

Assessment of serum and salivary defensin levels in primary Sjögren's syndrome: a preliminary report

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Abstract

Introduction: Defensins are described as antimicrobial peptides and innate immunity mediators. Their levels may be an important anti-inflammatory determinant in autoimmune diseases including Sjögren's syndrome (SS).

Aim of the research: The purpose of the study was to assess and compare human β -defensin-1 (hBD-1) levels in serum and saliva in patients with SS and healthy subjects.

Material and methods: The study group was composed of 20 SS patients and 14 healthy subjects (HS). Serum and parotid salivary hBD-1 levels were measured using the ELISA kit. The patients' health assessment included medical history and physical examination such as Schirmer's test. For xerostomia assessment all patients completed the visual analogue scale (VAS). Routine laboratory tests in the SS group included measurement of erythrocyte sedimentation rate and detection of antinuclear antibodies.

Results: There were no statistically significant differences in either serum or salivary hBD-1 levels between HS and SS patients. We observed a statistically significant difference between serum and salivary hBD-1 levels in SS patients but not in the HS group. Neither serum nor salivary concentrations of hBD-1 significantly correlated with the age of SS patients, disease duration, erythrocyte sedimentation rate, and xerostomia and dry eye severity.

Conclusions: Further studies on a larger group of SS patients are needed to assess whether hBD-1 contributes to both SS pathogenesis and course.

Introduction

Defensins are antimicrobial peptides divided into three subfamilies α -, β -, θ -defensins [1]. The dual function of defensins including a direct broad antimicrobial activity and an immunomodulatory effect suggests their potential contribution to the pathogenesis of autoimmune diseases such as Sjögren's syndrome (SS) [2–4]. Human β -defensins (hBDs) are expressed mainly by epithelial cells and keratinocytes [2, 4]. Their expression was detected in ductal epithelial cells of salivary glands, tongue and oral mucosa [5–8]. Thus, hBDs may play a vital role in the innate defence against oral microorganisms and protect salivary glands from retrograde infection [7]. Expression of hBDs in salivary glands justifies the choice of salivary hBD levels to assess autoimmune diseases that affect salivary glands and predispose to local oral infections, like SS. SS is characterized by periductal lymphocytic infiltrates in the salivary and lacrimal glands, leading to xerostomia and xerophthalmia, which predominate in SS [9, 10]. Lack of saliva and its antibacterial properties results in predisposition

to opportunistic infections [11]. Higher risk of infections in the oral cavity in pSS patients may result not only from hyposalivation but also qualitative changes of salivary antimicrobial peptides [2]. These findings suggest that defensins may be an important component of the local defence mechanism in pSS patients.

Although pSS is characterized by local changes in the exocrine glands, it may give systemic symptoms resulting from the pathomechanism referred to as autoimmune epithelitis. Therefore, serum defensin levels may be helpful in assessing systemic changes brought about in SS. Epithelial damage and subsequent apoptosis may cause a breakdown of epithelium homeostasis and full-blown SS manifestations. Some viruses that display strong tropism to salivary glands such as the Epstein-Barr virus, cytomegalovirus, and herpes simplex virus may trigger the initial damage of the glandular epithelium and subsequent immunological processes and formation of infiltrates in the involved exocrine glands [12, 13]. Defensins in pSS patients are able to induce IFN-stimulated genes and play a role in local antiviral defence [2]. Their

potential increase in salivary glands in pSS may support the hypothesis that viruses are one of the most likely underlying factors driving to SS development. Therefore, defensin levels in pSS patients compared to healthy subjects are worth analysing, considering their immunological properties.

Aim of the research

The aim of the study was to assess and compare serum and saliva hBD-1 levels in SS patients and healthy subjects and to explore possible correlations between serum and saliva hBD-1 levels and selected SS laboratory and clinical parameters.

Material and methods

Study groups

We enrolled in this pilot study 20 consecutive women with SS (mean age: 48.2 ± 14.9) who fulfilled the 2016 ACR-EULAR Classification Criteria for pSS [14]. The patients were recruited from the Department of Rheumatology and Clinical Immunology at Poznan University of Medical Sciences, Poland. Fourteen age- and gender-matched healthy subjects from the Regional Center of Blood Donation and Blood Treatment in Poznan formed the control group (mean age: 50.4 ± 10.1). Exclusion criteria included the presence of IgG4 syndrome, previous radiotherapy to the head and neck, lymphoma, sarcoidosis, graft-versus-host disease, infections of the hepatitis C virus, human T-lymphotropic virus type I and HIV.

Ethical issue

The protocol of this study was approved by the Institutional Review Board at Poznan University of Medical Sciences (number 211/13) and informed consent had been obtained from all subjects before any procedure was carried out. This study was performed in accordance with an appropriate version of the World Medical Association Declaration of Helsinki.

Patient examination

The patients' health assessment comprised complete medical history and physical examination including assessment of xerostomia and dry eye and determination of any oral symptoms. To assess xerostomia all patients completed visual analogue scale (VAS) as presented [15]. Moreover, unstimulated whole saliva flow rate (USWSF) was measured in eleven patients as described [16]. Volumes of ≤ 1.5 ml/15 min in USWSF were marked as abnormal saliva secretion and as a diagnostic criterion of xerostomia. The Schirmer's test was carried out to assess the severity of dry eye as presented [15]. Lacrimation in Schirmer's test ≤ 5 mm in one eye was marked as abnormal tear secretion and a positive result of Schirmer's test.

Blood and saliva sampling

Peripheral blood samples were collected from the antecubital vein in BD Vacutainer Rapid Serum Tubes (Becton, Dickinson and Company, Franklin Lakes, USA). After 5 min, clots were removed by centrifugation at 4000 rpm for 15 min at room temperature. Parotid saliva was collected directly from the parotid gland by opening with Lashley cups into Eppendorf tubes after stimulation with 3% citric acid [17]. Parotid saliva samples were collected from all patients between 8:00 and 10:00 a.m. Two hours before sampling, patients and healthy controls refrained from eating, drinking, mouth rinsing, and teeth brushing. The sera and parotid saliva samples had been stored at -20°C before estimations were made.

Laboratory tests and research methods

Routine laboratory tests included erythrocyte sedimentation rate (ESR, Westergren method), and the detection of antinuclear antibodies (ANA) by indirect immunofluorescence on HEp-20-10 cells (Euroimmun, Germany) along with their specification using the blot type test (Euroimmun, Germany) in case of ANA positivity. Serum and salivary concentrations of hBD-1 were measured using the ELISA kit (GenWay Biotech, Inc., USA) and calculated using standard curves generated with specific standards according to the manufacturer's recommendations.

Statistical analysis

The statistical analysis was made using Statistica version 13 software (StatSoft Inc.). Patients' demographic data were analysed using descriptive statistics. The contiguous data were tested for normal distribution using the Kolmogorov-Smirnov test. For normally distributed data, results were presented as mean $\pm$ standard deviation (SD), and non-normally distributed data were expressed as a median (interquartile range – IQR). The differences between the groups were tested using the Mann-Whitney *U* test. The Spearman's rank correlation analysis was used to find the associations between serum and saliva hBD-1 levels and other laboratory and clinical parameters of SS activity. The differences were considered to be statistically significant at $p < 0.05$.

Results

Antinuclear antibodies were detected in 15 (88.2%) pSS patients. A detailed analysis of the antinuclear antibodies profile showed that anti-Ro/SSA antibodies and anti-Ro52 antibodies were detected in 18 (90%) and 19 (95%) pSS patients, respectively. Anti-La/SSB antibodies were found in 11 (55%) patients with pSS. Predominant symptoms and organ involvement in pSS patients was arthritis (50%) found in 10 subjects, followed by lymph-

adenopathy (25%) and cutaneous symptoms (25%) revealed in 3 pSS patients. Raynaud's phenomenon and generalized weakness were observed in 3 (15%) pSS patients. Peripheral nervous system involvement, pulmonary involvement and weight loss were detected in 3 (15%) patients, 2 (10%) patients and 2 (10%) patients, respectively. Either mild fever or sweating was diagnosed in 1 pSS patient. The Schirmer's test was positive in 7 pSS subjects (35%). The detailed analysis of the oral symptoms revealed that 8 patients with pSS reported dysphagia (40%), 4 reported cheilitis simplex (20%), 2 reported aphthae (10%) and 1 patient reported cheilitis angularis (5%). The pSS group included 2 current smokers (10%). The mean unstimulated whole salivary flow (USWSF) in the pSS group was 0.5 ml/15 min (SD = 0.5 ml/15 min). The median of Erythrocyte Sedimentation Rate (ESR) in the pSS group was 28 mm/h (interquartile range (IQR) = 35 mm/h). The disease duration in the pSS group was 5 years (IQR = 8 years). The median of xerostomia assessed using Visual Analogue Scale in the pSS group was 52 mm (IQR = 53 mm). The serum level of hBD-1 in patients with pSS was 13.4 ng/ml (IQR = 9.7 ng/ml). The serum level of hBD-1 in the control group was 14.9 (IQR = 16.1 ng/ml). The salivary level of hBD-1 in patients with pSS was 7.2 ng/ml (IQR = 7.3 ng/ml) and salivary level of hBD-1 in healthy subjects was 10.8 ng/ml (IQR = 15.5 ng/ml).

There were no statistically significant differences between the controls and SS patients according to serum or saliva hBD-1 concentration ($p = 0.12$, $p = 0.47$, respectively, Figure 1). However, we observed a statistically significant difference between serum and saliva levels of hBD-1 in SS patients ($p < 0.01$, Figure 1), but not in the control group ($p = 0.15$). The comparison of serum and salivary levels of hBD-1 between the SS group and the control group is presented in Figure 1. Neither serum nor saliva concentrations of hBD-1 significantly correlated with the age of SS patients, disease duration, ESR, and the severity of xerostomia.

Discussion

Data regarding the role of defensins in SS are scarce. The presence of hBD-2 in the saliva of pSS patients following pilocarpine treatment indicated that defensins may represent markers of inflammation in SS [18]. It is a quite common and universal relationship in many autoimmune and inflammatory diseases [19–21]. It seems that α -defensins are less SS-specific than β -defensins that are released mainly by salivary glands and ductal epithelial cells. The main sources of hBDs are salivary glands and epithelial cells that are involved in the pathology of SS [20, 21]. A study of the expression profile of human defensins in oral tissues suggested an important role for hBD-1 and hBD-2 in the innate oral epithelial host defense [22, 23]. Moreover, the results of a study of global gene expression patterns in conjunctival

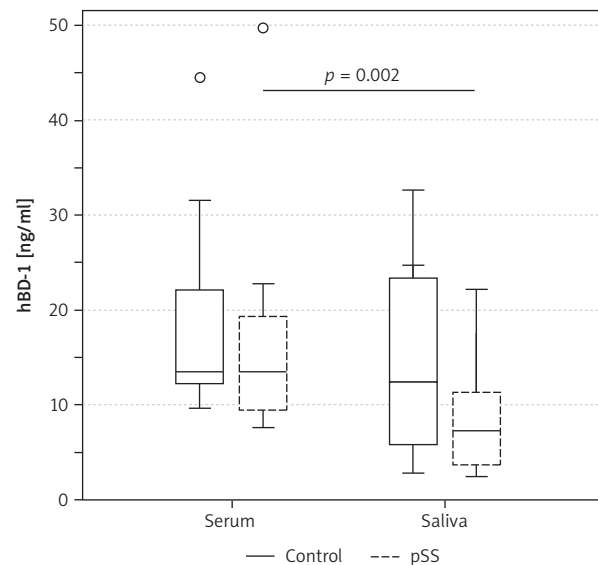


Figure 1. The comparison of serum and salivary levels of β -defensin-1 (BD-1) in SS patients and the controls

epithelial cells from SS patients showed up-regulation of hBD-2 when compared to healthy controls [24]. The origin of hBD-1 and hBD-2 justifies their use in the assessment of patients with salivary and lacrimal glands involvement, such as SS patients. In the present study we assessed hBD-1 concentrations in pSS patients both in serum and in saliva and we found significantly higher levels of hBD-1 in serum than in saliva of the SS patients. The finding could be a result of the complex nature of SS as it was shown that the gene for hBD-1 is up- or down regulated by several mediators associated with inflammation [25]. Moreover, the limitations associated with saliva collection and the masking effect of other salivary antimicrobial proteins on the level of β -defensins should be noted. Sahasrabudhe *et al.* observed that the salivary hBD-1 level may be present but undetectable by the dot-blot assay due to the masking effect of salivary mucins [26]. Mucins can interact with hBD-1 and form complexes that enhance their antimicrobial activity but worsen sole detection of defensins in saliva [26]. It could be partially the reason for decreased salivary hBD-1 concentrations in the SS patients in comparison to the serum hBD-1 levels. Moreover, in our study there were no differences in serum hBD-1 levels between pSS patients and the control group. It agrees with the results of a recent study in another autoimmune disease, namely systemic sclerosis (SSc) [27], where no difference in serum hBD-1 and hBD-2 levels in SSc patients compared to healthy subjects has been found. Our observations could also result from local predominance of pathological changes in pSS limited to exocrine glands. The hBDs are released from epithelial ductal cells and keratinocytes and they act locally to stop invading bacteria, viruses and

fungi, and the limited systemic organ involvement in pSS patients was reflected by the lack of changes in the serum level of hBD-1 in pSS patients compared to healthy subjects. Previous studies showed a strong correlation of immunohistochemical hBD-1 staining and inflammation in salivary glands [25]. Moreover, hBD-1 expression was the strongest in salivary ducts with periductal inflammation [27]. Sahasrabudhe *et al.* postulated that immunolocalization of hBD-1 near the lumen in the duct cells provides likely protection against retrograde infections in the oral cavity [26]. The expression levels of defensins were higher in the inflamed salivary glands and inflamed oral epithelial cells when compared with the non-inflamed specimens [28]. Furthermore, it is suggested that the differences in hBD-1 and hBD-2 expression patterns in minor salivary glands in SS patients result from varying disease progression and duration [29]. Kaneda *et al.* showed that the decreased expression of hBD-2 occurred faster than that of hBD-1 and the decrease in hBD-2 expression in minor salivary glands might begin before infiltration of lymphocytes around ductal cells in SS subjects [29]. It seems that serum hBD-1 plays a limited role as a systemic biomarker specific to pSS and accompanying inflammation. However, based on the literature it may play a function of a local biomarker related to exocrine glands. These observations are consistent with Yamane *et al.* who revealed that even SS-specific proteins present in saliva are not detected in serum samples [30]. There was a little overlap of immune complexes formed by specific proteins between salivary and serum samples, suggesting that many immune complexes are formed locally and independently of the circulation [30]. Detection of these proteins in saliva and not in serum resulted from SS pathogenesis. For comprehensive assessment of hBD-1 and its impact on pSS it is necessary to explore a possible correlation between hBD-1 expression in salivary glands and severity of local infiltration in involved salivary glands.

Although there were no differences in salivary hBD-1 levels between pSS patients and healthy controls, decreased salivary hBD-1 levels in pSS in comparison to serum hBD-1 levels indicate that pSS patients may be more prone to local bacterial infections involving the oral cavity. Moreover, no correlations between hBD-1 levels and selected clinical and laboratory parameters indicate that hBD-1 is involved mainly in local defense mechanisms. The differences between serum and saliva in pSS patients may be explained by the findings obtained by Takeshita *et al.* who revealed that the bronchoalveolar fluid in SS patients contains higher titers of disease-related autoantibodies than serum [31]. Salivary glands are the sites of autoimmunity in SS and this fact can be reflected in decreased salivary hBD-1 levels in pSS in comparison to serum hBD-1 levels.

Limitations of this project arise from the small study group. Thus, it should be considered a pilot study. Our findings need to be verified in a larger SS population. We sampled parotid saliva to reduce potential disturbing impact of oral hygiene and local infections on the salivary level of defensins. However, this kind of impact cannot be definitively excluded. Moreover, other potential differences resulting from various saliva sampling methods and differences between stimulated and unstimulated saliva should be considered. Another limitation of the study was the lack of consideration of the role of polymorphisms in the gene encoding for human β -defensins on their serum levels in autoimmune diseases.

Conclusions

To determine whether hBD-1 contributes to the pathogenesis and the course of SS, further studies on a larger group of patients and detailed molecular tests, preferably on a tissue level, are needed.

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Ethical approval

The protocol of this study was approved by the Institutional Review Board at Poznan University of Medical Sciences (number 211/13).

Conflict of interest

The authors declare no conflict of interest.

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