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Saliva levels of caspase-1, TNF-α, and IFN-γ in primary Sjögren's syndrome: oral mucosal abnormalities revisited

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Background/aim: Abnormalities in oral mucosal immunity contribute to complex pathogenesis of primary Sjögren's syndrome (pSjS). We aimed to measure saliva and serum levels of caspase-1, tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) in patients with pSjS.

Materials and methods: We studied 43 pSjS patients fulfilling the AECG criteria and 30 age/sex-matched healthy controls, as well as 39 rheumatoid arthritis (RA) patients as a disease control group. ESSDAI scores were less than seven in all patients with pSjS, indicating low disease activity. Quantitative analyses were made in serum and whole saliva samples. The statistical analysis was performed using SPSS 19.0.

Results: While no significant difference was found in serum measurements, saliva levels of TNF-α and caspase-1 were significantly higher in pSjS patients versus healthy controls when using the Mann–Whitney U test. On the other hand, in the pSjS group, saliva levels of TNF-α and caspase-1 were also significantly higher compared to the RA group using Student's t-test. In the pSjS group, those parameters did not show any correlation with disease duration, seropositivity, and smoking.

Conclusion: Despite low disease activity, saliva TNF-α and caspase-1 levels were found to be significantly higher in the pSjS group, which may suggest a possible advantage of local anticytokine treatments in selected cases.

Key words: Primary Sjögren's syndrome, caspase-1, tumor necrosis factor-alpha, interferon gamma, local treatment

1. Introduction

Sjögren's syndrome (SjS) is a chronic inflammatory autoimmune disease of unknown etiology, characterized by lymphocytic infiltration of exocrine glands resulting in dryness of the eyes and mouth. Extraglandular involvements such as arthritis, vasculitis, and hematological, pulmonary, and renal diseases may also occur. This disease is classified as 'primary' (pSjS) when it occurs by itself and 'secondary' when it occurs with another autoimmune disease (1,2). Despite extensive investigations, the disease-initiating events in the target exocrine glands are not known (1,2).

Rheumatoid arthritis (RA) manifests clinically as symmetric inflammation of the small joints in the hands and feet particularly. Chronic synovial inflammation leads to progressive destruction of joint cartilage and underlying bone (3).

Cytokines are small soluble peptides. They are used by the immune system to communicate and influence cellular function. Cytokines are released mostly by immune cells. Their functions can be autocrine, paracrine, or endocrine (4). Cytokines are regulators of the innate and adaptive immune system. They control the direction, amplitude, and duration of the inflammatory response (5).

Traditionally, the dominant role of the adaptive immune system in the pathogenesis of pSjS has been attributed mainly to the presence of lymphocytic infiltrates in the glands, and to the positivity of specific autoantibodies such as anti-Ro/SSA and anti-La/SSB. The exact cause of exocrine dysfunction in pSjS remains unclear. It has recently been suggested that not only the adaptive immune system but also the innate immune system may contribute significantly to the glandular damage (6). Since the

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salivary glands are the main target organs in pSjS, saliva may be better than serum as an ideal biological fluid to be investigated that closely reflects the underlying pathology of autoimmune glandular exocrinopathy in pSjS (7). In line with this assumption, increased saliva levels of various cytokines, including interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ), had already been shown in previous studies, reflecting the possible contribution of these cytokines in oral mucosal pathogenesis in pSjS. However, data regarding salivary levels of caspase-1, which is a very important enzyme contributing to local mucosal innate immunity, are limited. Therefore, we intended to investigate salivary levels of caspase-1, in addition to two prominent inflammatory cytokines, namely IFN-γ and TNF-α. Although because of technical problems we could not include IL-1, we believe that the triple combination of caspase-1, IFN-γ, and TNF-α may reflect the contribution of innate and adaptive immunity in local mucosal pathogenesis in pSjS. The present study was thus conducted to determine both saliva and serum levels of caspase-1, TNF-α, and IFN-γ in patients with pSjS, compared to both healthy controls and to patients with RA as a disease control group. We also aimed to investigate whether there is any correlation between the levels of these cytokines/enzymes and various clinical/laboratory parameters of pSjS.

2. Materials and methods

2.1. Patients and controls

This cross-sectional study was conducted by the enrollment of 43 patients with pSjS (F/M: 40/3, mean age ± SD: 51.1 ± 14.5 years, mean disease duration: 10.7 ± 7.1 years), all fulfilling the United States-European Consensus Criteria (8), and 30 healthy controls age- and sex-matched with the pSjS group. All of the healthy controls were recruited from the staff of Ege University Hospital and special attention was paid to exclude the relatives of the patients as healthy controls. While serum samples were obtained from all of the healthy controls, unfortunately saliva samples could not be obtained from 3 healthy controls. Besides, 39 patients with RA (F/M: 29/10, mean age ± SD: 54.6 ± 12.9 years, mean disease duration: 10.8 ± 6.0 years) all fulfilling the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria were also included as the disease control group (9).

The study patients were followed up at the Outpatient Clinic of the Department of Rheumatology at Ege University Hospital. All the medical records of the patients were carefully noted and a physical examination was performed for each patient. Exclusion criteria included the following: 1) pregnancy; 2) presence of any other known systemic diseases; 3) recent usage of antibiotics; 4) presence of periodontitis; 5) being edentulous.

None of the study participants, including both study groups and healthy controls, had oral mucosal lesions or dental problems.

None of the RA patients had clinical or laboratory features suggesting the presence of secondary SjS. Among our patents with pSjS, 24/43 had extraglandular involvement, and the distribution of extraglandular involvements is given in Table 1. All of the pSjS patients had been under usual corticosteroid and immunosuppressive treatment, and medications were not changed or stopped before enrollment in the study. Disease activity was determined according to the standard disease activity index, defined by EULAR, known as the Sjögren's Syndrome Disease Activity Index (ESSDAI) (10). ESSDAI scores of 0–7 were defined as mild disease, whereas scores above seven were defined as active disease. Disease activity in RA patients was determined on the basis of a disease activity score known as DAS 28 (11). RA patients having DAS 28 scores of 3.2 or higher were accepted to have active disease. Smoking status was determined according to self-reports of the study subjects.

2.2. Laboratory analysis

2.2.1. Serum sampling

Five milliliters of venous blood were taken from the antecubital vein by a standard venipuncture method, and serum was separated from blood by centrifugation at 1500 × g for 10 min. The serum samples were then stored at –40 °C until subsequent biochemical analyses and thawed immediately before assay.

2.2.2 Saliva sampling

Whole saliva samples were obtained from all patients and 27 out of thirty healthy controls, simply by expectorating into polypropylene tubes before clinical periodontal measurements or any periodontal intervention, in the morning time after an overnight fasting. The participants were requested not to drink (except water) or chew gum during sample collection. The samples were then frozen immediately and stored at –40 °C until the sample collection period was completed and thawed immediately before assays.

Antinuclear autoantibodies (ANAs) were determined and quantified using indirect immunofluorescence technology (Hep2 cells; Diamedix, Miami, FL, USA). Titers of 1/80 or higher were considered as positive. Anti-Ro/SSA and anti-La/SSB autoantibodies were detected by an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of IgG autoantibodies (ORGENTEC Diagnostica, Mainz, Germany). The cut-off value was 15 U/mL, as recommended by the manufacturer. Quantitative determination of serum rheumatoid factor (RF) was performed using an immunonephelometry test (Siemens, Marburg, Germany). RF was considered to be

Table 1. Clinical and laboratory features of the patients with pSjS.

Sex	N	%
Female/Male	40/3	93.0/7.0
Subjective symptoms		
Dry mouth	41	97.6
Dry eyes	39	92.9
Extraglandular manifestations	24	55.8
Raynaud phenomenon	9	20.9
Arthritis	13	30.2
Cutaneous manifestations	4	9.3
Pulmonary involvement	7	16.3
Hematological involvement	4	8.9
Liver involvement	8	18.6
Renal involvement	1	2.3
Neurological involvement	1	2.3
Vasculitis	2	4.7
Myositis	2	4.7
Eye findings		
Positive Schirmer's I test	25	58.1
Immunological features		
Antinuclear antibody positivity	35	72.9
Anti Ro/SS-A positivity	30	71.43
Anti La/SS-B positivity	16	38.15
Rheumatoid factor positivity	17	36.2
Cryoglobulin positivity	1	2.6
Low complement levels		
Serum complement 3	4	10.5
Serum complement 4	1	2.6
Minor salivary gland biopsy *		
Chisholm I	1	2.5
Chisholm II	1	2.5
Chisholm III	14	35.0
Chisholm IV	24	60.0
Cigarette smoking		
Current smokers	5	11.6
Nonsmokers	31	72.1
Former smokers	7	16.3

*Minor salivary gland biopsy was available for 40 out of 43 patients with pSjS.

positive when the concentration was higher than the cut-off value of the kit (15.9 IU/mL).

Specific ELISA kits for TNF-α and IFN-γ (eBioscience, San Diego, CA, USA) and caspase-1 (Sunredbio, Shanghai, China) were used for quantitative analyses. The assays were performed according to the manufacturer's recommendations. The minimum detection limits were 2.3 pg/mL, 0.99 pg/mL, and 0.117 ng/mL for TNF-α, IFN-γ, and caspase-1, respectively. Fifty (TNF-α, IFN-γ) or forty (caspase-1) microliter aliquots of saliva or serum were used for all assays and each assay was performed in duplicate.

Objective evaluation of ocular involvement was considered on the basis of a positive result for Schirmer's I test (without anesthesia; ≤5 mm/5 min).

This study was performed according to the principles of the Declaration of Helsinki and was approved by the local ethics committee; written informed consent was obtained from all the participants.

2.3 Statistical analysis

The statistical analysis was performed using SPSS 19.0. The Mann–Whitney U or Kruskal–Wallis test was used for the parameters that did not show a normal distribution. Student's t-test was used for the parameters that showed a normal distribution. $P < 0.05$ was accepted as statistically significant.

3. Results

Clinical and laboratory features of the patients with pSjS are shown in Table 1. As shown in detail in Table 1, at least one extraglandular involvement was present in 24/43 (55.8%) of the patients with pSjS. Among patients with pSjS, there were no cases having an ESSDAI score of higher than seven. In other words, all of the patients with pSjS, including those with extraglandular involvement, had low disease activity. During enrollment in the study, treatment agents that the patients had been receiving included corticosteroids (n: 7; 16.3%), hydroxychloroquine (n: 24; 60.0%), methotrexate (n: 5; 11.6%), azathioprine (n: 4, 9.3%), and leflunomide (n: 1; 2.3%). None of the patients had received any biologic treatment. The frequency of RA patients having DAS 28 score higher than 3.2 was 26.3% (n: 10). Rheumatoid factor positivity was present in 76.9% (n: 30), anti-CCP positivity in 66.7% (n: 24), and ANA positivity in 34.2% (n: 13). The frequency of current smokers among RA patients was 18.4%, nonsmokers 68.4% (n: 26), and former smokers 13.2% (n: 5).

Serum levels of caspase-1, TNF-α, and IFN-γ were not significantly different between the pSjS and RA groups and healthy controls. However, there were significant differences in saliva measurements. When we compared the saliva levels of caspase-1, TNF-α, and IFN-γ between the three groups, using the Kruskal–Wallis test, we found

that saliva levels of caspase-1 (P = 0.005) and TNF-α (P < 0.0001) showed significant differences, as delineated in Table 2.

When we compared the pSjS group with healthy controls using the Mann–Whitney U test, with respect to saliva levels of caspase-1, TNF-α, and IFN-γ, we found that only saliva levels of TNF-α (P < 0.0001) and caspase-1 (P = 0.031) were significantly higher in patients with pSjS.

When we compared the pSjS and RA groups using Student's t-test, saliva levels of TNF-α (P < 0.0001) and caspase-1 (P = 0.0003) were also significantly higher in the pSjS group compared to the RA group. Although saliva IFN-γ levels were higher in the RA group compared to the pSjS group, this did not reach statistical significance.

Patients with pSjS and RA were investigated as to whether serum and saliva levels of IFN-γ, caspase-1, and TNF-α showed positive correlations with various clinical and laboratory parameters. There were no significant correlations with disease duration in both patient groups. Similarly, when both patient groups were classified according to ANA or RF positivity, or smoking habit, there were no statistically significant differences between the serum and saliva levels of IFN-γ, caspase-1, and TNF-α in both groups. Since all of the patients with pSjS had low disease activity, we could not investigate a difference between pSjS patients with and without disease activity. In the RA group, serum and saliva levels of these three cytokines/enzymes were not significantly different between patients with and without active disease.

4. Discussion

In the literature, although there are studies investigating serum and saliva levels of various cytokines, including IL-1β, IFN-γ, and TNF-α, in patients with pSjS, studies focusing on saliva caspase-1 levels are remarkably rare. In the present study, we measured saliva and serum levels of caspase-1, TNF-α, and IFN-γ in patients with pSjS and compared them to both healthy controls and the RA group. Although there was no significant difference in serum measurements between the three groups, saliva levels of TNF-α and caspase-1 in the pSjS group were significantly higher than in both healthy controls and the RA group. However, saliva levels of these parameters in the pSjS group did not show any correlation with disease duration, ANA or RF positivity, or smoking. The patients included in the pSjS group were notable for having low disease activity.

Elevated saliva levels of TNF-α and caspase-1 found in the present study may support the well-known role of innate immunity in mucosal inflammation of pSjS, and might contribute to pathogenesis of pSjS by inducing apoptosis of epithelial cells in the oral and ocular mucosa (12). Elevated TNF-α saliva levels in pSjS were also reported in the literature (13,14). Kang et al. reported increased levels of saliva TNF-α in patients with pSjS compared to patients having only sicca symptoms (13). On the other hand, Zhou et al. addressed the importance of locally produced TNF-α in an experimental model, using NOD mice. They demonstrated that neutralization of TNF-α prior to disease onset impeded the onset and development of Sjögren-like salivary gland inflammation, as well as secretory function (15).

The data of significantly increased saliva levels of caspase-1 in pSjS are also in line with the literature. However, there are studies investigating the expression of the P2X7 receptor (P2X7 R)-NLRP3 inflammasome complex in the salivary glands (6,7), rather than measuring directly caspase-1 saliva levels in pSjS. The prominent components of this inflammasome complex are NLRP3, caspase-1, and IL-18, and this complex modulates the release of IL-1β and IL-18. Baldini et al. (6) found that the expressions of this inflammasome complex and caspase-1 were not only significantly higher in pSjS gland specimens compared to control groups, but also correlated with

Table 2. Saliva levels of IFN-γ, caspase-1, and TNF-α in patient groups and healthy controls.

Saliva	pSjS n: 43	RA n: 39	HC n: 27	Statistics P*
IFN-γ (pg/mL) Mean (min–max) ±SD	2.9 (0.4–13.5) ±3.5	4.5 (0.5–45.4) ±7.0	3.3 (1.9–9.4) ±1.7	0.306
Caspase-1 (ng/mL) Mean (min–max) ±SD	9.3 (2.8–16.1) ±2.3	7.7 (3.0–14.1) ±2.3	8.3 (5.4–10.9) ±1.7	0.005
TNF-α (pg/mL) Mean (min–max) ±SD	20.8 (3.0–88.2) ±16.9	6.0 (1.8–24.0) ±5.4	4.1 (2.1–8.6) ±1.7	0.0001

HC: Healthy controls.
*Kruskal–Wallis test.

anti-Ro/SSA positivity and with focus score. In another study, Baldini et al. (7) reported that salivary gland gene expressions of P2X7R, caspase-1, and IL-18 were strongly higher in patients with pSjS developing MALToma over the follow-up. In the literature, potential involvement of other inflammatory caspases in the pathogenesis of pSjS is also reported (16). Based upon studies performed in NOD autoimmune-prone mice, Bulosan et al. (16) showed upregulation of caspase-11 in macrophages, supporting the role of abnormal innate immunity in pSjS.

Given that saliva caspase-1 is found to be elevated in the present study and in previous studies reported in the literature, similar elevation in saliva levels of IL-1β is also expected. Although we could not investigate saliva IL-1β levels, increased concentrations of IL-1 in the salivary fluid and peripheral blood of patients with pSjS were reported by Willeke et al. (17). Furthermore, Dubost et al. (18) suggested that the salivary IL-1/IL-Ra imbalance might promote inflammatory lesions in the mouth. Muraki et al. (19) reported that IL-1β is involved in the destruction of salivary and lacrimal glands. In fact, IL-1β may have a proteolytic activity, leading to acinar and ductal structure disruption in the salivary glands of patients with pSjS.

In previous studies, saliva IFN-γ levels were found to be elevated and an IFN-γ signature was found to be present in patients with pSjS (13,14). Kang et al. (13) reported increased levels of saliva IFN-γ, IL-1, IL-6, and IL-10 levels in patients with pSjS compared to patients having only sicca symptoms. Fox et al. (14) also investigated saliva levels of those four cytokines in patients with pSjS using an ELISA assay. However, they used a different methodology; they collected saliva directly from Stensen's duct, i.e. the opening of the parotid gland. They found significantly elevated levels of all these cytokines compared to healthy controls. These investigators also studied mRNA expression of these cytokines in salivary gland biopsies using PCR and found higher levels (14).

The controversial finding of low saliva IFN-γ levels in the present study may be explained by low disease activity, as indicated by low ESSDAI scores. On the other hand, high IFN activity is not a rule in pSjS. In a previous study, the pattern of cytokine mRNA expression was studied in frozen minor salivary gland tissues from patients with pSjS. Interestingly, increased expression of IFN-γ mRNA was detected in only three out of 12 biopsy specimens (20). Similarly, Hall et al. (21) demonstrated high IFN activity in only 31 out of 53 pSjS patients and reported that high IFN activity was associated with a more severe disease phenotype in pSjS. The same authors also suggested that there are three different subgroups of patients with pSjS, based upon interferon activities: type I–predominant subgroup, type II–predominant subgroup, and mixed type I/II IFN activity subgroup (21). Therefore, our patients with low disease activity might have been in the type I–predominant subgroup.

Findings of elevated saliva levels of TNF-α and caspase-1 in the present study and of additional other cytokines reported in literature such as IL-1β and IFN-γ may have implications in tailoring local treatment for patients with pSjS. It may be speculated that systemic immunosuppressive treatment and achievement of low disease activity in pSjS may decrease systemic inflammatory cytokine and enzyme levels more readily compared to local mucosal microenvironment, which may be more resistant. The lack of differences in serum levels of some cytokines and enzymes despite significant differences in saliva levels, as found in the present study, might support this speculation. Data from a prospective double-blind randomized trial demonstrated that targeting IL-1 by topical application of anakinra is effective in reducing dry eye disease-related symptoms and corneal epitheliopathy (22). Likewise, although systemic anti-TNF treatment is ineffective and rather may cause exacerbation of Sjögren's activity (23,24), local TNF-α inhibition in oral mucosa may be effective, as suggested by previous experimental data (NOD mice) (15).

Our study has some limitations. First, the numbers of patients with pSjS and healthy controls were rather low. Additionally, immunosuppressive medications were not stopped before serum and saliva measurements were made, and all of the pSjS patients had displayed low disease activity when they were enrolled in this study. This might have also affected some of our results. On the other hand, we focused on individual time points to describe the cytokine profiles of the patients, although fluctuations in cytokine levels sometimes accompany disease flares or remissions. We also admit that measuring saliva levels of certain cytokines by ELISA method may not be as sensitive as PCR analysis. Finally, for technical reasons, IL-1β and IL-18 could not be studied in the present study.

In conclusion, despite low disease activity, saliva caspase-1 and TNF-α levels were found to be significantly higher in patients with pSjS compared to both healthy controls and the RA group. In selected cases with increased local mucosal production of certain proinflammatory cytokines, it may be an alternative approach to use local anticytokine treatments, rather than using systemic anticytokine treatments. Better understanding of the abnormalities in the cytokine network and their role in the pathogenesis of pSjS will likely lead to the identification of the best therapeutic targets for this disease.

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