Androgens and Integrins in Salivary Glands in Sjögren's Syndrome

PAULIINA POROLA, MIKAEL LAINE, ISMO VIRTANEN, RAIMO PÖLLÄNEN, BEATA D. PRZYBYLA, and YRJÖ T. KONTTINEN

ABSTRACT. ***Objective.*** Laminin α1-chain normally induces intercalated duct progenitors to differentiate to acinar cells through integrin (INT) α1ß1 and α2ß1 receptors. Maintenance of acinar cells is impaired in Sjögren's syndrome (SS), which is also characterized by low levels of serum and salivary androgens. We hypothesized that androgens normally support salivary gland remodeling by upregulating either laminin α1 chain or its cellular α1 or α2 INT subunit-containing receptors.

Methods. Intercalated duct and acinar human salivary gland (HSG) cells and labial salivary gland (LSG) biopsies from healthy controls and patients with SS were cultured without or with sex steroids. Laminin α1 chain and INT α1 and α2 subunits were studied using quantitative reverse-transcription real-time polymerase chain reaction and INT α1 and α2 subunits using immunofluorescence staining.

Results. INT α1-subunit and α2-subunit messenger RNA (mRNA) levels were increased in intercalated duct and acinar cells by DHEA and testosterone. In contrast, laminin α1-chain mRNA levels were not affected. The upregulating effect of DHEA on INT subunits was also seen at the protein level. DHEA also increased mRNA levels of both INT subunits in healthy but not SS LSG.

Conclusion. Androgens increased INT α1 and α2 subunits in tubuloepithelial cells and in healthy LSG, but in SS salivary glands this androgen regulation was defective, which is likely to contribute to defective outside-in signaling, acinar atrophy, and ductal cell hyperplasia. (J Rheumatol First Release May 1 2010; doi:10.3899/jrheum.091354)

Key Indexing Terms:
SJÖGREN'S SYNDROME ANDROGENS INTEGRINS LAMININ SALIVARY GLANDS

Sjögren's syndrome (SS)[1] is a complex women-dominant autoimmune disorder affecting salivary and lacrimal glands. The acinar cells of the severely affected glands become atrophic, but the factors responsible for their impaired maintenance are unclear. Normal salivary glands undergo continuous remodeling. To be able to do so, acinar cells undergo apoptosis, detach, and are carried away with salivary flow, leaving a space in the acinar basement membrane. This loss of acinar cells can be compensated by intercalated duct cells. These morphologically simple, undifferentiated progenitor cells undergo asymmetric divisions so that the progenitor cell pool is maintained at the same time that one of the daughter cells migrates to the acinus and receives site-specific signals for acinar differentiation to replace the lost acinar cell[2,3]. Cloned intercalated duct cells have been shown to possess such a differentiation potential[4,5]. In this acinar differentiation, process signaling between extracellular matrix (ECM) and cells is essential. Signaling between laminin α1 chain and its cellular ß1 subunit containing integrin (INT) receptors is especially important[6].

It has recently been shown that the major acinar cell INT able to mediate binding to acinar basement membrane-confined laminin-111 (LM-111) are α1ß1 and α2ß1 integrins, which both contain the ligand-binding I domain[7,8]. Thus it seems that in addition to LM-332-INT α6ß4 interaction[7,9], one of the central interactions necessary for the maintenance of acini occurs between the laminin α1-chain containing LM-111 in the acinar basement membrane and its α1ß1 and α2ß1 integrin receptors on the tubuloacinar epithelial cells. Interestingly, SS is characterized by low levels of acinar

From the Department of Medicine, Helsinki University Central Hospital; Department of Anatomy, University of Helsinki, Helsinki; ORTON Orthopaedic Hospital of the ORTON Foundation, Helsinki; COXA Hospital for Joint Replacement, Tampere, Finland; and the Institute of Aging, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA.

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P. Porola, MSc, Department of Medicine, Helsinki University Central Hospital, Department of Anatomy, University of Helsinki; M. Laine, MD, DDS; I. Virtanen, MD, PhD, Department of Anatomy, University of Helsinki; R. Pöllänen, PhD, Department of Medicine, Helsinki University Central Hospital; B.D. Przybyla, PhD, Institute of Aging, University of Arkansas for Medical Sciences; Y.T. Konttinen, MD, PhD, Department of Medicine, Helsinki University Central Hospital, ORTON Orthopaedic Hospital of the ORTON Foundation, COXA Hospital for Joint Replacement.

Address correspondence to Prof. Y.T. Konttinen, Department of Medicine, Biomedicum 1, Helsinki, Helsinki University Central Hospital, PO Box 700, FIN-00029 HUS, Helsinki, Finland. E-mail: yrjo.konttinen@helsinki.fi

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compartment-specific α1 laminin[10] and low levels of its integrin α1ß1 and α2ß1 receptors on acinar cells[8]. Additionally, in the early stages of SS, the ECM is actively remodeled, possibly as an attempt to diminish the acinar cell loss by enhancing the interaction between those cells and the acinar basement membrane[9]. In agreement with the intercalated duct progenitor cell hypothesis, advanced SS is characterized by diminished ECM remodeling, loss of acinar cells, and a reciprocal ductal cell hyperplasia[9,11-13].

Patients with SS have low concentrations of the prohormones DHEA and androstenedione, and the active androgens testosterone and dihydrotestosterone (DHT) in serum and saliva[14,15]. Humans and other primates are unique in being able to synthesize active sex steroids both in gonads and locally in peripheral tissues. This is possible because adrenal glands secrete high amounts of prohormones, which are further processed into active androgens or estrogens by intracrine steroidogenic enzymes, according to local tissue needs[16]. Thus, concentrations of sex steroids in peripheral tissues can differ from the corresponding systemic levels. It has recently been shown that salivary glands possess organized enzymatic functions needed for local production of sex steroids for intracrine use. But in SS salivary glands the androgen-processing enzymes are deranged further, deteriorating the local androgen depletion[17].

Signaling between extracellular matrix and cells through acinar-specific laminin α1 (or actually LM-111)–INT α1ß1/α2ß1 interactions is essential for salivary glands[6]. Results from our studies[8,10] led to the hypothesis that these molecules, laminin α1 and/or its INT receptors α1 and α2, which are expressed in low numbers in androgen-deficient salivary glands in SS[8,10], are normally upregulated by sex steroids. In other words, androgens would help to maintain acini, the secretory end pieces, in healthy salivary glands, but fail to do this in SS. In our study we tested our hypothesis at the cellular level, using a well established *in vitro* human submandibular gland cell model, the human salivary gland (HSG) cell line[18], and tried to extend this to the tissue level with labial salivary gland (LSG) obtained from healthy controls and patients with SS.

MATERIALS AND METHODS

Cell culture and stimulation. HSG cells[18] were cultured without or with Matrigel (BD Biosciences, San Jose, CA, USA) in DMEM/F-12 Nut Mix medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C. Without Matrigel these cells maintain their intercalated duct phenotype, but on LM-111-containing Matrigel they differentiate to acinar cells[19]. The HSG cells, with either ductal or acinar cell phenotype, were cultured in a fetal calf serum (FCS)-free medium without or with 1, 10, and 100 μM DHEA (Sigma-Aldrich, St. Louis, MO, USA), 1 μM testosterone (Sigma-Aldrich), 1 and 10 nM and 1 μM DHT (Sigma-Aldrich), or 100 nM 17ß-estradiol (Sigma-Aldrich) for 48 or 72 hours.

Tissue culture and stimulation. LSG biopsies were obtained from 4 healthy controls who were treated for mucocele (3 women, 1 man, ages 17, 17, 21, and 57 yrs) and 3 patients with SS (all women, ages 44, 58, and 65 yrs). Glands were split into pieces of about 2 mm^3 and cultured overnight in Dulbecco modified Eagle (DME)-Ham's F-12 medium (Gibco) containing 10% FCS, L-glutamine, 10× antibiotics (1000 U/ml penicillin and 1000 μg/ml streptomycin), and 2.5 μg/ml amphotericin B (Gibco). The next day the media were replaced with basal DME-Ham's F-12 medium containing 10% FCS, L-glutamine, 1× antibiotics, and 2.5 μg/ml amphotericin B, and after 1 more day with serum-free media for stimulation with 100 μM DHEA for 72 hours.

RNA isolation and cDNA synthesis. Cells grown on Matrigel were detached from the Matrigel with dispase (BD Biosciences). Total RNA was isolated using the Trizol protocol (Invitrogen, San Diego, CA, USA) or with RNeasy Mini kit (Qiagen, Hilden, Germany) and messenger RNA (mRNA) was purified from total RNA using the Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway). From tissue samples total RNA was isolated with High Pure RNA Tissue kit (Roche, Basel, Switzerland). cDNA was synthesized using SuperScript First Strand cDNA Synthesis System for quantitative reverse-transcription realtime polymerase chain reaction (RT-PCR; Invitrogen).

Quantitative RT-PCR. Quantitative RT-PCR was done using a Light Cycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany), SYBR Green I label, and purpose-designed primers (laminin α1 forward 5'-GCT CTG TGA CTG CAA ACC AA-3' and reverse 5'-TTT CTG GGT CGC AGG TAT TC-3'; integrin α1 forward 5'-TCC ACC GAA GAG GTA CTT GTT GCA-3' and reverse 5'-CCA AGC ATG ACC CAG TCC TGT GA-3'; and integrin α2 forward 5'-GGT GAG GAT GGA CTT TGC AT-3' and reverse 5'-GGC TTG GAA ACT GAG AGA CG-3'). Porphobilinogen deaminase (PBGD) and ß-actin housekeeping genes were used for standardization of the results (in cell and tissue samples, respectively) by using 5'-ACA TGC CCT GGA GAA GAA TG -3' and 5'-AGA TGC GGG AAC TTT CTC TG-3' and 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3' primers for PBGD and ß-actin, respectively.

Indirect immunofluorescence of integrin receptors. For indirect immunofluorescence staining of the integrin-type laminin receptors, which have been described in human LSG[8], the following monoclonal antibodies were used: TS2/7 against integrin α1-subunit[20] and 10G11 against integrin α2-subunit[21]. Cells were washed in 10 mM phosphate-buffered saline, 150 mM saline, pH 7.4, containing 0.1% Triton X-100. After incubation with primary antibodies, cells were washed in Triton X-100 containing phosphate buffer and the bound antibodies were visualized using FITC-conjugated secondary goat anti-mouse IgG antibody (Alexa Fluoro 488, Molecular Probes, Eugene, OR, USA). Propidium iodide diluted 1:1000 in phosphate buffer was used for nuclear counterstaining. After washes in Triton X-100 containing phosphate buffer, the specimens were embedded in fluorescent mounting medium (Dako, Glostrup, Denmark) and examined under an Olympus AX70 (Tokyo, Japan) microscope coupled with a CCD camera (Olympus DP71). Two different types of filters were used in order to show the FITC-positive staining with or without propidium iodide. Control immunostainings were performed using irrelevant primary monoclonal antibodies at the same concentration as and instead of the primary specific antibodies and by using conjugated secondary antibodies alone.

Statistical analysis. Statistical analysis was done using SPSS for Windows V.16.0 software (SPSS Inc., Chicago, IL, USA). Integrin α1 and α2 mRNA levels without and with hormone treatments were compared using the Mann-Whitney U test and the overall effects of stimulations with different androgen concentrations studied with the Kruskal-Wallis test. The level of significance throughout the study was set at 0.05.

RESULTS

Effect of Matrigel on expression of laminin α1 chain and integrin α1 and α2-subunits in salivary gland cells. Culture on Matrigel causes differentiation of HSG cells of intercalated duct phenotype to acinar cells, which increased the

mRNA levels of laminin α1 constantly over 72 hours [copy numbers per 10^5 PBGD, 780 ± 668 (n = 3), 1262 ± 505 (n = 3), and 3233 ± 3512 (n = 4) for 24-hour, 48-hour, and 72-hour timepoints, respectively].

Also, levels of INT α1 (p = 0.013; Figure 1A) but not those of α2-subunit mRNA (p = 0.333; Figure 1B) increased upon culture on Matrigel. The corresponding phenomenon was seen at the protein level in INT α1-subunits (Figure 2).

Effect of sex steroids on expression of laminin α1 and INT α1 and α2-subunits in salivary gland cells. Culture in the presence of sex steroids did not affect laminin α1-chain mRNA levels in ductal or acinar HSG cells or LSG biopsies (data not shown).

Expression of the mRNA levels of INT α1 and α2-subunits in ductal (cultured without Matrigel) and acinar (cultured on Matrigel) HSG cells was significantly increased after 72-hour stimulation by DHEA.

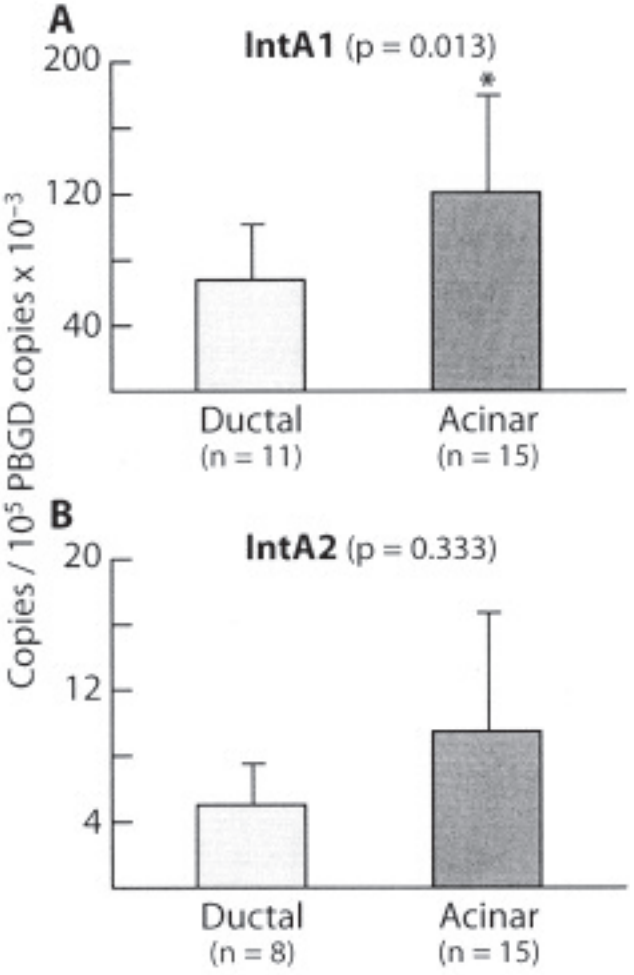

Figure 1. Effect of 72-hour culture on Matrigel on mRNA levels of integrin (INT) α1 (A) and α2 (B) chains in human submandibular gland HSG cells. Levels of INT α1-chain mRNA (scale to 200,000) were much higher in ductal (without Matrigel) and acinar (with Matrigel) cells than levels of INT α2-chains (scale to 20,000). *Statistically significant differences, p < 0.05.

In intercalated duct cells, 1, 10, and 100 μM DHEA increased INT α1-subunits by 37% (p = 0.312), 46% (p = 0.016), and 449% (p = 0.004), respectively. The corresponding increases in INT α2-subunits were 54% (0.153), 640% (p = 0.003), and 1100% (p = 0.007) (Figure 3).

In acinar cells 1, 10, and 100 μM DHEA induced changes of INT α1-subunits by 90% (0.053), 102% (p = 0.009), and 113% (p = 0.655), respectively, and of INT α2-subunits by 39% (p = 0.551), 473% (p = 0.046), and 3% (p = 0.594), respectively (Figure 3).

Stimulation with 1 μM testosterone increased INT α1 and α2-subunits by 79% (p = 0.009) and 120% (p = 0.020), respectively, in intercalated duct cells. In acinar cells, testosterone changed INT α1 and α2-subunits by 72% (p = 0.026) and 96% (p = 0.086), respectively (Figure 4).

Stimulation with 1 nM, 10 nM, or 1 μM DHT showed only a tendency by changing the expression of INT α1-subunit mRNA levels/10^5 PBGD in ductal cells by 33%, 31%, and 55% (66,620 ± 35,175, n = 11, vs 88,619 ± 20,818, n = 3; 87,172 ± 19,015, n = 3; and 102,971 ± 7663, n = 3, respectively). The corresponding changes of INT α2-subunit were 41%, 3%, and 62% (5008 ± 2467, n = 8, vs 7064 ± 1231, n = 3; 5170 ± 470, n = 3; and 8137 ± 1298, n = 3). In acinar cells the changes in INT α1-subunit were 16%, 94%, and 70% (120,926 ± 59,627, n = 8, vs 140,696 ± 99,367, n = 2; 234,734 ± 122,191, n = 3; and 205,284 ± 95,411, n = 3) and for INT α2-subunit 135%, 89%, and 90% (9422 ± 7167, n = 15 vs 22,135 ± 19,277, n = 2; 17,835 ± 11,051, n = 3; and 17,932 ± 6692, n = 3). Despite consistent upregulation at all concentrations used, these increases were not statistically significant. Estradiol did not have significant effects on INT subunits (data not shown).

At the protein level, both ductal and acinar HSG cells cultured 48 and 72 hours in the presence of DHEA contained more cells intensively positive for INT α1-subunit, compared to the cells cultured in the absence of DHEA (shown for acinar cells in Figure 2). According to results from qRT-PCR, INT α2-subunit immunostaining was much weaker than that of the INT α1-subunit (not shown). Staining controls were negative, confirming the specificity of the staining with the monoclonal antibodies used in our study.

Effect of DHEA stimulation on laminin α1 chain and INT α1 and α2-subunits in salivary gland biopsies. Extending these observations to tissue samples, we observed that in agreement with the cell stimulations, the expression of laminin α1-chain in LSG of healthy controls and patients with SS was unaffected by stimulation with androgens (data not shown). The expression of INT α1 and α2-subunits was increased by DHEA stimulation in LSG from healthy controls, but in LSG from patients with SS such an increase was not seen (Figure 5). The effect of DHEA on the expression of INT α1 and α2 subunits showed a slight tendency to diminish with increasing age in both healthy and SS labial salivary glands (data not shown).

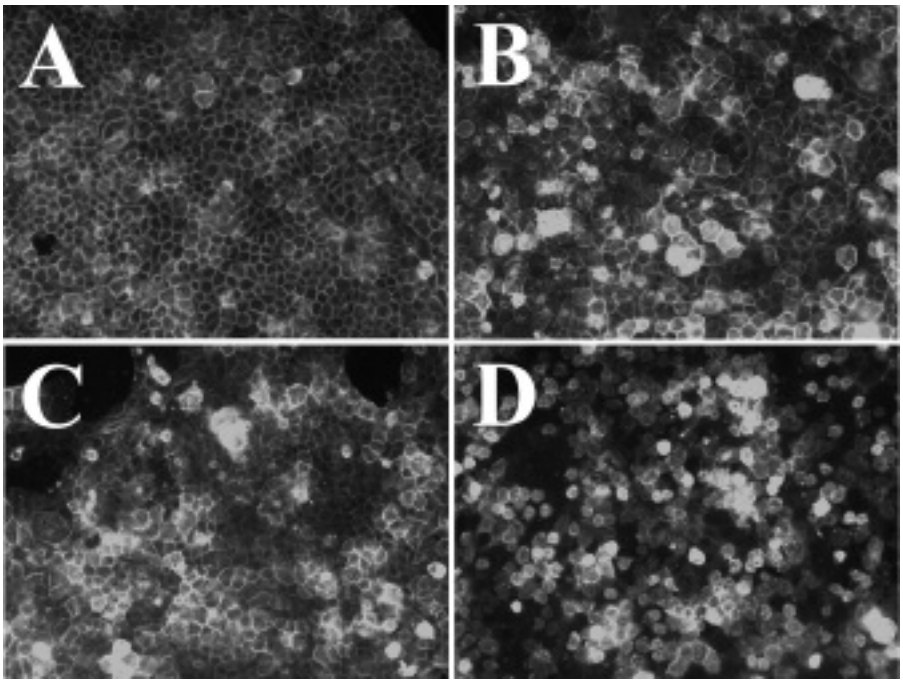

Figure 2. Immunofluorescence staining of integrin (INT) α1-subunit in human submandibular gland HSG cells cultured on Matrigel without or with DHEA for 48 and 72 hours (B and D, respectively). Compared to non-DHEA-stimulated cells (A, 48 h; C, 72 h), a clear increase of INT α1-subunit staining intensity is seen upon DHEA costimulation (DHEA and Matrigel). Some increase in the intensity of INT α1-subunit staining is also seen in nonstimulated cells (A compared to C). Original magnification 200×.

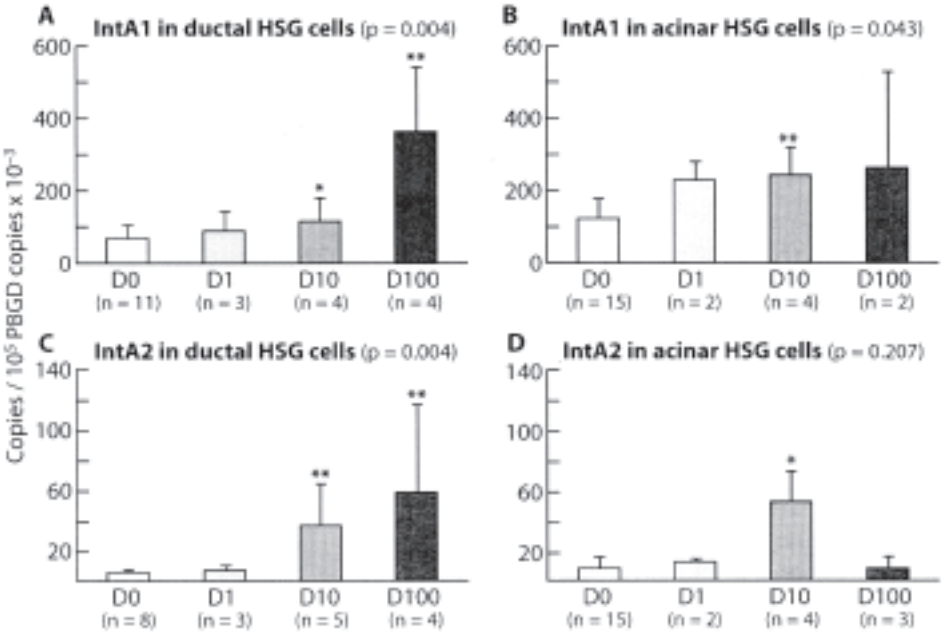

Figure 3. Effect of DHEA stimulations on the expression of integrin (INT) α1 (A and B) and α2 (C and D) subunits in ductal and acinar human submandibular gland HSG cells (cultured without or with Matrigel, respectively). Stimulations were done using 1, 10, or 100 μM DHEA for 72 hours (D1, D10, D100, respectively; D0: nonstimulated samples). Overall significance (Kruskal-Wallis test) is shown in the heading for each panel. *$p < 0.05$, ** $p < 0.01$, individual statistically significant differences with nonstimulated samples.

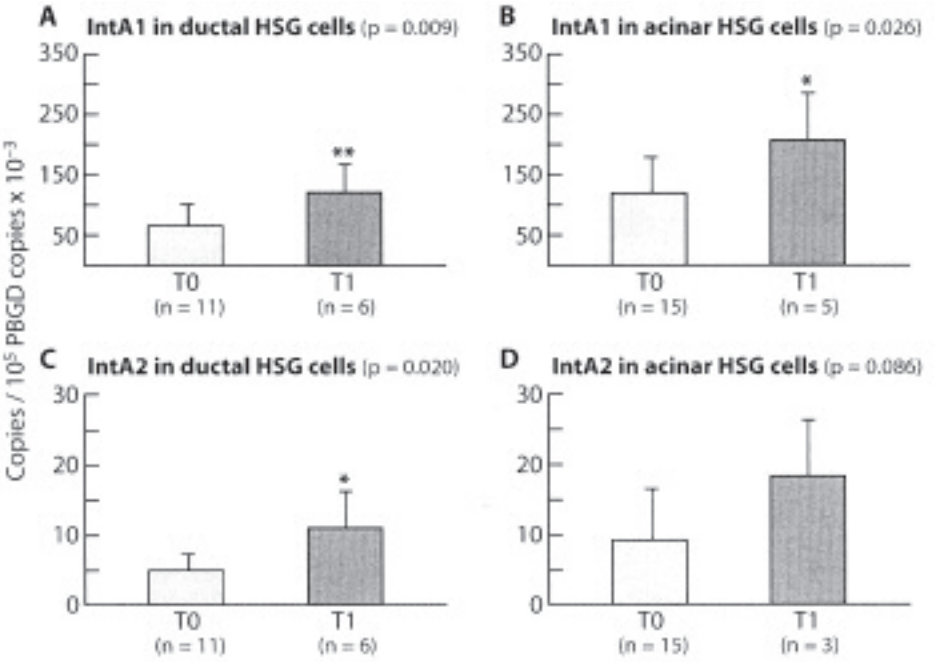

Figure 4. Effects of testosterone stimulations on the expression of integrin (INT) α1 (A and B) and α2 (C and D) subunits in ductal and acinar human submandibular gland HSG cell line (cultured without or with Matrigel, respectively). Stimulations were done using 1 μM testosterone for 72 hours. *p < 0.05, **p < 0.01.

DISCUSSION

Sjögren's syndrome is characterized by low levels of androgens both at the endocrine level in the systemic circulation and at the intracrine level locally in salivary glands[14,15]. Earlier findings also suggested deficiencies in acinar-specific laminin molecules signaling from basement membrane to tubuloacinar epithelial cells, namely LM-111 signaling through the integrin receptors α1ß1 and α2ß1, in salivary glands in SS[8,10]. Our hypothesis was that these characteristic features are interconnected. Results confirm our hypothesis and show that sex steroids increase the expression of α1ß1 and α2ß1 INT receptors for LM-111 in ductal and acinar cells. Interestingly, of the androgens used for stimulations, DHEA proved to be the most effective upregulator of both INT subunits and showed a dose-dependent behavior (except for INT α2-subunit in acinar HSG cells). That DHT was not as effective could be for several reasons relating to dose, timing, passive cellular uptake by diffusion, intracrine balance between different sex steroids, or their nongenomic actions. Nevertheless, physiological concentrations of androgens elevated the levels of both INT. Further, these INT receptors were relatively low in intercalated duct cells, but increased about 2-fold upon differentiation to acinar cells on Matrigel, suggesting that contact with laminin α1 and in particular with DHEA and its metabolites increases these tubuloacinar cell-specific INT receptors[8].

In some experiments, DHEA upregulated INT subunits in healthy salivary glands. However, in SS salivary glands this androgen-INT link was interrupted, suggesting faulty intracrine DHEA processing[15,17]. Since the composition and remodeling of ECM is affected by age[22,23], we also studied DHEA regulation of INT α1 and α2 in relation to age. The effect of DHEA treatment on the expression of these integrin subunits was perhaps slightly more remarkable in younger individuals and diminished in older tissue, but interindividual variation was also quite remarkable. Additionally, the upregulating effect of DHEA seen in healthy LSG was minor compared with effects seen at the cellular level, especially for INT α1, and this is possibly explained by the presence of INT α1 in cells other than salivary epithelial cells. This effect is also indicated by the finding that the mRNA levels of both INT subunits were higher in LSG in patients with SS than in healthy individuals. INT α1 and α2-subunits are expressed in CD4-positive and CD8-positive T lymphocytes and lymphocyte infiltrates[24]. The expression of INT α1 and α2 has been shown in infiltrating T cells in the chronic inflammatory environment and has been suggested as important for generation of inflammatory responses in tissues[25] in, for example, rheumatoid arthritis[26,27].

Current observations demonstrate for the first time a link between 2 features of SS, androgen depletion[15,17] and extracellular matrix-INT signaling[8,10], by suggesting that androgens regulate molecules involved in matrix-cell signaling. These results highlight the importance of locally balanced intracrine processing and the effects of DHEA. DHEA was incapable of increasing the expression of INT in LSG of

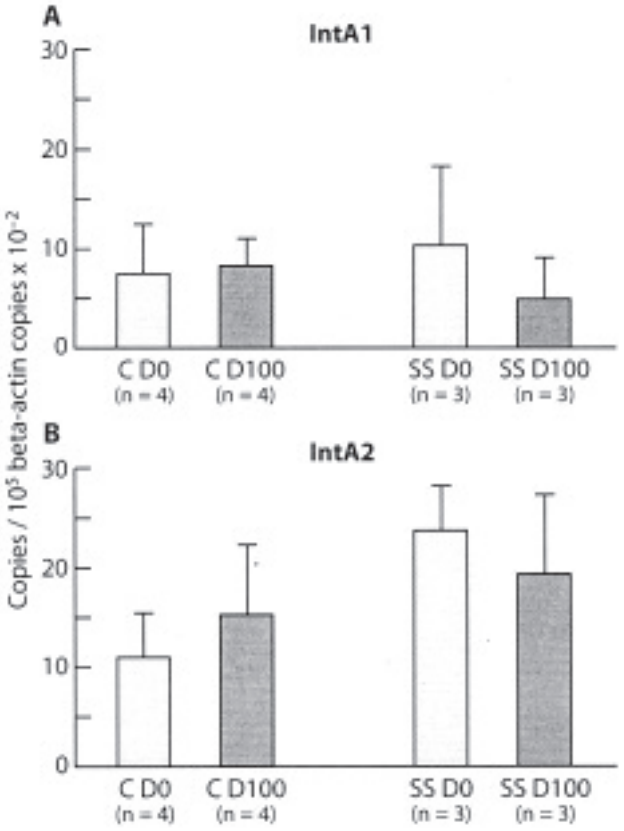

Figure 5. The effect of 100 μM DHEA stimulation for 72 hours on expression of INT α1 (A) and α2 (B) subunits in labial salivary glands of patients with SS (n = 3) and healthy controls (C, n = 4). Nonstimulated samples are marked C D0 (controls, no DHEA) or SS D0 (no DHEA), and DHEA-stimulated samples C D100 (100 μM DHEA) or SS D100.

patients with SS. In addition to the LM-111-INT α1ß1/α2ß1 connection, the connection between LM332 and INT α6ß4 is vital for acinar cells. These molecules form adhesion complexes and thus cooperate in attachment of acinar cells to ECM. In SS, disturbances in the expression and localization of these INT molecules have been reported[28] and may lead to detachment of acinar cells and further contribute to acinar cell loss. In our study we concentrated on the effects of androgens on acinar-specific INT subunits α1 and α2, which have been shown to be decreased in SS[8], but it would be interesting to study the eventual androgen regulation of INT α6ß4 as well. If such a regulation were found, it would mean that androgen depletion in SS deteriorates the attachment, differentiation, and survival of salivary gland acinar cells.

We observed that androgens upregulate molecules essential for progenitor cell migration along, and matrix cell signaling from, LM-111 in the basement membrane to tubuloacinar epithelial cells in human salivary glands. Through this mechanism, low levels of DHEA and its metabolites in salivary glands in patients with SS could impair maintenance of acini.

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