

Low Salivary Dehydroepiandrosterone and Androgen-Regulated Cysteine-Rich Secretory Protein 3 Levels in Sjögren's Syndrome

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Objective. Sjögren's syndrome (SS), an autoimmune disease of exocrine glands, typically starts at the time of adrenopause. We undertook this study to test the hypothesis that SS is characterized by an insufficient androgen effect at the target tissue level.

Methods. We searched for androgen response elements (AREs) in the cysteine-rich secretory protein 3 (*crisp-3*) gene. Dehydroepiandrosterone (DHEA) responsiveness was experimentally studied using quantitative reverse transcriptase–polymerase chain reaction and immunofluorescence staining of human submandibular gland–derived acinar cells and labial salivary gland explants with or without DHEA. Finally, glandular and salivary CRISP-3 in healthy controls and SS patients was analyzed using immunohistochemistry, in

situ hybridization, and enzyme-linked immunosorbent assay. Serum DHEA sulfate (DHEAS) and salivary DHEA levels were measured using a radioimmuno-metric method.

Results. Literature analysis and a search for AREs in gene banks suggested androgen dependency of human CRISP-3, and this was verified by studies of human submandibular gland acinar cells cultured with or without DHEA, in which DHEA increased CRISP-3 messenger RNA (mRNA) levels ($P = 0.018$). This finding was confirmed by the results of DHEA stimulation of labial salivary gland explants. Glandular CRISP-3 mRNA and protein labeling was weak and diffuse, coupled with low secretion in saliva (mean \pm SEM $21.1 \pm 2.7 \mu\text{g}$ CRISP-3/15 minutes in SS patients versus $97.6 \pm 12.0 \mu\text{g}$ CRISP-3/15 minutes in healthy controls; $P < 0.0001$). Compared with healthy controls, SS patients had low serum levels of DHEAS ($P = 0.008$) and also low salivary levels of DHEA (mean \pm SEM 224 ± 33 pmoles versus 419 ± 98 pmoles; $P = 0.005$).

Conclusion. CRISP-3 pathology was seen in acini remote from lymphocyte foci and is apparently not secondary to local inflammation, but may represent some systemic effect in SS. Indeed, androgen deprivation in the salivary glands of SS patients is evidenced both by low salivary levels of DHEA and by low levels of DHEA-regulated CRISP-3. This may explain some of the characteristic features of SS.

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Sjögren's syndrome (SS) is a common autoimmune disease of the exocrine glands leading to dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia) (1). The reason for this organ-specific involvement of exocrine glands is not known. SS is also characterized by a predominance among women and by a late age at onset (2). The reason for this predominance

among women is not known. Usually, estrogens have been considered to contribute to autoimmunity, whereas androgens are supposed to be protective (3–6). Most of the female patients with SS contract the disease at age 40–50 years, at the time of menopause, which is difficult to reconcile with the general paradigm of estrogens favoring autoimmunity. Therefore, it was hypothesized that the female predisposition for SS and the late age at onset of the disease could be explained by androgen deficiency developing at age 40–50 years, during menopause and adrenopause (7–9).

Reduced concentrations of dehydroepiandrosterone sulfate (DHEAS) have also been described in both male and female patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) (10–12). Since stress and cytokines, like interleukin-6, should up-regulate the hypothalamic–pituitary–adrenal axis, serum levels of DHEAS should be high rather than low in these diseases. In addition, in SS, only DHEAS (but not, for example, testosterone) is affected. Low serum levels of DHEAS in both SLE and RA as well as in primary SS might be the common denominator and a shared pathogenetic factor contributing to salivary gland involvement and generation of either the secondary (in SLE and RA) or the primary form of SS.

Ovaries produce small amounts of testosterone, driven by luteinizing hormone. Removal of the ovaries in fertile women decreases testosterone levels by ~50%, and testosterone levels also decrease at the time of menopause. The other significant source of androgens in women is the cortex of the adrenal glands. Cells in the zona reticularis produce weakly androgenic DHEA, which is the major steroid hormone produced in that gland (13). DHEA concentrations reach their highest levels in adults after the adrenarche at age ~25 years. Thereafter, DHEA and DHEAS concentrations decline, a phenomenon referred to as adrenopause. This is a normal physiologic aging phenomenon. Valtysdottir and coworkers made the important keystone observation that patients with SS have low serum DHEAS concentrations compared with age- and sex-matched healthy controls (14). Low DHEAS concentrations in SS correlate with a low quality of life and may contribute to the most difficult general symptom of the syndrome, fatigue (15,16). In conclusion, androgen levels normally decrease in women at the time of menopause and adrenopause and are particularly low in SS patients.

Using cell- and tissue-specific DHEA processing, higher-level primates locally tailor their organ-specific sex steroid profile according to the local tissue requirements and age (17). Thus, apart from its direct androgen

effect, DHEA and its sulfate also form a circulating prohormone pool, which in part is converted to androstenedione in tissues and then either to testosterone (and further to 5 α -dihydrotestosterone) or estrone (and further to 17 β -estradiol) in a tissue-specific manner. Conversion of testosterone to 5 α -dihydrotestosterone by 5 α -reductase is important in benign prostatic hyperplasia, while aromatase-catalyzed formation of estrone and 17 β -estradiol is important in breast cancer. Intracrine processing does not exist in commonly used experimental animals, such as rats and mice. Due to this intracrine processing in higher-level primates, it is difficult to judge, based on circulating systemic hormone levels, whether a certain peripheral tissue has androgen deficiency, since the contribution of the local intracrine processing of the DHEA prohormone into its various derivatives should also be taken into account.

There have been many studies on the more potent sex steroids, such as dihydrotestosterone or 17 β -estradiol, but the present study focuses on the prohormone DHEA, which in peripheral tissue can be converted to, for example, dihydrotestosterone or 17 β -estradiol (although its fate in the salivary glands is not known). In peripheral tissues, androgen binds to a cytoplasmic androgen receptor. This leads to structural changes and translocation of the androgen–receptor complex to the cell nucleus, where this transcription factor binds to androgen response elements (AREs) in the promoter regions of androgen-regulated genes. Therefore, use of androgen-regulated molecules as biomarkers at the local tissue level would seem to overcome the obstacle mentioned above and could allow direct assessment of the peripheral androgen effects. We therefore decided to test our hypothesis of diminished DHEA effect on salivary glands in SS by using cysteine-rich secretory protein 3 (CRISP-3) as a potential DHEA-regulated biomarker. DHEA was used to stimulate human submandibular gland cells and human labial salivary gland explants, in order to assess its effects on CRISP-3. CRISP-3 was originally described as an androgen-dependent protein in mouse salivary (18) and lacrimal (19) glands, based on the findings of structural and functional studies (20,21).

PATIENTS AND METHODS

Search for AREs. The sequence of the *crisp-3* gene, using RefSeq code NM_006061, was retrieved from the National Cancer Institute BLASTN Web site (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Search&DB=nucleotide>). The flanking sequences of 10,000 basepairs upstream and 2,000 bp downstream from the transcription initiation site,

including the introns of the *crisp-3* gene complex, were retrieved from <http://dbtss.hgc.jp/index.html>. The two 6-bp asymmetric half-sites of the ARE sequence (5'-GGA/TACAnnTGTTCT-3') were obtained from the literature (22). The Fuzznuc program was used to search for nucleic acid patterns (www.csc.fi), and the National Center for Biotechnology Information BL2SEQ program was used to perform comparisons between 2 sequences.

Human submandibular gland cell stimulation. Human submandibular gland cells established from neoplastic ductal epithelial cells from an irradiated human submandibular gland (23) were a gift from Dr. Marc R. Kok, Amsterdam, The Netherlands. These cells undergo terminal differentiation into amylase-positive acinar cells and glandular-like structures when cultured on basement membrane-like growth factor-depleted Matrigel substrate (24). Before the experiments were performed, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to demonstrate that human submandibular gland cells cultured in suspension contain androgen receptors (data not shown). Eighty thousand human submandibular gland cells in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 Nut Mix medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin were added overnight to growth factor-depleted Matrigel (BD Biosciences, San Jose, CA) in 24-well plates, followed by 24-hour culture with or without 100 µM DHEA in DMEM-Ham's F-12 Nut Mix medium with 10% sex hormone-depleted FCS.

Tissue stimulation. Labial salivary gland tissue samples were obtained from 2 healthy controls (1 male and 1 female, age 17 years) who were treated for mucocele. The samples were minced into pieces (~2 mm³), put into a 6-well plate, and left overnight in DMEM-Ham's F-12 medium (Gibco BRL) containing 10% FCS with 1,000 units/ml penicillin, 2 mM L-glutamine, 1 mg/ml streptomycin (10×), and Fungizone (2.5 µg/ml; Gibco BRL) solution. The next day, the media were changed to basal DMEM-Ham's F-12 media with 10% serum-stripped FCS, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin (1× solution), and 2.5 µg/ml Fungizone. Tissues were cultured with or without 100 µM DHEA for 72 hours before quantitative RT-PCR analysis of CRISP-3.

DHEA measurements. A radioimmunoassay was used for the quantitative determination of serum levels of DHEAS (Thermo, Waltham, MA). DHEAS levels were measured in 41-60-year-old women with SS (n = 58) and compared with those in 22 healthy control women in the same age range. Using a Salivary DHEA Enzyme immunoassay kit (Salimetrics, State College, PA), DHEA levels were measured in resting saliva from 45 SS patients (all women, age 41-60 years) and from 10 age- and sex-matched healthy controls. The controls did not have any signs or symptoms of SS, and although testing for serum SSA and SSB antibodies and labial salivary gland biopsies were not performed (since the controls did not have any symptoms), they are referred to as healthy controls.

Analysis of CRISP-3. For quantification of CRISP-3 messenger RNA (mRNA), total RNA was isolated from human submandibular gland cells detached from Matrigel with Dispase (BD Biosciences) and subjected to quantitative RT-

Table 1. Primers used for cysteine-rich secretory protein 3 (CRISP-3) and porphobilinogen deaminase (PBGD) mRNA

CRISP-3	
Sense	5'-TTCACCCTTCTTCCCAATCA-3'
Antisense	5'-CCTCCTTGAGTGGAGGGTATT-3'
PBGD	
Sense	5'-ACATGCCCTGGAGAAGAATG-3'
Antisense	5'-AGATGCGGGAACCTTCTCTG-3'

PCR using a LightCycler PCR machine (Roche Molecular Biochemicals, Mannheim, Germany), fluorescent SYBR Green I labeling, and primers specifically designed for CRISP-3 (240-bp product) or porphobilinogen deaminase (237-bp product). The latter was used as a housekeeping gene for standardization of the results (Table 1).

Translation of CRISP-3 mRNA into the corresponding protein was ascertained by indirect immunofluorescence staining of the cultured human submandibular gland cells. Cells were first fixed with 3% paraformaldehyde, then permeabilized in 0.5% Triton X-100 in phosphate buffered saline followed by staining using 0.6 µg/ml rabbit anti-human CRISP-3 immunoglobulin and Alexa Fluor 568-conjugated goat anti-rabbit immunoglobulin before inspection under an immunofluorescence microscope. For the rabbit anti-human CRISP-3 antibody, the complementary DNA (cDNA) for recombinant CRISP-3Δ (residues 20-95 in the coding region) was amplified from a λ-phage human bone marrow cDNA library (Clontech, Palo Alto, CA), cloned into pTrcHisB (Invitrogen, San Diego, CA), and expressed as a fusion protein with a polyhistidine (His₆) tag. The protein was affinity purified with TALON Metal Affinity Resin (Clontech), concentrated, and dialyzed. The purified protein was used for immunization of rabbits. The specificity of the antibody was confirmed by immunoblotting of a postnuclear supernatant of neutrophils that gave 2 bands (27-kd and 29-kd bands corresponding to unglycosylated and glycosylated forms of CRISP-3, respectively). In addition, immunohistochemical staining of leukocytes demonstrated that the antibody could recognize CRISP-3 in paraformaldehyde-fixed neutrophils (25).

Participants and samples. Labial salivary glands were obtained from 11 patients with primary SS (all women, mean age 47 years) and from 11 normal healthy subjects (all women, mean age 50 years). Five to 10 labial salivary glands were removed under local anesthesia and processed for histology (26). Resting and stimulated saliva was collected (26) from another set of 29 SS patients (27 women and 2 men, mean age 53 years) and 32 healthy controls (13 women and 19 men, mean age 45 years). The study was approved by the ethics committee. All subjects gave their informed consent. The diagnosis of SS was based on the modified European criteria (1).

Immunohistochemistry. Paraffin sections (4 µm) were deparaffinized, rehydrated in decreasing ethanol series, and washed in 10 mM Tris, 150 mM NaCl, pH 7.5 buffer (Tris buffered saline [TBS]). Hidden antigenic epitopes were revealed after pretreatment with Buffer for Antigen Retrieval (TechMate; Dako, Glostrup, Denmark) using a microwave oven twice for 5 minutes each at 700W. Slides were installed in

a Dako TechMate Horizon Immunostainer robot and stained automatically at 22°C using the following protocol: 1) rabbit anti-human CRISP-3 immunoglobulin (0.6 µg/ml) (described above), 25 minutes; 2) biotinylated goat anti-rabbit immunoglobulin in TBS containing carrier protein and sodium azide, 25 minutes; 3) peroxidase blocking solution, 3 times for 3 minutes each; 4) peroxidase-conjugated streptavidin, 25 minutes; and 5) Substrate Working Solution containing 3,3'-diaminobenzidine tetrahydrochloride (ChemMate detection kit; Dako), 5 minutes. The sections were washed with Dako ChemMate washing buffer 3 times for 5 minutes each between these steps. Nonimmune rabbit serum immunoglobulin was used at the same concentration as (and instead of) the primary antibody as a negative staining control.

In situ hybridization. A CRISP-3 cDNA insert comprising nucleotide 1179–1569 (RefSeq code NM_006061) (27) was inserted into pBluescript II KS (Stratagene, La Jolla, CA) and, after linearization with *Xho* I and *Spe* I, was used as a template for in vitro transcription to generate ³⁵S-labeled antisense and sense probes, respectively. After transcription, the RNA probes were ultrafiltrated (Micron 100; Amicon, Beverly, MA) before hybridization. Deparaffinized, rehydrated sections (5 µm) were hybridized overnight with 25–50 × 10⁶ counts per minute of ³⁵S-labeled RNA probes at 55°C. After hybridization, the slides were washed under stringent conditions, including incubation with 50 µg/ml RNase A (Sigma, St. Louis, MO) for 30 minutes at 37°C, and processed for autoradiography for 3–4 weeks (28).

Sandwich enzyme-linked immunosorbent assay. Flat-bottomed plates (96-well) were coated overnight with rabbit anti-human CRISP-3 antibodies (described above) in coating buffer. Nonspecific binding was blocked with sample buffer (0.5M NaCl, 3 mM KCl, 8 mM Na₂HPO₄/KH₂PO₄, 1% bovine serum albumin, 1% Triton X-100, pH 7.2). CRISP-3 standard (affinity-purified native CRISP-3 derived from isolated neutrophils) and samples were prediluted in sample buffer, supplemented with 2% sodium dodecyl sulfate and 4 mM dithiothreitol, and incubated for 20 minutes at 37°C, followed by 2 hours of incubation at room temperature. Four-fold diluted standards (1.56–100 ng/ml) and samples were added. This was followed by incubation with biotinylated anti-CRISP-3 antibodies (3 µg/ml) and avidin-peroxidase (1:2,000) at room temperature before color was developed by a 15-minute incubation in 0.1M sodium phosphate, 0.1M citric acid, pH 5.0, containing 0.04% *o*-phenylenediamine and 0.006% H₂O₂ (25). Absorbance was read at 492 nm. All assays were performed in duplicate.

Statistical analysis. SPSS statistical software, version 13.0 (SPSS, Chicago, IL) was used for the statistical analysis, except for the CRISP-3 measurements in saliva, which were done using BMDP-PC 7.01 software (BMDP Statistical Software, Los Angeles, CA). Normality of the distribution of the variables was tested with the Shapiro-Wilk statistic. Results are expressed as the mean ± SEM. Student's *t*-test or the Mann-Whitney U test was used for pairwise comparison of normally and non-normally distributed variables, respectively.

RESULTS

AREs in the human *crisp-3* gene complex. The human *crisp-3* gene, including 10,000 bp upstream and

2,000 bp downstream from the transcription initiation site, contained in the (+) and (–) strands 10 direct and 3 indirect sequences, which differed at most in 2 nucleotides (83.3% homology) from the palindromic ARE 5'-GGA/TACAnnnTGGTCT-3' (data not shown).

DHEA and DHEAS concentrations. Fifty-eight SS patients age 41–60 years had low serum levels of DHEAS compared with 22 controls (mean ± SEM 2.0 ± 0.2 µmoles versus 3.4 ± 0.3 µmoles; *P* < 0.0001). Forty-eight of the 58 SS patients enrolled in this study did not use glucocorticoids, but 10 used low-dose glucocorticoids (<10 mg/day prednisolone or equivalent). The corresponding serum levels of DHEAS were higher in those patients who did not use glucocorticoids than in those who did (mean ± SEM 2.3 ± 0.19 µmoles versus 0.9 ± 0.22 µmoles; *P* = 0.001). Serum levels of DHEAS in SS patients who did not use glucocorticoids were significantly lower than those in healthy controls (2.3 ± 0.19 µmoles versus 3.4 ± 0.3 µmoles; *P* = 0.008). Thirty-seven of the 58 SS patients were premenopausal, 3 had undergone ovariectomy, and 18 were postmenopausal. The corresponding serum levels of DHEAS in these patient groups were 2.3 ± 0.23 µmoles, 1.3 ± 0.17 µmoles, and 1.8 ± 0.3 µmoles, respectively. Also, salivary levels of DHEA were significantly lower in SS patients (*n* = 45) than in age- and sex-matched healthy controls (*n* = 10) (224 ± 33.3 pmoles versus 419 ± 98.1 pmoles; *P* = 0.005).

Androgen responsiveness of human submandibular gland salivary cells and labial salivary gland tissue. There was a consistent increase in human CRISP-3 mRNA copy numbers in human submandibular gland cells cultured on growth factor-depleted Matrigel and stimulated with 100 µM DHEA for 24 hours (Figure 1). A clear-cut concomitant increase in the immunofluorescence staining intensity suggested that this mRNA was also translated to the corresponding protein (data not shown). The CRISP-3 mRNA level in human submandibular gland cells cultured without Matrigel was 99 ± 56 copies/10⁵ porphobilinogen deaminase copies. Transdifferentiation of these intercalated duct epithelial cells into secretory acinar cells upon culture on growth factor-depleted Matrigel increased the CRISP-3 mRNA level to 256 ± 207 copies/10⁵ porphobilinogen deaminase copies. CRISP-3 mRNA levels were further up-regulated by DHEA so that the cells cultured in the presence of 100 µM DHEA had 1,274 ± 1,085 CRISP-3 mRNA copies/10⁵ porphobilinogen deaminase copies. Although the range of values in individual experiments varied widely, the direction of the change was always the same, and the rise in CRISP-3 mRNA was significant

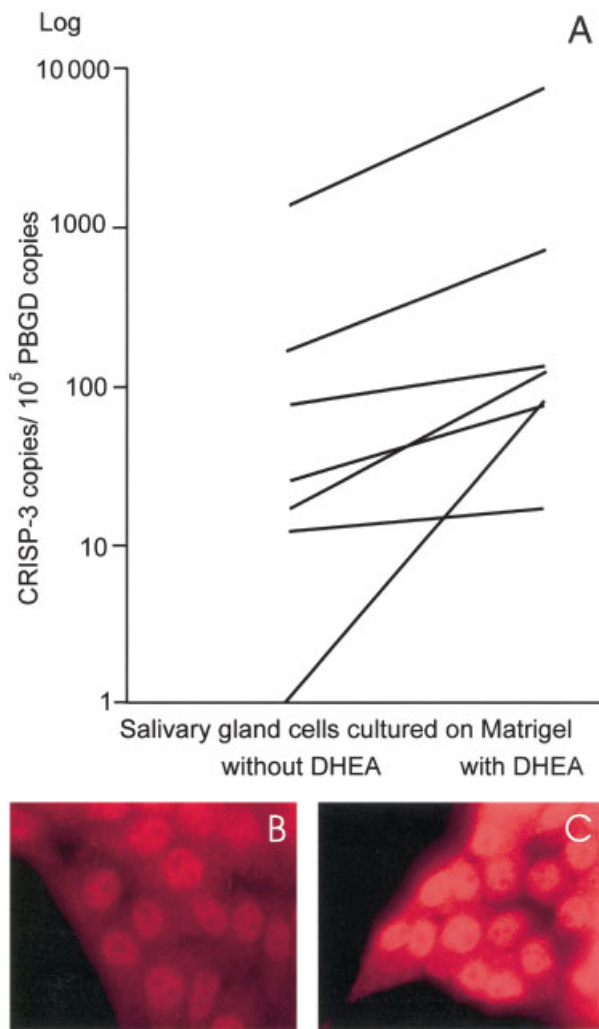


Figure 1. Up-regulation of cysteine-rich secretory protein 3 (CRISP-3) mRNA and protein by dehydroepiandrosterone (DHEA) in cultured human submandibular gland acinar cells in 7 independent experiments. **A**, Number of CRISP-3 mRNA molecules per 10^5 porphobilinogen deaminase (PBGD) mRNA molecules. CRISP-3 was always up-regulated by $100 \mu\text{M}$ DHEA in human submandibular gland cells, which had the acinar cell phenotype since they were cultured on growth factor-depleted Matrigel ($P = 0.018$ versus unstimulated cells, by Wilcoxon's signed rank test). **B** and **C**, Immunofluorescence staining showing up-regulation of the corresponding CRISP-3 protein without DHEA (**B**) or with DHEA (**C**). (Original magnification $\times 100$.)

($P = 0.018$ by Wilcoxon's signed rank test). Explants of human labial salivary glands were stimulated with $100 \mu\text{M}$ DHEA for 72 hours, and these treatments also increased the CRISP-3 mRNA levels (Figure 2).

CRISP-3 staining. In glands of healthy controls, CRISP-3 protein was observed in half-moon-like struc-

tures (demilunes) in the acini, whereas mature mucous cells stained only weakly (Figure 3A). In contrast, in glands of SS patients, CRISP-3 staining was weaker and had lost its polarized organization in the acini (Figure 3B). Salivary ductal epithelial staining was weak in healthy controls (Figure 3A, inset), although CRISP-3 protein in SS patients was found as mucin clots in the lumen of the salivary ducts (Figure 3B, inset). Lymphocytes in the interstitium in healthy glands (Figure 3A) and in lymphocyte foci in SS (Figure 3B) were CRISP-3 negative in paraffin sections. Although labial salivary glands in SS are typically characterized by focal lymphocyte infiltrates, the CRISP-3 staining pattern was pathologic in all acinar cells in all acini in all labial salivary

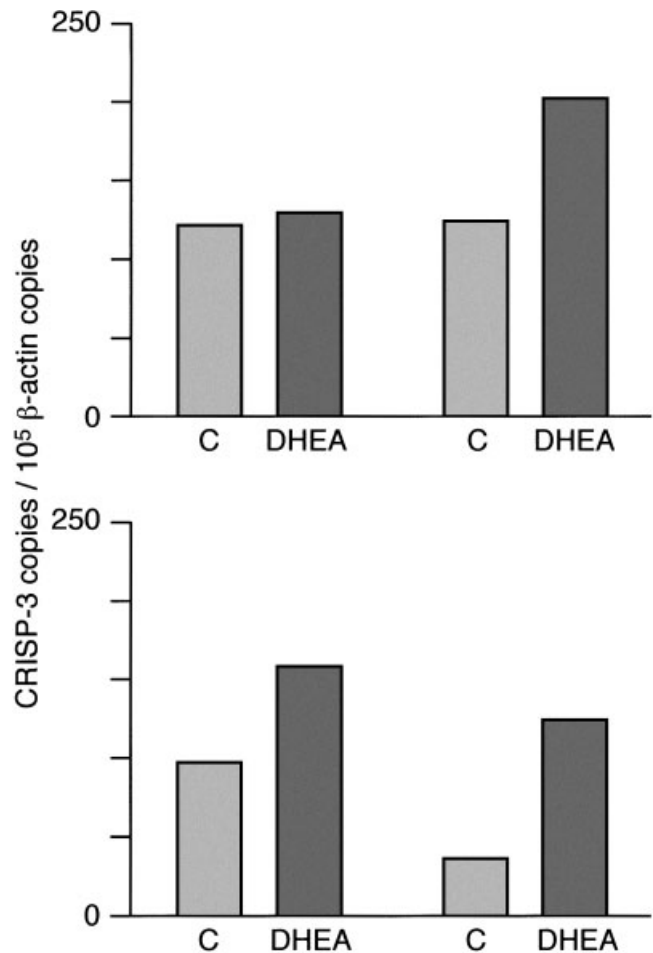


Figure 2. Up-regulation of CRISP-3 mRNA by DHEA in cultured human labial salivary gland tissue explants. Shown are the number of CRISP-3 mRNA molecules per 10^5 β -actin mRNA molecules. Bars show CRISP-3 mRNA values without DHEA (negative controls [C]) and with DHEA in 2 different healthy donors in 2 parallel experiments. See Figure 1 for definitions.

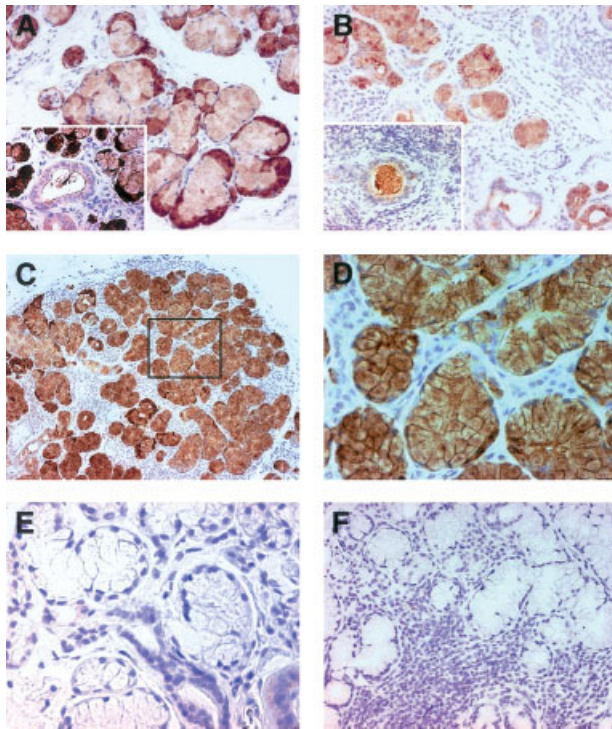


Figure 3. **A,** In healthy labial salivary gland acini, cysteine-rich secretory protein 3 (CRISP-3) staining was strong and practically confined to half-moon-like structures. Salivary ductal epithelial cells did not stain or stained only weakly (**inset** in **A**). **B,** In Sjögren's syndrome (SS), acinar cell staining was weak and disorganized. In SS, the lumen of the salivary ducts sometimes contained intraductal mucin clots, which stained intensely (**inset** in **B**). **C,** Even acini in regions without inflammatory cells clearly exhibited weak and disorganized CRISP-3 staining, with a loss of the polarized organization of CRISP-3 protein into half-moon-like structures. **D,** Higher magnification of boxed area in **C**. **E** and **F,** Negative control staining experiments confirmed the specificity of the staining both in healthy controls (**E**) and in SS patients (**F**). (Hematoxylin counterstained; original magnification $\times 200$ in **A**, **B**, **E**, and **F**; $\times 100$ in **C**; $\times 400$ in **D** and **insets**.)

glands studied in SS. Even acini in regions without inflammatory cells (Figures 3C and D) clearly disclosed weak and disorganized CRISP-3 staining.

Negative staining controls using nonimmune normal rabbit immunoglobulin at the same concentration as (and instead of) the specific primary antibodies verified the specificity of staining in healthy controls and in SS patients (Figures 3E and F). Erythrocytes did not stain, showing successful blocking of the endogenous peroxidase. Occasional CRISP-3-immunoreactive stromal neutrophils served as a positive internal staining control (results not shown).

CRISP-3 mRNA. In glands of healthy controls, CRISP-3 mRNA was observed in the acinar demilunes,

whereas mature mucous cells (Figures 4A and D) and ductal epithelial cells (results not shown) did not label over the background. In contrast, in glands of SS patients, acinar labeling was weak and diffuse and not organized into demilunes. Ductal epithelial cell labeling did not exceed that of the background (Figures 4B and E). Negative labeling controls using sense RNA probes (instead of the CRISP-3-specific antisense RNA probe) disclosed specificity of the in situ hybridization (Figures 4C and F). No labeling was detected in adipocytes, fibroblasts, striated muscle cells, or endothelial cells.

CRISP-3 in saliva. In resting mixed whole saliva, the CRISP-3 concentration (in $\mu\text{g}/\text{mg}$ salivary protein) was low in SS patients compared with that in healthy controls ($P < 0.005$). When the salivary flow was taken into consideration, the difference in the CRISP-3 output between SS patients and healthy controls became highly significant ($P < 0.0001$). In stimulated mixed whole saliva, the CRISP-3 concentration and the CRISP-3 output were both low in SS patients compared with those in healthy controls (both $P < 0.0001$) (Table 2).

DISCUSSION

We used CRISP-3 as a biomarker for local androgen effects (29). The activity of the *crisp-3* gene in the mouse salivary gland is strongly androgen dependent (18). CRISP-3 mRNA levels are much higher in the lacrimal glands of male than female BALB/c and NOD mice (19). Accordingly, structural analysis of 800 bp of the mouse *crisp-3* gene promoter region disclosed 2 regions almost exactly matching the androgen-responsive consensus element (20). Functional studies showed that gonadotropin-releasing hormone antagonist in male mice decreased CRISP-3 expression in the salivary glands in spite of an up-regulation of androgen receptor transcript levels (21). Testosterone treatment of ovariectomized female mice increased CRISP-3 mRNA levels significantly, and this effect was prevented by concomitant treatment with an antiandrogen (21). DHEA-androgen receptor complex binds to the AREs (22). A gene bank search disclosed a total of 13 direct or indirect putative AREs in the human *crisp-3* gene complex. This suggests that the human *crisp-3* gene, quite like its murine equivalent, is controlled by androgens.

Although the structural basis for androgen regulation of the *crisp-3* gene exists, it was necessary to confirm these findings and to determine whether DHEA stimulation works in functional studies. Intercalated duct cells have been considered to represent a potential pool of undifferentiated progenitor cells, which help to

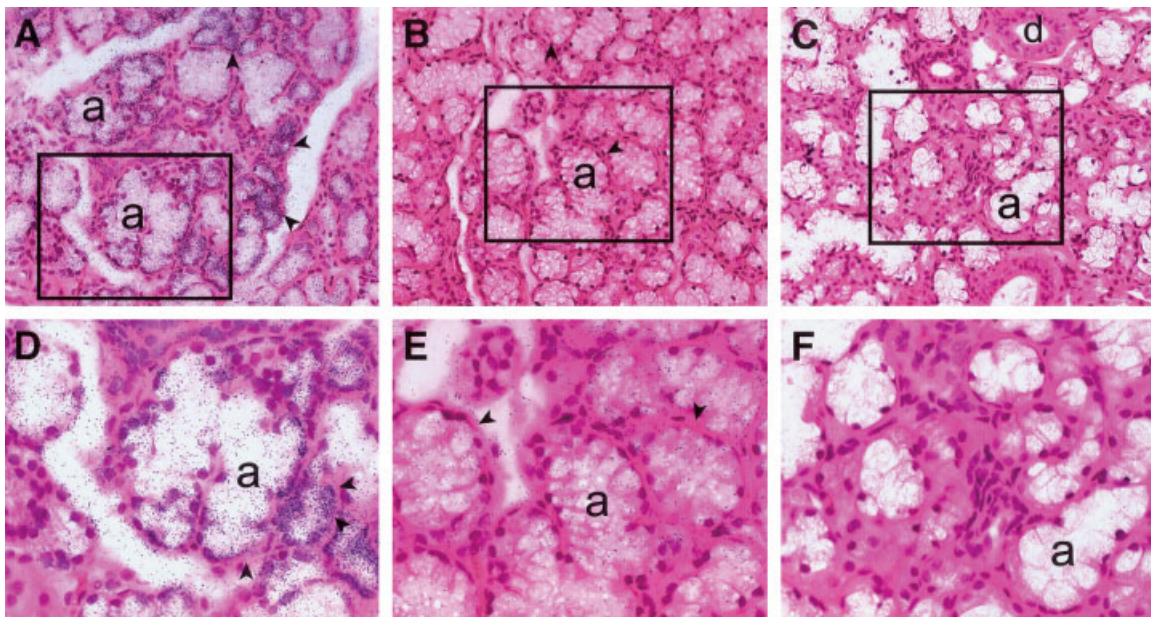


Figure 4. Cysteine-rich secretory protein 3 (CRISP-3) mRNA in healthy and diseased salivary glands. **A**, In situ hybridization disclosed relatively intense accumulation of autoradiography grains (CRISP-3 mRNA) in healthy labial salivary glands into half-moon-like structures (**arrowheads**). **B**, In contrast, labeling of acini (**arrowheads** in **B** and **E**) was weak and diffuse in the glands of patients with Sjögren's syndrome. **C**, The negative labeling control, in which a sense probe was used, confirmed the specificity of the hybridization. **a** = acini; **d** = duct. **D-F**, Higher magnification of boxed areas in **A-C**, respectively. (Hematoxylin counterstained; original magnification $\times 200$ in **A-C**; $\times 300$ in **D-F**.)

renew acinar cells (23,30). In accordance with this hypothesis, intercalated human submandibular gland duct cells were shown to differentiate to acinar cells (24) when cultured on basement membrane-like growth factor-depleted Matrigel, similar to the acinar basement membrane of the human labial salivary glands (31). CRISP-3 mRNA copy numbers consistently increased when the human submandibular gland cells were stimulated with DHEA. Although the number of CRISP-3 mRNA molecules varied 1,000-fold in different experiments, the direction of the change was always the same. These structural and functional findings show that, quite like the mouse *crisp-3* gene, the human *crisp-3* gene is also regulated by androgens.

The effect of DHEA on CRISP-3 mRNA induction in the human submandibular gland cell line is an interesting result. However, it is not quite certain whether this finding can be extrapolated to the labial salivary glands in SS patients, considering all the autocrine and paracrine effects that control glandular function. It is therefore of interest that DHEA stimulation of explants of labial salivary glands from 2 different healthy individuals in 2 parallel experiments resulted in a clear increase in the CRISP-3 mRNA copy number. Together with the human submandibular gland results, this further strengthens the impression that CRISP-3 is regulated by DHEA.

After these experiments, work was continued by

Table 2. CRISP-3 levels in resting and stimulated mixed saliva*

	Healthy controls	SS patients	<i>P</i>
Resting			
CRISP-3, $\mu\text{g}/\text{mg}$ salivary protein	14.0 ± 1.4	8.4 ± 1.4	<0.005
CRISP-3 output, $\mu\text{g}/15$ minutes	97.6 ± 12.0	21.1 ± 2.7	<0.0001
Stimulated			
CRISP-3, $\mu\text{g}/\text{mg}$ salivary protein	13.1 ± 0.9	3.7 ± 0.4	<0.0001
CRISP-3 output, $\mu\text{g}/15$ minutes	240.1 ± 26.2	53.8 ± 7.1	<0.0001

* Values are the mean \pm SEM. CRISP-3 = cysteine-rich secretory protein 3; SS = Sjögren's syndrome.

using CRISP-3 as a biomarker for local androgen effects in human salivary glands in healthy controls and in SS patients. In normal labial salivary glands, acinar demilunes stained very strongly, whereas mature mucous cells in the resting phase, filled with mucins, did not stain. In situ hybridization confirmed these findings regarding acinar organization of CRISP-3 mRNA in healthy controls and in SS patients. While both CRISP-3 mRNA and protein were found in the secretory acinar cells, CRISP-3 output to saliva was also high in healthy controls compared with that in SS patients. In clear contrast to that in healthy controls, CRISP-3 staining in SS patients was not confined to the acinar demilunes but was found in all acinar cells, which stained weakly in a diffuse manner. In in situ hybridization, CRISP-3 mRNA expression was very weak, and the autoradiography grains were not organized into demilunes but were diffusely distributed. Accordingly, CRISP-3 output to saliva was very low in SS patients. All these findings indicate that the expression level of CRISP-3 is pathologically low in the exocrine target tissue of SS patients.

In principle, the disturbed and diminished acinar cell CRISP-3 in SS could be due to locally produced cytokines, but this is refuted by the generalized nature of these changes (i.e., all acinar cells seemed to be affected). Our findings would be in accordance with a lack of some systemic factor, which is supposed to be DHEA. In fact, the level of DHEAS, which was shown to be low in serum in the present study, has already been shown to be low in SS patients (14). The present study demonstrates that DHEA regulates acinar cell CRISP-3. As a totally new observation, the present study demonstrates that not only serum but also salivary DHEA concentrations are clearly diminished in SS patients compared with healthy controls. Accordingly, CRISP-3 mRNA and protein levels are also low in acinar cells, which are not able to produce normal concentrations of this DHEA marker protein. The different ages at onset in SLE and SS might relate to other associated changes in the sex steroid profile. In women with SLE, the level of estradiol has been shown to be increased and that of testosterone decreased (11), while these values have been reported to be normal in SS patients (8,14).

CRISP-3 expression and output in salivary glands from SS patients are low, and the fact that those patients also have low serum levels of DHEAS does not prove that there is a causal connection between the 2 findings. It should also be noted in this context that concentrations of salivary DHEA, more specifically, its activated or desulfated form, were low in SS patients compared with healthy controls, indicating that local salivary gland

DHEA concentrations are low in SS. To test whether the association between the low levels of DHEA and CRISP-3 could be causal, cultured salivary gland acinar cells were exposed to DHEA. These experiments clearly demonstrate that DHEA, at least in vitro, is an important factor able to up-regulate CRISP-3 expression. In principle, the decrease in CRISP-3 concentration in saliva could be a consequence of other factors responsible for the hyposalivation observed in SS patients. Lack of, or at least greatly diminished, expression of laminin α 1-chain has recently been shown in the acinar basement membrane in SS (31). The present study demonstrates that laminin α 1-chain containing Matrigel alone increased the number of CRISP-3 mRNA copies from 99 to 256, but that addition of DHEA further increased the number to 1,274. These experiments clearly demonstrate a dominant up-regulatory effect of DHEA on CRISP-3 mRNA levels.

Interestingly, it has earlier been described that CRISP-3 is found in B lymphocytes in the labial salivary glands of SS patients, while CRISP-3 was not observed at all in B lymphocytes or in acinar or any other epithelial cells in normal labial salivary glands (32). The primers used in that study for RT-PCR and also used to produce in situ hybridization probes recognize something that was found only in SS and was absent from healthy control glands. However, a detailed analysis of the primers used in that study shows that they do not recognize human CRISP-3 or any other known member of the human CRISP family. Interest in this molecule of obscure identity was raised by a differential expression of that molecule between 1 SS patient and 1 healthy control (32). In the present study, CRISP-3 was found, quite as expected, also in normal healthy human salivary gland acinar cells, at both the mRNA and protein levels, as well as in saliva.

Our findings are in accordance with those of Sullivan and coworkers, who have worked with lacrimal glands in an experimental mouse model of SS. Androgens effectively increase the expression of their own receptors (33) and have an immunosuppressive impact on lymphocyte infiltration (34). Some of these effects represent direct effects on lacrimal gland acinar cells and their function (35). These investigators also first reported that CRISP-3 in mouse lacrimal glands is regulated by androgens (19).

In conclusion, CRISP-3 deficiency in salivary glands in SS might be caused by low levels of the circulating and salivary DHEA prohormone and/or by its inefficient intracrine processing in salivary glands. These findings suggest that the prime target tissue of this

syndrome has a deficiency of DHEA effect. This may influence the fate of the acinar cells (potential autoantigen depots) and subsequent activation and expansion of autoreactive B lymphocytes. The present observations provide novel insight into the endocrine effects at the target tissue level in this autoimmune disease predominantly affecting females and with a late age at onset. More attention should be paid to disease-specific and organ-targeting pathomechanisms, which may contribute to typical end-organ involvement and autoantibody profiles.

AUTHOR CONTRIBUTIONS

Dr. Konttinen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Laine, Porola, Udby, Kjeldsen, Konttinen.

Acquisition of data. Laine, Porola, Udby, Kjeldsen, Cowland, Borregaard, Hietanen, Ståhle, Pihakari, Konttinen.

Analysis and interpretation of data. Laine, Porola, Hietanen, Konttinen.

Manuscript preparation. Laine, Porola, Udby, Kjeldsen, Cowland, Borregaard, Hietanen, Konttinen.

Statistical analysis. Laine, Porola.

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