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Original Article

Chemokine (C-X-C Motif) Ligand 1 (CXCL1) Expression in the Minor Salivary Glands of Sjögren's Syndrome Patients

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Objective. To evaluate the laboratory and clinical manifestations of Sjögren's syndrome (SS) association with chemokine (C-X-C motif) ligand 1 (CXCL1) expression in the ductal and acinar salivary gland epithelial cells (SGEC) of the minor salivary glands. **Methods.** The sociodemographic data of 106 SS patients was obtained, and the glandular and extraglandular manifestations of the disease documented. The minor salivary glands were biopsied and the laboratory findings analyzed. European League Against Rheumatism SS disease activity index (ESSDAI) and SS disease damage index (SSDDI) scores were obtained during biopsy. An immunohistochemical approach was used to define the expression of CXCL1 in the salivary glands. **Results.** Of 106 patients, the minor salivary glands of 22 patients (20.7%) stained positively for CXCL1. Such CXCL1-positive patients exhibited higher ESSDAI scores at the time of biopsy than the CXCL1-negative patients (3.86 ± 2.27 vs. 2.64 ± 1.62, $p = 0.015$). Lymphadenopathy was more frequently observed in CXCL1-positive patients, compared with CXCL1-negative patients (31.8% vs. 9.5%, $p = 0.014$). No differences between groups were identified in terms of sociodemographic characteristics, laboratory data, or the extent of the glandular manifestation of SS. **Conclusion.** The expression of CXCL1 within the ductal and acinar SGEC of SS patients is associated with lymphadenopathy and elevated clinical disease activity. CXCL1 may play an important role in the disease activity and prognosis of SS. **(J Rheum Dis 2016;23:297-303)**

Key Words. Chemokines, CXCL1, Disease activity, Sjögren's syndrome

INTRODUCTION

Sjögren's syndrome (SS) is a chronic inflammatory disease that causes clinical functional impairment of the exocrine glands and pathological periductal lymphoid infiltrations in the affected glands [1,2]. These inflammatory lymphoid proliferations are frequently accompanied by the invasion and destruction of ductal epithelia and acini, suggesting a close interaction between epithelial and immune cells [3]. Epithelial cells are thought to participate in and modulate the inflammatory process in SS, as supported by previous immunohistochemical studies of the salivary gland tissue of SS patients that demonstrated an increased expression of various immunoactive molecules in the ductal and acinar salivary gland epithelial cells (SGEC) [4-9]. Additionally, several studies of cultured SGEC from SS patients found that various molecules associated with innate and acquired immune responses were expressed [10-14]. These results strongly suggest the implication of epithelial cells in the induction and promotion of chronic inflammation and that they have an important role to play in the pathogenesis of SS.

Chemokine (C-X-C motif) ligand 1 (CXCL1), previously called growth related oncogene-alpha, is a small cytokine belonging to the CXC chemokine family and interacts

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with its receptor, CXC chemokine receptor 2 (CXCR2). CXCL1 was initially characterized by its stimulatory action in malignant melanoma cells [15] and is known to be expressed by macrophages, neutrophils, and epithelial cells [16,17]. CXCL1 has neutrophil chemoattractant properties [18] and has a reported association with the processes of angiogenesis, atherosclerosis, inflammation, and tumorigenesis [19-21]. There has been recent and growing interest in the role of CXCL1 in several autoimmune diseases, such as rheumatoid arthritis [22], systemic sclerosis [23], and SS [24]. In these studies, the expression of CXCL1 was found to be increased in diseased tissues, including the SGEC of SS patients. However, the role of CXCL1 in SS and its relationship with the clinical findings of SS are still poorly understood. In the current study, we investigated the association between CXCL1 expression in the minor salivary glands and the laboratory and clinical manifestations of SS patients.

MATERIALS AND METHODS

Patients and tissue samples

A total of 106 SS patients who were treated between January 2006 and April 2013 and who fulfilled both the revised criteria proposed by the American-European Consensus Group [25] and the histopathologic criteria for a diagnosis of SS, i.e., ≥ 1 lymphocytic focus containing at least 50 mononuclear cells per 4 mm^2 (the focus score) [26], were retrospectively evaluated. This study was approved by the Institutional Review Board of Chonnam National University Hospital (CNUH-2015-049), Republic of Korea. Although the requirement for informed consent was waived due to the retrospective nature of the study, additional data collection carried out during the study preserved the patients' anonymity and confidentiality.

Clinical and laboratory manifestations

Baseline demographic, laboratory, and clinical findings were collected at the time the minor salivary gland biopsy was performed. An experienced rheumatologist assessed the clinical symptoms, including dry eye, dry mouth, enlargement of parotid glands, and experience of extraglandular manifestations. All patients underwent an ophthalmologic test, including Schirmer's test and a tear film break-up time test, conducted by an appointed ophthalmologist. Salivary gland scans with $^{99\text{-}m}$Tc were considered positive when the tracer showed delayed uptake, reduced concentration, or delayed excretion. Gastroesophageal reflux was diagnosed by both subjective symptoms and the objective results of gastroscopy. Lymphadenopathy was defined when lymph nodes were palpable in physical examination or abnormal in number, size or consistency in radiographic results including ultrasonography, computed tomography (CT), or magnetic resonance imaging and ruled out if there was any evidence of infection. Autoimmune thyroiditis was diagnosed from both hypothyroidism and increased autoantibody levels such as anti-thyroid peroxidase or anti-thyroglobulin antibodies. The assessment of interstitial lung disease or pulmonary fibrosis was based on chest radiographs or high-resolution CT. Renal involvement was defined if the patient showed active urine sediment, proteinuria greater than 500 mg/day, a decreased glomerular filtration rate less than 60 mL/min, or renal tubular acidosis. We routinely performed urinalysis for all patients and carefully reviewed all examined radiographic results, including plain abdominal radiography, ultrasonography, and abdominal CT, to evaluate nephrocalcinosis. Serositis was defined as pleural effusion detected by radiography or pericardial effusion on echocardiography. Carpal tunnel syndrome was defined as a complaint of abnormal sensation in combination with the results of a nerve conduction study. Confirmation of lymphoma was based on the results of a lymph node biopsy. The European League Against Rheumatism SS disease activity index (ESSDAI) [27] and the SS disease damage index (SSDDI) [28] were used to assess disease activity and the degree of damage, respectively.

Laboratory profiles were measured at the time of the labial salivary gland biopsy, including white blood cell count, lymphocytes, hemoglobin, platelets, erythrocyte sedimentation rate, C-reactive protein, immunoglobulin G, and complement level. Autoantibodies against SS-A/Ro and SS-B/La were determined by enzyme-linked immunosorbent assay (ELISA); rheumatoid factor (RF) was assessed by nephelometry at the time of the minor salivary gland biopsy.

Immunohistochemistry and assessment of chemokine expression

Formalin-fixed, paraffin-embedded, 3-μm-thick tissue sections placed on poly-L-lysine-coated slides were deparaffinized in xylene and rehydrated gradually in ethanol. Microwave antigen retrieval was performed in a citrate buffer prior to immunohistochemical staining,

and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. For immunohistochemistry, we used goat anti-human CXCL1 polyclonal antibody (R&D Systems, Minneapolis, MN, USA; catalog number AF275, 1:100) for the primary antibody. The secondary antibody was rabbit anti-gout biotin (DAKO, Carpinteria, CA, USA). A subsequent reaction to decrease possible false-positive staining owing to endogenous biotin was performed with a biotin-free system (Envision peroxidase detection system; DAKO). The sections were incubated in a chromogenic solution with diaminobenzidine (DAKO), and counterstained with hematoxylin. Tissue sections sequential to those used to assess the foci were stained for CXCL1. The biopsy tissue was considered positive for CXCL1 if the cytoplasm and membrane of the acini and ductal cells showed positive staining (Figure 1). All sections were independently analyzed by two observers who were blinded to the patients' data. In cases of disagreement, the biopsy tissues were reviewed by another independent observer; any discrepancies were resolved by consensus between the observers.

Statistical analysis

The demographic, laboratory, and clinical manifestations were summarized using means and standard deviations for continuous variables and numbers and percentages for categorical variables. Comparisons between the groups were performed using the Mann–Whitney U-test for continuous variables and the chi-squared test for the categorical data. Statistical analysis was performed using the DASW statistics ver. 18.0 (IBM Co., Armonk, NY, USA). p-values <0.05 were considered to indicate statistical significance.

RESULTS

The minor salivary glands stained positively for CXCL1 in 22 (20.8%) out of 106 SS patients. The mean age of the patients was 45.4±11.3 years at the time the minor salivary gland biopsy was performed, and their sicca symptom onset age was 42.8±10.9 years. Of the total number of patients, 103 patients (97.2%) were female and most of the patients displayed glandular subjective symptoms and positivity for objective tests.

The baseline sociodemographic, glandular, and extraglandular manifestations of the 106 patients at the time of the minor salivary gland biopsy are summarized in Table 1. The age at the time of the biopsy and the age at the onset of sicca symptoms did not differ significantly between CXCL1-positive and CXCL1-negative patients. Glandular manifestations including dry eye, dry mouth, positive rates of Schirmer's test, positive rates of the tear film break-up time test, and salivary scan positive rates were identified in most of the patients and did not differ between the groups. Enlargement of the parotid gland also did not differ between the groups. As an extraglandular manifestation, lymphadenopathy was more frequently observed in CXCL1-positive patients compared with CXCL1-negative patients (31.8% vs. 9.5%, respectively; p=0.014). Other extraglandular manifestations, includ-

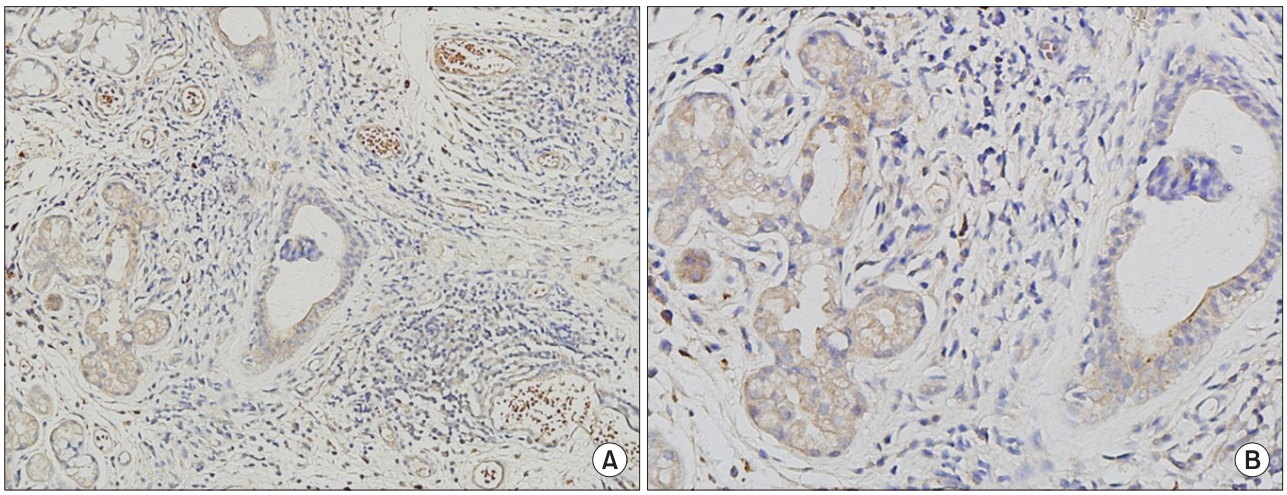

Figure 1. Immunohistochemistry of the expression of chemokine (C-X-C motif) ligand 1 (CXCL1) in a biopsy of the minor salivary gland of a patient with Sjögren's syndrome. Cytoplasm and membrane of acini and ductal cells from tissue sections show positive staining for CXCL1 (brown) in (A) and (B) (A, ×200; B, ×400).

Table 1. Comparison of the baseline sociodemographic characteristics and clinical manifestations at the time of the minor salivary gland biopsy in patients with Sjögren's syndrome

Characteristics of patient	CXCL1 positive (n=22)	CXCL1 negative (n=84)	p-value
Age (yr)	43.64±12.75	45.87±10.95	0.458
Sicca symptom onset age (yr)	40.41±12.20	43.46±10.46	0.291
Women	22/22 (100)	81/84 (96.4)	1.000
Glandular manifestations			
Dry eye	20/22 (90.9)	82/84 (97.6)	0.190
Dry mouth	20/22 (90.9)	81/84 (96.4)	0.276
Schirmer's test positive rates	15/19 (78.9)	64/74 (86.5)	0.474
BUT positive rate	20/20 (100)	75/76 (98.7)	1.000
Salivary scan positive rates	21/21 (100)	73/79 (92.4)	0.338
Enlargement of parotid glands	2/22 (9.1)	8/84 (9.5)	1.000
Extraglandular manifestations			
Arthralgia	18/22 (81.8)	51/84 (60.7)	0.081
Raynaud's phenomenon	5/22 (22.7)	16/84 (19.0)	0.765
GER	1/22 (4.5)	12/84 (14.3)	0.294
Lymphadenopathy	7/22 (31.8)	8/84 (9.5)	0.014
Autoimmune thyroiditis	2/22 (9.1)	8/84 (9.5)	1.000
ILD/pulmonary fibrosis	3/22 (13.6)	3/84 (3.6)	0.102
Renal involvement	2/22 (9.1)	3/84 (3.6)	0.276
Psychosis	0/22 (0)	3/84 (3.6)	1.000
Serositis	1/22 (4.5)	2/84 (2.4)	0.506
Carpal tunnel syndrome	0/22 (0)	2/84 (2.4)	1.000
Lymphoma	0/22 (0)	1/84 (1.2)	1.000
Sclerodactyly	0/22 (0)	1/84 (1.2)	1.000

Unless otherwise specified, data are shown as means±standard deviations and number (%). CXCL1: chemokine (C-X-C motif) ligand 1, BUT: tear film break-up time, GER: gastro-esophageal reflux, ILD: interstitial lung disease.

ing arthralgia, Raynaud's phenomenon, gastro-esophageal reflux, autoimmune thyroiditis, photosensitivity, interstitial lung disease, pulmonary fibrosis, renal involvement, psychosis, serositis, carpal tunnel syndrome, lymphoma, and sclerodactyly, did not differ between the two groups.

The laboratory manifestations, histologic focus scores, and clinical indices are described in Table 2. Laboratory manifestations, including RF, and anti-SS-A and -SS-B titer, did not differ between the two groups. Pathologically, the focus scores also did not differ between the groups. In terms of the clinical index, the ESSDAI score of the CXCL1-positive patients was higher than that of CXCL1-negative patients (8.36±4.10 vs. 4.96±3.24; p=0.001), but the SSDDI score did not differ according to the expression of CXCL1. Moreover, when the difference in ESSDAI domain between the groups was analyzed, no significant difference was identified (data not shown).

In our study, 10 patients (9.4%) had secondary SS. Of those patients, 2 patients had rheumatoid arthritis and 8 patients had systemic lupus erythematosus. Of the eight patients with SLE, two patients was included in CXCL1 positive group (2/22, 9.1%) and six was in CXCL1 negative group (6/84, 7.1%). There were no statistically significant differences in the demographic, pathological, laboratory and clinical manifestations between patients with primary SS and secondary SS (data not shown).

DISCUSSION

In this study, 20.7% of SS patients stained positively for CXCL1 in the minor salivary glands. Interestingly, the expression of CXCL1 was associated with a higher prevalence of lymphadenopathy and a greater ESSDAI score at the time of the minor salivary gland biopsy. In terms of the sociodemographic characteristics, laboratory data, and the extent of the glandular manifestations of SS, no significant difference was identified between the two groups.

Considering the clinical manifestations, the prevalence of lymphadenopathy was increased in the CXCL1 positive patients. Lymphadenopathy is one of the hallmarks of

Table 2. Comparison of the laboratory manifestations, focus score, and clinical index by CXCL1 level in patients with Sjögren's syndrome

Characteristics of patient	CXCL1 positive (n=22)	CXCL1 negative (n=84)	p-value
Laboratory manifestations			
WBC (/mm^3)	5,555±2,028	5,345±2,002	0.794
Lymphocyte (/mm^3)	1,600±524.5	1,771±678.7	0.335
Hemoglobin (g/dL)	11.80±1.894	12.33±1.389	0.348
Platelet (×10^3/mm^3)	245.7±62.41	227.9±69.11	0.117
C3 (mg/dL)	90.29±28.32	101.5±22.76	0.098
C4 (mg/dL)	21.59±11.30	21.63±10.12	0.989
CH50 (U/mL)	47.70±18.95	51.36±15.06	0.801
Ig G (mg/dL)	2209±856.1	1980±586.7	0.295
ESR (mm/hr)	48.91±33.58	39.62±26.35	0.272
CRP (mg/dL)	0.534±0.866	0.357±0.611	0.776
RF (IU/mL)	52.63±110.0	35.91±55.66	0.848
Anti-SS-A/Ro (U/mL)	148.5±78.85	156.4±80.67	0.680
Anti-SS-B/La (U/mL)	50.62±68.15	40.60±67.48	0.264
Focus score	1.70±0.93	1.95±0.98	0.281
ESSDAI	8.36±4.10	4.96±3.24	0.001
SSDDI	1.05±0.84	0.92±0.95	0.320

Data are shown as means±standard deviations. CXCL1: chemokine (C-X-C motif) ligand 1, WBC: white blood cell, Ig: immunoglobulin, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, RF: rheumatoid factor, ESSDAI: EULAR Sjögren's syndrome disease activity index, SSDDI: Sjögren's syndrome disease damage index.

acute or chronic inflammatory conditions. CXCL1 is well known to attract neutrophils to areas of active inflammation [29]. Moreover, as demonstrated elsewhere, it is active in T-lymphocyte chemotaxis [30] and potent monocyte chemotaxis [31]. In a recent study, the CXCL1/CXCR2 expression in SGEC was strongly affected by the stimulation of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α [24], which indicated a close relationship between CXCL1 and the inflammatory process. Our results suggest that increased expression of CXCL1 in ductal or acinar SGEC results in excessive leukocyte recruitment, aggravates the immune reaction in SS, and causes a prevalence of lymphadenopathy. In addition, difference in CXCL1 expression was not associated with age, gender ratio, laboratory results, glandular symptoms, or salivary dysfunction. As glandular functions tend to be decreased across all SS patients, our data imply that various mechanisms other than CXCL1 in salivary gland serve as major cause of glandular manifestations and glandular enlargement.

Additionally, our results revealed that the ESSDAI score at the time of biopsy was increased in CXCL1 positive patients. However, we were unable to identify a dominant domain with a statistical difference. As mentioned above, CXCL1 is a chemoattractant peptide and the interaction between CXCL1 and CXCR2 is known to induce the destruction of SGEC, aggregate inflammatory cells, and produce angiogenesis in inflamed tissue. Lisi et al. [24] demonstrated that the expression of CXCR2 in the salivary gland biopsies of SS patients was increased compared with healthy controls at both the gene and protein levels, and that it was simultaneously correlated with a higher level of CXCL1 expression. They also found an over-expression of pro-angiogenic factors such as CXCL1 in human SGEC treated with anti-Ro/SSA and identified a close association between angiogenesis and inflammation in SS [32-34]. Although a precise mechanism has yet to be fully established, the CXCL1/CXCR2 system should play a key role in inflammatory processes in SS through the recruitment of inflammatory cells and angiogenesis. In accordance with previous data, the increased ESSDAI score in our study also supports the role of CXCL1 in inducing and deteriorating inflammation in SS. We suggest that the expression of CXCL1 activates inflammation via the complex interactions of various molecules associated with an innate and acquired immune response but not through specific pathogenic functions, and that this may imply that there is no significant difference between the domains of disease activity.

As histopathologic finding, focus score did not differ between two groups. Focus score may be associated with periductal lymphocytic aggregations rather than activation of salivary gland epithelial cells which results from CXCL1. As mentioned above, it is suggested that CXCL1 may participate in the pathogenesis of SS mainly by leukocyte recruitment and subsequent activation of inflammation.

This is the first study to investigate the clinical significance of the expression of CXCL1 in the SGEC of SS patients. The destruction and impaired function of epithelial cells in the exocrine glands of SS patients was initially considered to be induced by humoral and cellular immune reactions against epithelial tissues, but several studies have since indicated that epithelial cells play a role not only as the target but also as inducers or intensifiers of the inflammatory process [35-37]. As mentioned above, the SGEC of SS patients are thought to functionally participate in and intrinsically activate immune reactions, in agreement with use of the term 'autoimmune epithelitis' for SS. However, until now, the effect of activated SGEC on the clinical manifestations and disease activity of SS was poorly evaluated. Therefore, we focused on assessing the clinical significance of the expression of CXCL1 as a result of the activation of SGEC in SS patients. Furthermore, the blockade of CXCR2 was found to reduce leukocyte recruitment, tissue damage, and mortality in several inflammatory diseases [38]. The CXCL1 expression in the epithelia of SS is a challenging issue for understanding the pathogenesis of this disease and for developing new approaches to treatment.

Several limitations of our study must be noted. First, this was a retrospective study and our data were collected mainly by a review of the patients' medical records; therefore, recall bias cannot be ruled out. Second, the results may have been enhanced if we had evaluated the serum CXCL1 level at the time of the minor salivary biopsy to practically assess the systemic effect beyond the affected tissue.

CONCLUSION

In conclusion, the expression of CXCL1 within the ductal and acinar SGEC of SS patients is associated with lymphadenopathy and the aggravated activity of clinical disease. CXCL1 should play an important role in terms of the disease activity and further prognosis of SS. Additional studies are needed to further define the role of CXCL1 in SS.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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