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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

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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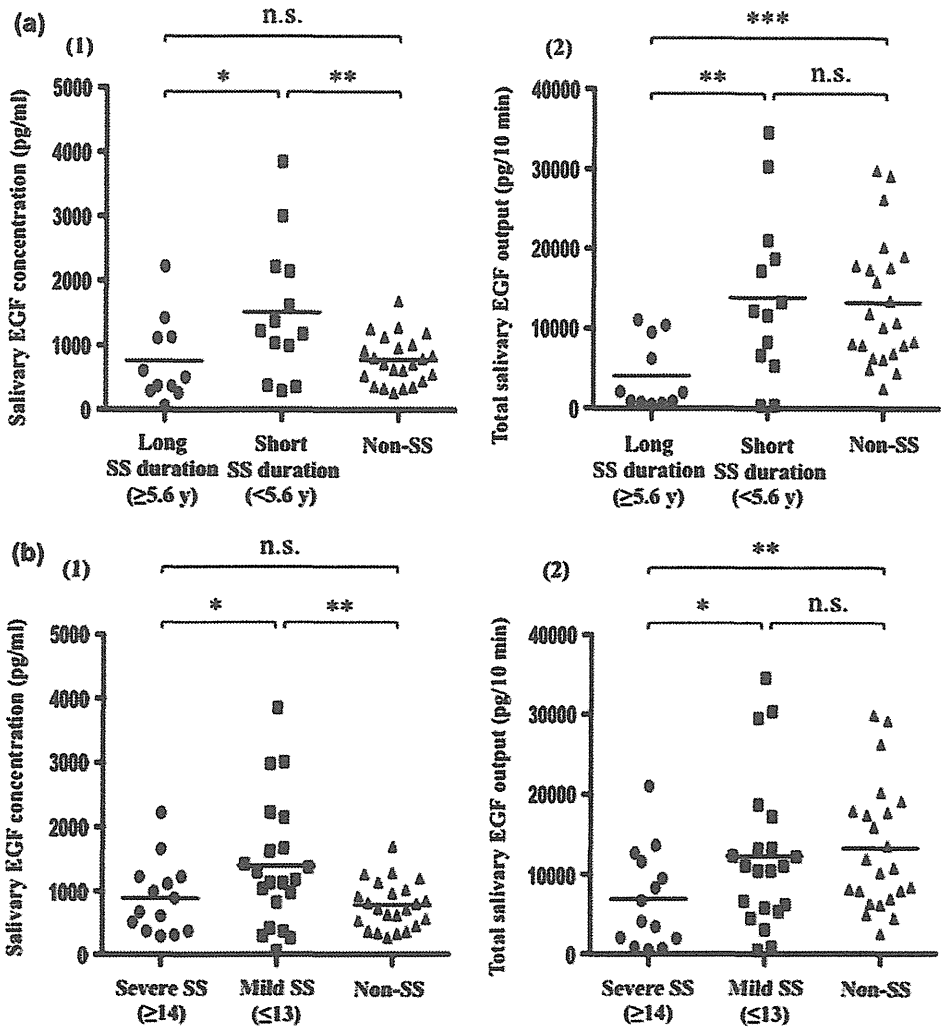

Figure 1. Salivary EGF levels of the SS and non-SS groups. (**a**) The SS group was divided into the long-duration group and short-duration group depending on disease duration, and salivary EGF levels were compared among these groups and the non-SS group. (1) Salivary EGF concentration. (2) Total salivary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

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

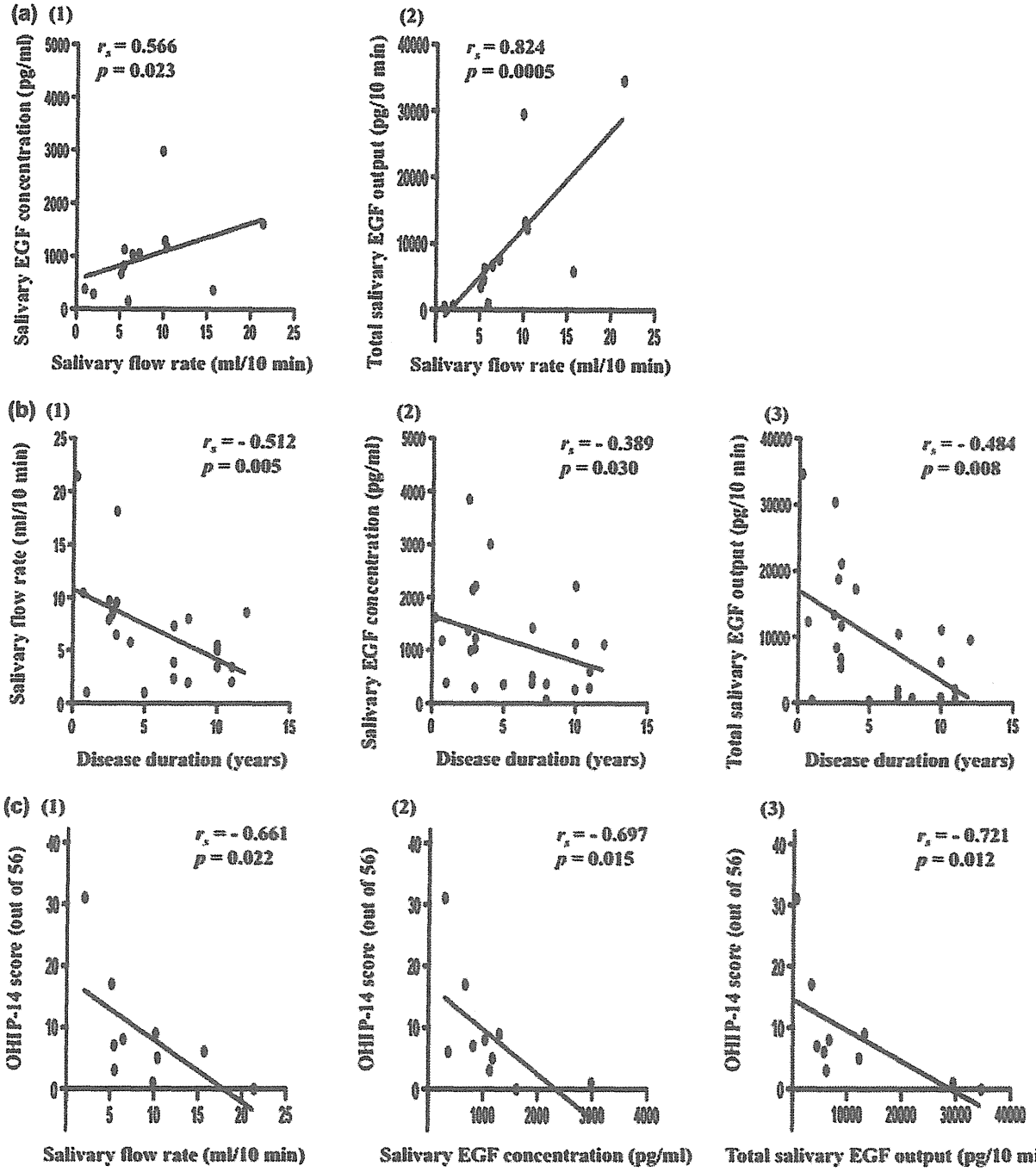

Figure 2. Correlations between each factor in the SS group. (a) Correlation of salivary flow rate with salivary EGF concentration (1) and total salivary EGF output (2). (b) Correlation of disease duration with salivary flow rate (1), salivary EGF concentration (2) and total salivary EGF output (3). (c) 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.

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Conflict of interest

None.

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Aryl Hydrocarbon Receptor-Mediated Induction of EBV Reactivation as a Risk Factor for Sjögren's Syndrome

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a variety of biological effects by binding to environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin). Although numerous animal studies have demonstrated the harmful effects of dioxins, it remains controversial whether dioxins pose a risk to human health. Enhanced lytic replication of EBV is a risk factor for the development of autoimmune diseases and cancers. This study evaluated the possibility that ligand-activated AhR reactivates EBV. EBV reactivation and AhR transactivation were evaluated with luciferase assays. Saliva samples were collected from 19 patients with primary Sjögren's syndrome (SS). Control saliva samples were obtained from 10 healthy individuals and nine patients with severe dry mouth. TCDD enhanced BZLF1 transcription, which mediates the switch from the latent to the lytic form of EBV infection in EBV-positive B cell lines and in a salivary gland epithelial cell line. Moreover, TCDD-induced increases in BZLF1 mRNA and EBV genomic DNA levels were confirmed in the B cell lines. Saliva from SS patients activated the transcription of both CYP1A1 and BZLF1. Additionally, there was a positive correlation between CYP1A1 and BZLF1 promoter activities. AhR ligands elicited the reactivation of EBV in activated B cells and salivary epithelial cells, and these ligands are involved in SS. Our findings reveal novel aspects of the biological effects of dioxin and the AhR-dependent pathogenesis of autoimmune diseases. *The Journal of Immunology*, **2012, 188: 4654–4662.**

Halogenated aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin), are chemical carcinogens that are widely distributed in the environment, and these chemicals are persistent environmental contaminants. Human exposure to mixtures of halogenated aromatic hydrocarbons and polynuclear aromatic hydrocarbons occurs mainly through diet; thus, exposure is universal and can accumulate throughout a lifetime. Most of the harmful effects of dioxins are thought to be mediated by the aryl hydrocarbon receptor (AhR) (1–3). The binding of ligands to the AhR triggers its translocation into the nucleus, where the AhR forms a heterodimer with the AhR nuclear translocator (Arnt). The AhR/Arnt heterodimer binds to specific xenobiotic response elements located in the promoter and enhancer regions of target genes, thus regulating the transcription of these genes.

Dioxins directly induce the replication of the influenza virus (4), human CMV (5), and HIV type 1 (6) in vitro. Moreover, viral titers in the salivary glands of rats infected with CMV are elevated by dioxin in vivo (7). Although numerous animal studies have demonstrated the harmful effects of dioxins, the dioxin-related risks to human health remain controversial.

EBV is a ubiquitous herpes virus that infects >90% of the world's population. Infection is usually asymptomatic and associated with lifelong persistence of the virus in resting recirculating memory B cells (8). The induction of lytic replication results in new viral infection and EBV-associated cellular transformation, and this induction may be a risk factor for both malignant transformation and the development of autoimmune diseases. The pathogenesis of many autoimmune diseases, including multiple sclerosis, systemic lupus erythematosus, Sjögren's syndrome (SS), and rheumatoid arthritis, is not fully understood. Both genetic predisposition and environmental factors, including EBV infection, contribute to the development and/or promotion of these diseases (9).

During viral replication, the EBV BZLF1 is the first gene to be transcribed. Its gene product Zta (also called ZEBRA, EB1, and Z) is considered both necessary and sufficient to induce the EBV lytic cycle (10, 11). The regulatory elements within the BZLF1 promoter (Zp) are divided into three groups: positive elements (ZI, ZII, and ZIIIA), negative elements (H1, ZIIR, ZIV, and ZV), and autoreactivation elements (ZIIIA and ZIIIB) (12). The region from −221 to +12 bp, which carries the ZI, ZII, ZIII, and ZV motifs, may be necessary for maintaining basal activity and transcriptional activation by lytic cycle-inducing agents (13). Conversely, the distal region from −554 to -221 bp contains repressive elements (14). In latently infected B cells, BZLF1 expression leading to viral replication is induced by various reagents, including phorbol ester (15), anti-Ig Abs (16), and butyrate (17). However, the physiological

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Abbreviations used in this article: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; CALUX, chemically activated luciferase gene expression; DM, dry mouth; HA, hemagglutinin; NML, normal healthy individual; SS, Sjögren's syndrome; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; XBP-1, X-box binding protein-1; Zp, BZLF1 promoter; Zta, EBV BZLF1 gene.

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stimulant that controls the initiation of viral replication and the switch from latency in vivo is unknown.

B cells are a major component of humoral immunity and are a sensitive immune target of TCDD (18). Resting human B cells are also the main sites of primary EBV infection in vivo. In healthy individuals, EBV persists in the memory B cells of the mucosal lymphoid tissue in the Waldeyer's ring (tonsils/adenoids) and in the peripheral blood for the duration of the individual's lifespan. EBV-infected memory B cells from this reservoir can be induced to enter the lytic phase of infection, which is accompanied by the differentiation of the B cells into plasma cells (19).

In this study, we demonstrate that BZLF1 is a novel target gene of dioxin-activated AhR. Infectious EBV is present in both the saliva of SS patients (20–22) and culture supernatants of B cell lines established from SS patients (23). Mariette et al. (24) previously used in situ hybridization to detect EBV DNA in a substantial proportion of lymphoid cells and epithelial cells in salivary glands from patients with SS. In our study, saliva from SS patients transactivated both BZLF1 and CYP1A1, which are target genes of AhR. Taken together, these findings demonstrate that the AhR ligands in SS saliva may be involved in EBV reactivation. Although dioxins are known to modulate the immune system, resulting in the development of autoimmune diseases, our study suggests a new role for dioxins as a pathogenetic factor for autoimmune disease via EBV reactivation.

Materials and Methods

Cell culture

The EBV-positive B cell lines B95-8 (an EBV-transformed marmoset B cell line) and P3HR1 (a Burkitt's lymphoma cell line) were maintained in RPMI 1640 supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml), and 10% FCS. These cell lines promptly and synchronously activated latent EBV genomes after 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) stimulation. The cells were suspended in fresh medium to produce a final concentration of 1×10^6 cells/ml and were cultured for the indicated time in the presence or absence of TPA. The salivary adenocarcinoma cell line HSY (25) (provided by Dr. M. Sato, Tokushima University) was cultured in MEM containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% FCS.

Plasmids

The promoter region (−552 to +13) of BZLF1 was amplified by PCR and cloned into pGL3 basic luciferase vector (Promega) using the primers pZp+13Hind (5′-CGAAGCTTGCCGGCAAGGTGCAATGTTT-3′) and pZp−551Xho (5′-AGCTCGAGGGATCCCTAACGCCAG-3′). The following combinations of enzymes were used to construct the pZp221-Luc and pZp130-Luc plasmids: XhoI-SphI for Zp221 and XhoI-NsiI for Zp130. Constructs pZp552Δ130, MI, MII, and MIII were synthesized from pZp552-Luc by temperature cycling using the primers and PfuTurbo DNA polymerase. These constructed pZp plasmids are shown in Fig. 1. Reporter DNA construct containing the human CYP1A1 promoter was generated by inserting a 1.9-kb CYP1A1 promoter PCR fragment into the pGL3 basic luciferase vector.

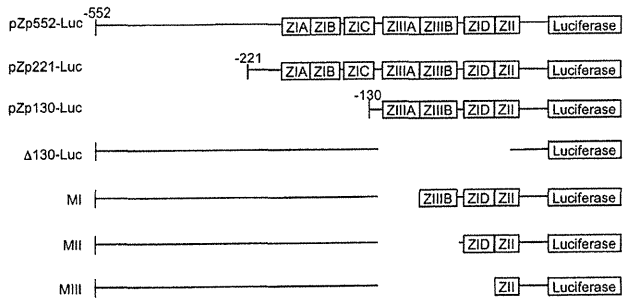

FIGURE 1. Schematic description of the relevant plasmids used in transfection experiments to analyze the transcriptional *cis*-elements.

To construct the expression vectors pCI-AhR-hemagglutinin (HA) and pCI-Arnt-HA, the AhR and Arnt cDNAs were amplified by RT-PCR and subcloned into pCI-neo (Promega), and an HA tag was fused to the C-terminal end of the gene. The RT-PCR primers used were as follows: AhR, 5′-CTCGAGACCATGAACAGCAGC-3′ and 5′-ACGCGTCAGGAATCCACTG-3′; and Arnt, 5′-CGCTCGAGGCCATGGCGGCGACT-3′ and 5′-GTACGCGTTTCTGAAAAGGGGGGA-3′. To construct Ad-AhR-HA and Ad-Arnt-HA, HA-tagged cDNAs were excised from pCI-AhR-HA and pCI-Arnt-HA and inserted into the cosmid pAxCAwt (TaKaRa, Shiga, Japan). Viral titers were measured in a limiting dilution bioassay using HEK293 cells.

Reporter assay

Cells were seeded in 12- or 24-well plates at a density of 10^6 cells per well and grown to subconfluence. HSY cells were seeded in 12-well plates 24 h prior to transfection. Transient transfection experiments were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were cotransfected with the pZp552-Luc, pZp221-Luc, pZp130-Luc, or CYP1A1-Luc reporters using 1 ng *Renilla* luciferase reporter (pRL-TK) driven by the HSV thymidine kinase (HSV-tk) promoter as an internal control and the pCI-AhR, pCI-Arnt, or pCI-Zta plasmid using Lipofectamine 2000. After 4 h, transfected cells were treated with either TCDD or a vehicle control (DMSO) for 48 h. Final DNA concentrations were adjusted using empty expression vectors to ensure that equal amounts of DNA were used in each well. Cells were harvested and lysed in a passive lysis buffer (Promega). Lysates were sequentially assayed for firefly and *Renilla* luciferase activity in a luminometer using the dual luciferase reporter assay system (Promega) and an ARVO MX multilabel counter (PerkinElmer). The results are presented in terms of relative luciferase activity, which represents the ratio of stimulated activity to nonstimulated activity in each experiment. All transfection experiments were performed in triplicate.

Quantitative PCR

Total RNA (2 μg) was used for cDNA synthesis with the SuperScript III first-strand synthesis system (Invitrogen). Quantitative PCR was performed using the SYBR Premix Ex Taq II kit (TaKaRa) and analyzed on a StepOnePlus instrument (Applied Biosystems). The PCR primers used were as follows: BZ1, 5′- TTCCACAGCCTGCACCAGTG-3′; BZ2, 5′-GGCAGCAGCCACCTCACGGT-3′; BRLF1 up, 5′-CATCACTATAGGGCACGCGA-3′; BRLF1 down, 5′-TAATGGCCACGCTCAACATC-3′; BALF5 up, 5′-CGGAAGCCCTCTGGACTTC-3′; and BALF5 down, 5′-CCCTGTTTATCCGATGGAATG -3′. The latter two primers recognize the EBV viral DNA BALF5 gene that encodes the viral DNA polymerase. Briefly, each 20-μl reaction sample contained 2 μl cDNA or genomic DNA, 10 μl SYBR Premix Ex Taq II (TaKaRa), 0.4 μl ROX reference dye, 0.8 μl each primer at 10 μM, and 6 μl double-distilled H_2O. Each experiment was run in triplicate. The PCR reaction was conducted as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

EMSA

HSY cells were infected with Ad-AhR and Ad-Arnt and then stimulated with TCDD for 2 h. Cell lysates were extracted from Ad-AhR– and Ad-Arnt–infected HSY cells using the CelLytic M cell lysis reagent (Sigma-Aldrich) and stored at −80°C until the assay was performed. EMSA was carried out with the LightShift chemiluminescent EMSA kit (Pierce Chemical). Oligonucleotide probe for Zp130 was biotinylated using the biotin 3′ end-labeling kit (Pierce Chemical). Briefly, cell lysates were incubated with 20 fmol biotin-labeled oligonucleotide for 20 min at room temperature in a binding buffer (10 mM Tris, 1 mM EDTA, 1 mM DTT, 100 mM KCl, and 10% [v/v] glycerol) and 1 μg nonspecific inhibitor Poly (deoxyinosinic-deoxycytidylic) in a final volume of 20 μl and incubated for 20 min at 25°C, followed by nondenaturing gel electrophoresis using 0.5× TAE as the running buffer. After electrophoresis, the biotinylated probes were transferred to a Hybond N+ membrane (GE Healthcare) followed by cross-linking with UV light. The biotinylated DNA was imaged using a VersaDoc 5000 (Bio-Rad). The specificity of AhR DNA binding was determined by competition reactions in which a 200-fold molar excess (4 pmol) of unlabeled oligonucleotide was added to the binding reaction.

Saliva samples

All SS patients were evaluated at the outpatient clinic of the Tsurumi University School of Dental Medicine (Yokohama, Japan) and diagnosed based on criteria proposed by the Japan Ministry of Health, Labor, and Welfare (Table I). Saliva samples were collected from 19 patients with SS (mean age, 61.9 y) at the initial medical examination, prior to the ad-

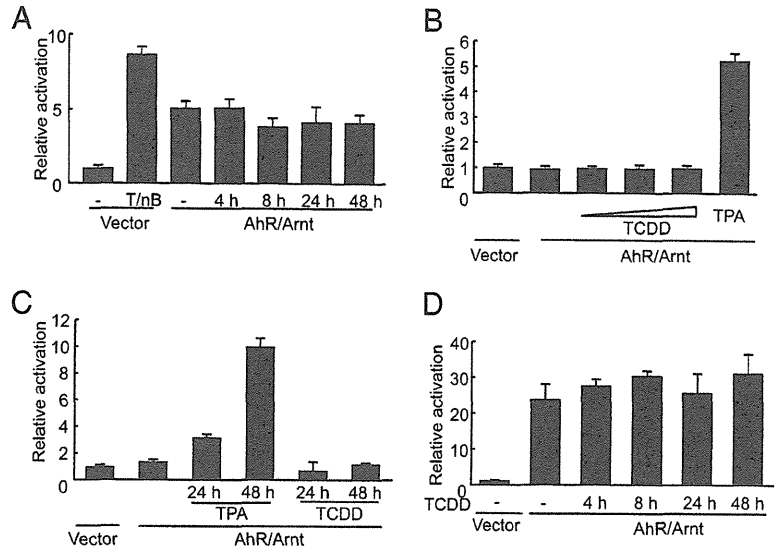

FIGURE 2. TCDD did not activate Zp or CYP1A1 transcription in unstimulated B cell lines. (**A**) pZp552-Luc was transiently cotransfected with human AhR and Arnt into P3HR1 cells cultured for the indicated times in the presence of 100 nM TCDD, TPA/n-butylate, TPA, and *n*-butylate. (**B** and **C**) Human AhR and Arnt were cotransfected into the Zp552-B95-8 stable cell line. Zp activities were measured after 24 and/or 48 h stimulation with either TPA (100 ng/ml) or TCDD [1–100 nM (B) or 100 nM (C)]. (**D**) CYP1A1-Luc was transiently cotransfected with human AhR and Arnt into P3HR1 cells and cultured for the indicated times in the presence of 100 nM TCDD. Values represent means ± SD derived from the normalized data, with transfections performed in triplicate in each experiment. Data were averaged over at least three experiments.

ministration of any medication. Control saliva samples were obtained from 10 age-matched normal healthy individuals (NML) (mean age, 63.7 y) and 9 patients with severe dry mouth (DM; mean age, 58.1 y). Informed consent was obtained from all patients. The average stimulated and unstimulated saliva flow rates in both SS and DM individuals were <5 ml/10 min and <0.5 ml/15 min, respectively. Samples were centrifuged at 12,000 × *g* for 45 min and filtered through a 0.22-μm filter to remove cells, viruses, and particulate debris. Aliquots of the samples were then stored at −80°C.

Ethics

Informed consent was obtained from all patients, and the Ethical Committee of Tsurumi University approved this study.

Results

TCDD does not change the EBV infectious status in B cell lines

To test the effect of TCDD on EBV reactivation in B lymphocytes, we used the EBV-infected B cell lines B95-8 and P3HR1, in which the EBV lytic cycle can be induced by TPA stimulation in vitro. Because BZLF1 expression following Zp reactivation is sufficient for this switch (10), we initially conducted a Zp assay to confirm EBV reactivation in B cells. pZp552-Luc, indicated in Fig. 1, was transiently transfected into P3HR1, and TCDD had no effect on Zp552 activation, even in the presence of AhR and Arnt expression (Fig. 2A). We also tested the stably transfected cell line Zp552-B95-8. Zp552 activation was strongly enhanced in Zp552-B95-8 cells following TPA stimulation, but the cells did not respond to TCDD in the presence of AhR and Arnt (Fig. 2B, 2C). Moreover, the promoter activity of CYP1A1, a target gene of AhR, was unchanged following TCDD stimulation, even in the presence of AhR and Arnt (Fig. 2D). Although B cells have previously demonstrated an inappropriate activation response to TCDD (26), our results show that TCDD stimulation alone is not sufficient for AhR activation or for BZLF1 and CYP1A1 transcription in P3HR1 and B95-8 cells.

TCDD induces EBV reactivation in TPA-activated B cell lines

Several studies have demonstrated that activated B cells are more sensitive to TCDD than are nonactivated cells (27–29). TPA-activated splenocytes show increased AhR expression and enhanced dioxin response element binding to AhR in the presence of TCDD (28). Thus, we studied the effect of TCDD on B cells under moderate activation by TPA. P3HR1 and B95-8 cells were stimulated with a low concentration of TPA (1 ng/ml) prior to incubation with TCDD. Simultaneous TPA and TCDD treatment enhanced the transactivation of pZp552 compared with treatment with TPA alone (Fig. 3). TPA combined with *n*-butylate stimulated Zp552, and the greatest activity was detected after 48 h incubation (Fig. 2C). In contrast, under moderate TPA stimulation, TCDD increased Zp552 activity after as little as 6 h stimulation, and the maximum activity was observed after 24 h (Fig. 3). The increased expression of BZLF1 mRNA was confirmed by RT-PCR (Fig. 4A) and real-time PCR (Fig. 4B), and significant expression was detected after 24 h combined TCDD/TPA treatment (Fig. 4A, 4B). EBV genomic DNA was amplified by PCR using primers for the BALF5 gene, and the levels of this DNA were increased in B95-8 and P3HR1 cells following treatment with TCDD combined with TPA (Fig. 4C). Real-time PCR revealed significant EBV replication after 24 or 48 h treatment in P3HR1 and B95-8 cells, respectively (Fig. 4D). At the point when virus replication was confirmed, the mRNA expression of an alternative virus gene, BRLF1, was also increased in these cells (Fig. 4E, 4F). These results suggest that the activation of B cells is required for TCDD

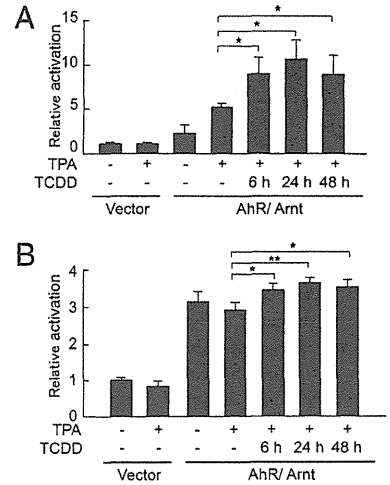

FIGURE 3. Transactivation of Zp by TCDD in TPA-activated B cell lines. Human AhR and Arnt were cotransfected into P3HR-1 (**A**) and B95-8 cells (**B**). Zp activities were measured after 6, 24, and 48 h stimulation with TCDD (100 nM) in the presence of TPA (1 ng/ml). Values represent means ± SD derived from normalized data, with transfections performed in triplicate in each experiment. Data were averaged over at least three experiments. Statistical analysis was performed using a one-way ANOVA. $*p < 0.05$, $**p < 0.005$.

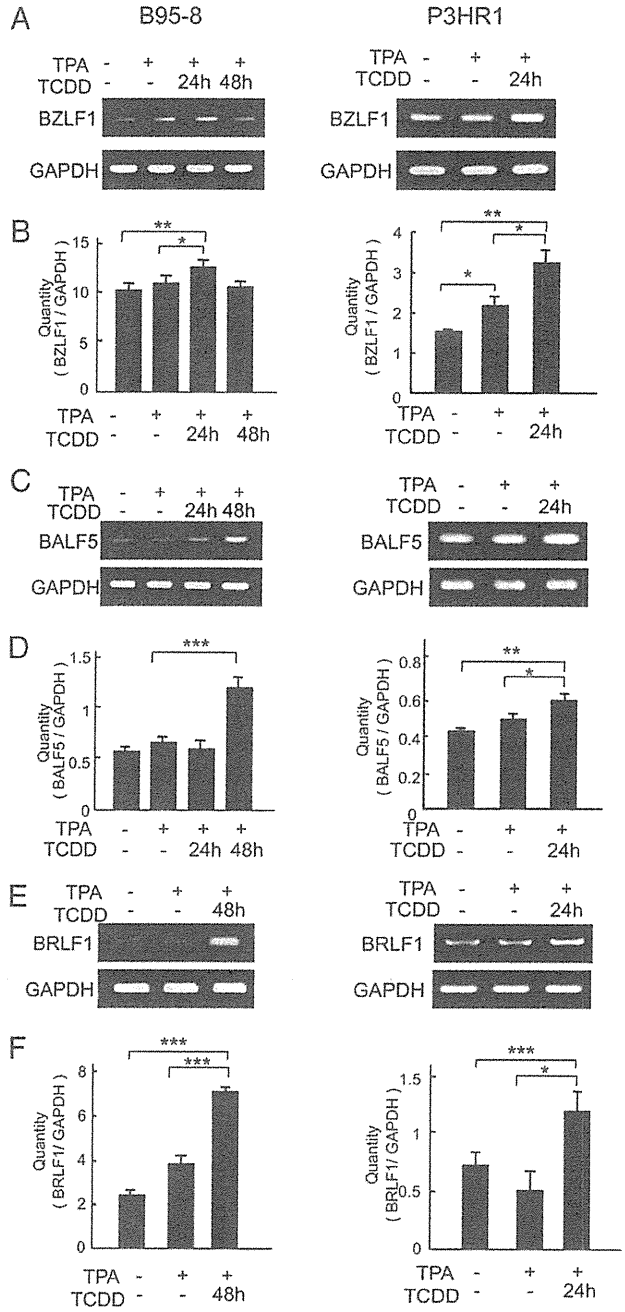

FIGURE 4. EBV gene expression and replication by TCDD in TPA-activated B cell lines. mRNA and genomic DNA were obtained from B95-8 (*left panels*) and P3HR1 (*right panels*) cells stimulated with TCDD (100 nM) in the presence of TPA (0.01 ng/ml). RT-PCR (**A**, **E**) and real-time RT-PCR (**B**, **F**) analysis of BZLF1 (A, B) and BRLF1 (E, F) transcripts. PCR (**C**) and real-time PCR (**D**) analyses were conducted for BALF5 to represent EBV genomic DNA. Real-time PCR results are presented relative to GAPDH mRNA expression (B, F) or genomic DNA (D). Values represent means ± SD derived from normalized data, with transfections performed in triplicate in each experiment. Data were averaged over at least three experiments. Statistical analysis was performed using a one-way ANOVA. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

to reactivate EBV and emphasize that Zp activity reflects BZLF1 mRNA expression and EBV replication.

TCDD induces Zp activation in salivary gland epithelial cells

EBV replication only occurs in a small percentage of B cells (30), and the levels of virus released from B cells alone differs considerably from the actual virus levels in the saliva (31). Thus, EBV reactivation in B cells is thought to be only the initial event in the spread of virus particles in vivo. The actual physiological site of virus production is thought to be the epithelial cells of the nasopharynx and parotid glands (31–34). The calculated potential virus production from these cells is sufficient to account for the observed levels of shedding in saliva (31). Thus, we tested Zp activation in the salivary epithelial cell line HSY.

HSY cells were cotransfected with pZp552-Luc or CYP1A1-Luc and AhR and Arnt, and the cells were then exposed to TCDD stimulation. Clear TCDD-induced CYP1A1 promoter activity was observed in HSY cells (Fig. 5A), and Zp552-Luc activity was also enhanced by TCDD (Fig. 5B).

To confirm the role of the AhR/Arnt complex, we transfected different quantities of AhR and Arnt into HSY cells. The TCDD (10 nM) treatment-induced Zp activity was dependent on the levels of transfected AhR and Arnt (Fig. 5C). The range of TCDD required

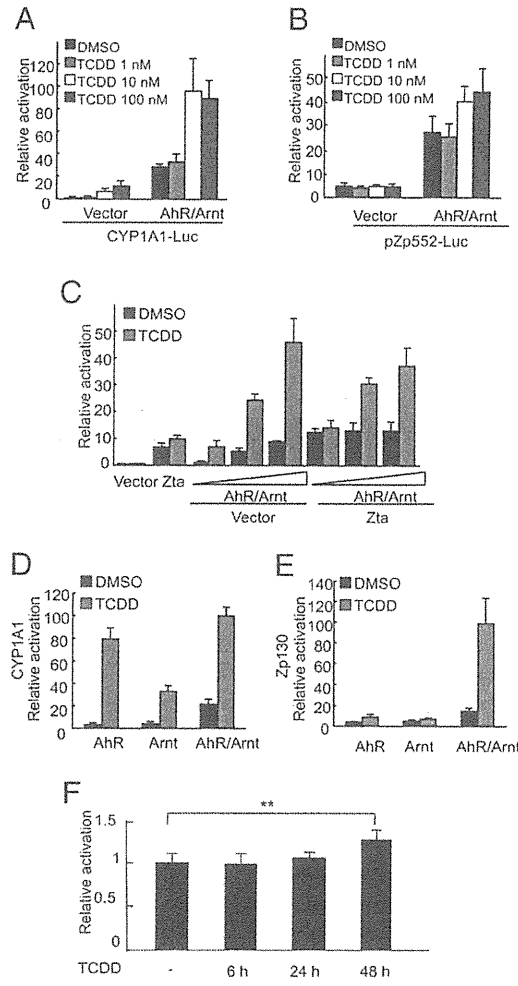

FIGURE 5. Transcriptional activation of the CYP1A1 promoter or Zp in response to TCDD in salivary epithelial cells and HepG2 cells. (**A–E**) HSY cells transiently cotransfected with CYP1A1-Luc (A, D), pZp552-Luc (B, C), or pZp130-Luc (E) and plasmids encoding human AhR and/or Arnt. (C) Increasing amounts of plasmids encoding human AhR and Arnt (0.05–0.25 μg) were cotransfected with or without Zta. (**F**) HepG2 cells were transfected with Zp130-Luc. Luciferase activities were measured after 48 h stimulation with increasing amounts of TCDD (1–100 nM) (A, B) or 10 nM TCDD (C–F). Data were averaged over at least three experiments. Values represent means ± SD derived from normalized data, with transfections performed in triplicate in each experiment. $**p < 0.01$.

for both the activation of the CYP1A1 promoter and for the activation of Zp was similar. Although CYP1A1 promoter activity was increased by either AhR or Arnt (Fig. 5D), an increase in Zp130 activity was observed only upon the exogenous expression of both AhR and Arnt (Fig. 5E). Zp contains the autoreactive sequence sites ZIIIA and ZIIIB; therefore, the exogenous expression of the BZLF1 gene product Zta increased the activity of Zp (Fig. 5C). The Zp activity induced by TCDD-activated AhR/Arnt was not enhanced by Zta expression (Fig. 5C). These results demonstrate that Zp activity is stimulated by TCDD via the AhR/Arnt complex and that this stimulation represents a distinct transcriptional mechanism that occurs via CYP1A1 promoter activation. Moreover, the endogenous expression of AhR/Arnt in HepG2 cells is also responsive for Zp transactivation by TCDD (Fig. 5F). This activity was not dramatic but was still significant.

Identification of the cis*-acting AhR-responsive region in Zp*

To investigate the activation of the AhR-responsive regions in Zp, we generated a series of deletion plasmids encompassing the −551- to +13-bp region of Zp flanked by the luciferase-coding region (Fig. 1).

We cotransfected HSY cells with pZp130-Luc, pZp221-Luc, or pZp552-Luc and AhR and Arnt. The TCDD-induced Zp552-Luc and Zp221-Luc activities were similar, whereas the shortest plasmid, pZp130-Luc, showed the highest level of activation in response to TCDD (Fig. 6A). A TCDD concentration of >10 nM was required to activate pZp130-Luc (Fig. 6B); the same concentration was required to activate pZp552-Luc and CYP1A1-Luc (Fig. 5A, 5B). To confirm the presence of AhR *cis*-response elements in Zp130, we generated the deletion mutant plasmid pZp552Δ130-Luc by eliminating the −130- to −44-bp region from the intact pZp552-Luc plasmid. TCDD failed to activate pZp552Δ130-Luc in HSY cells (Fig. 6C). Exogenously expressed Zta also failed to stimulate this promoter because the Zta binding sites ZIIIA and ZIIIB are located between −134 and −104 bp (Fig. 6C). The serially mutated reporter plasmids MI, MII, and MIII (Fig. 1) exhibited an incomplete loss of promoter activity in comparison with intact pZp552-Luc (Fig. 6D). These results indicate that the putative location of the AhR response element is proximal to the transcription start site within a 130-bp segment of the Zp.

Coexpression of AhR and Arnt is required for Zp activation (Fig. 5E). The AhR/Arnt heterodimer binds specific xenobiotic response elements containing core motifs (5′-GCGTG-3′) in responsive genes, modulating their expression. Although there is no core sequence for AhR in the Zp region, we attempted to evaluate the binding of TCDD-activated AhR/Arnt to the Zp130 fragment using an EMSA. Nuclear extracts from TCDD-treated HSY cells that ectopically expressed AhR and Arnt showed that AhR/Arnt bound to Zp130. This binding was specifically inhibited by nonlabeled Zp130 (Fig. 6E). This result suggests a physical interaction between the AhR/Arnt complex and Zp130. A luciferase assay conducted using a deletion mutant of pZp552-Luc demonstrated that ligand-activated AhR interacts primarily with the ZII element (Fig. 6D), which also binds to basic leucine zipper transcription factors such as ATF1, CREB, and ATF2 (35). Future studies will be necessary to define the AhR binding sites within Zp.

AhR activation is associated with EBV reactivation in SS

SS is a systemic autoimmune disorder that affects the salivary and lacrimal glands and causes mononuclear cell infiltration, causing

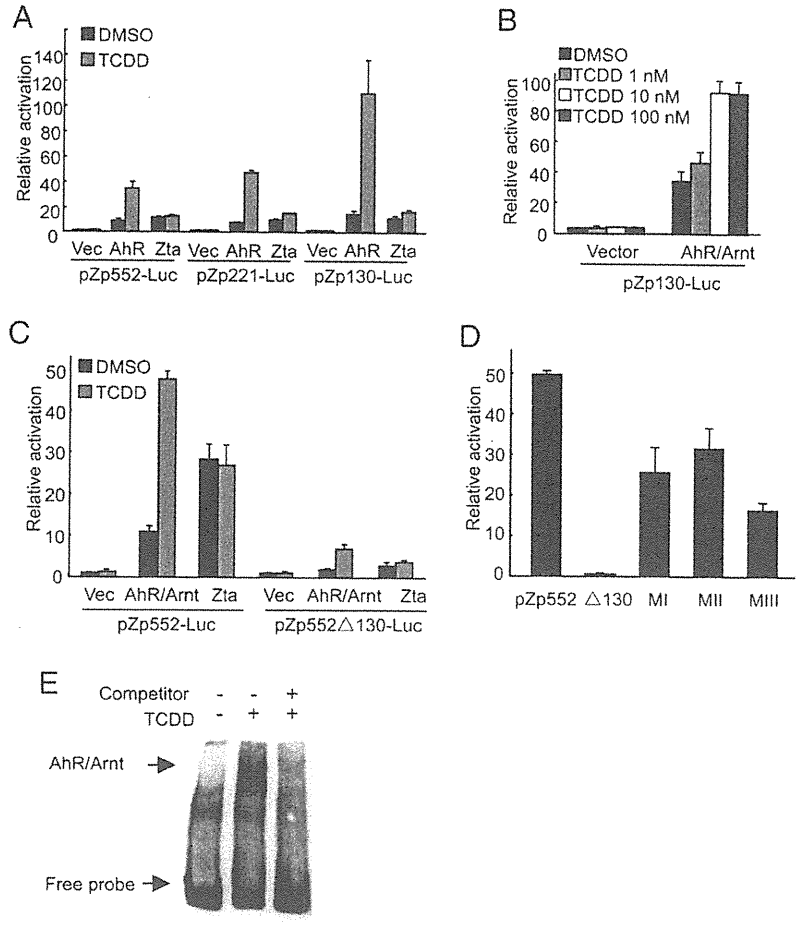

FIGURE 6. The region of Zp required for response to TCDD-activated AhR in HSY cells. (**A–D**) Luciferase activities were measured after 48 h stimulation with TCDD with or without exogenous human AhR and Arnt or Zta in HSY cells. The plasmid pZp552Δ130-Luc, which lacked bases −130 to −40 from the original pZp553-Luc (C, D), and deletion mutants MI, MII, or MIII (D) are shown in Fig. 1. Values represent means ± SD derived from normalized data, with transfections performed in triplicate in each experiment. Data were averaged over at least three experiments. (**E**) Ad-AhR– and Ad-Arnt–infected HSY cells were incubated for 2 h in the presence of vehicle (0.001%) or TCDD (10 nM). The nuclear extract was incubated for 20 min with 20 fmol biotin-labeled Zp130 probe and then subjected to EMSA. The specificity of AhR DNA binding was determined by competition reactions with 4 pmol of unlabeled Zp130.

the clinical symptoms of dryness of the mouth and eyes. The pathogenesis of SS remains unclear; however, a high incidence of EBV reactivation in SS has been reported. EBV Ags and DNA are present in the infiltrating lymphocytes and salivary gland epithelial cells of SS patients (22, 24). Infectious EBV is present in the saliva of SS patients (21, 36) and in the culture supernatants of B cell lines established from SS patients (23). Thus, EBV reactivation in SS is thought to contribute to the initiation or perpetuation of tissue destruction in target organs such as the salivary and lacrimal glands. To detect AhR ligands in the saliva of SS patients, we conducted a reporter assay for the CYP1A1 promoter and Zp. As disease controls, we assessed DM patients with levels of saliva secretion similar to those of SS patients. In this study, we found that Zp130 was strongly activated by the saliva of SS patients in the presence of exogenous AhR expression, whereas activation in the saliva of NML or DM individuals was basal level (Fig. 7A). The saliva from SS patients also activated the CYP1A1 promoter in the presence of exogenous AhR expression (Fig. 7B), and this activity was significantly weaker in the saliva of NML and DM individuals. Moreover, there was a positive correlation between the Zp and CYP1A1 promoter activities (Fig. 7C). These results indicate that the AhR ligand in the saliva of SS patients may stimulate BZLF1 transactivation.

The relationship between AhR activation and autoantibodies in SS

The most common autoantibodies in patients with SS are those directed against the SSA/Ro and SSB/La ribonucleoprotein complexes (37, 38). These Abs are detected in serum and can be produced locally in the affected salivary glands (39). Anti-SSB/La Abs are accompanied by anti-SSA/Ro Abs, whereas anti-SSA/Ro Abs can be found either with or without anti-SSB/La Abs (40). In this study, serum titers of anti-SSA/Ro and anti-SSB/La Abs were evaluated relative to Zp and CYP1A1 promoter activities. Although a high titer of anti-SSB/La Ab was detected in only 6 of 19 patients (Table I), the Ab titer was correlated with Zp and CYP1A1 promoter activities in these patients (Fig. 8A, 8B).

Discussion

Several epidemiological studies suggest an association between dioxin exposure and an increased incidence of certain human diseases. After an industrial accident in Seveso, Italy, in which workers were acutely exposed to high levels of TCDD, a high incidence of gastrointestinal, lymphatic, and hematopoietic cancers and endocrine and immunological effects was observed (41). The long-term effects of dioxin exposure include an excess of lymphohematopoietic neoplasms, such as Hodgkin's and non-Hodgkin's lymphoma (42). EBV has been associated with certain types of non-Hodgkin's lymphoma, such as Burkitt's lymphoma and lymphomas related to severe immunosuppression (43). Dioxins in adipose tissue and Abs against Epstein–Barr early Ag were investigated in non-Hodgkin's lymphoma patients. An increased risk of non-Hodgkin's lymphoma was found for patients in the high-concentration and high-titer group (44). The level of early Ag Abs reflects the degree of EBV viral replication, a relationship that was also observed in SS patients. Moreover, a high early Ag Ab titer was frequently detected in SS patients who developed non-Hodgkin's lymphoma (45, 46). The present study demonstrates that environmental contaminants such as dioxins may induce EBV reactivation and thus act as risk factors for SS.

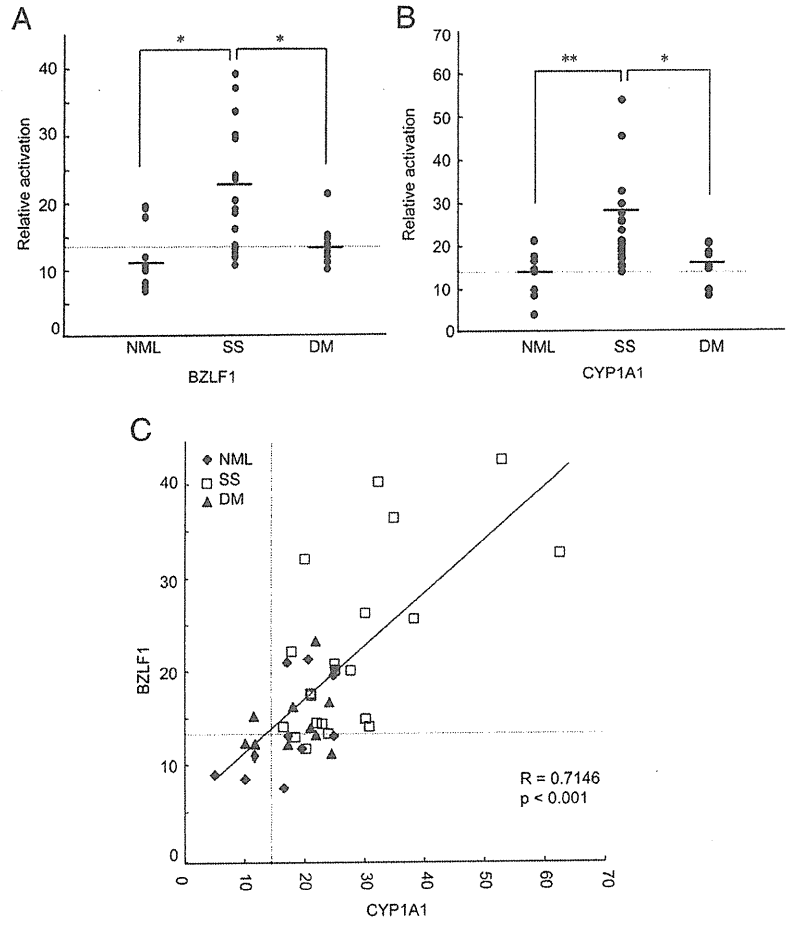

FIGURE 7. Zp and CYP1A1 promoter activation by SS saliva. pZp130-Luc (**A**) or CYP1A1-Luc (**B**) was cotransfected with plasmids encoding human AhR and Arnt into HSY cells, followed by stimulation with NML, SS, and non-SS DM saliva. Luciferase activities were measured after 48 h stimulation. (**C**) A statistically significant correlation between Zp130 (A) and CYP1A1 activities (B). The activities of each promoter were expressed as the relative activity with respect to the activity induced by 100 nM TCDD, which was set to a value of 100. The baseline of this assay, derived from DMSO alone, is indicated by the dotted lines. Statistical analysis was performed using one-way ANOVA and the Pearson correlation test. $*p < 0.05$, $**p < 0.005$.

Table I. Clinical information on patients and summary of data obtained by luciferase assay

Patient No.	Age (y)	Sex	Autoantibody		Luciferase Assay	
			SSA/Ro	SSB/La	BZLF1	CYP1A1
SS 01	55	F	23.8	6.4	23.4	39.2
SS 02	34	F	ND	ND	38.8	54.3
SS 03	76	F	500.0	7.0	13.6	30.9
SS 04	72	F	500.0	7.0	10.7	20.6
SS 05	70	F	ND	ND	19.0	25.6
SS 06	70	F	500.0	7.0	36.7	33.0
SS 07	72	F	7.0	7.0	20.2	18.2
SS 08	72	F	ND	ND	18.3	28.3
SS 09	69	F	107.7	7.0	13.0	16.8
SS 10	68	F	500.0	474.6	24.0	30.8
SS 11	58	F	500.0	132.2	29.3	20.4
SS 12	52	F	500.0	341.0	29.8	64.2
SS 13	52	F	500.0	ND	12.1	24.5
SS 14	58	F	500.0	500.0	33.3	35.8
SS 15	71	F	476.0	32.0	13.1	23.4
SS 16	66	F	500.0	27.1	16.1	21.4
SS 17	37	F	500.0	7.0	11.8	18.8
SS 18	58	F	307.0	ND	13.2	22.5
SS 19	70	F	ND	ND	12.5	31.1

In this study, we studied ligand-activated AhR using a Zp reporter assay that involved the modification of chemically activated luciferase gene expression (CALUX). The CALUX method is becoming increasingly common for the evaluation of dioxins in blood (47), milk (48), food (49), and environmental samples (50). The conventional method, high-resolution chromatography/mass spectrometry, is time-consuming, costly, and requires large sample volumes. A previous comparison of results obtained using the two assays revealed few differences (51). For our assessments, involving limited volumes of saliva, the CALUX bioassay was a useful tool for evaluating dioxins.

In B cells, the observed mechanisms of EBV reactivation in vitro are hypothesized to involve the activation of BZLF1 transcription by TPA, *N*-butyric acid, or Ig–cross-linking Abs. However, the priming factor for EBV reactivation in vivo is poorly understood. The plasma cell differentiation factor X-box binding protein-1 (XBP-1) binds and transactivates Zp in a plasma cell line and in lymphoid cell lines but not in epithelial cell lines (52). XBP-1 also induces BZLF1 transcription and is involved in B cell differentiation into plasma cells. In this study, we propose a new candidate dioxin that induces BZLF1 in both activated B cells and salivary epithelial cells. In contrast to XBP-1, the response to TCDD-activated AhR was higher in salivary epithelial cells than in activated B cells. Activated B cells have increased AhR mRNA levels and protein expression (27) and are more responsive to TCDD than are nonactivated B cells (29). Chen and Tukey (53) reported that TPA, as a protein kinase C activator, enhances CYP1A1 gene transcription by AhR, indicating that protein kinase C promotes nuclear events that work in concert with or precede AhR binding to the gene. This result suggests that the B cells that are responsible for TCDD after TPA stimulation may use the protein kinase C signaling pathway.

EBNA-3 is an EBV-encoded nuclear Ag that is indispensable for B cell transformation and is implicated in the maintenance of lymphoblastoid cell line proliferation. The overexpression of EBNA-3 induces G_0/G_1 arrest (54), and the elimination of EBNA-3 results in cell death (55) in lymphoblastoid cell lines. EBNA-3 directly interacts with AhR and enhances its transactivational function via the ligand-activated AhR (56). TCDD-activated AhR induces cell cycle arrest at G_1, accompanied by the increased expression of the CDK2 inhibitor $p27^{kip1}$ (57). Studies of murine B cell development have shown that AhR ligands, such as polycyclic aromatic compounds, induce apoptosis in pro- and pre-B cells (58). The function of AhR in cell cycle progression is also consistent with its interaction with proteins such as pRb (59) and the p65 subunit of NF-κB RelA (60). Although the molecular mechanisms underlying its activity are not understood, EBNA-3 compromises the inhibitory effect of TCDD on the growth of EBV-positive lymphoblasts (56). All of the latent EBV genes, including

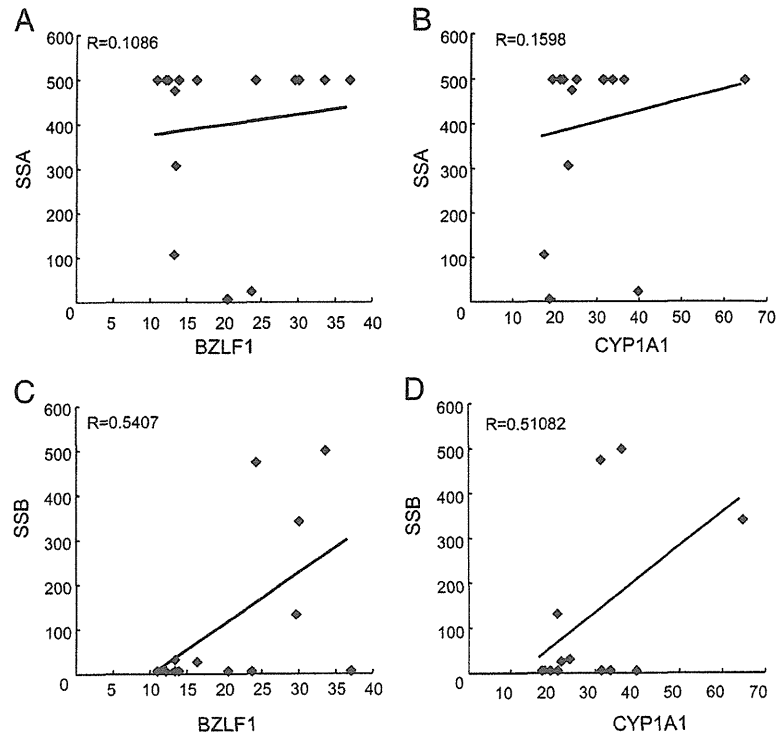

FIGURE 8. Analysis of the correlation between autoantibody titer and AhR transactivation in SS patients. The titer of anti-SSA/Ro (**A**, **B**) or SSB/La (**C**, **D**) autoantibodies in SS sera was compared with the corresponding Zp (A, C) (Fig. 7A) or CYP1A1 (B, D) (Fig. 7B) promoter activities. Statistical analysis was performed using a Pearson correlation test.

EBNA-3, are expressed in EBV$^+$ naive (IgD$^+$) B cells (latency III). Latency III is known as the growth program because EBV-transformed B cells proliferating in vitro exhibit this expression pattern (61). In germinal center B cells, EBV gene expression is restricted to EBNA1, LMP1, and LMP2 (latency II) (62). In the peripheral blood, EBV exists within the IgD$^-$ memory B cell pool, with expression more restricted to LMP2A (latency 0) (8). However, EBV status in the salivary glands has not been elucidated. In this study, we used EBV-positive B cell lines B95-8 and P3HR1, which express latency III and latency II patterns, respectively. This finding suggests that EBNA-3 may cause different Zp sensitivity toward TCDD-activated AhR in B95-8 and P3HR1 (Figs. 3, 4).

Our previous study demonstrated that Zp221 activity and BZLF1 transcription were induced by saliva from SS patients (63). TGF-β1, which is expressed in SS salivary glands, induced Zp221 activity via the MAPK signaling pathway. These data suggest that EBV reactivation at the inflammatory site may occur because of the presence of TGF-β1. In the present study, we observed TCDD-induced AhR-dependent Zp activity. Thus, TCDD may be a trigger for EBV reactivation without inflammation.

AhR has been shown to regulate the level of T regulatory and Th17 cell differentiation and to modulate the severity of experimental autoimmune encephalitis in a ligand-dependent manner in mice (64–66). Human CD4 cells derived from healthy donors produced IL-22 but not IL-17 in response to ligand-activated AhR (67, 68). Human exposure to an extremely high dose of TCDD in vivo induced a selective increase in the frequency of T cells producing IL-22 but not IL-17, IL-10, or IFN-γ (69). Activated AhR also promoted T cell differentiation into functional Foxp3$^+$ human induced regulatory T cells, which produce IL-10 in vitro (70). More recently, Li et al. (71) showed that intraepithelial lymphocytes express high levels of AhR and that receptor activation directly affects the maintenance but not the development, homing, or proliferation of intraepithelial lymphocytes. AhR activity can be regulated by certain dietary components derived from vegetables, which may regulate the intestinal immune system. Currently, it is not clear why AhR activation can either increase or reduce inflammatory reactions. We hypothesize that the immunological condition of the host may contribute to the pathogenesis of autoimmune diseases involving EBV reactivation caused by activated AhR.

SS is a chronic autoimmune disease that is characterized by the presence of a variety of autoantibodies directed against organ-specific and non–organ-specific autoantigens. The most common of these Abs are directed against two ribonucleoprotein Ags known as SSA/Ro and SSB/La. It is still unknown whether any of the autoantibodies have a pathogenic potential or whether they are all part of a secondary response to salivary glands already damaged by other processes. Anti-SSA/Ro and anti-SSB/La are found in the saliva of SS patients (72), and the B cells that infiltrate the salivary glands contain intracytoplasmic Igs with anti-SSA/Ro and anti-SSB/La activity (39, 73, 74). In this case, ectopic lymphoid germinal centers that contain Ag-presenting dendritic cells, T cells, and B cells have been found, providing a microenvironment that is conducive to the propagation of the autoimmune response (39). Finally, an increased production of SSB/La mRNA in acinar epithelial cells has been observed (75), and translocation and membrane localization of the SSB/La protein has been observed in conjunctival epithelial cells of SS patients (76). These reports suggest that anti-SSA/Ro and anti-SSB/La Abs may participate in the local immune response in the affected exocrine glands. In the present study, we found a correlation between anti-SSB/La in the sera and AhR activation by saliva in SS patients. These data lead us to speculate that EBV reactivation induced by dioxins may cause an immune response in the salivary glands of SS patients.

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Disclosures

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RESEARCH ARTICLE Open Access

Regulatory mechanisms for the production of BAFF and IL-6 are impaired in monocytes of patients of primary Sjögren's syndrome

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Abstract

Introduction: In this study, we investigated possible aberrations of monocytes from patients with primary Sjögren's syndrome (pSS). We focused on B-cell-activating factor of the TNF family (BAFF) and IL-6 because they are both produced by monocytes and are known to be involved in the pathogenesis of pSS.

Methods: Peripheral monocytes were prepared from both pSS patients and normal individuals. The cells were stimulated *in vitro* with IFN-γ, and the amounts of IL-6 and soluble BAFF (sBAFF) produced by the cells were quantitated. The effect of sBAFF itself on the production of IL-6 was also studied. To investigate the response of pSS monocytes to these stimuli, the expression levels of the genes encoding BAFF receptors and IL-6-regulating transcription factors were quantitated.

Results: Peripheral pSS monocytes produced significantly higher amounts of sBAFF and IL-6 than normal monocytes did, even in the absence of stimulation. The production of these cytokines was significantly increased upon stimulation with IFN-γ. The elevated production of IL-6 was significantly suppressed by an anti-BAFF antibody. In addition, stimulation of pSS monocytes with sBAFF induced a significant increase in IL-6 production. Moreover, the expression levels of a BAFF receptor and transcription factors regulating IL-6 were significantly elevated in pSS monocytes compared to normal monocytes.

Conclusions: The results of the present study suggest that the mechanisms underlying the production of sBAFF and IL-6 are impaired in pSS monocytes. Our research implies that this impairment is due to abnormally overexpressed IL-6-regulating transcription factors and a BAFF receptor. These abnormalities may cause the development of pSS.

Introduction

Sjögren's syndrome (SS) is an autoimmune disease which primarily affects the salivary and lachrymal glands. Major clinical manifestations of primary SS (pSS) are xerostomia and keratoconjunctive sicca, which are consequences of lesions of the salivary glands and lachrymal glands, respectively. Accumulating evidence suggests that lymphocytic infiltrate of exocrine glands plays a key role in lesion formation and the subsequent dysfunction of the glands [1].

B-cell-activating factor of the TNF family (BAFF) (tumor necrosis factor ligand superfamily, member 13b) is a cytokine which is primarily produced by monocytes and dendritic cells [2-4] in addition to T cells [5,6]. It plays a crucial role in the proliferation, differentiation and survival of B cells [2,4,5,7]. BAFF is a type II membrane-bound protein of 285 amino acid residues. A C-terminal fragment of 152 amino acid residues is released from cells as soluble BAFF (sBAFF) [5]. sBAFF binds to its receptors (that is, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA) and B cell activating factor receptor (BAFF-R) [8-14]), possibly as a trimer [8,11,13], and elicits signal transduction through

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several pathways [10,11,13,15,16]. It is noteworthy that transgenic mice that overexpress BAFF in lymphoid cells develop hyperplasia of mature B cells [8,17,18] or pSS-like pathology [19]. BAFF is also elevated in the serum of pSS patients [20,21] and strongly expressed in the lymphocytes infiltrating the salivary glands [22,23]. Moreover, elevated production of BAFF has been linked to the development of another autoimmune disease, systemic lupus erythematosus [24-26].

Notably, systemic and/or local concentrations of several other cytokines, such as IL-6, are also significantly elevated in pSS patients compared to normal individuals [27,28]. IL-6 promotes the differentiation of B cells [29], which play a pivotal role in the production of autoantibodies and hence in the development of pSS. Since monocytes produce both IL-6 [30] and BAFF [2,4,31], we hypothesized that the production of these cytokines is dysregulated in pSS monocytes. If that is the case, aberrations of pSS monocytes may be implicated in the abnormal production of autoreactive immunoglobulin G (IgG) by B cells, which is involved in the pathogenesis of autoimmune diseases such as pSS [32]. In the present study, we demonstrate that the regulatory mechanisms for the production of these cytokines are impaired in pSS monocytes.

Materials and methods

Patients and controls

Venous blood samples were collected from pSS patients (n = 13 females ages 32 to 64 years (average age = 50.5)) and normal individuals (n = 12 females ages 26 to 60 years (average age = 43.5)) after receiving their informed consent. Patients fulfilled the American-European Consensus Group criteria for pSS [33]. At the time of the collection of blood samples, two patients (15.4%) were receiving prednisolone at a daily dose < 5 mg. The remaining patients were free of medication. This study was approved by the ethics committees at Keio University School of Medicine and Saitama Medical University.

Stimulation of peripheral monocytes *in vitro*

Peripheral monocytes were isolated as follows: Whole blood was mixed with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, BC, Canada) and centrifuged over Ficoll-Hypaque (Beckman Coulter, Fullerton, CA, USA). A monocyte-enriched fraction was collected and cultured overnight in RPMI 1640 (American Tissue Culture Collection, Manassas, VA, USA) supplemented with 10% FCS in a humidified incubator (7% CO_2) at 37°C so that the expression of various stress-induced genes subsided. The cells were then washed once with the medium to remove debris. Fluorescence-activated cell sorting (FACS) analysis of the cells demonstrated that > 96% of the living cells were CD14-positive.

The monocytes were cultured in the absence or presence of various concentrations of IFN-γ or sBAFF, and the cumulative production of sBAFF and/or IL-6 was examined by ELISA. The production was dependent on the incubation period. The optimal incubation period was found to be 96 hours. The production of the cytokines increased almost in proportion to the concentration of stimuli up to 200 ng/ml IFN-γ or 2 μg/ml sBAFF.

Antibodies and recombinant proteins

An anti-BAFF mAb for ELISA was prepared in our laboratory [6]. A rabbit polyclonal anti-BAFF antibody and recombinant human sBAFF were purchased from Chemicon International (Temecula, CA, USA). Recombinant human IFN-γ, a control mouse IgG1, and mAbs for measurement of the amount of IL-6 by ELISA (MQ2-13A5 and MQ2-39C3 for capture and detection, respectively) and for FACS analysis (CD4-APC (RPA-T4) for T cells, CD14-PE-Cy7 (M5E2) for monocytes, CD20-APC-Cy7 (L27) for B cells and CD268-FITC (11C1) for BAFF-R) were purchased from BD Biosciences/Pharmingen (San Diego, CA, USA). An anti-TACI antibody for FACS analysis (CD267-PE (FAB1741P)) was purchased from R&D Systems (Minneapolis, MN, USA).

ELISA

Monocytes were cultured at 2.5 × 10^5/ml for 96 hours in a 24-well plate (2 ml/well) in the presence of stimuli (that is, recombinant human IFN-γ or recombinant human sBAFF). The amounts of sBAFF (in response to IFN-γ as a stimulus) and IL-6 (in response to IFN-γ or sBAFF as stimuli) in the culture supernatants were measured by sandwich ELISA according to previously described methods [6], except for the concentrations of capture and detection antibodies for IL-6, which were prepared at 0.5 μg/ml.

For quantitation of sBAFF, we used our own anti-BAFF mAb, which specifically detects sBAFF and does not react with a proliferation-inducing ligand (APRIL) [6]. In our hands, the sensitivity of our ELISA system was better than that of commercially available ELISA kits (R&D Systems) in the range of 0.4 to 100 ng/ml sBAFF (data not shown).

Quantitation of the gene expression levels

The expression levels of BAFF, BAFF-R, TACI, NF-IL6, NF-IL6β, NF-κB1, NF-κB2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantitated by using a method described previously [6]. The following oligonucleotides were used as primers for PCR: 5'-