Abstract

Background. Aberrant angiogenesis plays a role in the pathogenesis of Sjögren’s syndrome (SS).

Objectives. The aim of this study was to compare the levels of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in stimulatory parotid saliva and in serum in healthy subjects (HS), patients with primary SS (pSS) and secondary SS (sSS) and to evaluate the expression of EGF, proangiogenic VEGF165 and antangiogenic VEGF165b mRNA isoforms. Additionally, we determined the salivary levels of serine/arginine splicing factor (SRSF1), which regulates VEGF165 and VEGF165b expression.

Material and methods. The study comprised 34 women (16 with pSS and 18 with sSS) and healthy subjects for blood and saliva sampling. EGF and VEGF levels in saliva and serum and salivary SRSF1 levels were determined by enzyme-linked immunosorbent assay (ELISA). The expression of VEGF165, VEGF165b and EGF in peripheral blood mononuclear cells (PBMC) was evaluated by quantitative polymerase chain reaction (qPCR).

Results. There were no differences in the levels of EGF, VEGF, SRSF1 and in the expression of the EGF, VEGF165 and VEGF165b between HS and SS patients, or between pSS and sSS patients. The salivary levels of VEGF165 and EGF were significantly higher in pSS, sSS and HS than serum levels. Levels of SRSF1 correlated positively with VEGF and EGF levels. Levels of EGF, VEGF and SRSF1 correlated with each other.

Conclusions. The balance of VEGF isoforms is not disturbed in SS. Saliva is more sensitive for the detection of EGF and VEGF than serum, but salivary levels of those proteins are not representative for SS.

Key words: Sjögren’s syndrome, angiogenesis, vascular endothelial growth factor, epidermal growth factor
Introduction

Sjögren’s syndrome (SS) is a systemic autoimmune disease characterized by periductal mononuclear cell infiltrate in the salivary and lachrymal glands, autoimmune-injuries to endothelial cells and their subsequent apoptosis.1,2 Inflammatory cells secrete growth factors, proteases and cytokines that induce extracellular matrix degradation, endothelial cell growth and migration promoting angiogenesis. Neovessels may contribute to the perpetuation of inflammation by recruiting more inflammatory cells to the inflammation site. Angiogenic factors induce endothelial cells to express adhesive molecules, cytokines and chemokines, which have additional stimulatory effects on chronic inflammation.3

One of the major proangiogenic regulators is vascular endothelial growth factor (VEGF). It promotes the migration of inflammatory cells into the extracellular matrix by inducing vascular permeability and endothelial cell expression of adhesion molecules, of which elevated levels are observed in SS.3 VEGF exerts its biological function by binding to its receptors: VEGFR-1, VEGFR-2 and VEGFR-3.4,5 The main isoform VEGF165 has 165 amino acids in the mature structure. Two families of VEGF proteins are formed by an alternative splice-acceptor-site to give to 2 distinctive C-terminal sequences differing in their angiogenic properties.6–8 These 2 isoforms bind to the VEGFR-2 with the same affinity, but the binding of VEGF165b results in an insufficient tyrosine phosphorylation/activation of VEGF-2 and incomplete or transient downstream signaling, which leads to an impaired angiogenic response.6,9 The isoforms VEGF165 and VEGF165b account for a substantial proportion of the total VEGF.9 The balance of the VEGFxxx (proangiogenic) and VEGFxxxb (antiangiogenic) families may have a crucial role in controlling angiogenesis in health; an imbalance can underpin pathological angiogenesis. Primarily, the influence of VEGF165 and VEGF165b on cancerogenesis was examined.4,10–12 Similar pathomechanisms between pro- and antiangiogenic also seem to occur in autoimmune diseases and coexist chronic inflammation influencing their development by stimulating or inhibiting angiogenesis. The mechanism regulating the expression of pro- and antiangiogenic isoforms of VEGF is not known. Many growth factors and other proteins can change the proportion of VEGF165 and VEGF165b and regulate alternative splicing, e.g., insulin-like growth factor 1 (IGF-1), transforming growth factor alpha 1 (TGF-α1), transforming growth factor beta 1 (TGF-β1) and its co-regulator-serine-rich protein splicing factor 1 (SRSF1).9 The serine/arginine rich proteins regulate binding to exon-splicing enhancers and silencers and intronic enhancers and silencers in pre-mRNA. Exon splicing depends on the balance of splicing factors activities.

So far, most studies have focused on the serum levels of VEGF and epidermal growth factor (EGF) and their local expression in salivary gland biopsy specimens in SS patients. Therefore, this is also the first trial assessing the possible role of VEGF splice variants: VEGF165 and VEGF165b and SRSF1 in SS. In SS, pathological changes are more advanced locally in comparison to systemic ones. Proinflammatory cytokine production is disordered in salivary glands as well as in peripheral blood. In SS patients there are particular differences between leukocytes in the peripheral blood and in the salivary glands.13

The aim of this study was to compare the levels of VEGF and EGF in stimulated parotid saliva and in serum in patients with primary SS (pSS) and secondary SS (sSS) and in healthy subjects (HS), as well as to evaluate the expression of EGF and isoforms VEGF165 and VEGF165b in PBMC in pSS, sSS and HS. Furthermore, the levels of SRSF1 in parotid saliva were compared.

Material and methods

Study groups

The study comprised 34 women with SS (16 with pSS and 18 with sSS) aged 41.5 (interquartile ranges (IQR): 28.5) in pSS group and 56.0 (IQR: 21.0) in sSS group, respectively (Table 1), fulfilling the 2002 American-European Consensus Group (AECG) classification criteria. Our study diagnosis of pSS required 4 out of 6 criteria, including the presence of the antibody to SS-A/SS-B. The diagnosis of sSS has not yet been addressed by the AECG. In practice, we required the patients to fulfill the criteria for pSS and also to meet the American College of Rheumatology (ACR) criteria for an established connective-tissue disease (CTD), such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD).1,2,14 Patients were recruited consecutively in 2013 from the Department of Rheumatology and Clinical Immunology. Exclusions to the diagnosis of SS included previous radiotherapy to the head and neck, lymphoma, sarcoidosis, graft-versus-host disease, infections with hepatitis C virus (HCV), human T-cell lymphotropic virus type 1 (HTLV-1) and human immunodeficiency virus (HIV). The patient’s history was taken and physical and dental examinations were performed in the Department of Oral Surgery at Poznan University of Medical Sciences by dentist for each subject. Laboratory assessments included routine measurements of erythrocyte sedimentation rate (ESR) (Westergren method)15 and detection of antinuclear antibodies (ANA) by indirect immunofluorescence (IIF) on HEP-20-10 cells (Euroimmun, Lubeck, Germany) and their differentiation using ANA Profile3 (Euroimmun, Lubeck, Germany). Xerostomia (assessed by patients) was measured by a visual analogue scale (VAS) and Fox’s test. To assess ocular sicca symptoms, Schirmer’s test was carried out. Both eyes were tested at the same time. Schirmer’s test was performed without anesthesia before the procedure. Special paper strips (Alcon Laboratory, Fort Worth, USA)
Blood and saliva sampling

Peripheral blood samples were collected from the antecubital vein into BD Vacutainer Rapid Serum Tubes (Becton; Dickinson and Company, New York, USA). Peripheral blood mononuclear cells (PBMC) were isolated from 3 mL of fresh EDTA whole blood with the use of Lymphocyte Separation Medium 1077 (PAA, Pasching, Austria), in accordance with the manufacturer’s protocol. Next, the cells were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, USA).

Parotid saliva was collected directly from the parotid gland opening with Lashley cups into Eppendorf tubes after stimulation with 3% citric acid. Gauze swabs soaked with citric acid were put on the tongue every minute. Saliva sampling lasted as long as was needed to collect 2 mL saliva. Saliva was not centrifuged according to the instruction recommended in the applied tests. We did not add any proteinase inhibitors. About 2 h before sampling, the patients refrained from eating, drinking, rinsing their mouths and brushing their teeth. The saliva, sera and lysed PBMC were stored at −70°C.

RNA extraction, reverse transcription and quantitative polymerase chain reaction

Extraction of RNA, reverse transcription and quantitative polymerase chain reaction (qPCR) were carried out as described earlier.\textsuperscript{18} The sequences used for EGF amplification were: F: 5’CCTGATGGGAAACGATGTC3’, R: 5’GTGAGGAAACAACCGCTAC3’. The sequences for VEGF\textsubscript{165} and VEGF\textsubscript{165,b} were the same as described previously.\textsuperscript{19}

Measurement of studied proteins

Circulating EGF and VEGF\textsubscript{165} levels in the saliva and serum were determined by ELISA (R&D Systems, Minneapolis, USA) with a sensitivity of less than 5.0 pg/mL for VEGF\textsubscript{165} and 0.7 pg/mL for EGF. Soluble SRSF1 levels in parotid saliva were determined by ELISA (EIAab SCIENCE, Wuhan, China) with a sensitivity of less than 0.078 ng/mL. Absorbance was measured with a ELx800 96-well Microplate Reader and KC junior 1.11 (Bio-Tek Instruments, Vermont, USA).

Statistical analysis

The calculations were carried out with Microsoft Excel 2010 and STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The distributions obtained at each step of data processing were evaluated for normality using the Shapiro-Wilk test. Depending on the number of groups analyzed, the differences between them were tested using the Mann-Whitney U test or the Kruskal-Wallis one-way analysis of variance (ANOVA) by ranks, followed by post hoc multiple comparisons of the mean ranks. Spearman’s rank correlation analysis was used to find the associations between the levels of selected cytokines and other laboratory and clinical parameters of SS activity. All the data is expressed as medians with interquartile ranges (IQR). Differences were considered to be statistically significant at p < 0.05.

Results

Demography

All the subjects were of Caucasian origin. Table 1 and 2 present a demographic, laboratory and clinical profile of SS patients. The median age of the HS group for serum sampling (14 women and 1 man) was 49 (IQR: 14). The median age of the control group for saliva sampling (10 women and 5 men) was 23 (IQR: 5). The correlation between VEGF levels and age was studied in cancerogenesis. The VEGF expression was not correlated with age in patients with colon cancer.\textsuperscript{20} There were no differences in VEGF levels between the young control group and the elder group with prostate patients.\textsuperscript{21} There was no correlation between the age of healthy people without immunological, inflammatory and neoplastic disease and the VEGF concentration.\textsuperscript{22} The main criterion to classify patients to the control group was the elimination of the possible role of inflammatory, immunological and other diseases and drugs on the VEGF levels.
Expression levels of VEGF, EGF, SRSF1, and transcript levels of \( VEGF_{165} \), \( VEGF_{165b} \) and \( EGF \) in pSS, sSS and HS levels of VEGF and EGF in saliva were significantly higher than in serum. There were no differences between the pSS and sSS group for EGF and VEGF levels. There was also no significant difference in salivary SRSF1 concentration between pSS, sSS and HS. \( VEGF_{165} \), \( VEGF_{165b} \) and \( EGF \) mRNA expression in pSS patients, sSS patients and HS were at similar levels. Detailed results are presented in Table 3.

There was a statistically significant correlation between VEGF and EGF serum levels in SS patients (Fig. 1). The levels of SRSF1 in parotid saliva correlated positively with the salivary levels of VEGF and EGF (Fig. 2). No statistically significant correlations were found between the gene expression and the other SS activity parameters.

### Discussion

VEGF contributes to the pathomechanism of SS in many aspects. Recent studies demonstrated a close interplay between VEGF and its receptors in various autoimmune diseases, including SLE, RA, and multiple sclerosis. Increased levels of VEGF and VEGFR-1 in plasma were detected in patients with active SLE as compared to the levels in patients with inactive disease and in controls. Moreover, VEGF was overexpressed in the skin in systemic sclerosis (SSc) and its increased levels correlated with the severity of nailfold capillary loss in SSc. Considerable amounts of VEGF in both glandular epithelia and inflammatory cells of inflamed glands in SS patients as compared with those from HS were observed. Additionally, an immunohistochemistry examination revealed a strongly positive staining for VEGF and

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**Table 1. Characteristics of SS patients**

| Clinical and laboratory data       | pSS (n = 16) | sSS (n = 18) |
|-----------------------------------|-------------|-------------|
| Age (years)                       | 41.5 (28.5) | 56.0 (21.0) |
| BMI [kg/cm²]                      | 24.65 (76.0)| 23.55 (5.30)|
| Disease duration (years)          | 4.5 (4)     | 3 (9)       |
| RA, n [%]                         | –           | 7 (38.89)   |
| SLE, n [%]                        | –           | 4 (22.20)   |
| Others, n [%]                     | –           | 7 (38.89)   |
| Westergen ESR [mm/h]              | 23.5 (31)   | 19 (15)     |
| ANA titer >1:160, n [%]           | 11 (68.75)  | 15 (83.33)  |
| SS-A, n [%]                       | 10 (62.50)  | 10 (55.55)  |
| SS-B, n [%]                       | 7 (43.75)   | 4 (22.22)   |
| Ro-52                             | 10 (62.50)  | 11 (61.11)  |
| dsDNA                             | 0           | 3 (16.67)   |
| Sm                                | 0           | 1 (5.55)    |
| PCNA                              | 0           | 1 (5.55)    |
| Ribosomal-P-protein               | 0           | 3 (16.67)   |
| Centromeres B                     | 0           | 1 (5.55)    |
| PM-ScI                            | 0           | 1 (5.55)    |
| Histones                          | 0           | 1 (5.55)    |
| Nucleosomes                       | 0           | 1 (5.55)    |
| RNP                               | 0           | 1 (5.55)    |

**Organ involvement, n [%]**

| Articular                         | 11 (68.75)  | 11 (61.11)  |
| Peripheral nervous system         | 5 (31.25)   | 3 (16.67)   |
| Cutaneous                         | 4 (25.00)   | 3 (16.67)   |
| Pulmonary                         | 4 (25.00)   | 1 (5.55)    |
| Lymphadenopathy                   | 3 (18.75)   | 2 (11.11)   |
| Glandular                         | 1 (6.25)    | 1 (6.25)    |

**Current treatment, n [%]**

| MTX                              | 0           | 5 (27.78)   |
| NSAID                            | 4 (25.00)   | 4 (22.22)   |
| Methylprednisolone               | 4 (25.00)   | 6 (33.33)   |

**Table 2. Oral and ocular characteristics of SS patients**

| SS patients (n = 34) | pSS (n = 16) | sSS (n = 18) |
|----------------------|-------------|-------------|
| PtXer-VAS [mm]       | 46.5 (52.5) | 28.0 (47.0) |
| Fox’s test score [%] | 45 (40)     | 55 (30)     |

**Oral symptoms, n [%]**

| Dysphagia             | 4 (25.0)    | 6 (33.33)   |
| Xerostomia            | 9 (56.25)   | 12 (66.67)  |

**Ocular symptoms**

| Schirmer’s test [mm] (pSS n = 11, sSS n = 12) | 4 (8) | 10.75 (12) |
| Dryness of the eyes (subjective assessment), n [%] | 11 (68.75) | 13 (72.22) |

pSS – primary Sjögren’s syndrome; sSS – secondary Sjögren’s syndrome; BMI – body mass index; MTX – methotrexate; NSAID – nonsteroidal anti-inflammatory drugs; RA – rheumatoid arthritis; SLE – systemic lupus erythematosus; ESR – erythrocyte sedimentation rate. Unless otherwise stated, data is expressed as median (IQR).
VEGFR-2 proteins in the biopsy specimens from SS. Furthermore, anti-Ro/SS-A antibodies enhanced VEGF expression in SS salivary glands biopsy specimens.\textsuperscript{26,27} We obtained contradictory results to the previous studies, because even sSS patients with SLE or RA had no increased levels of VEGF when compared to the controls. Levels of VEGF in parotid saliva were elevated compared to serum levels. This means that the increased levels of VEGF in salivary gland specimens are not reflected in their elevated serum levels. Levels of VEGF and EGF cannot be diagnostic parameters in SS.

Our results confirm that serum and salivary levels of VEGF, EGF as well as PBMC expression of VEGF\textsubscript{165} and VEGF\textsubscript{165b} are independent of the type of syndrome and other connective tissue diseases in sSS. In recent studies comparing pSS and sSS, it was found that the intensity and frequency of some symptoms can vary. Similar clinical, serological and histological features with the exception of perivascular infiltrates in the salivary gland biopsies suggest that pSS and sSS are of the same entity.\textsuperscript{28,29} In previous studies, different levels of VEGF and its receptors in SSc and SLE were observed. Ambiguous results can arise from the different classification of SSS.\textsuperscript{28} In our opinion, vasculitis and vasculitic changes are more important issues which should be taken into consideration when VEGF and its isoforms are compared in pSS and sSS. According to Manetti et al., increased plasma levels of VEGF\textsubscript{165b} isoform are associated with the severity of capillary architectural derangement in SSc patients.\textsuperscript{6} Elevated plasma levels of VEGF\textsubscript{165b} correlated significantly with the absence of microhemorrhages and the presence of ramified/bushy capillaries and avascular areas. The authors suggested that in SSc patients, the VEGF\textsubscript{165b} might actively participate in the loss of microvessels. Additionally, the anti-angiogenic VEGF\textsubscript{165b} splice variant was selectively upregulated at both mRNA and protein levels in the skin biopsy samples from patients with SSc.\textsuperscript{5} No significant difference in skin expression of VEGF\textsubscript{165b} was found between patients with limited cutaneous SSc and those with diffuse cutaneous SSc.\textsuperscript{6} In our study, the group of patients with vasculitis was too small to include this symptom in statistical analysis and comparison between patients.

In our study, serum levels of VEGF significantly corresponded with the serum levels of EGF. In previous studies, a similar role in the endothelial cell activation by VEGF and fibroblast growth factor 1 and 2 was observed.\textsuperscript{3} Moreover, IGF-1 and TGF-β1 have previously been shown to enhance total VEGF expression.\textsuperscript{9} According to Nowak et al.,
IGF-1 and TGF-β1 and β2 differentially affect the expression of VEGF<sub>165</sub> and VEGF<sub>165b</sub>. EGF is responsible for the growth of epithelial cells, which are the main targets in the initiation of inflammation and angiogenesis in SS. Our results confirm that an evaluation of both EGF and VEGF is helpful for gaining a comprehensive view of angiogenesis in SS.

There were no differences in the levels of VEGF<sub>165</sub> and VEGF<sub>165b</sub> between pSS, sSS and HS. There were also no statistically significant correlations between the salivary levels of SRSF1 and PBMC expression of VEGF<sub>165</sub> and VEGF<sub>165b</sub>. But SRSF1 levels correlated positively with total VEGF salivary levels. According to Manetti et al., TGF-β1 upregulates the expression of VEGF<sub>165</sub>b and serum/arginine protein 55 in both SS and healthy microvascular endothelial cells. The binding of them to VEGF pre-mRNA or their interaction with VEGF pre-mRNA sequences has been implicated in growth-factor-mediated alternate splice-site selection. Nowak et al. even suggested that known splicing factors differentially affect the expression of the VEGF<sub>xx</sub> and VEGF<sub>xxb</sub> isoform families. Additionally, VEGFR-1 and neuropilin modulate VEGF<sub>165</sub> signaling. Furthermore, VEGFR-1, fibronectin and collagen also have differentially spliced inhibitory isoforms. A universal mechanism may, therefore, exist for the regulation of these antiangiogenic splicing events.

There were no statistically significant differences in the salivary levels of the selected proteins determined in pSS and sSS patients. According to Hernández-Molina et al., the salivary levels of the pro-inflammatory cytokines were similar in pSS, sSS and pre-clinical SS and systemic autoimmune diseases, but there were differences between HS and patients with other autoimmune diseases. Mori et al. observed higher levels of pro-inflammatory cytokines in the saliva of SS patients than in those from controls. According to Bertorello et al., only increased salivary levels of interleukin 10 in SS patients correlate with the severity of the disease. These differences in the salivary levels of the main cytokines can also stem from vasculitis in SS. Thus, in saliva it is difficult to find a representative marker with a suitably high specificity to SS.

The limitations of this study can arise from the choice of the type of saliva and the methods of saliva sampling. The levels of salivary peptides vary depending on the type of salivary glands and methods of saliva sampling. Resting and mixed saliva seem to be the most representative oral fluids. On the other hand, mixed saliva consists of crevicular fluid, which can increase the total salivary concentration of proinflammatory cytokines and markers. Stimulation mainly produces parotid saliva, which is easily available for sampling, especially in dry mouth conditions in SS patients. Lack of the measurement of VEGF2 levels does not provide a comprehensive view of the VEGF<sub>165</sub> and VEGF<sub>165b</sub> relationship. In our study, a few patients were taking methylprednisolone, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive drugs. There were no naïve patients regarding both disease-modifying antirheumatic drugs (DMARDs) and NSAIDs. According to Nagashima et al., serum levels of VEGF are reduced by combined therapy with corticosteroids and MTX. Current and previous therapy and co-existing diseases may interfere with our results, but it is not clear whether these drugs can affect serum and salivary VEGF and EGF levels in SS patients. The main limitations of this study arise from the small sample size. It should, therefore, be considered as a pilot study and our findings have to be verified in a larger SS cohort.

**Conclusions**

The balance of VEGF isoforms is not disturbed in SS. Saliva is more sensitive as a medium for detecting EGF and VEGF than serum, but salivary levels of these proteins are not representative for SS group.

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