Anti-MDA5 Ab, anti-melanoma differentiation-associated gene 5 (MDA5) antibody; CADM, Clinically amyopathic dermatomyositis; ILD, interstitial lung disease; RPILD, Rapidly progressive interstitial lung disease; IFN, Interferon; IL-18, interleukin-18.

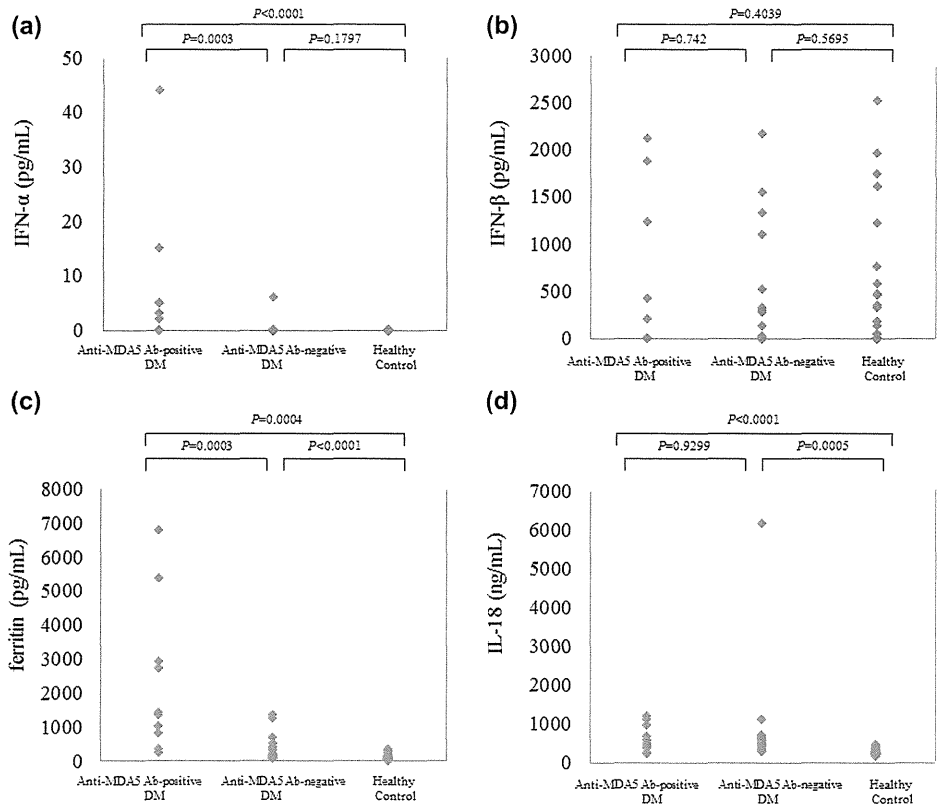

Figure 1. Serum levels of IFN-α, IFN-β, ferritin, and IL-18 in anti-MDA5 Ab-positive DM, anti-MDA5 Ab-negative DM, and healthy controls. *P* value was estimated using the Mann–Whitney U-test.

between IFN-α and IL-18 in these patients (Figure 2). Significant correlations were not observed between INF-α and with the other three variables (Figure 2).

Discussion

The importance of anti-MDA5 Ab in patients with CADM has been established. Authors and other investigators have found an extremely high prevalence of anti-MDA-5 Ab in patients with CADM complicated with RPILD [5,8,14]. In these reports, the titer of anti-MDA5 Ab at the first visit was higher in the CADM patients who died of the disease compared to that of the surviving patients [8], though we did not find the difference probably due to very small sample size. In addition, sustained high levels of anti-MDA5 Ab during therapy were associated with death in patients with anti-MDA5 Ab-positive CADM [8]. Although 2 of the 10 anti-MDA5 Ab-positive DM patients in the present study were classified as having classical DM, the cases of all 10 anti-MDA5 Ab-positive DM patients were complicated by RPILD.

Table 2. Comparison of serum cytokine levels between the anti-MDA5 Ab positive patients and anti-MDA5 Ab negative patients with ILD.

	Patients with ILD ($n = 19$)		
Variable	anti-MDA5 positive ($n = 10$)	anti-MDA5 negative ($n = 9$)	*p*-value
IFN-α (pg/mL), median	4 (0–44)	0 (0–6)	0.0435
IFN-β (pg/mL), median	100 (0–1873)	25 (0–2167)	1.0318
Ferritin (pg/mL), median (normal range 45–163)	1376 (241–6775)	318 (77–1235)	0.003
IL-18 (ng/mL), median (normal range 18–121)	539.5 (239–1210)	617 (439–6170)	0.447

Anti-MDA5 Ab, anti-melanoma differentiation-associated gene 5 (MDA5) antibody; IFN, Interferon; IL-18, interleukin-18, ILD, interstitial lung disease.

Anti-MDA5 Ab is detected in a small group of patients with classical DM, and all these classical DM patients complicate RPILD [15]. Therefore, all 10 anti-MDA5 Ab-positive DM patients in the present study are considered as having the characteristic feature of DM-complicated RPILD. The present study revealed for the first time that serum concentrations of IFN-α are high in the cases of anti-MDA5 Ab-positive DM patients complicated by RPILD compared to those of anti-MDA5 Ab-negative DM patients. It is undeniable that elevated serum IFN-α and ferritin levels in anti-MDA5 Ab-positive patients than in anti-MDA5 Ab-negative DM might associate with ILD. It is known that almost all anti-MDA5 Ab-positive patients complicated ILD [14], especially RPILD, during their clinical courses that is also revealed in the present study. We have found the higher levels of IFN-α and ferritin in anti-MDA5-positive patients with ILD than those in anti-MDA5-negative patients with ILD. Therefore, it might be considered that IFN-α and ferritin levels are reflective toward the presence of anti-MDA5 Ab and RPILD. In addition, Gono et al. have reported the hyperferritinemia with an accumulation of ferritin-producing macrophages in the lung in an autopsied case of anti-MDA5 Ab-positive CADM complicated by RPILD [9], suggesting that some stimuli trigger the activation of macrophages leading to hyperferritinemia. Because MDA5 is expressed in macrophage-lineage cell types [16] and a member of the RLR family that recognizes dsRNA derived from viruses and trigger antiviral response [6], our present data may explain a putative implication of dsRNA virus infection in patients with anti-MDA5 Ab-positive DM. Viral infection may induce the production of IFN-α and previous report suggests that IFN-α

Table 3. Comparison of serum cytokine levels between the alive and the deceased in anti-MDA5 Ab-positive patients.

	Anti-MDA5 Ab positive ($n = 10$)		
Variable	Alive ($n = 7$)	Deceased ($n = 3$)	p value
Anti-MDA5 Ab (U/mL), median	101.841 (16.946–406.473)	207.006 (21.797–1448.155)	0.569
IFN-α (pg/mL), median	5 (0–44)	3 (0–5)	0.667
IFN-β (pg/mL), median	200 (0–1873)	0 (0–2113)	1.000
Ferritin (pg/mL), median (normal range 45–163)	1329 (241–6775)	1423 (1004–5357)	0.667
IL-18 (ng/mL), median (normal range 18–121)	502 (268–1210)	577 (239–967)	0.833

Anti-MDA5 Ab, anti-melanoma differentiation-associated gene 5 (MDA5) antibody; IFN, Interferon; IL-18, interleukin-18.

stimulates the synthesis or secretion of ferritin [17]. Considering IFN-α and ferritin levels were higher in patients with anti-MDA5 Ab-positive DM complicated with RPILD, IFN-α might play an important role in inflammation triggered in the lung.

Serum IL-18 is reported previously to be high in DM patients complicated with ILD compared to those without ILD [10]. In a study of anti-MDA5 Ab-positive DM patients, sustained high IL-18 levels during therapy were observed in the deceased patients compared to those of the survived ones, indicating that IL-18 could also reflect disease activity in patients with DM, especially those who are anti-MDA5 Ab-positive [8]. Interestingly, a positive correlation was found between IFN-α and IL-18 in the anti-MDA5 Ab-positive DM patients of present study, which may support our hypothesis that IFN-α is a useful biomarker in patients with anti-MDA5 Ab-positive DM. In contrast, we did not find the difference of serum IL-18 levels at first visit between anti-MDA5 Ab-positive and anti-MDA5 Ab-negative DM patients, though the serum concentrations of IL-18 from both groups were higher than those of healthy controls. Therefore, the mechanisms to stimulate the production of IL-18 might be different in anti-MDA5 Ab-negative DM patients as compared to those of anti-MDA5 Ab-positive DM patients.

We did not observe a difference in the serum concentrations of another type I IFN, IFN-β, between our anti-MDA5 Ab-positive and anti-MDA5 Ab-negative DM patients. Although IFN-α, IFN-β, and IL-18 are produced in muscle tissues of patients with DM [18,19], the organs responsible for the predominant expression of IFN-α in anti-MDA5 Ab-positive DM patients may not be muscles, since the muscular involvement is less in these patients compared to those of anti-MDA5 Ab-negative DM patients.

In summary, our present data indicate that serum IFN-α is a useful biomarker in patients with anti-MDA5 Ab-positive DM complicated by RPILD. However, other investigators recently reported that serum levels of IFN-α do not correlate with disease activity in DM patients [20]. Since the patterns of organ involvement of DM patients differ widely according to myositis-specific

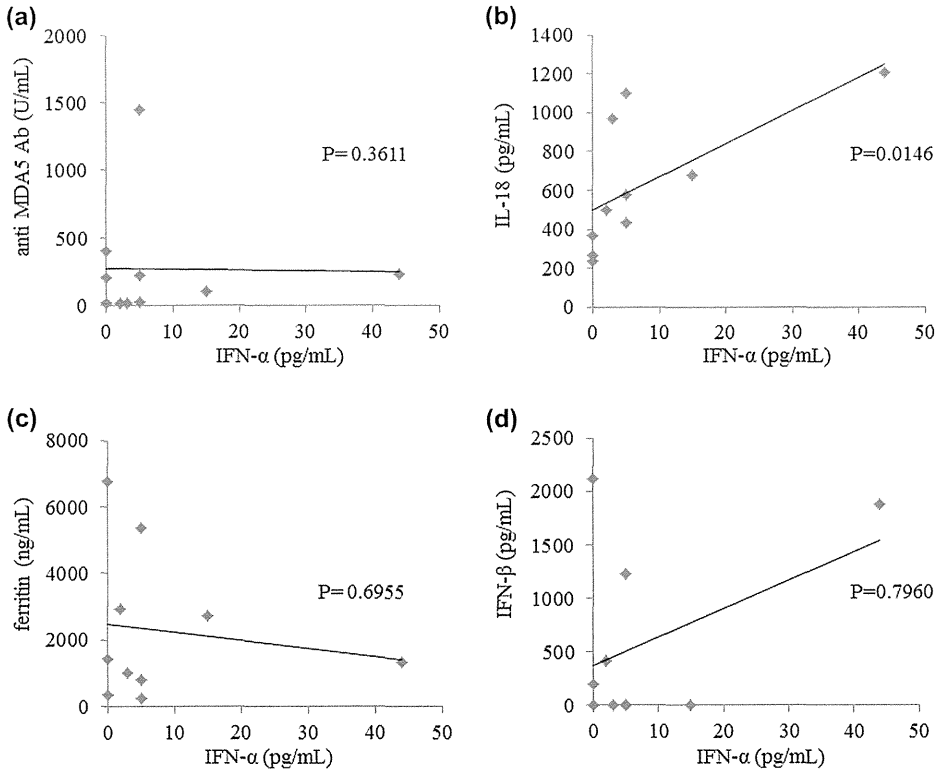

Figure 2. Correlations of serum IFN-α with anti-MDA5 Ab titer, serum IL-18, serum ferritin or IFN-α in the anti-MDA5 Ab-positive DM patients. (a) IFN-α with anti-MDA5 Ab titer, (b) IFN-α and IL-18, (c) IFN-α and ferritin, (d) IFN-α and IFN-β. Positive correlations were found between IFN-α and IL-18 ($p = 0.0146$).

autoantibodies [4,5,14,15,21], further replicative investigations may confirm the pathologic role of IFN-α in patients with DM.

Acknowledgments

We thank Kaori Furukawa (Nagasaki University) for her excellent work measuring IFN-α, IFN-β, ferritin and IL-18 by ELISA.

Conflict of interest

None.

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ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; Early Online: 1–6

DOI: 10.3109/14397595.2013.843750

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CASE REPORT

Comorbid case of IgG4-related disease and primary Sjögren's syndrome

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Abstract

A 63-year-old man with enlargement of the bilateral submandibular glands visited with elevated serum IgG4. A biopsy specimen showed plasma cell infiltration with more than 50% IgG4/IgG staining, suggesting the existence of IgG4-related disease (IgG4-RD). Positive anti-SS-A/Ro antibody and the labial salivary gland's biopsy suggested existence of primary Sjögren's syndrome (pSS). Administration of glucocorticoid improved the serum IgG4 level while reducing the submandibular gland lesions. Positive TUNEL staining suggested the coexistence of IgG4-RD and pSS.

Keywords

Apoptosis, IgG4-related disease, Sjögren's syndrome, TUNEL

History

Received 25 January 2013
Accepted 8 May 2013
Published online 24 February 2014

Introduction

IgG4-related disease (IgG4-RD) is a newly confirmed disorder that demonstrates Mikulicz's disease (MD) and entails the involvement of various organs including pancreas, kidney, pituitary gland and retroperitoneum [1]. Contrarily, primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by sicca symptoms with the infiltration of mononuclear cells (MNCs) into exocrine glands such as the labial salivary gland (LSG) [2]. Although both disorders have similar clinical manifestations, the elevation of serum IgG4 and IgG4-positive plasma cell infiltration are obvious characteristics of IgG4-RD. Here, we report a clinically comorbid case of IgG4-RD and pSS and demonstrate the pathological difference between these diseases with respect to cell death.

Case report

A 63-year-old man presented with a 1-year history of submandibular gland enlargement. He also complained of severe xerostomia and xerophthalmia. He had a history of allergic rhinitis, but had no bronchial asthma. On physical examination, his vital signs were normal. The respiratory and heart sounds and abdominal findings were normal. The laboratory findings on admission are shown in Table 1. The elevation of eosinophils, serum IgG4 and IgE was shown along with the elevation of sIL-2R. Elevation of the serum β2-MG level, urine β2-MG level and urine NAG level was also shown. Urinalysis was normal. The Saxon test and Schirmer test were positive. Regarding the significant accumulation of gallium, there was uptake in the bilateral submandibular glands and the bilateral kidneys and prostate (Figure 1). The biopsy of the submandibular glands showed diffuse fibrosis of the glands with diffuse plasmacytic infiltration, in which 61% of IgG-positive infiltrating cells were IgG4-positive (Figure 2). Although vague storiform fibrosis and destruction of glandular cells were seen, obstructive phlebitis and germinal center formation were not observed. According to the elevation of serum IgG4 and IgG4-positive plasmacytic cell infiltration, the patient was diagnosed with IgG4-RD on the basis of the criteria. Moreover, anti-SS-A antibody was positive with the infiltration of more than 50 mononuclear cells into LSGs (Figure 2). Presence of anti-SS-A antibody was also confirmed by double-immunodiffusion (DID). These findings fulfilled the American–European Consensus Group classification criteria of pSS. Prednisolone (PSL) was started at a dose of 40 mg per day. The serum IgG4 level decreased from 1010 mg/dl to 213 mg/dl at 1.5 months after treatment, and a reduction in the submandibular gland size was observed (Figure 3). On the ultrasonography of the submandibular gland, the hypoechoic areas were also smaller than before the treatment (Figure 4). The PSL dosage was tapered gradually every 2 weeks without exacerbation of the serum IgG4 level or glandular swelling. To investigate whether or not this case was pSS- or IgG4-RD -dominant, we employed terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining to show double-stranded DNA breaks,that is different from nick translation method which represents single strand DNA break [3], resulting in positive TUNEL staining in the present case (Figure 5). The frequency of TUNEL-positive cells in this case was greater than that of the typical pSS case. To demonstrate that apoptosis is mediated by Fas system, immunostaining of Fas was also performed, resulting in expression of Fas antigen in salivary glands of this patient and a pSS patient with anti-SS-A/SS-B antibodies (Figure 6). Fas expression as well as positive TUNEL staining was dominantly observed in this case compared with the typical pSS patient.

Discussion

The presence of exocrine dysfunction including positive Saxon and Schirmer tests, a high serum level of immunoglobulin and MNCs infiltration in the LSG in this case were a common conditions in

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Table 1. Laboratory findings.

Hematology	
WBC (3900–9800)	4800/μl
Stab + Seg (40–70)	51%
Lymph (25–50)	28%
Mono (1–14)	6%
Eosino (0–5)	14%
Hb (13.5–17.6)	13.6 g/dl
Plt (13.1–36.2)	20.6×10^4 /μl
ESR (2–10)	60 mm/h
Coagulation	
PT (82–127)	109%
APTT (25.2–34.4)	23.6 sec
Fibrinogen (168–329)	387 mg/m
Biochemistry	
TP (6.7–8.3)	8.4 g/dl
Alb (4.0–5.0)	3.4 g/dl
T.bil (0.3–1.5)	0.8 mg/dl
AST (13–33)	21 IU/l
ALT (8–42)	15 IU/l
γ-GTP (10–47)	34 IU/l
ALP (115–359)	219 IU/l
LDH (119–229)	171 IU/l
CK (62–287)	55 IU/l
BUN (8–22)	13 mg/dl
Cr (0.4–1.1)	0.95 mg/dl
Na (138–146)	140 mEq/l
K (3.6–4.9)	4.0 mEq/l
Cl (99–109)	106 mEq/l
UA (2.3–8.0)	6.9 mg/dl
AMY (40–130)	70 mg/dl
Glu (70–110)	106 mg/dl
Ferritin (40–465)	87 ng/ml
Immunology	
CRP (< 0.17)	0.16 mg/dl
RF (≤ = 15)	9.2 IU/ml
Antinuclear antibody (< 20)	< 20
Anti-SS-A antibody (10–30)	35.1 U/ml
Anti-SS-B antibody (15–25)	8.6 U/ml
Anti-RNP antibody (15–22)	Negative
CH50 (30–45)	44.6 U/ml
C3 (65–135)	85.0 mg/ml
C4 (13–35)	19.7 mg/ml
IgA (110–410)	224 mg/dl
IgG (870–1700)	2949 mg/dl
IgG1 (320–748)	1550 mg/dl
IgG2 (208–754)	1210 mg/dl
IgG3 (6.6–88.3)	199 mg/dl
IgG4 (4.8–105)	1060 mg/dl
IgM (35–220)	24.3 mg/dl
IgE (≤ = 269.1)	1435.3 IU/ml
sIL-2R (y127–582)	1513 U/ml
IL-6 (≤ = 4.0)	2.0 pg/ml
β2-MG (serum:0.68–1.65)	2.89 μg/ml
β2-MG (urine:14–329)	2679 μg/l
NAG (urine:1.0–7.0)	15.6 U/l
PSA (≤ = 4.0)	0.762 ng/ml
KL-6 (< 500)	349 U/ml
Urinalysis	Normal
Saxon test (> 2)	0.45 g/2 min
Schirmer test (> 5)	Rt : 4 mm
	Lt : 5 mm

Abbreviations: Alb, albumin; ALP, alkaline phosphatase; ALT, L-alanine aminotransferase; AMY, amylase; APTT, activated partial thromboplastin time; AST, L-aspartate aminotransferase; BUN, blood urea nitrogen; C3, complement 3; C4, complement 4; CH50, homolytic complement activity; CK, creatine kinase; Cl, chlorine; Cr, creatinine; CRP, C-reactive protein; Eosino, eosinocyte; ESR, erythrocyte sedimentation rate; Glu, glucose; Hb, hemoglobin; IL-6, interleukin-6; K, potassium; LDH, lactate dehydrogenase; Lymph, lymphocyte; Mono, monocyte; Na, sodium; NAG, N-acetyl-β-glucosaminidase; Plt, platelet; PSA, prostate specific antigen; PT, prothrombin time; RF, rheumatoid factor; sIL-2R, soluble interleukin-2 receptor; Stab + Seg, stab neutrophil + segmented; T.bil, total bilirubin; TP, total protein; UA, uric acid; WBC, white blood cell; β2-MG, β2-microglobulin; γ-GTP, γ-glutomyl transpeptidase.

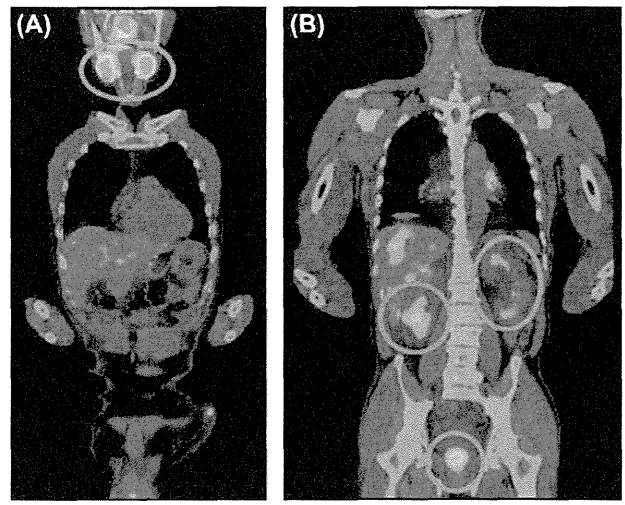

Figure 1. Gallium scintigraphy findings before treatment. Significant accumulation of gallium was observed in the bilateral submandibular glands, the bilateral kidneys and the prostate. Red circled sites demonstrate positive uptake lesions.

both MD and pSS. However, this case also showed a greater than 40% IgG4/IgG ratio in the LSG with bilateral submandibular gland swelling. Furthermore, a prompt response to glucocorticoid was observed with regard to reduction of the submandibular gland size. Although the usefulness of ultrasonography for the examination of salivary glands in pSS was already shown [4], the change over time with respect to IgG4-RD has rarely been reported [5]. Therefore, the improvement of the ultrasonography image resulting from glucocorticoid therapy in our case seems to be useful. These clinical manifestations would have corresponded to IgG4-related MD if not for the positive anti-SS-A/Ro autoantibody finding. This case is an atypical male patient with SS and overlapped with IgG4-RD. Autoantibody polarized toward Ro/SS-A 52kd antigen may relate with developing atypical male patient with SS because it was reported [6] that Ro/SS-A antigen consists of 52-kd and 60-kd proteins and the former mainly exists in cytoplasm whereas the latter localizes in nucleus. Furthermore, Barcellos et al. reported that 52-kd Ro/SS-A antigen expression was mainly detected in cytoplasm of ductal cells in LSG from pSS patients. They also showed 52-kd Ro/SS-A antigen was weakly detected in the nucleus of ductal cells and cytoplasm of acinar cells although 60-kd Ro/SS-A antigen was found in both cytoplasm and nucleus of ductal cells. Destruction of ductal cells and/or acinar cells might be different in each case of pSS. Considering a present atypical male pSS patient complicated with IgG4-RD, autoimmune reaction might be dominated toward not 60-kd Ro/SS-A but 52-kD Ro/SS-A, producing anti-52-kD Ro/SS-A antibody in the absence of ANA [7].

Recently, new classification criteria for SS were dispatched by SICCA research groups [2] in which the presence of IgG4-RD is one of the exclusion criteria. The reason IgG4-RD was one exclusion criterion was that overlapping clinical features were observed in conditions including neck radiation treatment, hepatitis C infection, acquired immunodeficiency syndrome, sarcoidosis, amyloidosis, graft versus host disease and IgG4-RD. Baer et al. [8] reported that there was only one IgG4-related disease case in 2594 Sjögren's syndrome cases of the SICCA registry database. Masaki et al. [1] reported that the incidence of anti-SS-A/Ro antibody and anti-SS-B/La antibody were significantly lower in patients with IgG4 + MOLPS than in those with typical Sjögren's syndrome (1.6% and 0.0% vs. 100% and 100%). Meanwhile, Umehara et al. recently demonstrated the following diagnostic

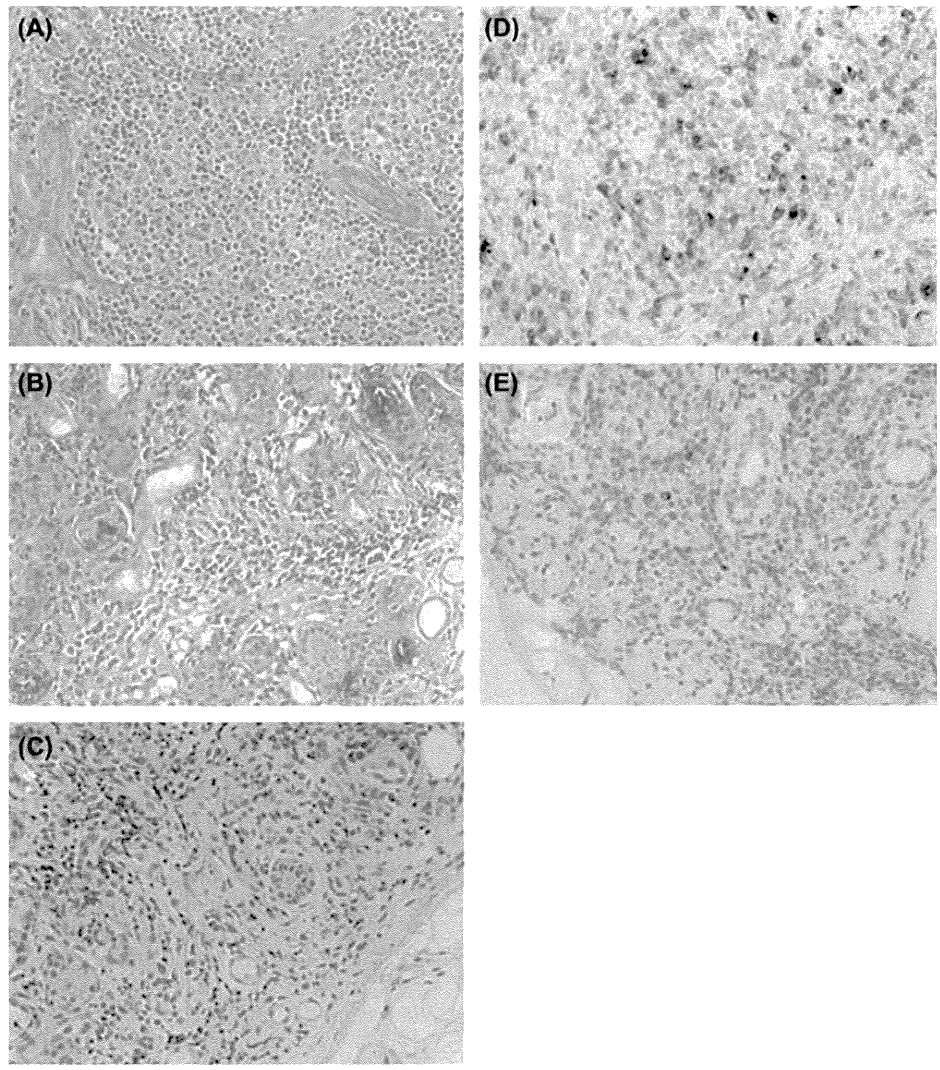

Figure 2. Immunohistochemistry of salivary glands. Submandibular glands (A,C,D): These salivary gland lobules exhibit diffuse fibrosis. There are multiple germinal centers surrounded by infiltrating mononuclear cells. Labial salivary glands (B,E): Many IgG4-positive plasma cells are distributed diffusely. (A,B: periodic acid-Schiff stain, C: Hematoxylin–Eosin staining; original magnification: ×200). (D,E: IgG4 immunostain; original magnification: ×200).

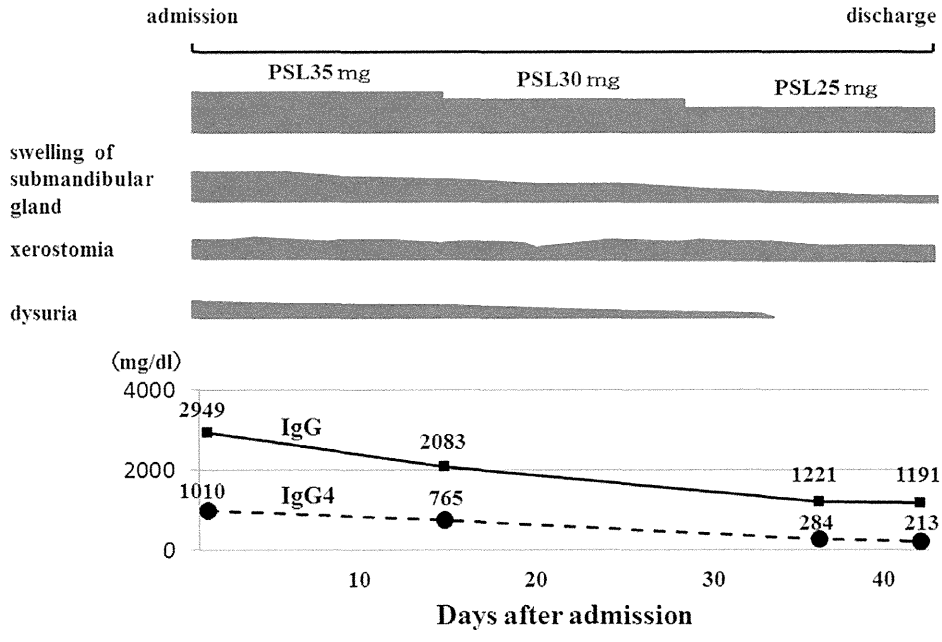

Figure 3. Clinical course during hospitalization. PSL was initiated at a dose of 40 mg per day. The serum IgG4 level decreased from 1010 mg/dl to 213 mg/dl after steroid therapy with a reduction in the size of the submandibular glands.

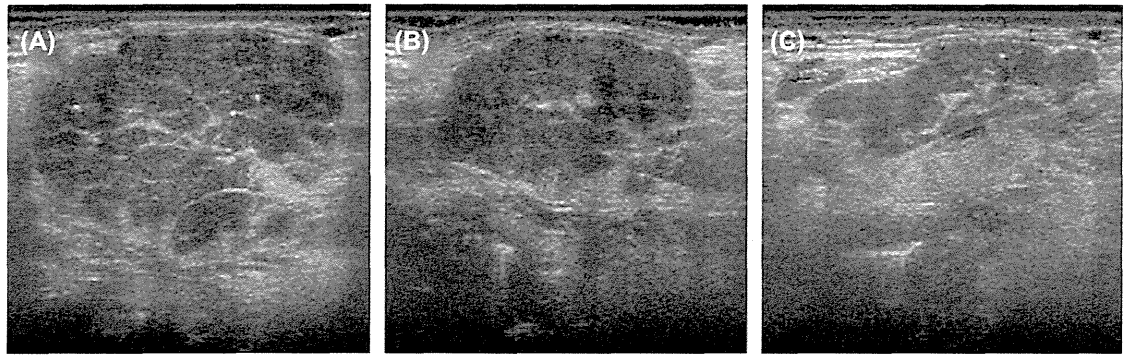

Figure 4. Ultrasonography findings of submandibular glands. A huge nodular lesion of the submandibular gland was observed (A). Two weeks after glucocorticoid therapy, the size of the hypoechoic area was slightly diminished (B). Two months after the initiation of treatment, the size of the hypoechoic area decreased (C).

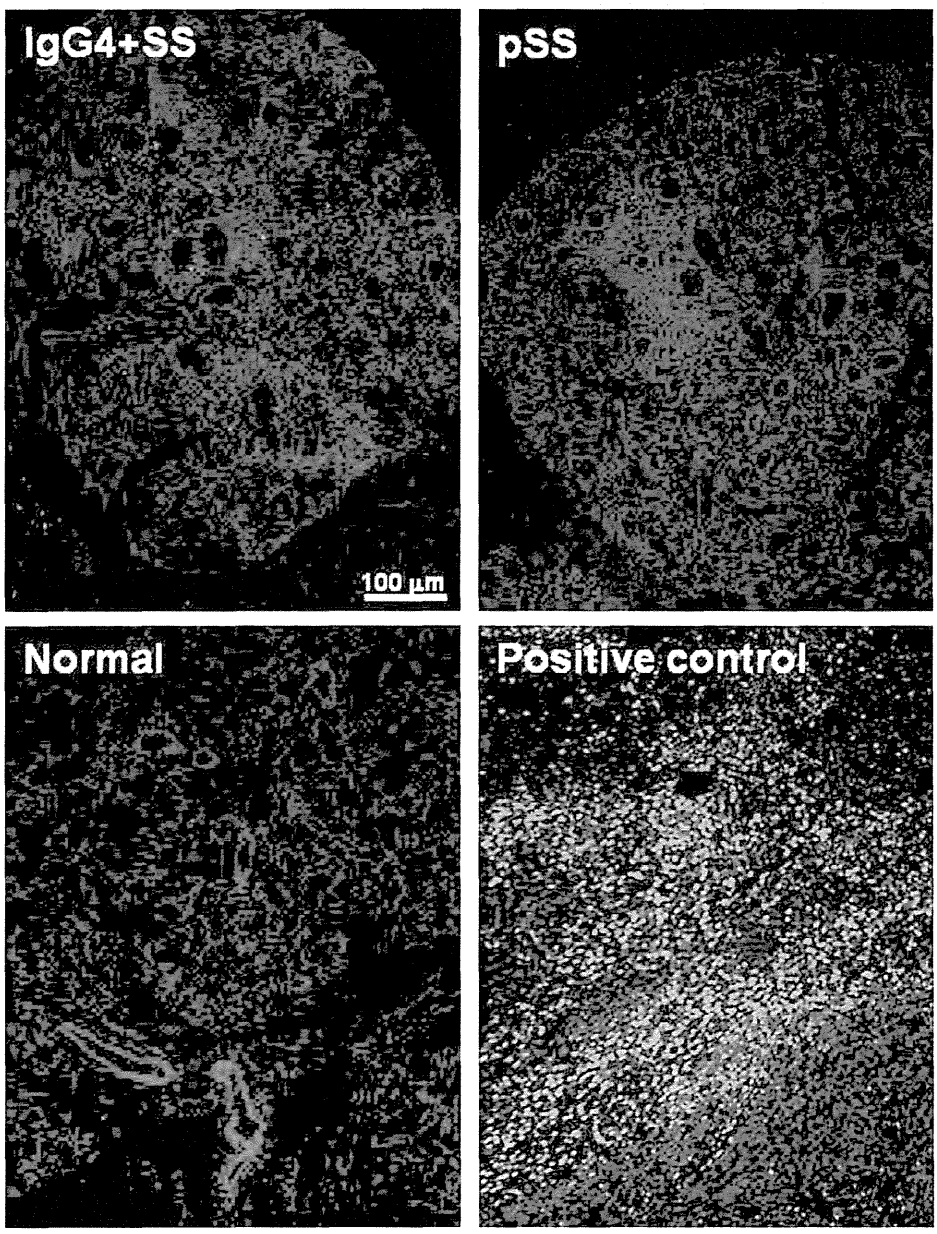

Figure 5. TUNEL staining. To detect apoptosis, TUNEL staining kit (MEBSTAIN Apoptosis kit Direct; MBL, Nagoya, Japan) was employed to show double-stranded DNA breaks. After the tissue sections of the labial salivary glands were incubated in proteinase K solution, they were incubated with terminal deoxynucleotidyl transferase solution at 37°C for 1 h. The fluorescein isothiocyanate signal of dUTP was captured by fluorescence microscopy using the KEYENCE BIOREVO BZ-9000. As a positive control, hepatocellular carcinoma tissue was used. The normal control subject had xerostomia; however, the patient did not fulfill the diagnostic criteria of pSS. A typical pSS patient would show positive anti-SS-A/Ro and SS-B/La antibodies with positive sialography and salivary gland biopsy.

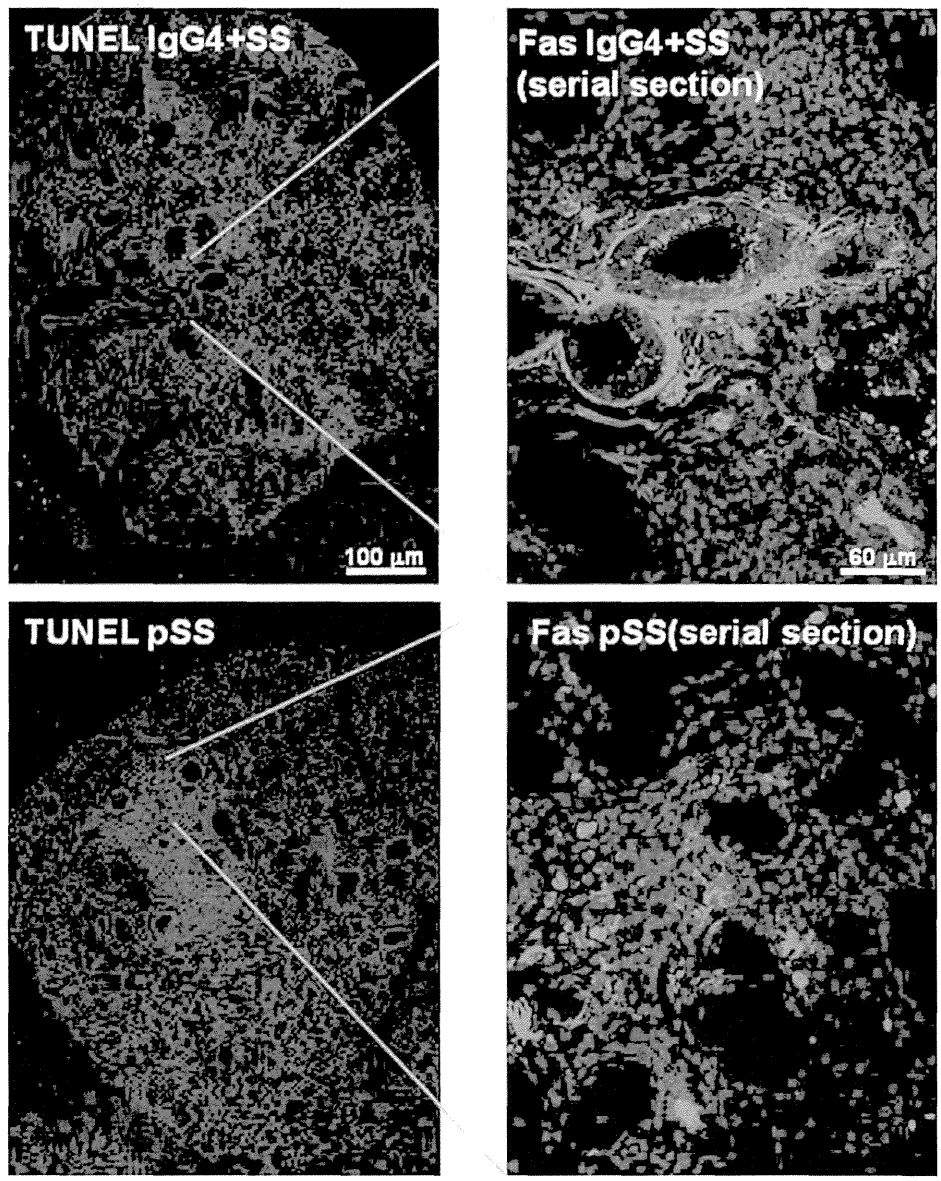

Figure 6. Fas immunostaining of LSGs. To show whether TUNEL-positive apoptosis is derived from Fas/Fas ligand system, we performed immunostaining of Fas toward this patient and a typical pSS patient. Using mouse monoclonal antibody against Fas (UB2; MBL, Nagoya, Japan), indirect immunostaining was done. Fas immunostaining in a serial section showed the location that nearly corresponds to that of TUNEL staining. FITC-labeled anti-mouse secondary antibody was used as a secondary antibody. FITC signal was captured by fluorescence microscopy using the KEYENCE BIOREVO BZ-9000.

criteria for IgG4-RD: 1) A serum IgG4 concentration > 135 mg/dl; and 2) > 40% of IgG-positive plasma cells being IgG4-positive and > 10 cells/high-powered field of biopsy sample [9,10]. In the diagnostic criteria, SS and other diseases such as sclerosing cholangitis, multicentric Castleman's disease and idiopathic retroperitoneal fibrosis were included in the exclusion criteria for IgG4-RD. Therefore, it is necessary to continue to observe the patient for further signs of IgG4-RD or pSS when the patient possesses manifestations of both IgG4-RD and pSS.

Tsubota et al. showed that the difference between MD and pSS was pathologically explained by the frequency of apoptosis by comparing APO2.7 staining of the lacrimal glands in both MD and SS [11–13]. They showed that apoptosis was observed in the lacrimal glands in SS but not MD. According to their suggestion, we performed TUNEL staining of LSGs in this patient, and positive TUNEL staining was observed. In our patient, the frequency of TUNEL-positive staining of the LSGs was greater than that in a typical pSS patient. If our patient had typical IgG4-RD characteristics, the TUNEL results ought to be those of a normal control. These results suggest that our patient showed a mixture of IgG4-RD and pSS. Furthermore, results of Fas immunostaining of this patient and a typical pSS patient suggested possible existence of Fas-mediated apoptosis in both patients. However, there are other candidates of apoptosis-inducible autoantigens [14] such as cytotoxic granules, tumor necrosis factor-related apoptosis-inducing ligand except for Fas. Therefore, we should avoid giving a straight answer to indicate that frequency of TUNEL-positive cells could be explained by existence of Fas. One possibility might be existence of somewhat mutual immune-antigen for both IgG4-RD and pSS.

In summary, the present case is considered to exhibit the coexistence of two different diseases. The accumulation of similar cases is required to elucidate the mechanism of IgG4-RD and pSS [8].

Conflict of interest

None.

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Japan College of Rheumatology
MODERN RHEUMATOLOGY

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

Mod Rheumatol, 2014; 24(4): 626–632
© 2014 Japan College of Rheumatology
DOI: 10.3109/14397595.2013.850766

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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

Received 5 July 2013
Accepted 27 September 2013
Published online 31 October 2013

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

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.

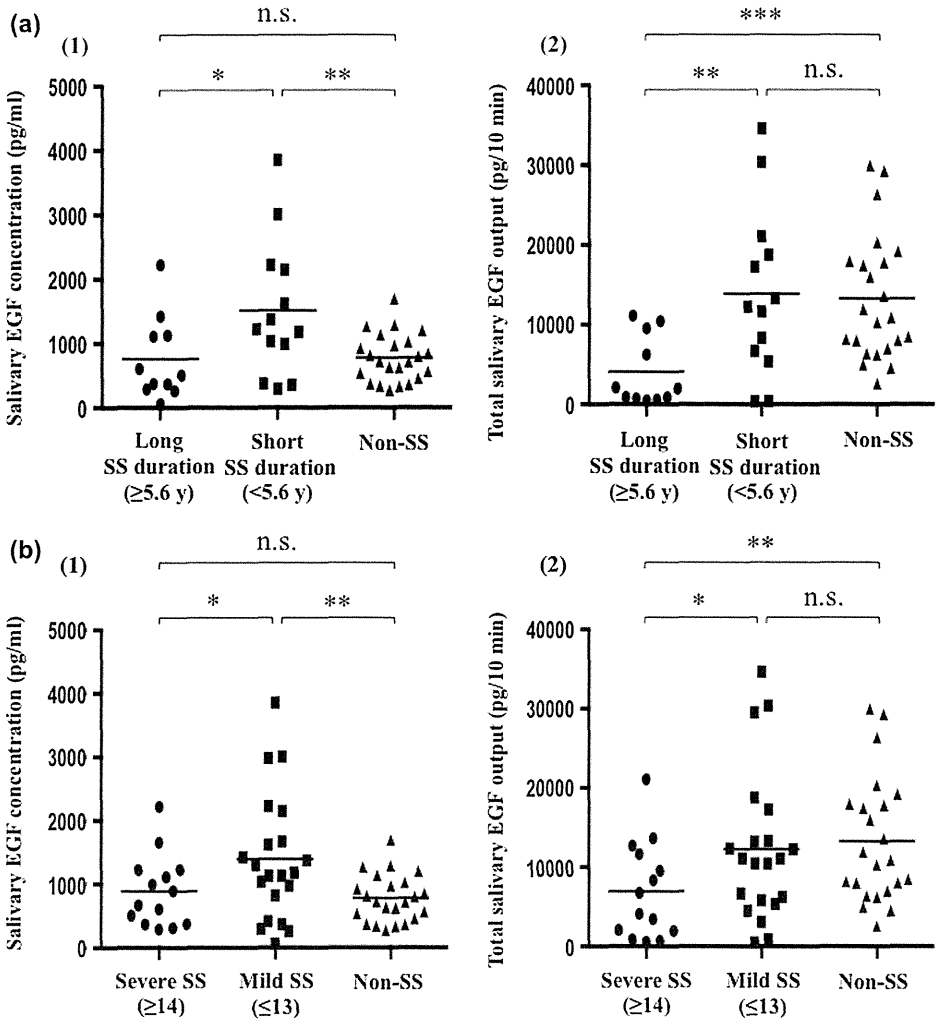

Figure 1. Salivary EGF levels of the SS and non-SS groups. (**a**) The SS group was divided into the long-duration group and short-duration group depending on disease duration, and salivary EGF levels were compared among these groups and the non-SS group. (1) Salivary EGF concentration. (2) Total salivary 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.

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

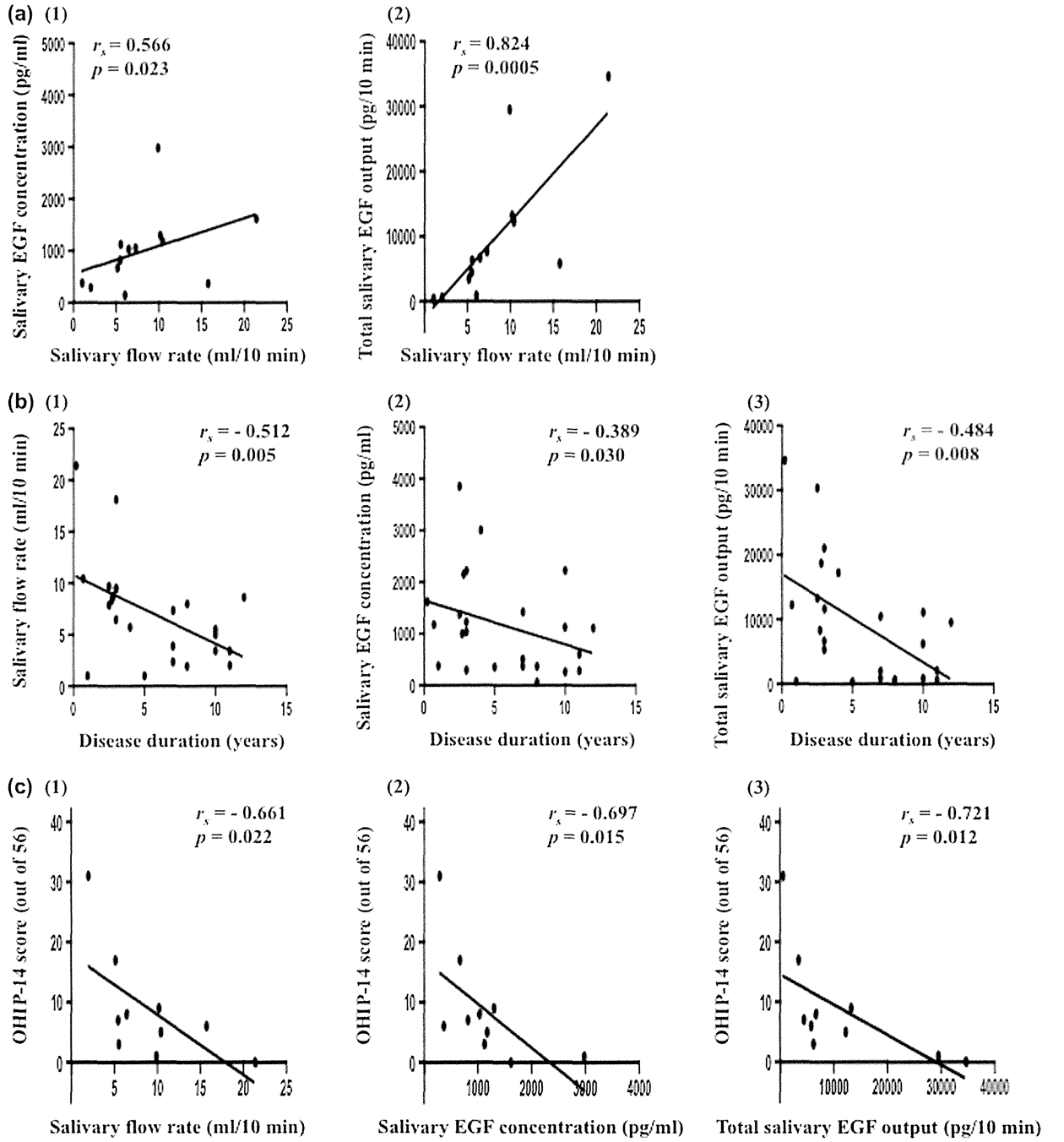

Figure 2. Correlations between each factor in the SS group. (**a**) Correlation of salivary flow rate with salivary EGF concentration (**1**) and total salivary EGF output (**2**). (**b**) Correlation of disease duration with salivary flow rate (**1**), salivary EGF concentration (**2**) and total salivary 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

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

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.

Acknowledgements

We thank Professor Seiichi Hirota (Department of Surgical Pathology, Hyogo College of Medicine) and clinical laboratory technicians (Department of Surgical Pathology, Hyogo College of Medicine Hospital) for assisting with the pathological examinations.

Funding

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

Conflict of interest

None.

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Original Article

Therapeutic effects of isoflavones on impaired salivary secretion

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(Received 1 April, 2014; Accepted 30 April, 2014; Published online 30 September, 2014)

Dry mouth, which is characterized by decreased salivation, has a number of causes; the involvement of estrogen has been suggested as symptoms typically develop in middle-aged females. However, there is a lack of consensus regarding the treatment of this condition. Soy isoflavones, a subgroup of flavonoids, are abundantly found in the soy germ. They are thought to exert a number of effects by specifically binding to estrogen receptors due to their structural similarity to estrogen. Recently, soy isoflavones have been found to exert antioxidant effects, ameliorating disorders caused by reactive oxygen/free radicals. Based on these observations, the effects of soybean isoflavones on impaired salivary secretion were studied in patients with dry mouth. Soy isoflavone aglycones were administered at 25 mg per day to 15 subjects with an average age of 67.9 ± 8.0 years for 2 months, and salivary secretion was analyzed. The results showed a significant improvement based on the saliva flow rate and self-completed questionnaire, thus suggesting the usefulness of isoflavones in improving the symptoms of salivary gland hypofunction.

Key Words: **isoflavones, dry mouth, estrogen, salivary secretion, reactive oxygen species**

Studies have shown that dry mouth is a multi-factorial disease in which the saliva flow decreases, thereby resulting in not only mouth dryness, feelings of thirst, and difficulty in food ingestion but also dysgeusia, dental caries, periodontal disease, burning mouth, and reduced quality of life (QOL).[1] Although the pathogenesis of this disease is not yet fully elucidated, its incidence increases with age. The disease has been reported to be more common in postmenopausal women.[2] In addition, an *in vivo* mouse experiment demonstrated that ovariectomy resulted in decreased salivary secretion and that estrogen replacement ameliorated this decrease.[3] Although feelings of thirst have been reported to improve upon estrogen replacement therapy (ERT) in patients with menopausal disorders,[4] an alternative method to ERT has been sought due to concerns of the Women's Health Initiative (WHI) regarding the link between ERT and tumorigenesis.[5]

In recent years, oxidative stress has been proposed to play a role in accelerated aging[6] and in the pathogenesis of Sjogren's syndrome (SS), which is characterized by severe mouth dryness in middle-aged females.[7] Particular oxygen radicals, such as superoxides and peroxides, mediate apoptosis,[8] thus causing oxidative damage to membrane lipids and proteins and reducing their function.[9] Furthermore, pathophysiological mechanisms involving oxidative damage have been reported.[10,11]

In addition, the various physiological effects of soybeans and a variety of soybean bioactive compounds have been investigated. Specifically, research on soy isoflavones has been undertaken, and estrogenic effects on bone metabolism and anti-cancer activity have been reported.[12–15]

Isoflavones are a subclass of flavonoids that are abundantly found in the pulse family, including soybeans, soy foods, and Japanese arrowroot, and exist in the glycoside or aglycone form. A total of 12 types of soy isoflavones are contained in soybean and soy germ (hypocotyl), including the aglycones (genistein, daidzein, and glycitein), three types of glycosides, and their acetyl and malonyl glycosides.[16] Although many aglycones are contained in fermented soybean products, such as miso and fermented soybeans, the isoflavones found in soybean or soybean products are typically glycosides. After ingestion, isoflavone glycosides are hydrolyzed by salivary enzymes,[17] enzymes found in the small intestinal mucosa,[18] and β-glucosidases from *Enterobacteriaceae*, thereby generating isoflavone aglycones[19,20] that are absorbed in the intestine to exert their effects.

Although the binding capacity of aglycones to estrogen receptors (ERs) differs according to the test method employed, it is reported to be weaker than that of estrogen.[21,22] Because of structural similarities to estrogen, aglycones specifically combine with ERs (α and β) and demonstrate estrogenic effects.[23,24] ER-α, which is abundant in the female genitalia, is also abundant in mammary glands, the hypothalamus, endothelial cells, and vascular smooth muscle. ER-β is abundant in the prostate gland, ovary, lung, brain, blood vessels, bone,[25] and salivary glands.[26–28]

In addition, isoflavones exhibit antioxidative properties and ameliorating effects against free radical-induced damage of cell membrane lipids, lipoproteins,[29–33] and DNA.[34–37] In this study, the effects of soy isoflavones on dry mouth patients are examined.

Materials and Methods

Evaluation of isoflavone effects in subjects. Tablets containing 25 mg of isoflavones extracted from soy (aglycone equivalent, 23.6 mg) were provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Fifteen patients of the Dry Mouth Clinic of Tsurumi University Dental Hospital were enrolled in this study. The subjects included one male and fourteen females, ranging in age from 49 to 81 years (67.9 ± 8.0 years; mean ± SD). All fifteen subjects with dry mouth had a salivary secretion of less than 10 ml, as determined by the stimulated saliva flow rate (gum test). Ten patients were assigned to the SS group, and the remaining five to the non-SS group (Table 1). The diagnosis of SS was based on the diagnostic criteria.[38] Patients who had a medical history of significant cardiovascular disease, significant pulmonary obstructive disease, gastrointestinal obstructive disease, epilepsy, or

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doi: 10.3164/jcbn.14-49