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Comparison of salivary calmodulin binding proteins in Sjögren's syndrome and healthy individuals

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BACKGROUND: Reduction in salivary secretion is the hallmark of Sjögren's syndrome (SS). Calmodulin (CaM) and calmodulin binding proteins (CaMBPs) play a key role in the secretory process of saliva. Recent studies have suggested that SS-B, an autoantibody associated with SS, is a CaMBP. This finding suggests that CaMBP may contribute to the loss of saliva in SS. To better understand the role(s) of these proteins in SS, the purpose of this study was to compare salivary CaMBPs in Sjögren's patients and controls.

METHODS: Saliva samples were collected from 20 patients and 20 age-, race-, and gender-matched controls. CaM overlay was used to identify CaMBPs in saliva of patients and controls.

RESULTS: Higher number of salivary CaMBPs was observed among patients than controls.

CONCLUSIONS: The increased number of salivary CaMBPs in SS may suggest a potential role for these proteins in the pathogenesis of the disease.
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Introduction

Sjögren's syndrome (SS) is a chronic inflammatory disease of the exocrine glands that may be associated with extra-glandular involvement. There are two forms of SS, primary, and secondary. Primary SS affects mainly the exocrine glands whereas secondary SS is accompanied by another autoimmune disease or connective tissue disease. The etiology of the disease is not well understood; however, the inflammatory process is thought to be the primary cause of tissue damage and functional impairment of the exocrine glands (1).

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A definitive diagnosis of SS often depends on combined clinical and laboratory findings that include positive salivary gland biopsy and serum autoantibodies. The autoantibodies often associated with SS are antinuclear antibody (ANA), rheumatoid factor (RF), anti-Ro (SS-A), and anti-La (SS-B) antibodies (2). These auto-antibodies are used for the diagnosis of SS, but their involvement in the etiology of the disease remains unclear.

Reduction in salivary secretion is the hallmark of SS. The secretion of saliva is regulated by neurotransmitters, which exert their effects by modulating the concentration of calcium in the acinar cells. The salivary acini has several mechanisms that tightly regulate the levels of intracellular Ca^{2+} via ATP-dependent Ca^{2+} pumps, agonist sensitive Ca^{2+} pool (ER pump) (3), calmodulin (CaM), and protein kinases A, and C (4). CaM is a Ca^{2+}-binding protein which is a key component of Ca^{2+} second messenger system and it is involved in controlling many of the biochemical processes of the cells. CaM is a small acidic protein with a molecular weight of approximately 17 kDa and contains four Ca^{2+} domains. It typically binds zero, two, or four calcium ions, and binds and regulates different proteins in each state. The binding of Ca^{2+} to CaM induces a conformational change in the protein that allows CaM to bind with specific proteins known as calmodulin-binding proteins (CaMBPs); this binding is a key for cellular functions (5). There are over a 100 proteins that are known to bind CaM. Studies have suggested that La/SS-B, an autoantibody associated with SS, is a CaMBP. La/SS-B is an RNA-binding nuclear phosphoprotein that has been associated with autoimmune response in patients with systemic lupus erythematosus and SS (6). La/SS-B production was found to be upregulated in patients with primary SS and it is believed that these autoantibodies play a role in the inflammatory immune response within the salivary glands (7). The finding that La/SS-B is a CaMBP raises the question that CaMBPs may play a role in the pathogenesis of SS, and/or contribute to the observed reduction in salivary output in patients with SS. To better understand the role(s) of CaMBPs in SS, the

purpose of this study was to compare CaMBPs in saliva of patients with SS to that of healthy controls.

Materials and methods

A total of 40 individuals participated in this study; 20 had SS and 20 age-, sex-, and race-matched healthy controls. Of the SS individuals, there were four men and 16 women ranging in ages from 38 to 74 years (mean age: 58 ± 2.5 years) (Table 1). For healthy controls, there were four men and 16 women ranging in ages from 38 to 79 years (mean age: 58 ± 2.4 years) (Table 1).

The diagnosis of SS was based on the European Community Criteria for the Diagnosis of SS (2), which includes dry mouth, dry eyes, at least one positive autoantibody and/or a positive salivary gland biopsy with a focus score of at least one (50 lymphocytes per 4 mm^2). Each study participant completed an informed consent approved by the Institutional Review Board at Baylor College of Dentistry and filled out a questionnaire relative to symptoms associated with dry mouth and dry eyes. Exclusion criteria included patients with history of radiation for head and neck malignancies, amyloidosis, sarcoidosis, graft versus host disease, pre-existing lymphoma, and HIV/AIDS (acquired immunodeficiency syndrome) infection.

Salivary flow rate and total salivary protein

Stimulated human parotid saliva was collected using Carlson–Crittenden cup, as previously described (8). In brief, 2% citric acid (~200 μl) was applied to the dorsum of the tongue at 30-s intervals. Ten-minute samples were collected into chilled, pre-weighed microfuge tubes, and stored at 50°C, until used. Salivary flow rate was expressed as ml/min/gland. Total salivary protein was determined using the Bicinchoninic acid method according to the manufacturer instructions (Pierce, Rockford, IL, USA) with a modification to decrease the total volume used (9). Total protein was recorded as mg%.

SDS-PAGE and calmodulin overlay

Salivary proteins (20 μg) aliquots were separated on 10% SDS-PAGE at room temperature. Each gel contained parallel samples for patients and controls, and broad range (10–250) kDa molecular weight standards (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA) (Pierce) and calcineurin (Sigma–Aldrich, St Louis, MO, USA) served as negative and positive controls, respectively. Following gel electrophoresis, the proteins were transferred electrophoretically onto a 0.2 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) overnight at 20 V at 4°C. The blots were then blocked, with a blocking buffer (Tris Base/Ca–Mg (50 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM $CaCl_2$, and 50 mM $MgCl_2$) + 1% BSA), for 1 h at room temperature, followed by incubation with biotinylated CaM, according to the manufacturer's instructions, (Calbiochem, La Jolla, CA, USA) at 100 ng/ml in blocking buffer then for 2 h at room temperature. Blots were then washed with TBS/Ca–Mg buffer + 0.05% Tween 20 protein grade (Calbiochem) 2X for 10 min each. The blots were then incubated for 30 min with streptavidin, conjugated with alkaline phosphatase (Calbiochem), and washed with TBS/Ca–Mg, 3X for 15 min each. This was followed by 15 min incubation in substrate buffer (100 mM Tris, 100 mM NaCl, pH 9.0, 50 mM $MgCl_2$, 0.5 mM $CaCl_2$). Finally, CaMBPs were visualized by incubating the blots with 5-bromo-4-chloro-3-indolyphosphate/nitroblue tetrazolium (BCIP/NBT) for 20–30 min (Calbiochem) protected from light. All procedures were performed at room temperature. Blots were then washed extensively with distilled water and dried.

Statistical analyses

The non-parametric Mann–Whitney *U*-test was used to examine the differences in salivary flow rates, total salivary protein, and CaMBPs between patients and controls. Chi-square test was used to determine the frequency of oral and ocular symptoms, and CaMBPs among patients and controls. Spearman rank correlation was used to compare the symptoms and laboratory findings of Sjögren's individuals with the salivary proteins and CaMBPs on the gels and the blots. A level of significance was set at 0.05 to be considered statistically significant. (Stat View for Windows, SAS Institute, Inc., Version 5.0.1, Cary, NC, USA).

Table 1 Demographics of the study population for Sjögren's syndrome (SS) and healthy controls (HC)

Parameter	*SS*	*HC*
Age (years)*	57.8 ± 2.5	57.6 ± 2.4
Gender		
Female	16	16
Male	4	4
Race		
White	15	15
Hispanic	1	1
Black	3	3
Asian	1	1

*Mann–Whitney *U*-test $P = 0.87$.

Results

Demographics of the study population

All the SS patients had combined symptoms of dry mouth, dry eyes, reduced salivary flow rate, and at least one positive autoantibody and/or positive minor salivary gland biopsy (12 patients had positive ANA; 8 positive RF, 13 positive SS-A, and 9 positive SS-B). Nineteen of the SS group had positive minor salivary gland biopsy (18 of them had grade 4 biopsy and one had grade 3) (Table 2). One of the patients was unable to undergo minor salivary gland biopsy because of blood coagulation complications. Seventeen of the patients had primary SS and three had secondary SS (two with systemic lupus erythematosus and one with rheumatoid arthritis). None of the controls had combined symptoms of dry eyes and dry mouth. However, four of the controls had dry eyes and one had dry mouth (Table 2).

Table 2 Medical history and laboratory findings

	Sjögren's syndrome	*Healthy controls*
Symptoms		
Ocular	20	4
Oral	20	1
Joint	14	5
Muscle	10	3
Other[a]	17	10
Autoantibodies		
ANA	12	N/A
RF	8	N/A
SS-A	13	N/A
SS-B	9	N/A
Biopsy score[b]		
Score of 4	18	N/A
Score of 3	1	N/A

ANA, antibody antinuclear; N/A, not tested; RF, rheumatoid factor.
[a]Nose, ears, vagina, digestive tract, lungs, and skin.
[b]One participant could not have a biopsy because of blood coagulation complications.

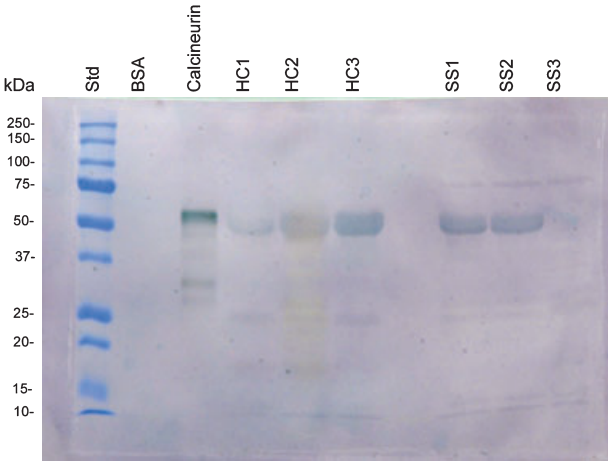

Figure 1 Calmodulin overlay. BSA, bovine serum albumin; HC, healthy controls #1–3; kDa, kiloDaltons; SS, Sjögren's syndrome patients #1–3; Std, molecular weight standard.

Salivary findings

The mean ± SEM salivary flow rate for SS patients was 0.24 ± 0.03 ml/min/gland (range 0.03–0.57 ml/min/gland) vs. 0.27 ± 0.04 ml/min/gland (range 0.07–0.71 ml/min/gland) for healthy controls. There was no statistically significant difference in salivary flow rate between these groups ($P = 0.66$) (Table 3). The mean ± SEM total salivary protein was 158 ± 18 mg% (range 80–430 mg%) for SS patients and 117 ± 13 mg% (range 60–310 mg%) for healthy controls. Significantly higher levels of total salivary protein were observed among SS patients than healthy controls ($P = 0.03$) (Table 3).

Calmodulin binding proteins

Similar number of CaMBPs was observed at approximately 13, 25, 40, and 60 kDa in both patients and controls. In addition, there was another CaMBP at approximately 90 kDa; this protein was present in 11(55%) of SS patients and only 2 (10%) of the controls. Statistical analyses revealed a significantly higher number of CaMBPs among SS patients than healthy controls ($P = 0.01$) (Fig. 1).

Spearman-rank correlation analysis showed a significant correlation between the number of CaMBPs and dry mouth. However, there was no correlation between the number of CaMBPs and other symptoms or any of the autoantibodies (ANA, RF, SS-A and SS-B).

Table 3 Salivary flow rate and protein concentration between Sjögren's syndrome (SS) and healthy controls (HC)

	Mean ± SEM		
Parameters	*SS*	*HC*	*P**
Flow rate (ml/min/gland)	0.24 ± 0.03	0.27 ± 0.04	0.66
Protein (mg%)	158.00 ± 18.00	117.25 ± 13.84	0.03

*Mann–Whitney *U*-test.

Discussion

Sjögren's syndrome is a chronic inflammatory disease of the exocrine glands that causes damage of the exocrine glands and reduction in their secretion. Previous studies, from our laboratory, have shown that damage of the salivary glands is associated with increased levels of CaM (10). The intracellular roles of CaM are modulated through its interaction with CaMBPs. Previous studies have shown that CaMBPs are present in extracellular fluid including saliva (5). The purpose of this study was to examine CaMBPs in saliva of SS patients and healthy controls.

Damage of the salivary glands is associated with the reduction in their secretion. It has been estimated that the median salivary flow rate of healthy adults is approximately 0.48 ml/min (range 0.1–2 ml/min) (11). A reduction in salivary flow rate to approximately 0.1 ml/min is often associated with dry mouth (12). By definition, patients with SS have dry mouth and they have reduced salivary output. In this study, there was no significant difference in salivary flow rate between patients and controls (Table 3). It is important to point out that the salivary flow rate of controls was lower than normal. The observed low salivary flow rate among controls could be because of age. Many of the individuals in this study were elderly patients and studies have suggested that salivary flow rate is decreased with increasing age 65–85 years (13).

Both total salivary protein and the number of CaMBPs were significantly higher among patients than controls. Both patients and controls had four CaMBPs that were consistent on all of the overlays.

Five salivary CaMBPs were identified in this study. Four (~13, 25, 40, and 60 kDa) were consistently present in both patients and controls (Fig. 1); three of them (13, 25, and 60 kDa) corresponded closely with previously reported salivary CaMBPs (5). However, the other salivary CaMBPs (~ 90 kDa) was more prevalent among the patients group. The 90 kDa was observed in

majority of the Sjögren's patients but only two of the controls. Results of Western blot analyses suggest that the 90 kDa protein is lactoferrin (data not shown). Lactoferrin is a normal component of saliva but its level is increased in patients with SS (14). Therefore, the absence of the 90 kDa in the control saliva may be because of the low sensitivity of the overlay technic to detect small amount of lactoferrin. To our knowledge, this is the first study that shows lactoferrin as a CaMBP.

In conclusion, this is the first study to examine salivary CaMBPs in SS. Our results suggest that there are higher numbers of CaMBPs in SS patients than healthy controls. Specifically, the 90 kDa CaMBP was present at higher frequency among SS patients as compared with healthy controls. The role of CaMBPs in the pathogenesis of the disease remains unclear.

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