Salivary Resistin Reflects Local Inflammation in Sjögren's Syndrome

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ABSTRACT. ***Objective.*** To assess the role of resistin in primary Sjögren's syndrome (pSS) and its relation to local inflammation.

Methods. Blood and saliva were collected from 37 patients with pSS (duration of symptoms 12.6 ± 1 yrs) and 32 healthy controls. Expression of resistin in salivary glands was visualized immunohistologically, and levels of resistin were detected by ELISA. Levels of resistin were evaluated at baseline and following oral dehydroepiandrosterone (DHEA) treatment (50 mg/day). The effect of DHEA treatment on the secretion of resistin was assessed *in vitro* in human leukocytes after challenge with insulin and lipopolysaccharide.

Results. Levels of resistin in saliva were significantly higher in patients with pSS than in controls, while circulating levels of resistin were similar in both groups. Resistin was expressed in the epithelial cells of striated ducts and in the lymphocytic foci. Resistin levels in saliva were related to the intensity of inflammation in the minor salivary glands of pSS patients. No changes of the levels of resistin in blood or saliva were observed during DHEA treatment. Exposure of naive leukocytes to DHEA *in vitro* induced significant expression of resistin compared to nonstimulated peripheral blood mononuclear cells ($p = 0.031$).

Conclusion. We showed that levels of resistin are upregulated locally in the salivary glands of patients with pSS; and that the levels of resistin correspond to the intensity of lymphocytic inflammation in patients with pSS. We suggest that resistin is expressed in the salivary glands of patients with pSS and may be a driving factor of local inflammation. (First Release Aug 15 2008; J Rheumatol 2008;35:2005–11)

Key Indexing Terms:
RESISTIN INFLAMMATION SJÖGREN'S SYNDROME SALIVARY GLAND DHEA

Resistin, FIZZ3, is a small cystein-rich peptide belonging to the family of FIZZ proteins, found in inflammatory zones[1]. Resistin is secreted as a dimer of 9.5 kDa polypeptide chains and assembled into multimers connected by a disulfide bond[2]. Tissue distribution and the function of resistin differ between species. In mice, high levels of resistin are produced in adipose tissue[3]. The main known effect of resistin in mice is related to its regulation of carbohydrate metabolism, reducing the sensitivity of cells to insulin. In humans, blood mononuclear leukocytes are the main source of resistin[4,5]. Human resistin is expressed at the highest level in the bone marrow[4]. In humans, resistin acts as a modulator of inflammation, promoting release of nuclear factor-κB-dependent cytokines and chemokines from leukocytes[6]. Hormonal regulation of resistin production has attracted attention. Resistin expression in mouse adipocytes is remarkably reduced by insulin and insulin-like growth factor 1 (IGF-1)[3,7]. In contrast, insulin stimulates resistin secretion in humans[8]. Estrogens induce the expression of resistin in human[9] and in mouse adipocytes[10]. At the transcriptional level, the production of resistin in mice is regulated by CCAAT/enhancer-binding protein pathway[11,12] and peroxisome proliferators activated receptor (PPAR)[13,14].

Dehydroepiandrosterone (DHEA) is a sex steroid hormone serving as a precursor of androgens and estrogens in nonreproductive tissues[15]. Stimulation with DHEA upregulates cytosolic/nuclear hormone receptors such as estrogen receptor-ß, androstenol receptor, PPAR-α, and pregnane X receptor, and increase their ligand-specific sensitivity[16].

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These properties of DHEA address a potentially interesting regulator of resistin expression. DHEA is produced by the adrenal glands in humans and measured as its metabolite, DHEA sulfate (DHEA-SO_4) in circulation. This production peaks during the third decade of life followed by an age-dependent decline[17].

Primary Sjögren's syndrome (pSS) is an autoimmune condition characterized by a progressive lymphocytic infiltration of the exocrine glands. The predominant cells in minor labial salivary glands are T cells, while B cells, macrophages, and dendritic cells appear in advanced chronic lesions[18]. The functions of antigens triggering the expansion and differentiation of T cells in pSS are not completely characterized. The pathogenic role of cytokines and other signal molecules regulating interaction between subsets of T cells and propagating inflammation has been described[19].

We evaluated salivary and circulating levels of resistin and its expression in the minor salivary gland in patients with pSS. A regulatory role of DHEA on resistin secretion was evaluated *in vivo* and *in vitro*. We demonstrate that resistin expression is a part of local inflammation in the salivary glands and that DHEA treatment does not interfere with resistin expression.

MATERIALS AND METHODS

Thirty-seven patients (33 women, 4 men, ages 25–75 yrs) with the diagnosis of pSS fulfilling the American-European diagnostic criteria[20] were recruited from the outpatient cohort at the Rheumatology Clinics of Göteborg and Borås, Sweden. Clinical and demographic characteristics of the patients are presented in Table 1. All patients had ocular and oral symptoms of mucosal dryness supported by reduced unstimulated salivary flow and the Schirmer test. Histological evaluations of the minor salivary glands were performed in 31 patients (see below). One patient underwent sialography of the parotid gland. No information about histological changes in the salivary glands was available from the remaining 5 patients. The control group comprised 32 healthy individuals (31 women, 1 man, ages 26–65 yrs). None of the controls had any history of rheumatic disease, or subjective symptoms of oral or ocular mucosal dryness. At the time of blood and saliva sampling, no control had symptoms of inflammation in the oral cavity or used antibiotics.

Table 1. Clinical and demographic characteristics of patients with primary Sjögren's syndrome (SS) and healthy controls.

	SS, n = 37	Control, n = 32
Age, yrs (range)	58 ± 2 (25–75)	50 ± 2 (26–65)
Sex, male/female, n	4/33	1/31
Duration of disease, yrs	5.2 ± 0.8	—
Duration of symptoms, yrs	12.6 ± 1	—
Antinuclear antibodies, n (ANA, SSA, SSB)	21	Not assessed
Premenopausal/ postmenopausal, n	12/21	14/17
Saliva production, unstimulated/ stimulated, n	11/25	32/–
Treatment with DMARD, n	7*	
Prednisolone	4	
None	26	32

*Seven patients received disease-modifying antirheumatic drugs (DMARD). One patient was treated with cyclosporine, one with chloroquine, 4 with hydroxychloroquine, and one with azathioprine, as well as prednisolone; 3 patients received prednisolone.

DHEA treatment of pSS patients. Patients with pSS were enrolled in a double-blind crossover study of oral treatment using low doses of DHEA (registration no. NCT00543166). The study was approved by the Ethics Committee at Uppsala University, Uppsala, Sweden. Written informed consent was obtained from all subjects. Patients were randomized to either active treatment (DHEA 50 mg/day; Group 1, n = 18) or placebo (Group 2, n = 19) during a 4-month period. Samples of serum and saliva were taken at baseline and following 4 months of treatment. After a washout period of 1 month new baseline samples of serum and saliva were taken and the patients who had received active drug continued with placebo (Group 1, n = 18) and the patients in the placebo group received DHEA (Group 2, n = 19) for the next 4 months. Low circulating DHEA-sulfate levels at inclusion were prerequisite for participation in the study. Thirty-four patients completed the study; 3 discontinued their medication and were dismissed.

Collection of saliva and blood samples. Samples of unstimulated and stimulated saliva were collected at the Institute of Odontology, Göteborg, by experienced dentists (UD, CSM, BH) at baseline and during the followup visits as described[21]. Unstimulated saliva was collected for 15 min by the draining method and stimulated saliva was collected for 5 min during stimulation by paraffin chewing. Due to obvious difficulties to produce unstimulated saliva for pSS patients, only samples of stimulated saliva were measured and used for the analysis. Matched venous blood samples were obtained simultaneously and collected into heparinized tubes. Blood and saliva samples were centrifuged at 800 g for 15 min, aliquoted, and stored frozen at –20°C until used.

Histopathological and immunohistological examination of salivary glands. Histological evaluations of the minor salivary glands were performed after routine fixation in formalin and paraffin embedding of the tissue. Tissue sections were cut and stained with hematoxylin and eosin. The intensity of sialoadenitis was evaluated by the presence of focal lymphocytic infiltration per 4 mm^2 of glandular tissue. For immunohistological examination, 4 mm thin paraffin sections were prepared. The antigen retrieval was performed by heating the sections in a microwave oven in citrate buffer (pH 6.0). Sections were incubated with monoclonal mouse anti-human resistin antibodies (R&D Systems, Abingdon, UK); mouse IgG of identical isotype were used in parallel tissue sections as a control. Staining was followed by secondary alkaline phosphatase-coupled rabbit anti-mouse antibodies (Dako, Glostrup, Denmark) and NBT/X-phosphate (Sigma, St. Louise, MO, USA) as substrate. Immunohistological sections were counterstained with hematoxylin.

Laboratory analyses. Concentrations of resistin in blood and saliva were determined by a sandwich ELISA using a matched antibody pair (R&D Systems) following the manufacturer's recommendations. Serum samples were analyzed in dilution 1:10 in 1% bovine serum albumin-phosphate buffered saline (BSA-PBS), and saliva samples in dilution 1:2. Levels of resistin were calculated using serial dilutions of human recombinant resistin and expressed in ng/ml. Total protein in saliva was measured with Bradford reagent (Sigma) using sample dilution 1:20 in distilled water. Colorimetric reaction was measured at wavelength 595 nm and compared to a serial dilution of BSA, and presented as mg/ml. Circulating levels of DHEA-sulfate were measured in blood samples by radioimmune assay (Diagnostic Products, Los Angeles, CA, USA). The lowest detectable level was 0.14 µmol/l. Serum levels of C-reactive protein (CRP) were measured by nephelometry with established normal range 0–5 mg/l.

In vitro exposure of human leukocytes to DHEA. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood of healthy individuals by density gradient separation on Lymphoprep (Axis-Shield, Oslo, Norway). Human promyelocytic leukemia cells, HL60 cells, originally obtained from the American Type Culture Collection (Manassas, VA, USA)

were cultured in Iscove's medium containing 1% L-glutamine, 5×10^{-5} M ß-mercaptoethanol, 50 µg/ml gentamycin sulfate, and 10% heat-inactivated fetal calf serum in a humid atmosphere of 5% CO_2 at 37°C. Cells (1×10^6/ml) were stimulated with DHEA (Sigma; dose range 10^{-7}–10^{-9} M) followed by lipopolysaccharide (LPS; Sigma) or insulin (Novo, Bagsvard, Denmark) as indicated. Supernatants were collected after 48 h incubation and kept frozen until analyzed.

Statistical analyses. Levels of resistin in blood and saliva samples were expressed as mean ± SEM. Comparison of resistin levels in the paired saliva and blood samples and during DHEA treatment was by paired t–test. Comparisons between different patient groups and controls were by Mann-Whitney statistics. Simple regression analysis was employed to test the correlation between resistin levels and age. SPSS® software (SPSS, Chicago, IL, USA) was used. The p values < 0.05 were regarded as statistically significant.

RESULTS

Resistin levels in saliva of pSS patients. Levels of resistin in blood and in saliva of pSS patients (n = 37) and healthy controls (n = 32) were compared (Figure 1). Twenty-five of 37 patients were able to produce stimulated saliva. The levels of salivary resistin were significantly lower than those in blood of pSS patients (5.1 ± 1.1 vs 7.9 ± 0.4 ng/ml; p = 0.01) and in controls (2.2 ± 0.5 vs 8.2 ± 0.7 ng/ml; p < 0.0001; Figure 1). Levels of resistin in saliva were significantly higher in pSS patients compared to healthy controls (p = 0.001), while levels of resistin in blood were similar between the 2 groups. To investigate the relationship between levels of resistin and the intensity of inflammation, levels of resistin were analyzed in relation to morphological findings in the biopsies of minor salivary glands of pSS patients. The results of salivary gland biopsies were available from 31 of 37 pSS patients. The levels of resistin in pSS patients having high grade (foci score > 1/4 mm^2) and low grade (foci score ≤ 1/4 mm^2) of inflammation in the salivary gland biopsies were compared (Table 2). Patients with low grade inflammation had significantly lower levels of resistin in saliva than patients with high grade of local inflammation (p = 0.023). The levels of resistin in blood of pSS patients showed no difference between the groups with low and high inflammation scores. The levels of resistin in blood correlated with the level of acute-phase protein CRP (r = 0.35, p = 0.032), but not with the sedimentation rate or the white blood cell count.

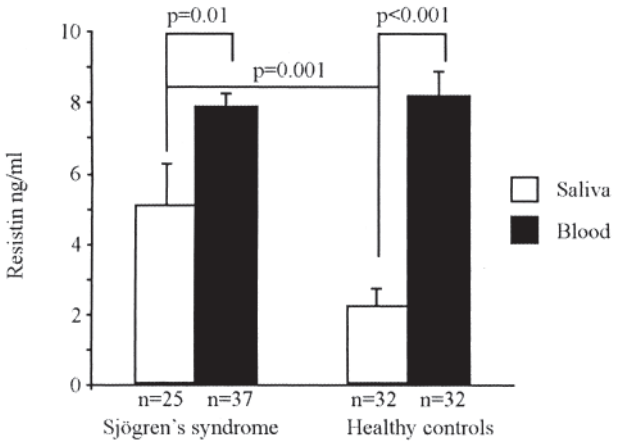

Figure 1. Resistin levels in blood and saliva of patients with Sjögren's syndrome and matched healthy controls.

Resistin expression in minor salivary glands of patients with pSS. To elucidate the morphological structures responsible for the extensive expression of resistin in pSS patients, immunohistological examination of minor salivary glands for resistin was performed. Resistin expression was visualized in the lymphocytic foci (Figure 2A). Accumulation of resistin was also observed in the epithelial cells of the striated ducts, representing another source of resistin in saliva (Figure 2B). Resistin expression was visualized in the mononuclear cells infiltrating the stromal tissue of salivary glands (Figure 2C). These observations present multiple cellular sources of resistin existing in the salivary gland tissues of patients with pSS. In salivary gland tissue of healthy individuals we observed accumulation of resistin in the cells of striated ducts (Figure 2D), a potential source of resistin in saliva even in the absence of inflammation.

Influence of DHEA treatment on resistin levels in pSS patients. At inclusion, patients had serum levels of DHEA-sulfate below the mean levels of the age and sex-matched healthy controls (1.96 ± 0.41 ng/ml). The circulating levels of DHEA-sulfate and the levels of resistin of nontreated pSS patients correlated significantly (r = 0.38, p = 0.02). Levels of DHEA-sulfate correlated neither to the level of acute-phase proteins nor to the intensity of inflammation in the salivary gland biopsies. To evaluate the regulatory effect of DHEA on resistin during inflammation, levels of resistin were measured in blood and saliva of pSS patients before and 4 months after oral DHEA intake. Patients were randomized into 2 groups for the consecutive use of DHEA, followed by placebo treatment (Group 1) or placebo followed by DHEA treatment (Group 2), as described above. The levels of resistin in blood and saliva were measured before and after 4 months of DHEA treatment (Table 3). This was compared to the change of resistin levels during the placebo period. After randomization, the 2 groups of patients differed significantly in the baseline levels of resistin in saliva (6.5 ± 2.2 vs 3.9 ± 0.9 ng/ml; p = 0.001), while the levels of resistin in blood were similar. This discrepancy was not due to the difference in salivary gland inflammation, volume of secreted saliva, or level of DHEA-sulfate before treatment (1.82 ± 0.43 ng/ml, patients treated with DHEA followed by placebo; 2.10 ± 0.4 ng/ml, patients treated with placebo followed by DHEA). This discrepancy enabled sequential analyses of the changes of resistin levels in these 2 groups. Group 1 showed no significant changes in the levels of resistin in blood or saliva, neither during DHEA treatment nor during the placebo period. Group 2 showed an increase of the levels of resistin in saliva during both DHEA treatment and the placebo period (Table 3). The clinical effect of DHEA treatment resulted in significantly increased

Table 2. The relation between salivary resistin and the intensity of inflammation in salivary gland biopsies of patients with pSS.

	Low Grade Inflammation, foci ≤ 1/4 mm^2 (n = 20)	High Grade Inflammation, foci > 1/4 mm^2 (n = 11)	p
Inflammation in salivary gland, foci/4 mm^2	0.81 ± 0.1	3.5 ±0.5	< 0.0001
Resistin in saliva, ng/ml	3.6 ± 1	7.9 ± 2	0.023
Volume of stimulated saliva, ml/5 min	3.0 ± 0.7	1.35 ± 0.5	NS
Total protein in saliva, mg/ml	5.3 ± 2	10.4 ± 3.0	0.015
Resistin in blood, ng/ml	8.1 ± 0.5	8.73 ± 0.5	NS
C-reactive protein, mg/ml	5.6 ± 0.4	5.00 ± 0.00	NS
WBC count, × 10^6/ml	6.0 ± 0.3	6.0 ± 0.5	NS
DHEA sulfate in blood, µmol/l	1.8 ± 0.4	1.3 ± 0.2	NS

WBC: white blood cells; NS: nonsignificant.

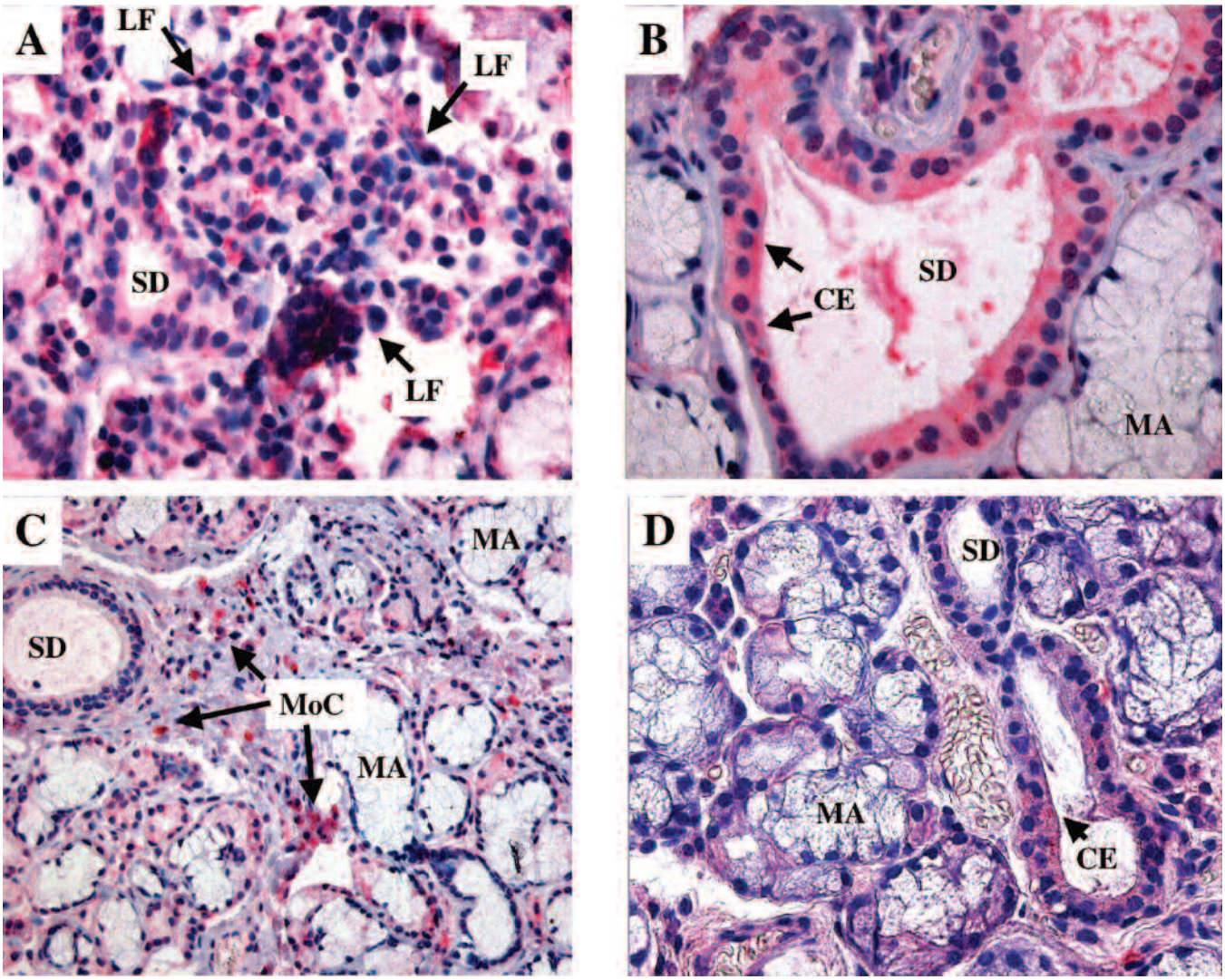

Figure 2. Resistin expression in minor salivary gland of patients with primary Sjögren's syndrome and healthy individuals. A. Resistin (stained orange) is expressed in mononuclear cells within the lymphocytic focus, indicated by arrows (original magnification ×40). B. Secretion of resistin into the saliva is observed in the cells of columnar epithelium within striated ducts (magnification ×40). C. Mononuclear cells infiltrating stromal tissue within salivary gland express resistin (original magnification ×20). D. Salivary gland of a healthy individual. Resistin is expressed in the cells of columnar epithelium (arrow; original magnification ×40). MoC: mononuclear cells; LF: lymphocytic focus; SD: striated ducts; CE: columnar epithelium; MA: mucus-secreting acini.

Table 3. Changes of resistin levels in saliva and blood of patients with pSS during DHEA treatment and placebo.

	Resistin					
	Saliva			Blood		
	Start	p	4 Months	Start	p	4 Months
DHEA						
Group 1, n = 19	6.5 ± 2.2	NS	7.9 ± 0.4	8.1 ± 0.5	NS	8.0 ± 0.5
Group 2, n = 18	3.9 ± 0.9*	NS	5.6 ± 1.3	7.4 ± 0.5	NS	7.6 ± 0.6
Placebo						
Group 1, n = 19	6.2 ± 1.5	NS	6.3 ± 1.4	7.9 ± 0.6	NS	8.5 ± 0.7
Group 2, n = 18	4.6 ± 1.2	0.049	5.6 ± 1.3	7.7 ± 0.5	NS	6.9 ± 0.7

* At baseline, Groups 1 and 2 differed significantly in resistin levels in saliva (p = 0.001). NS: nonsignificant.

volumes of stimulated saliva in patients receiving placebo followed by DHEA (2.7 ± 0.8 vs 3.2 ± 0.9 ml/5 min; p = 0.003). No significant difference in salivary volume was observed in the other group. In summary, DHEA did not affect resistin secretion in our *in vivo* study of patients with pSS.

Influence of DHEA on resistin secretion by human leukocytes in vitro. In noninflammatory conditions, exposure of human PBMC to DHEA led to a significant dose-dependent increase of the levels of resistin in supernatants (0.4 ± 0.1 vs 0.8 ± 0.2 ng/ml; p = 0.03; Figure 3A). Analogously, DHEA induced resistin production by human myelocytic HL60 cells (p = 0.001; Figure 3B). LPS has been reported to stimulate resistin secretion in monocytes[5], and insulin to stimulate resistin secretion in adipocytes[8]. To evaluate whether DHEA prevents resistin production in inflammatory conditions, HL60 cells were exposed to DHEA prior to LPS stimulation. For this experiment a single DHEA dose (10^{-7} M) was chosen. Exposure of HL60 cells to DHEA prior to LPS stimulation significantly potentiated production of resistin

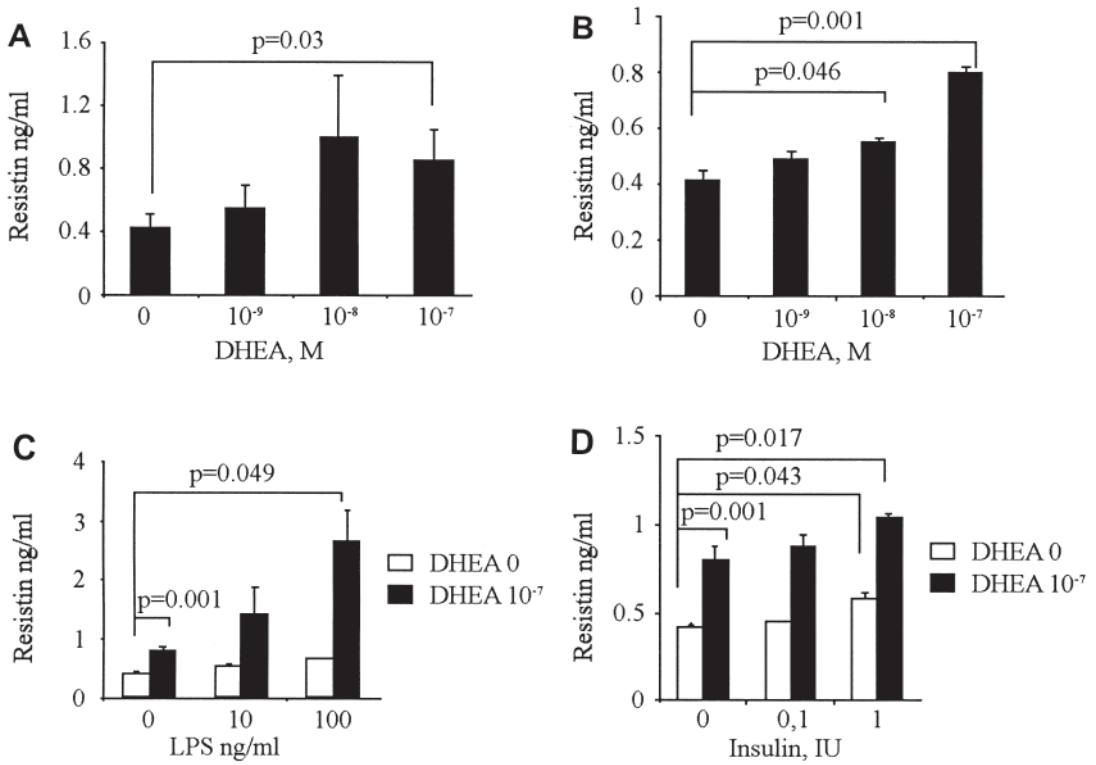

Figure 3. Resistin secretion by human leukocytes following DHEA treatment *in vitro*. A. Human PBMC (1×10^6/ml, n = 4) were treated with DHEA (dose range 0–10^{-7} M). Resistin levels were measured in supernatants following 48 h of DHEA exposure. B. HL60 myelocytic cells (1×10^6/ml, n = 3) were exposed to DHEA (dose range 0–10^{-7} M). Resistin levels were measured in supernatants following 48 h of DHEA exposure. C. HL60 myelocytic cells (1×10^6/ml, n = 3) were exposed to DHEA (10^{-7} M) for 1 h followed by addition of increasing dose of LPS (dose range 0–100 ng/ml). Resistin levels were measured in supernatants after 48 h. D. HL60 myelocytic cells (1×10^6/ml, n = 3) were exposed to DHEA (10^{-7} M) for 1 h, followed by addition of increasing dose of insulin (dose range 0–1 IU/ml). Resistin levels were measured in supernatants after 48 h.

as compared to DHEA or LPS separately ($p = 0.049$; Figure 3C). To evaluate whether DHEA prevents resistin production in metabolic conditions, HL60 cells were exposed to DHEA prior to insulin. An additive effect of DHEA and insulin on the production of resistin was observed *in vitro* ($p = 0.017$; Figure 3D).

DISCUSSION

We evaluated the expression of resistin in salivary gland and measured concentrations of resistin in blood and saliva from patients with pSS. We found that the levels of resistin in saliva were significantly higher in pSS patients than in saliva from healthy controls. Levels of resistin in blood correlated to the levels of CRP. In saliva, resistin levels were higher in the patients with a higher grade of salivary gland inflammation. In contrast, circulating levels of resistin in pSS patients and the controls were similar, suggesting that the salivary resistin was produced locally in the glands. Indeed, immunohistological examination of salivary glands clearly showed extensive expression of resistin within the lymphocytic foci as well as by the mononuclear cells infiltrating stromal tissues (Figure 2). This supports the finding of a relationship between the increased levels of resistin in saliva of pSS patients compared to healthy controls. Epithelial cells of striated ducts of salivary glands also showed accumulation of resistin, presenting another source of resistin in saliva. However, the levels of resistin were not dependent on the volume or the protein content of the saliva in patients with pSS, suggesting that infiltrating lymphocytes play a major role in regulation of resistin production by macrophages and ductal epithelial cells[6,22-27]. Resistin is known as a potent regulator of inflammation, initiating and modulating production of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6). In this context, CD4+ T cells and epithelial cells in minor salivary glands of patients with pSS express proinflammatory cytokines such as TNF-α and IL-1ß, as well as IL-6[28]. Thus, exposure of the above cell types to resistin produced by macrophages infiltrating the tissues of salivary gland might have triggered increased cytokine expression and potentiated the inflammatory process.

We evaluated the role of DHEA treatment on resistin secretion by human leukocytes *in vitro* and on salivary and blood levels of resistin *in vivo*. The rationale for our study was the potential antiinflammatory properties of DHEA[29]. However, *in vitro* exposure of human naive leukocytes to DHEA resulted in a dose-dependent increase of resistin production. These stimulatory effects of DHEA were supported *in vivo* by increased secretion of resistin in saliva of pSS patients following 4 months of DHEA intake, although this increase did not reach a statistically significant level. Importantly, this increase of resistin concentration was observed despite a significant improvement of salivary production occurring during DHEA treatment. The improvement of clinical signs of pSS following DHEA treatment is in agreement with reports of beneficial effects of DHEA on mouth dryness[30].

We have demonstrated that resistin is upregulated locally in salivary glands of patients with pSS. Further, resistin corresponded to the intensity of local inflammation in these patients. Our hypothesis suggesting DHEA as an anti-inflammatory agent downregulating synthesis of resistin was not supported in our clinical study on patients with pSS or in *in vitro* experiments.

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