Pathological Changes at the Target Tissue Level in Sjögren’s Syndrome and Their Effect on the Exocrine Function of the Salivary Glands

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals I–IV.


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2. ABSTRACT

Sjögren’s syndrome (SS) is a common autoimmune disease affecting the exocrine salivary and lacrimal glands. Histopathological findings in salivary glands include lymphocyte infiltrates, acinar cell atrophy and ductal cell hyperplasia. Clinical symptoms of SS include dry eyes and mouth as well as fatigue, Raynaud’s phenomenon and arthralgia. Other characteristic clinical features are a considerable female predominance, with 9:1 female-to-male ratio, and a late age of onset, commonly at the time of menopause at age of 40-50 years. The levels of the androgen prohormone dehydroepiandrosterone-sulphate (DHEA-S) in the serum are lower in patients with SS than in age- and sex-matched healthy control subjects. The eventual systemic effects of low androgen levels in SS are not currently well understood.

Basement membranes (BM) are highly specialized layers of extracellular matrix underneath the epithelial cells present in all epithelial structures. The BMs are composed of two independent matrix networks, namely αβγ-heterotrimeric laminin (LM) glycoproteins and type IV collagens interconnected together by nidogen-1 link protein. BMs are able to deliver messages to the above lying epithelial cell via cellular laminin-receptors. These receptors include the αβ-heterodimeric transmembrane proteins called integrins (Int) as well as some non-integrin receptors such as α- and β-dystroglycan and Lutheran blood group antigen (Lu).

The laminin composition of BMs is tissue specific and the first aim of this study was to assess the distribution of laminins in labial salivary glands (LSGs) of normal healthy controls and patients with SS. This profiling was done using labeling of laminin chains with a wide selection of chain-specific monoclonal antibodies (MAb) and pattern recognition analysis of the labeled specimen. Results show that laminins have a complex distribution in LSGs. Because differences exist in the distribution and expression of laminins between healthy controls and patients with SS, laminins are suggested to have specific tasks in the dynamic regulation of the acinar cell function. Acinar BM LM-111 seems to be important for the normal acinar cell differentiation. Its expression was clearly diminished in patients with SS. Also LM-211 and -411 seem to have acinar specific but somewhat less clear functional tasks in the LSGs. Other laminins such as LM-311, -332 and -511 seem to have more general structure maintaining and supporting roles in LSGs and were found to be relatively intact also in SS.

The study was continued by the assessment of the integrin and non-integrin laminin receptors using immunohistochemistry with monoclonal antibodies to Int subunits, Lu and α-dystroglycan. The results show that also the integrin and non-integrin receptors of laminins have complex distribution in LSGs. Ints α3β1, α6β1
and α6β4 as well as the Lutheran blood group antigen seem to have glandular architecture maintaining role in the LSGs supplying structural basis for the firm attachment of epithelial cells to the BM. The expression of Ints α1β1 and α2β1 differed clearly from other Ints in that they were found almost exclusively on the acinar and intercalated duct cells in salivons. This suggests some type of acinar cell compartment-specific or -dominant function. Expression of these integrins was lower in SS compared to healthy controls. This suggests that the LM-111 and -211-to-Int α1β1 and α2β1 interactions are defective in SS and that these interactions are necessary for the maintenance of the acini in LSGs.

The third aim of the study was to test the hypothesis that SS is characterized by an insufficient androgen effect on the salivary glands. First search for androgen responsive elements (AREs) in the crisp-3 gene was performed. Then dehydroepiandrosterone (DHEA) responsiveness was studied experimentally using quantitative real time-polymerase chain reaction (qRT-PCR) and immunofluorescence staining of human submandibular gland-derived acinar cells and LSG explants with and without DHEA. Glandular and salivary cysteine-rich secretory protein-3 (CRISP-3) in healthy controls and patients with SS was analyzed using immunohistochemistry, in situ hybridization, and enzyme-linked immunosorbent assay (ELISA). Finally, serum DHEA-S and salivary DHEA levels were measured using a radioimmunometric method. The results show that low DHEA/DHEA-S concentrations in serum and locally in saliva of patients with SS seem to affect the salivary glands. This effect on salivary glands was detected using the androgen-dependent CRISP-3 protein, the production and secretion of which were clearly diminished in SS. This may be due to the impaired function of the intracrine DHEA prohormone metabolizing machinery, which fails to convert DHEA into its active metabolites.

Finally the effects of androgens on laminins and integrins in salivary cells and glandular explants were assessed. Progenitor epithelial cells from the intercalated ducts of LSGs are thought to migrate to the acinar compartment and then to undergo a phenotypic change from progenitor cells into secretory acinar cells. This migration and subsequent phenotype change seem to be regulated by the LM-111-to-Int α1β1 interaction. In this study HSG cells and LSG explants from healthy controls and patients with SS were cultured with or without sex steroids. Laminin α1 and Ints α1β1 and α2β1 were studied using qRT-PCR and immunofluorescence staining. In conclusion, lack of appropriate LM-111-to-Int α1β1 interaction seems to limit the remodelling process. Androgens are effective stimulators of Int α1β1 and α2β1 expression. DHEA had the most effective stimulating effect of the androgens tested and this effect may be deficient in the LSGs of patients with SS.
3. ABBREVIATIONS

α-DG  α-dystroglycan  
αSMA  alpha smooth muscle actin  
3β-HSD  3β-hydroxysteroid dehydrogenase  
4-dione  androstenedione  
5-diol  androstenediol  
17β-HSD  17β-hydroxysteroid dehydrogenase  
ABC  avidin-biotin-peroxidase complex  
ACTH  adrenocorticotropic hormone  
AIDS  acquired immune deficiency syndrome  
AQP  aquaporin  
ARE  androgen responsive element  
B-CAM  basal cell adhesion molecule  
BM  basement membrane  
BSA  bovine serum albumin  
cDNA  complementary DNA  
CRH  corticotropin-releasing hormone  
CRISP-3  cysteine-rich secretory protein -3  
DGC  dystrophin glycoprotein complex  
DHEA  dehydroepiandrosterone  
DHEA-S  dehydroepiandrosterone-sulphate  
DHT  dihydrotestosterone  
DNA  deoxyribonucleic acid  
E1  estrone  
E1-S  estrone-sulphate  
E2  estradiol  
ECM  extracellular matrix  
EHS tumor  Engelbreth-Holm-Swarm tumor  
ELISA  enzyme-linked immunosorbent assay  
FCS  fetal calf serum  
FTTC  fluorescein isothiocyanate  
HCQ  hydroxychloroquine  
HIV  human immunodeficiency virus  
HPA  hypothalamic-pituitary-adrenal axis  
HTLV-1  human T-lymphotropic virus type-1  
HSG  human submandibular gland cell line  
Int  integrin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>JAR</td>
<td>human choriocarcinoma trophoblast cells</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LG</td>
<td>laminin G</td>
</tr>
<tr>
<td>LM</td>
<td>laminin</td>
</tr>
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<td>LSG</td>
<td>labial salivary gland</td>
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<td>Lu</td>
<td>Lutheran blood group antigen</td>
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<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MFI-20</td>
<td>Multiple Fatigue Inventory-20 questionnaire</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NOD mice</td>
<td>non-obese diabetic mice</td>
</tr>
<tr>
<td>PBGD</td>
<td>porphobilinogen deaminase</td>
</tr>
<tr>
<td>PBS</td>
<td>10 mM phosphate buffered, 150 mM saline, pH 7.4</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real time-PCR</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid cell adhesion sequence</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>SS</td>
<td>Sjögren’s syndrome</td>
</tr>
<tr>
<td>TBS</td>
<td>10 mM Tris buffered, 150 mM NaCl, pH 7.5</td>
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4. INTRODUCTION

Sjögren’s syndrome (SS) is a common autoimmune disease characterized by dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia) as well as fatigue, Raynaud’s phenomenon and arthralgia (Vitali et al., 2002). The typical histopathological findings in the exocrine salivary glands such as the lymphocyte infiltrates, acinar cell atrophy and ductal cell hyperplasia are well characterized and known but the mechanisms behind these changes are largely unclear (Fox et al., 2000). SS has also two quite characteristic features, namely the female predominance and the late age of onset (Talal, 1987). Most female patients with SS contract their disease at age of 40–50 years, at the time of menopause. This female dominance in SS leads to an assumption that there must be a systemic factor able to effectively segregate sexes. Indeed, the levels of the androgen prohormone dehydroepiandrosterone-sulphate (DHEA-S) in serum are lower in patients with SS than in age- and sex-matched healthy control subjects (Valtysdottir et al., 2001). Patients with SS have often chronic stress symptoms such as fatigue, anxiety and depressed mood and these symptoms may correlate to various disturbances in the neuroendocrine reactivity including the low DHEA-S concentrations in serum (Valtysdottir et al., 2001). In conclusion, androgen levels normally decrease in women at the time of menopause and adrenopause and are particularly low in SS patients. The eventual systemic effects of these factors in SS are not currently well understood.

The aim of this study was to clear mechanisms behind the histopathological changes mentioned above. In this study, the composition of the basement membrane (BM) in labial salivary glands (LSG) both in healthy control subjects and in patients with SS was assessed in some detail. Of course this study is unable to fully characterize the complex BM composition but it suggests that in SS the structure of the BM is impaired and that some systemic factors might at least in part be responsible for this impairment.

In this study also the differentiation process of the intercalated duct cells to secretory acinar cells was studied. It was analyzed why this differentiation does not function properly in SS. Our hypothesis was that because ductal progenitor cells are unable to differentiate to healthy secretory acinar cells, ductal hyperplasia develops as a consequence. Indeed, acinar atrophy and ductal hyperplasia are well known histopathological characteristics of advanced SS (Fox, 2005). The reason for this defect must be a factor that in healthy glands is locally confined in the acini, but which is absent (or weak) in the acini in SS. To be able to understand the process underneath the phenotype change of the salivary gland epithelial cell, one must know the structures in the immediate vicinity of the epithelial cells during this process.
In this study, the profiles and distribution of the laminins of the BM of LSGs were studied, followed by an analysis of their cellular receptors.

The diminished production of dehydroepiandrosterone (DHEA) by the reticular zone of the adrenal glands and its impaired intracellular intracrine conversion to androgens and estrogens such as dihydrotestosterone (DHT) could be one of the reasons for the histopathological changes mentioned above. These mechanisms could lead to the atrophy and loss of acinar cells and could direct a systemic immune aggression against the tubuloacinar epithelial cells. Ultimately these mechanisms could lead to the more or less severe destruction of the acinar cells and an accompanying diminished exocrine secretory function. The effect of DHEA and androgens was analyzed in this study by using a DHEA-regulated androgen marker cysteine-rich secretory protein-3 (CRISP-3) (Udby et al., 2002; Udby et al., 2005).
5. REVIEW OF THE LITERATURE

5.1. SJÖGREN’S SYNDROME (SS)

Sjögren’s syndrome (SS) is a common autoimmune disease with very characteristic clinical features, namely dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia) accompanied by fatigue and arthralgia (Vitali et al., 2002). SS is an eponym after Swedish ophthalmologist Henrik Sjögren (1899–1986). He described female patients suffering from keratoconjunctivitis sicca and xerostomia with arthralgia or arthritis in his doctoral thesis entitled “Zur Kenntniss der Keratoconjunctivitis sicca” in 1933 (Sjögren, 1933). SS is currently classified as a systemic chronic autoimmune inflammatory disease and it is divided in primary form, where lacrimal and salivary glands are affected but there is no associated basic autoimmune disease, and in secondary form where an associated autoimmune disease, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or scleroderma, is present (Rehman, 2003). Although SS can appear at any age, there is a clear-cut peak incidence between 40–50 years (Talal, 1987; Vitali et al., 2002; Delaleu et al., 2005). Furthermore, SS has one of the most prominent female predominances among the autoimmune rheumatic diseases, 9 out of 10 patients being women (Ahmed et al., 1985; Konttinen et al., 2009). The prevalence in population varies between 0.5% of all adult females up to 3–4% of total population, depending on the criteria used and report studied, but SS is anyway considered as one of the most frequent autoimmune diseases worldwide (Fox et al., 1986; Thomas et al., 1998; Vitali et al., 1993; Vitali et al., 2002).

The etiology of SS, as well as most of the other autoimmune inflammatory diseases, remains to be revealed. There have naturally been numerous different suggestions considering the etiology of the syndrome such as infectious agents e.g. the Epstein-Barr virus, human immunodeficiency virus (HIV) or the cell-free human T-lymphotropic virus type-1 (HTLV-1) attacking the salivary glands. These hypotheses have been unsuccessful in explaining the female predominance or the relatively late onset of the disorder. Yet they have a role in the differential diagnostics of SS (Vitali et al., 2002).
5.1.1. CLINICAL FEATURES

Dry mouth and dry eyes are the most commonly acknowledged symptoms of SS. The greatly reduced saliva production of patients with SS is often associated with a general discomfort of the oral cavity. Xerostomia is accompanied by difficulties in swallowing, speaking, alterations in taste and difficulties in wearing dentures. The deficiency in the quality and quantity of the saliva is reflected in the general dental and oral health of SS patients and leads to increased dental caries, mucositis, oral candidiasis and swelling of the salivary glands, especially the parotid glands (Soto-Rojas and Kraus, 2002). Dryness of the oral cavity is closely related to the general dryness of the pharynx and the respiratory tract. Dryness of the mucosa in the nasopharynx can cause alterations in the sense of smell and even recurrent sinusitis. Mucosal dryness of the larynx and the trachea can lead to dry irritating cough and predisposes the patient to respiratory infections. It should be mentioned, however, that these sicca, i.e. dryness symptoms, may vary greatly from being mild to extremely disabling (Soto-Rojas and Kraus, 2002).

In patients with SS the autoimmune functional impairment and destruction of the lacrimal glands result in a reduced production of the aqueous component of the tear fluid. The inflammatory cytokines released from the lymphocyte infiltrates in the lacrimal glands cause changes at the ocular surfaces and ultimately lead to destruction of the neural network responsible for reflex tear secretion (Stern et al., 1998; Pflugfelder et al., 1999). The ocular sicca symptoms are commonly presented as photosensitivity and itching of the eyes as well as fluctuating vision.

Apart from the symptoms mentioned above, SS can broaden its spectrum from an autoimmune exocrinopathy to systemic and extra-glandular manifestations. These may involve the musculoskeletal, pulmonary, gastrointestinal, hepatobiliary, hematological, vascular, dermatological, renal, genital, and nervous systems (Hietaharju et al., 1993; Pertovaara et al., 2001; Leppilahti et al., 2003). Joint and muscle pains are very common in SS. Secondary SS is most often related to RA or SLE, but joint and muscle pains are often present in primary SS as well. Secondary Raynaud’s phenomenon, a three-phase condition that causes numbness and cooling of extremities such as fingers in response to cold temperatures or stress, is a very common symptom in patients with SS (Fox, 2005).

Even though xerostomia and keratoconjunctivitis sicca can cause a major decrease in the quality of life, patients with SS often experience the fatigue as the most morbid symptom of their disease. Fatigue can also be considered as one of the most common symptoms of SS (Tensing et al., 2001).

Patients with SS have been reported to have up to 40 times increased risk of developing non-Hodgkin’s lymphoma, which is primarily of B cell origin (Kassan et al., 1978). One peculiar and clinically important finding is that SS mothers having SS-A/Ro and/or SS-B/La autoantibodies in serum have an increased risk of having
children with neonatal lupus with a congenital heart block requiring pacemaker therapy as a result of transplacental passage of these antibodies from the mother to the fetus (Reed et al., 1983).

5.1.2. DIAGNOSIS

SS as its name implies is a syndrome, i.e. a condition that contains numerous different symptoms, clinical and/or laboratory findings that have to be met for the diagnosis. Usually the diagnosis is nowadays based on the American-European consensus criteria including the presence of the sicca symptoms occurring in an autoimmune context (Vitali et al., 2002). Primary SS is characterized by at least four typical features, which include dry mouth and impaired salivary gland function, dry eyes and impaired lacrimal gland function, but these symptoms and findings must always be associated with signs of autoimmunity in form of focal sialadenitis and/or SS auto-antibodies, according to the currently prevailing criteria SS-A/Ro and/or SS-B/La. It should be pointed out that some features important for an individual patient, like fatigue (Bjerrum and Prause 1990), are absent from the diagnostic criteria as they are too non-specific to be useful in the diagnostic work up. The American-European consensus inclusion and exclusion criteria as well as the classification rules are presented in Table 1. In order for the clinician to make the diagnosis of primary SS, biopsy of the labial salivary glands and/or patient serum is needed for the demonstration of autoimmune features. In addition to inclusion criteria, certain other diseases must be excluded, especially when the syndrome presents with uncommon or uncharacteristic features, such as a young man lacking auto-antibodies. The most important exclusion criteria acknowledged by the American-European consensus group are past head and neck cancer treatment with radiation, hepatitis C virus infection, acquired immune deficiency syndrome (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease and usage of anti-cholinergic drugs (Vitali et al., 2002). However, also many other conditions may simulate the symptoms or signs of SS such as allergic conjunctivitis, fungal infections, Stevens-Johnson syndrome, post-Lyell syndrome conditions, blepharitis and sialosis (Fox, 2005). The classification rules require the presence of any 4 of the 6 inclusion items for primary SS, as long as one of the items is the histopathological finding of focal sialoadenitis in salivary glands or the presence of auto-antibodies in patient serum. Also the presence of any 3 of the 4 objective criteria items (ocular signs, histopathology, salivary gland involvement or serology) is indicative of primary SS.
Table 1. The American-European consensus about six inclusion criteria, seven exclusion criteria and classification rules for primary and secondary Sjögren’s syndrome (modified from Vitali et al., 2002).

<table>
<thead>
<tr>
<th>Symptoms / Clinical findings</th>
<th>Inclusion criteria</th>
</tr>
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<tbody>
<tr>
<td>Ocular symptoms (I)</td>
<td>Dry eyes over 3 months or sensation of sand or gravel in the eyes or usage of tear substitutes more than 3 times a day</td>
</tr>
<tr>
<td>Oral symptoms (II)</td>
<td>Dry mouth over 3 months or recurrently or permanently swollen parotid glands or need to drink liquids in order to aid swallowing</td>
</tr>
<tr>
<td>Ocular signs (III)</td>
<td>Schirmer’s I test, performed without anesthesia, ≤ 5 mm in 5 minutes or Rose Bengal score ≥ 4</td>
</tr>
<tr>
<td>Histopathological findings (IV)</td>
<td>Focal lymphocytic sialoadenitis in labial salivary glands, with a focus score ≥ 1, defined as number of lymphocytic foci containing over 50 lymphocytes per 4 mm² of glandular tissue</td>
</tr>
<tr>
<td>Salivary gland involvement (V)</td>
<td>Unstimulated whole saliva flow ≤ 1.5 ml in 15 minutes or parotid sialography indicating presence of diffuse sialectasia or salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer</td>
</tr>
<tr>
<td>Auto-antibodies (VI)</td>
<td>Presence of SS-A/Ro and/or SS-B/La auto-antibodies in the serum</td>
</tr>
</tbody>
</table>

Exclusion criteria
- Past head and neck radiation therapy
- Hepatitis C infection
- Acquired immunodeficiency syndrome (AIDS)
- Pre-existing lymphoma
- Sarcoidosis
- Graft versus host disease
- Use of anti-cholinergic drugs

Classification rules for primary Sjögren’s syndrome
- a) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (histopathology) or VI (serology) is positive
- b) The presence of any 3 of the 4 objective criteria items (III, IV, V, VI)

Classification rules for secondary Sjögren’s syndrome
- In patients with potentially associated disease (e.g. another connective tissue disease), the presence of item I or item II plus any 2 from among the item III, IV and V may be considered as indicative of secondary Sjögren’s syndrome

5.1.3. TREATMENT

To date there is no curative treatment for SS. However, after the diagnosis has vigilantly been done, patients with SS can be helped with several approaches. These treatments can be divided to local and systemic. Local treatments are mostly symptomatic such as moisturizing mouthwashes, chewing gums and salivary substitutes used in the treatment of xerostomia. Infections in the oral cavity should be treated actively and effectively. Oral candidiasis can be treated with topical antifungal treatment followed by systemic treatment if necessary (Mathews et al.,...
In the management of xerostomia, good individualized dental hygiene is useful, but the patient needs professional help from a dentist and a dental hygienist. Healthy eating habits, regular tooth brushing and topical application of fluoride on the surfaces of the teeth are important in the prevention of caries lesions (Pedersen et al., 2005). The maintenance of the permanent teeth is important for the patients with SS because dentures can be difficult to wear and may cause recurrent ulcerations, pain and candidiasis in the dry oral cavity. Furthermore it should be kept in mind that prosthodontic treatment is much more difficult and expensive than prophylactic care of the teeth. A new experimental approach in the treatment of hyposalivation and xerostomia in SS is a salivary pacemaker, which is a device capable of neuronal stimulation with electric impulses (Fedele et al., 2008). The new third-generation implant-supported neuro-stimulating devices can be permanently applied into the oral cavity as it is built into an osteointegrated dental implant inserted near the lingual nerve in the third molar area. The stimulator gives small electric stimuli to the lingual nerve, which carries salivary impulses (Fedele et al., 2008). These stimulators are not yet currently available for commercial use, however, and controlled clinical trials are currently undergoing.

For the treatment of keratoconjunctivitis sicca (dry eyes) artificial eye moisturizing drops are useful as also mechanical shielding of the eyes against wind using special eyeglasses. Surgical treatment for keratoconjunctivitis sicca such as congestive treatment of the nasolacrimal duct is possible, but is an irreversible maneuver for which collagen or silicon plugs offer an alternative. Infections of the eye and surrounding structures such as the eyelids should be treated effectively and rapidly with local antibiotic eye drops with or without glucocorticosteroids (Fox, 2005).

Systemic approaches in the treatment of xerostomia include muscarinic agonists such as pilocarpine or cevimeline. These drugs, approved for the treatment of SS related xerostomia in many countries, stimulate muscarinic M1 and M3 receptors in the salivary and lacrimal glands and thus increase the secretory function of the glands (Fox et al., 2001). However, they can cause certain cholinergic side-effects, such as urinary frequency, excessive sweating, flushing and headache (Mavragani et al., 2006), which may limit their use in clinical practice. Hydroxychloroquine (HCQ), a commonly used anti-rheumatic drug, has been shown to be effective in the treatment of xerostomia in patients with SS who lack severe organ manifestations (Rihl et al., 2009). However, the mode of action of HCQ in SS is not clear. In vitro data postulate an effect mediated by an inhibition of the glandular cholinesterase activity (Rihl et al., 2009). Naturally also corticosteroids have been used in the conventional treatment of SS, but they are mainly reserved for severe extraglandular manifestations of SS such as vasculitis, myositis and renal and pulmonary manifestations. The evidence for alleviation of the sicca symptoms with the use of corticosteroids is limited (Mavragani et al., 2006). In addition to the systemic therapies mentioned above, several other approaches have also been suggested such
as bromhexine, etanercept, tumor necrosis factor (TNF) inhibitors (infliximab) and rituximab (Mathews et al., 2008). In a randomized controlled trial TNF-inhibitor infliximab was found to be ineffective in SS (Mariette et al., 2004). Rituximab is a chimeric monoclonal antibody targeting the B lymphocyte antigen CD20 and it destroys both normal and malignant CD20 positive B cells. The use of rituximab for SS has been reported to exert some therapeutic effect (Ramos-Casals et al., 2008) or even good clinical responses (Meijer et al., 2009). Rituximab may find a place in the treatment of selected cases of SS with systemic manifestations as well as in prevention and treatment of SS associated lymphoma (Alcântara et al., 2009). However, these drugs have yet to establish their place in the routine treatment of SS.

Apart from the oral and ocular symptoms of the SS, fatigue is a very disabling symptom. It has been proposed that supraphysiological or substitution doses of DHEA could be used in the treatment of fatigue, but scientific evidence for this could not be found (Hartkamp et al., 2008; Virkki et al., 2010). Fatigue in patients with SS can also be caused by other reasons, for example arthralgia, muscle pain, restless feet syndrome or hypothyroidism. These conditions are commonly treatable and should be treated accordingly.

5.2. SALIVARY GLANDS

Three pairs of major (extrinsic) salivary glands and several minor (intrinsic) salivary glands are responsible for the human saliva production. The major salivary glands, the parotid, submandibular and sublingual glands, are paired. The minor salivary glands are located around the oral cavity in specific parts including the glands of the tongue (anterior and posterior lingual glands), the labial glands, the buccal glands, the molar glands, the incisive glands, and the palatine glands. The largest of the salivary glands are the parotid glands, which are located on the side of the face in the retromandibular fossa. They have long ducts that open into the oral cavity close to the upper first molars. Submandibular and sublingual glands are located in the floor of the oral cavity, in the submandibular fossa and in the sublingual eminence, respectively. The ducts of the submandibular gland open in the oral cavity underneath the tongue and the sublingual glands have multiple small ductal openings in the floor of the mouth (Saracco and Crabill, 1993).

Salivary gland is composed of several different cell types, with the two main components being the parenchymal and stromal components. Even though the composition of saliva produced by different salivary glands differs considerably, the universal structure of the salivary gland is fairly similar in all glands. The gland structure can be referred to as a bunch of grapes in which the ducts represent the branching stems and the secretory acini represent the grapes (Ross et al., 2006), although the structure of the parotid glands has sometimes been compared to a tree
rather than to a bunch of grapes (Tucker, 2007). The smallest parenchymal unit of a salivary gland is referred to as a salivon, which consists of a bunch of secretory acini and ductal segments connected to it. Acinar cells are responsible for the water and protein secretion (primary saliva) and they belong to two different cell types, either serous or mucous, defined by the composition of their secretions. Mucous cells secrete mostly large negatively charged glycoproteins with carbohydrate chains or mucins, which are responsible for the viscosity of the saliva. Serous acinar cells secrete mostly proteins, such as the starch breaking enzyme amylase. Several acinar and myoepithelial cells together form a unit called acinus, which is mixed if it contains both mucous and serous acinar cells. The acini are connected to the saliva carrying and modifying striated ducts by the intercalated ducts (Figure 1). Acinar cells are surrounded by alpha smooth muscle actin (αSMA) and myosin filaments containing myoepithelial cells, which are able to contract and thus mechanically press the acinar secretion into salivary ducts. The ductal cells modulate the composition of the saliva via various ion pumps and channels. The ductal system consists of intercalated, striated/granular convoluted tubule and excretory intra- and interlobular ducts. The excretory ducts contain a cytokeratin 14 positive basal cell layer. Basal cells are conceived as reserve cells of the epithelium. They are also scattered around the salivon. All the epithelial cells are attached to sheet of extracellular matrix material called basement membrane (BM) (Ross et al., 2006).

Figure 1. Schematic view of the acinus and its ducts as discussed in the text. Figure does not show the distribution of the basal cells, which form a basal cell layer in excretory ducts but are also scattered around the salivon (Redrawn and modified from Ross et al., 2006; original diagram based on Johnson LR (ed) Gastrointestinal Physiology, 2nd ed., CV Mosby, 1981, p.47).
The parotid glands contain only serous acinar cells, sublingual glands contain only mucous acinar cells but the submandibular glands contain both serous and mucous or so called mixed acini. The minor salivary glands are mainly mucous, with an exception of the tip of the tongue and the von Ebner’s glands in the circumvallate papillae, which are serous (Ross et al., 2006).

5.2.1. DEVELOPMENTAL BIOLOGY

During the developmental process of the salivary glands, epithelial-mesenchymal interactions and branching morphogenesis play important roles. The salivary gland development occurs through certain well-defined stages and all salivary glands develop in a similar manner (Figure 2). The submandibular gland of the mouse serves here as a model because it has been used as a model for organ culture for almost 60 years (Borghese, 1950). The first developmental stage is known as a prebud stage, evident in the mouse embryo as a thickening of the epithelium next to the tongue, which then protrudes into the underlying mesenchyme (Figure 2, panel 1). During this process a bud is formed, which is linked to the overlying oral surface by a duct (Figure 2, panel 2). This duct later forms the main duct of the salivary gland and is visible as a slight pit on the oral surface. The next process is called pseudoglandular stage, where the bud continues to branch into a cluster of branches and buds (Figure 2, panel 3). During this stage of development the proliferation rate is high in the epithelium and low in the mesenchyme (Jaskoll and Melnick, 1999; Tucker, 2007). The branching procedure then continues eventually leading to the development of a multi-lobed gland. As the development process of the salivary gland proceeds, the epithelial cells form a solid core that needs to be tunneled in order to form a route from the secreting acini to the oral cavity. This tunnel or lumen is formed with the aid of the programmed cell death, i.e. apoptosis, of the core epithelial cells, which leads to the formation of the ducts (Figure 2, panel 4). After this stage the distinct well-formed lumen in the terminal end buds develops (Figure 2, panel 5). However, the continuity between the ducts and lumen is not complete but it continues to form postnatally and the final differentiation involves in many species formation of the granular convoluted tubules arising at puberty (Gresik, 1994; Melnick and Jascoll, 2000; Tucker, 2007). In humans the parotid gland begins to develop at 4–6 weeks of embryonic life, the submandibular gland at 6 weeks, and the sublingual and minor salivary glands at 8–12 weeks (Loureño and Kapas, 2005).
Figure 2. A schematic view of the salivary gland development stages: 1. Prebud stage, 2. Initial bud stage, 3. Pseudoglandular stage, 4. Canaliculur stage, 5. Terminal bud stage (Modified from the original figure published by Tucker, 2007).

Acini in salivary glands are constantly and rather rapidly remodeled. In rodents salivary gland cell life span is approximately 200 days (Schwarz-Arad et al., 1988; Denny and Denny, 1993). This type of rapid remodeling is enabled by the acinar epithelial cell apoptosis, which leaves an empty lot in the acinus. The regeneration is enabled by the progenitor cells residing at least in part in the intercalated duct compartment. These undifferentiated salivary gland ductal cells do not have any specialized secretory or salivary modifying function but they can migrate from the intercalated duct area to the acinus, where they receive their differentiation signals. These differentiation signals at least partly engage integrins (Lourenço and Kapas, 2005). It have been implicated that the specific messages of differentiation and normal function come from the underlying BM, delivered by the LM-111-Intα1β1/α2β1 interactions, which were studied in this thesis work. In submandibular epithelial morphogenesis the role of laminin α5 signaling through integrin β1 has been shown to be significant further emphasizing the important role of the BM in the salivary gland development (Rebustini et al., 2007).
5.2.2. FUNCTION

The main function of the salivary glands is production of saliva, which moistens the oral and pharyngeal mucosa. Saliva is also important in the maintenance of pH in the oral cavity and the parotid saliva in particular has a significant buffering capacity (Mathews et al., 2008). Impairment of these functions becomes evident in an increase of dental caries rate and other complications. Saliva contains mostly water (98%), the remaining 2% being composed of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate and phosphates), mucus composed mainly of mucopolysaccharides and glycoproteins, antiseptic substances (hydrogen peroxide, IgA etc.) and various enzymes and proteins (amylase, lysozymes, lingual lipase, epidermal growth factor etc.) (Pink et al., 2009). Calcium and phosphate are important in the tooth surface remineralization process after acid exposure, whereas bicarbonate, phosphate and organic components contribute to the buffering capacity of the saliva (Mathews et al., 2008). The compositions of saliva and plasma are compared in Table 2.

Table 2. Composition of stimulated adult saliva compared to adult plasma (modified from Roth and Calmes, 1981 and from the HUSLAB analysis instruction manual, Hospital District of Helsinki and Uusimaa [http://huslab.fi/ohjekirja/index.html]).

<table>
<thead>
<tr>
<th></th>
<th><strong>Saliva</strong></th>
<th><strong>Plasma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (Na⁺) *</td>
<td>2–21 mmol/L *</td>
<td>137–145 mmol/L</td>
</tr>
<tr>
<td>Potassium (K⁺) **</td>
<td>10–36 mmol/L **</td>
<td>3.3–4.9 mmol/L</td>
</tr>
<tr>
<td>Calcium (Ca²⁺)</td>
<td>1.2–2.8 mmol/L</td>
<td>2.15–2.51 mmol/L</td>
</tr>
<tr>
<td>Magnesium (Mg²⁺)</td>
<td>0.08–0.5 mmol/L *</td>
<td>0.71–0.94 mmol/L</td>
</tr>
<tr>
<td>Chloride (Cl⁻) *</td>
<td>5–40 mmol/L *</td>
<td>96–107 mmol/L</td>
</tr>
<tr>
<td>Bicarbonate (HCO₃⁻)</td>
<td>25 mmol/L</td>
<td>21–28 mmol/L</td>
</tr>
<tr>
<td>Phosphate (HPO₄²⁻)</td>
<td>1.4–39 mmol/L</td>
<td>0.76–1.53 mmol/L</td>
</tr>
<tr>
<td>Uric acid</td>
<td>2–3 mmol/L</td>
<td>0.155–0.48 mmol/L</td>
</tr>
<tr>
<td>Urea</td>
<td>3–5mmol/L</td>
<td>2.6–8.1 mmol/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>&lt;1.0 mmol/L</td>
<td>4.0–6.1 mmol/L</td>
</tr>
<tr>
<td>Total proteins (mg/ml)</td>
<td>1.5–2.5</td>
<td>60–90</td>
</tr>
</tbody>
</table>

* lower than plasma  
** higher than plasma

Water is secreted through the acinar cell lipid bi-layer via highly permeable and water-selective channels, namely aquaporins (AQP). AQP1, AQP5 and AQP8 are present in salivary glands, whereas the presence of other AQPs remains controversial. AQP5 seems to have a major functional role in the salivary gland secretory process (Delporte and Steinfeld, 2006). In acini osmotic and hydrostatic gradients regulate the flow of watery saliva. Osmotic gradient is built up by electrolytes, and the ATPase and actin-myosin containing myoepithelial cells contract producing the hydraulic
pressure which squeezes the secretory salivon (Pinkstaff, 1993). The selective localization of AQP5 channels at the apical cell membrane is necessary for the normal water secretion and in SS and in the NOD mouse (non-obese diabetic mice) model for SS the abnormal distribution of these channels is thought to associate with impairment of salivary flow rate (Steinfeld et al., 2001; Konttinen et al., 2005), although this is not quite unequivocal (Beroukas et al., 2001). The initial secretion of the acinar cells and plasma contain potassium and sodium in equivalent concentrations, but as the saliva passes through the duct system, saliva becomes hypotonic as sodium is reabsorbed. Sodium is mainly reabsorbed in striated ducts but also in part in the excretory ducts. While sodium is reabsorbed, potassium is secreted into the saliva by the duct cells. However, the amount of potassium secreted does not match with the amount of sodium reabsorbed, resulting in hypotonicity of saliva (Ross et al., 2006). Saliva contains several proteins, which have different functions. These functions include lubrication and protection (mucosal glycoproteins), digestive (amylase) and immunodefensive function (immunoglobulins IgA, IgG and other antimicrobial factors such as lysozyme). On the basis of research on general populations the minimum flow of unstimulated total mixed saliva exceeds 0.1 mL/min, whereas the minimum volume for the stimulated saliva is 0.2 mL/min. However, normal healthy control subject produces daily approximately 1 to 1.5L of whole saliva (Humphrey and Williamson, 2001). Because salivary flow is considered to have very individual basal rates, it has been suggested that values below 45% of normal salivary flow could be considered as hypofunction (Ghezzi et al., 2000). The secretion of saliva is controlled by the salivary nuclei in the medulla. Also stimuli, which are mechanical, gustatory and olfactory, affect saliva production (Humphrey and Williamson, 2001). Some psychic factors, medication and various local or systemic diseases affect saliva production. Salivary flow is not evenly distributed in the oral cavity. The mandibular lingual region is a site of high volume, whereas maxillary anteriors and interproximals are sites of low volume flow (Humphrey and Williamson, 2001).

5.3. THE BASEMENT MEMBRANE

BM is a thin layer of specialized extracellular matrix (ECM) lying underneath the tubuloacinar epithelial cells and is found in all epithelial structures including the lacrimal and salivary glands. BM also surrounds vascular endothelium and individual muscle cells, peripheral nerve Schwann cells and fat cells. The discovery of the BMs dates back to the 19th century when Robert Todd and William Bowman first described them, using light microscopy techniques (Kefalides et al., 1979). When BMs are studied with electron microscopy, three different layers can be identified according to their electron density: lamina lucida, lamina densa and lamina fibroreticularis
(Leblond and Inoue, 1989). Currently it is widely believed that BM is a highly specialized tissue able to modulate the glandular tissue development, growth and function (Aumailley and Smyth, 1998; Ekblom et al., 1998, Yurchenco et al., 2004) and the latest view of their ultra-structure and components is discussed below.

5.3.1 GENERAL STRUCTURE AND FUNCTION

BM s are cell-associated ECM structures, which can be outlined as a construct composed of two independent matrix networks connected by the link protein, nidogen-1 (Timpl et al., 1979; Colognato and Yurchenco, 2000; Pujuguet et al., 2000; Patarroyo et al., 2002). One of the networks is formed of laminins and the second one of type IV collagens. Schematically the laminin network faces the epithelial cells, whereas type IV collagen network faces the connective tissue. The other matrix components of the BMs serve as internal link components and include heparan sulphate proteoglycans such as perlecan and agrin (Colognato and Yurchenco, 2000; Pujuguet et al., 2000). In addition, also type XV and XVIII collagens, cysteine-rich collagen type XIX and fibronectin are found in the BMs, but laminins, type IV collagens and nidogen-1 are the major scaffoldings of the BMs (Yurchenco et al., 2004). Multiple interactions such as self-polymerizations, inter-component binding and cell surface adhesions enhance the biological diversity of the BMs (Yurchenco et al., 2004). BMs provide mechanical strength to tissues and enable the specific tissue architecture to form and to be maintained. BMs can function as semi-permeable membranes and control in part recruitment of inflammatory cells across the blood-brain and other BM barriers (Sixt et al., 2001; Hallmann et al., 2005). The BMs have been shown to have many diverse roles during and after the development of tissues. This impact is mainly due to an ability to deliver cell-signaling messages from the extracellular matrix to the cells in outside-in signaling via intracellular signaling pathways (Miner and Yurchenco, 2004). Particular emphasis in this thesis work is on the laminin-integrin and laminin-non-integrin-receptor interactions, but also many other interactions such as collagen type IV-integrin, nidogen-laminin and nidogen-collagen type IV interactions exist (Sasaki et al., 2004).

The effects of malfunction or incorrect assembly of the BMs are evident in certain diseases such as the Alport syndrome, which is an X-linked disease due to mutations in the gene encoding α5 chain of collagen type IV. This defect leads to an interference with the structure and permeability of the glomerular basement membrane in the kidneys. This results in severe nephritis, which is accompanied by sensorineural deafness and various eye disorders such as lens dislocation, posterior cataracts and corneal dystrophy (Alport, 1927; Gubler, 2008).
5.3.2. LAMININS (LM)

Laminins (LM) are αβγ-heterotrimeric glycoproteins with a 30 years long history (Miner, 2008). The first identified laminin, EHS-laminin (currently known as laminin-111) was isolated from the matrix of the mouse Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979). After this discovery, the three chains of EHS-laminin (α1, β1 and γ1) were cloned leading to further discoveries of several homologues of these chains. To date, five α-, four β-, and three γ-polypeptide chains, all of which are encoded by their own genes, have been identified (Miner and Yurchenco, 2004). These chains can combine to 15 different αβγ-laminin heterotrimers in mice and man, which are expressed in a tissue specific fashion (Patarroyo et al., 2002; Miner and Yurchenco, 2004; Aumailley et al., 2005). This forms the basis to the hypothesis that their signals are crucial to the overlying tissues to specialize and function appropriately. Fibroblasts and epithelial cells are able to produce laminins (Colognato and Yurchenco, 2000) and in exocrine glands these cells are abundant.

The laminin nomenclature has recently been revised, because the increasing number of different laminin-isomers made it difficult to remember the correct chain compositions. As a result a simplified nomenclature was proposed (Aumailley et al., 2005). In the new nomenclature the name of the laminin-isomer is based on the numbers of the different α-, β-, and γ-chains composing it. Laminin-1 is now called laminin-111, because it contains α1-, β1- and γ1-chains and laminin-5 is called laminin-332, because of its α3β3γ2 configuration et cetera. Table 3 presents the former and current nomenclatures of laminins.
Table 3. Nomenclature of 15 currently known laminins and some of their known integrin receptors (modified from Patarroyo et al., 2002 and from Aumailley et al., 2005).

<table>
<thead>
<tr>
<th>Chains</th>
<th>Abbreviated</th>
<th>Previous names</th>
<th>Integrin receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1γ1</td>
<td>111</td>
<td>Laminin-1, EHS-laminin</td>
<td>α1β1,α2β1,α7β1,α6β4</td>
</tr>
<tr>
<td>α2β1γ1</td>
<td>211</td>
<td>Laminin-2, merosin</td>
<td>α1β1,α2β1,α3β1,α6β1,α7β1,α6β4</td>
</tr>
<tr>
<td>α1β2γ1</td>
<td>121</td>
<td>Laminin-3, s-laminin</td>
<td>ND</td>
</tr>
<tr>
<td>α2β2γ1</td>
<td>221</td>
<td>Laminin-4, s-merosin</td>
<td>Presumably similar to laminin-211</td>
</tr>
<tr>
<td>α3Aβ3γ2</td>
<td>332, or 3A32</td>
<td>Laminin-5 (-5A), kalinin, nicein, epiligrin</td>
<td>α3β1,α6β1,α6β4</td>
</tr>
<tr>
<td>α3Bβ3γ2</td>
<td>3B32</td>
<td>Laminin-5B</td>
<td>ND</td>
</tr>
<tr>
<td>α3Aβ1γ1</td>
<td>311, or 3A11</td>
<td>Laminin-6 (-6A), k-laminin</td>
<td>ND</td>
</tr>
<tr>
<td>α3Aβ2γ1</td>
<td>321, or 3A21</td>
<td>Laminin-7 (-7A), ks-laminin</td>
<td>ND</td>
</tr>
<tr>
<td>α4β1γ1</td>
<td>411</td>
<td>Laminin-8</td>
<td>α3β1,α6β1,α7β4</td>
</tr>
<tr>
<td>α4β2γ1</td>
<td>421</td>
<td>Laminin-9</td>
<td>ND</td>
</tr>
<tr>
<td>α5β1γ1</td>
<td>511</td>
<td>Laminin-10</td>
<td>α3β1,α6β1,α7β1,α6β4,αVβ3</td>
</tr>
<tr>
<td>α5β2γ1</td>
<td>521</td>
<td>Laminin-11</td>
<td>α3β1,α6β1</td>
</tr>
<tr>
<td>α2β1γ3</td>
<td>213</td>
<td>Laminin-12</td>
<td>ND</td>
</tr>
<tr>
<td>α4β2γ3</td>
<td>423</td>
<td>Laminin-14</td>
<td>ND</td>
</tr>
<tr>
<td>α5β2γ3</td>
<td>523</td>
<td>Laminin-15</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not specified
αTo recombinant laminin α5 IVa domain

5.3.2.1 General structure of laminins

The laminin trimer types have several shapes including cruciform, T-, Y- or rod-shaped forms (Patarroyo et al., 2002; Miner, 2008). As stated above, there exists five α-, four β- and three γ-chains from which the so far known 15 laminin trimers are composed of in different heterodimeric combinations composed of one α-, one β- and one γ-chain each. It is highly probable that there exist even more trimer combinations yet to be revealed. The α-, β- and γ-chains form triple-helical coiled-coils linked by disulfide bridges. As a result, large heterotrimeric glycoproteins with molecular weight between 400 to 800 kilo Daltons (kDa) are formed (Colognato and Yurchenco, 2000; Patarroyo et al., 2002). All the different chains have characteristic domains, which serve as binding sites for different proteins such as integrins, heparin, dystroglycan and entactin/nidogen (Miner, 2008). The α-chains contain G domain, which is 100kDa COOH-terminal globular domain. This domain is further subdivided into five self-folding modules called laminin G (LG) modules (LG1-LG5) (Timpl et al., 2000; Miner, 2008). The G-domain contains several binding sites for cell-surface receptors such as integrins (Aumailley and Smyth, 1998).
5.3.2.2  In vivo functions of different laminins

Years of study of laminins have revealed that they have a tissue specific distribution (Patarroyo et al., 2002; Petäjäniemi et al., 2002) and are synthesized by several cell types. Mutations in laminins both in mice and humans and experimentally induced laminin chain mutations generated in mice clearly indicate that laminins have important tissue specific functions both in developing tissues and in the maintenance of tissue function and integrity. In mice the deletion of the LAMC1 gene encoding the laminin γ1 chain, which is found in most laminins, results in embryonic lethality due to the overall lack of BMs (Smyth et al., 1999). In this thesis work the specific laminin distribution is studied in the salivary glands in healthy controls and in patients with SS.

5.3.3.  LAMININ RECEPTORS

BM proteins such as laminins are unable to work on their own without receptors that are able to deliver their messages to cells. These receptors are transmembrane surface glycoproteins and belong mainly to the integrin protein family. Also some other proteins such as α- and β-dystroglycan and Lutheran blood group antigen are able to bind laminins and deliver their outside-in messages (Winder, 2001; Kikkawa and Miner, 2005). These above mentioned receptor proteins have many different specific tasks. They can function as anchorage proteins able to maintain tissue integrity and architecture as well as deliver extra-cellular signals. These signals regulate cell migration, growth and differentiation of the epithelial cells and they do so by using multiple intracellular signaling routes (Kumar et al., 2002).

5.3.3.1.  Integrins (Int)

Integrins (Int) are heterodimeric transmembrane proteins, the extracellular part of which binds to the BM and various parts of the ECM, including laminins, fibronectin and collagens, whereas the intracellular part is connected to the cellular cytoskeleton (Ruoslathi, 1991; Belkin and Stepp, 2000). With their earlier mentioned dual functions integrins provide cells both the capability to bind to and react with the ECM. The binding function is important in anchoring the cells to ECM and in the maintenance of tissue integrity. It is also known that integrins bind to their ligands with relatively low affinity compared to other transmembrane proteins. This is believed to allow the cells to explore their surroundings without losing attachment to it (Ruoslathi, 1991). Binding to ECM delivers regulatory signals of growth, migration and differentiation to the cell. These messages are delivered through multiple
intracellular signaling routes, which partly overlap with those utilized by growth factor receptors (Giancotti and Ruoslahti, 1999; Kumar et al., 2002). Integrins can signal through the cell surface in both directions, using inside-out (activity is regulated from the inside of the cell) or outside-in signaling (activity is regulated from the ECM to the cells). The signals themselves influence to cell signals, which regulate cellular proliferation and differentiation. These types of control signals are lost in neoplastic cells (Giancotti and Ruoslahti, 1999).

5.3.3.1.1. General structure of integrins
In mammals 24 distinct heterodimeric αβ integrins have been identified this far and these are composed of different combinations of 18 different α- and 8 different β-subunits (Giancotti and Tarone, 2003). These subunits are noncovalently bound and each subunit crosses the cell membrane once (Hynes, 2002). The integrin receptor family is illustrated in Figure 3, where their α- and β-subunit associations are shown. These integrins can be divided in subfamilies based on their evolutionary relationships, ligand specificity and restricted expression on white blood cells (β2 and β7 subunits) (Hynes, 2002). The ligand specificities, although mainly determined by the α-subunits, rely on both subunits of a given αβ heterodimer, and the ligand specificities are significantly more complex than shown in Figure 3. Some of the known ligand specificities to different laminin heterotrimers are evident from their names in Figure 3. The cytoplasmic domains are smaller in size than extracellular domains containing the binding areas, however, the cytoplasmic domain is equally important for the regulation of the activation of the interaction cascades inside the cell (Hynes, 2002).

Figure 3. The integrin receptor family (modified from Hynes, 2002). *Also other integrins such as Int α1β1 and α2β1 function as laminin receptors (Patarroyo et al., 2002; Tulla et al., 2008). RGD refers to arginine-glycine-aspartic acid cell adhesion sequence in molecules such as fibronectin and vitronectin in vertebrates (Hynes, 2002).
5.3.3.2. In vivo functions of different integrins
Integrins are cell anchoring and signaling devices related to different cell functions such as cell survival, control of transcription, cell proliferation, cell motility and cytoskeletal organization. Integrins are not constantly active. They can be expressed on cell surface in inactive state but are activated upon need. Integrins frequently intercommunicate in order to activate or inactivate each others, which is predominately seen in blood leukocytes and platelets. Inadequate activation could lead to inappropriate activation of the immune system or to thrombosis (Hynes, 2002). The functional ligand-receptor interactions can affect multiple signal transduction pathways and can have diverse effects on their target cells. For instance, Int α6β4 stimulation of normal epithelial cells can lead to their differentiation to breast or prostate cells (Giancotti, 1996; Mercurio et al., 2001). Because integrins play crucial role in the regulation of tissue and organ organization as well as cell proliferation and differentiation, they may also have important tasks in the salivary glands. For example, Int α6β4 can bind to LM -111, 211, -332 and -511, all of which are expressed in LSGs (Aumailley and Smyth, 1998). A recently published paper shows that in SS the lateral redistribution of Int α6β4 and the formation of new cell-cell adhesions help maintain the acinar organization and promote cell survival, whereas in severe BM alterations these functions are lost (Velozo et al., 2009). This finding points out the importance between the equilibrium between correct cell-cell and cell-BM attachments to promote acinar cell survival.

Earlier studies have also addressed the localization of the Int β1, β3 and β4 subunits during the salivary gland development and assessed their roles in the development process (Lourenço and Kapas, 2005). Because of the overlapping and co-operative functions of the integrins, extensive integrin profiling was performed in this study.

5.3.3.2. Non-integrin receptors for laminins
Integrins are not the only proteins able to bind laminins and deliver their messages. Also the Lutheran blood group antigen as well as α- and β-dystroglycan are known to function as receptors for laminins (Durbeej et al., 1998; Parsons et al., 2001).

5.3.3.2.1. Lutheran blood group antigen
The Lutheran blood group antigen is part of the Lutheran bloop group system, which contains two immunoglobulin superfamily proteins, namely Lutheran and basal cell adhesion molecules (Lu and B-CAM) (Eyler and Telen, 2006). The Lutheran blood group is composed of a complex set of antigens that are expressed on two integral membrane glycoprotein isoforms of 85 and 78 kDa. Lu/B-CAM is widely expressed in adult tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney
and pancreas (Eyler and Telen, 2006). Lutheran blood group antigen is a specific receptor for Laminin α5-chain (LM-511/521) (Parsons et al., 2001). LM-511 is present in the subendothelium and bone marrow sinusoids, which suggests a role in erythropoiesis (Parsons et al., 1997; Parsons et al., 2001). However, the role of the Lutheran blood group antigen in the LSGs is currently unknown.

5.3.3.2.2. α- and β- dystroglycan
Dystroglycan is a cell surface laminin/agrin receptor, which was first identified as a part of a multimeric protein complex from skeletal muscle, the so-called dystrophin glycoprotein complex (DGC) containing dystroglycan, dystrophin, sarcoglycans, sarcospan, syntrophins and dystrobrevin. DGC was shown to be disrupted or missing in Duchenne muscular dystrophy (Winder, 2001). Dystroglycan contains two proteins, α- and β-dystroglycan. α-dystroglycan is a peripheral membrane protein and the β-dystroglycan is a membrane spanning protein. α-dystroglycan is a laminin-binding receptor, whereas β-dystroglycan binds to the cytoskeletal protein dystrophin (Durbeej et al., 1998). Dystroglycan is not restricted to the muscle tissue, but is currently believed to have a ubiquitous expression pattern in human tissues, including the salivary glands, and to function as a mediator between the ECM and the cytoskeleton in a similar manner as the integrins (Durbeej et al., 1998; Winder, 2001).

5.4. ANDROGENS IN SS

As mentioned earlier in the chapter considering the clinical features of SS, this syndrome is commonly seen in female patients 40–50 years old. These patients have commonly undergone or are undergoing meno- and adrenopause. Furthermore, SS is very rarely diagnosed in younger males. These aspects suggest that androgens might be somehow protective against the syndrome.

5.4.1. GENERAL ASPECTS CONSIDERING ANDROGENS IN SS

Estrogens have been considered to favor autoimmune processes whereas androgens have been suggested to be protective against autoimmune diseases (Cutolo and Wilder, 2000). As the function of the ovaries in females cease during aging, one would expect that this should rather protect than predispose women from developing SS. Because androgens in general protect from autoimmune diseases, it might be that the deficiency of androgens in SS patients is the crucial factor involved in the development of SS in particular in women. Patients with SS have been reported to have low serum DHEA-sulphate concentrations (Valtysdottir et al., 2001).
DHEA and its sulphate metabolite DHEA-S are secreted by the human adrenal gland, the production of which also ceases during aging (Alesci and Bornstein, 2001). The reduced production of DHEA, DHEA-S and androstenedione (4-dione) prohormones in the reticular zone of the adrenal cortex start often before the age of 50 years and is known as adrenopause. The lack of prohormones in the circulation or androgen effects at the target tissue level might also contribute to the fatigue symptom common in patients with SS. This study concentrates in the endocrine production of DHEA and its sulphated form and their intracrine conversion in salivary gland tissue and cells into androgens.

5.4.2. DEHYDROEPIANDROSTERONE (DHEA) AND ITS NORMAL FUNCTION IN VIVO

DHEA and its sulphated form DHEA-S as well as 4-dione are weak androgenic hormones produced from cholesterol by the reticular zone of the adrenal cortex and secreted in large amounts to the circulation, a feature unique to humans and other higher primates. Small amount of DHEA is also produced in the gonads of premenopausal women, adipose tissue, brain and skin. DHEA is first and foremost a prohormone for the sex steroid synthesis: DHEA and 4-dione can be converted into more potent androgens and estrogens in at least some peripheral tissues (Labrie et al., 1998; Notelovitz, 2000; Spaan et al., 2009) (Figure 4). The secretion of DHEA/DHEA-S is in part under the control of the hypothalamic-pituitary-adrenal (HPA)-glucocorticosteroid axis. High concentrations of glucocorticosteroids in serum have inhibitory effect on the production of corticotropin-releasing hormone (CRH) by the hypothalamus and vice versa. The release of CRH from hypothalamus is also influenced by stress (mainly via stress related cytokines) and the sleep/wake cycle. CRH stimulates the anterior pituitary gland to produce pro-opiomelanocortin, a source of adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal gland to produce glucocorticosteroids and other corticosteroids such as DHEA from cholesterol. Orally ingested DHEA is converted into DHEA-S when passing through intestine and the liver. The serum levels of adrenal DHEA and DHEA-S are high in both sexes between 20–30 years of age and start to decrease progressively and profoundly during aging. The characteristic aspect in the function of DHEA/DHEA-S is their peripheral intracrine processing, i.e. they are locally processed and tailor-made in the target tissues to final potent end products, androgens and estrogens (Labrie et al., 1998; Spaan et al., 2009; Figure 4). DHEA is considered as a drive-producing hormone, and it is used as a nutritional supplement for antiaging, metabolic support and for increasing drive in the United States, but without strong evidence base (Arnold, 2009). DHEA has also been reported but not proven to inhibit breast cancer and to increase bone mineral density, muscle mass, and libido.
5.4.2.1. **Intracellular processing of DHEA**

After the menopause the secretion of estradiol by the ovaries ceases and almost 100% of estrogens in postmenopausal women are made locally in peripheral tissues in intracrine manner (Labrie et al., 2003). Prior to adrenopause, adrenal glands secrete DHEA, which is fairly weak androgen by itself but is largely a precursor of biologically more potent steroids such as testosterone and dihydrotestosterone (DHT). DHEA can be intracellularly processed to several biologically active androgens and estrogens. Transformation of the adrenal precursor steroids DHEA/DHEA-S to androgens and estrogens in peripheral tissues depends on the expression of different steroid-converting enzymes in tissues. Steroid sulphatase desulphates DHEA-S into DHEA and estrone sulphate (E1-S) into estrone (E1). 3β-hydroxysteroid dehydrogenase (3β-HSD) converts DHEA into 4-dione and androstenediol (5-diol) into testosterone. 17β-hydroxysteroid dehydrogenase (17β-HSD) converts DHEA into 5-diol, 4-dione into testosterone and E1 into estradiol (E2). Finally 5α-reductase-1 and -2 convert testosterone into DHT and aromatase converts testosterone into E2 and 4-dione to E1 (Labrie et al., 2003; Spaan et al., 2009). The intracrine cascades discussed above are schematically portrayed in Figure 4. In breast and uterine tissues, DHEA is mainly converted to 17-β-estradiol by the aromatase enzyme rather than metabolized to testosterone or DHT. Accordingly, aromatase inhibitors such as anastrozole are used clinically in the treatment of estrogen-sensitive breast cancer (Howell et al., 2005). Similarly in males DHEA in prostate is mainly converted first to testosterone and then by 5α-reductase-1 and -2 to DHT. DHT has strong effects on the prostate cells causing in the longterm benign enlargement of the prostate, which causes several urological symptoms in males. Accordingly 5α-reductase inhibitors such as finasteride (inhibits 5α-reductase-2) or dutasteride (inhibits both 5α-reductase-1 and -2) are used as effective drugs in the treatment of this condition as well as in the treatment of prostate cancer (Naslund et al., 2008). When DHEA is administrated to patients, the strongest effect is seen on the circulating levels of the glucuronide derivatives of the metabolites of DHT, which means that the administrated DHEA and DHEA-S are converted locally in peripheral tissues to DHT more than to E2 (Labrie et al., 2003). The steroid end-products act locally and mostly intracellularly on the cells of the tissues and do not diffuse into the circulation in large quantities, a hallmark for the intracrine mechanism of action. The local tailoring of androgens at the target tissue levels supplies cells with tools to meet their site-, gender- and
Figure 4. Schematic representation of the human steroid enzymes in peripheral intracrine tissues. Abbreviations: DHEA (dehydroepiandrosterone), DHEA-S (dehydroepiandrosterone-sulphate), 3β-hydroxysteroid dehydrogenase (3β-HSD), androstenedione (4-dione), androstenediol (5-diol), 17β-hydroxysteroid dehydrogenase (17β-HSD), dihydrotestosterone (DHT) (Modified from Labrie et al., 2003 and from Spaan et al., 2009).
5.4.2.2. **Cysteine-rich secretory protein -3 (CRISP-3)**

CRISP-3 (also known as specific granule protein of 28 kDa, SGP28) is a relatively recently discovered protein that may have a role in the innate immune defence (Kjeldsen et al., 1996). With the help of Northern blot analysis CRISP-3 has been found to be present in large quantities in salivary glands, pancreas and prostate. In smaller quantities it is present in the epididymis, ovaries, thymus and the large intestine (Kratzschmar et al., 1996). Also several body fluids such as saliva, sweat, blood and semen contain CRISP-3 (Udby et al., 2002).

CRISP-3 was originally described as an androgen-dependent protein in mouse salivary and lacrimal glands (Haendler et al., 1993). In mice the secretion of CRISP-3 is regulated by the androgens and mice have an androgen responsive element (ARE) in the regulatory region of the CRISP-3 encoding gene (Haendler et al., 1993). This is a very useful finding for SS research because postmenopausal women (the most common target group of SS) and patients with SS in particular have been reported to have clearly lowered concentrations of DHEA in serum (Alesi et al., 2001; Valtysdottir et al., 2001). Under these circumstances CRISP-3 could be used as a potential DHEA (androgen)-regulated marker. This on the other hand could help to explain the female predominance, the most common age of onset of the syndrome and the exocrine gland involvement in it.

5.4.2.3. **Effect of sex steroids on laminin and integrin production**

Prior to this study not much was known about the effect of sex steroids on the production of laminins and integrins in salivary gland cells. The aim of this study was to test if DHEA has stimulatory effects on the production of laminins and/or integrins.

5.4.3. **SS AND SALIVARY GLANDS**

In salivary glands the intracrino logical processing of DHEA occurs similarly as in other peripheral tissues. Salivary glands contain several corticosteroid-converting enzymes. In salivary glands DHEA/DHEA-S are primarily converted to androgens 4-dione and testosterone or can even further be metabolized to DHT (Spaan et al., 2009). In normal healthy salivary glands DHT is produced from testosterone by 5a-reductase-1, whereas patients with SS may have defects in this conversion process (Notelevitz, 2000; Porola et al., 2008).
5.5. SS IN MALE PATIENTS

As stated earlier, 9 out of 10 patients with SS are women (Ahmed et al., 1985). Accordingly, the mere 10% of patients with SS are males. The clinical manifestations of SS seem to be similar in both sexes (Vitali et al., 2002; Konttinen et al., 2009). It is also known from epidemiologic and clinical studies and from clinical experience that patients with SS are rarely young males. Furthermore, as there is a clear-cut peak incidence of SS at 40–50 years age (Talal, 1987; Vitali et al., 2002; Delaleu et al., 2005), it is evident that most male patients with SS are elderly. The reason for the clear-cut female predominance in SS is unknown. Women have relatively low levels of androgens as the result of menopause resulting from the gradual termination of steroid production in the ovaries and the lowering of the DHEA production in the adrenal cortex as the result of adrenopause. After the adulthood has been reached the DHEA and DHEA-S levels start to decline in both sexes so that at 70–80 years of age, the peak DHEA-S concentrations in the serum are only 10–20% of those of young adults (Genazzani et al., 2007). In addition to low DHEA-S levels, androgen deficiency in the aging male can be caused by hypogonadism. It can be treated with testosterone replacement therapies. In fact some elderly males can have low levels of both DHEA and testosterone as do elderly females. Hypogonadism is to some extent also related to obesity, especially to type 2 diabetes (Kapoor et al., 2007), but younger obese males tend to have normal function of their adrenal glands. These phenomena could explain why also males can be affected and why they tend to do so at a relatively high age (Ramos-Casals et al., 2008).
6. AIMS OF THE STUDY

1. To assess the distribution of laminins in the labial salivary glands of normal healthy control subjects and patients with Sjögren's syndrome.

2. To assess the distribution of integrin and non-integrin laminin receptors in the labial salivary glands of normal healthy control subjects and patients with Sjögren's syndrome.

3. To assess the DHEA/DHEA-S concentration in relation to the expression of CRISP-3 in salivary gland cells and salivary glands in healthy control subjects and patients with Sjögren's syndrome.

4. To assess the effect of androgens on laminins and integrins in salivary gland cells and salivary gland explants in healthy control subjects and patient's with Sjögren's syndrome.
7. MATERIALS AND METHODS

7.1. PATIENTS AND SAMPLES

The ethics committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS, Finland) approved the projects of this thesis study (I-IV). The diagnosis of primary SS was based on the American-European consensus criteria for SS (Vital et al., 2002). Healthy control subjects were either treated for other conditions, for example for mucocele, had a suspicion of SS that was not later confirmed by the diagnostic criteria or they were enrolled the study as volunteers or in a routine check up. One such a check-up was the Finnish Broadcasting company (YLE) study, where the employees underwent a normal physical examination, in association to which also saliva and blood samples were collected. LSG samples of all healthy controls had a focus score < 1, whereas the focus score in SS was always ≥ 1 (I-IV). All subjects were asked and confirmed their informed consent. In the text below the patients and samples are described in the context of the methods used to analyse them.

7.1.1. INCLUSION AND EXCLUSION CRITERIA FOR THE STUDY

The main inclusion criterion for this study was SS as diagnosed based on the American-European consensus criteria. For most of the saliva and serum samples three inclusion criteria were used, because the same samples were also used in a randomized clinical trial on the effect of DHEA substitution on fatigue in SS (Virkki et al., 2010). These three inclusion criteria were: 1) Primary SS according to the American-European consensus criteria, 2) General Fatigue ≥14 calculated from MFI-20 (Multiple fatigue inventory-20 questionnaire, d’Elia et al., 2008); the value was based on a pilot study of 239 members of the Finnish SS Patient Association, 3) subnormal serum DHEA-S values (the age and gender adjusted reference values were calculated based on a pilot study of 81 healthy women and 57 healthy men).

The exclusion criteria were 1) Age <18 years or >80 years, 2) prisoner, 3) individuals not able to give their informed consent, 4) history of breast cancer, 5) history of uterus cancer, 6) history of prostatic cancer, 7) history of stroke or prothrombotic coagulation disorders, 8) pregnant or lactating women, 9) fertile patients without adequate prevention, 10) difficult acne, 11) a significant liver disease,
12) patients with changes in their systemic medication taken for SS during the previous three months, 13) patients taking more than 10 mg prednisolone per day.

7.1.2. **COLLECTION OF THE LABIAL SALIVARY GLAND (LSG) SAMPLES (I-III)**

In the tissue sample collection five to ten individual LSGs were removed during an incision made to the lower lip mucosa under local anesthesia (Xylocain Dental Adrenalin®; lidocain-adrenalin 20 mg/ml / 12.5 μg/ml). After incision the glands were removed separately by blunt dissection (Daniels, 1984) and immediately snap frozen in liquid nitrogen, stored at -70°C and embedded in OCT™ (Sakura, Zoeterwoude, the Netherlands) before cutting in a cryostat. Some tissue specimens were fixed in formalin and embedded in paraffin. These tissue sections were later used for staining experiments after deparaffinization. An inflammatory focus score was calculated from 6 μm sections under the light microscope using a 400X magnification by counting the number of mononuclear cell infiltrates containing at least 50 inflammatory cells and is expressed as the number of such foci per 4 mm² glandular area (Daniels, 1984; Segerberg-Konttinen et al., 1986). In report I the LSGs were obtained from 8 patients with primary SS (7 female and 1 male; age 50 ± 7 [mean ± SEM] years [range 25–79]) and 11 healthy control subjects (all females; age 50 ± 2 [mean ± SEM] years [range 41–65]). In the report II the LSGs were obtained from 5 patients with primary SS and from 6 healthy control subjects. In the report III the LSGs were obtained from 11 patients with primary SS (all females, mean age 47 years) and from 11 normal healthy control subjects (all females, mean age 50 years). In the report IV the LSGs were obtained from 3 patients with SS (all females, ages 44, 58 and 65 years) and from 4 healthy controls (3 female and 1 male; ages 17, 17, 21 and 57 years).

For additional tissue explant stimulation experiments LSG tissue samples were obtained from 2 healthy controls (one male and one female, both aged 17 years) treated for mucocele (report III). These samples were minced into approximately 2 mm³ pieces, put into a 6-well-plate and left in DMEM/F-12 medium (Gibco BRL, Grand Island, NY) containing 10% foetal calf serum (FCS) with 1000 U/ml penicillin, 2nM L-glutamine, 1 mg/ml streptomycin and Fungizone (2.5 μg/ml) (Gibco BRL, Grand Island, NY) solution overnight.

7.1.3. **COLLECTION OF THE RESTING AND STIMULATED SALIVA (III)**

Resting and stimulated saliva was collected from 29 patients with SS (27 females and 2 males, mean age 53 years) and 32 healthy control subjects (13 females and 19 males, mean age 45 years). The collection of resting saliva was done between 9
a.m. and 2 p.m. Subjects rinsed their mouths thoroughly with water prior to the collection. Collection of stimulated saliva was done similarly, but the subjects chewed paraffin-wax for 30 seconds, expectorated and continued to chew paraffin for at least the 5 next minutes and the stimulated whole saliva was collected. Because patients with SS have very diminished secretion of saliva, the saliva collection time was often extended to 15 minutes in both resting and stimulated saliva collection. Salivary secretion rates are expressed as millilitres per minute. Immediately after collecting the saliva, the samples were centrifuged at 1000 g for 5 minutes and the supernatants were frozen at -70 °C until analyzed.

7.2. ANTIBODIES (I-IV)

The primary antibodies used in this study are listed in Table 4.

Table 4. Primary antibodies of this study and some of their characteristics

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Source</th>
<th>Reference</th>
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<tr>
<td>MAb 161EB7</td>
<td>LM α1 chain</td>
<td>Mouse</td>
<td>Virtanen et al., 2000</td>
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<tr>
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<td>Leivo et al., 1988</td>
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<td>Mouse</td>
<td>Rousselle et al., 1991</td>
<td>I</td>
</tr>
<tr>
<td>MAb FC10</td>
<td>LM α4 chain</td>
<td>Mouse</td>
<td>Petäjäniemi et al., 2002</td>
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<td>LM α5 chain</td>
<td>Mouse</td>
<td>Engvall et al., 1986</td>
<td>I</td>
</tr>
<tr>
<td>MAb 114DG10</td>
<td>LM β1 chain</td>
<td>Mouse</td>
<td>Virtanen et al., 1997</td>
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<tr>
<td>MAb S-Laminin</td>
<td>LM β2 chain</td>
<td>Mouse</td>
<td>Chemicon, Temecula, CA</td>
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<tr>
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<td>LM γ1 chain</td>
<td>Mouse</td>
<td>Määttä et al., 2001</td>
<td>I</td>
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<tr>
<td>MAb D485</td>
<td>LM γ2 chain</td>
<td>Mouse</td>
<td>Mizushima et al., 1998</td>
<td>I</td>
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<tr>
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<tr>
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<td>II</td>
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<td>Tamura et al., 1990</td>
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<tr>
<td>MAb IIH6 (IgM)</td>
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<td>Mouse</td>
<td>Ervasti and Campbell, 1993</td>
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<tr>
<td>MAb BRIC221</td>
<td>Lutheran ag</td>
<td>Mouse</td>
<td>Parsons et al., 1997</td>
<td>II</td>
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<tr>
<td>MAb CRISP-3</td>
<td>CRISP-3</td>
<td>Rabbit</td>
<td>Udby et al., 2005</td>
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7.3. INDIRECT IMMUNOFLUORESCENCE LABELING (I-II, IV)

Six μm thick cryostat sections were fixed with acetone at -20°C for 10 minutes (I-II). After fixation sections were stained using the following protocol: 1) wash in 10 mM phosphate buffered 0.15 M saline (PBS, pH 7.4) containing 0.1 % Triton X, 2) wash in PBS, 3) incubation in normal goat serum, 4) incubation in the primary monoclonal antibody. All of the monoclonal antibodies (MAbs, Table 4) were monoclonal mouse IgG antibodies except for GoH3, which is a monoclonal rat anti-human IgG antibody, and IIH6, which is a monoclonal mouse anti-human IgM antibody, 5) phosphate buffered saline and 6) incubation in fluorescein isothiocyanate-conjugated secondary antibodies, goat anti-mouse IgG for monoclonal mouse antibodies (Alexa Fluoro 488, Molecular Probes, Eugene, OR), goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for monoclonal rat anti-human Intα6 IgG and goat anti-mouse IgM (Jackson) for monoclonal mouse anti-human α-DG IgM for 60 minutes at +4°C. 7) Propidium iodide 1:1000 in PBS was used to visualize the cell nuclei. After staining the sections were washed twice in PBS, and once in dH₂O after which the specimens were embedded in fluorescent mounting medium (DAKO, Glostrup, Denmark) and examined under an Olympus AX70 (Olympus, Hamburg, Germany) microscope. Staining intensities of immunostaining reactions were graded to - = negative, + = weak, ++ = moderate and +++ = strong. Results of this crude grading are summarized by providing the mean score values. Particular and systematic attention was paid to the pattern of the staining (pattern recognition). Control immunostainings were performed using irrelevant primary MAbs, for example Mouse IgG (DAKO, X0931, 100 mg/l), of the same isotype at the same concentration as and instead of the primary specific antibodies or using conjugated secondary antibodies alone. Staining controls were negative conforming the specificity of the staining results, obtained using the monoclonal antibodies of this study.

7.4. ANALYSIS OF CRISP-3 (III)

In order to quantify the CRISP-3 messenger RNA (mRNA), total RNA was isolated from human submandibular gland cells (HSG) cultured without and with Matrigel and extracted using dispase (BD Biosciences, San Jose, CA). They were subjected to quantitative real time-polymerase chain reaction (qRT-PCR) using Light Cycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany), fluorescent SYBR Green I label and specifically designed primers for CRISP-3 (240 base pair product) or porphobilinogen deaminase (237 base pair product), which was used as a housekeeping gene for the standardization of the results (Table 5).
Indirect immunofluorescence staining of the cultured HSG cells was used to visualize the corresponding protein. Fixation with 3% paraformaldehyde was first performed to the detached cells and after that cells were permeabilized in 0.5% Triton-X in PBS. Before inspection under immunofluorescence microscope, cells were stained using 0.6μg/mL rabbit anti-human CRISP-3 immunoglobulin and Alexa Fluor 568-conjugated goat anti-rabbit immunoglobulin. As described elsewhere, 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 Laboratories, Palo Alto, CA), cloned into pTrcHisB (Invitrogen, San Diego, CA) and expressed as a fusion protein with a polyhistidine tag (6 x His) in order to produce the rabbit anti-human CRISP-3 antibody used in these experiments. TALON® Metal Affinity Resin (Clontech Laboratories, Palo Alto, CA) was used to affinity purify the protein, which was concentrated and dialyzed. The immunization of rabbits was done using a purified protein. Immunoblotting of a postnuclear supernatant of neutrophils gave two bands (27 kDa and 29 kDa bands corresponding to unglycosylated and glycosylated forms of CRISP-3, respectively) and was used to ascertain the specificity of the antibody. The ability of the antibody to recognize CRISP-3 in paraformaldehyde fixed neutrophils was further confirmed by immunohistochemical staining of leukocytes (Udby et al., 2002).

Table 5. Primers used for cysteine-rich secretory protein-3 (CRISP-3) and porphobilinogen deaminase (PBGD) mRNA.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>CRISP-3</td>
<td>5'-TTCACCCCTTCTCCCAATCA-3'</td>
<td>5'-CCTCCTTGAGTGAGGTATT-3'</td>
</tr>
<tr>
<td>PBGD</td>
<td>5'-ACATGCCCTGGAGAAGAATG-3'</td>
<td>5'-AGATGCCCTTTCCTCTCTG-3'</td>
</tr>
</tbody>
</table>

7.5. SEX STEROID MEASUREMENTS (III)

The quantitative determination of serum levels of DHEA-S (Thermo, Waltham, MA) was done using a radioimmunometric assay. DHEA-S levels in serum were measured from 58 patients with SS (all females, age 41–60 years) and from 22 age and sex-matched healthy control subjects. A salivary DHEA enzyme immunoassay kit (Salimetrics, State College, PA) was used for the measurement of salivary DHEA levels. DHEA levels were measured in resting saliva from 45 patients with SS (all females, age 41–60 years) and from 10 age- and sex-matched healthy control subjects.
7.6. HUMAN SUBMANDIBULAR GLAND CELL LINE (HSG) STIMULATIONS (III, IV)

HSG cells established from neoplastic ductal epithelial cells from an irradiated human submandibular gland (Shirasuna et al., 1981) were a gift from Dr Marc R. Kok in Amsterdam, the Netherlands. These cells with an intercalated duct cell phenotype undergo terminal differentiation into amylase-positive acinar cells and form acinar-like structures when cultured on BM-like but growth factor-depleted Matrigel substrate (Royce et al., 1993). Before the experiments, RT-PCR was used to demonstrate that HSG cells cultured in suspension contain androgen receptors. 80,000 HSG cells in DMEM/F-12 Nut Mix medium (Gibco BRL, Grand Island, NY) supplemented with 10 % FCS, 2 mM glutamine, 100 U/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 without or with 100 µM DHEA in DMEM/F-12 Nut Mix medium with 10 % active charcoal treated, sex hormone depleted FCS.

7.7. IMMUNOHISTOCHEMICAL AVIDIN-BIOTIN-PEROXIDASE COMPLEX (ABC) STAINING (III)

For immunohistochemical staining 4 µm thick paraffin tissue sections were used. Sections were deparaffinized with xylene and rehydrated in decreasing ethanol series. After rehydration, sections were washed in 10 mM Tris, 150 mM NaCl buffer (TBS, pH 7.5). Buffer for Antigen Retrieval (Techmate™, DAKO, Glostrup, Denmark) was used in a microwave oven pretreatment for 2 times 5 minutes at 700W to reveal hidden antigenic epitopes. Subsequently the DAKO Techmate™ Horizon Immunostainer robot was used to automatically stain the slides at 22°C using the following protocol: 1) the primary rabbit anti-human CRISP-3 immunoglobulin (0.6 µg/mL; Table 4) diluted in DAKO ChemMate™ antibody diluent, 25 minutes; 2) biotinylated goat anti-rabbit immunoglobulin in TBS containing carrier protein and sodium azide, 25 minutes; 3) Peroxidase Blocking Solution, 3 x 3 minutes; 4) peroxidase-conjugated streptavidin, 25 minutes, and 5) Substrate Working Solution containing H₂O₂ and 3,3-diaminobenzidine tetrahydrochloride (ChemMate™ detection kit), 5 minutes. During the staining procedure the sections were washed using the DAKO ChemMate™ washing buffer 3 x 5 minutes and dried in absorbent pads between these steps. Non-immune rabbit serum immunoglobulin served as a negative staining control at the same concentration as and instead of the primary immunoglobulin. After the staining procedure the sections were removed from the immunostainer robot, counterstained with hematoxylin, washed, dehydrated in an
increasing ethanol series, cleared in xylene and mounted in synthetic mounting medium (Diatex, Becker Industrifärg AB, Märsta, Sweden).

7.8. IN SITU HYBRIDIZATION (III)

A CRISP-3 cDNA insert comprising nucleotides 1179–1569 (RefSeq code NM_006061) (Udby et al., 2005) was inserted into pBluescript II KS (Stratagene, La Jolla, CA). After linearization with Xho I it was used as a template for in vitro transcription to produce 35S-labeled antisense probes and with Spe I to produce 35S-labeled sense probes. After transcription and prior to hybridization the ribonucleic acid (RNA) probes were ultrafiltrated (Micron 100; Amicon, Inc, Beverly, MA). Deparaffinized, rehydrated 5 μm thick sections were hybridized with 25–50 x 106 cpm of 35S-labeled RNA probes at 55°C overnight. The slides were washed under stringent conditions including incubation with 50 μg/mL RNase-A (Sigma, St Louis, MO) for 30 min at +37°C after the hybridization. Finally, the sections were processed for autoradiography for 3–4 weeks (Stähle-Bäckdahl et al., 1994).

7.9. SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (III)

96 well flat-bottom plates were coated overnight with rabbit anti-human CRISP-3 antibodies in coating buffer (Udby et al., 2005). Non-specific binding was blocked with sample buffer (0.5 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄ / KH₂PO₄, 1 % bovine serum albumin, 1 % Triton X-100, pH 7.2). Four-fold dilutions of CRISP-3 standards (affinity purified native CRISP-3 derived from isolated neutrophils, 1.56 - 100 ng/mL) and samples were diluted in sample buffer, supplemented with 2 % sodium dodecyl sulfate and 4 mM dithiothreitol, and incubated for 20 minutes at 37°C, followed by 2 hour incubation at room temperature. This was followed by biotinylated anti-CRISP-3 antibodies (3 μg/mL) and avidin-peroxidase (1:2000) at room temperature before color was developed by 15 minute incubation in 0.1 M sodium phosphate, 0.1 M citric acid, pH 5.0, and 0.006 % H₂O₂ containing 0.04 % o-phenylenediamine (Udby et al., 2002). Absorbance was read at 492 nm. All assays were performed in duplicate.
7.10. SEARCH FOR ANDROGEN RESPONSIVE ELEMENTS (AREs) (III)

By using RefSeq code NM_006061 the National Cancer Institute BLASTN website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Search&DB=nucleotide) was thoroughly inspected for the sequence of the *crisp*-3 gene. The http://dbtss.hgc.jp/index.html website was used to obtain the bordering sequences of 10,000 base pairs upstream and 2000 base pairs downstream from the transcription initiation site, including the introns of the *crisp*-3 gene complex. The literature search revealed the two 6-base pair asymmetrical half-sites of the ARE sequence (5′-GGA/TACA nnnTGTTCT-3′) (Roche et al., 1992). Finally nucleic acid pattern search was done with the Fuzznuc program and the comparisons between two sequences were executed with National Center for Biotechnology Information BL2SEQ program.

7.11. EXPLANT TISSUE CULTURE STIMULATIONS (III-IV)

For the report III LSG tissue samples were obtained from two and for the report IV from four healthy controls treated for mucocele as discussed above. The samples were ground into pieces (approximately 2 mm³), put into 6-well-plates and left in DMEM/F-12 medium (Gibco BRL, Grand Island, NY) containing 10 % FCS with 1000 U/ml penicillin, 2nM L-glutamine, 1 mg/ml streptomycin (10x) and Fungizone (2.5 µg/ml; Gibco BRL, Grand Island, NY) solution overnight. During the following day, the media were changed to basal DMEM/F-12 media with 10 % stripped FCS, 2 mM L-glutamine, 100 U penicillin, 0.1 mg streptomycin (1x solution) and 2.5 µg/ml amphotericin B. Tissue explants were cultured without and with 100 µM DHEA for 72 hours before subsequent qRT-PCR of CRISP-3 mRNA.

7.12. RNA ISOLATION AND cDNA SYNTHESIS (IV)

Cells grown on Matrigel were detached from the Matrigel with Dispase (BD Biosciences, San Jose, CA). Total RNA was isolated using the Trizol protocol (Invitrogen, San Diego, CA) and mRNA was purified from total RNA using the Dynabeads mRNA Purification Kit (Dynal, Oslo, Norway). cDNA was synthesized using SuperScript First Strand cDNA Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). An equal amount of mRNA from every sample was used for cDNA synthesis.
7.13. QUANTITATIVE REAL TIME-PCR (qRT-PCR) (IV)

Quantitative real-time PCR was done using Light Cycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany), SYBR Green I label and purpose designed primers (laminin α1 forward 5’-GCTCTGTGACTGCAAACAA -3’ and reverse 5’-TTTCTGGGTCGAGGTATTCC-3’; integrin α1 forward 5’-TCCACCGAGAGGTACTTGTTGCA-3’ and reverse 5’-CCAAGCATGACCCAGTCTGTGA-3’; integrin α2 forward 5’-GAGTGGATGAGACATTTTCAT -3’ and reverse 5’-GCTTGGAACTGAGAGCAG-3’). PBGD housekeeping gene was used for standardization of the results by using 5’-ACATGCCCTGGAGAAGATG-3’ as forward and 5’-AGATGCGGAACTTTCTCTG -3’ as reverse primers.

7.14. STATISTICAL ANALYSIS (I-IV)

For the statistical calculations SPSS statistical software, version 13.0 (SPSS, Chicago, IL) was used. As an exception BMDP-PC 7.01 software (Los Angeles, CA) was used for the analysis of the CRISP-3 saliva measurements. The normality of the distribution of the variables was tested with Saphiro-Wilk statistics. Results are expressed as mean ± standard error of the mean (SEM). Student’s t-test or the Mann-Whitney test was used for pair-wise comparison of the normally and non-normally distributed variables, respectively.
8. RESULTS

8.1. LAMININ-CHAIN DISTRIBUTIONS IN LSGs IN HEALTHY CONTROL SUBJECTS COMPARED TO PATIENTS WITH SS (I)

In the report I two separate comparisons between the staining patterns of LSGs from healthy control subjects and patients with SS were completed. First evaluation was the comparison between normal healthy control subjects against mildly (focus score < 2) inflamed LSGs. The second comparison was done against LSGs, which had heavier lymphocyte infiltrates. These procedures showed that in the heavily inflamed LSGs in SS all laminin isoforms were relatively weakly labelled and BMs were disorganized and discontinuous, with the exception of LM-α4 chain (see below), for other findings, see the text below.

8.1.1. LAMININ α1 AND α2 CHAIN

LM-α1 and LM-α2 chains were found to be limited in healthy human LSGs to the BMs surrounding the acini, whereas these LM-α chains were not found in the ductal BMs. In patients with SS the intensities of the staining of LM-α1 and LM-α2 chains were weak compared to normal healthy control subjects. The mean staining intensity score for LM-α1 in SS was 1.2 versus (vs.) 1.8 in healthy controls, and for LM-α2 1.0 vs. 1.6, respectively. Weak acinar LM-α1 and LM-α2 labelling was seen in SS glands compared to healthy control glands, not only in heavily inflamed areas, but in patients with mild focal sialoadenitis also in areas lacking lymphocyte infiltrates.

8.1.2. LAMININ α3 CHAIN

Characteristic to LM-α3 chain staining was an uninterrupted continuous line of staining around acini, ducts and blood vessels. The intensity of this staining was analogous in healthy control subjects and in patients with SS (apart from the heavily inflamed LSG areas) with the staining scores in SS and healthy controls being 2.5 vs. 2.7.
8.1.3. **LAMININ α4 CHAIN**

LM-α4 chain staining was detected in the acinar BMs, whereas BMs surrounding the ducts did not contain it. The staining pattern of LM-α4 chain was found to be interrupted, non-continuous, patchy and haphazard. Staining in the form of continuous uninterrupted lines around the acini was not detected. LM-α4 staining was faint in healthy controls, whereas in patients with SS this staining was stronger. Further, periductal stromal areas infiltrated by inflammatory mononuclear cells stained for LM-α4. Because LM-α4 chain expressed a largely interstitial staining pattern, the purposely designed grading system used for other laminins in this report (I) was unsuitable for grading of the LM-α4 staining. In contrast to the findings mentioned above, LM-α4 staining was relatively intense and widespread already in the mildly inflamed LSGs in SS compared to healthy controls and became stronger and more widely distributed as the degree of inflammation increased.

8.1.4. **LAMININ α5 CHAIN**

LM-α5 chain was found both in acinar and ductal BMs and formed a continuous, uninterrupted stained line adjacent to the tubuloacinar epithelial cells. Its staining pattern and intensity were analogous in patients with SS and in healthy control subjects, staining intensities being 2.2 vs. 2.45, respectively.

8.1.5. **LAMININ β- AND γ CHAINS**

LM-β1 chain was expressed in the BMs of the LSGs surrounding both acini and ducts. Staining was seen in form of a continuous, uninterrupted line, whereas no LM-β2 chain staining was found. LM-γ1 and -γ2 chains were found in the BMs surrounding the acini and ducts in LSGs, forming a continuous, uninterrupted line. Immunoreactivities of LM-β1, -γ1 and -γ2 chains were similar in the LSGs of the healthy control subjects and in the LSGs of the patients with SS with mild inflammation, but weaker in heavily inflamed LSGs (see above).
8.2. DISTRIBUTION OF INTEGRIN AND NON-INTEGRIN RECEPTORS OF LAMININS IN HEALTHY CONTROL SUBJECTS AND PATIENTS WITH SS IN THE LSGs (II)

8.2.1. DISTRIBUTION OF INTEGRIN $\alpha$ SUBUNITS

In the LSGs of the healthy control subjects, Int $\alpha_3$ and $\alpha_6$ subunits formed uninterrupted lines on the cellular surface of the acinar and ductal epithelial cells, facing the BMs. Immunoreactivities of the Int $\alpha_1$ and $\alpha_2$ subunits were found only on the acinar cell membranes. In HSG cells (see below) Int $\alpha_1$ and $\alpha_2$ subunits were detected in intercalated duct cells, but in this study (II) the intercalated ducts were not separately analysed due to the difficult histological identification with the immunofluorescence staining method. The staining pattern of the Int $\alpha_1$ and $\alpha_2$ subunits was often interrupted and irregular rather than continuous. Epithelial cell membranes of larger salivary ducts in the LSGs lacked these integrin subunits. Int $\alpha_7$ and $\alpha_V$ subunits were not found in human LSGs.

8.2.2. DISTRIBUTION OF INTEGRIN $\beta$ SUBUNITS

Continuous and strongly positive immunoreactive lines of Int $\beta_1$ and $\beta_4$ subunits were seen on the acinar and ductal epithelial cell membranes facing the BM in both SS and healthy controls. Int $\beta_3$ subunit was not found in the human LSGs.

8.2.3. DISTRIBUTION OF NON-INTEGRIN RECEPTORS

$\alpha$-dystroglycan immunoreactivity was weakly present in the cell membranes of the ductal cells, but absent in the acinar cell membranes. Lutheran blood group antigen staining immunoreactivity formed strong and continuous lines in the acinar and ductal cell membranes facing the BM in both SS and healthy controls.

8.2.4. COMPARISON BETWEEN THE HEALTHY CONTROL SUBJECTS AND PATIENTS WITH SS

Int $\alpha_3$, $\alpha_6$, $\beta_1$ and $\beta_4$ subunit immunoreactivities formed continuous, uninterrupted lines on the acinar and ductal epithelial cell membranes facing the BM in the LGSs.
No differences in their staining intensity or their distribution were detected between the healthy control subjects and patients with SS.

Int α1 and α2 subunits were found almost solely on the acinar cells, forming immunoreactive interrupted and irregular lines on the basal aspect of the cell membranes in the LSGs of the healthy control subjects. The corresponding staining was weak in patients with SS.

Immunoreactivities for Int α7, αV or β3 were not present in human LSGs, whereas α-dystroglycan stained weakly on the ductal, but not on the acinar cells, in a similar fashion in the LSGs of healthy control subjects and in the LSGs of patients with SS. Lutheran blood group antigen immunoreactivity was strong around the acini and the ducts both in the LSGs of healthy control subjects and in the LSGs of patients with SS.

8.3. DHEA CONCENTRATIONS AND ANDROGEN-REGULATED CRISP-3 IN SS AND HEALTHY CONTROL SUBJECTS (III)

8.3.1. AREs IN THE HUMAN CRISP-3 GENE COMPLEX

The human *crisp-3* gene, including 10,000 base pairs upstream and 2,000 base pairs 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/TACAnnnTGTTC3′.

8.3.2. DHEA AND DHEA-S CONCENTRATIONS

58 patients with SS (age 41–60 years) had low serum levels of DHEA-S compared with 22 healthy control subjects (mean ± SEM 2.0 ± 0.2 μM vs. 3.4 ± 0.3 μM; p < 0.0001). 48 of the 58 patients with SS who participated in this study did not use glucocorticosteroids, whereas 10 used low-dose glucocorticosteroids (<10 mg/day prednisolone or equivalent). The corresponding serum levels of DHEA-S were higher in those patients who did not use glucocorticosteroids than in those who did (mean ± SEM 2.3 ± 0.19 μM vs. 0.9 ± 0.22 μM; p = 0.001). In patients with SS who did not use glucocorticosteroids the serum levels of DHEA-S were significantly lower than in healthy control subjects (2.3 ± 0.19 μM vs. 3.4 ± 0.3 μM; p = 0.008). 37 of the 58 patients with SS were premenopausal, 3 had undergone ovariectomy, and 18 were postmenopausal. The corresponding serum levels of DHEA-S in these patient groups were 2.3 ± 0.23 μM, 1.3 ± 0.17 μM, and 1.8 ± 0.3 μM, respectively. Salivary
levels of DHEA were significantly lower in SS patients \((n = 45)\) than in age- and sex-matched healthy controls \((n = 10)\) \((224 \pm 33.3 \text{ pM} \text{ vs. } 419 \pm 98.1 \text{ pM}; \ p = 0.005)\).

### 8.3.3. ANDROGEN RESPONSIVENESS OF HSG CELLS AND LSG EXPLANTS

A significant increase in human CRISP-3 mRNA copy number was seen in HSG cells cultured on growth factor-depleted Matrigel and stimulated with 100 \(\mu\text{M}\) DHEA for 24 hours \((p = 0.018 \text{ vs. unstimulated cells, see below})\). The CRISP-3 mRNA level in HSG cells cultured without Matrigel was 99 \pm 56 copies/\(10^5\) PBGD 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 \pm 207 copies/\(10^5\) PBGD copies. CRISP-3 mRNA levels were further up-regulated by DHEA so that the cells cultured on Matrigel in the presence of 100 \(\mu\text{M}\) DHEA had 1274 \pm 1085 CRISP-3 mRNA copies/\(10^5\) PBGD copies. The range of values in individual experiments varied, however, the change was always in the same direction and the rise in CRISP-3 mRNA was significant \((p = 0.018\) by Wilcoxon signed rank test). The translation of this mRNA to the corresponding protein was suggested by a straightforward increase in the immunofluorescence staining intensity of CRISP-3 of the cultured and stimulated cells. Finally, explants of human LSGs were stimulated with 100 \(\mu\text{M}\) DHEA for 72 hours and an increase in the CRISP-3 mRNA levels was seen in cultured HSG tissue explants from two different healthy donors in two parallel experiments.

### 8.3.4. CRISP-3 STAINING

CRISP-3 protein was expressed in half-moon like demilunes in the acini of the LSGs of healthy control subjects, whereas mature mucous cells stained only weakly. On the contrary, in the LSGs of patients with SS, CRISP-3 staining was weaker and had lost its polarized organization in the acini. The staining intensity in the salivary ductal epithelial cells was weak in the LSGs of the healthy controls subjects, whereas CRISP-3 protein was often found as desiccated secretes in the lumen of the salivary ducts of patients with SS. Lymphocytes in the interstitium in healthy glands and in lymphocyte foci in SS were found to be CRISP-3 negative in paraffin sections. The CRISP-3 staining pattern was found to be pathologic in all acinar cells in all acini in all LSGs studied in SS and independent of the location of the lymphocyte infiltrates. Even acini in areas clear of inflammatory cells disclosed weak and disorganized CRISP-3 staining in patients with SS.

Nonimmune normal rabbit immunoglobulin at the same concentration as (and instead of) the specific primary antibodies was used as the negative staining
control and it verified the specificity of staining in healthy control subjects and in the patients with SS. Erythrocytes were negative, which implies successful blocking of the endogenous peroxidase. As a positive internal sample control the infrequent but CRISP-3-immunoreactive intravascular and/or stromal neutrophils were useful.

8.3.5. IN SITU HYBRIDIZATION OF CRISP-3

In the LSGs of the healthy control subjects, CRISP-3 mRNA was observed in the acinar demilunes, whereas mature mucous cells and ductal epithelial cells did not label over the background. In contrast, in the LSGs of the patients with SS, acinar labeling was weak and diffuse, and not organized to demilunes. Ductal epithelial cell labeling did not exceed that of the background. The specificity of the in situ hybridization was ascertained by the negative labeling controls using sense RNA probes instead of the CRISP-3 specific antisense RNA probes. Adipocytes, fibroblasts, striated muscle cells or endothelial cells did not label.

8.3.6. CRISP-3 PROTEIN IN SALIVA

The CRISP-3 concentration (in μg/mg salivary protein) in resting mixed whole saliva was low in patients with SS compared with that in healthy control subjects ($p < 0.005$). Furthermore, when the salivary flow was also taken into consideration, the difference in the CRISP-3 output between SS patients and healthy controls became exceedingly significant ($p < 0.0001$). CRISP-3 was also low in the stimulated mixed whole saliva ($p < 0.0001$, Table 6).

Table 6. CRISP-3 levels in resting and stimulated saliva, values are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Patients with SS</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRISP-3, μg/mg salivary protein</td>
<td>14.0 ± 1.4</td>
<td>8.4 ± 1.4</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>CRISP-3 output, μg/15 minutes</td>
<td>97.6 ± 12.0</td>
<td>21.1 ± 2.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Stimulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRISP-3, μg/mg salivary protein</td>
<td>13.1 ± 0.9</td>
<td>3.7 ± 0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CRISP-3 output, μg/15 minutes</td>
<td>240.1 ± 26.2</td>
<td>53.8 ± 7.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
8.4. EFFECT OF ANDROGEN STIMULATION ON THE SALIVARY GLAND CELL LAMININ AND INTEGRIN EXPRESSION (IV)

8.4.1. EFFECT OF MATRIGEL ON EXPRESSION OF LM α1 CHAIN AND INT α1 AND α2 SUBUNITS IN SALIVARY GLAND CELLS

Culture on Matrigel causes differentiation of human HSG cells of intercalated duct phenotype to acinar cells. This differentiation increased the mRNA levels of laminin α1 constantly over 72 hours [copy numbers per $10^6$ PBGD, 780 ± 668 (n=3), 1262 ± 505 (n=3) and 3233 ± 3512 (n=4) at 24, 48 and 72 hours, respectively]. Also levels of integrin α1 (p=0.013) and α2 (p=0.333) subunit mRNAs changed upon culture on Matrigel. The corresponding phenomenon was seen at protein level in form of slightly enhanced staining of integrin α1 and α2 subunits.

8.4.2. EFFECT OF SEX STEROIDS ON EXPRESSION OF LM α1 CHAIN AND INT α1 AND α2 SUBUNITS IN SALIVARY GLAND CELLS

Sex steroid stimulation did not affect LM α1-chain mRNA levels in ductal or acinar HSG cells or LSG explants.

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

In intercalated duct cells 1, 10 and 100 μM DHEA increased in 72 hours Int α1 subunit mRNA by 37 % (p=0.312), 46 % (p=0.016) and 449 % (p=0.004), respectively. The corresponding increases in Int α2 subunit mRNA were 54 % (p=0.153), 640 % (p=0.003) and 1100 % (p=0.007).

In acinar cells 1, 10 and 100 μM DHEA induced in 72 hours increases of Int α1 subunit mRNA by 90 % (p=0.053), 102 % (p=0.009) and 113 % (p=0.655), respectively, and of Int α2 subunit mRNA by 39 % (p=0.551), 473 % (p=0.046) and 3 % (p=0.594), respectively.

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

Stimulation with 1 nM, 10 nM or 1 μM DHT did not statistically significantly increase the expression of Int α1 subunit mRNA levels/10^6 PBGD in ductal cells because the observed changes were small: 33 %, 31 % and 55 % (66620 ± 35175,
n=11; compared with 88619 ± 20818, n=3; 87172 ± 19015, n=3; and 102971 ± 7663, n=3, respectively). The corresponding changes of Int α2 subunit mRNA levels were 41 %, 3 % and 62 % (5008 ± 2467, n=8; compared with 7064 ± 1231, n=3; 5170 ± 470, n=3; and 8137 ± 1298, n=3). In acinar cells the increases in Int α1 subunit mRNA levels were 16 %, 94 % and 70 % (120926 ± 59627, n=8; compared with 140696 ± 99367, n=2; 234734 ± 122191, n=3; and 205284 ± 95411, n=3) and for Int α2 subunit mRNA levels 135 %, 89 % and 90 % (9422 ± 7167, n=15 compared with 22135 ± 19277, n=2; 17835 ± 11051, n=3; and 17932 ± 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 protein level both ductal and acinar HSG cells cultured for 48 and 72 hours in the presence of DHEA contained slightly more cells intensively positive for Int α1 subunit compared to the cells cultured in the absence of DHEA. In line with the results from qRT-PCR, Int α2 subunit immunostaining was much weaker than that of the Int α1 subunit, but both showed similar effects on staining of testosterone or DHT stimulations. Staining controls were negative confirming the specificity of the staining with the monoclonal antibodies used in this study.

8.4.3. EFFECT OF DHEA STIMULATION ON LM α1 CHAIN AND INT α1 AND α2 SUBUNITS IN SALIVARY GLAND BIOPSIES

Extending the above mentioned observations to tissue samples, the expression of LM α1-chain in LSGs of healthy control subjects and patients with SS was unaffected by stimulation with androgens. This is in agreement with the cell stimulation results. The expression of Int α1 and α2 subunits was increased by DHEA stimulation in LSGs from healthy control subjects, but in LSGs from patients with SS such an increase was not seen. 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 LSGs.
9. DISCUSSION

9.1. PATIENTS AND SAMPLE SELECTION

The patients for the present study were selected using the modified American-European diagnostic criteria (Vitali et al., 2002) in which focal lymphocytic sialoadenitis in LSGs, with a focus score ≥1 and/or SS-A and/or SS-B autoantibodies are required for the diagnosis of SS (Table 1). Regarding the control samples, LSGs are not normally obtained from healthy subjects. However, most of the so called healthy clinical control subjects in this study had some types of symptoms and/or clinical signs of SS, which was the reason for their enrolment and biopsy was taken as part of routine diagnostic procedure; in retrospect, however, these patients did not fulfil the consensus criteria. They had often depression, anti-cholinergic medication, fibromyalgia, forme fruste of the syndrome (only partially fulfilling the diagnostic criteria set) or some unknown reasons for their symptoms. Healthy controls were in such instances not totally healthy, but one would guess that this would rather decrease than increase the differences between the study groups.

The results of this study are also affected by tissue sampling. Lower lip biopsy or actually nowadays LSG biopsy was used in the collection of the minor salivary glands. This operation is fairly easy to perform by the surgeon or the Rheumatologist and samples are always collected from the same anatomically defined area (Daniels, 1984). The LSG samples were in SS and healthy controls different according to the inflammatory focus score, which was calculated from 6 μm thick frozen tissue sections by counting the number of mononuclear cell infiltrates containing at least 50 inflammatory mononuclear cells per 4 mm² glandular area under light microscope, using a graticule and 400 x magnification as described (Segerberg-Konttinen et al., 1986). Focus score values <1 are typical for healthy controls and represent normal values (no focal sialadenitis). In addition to the focus score, the patients were evaluated using the other American-European consensus criteria (Table 1). The usage of the consensus criteria verified that the patients with SS were segregated from the healthy controls even though both groups might initially have had a clinical suspicion of SS.

The collection of saliva samples was done according to the widely accepted and used protocols (Konttinen et al., 1997). Several recent studies concentrating in the contents and biology of the saliva have used the same protocol. However, it must be remembered that saliva is easily affected by several clinical conditions
9.2. THE SPECIFICITY AND SENSITIVITY OF THE METHODS

In this study immunolabelling or immunohistological staining plays an important role. The applied staining methods were indirect staining methods, using initially a primary antibody, into which multiple secondary antibodies or reagents can attach. The secondary antibodies were either conjugated with a label or contained sites into which the colour producing enzyme or enzyme containing complex could later attach. The use of primary antibodies effectively and specifically reveals the immunoreactive sites in the tissue sections in the process. Indirect methods make this type of immunohistological staining very flexible and more sensitive.

Immunohistological methods in this study can be divided to immunofluorescence methods and immunohistochemical methods, e.g. the avidin-biotin-peroxidase complex (ABC) method. In the first method the secondary antibody is conjugated with fluorescein isothiocyanate (FITC) that after photonic excitation emits light under the fluorescent microscope, which for this purpose must be provided with appropriate excitation and emission filters. Immunofluorescence methods have become some type of standard methods in studies focusing on BMs, because real positive staining shows clearly the structure of the BM, whereas negative staining results as practically black field in the microscope, i.e., it produces a good signal-background ratio. Immunofluorescence has proven to be useful and effective also in the clinical practice, for instance in the diagnostics of renal diseases and skin diseases, e.g. mucous membrane pemphigoid (Daniel and Thorne, 2008). It is also fairly rapid method to perform. The immunohistochemical method mentioned above and used in this study was the ABC method, which is not very reliable when studying laminins and laminin-receptors, because interpretation of the results is difficult compared to indirect immunofluorescence staining. However, in the article III, where cellular staining was studied and the morphological differences in the tissues between patients with SS and healthy control subjects were important, the ABC method proved to be a more valid method because it can due to its higher sensitivity be used for formalin fixed, paraffin embedded tissues with good morphological details. With both methods the background staining was minimized and the possibility of false negative or positive staining results was excluded using several measures to improve the quality and specificity of the methods, including positive sample controls and negative staining controls. All antibodies (described in Table 4) were diluted at their pre-tested optimal concentrations to avoid non-specific background staining. Relatively high concentrations of hydrogen peroxidase
in methanol in the ABC method were used to inactivate endogenous peroxidase in tissue sections. The sites that could non-specifically bind secondary antibodies were blocked by the use of normal non-immune sera from the same species in which the secondary antibodies were produced. BSA was added to the primary and secondary antibody solutions to prevent their non-specific binding to the walls of the test tubes. As a negative staining control normal IgG (or IgM) of the same subtype, but of irrelevant specificity (e.g. against glucose oxidase of Aspergillus niger, not present, nor inducible in human tissues), was used at the same concentration as and instead of the primary antibodies. The specificity of most of the antibodies used in this study has been studied earlier using a series of methods, including the use of negative and positive tissue (sample) controls, antigen absorption tests, immunoprecipitation, and/or Western blotting. The references to some such reports are shown in Table 4. Numerous studies have revealed the reproducibility of these methods and thus it is presumed that these antibodies and methods are reliable and specific also in their current applications.

In the first two reports (I, II) only immunostaining was used. This might sound insufficient to reveal the LM and Int distribution at the tissue and cell level. In some studies also qRT-PCR and Western blotting methods have been used to reveal the expression and quantity of LMs or Ints in tissues (Kwon et al., 2006). However, the use of PCR or Western blotting in human tissues for the study of BM proteins is extremely difficult or even impossible for localization studies, because these methods require that the tissues are minced into pieces before extraction of the target molecules for further processing and analysis. Such samples therefore include also all adjacent “contaminating” structures such as the blood vessels, nerves, adipose cells etc. These structures have their own BMs, which makes it difficult to interpret the results from the topographical point of view. Because the focus of this study was the BMs around the acinar and ductal epithelial cells in particular, these methods were not used in reports I and II. In spite of this criticism against the use of PCR and Western methods, it could have been possible to isolate more or less pure salivary gland epithelial cells (Dimitriou et al., 2002) or by laser capture microdissection (Espina et al., 2007) obtain specific tissue structures for more detailed studies, which however were not used in the current study.

Of course immunohistochemistry by itself is not always an adequate method to ascertain the presence and localization of particular molecules in the tissues. The expression of various molecules of interest at the mRNA level can be checked using in situ hybridization methods. However, one must remember that not all mRNAs are translated into the corresponding protein and, in particular, they are not deposited as mRNA molecules, which just provide the nucleotide sequences necessary for the ribosomal translation to the corresponding protein. In this study, tissue localization of mRNA molecules coding CRISP-3 was checked to study if and where CRISP-3 is locally produced in LSGs. In order to quantify the corresponding
mRNA levels qRT-PCR could be used because according to the immunohistological staining CRISP-3 was found almost exclusively in the acinar cells in LSGs. This method was also used in reports III and IV to reveal the effect of DHEA stimulation on CRIPS-3 and LM/Int chain/subunit production.

9.3. LIMITATIONS OF THE STUDY

This study has been performed vigilantly using highly reliable and specific methods. Regardless of the efforts to diminish the artefacts and other confounding factors, all studies, including the present one, have their limitations. These limitations are discussed below.

9.3.1. THE RESTRICTED PALETTE OF ANTIBODIES

The antibodies used in this study were chosen mainly based on knowledge gained from previous localization studies of other tissues (Patarroyo et al., 2002; Virtanen et al., 2000; Petäjäniemi et al., 2004). Collaboration with Professor Ismo Virtanen’s research group enabled the use of a quite wide palette of laminin-chain and integrin-subunit reactive antibodies, as well as the use of other, non-integrin type laminin-receptor antibodies. Table 4 shows the antibodies used in this study. Laminins, for example, form a relatively new family of proteins and new chain combinations of these trimetric proteins have been reported almost annually. This means that this study might not include all possible laminins present in LSGs. However, every effort was made to ascertain selection of those antibodies, which allow recognition in LSGs of a wide spectrum of currently known laminins and integrins. Combinatorial rules between different laminin chains and integrin subunits are to a large extent known and were used in the interpretation of the staining results in this study (Patarroyo et al., 2002), which enabled conclusions about the presence of various laminin heterotrimeric and integrin heterodimeric in LSGs and their potential interactions. It is naturally possible that future studies will reveal new combinations for these protein chains, which can further extend the present findings.

9.4. DISTRIBUTION OF THE LAMININ CHAINS IN LABIAL SALIVARY GLANDS

Laminin α1-chain was detected exclusively in the acinar BMs in this study. Laminin α1-chains were not found in the ductal or vascular BMs. In contrast to these findings one earlier study reported that Lm α1-chain is present in both acinar and ductal
BMs (Strassburger et al., 1998). Closer evaluation of this report disclosed that those experiments were performed using the monoclonal mouse anti-human 4C7 antibody (Engvall et al., 1986). It was at that time considered to recognize laminin α1-chain, but it has later been conclusively shown to recognize LM α5-chain and not LM α1-chain (Engvall et al., 1986; Tiger et al., 1997; Table 4). Therefore, the results of this earlier study need to be re-interpreted because that study shows the presence of, not of LM α1-chain, but of LM α5-chain in both the acinar and ductal BMs in salivary glands. This is totally in accordance with the findings of the present study, in which the same monoclonal mouse anti-human 4C7 antibody was used, so we were able to confirm the earlier findings (Strassburger et al., 1998). In contrast, in this study the eventual presence and tissue localization of laminin α1-chain was analyzed using the only available LM α1-chain specific monoclonal mouse anti-human 161EB7 antibody, the specificity of which has been validated elsewhere (Virtanen et al., 2000). The staining results with 161EB7 antibody clearly showed that LM α1-chain is only present in BMs surrounding the salivary gland acini. Staining intensity of LM α1-chain varied in the acinar BMs and was not as strong and homogenous as it was for some other laminins, which was interpreted to possibly reflect some dynamic and regulatory rather than structural and supporting function. Furthermore, in line with this reasoning, the intensity of LM α1 labelling was clearly weaker in SS LSG samples indicating that whatever the above speculated regulatory function is, it may be impaired in SS. Staining with LM β2-chain recognizing MAb were also performed and showed that LSGs did not contain this chain. Using the current staining results and the known combinatorial rules for the laminin α, β and γ chains, it was concluded that LM α1-chain forms a component of LM-111 in the acinar BMs of the human LSGs (Patarroyo et al., 2002; Tables 3 and 4). This would mean that the LM α1-chain and the corresponding LM-111 form part of some dynamic regulatory machinery in the acinar BMs, which is at least structurally changed in LSGs in patients with SS.

Using the pattern recognition analysis, the LM α2-chain labelling was also found to be restricted to the acinar BMs and formed interrupted lines around the acini. It was concluded that this laminin α chain has such an interrupted staining pattern because it has been reported to be associated with the myoepithelial cells in healthy major salivary glands (Strassburger et al., 1998). Two to three myoepithelial cells with long cellular extensions, all provided with a BM of their own, surround the ball- or cylinder like acinus, which might explain the current staining pattern. This suggests that LM α2-chain and the corresponding LM-211 play a role in the myoepithelial cell function in healthy LSGs. Since myoepithelial cells contain αSMA and myosin filaments, they are able to contract thus pushing the secreted saliva and mucins further into the ducts. Myoepithelial cells probably provide dynamic support for acini during the secretory and recovery phases of their functional cycles and help to squeeze the mucin-rich and viscous acinar cell secrete from the acinar
lumen to salivary ducts (Konttinen et al., in press). The staining intensity of the LM α2-chain was low in patients with SS compared to the healthy control subjects. This suggests that also the corresponding acinus-related function has changed in SS and associated with the dry mouth symptom, which is a characteristic clinical feature in SS.

Pattern recognition analysis was also used in the interpretation of the results of the labelling with FC10 MAb specifically recognizing the LM α4-chain (Petäjäniemi et al., 2002). Pattern recognition analysis disclosed irregular, interrupted, patchy and haphazard acinar and interstitial labelling for LM α4-chain. This is in accordance with findings showing that in addition to epithelial cells, fibroblasts, adipocytes and endothelial cells produce LM α4-chain (Colognato and Yurchenko, 2000), which is also found as intercellular cementing substance between synovial lining cells (Poduval et al., 2010). LM α4-chain is in LSGs a component of LM-411, because LM β2-chain was not detected and, thus, LM-421 cannot be present. This staining pattern of LM α4-chain was particularly clear in SS LSG samples, in which LM-411 was mainly detected in the interstitial connective tissue. In contrast to the universal pattern of LM α1- and α2-chain distribution, LM α4-chain was mainly found in the lymphocyte infiltrated areas. This finding was interpreted as an increased local inflammation-stimulated synthesis and/or repair. Recently it has been shown that stimulation with pro-inflammatory cytokines TNF-α or IL-1β increase the LM-α4 mRNA levels (Poduval et al., 2010). This LM α4 staining pattern could represent a response to the damage of acinar BMs and interstitial stroma caused by inflammation and proteolysis in focal sialadenitis in SS. In this context it should be mentioned that earlier studies on laminins in salivary glands suggested abnormal periductal/interstitial laminin staining and increase of the laminin expression in SS (McArthur et al., 1993). Our results show that although the assumed regulatory (minor) LM α1- and α2-chain expressions were decreased in LSGs of patients with SS, the much more abundant LM α4-chain expression was increased in SS. In the aforementioned study the antibody used was a commercial anti-human laminin antibody (LAM-89), the exact specificity of which is unknown. It might be that the increase of laminin expression detected in that study represents an increase in the expression of LM-411, which is secreted by fibroblasts, adipocytes and endothelial cells. The study by McArthur and coworkers could therefore be interpreted so that these cells start to secrete LM-411 early in the course of the syndrome, possibly as an effort to repair the damages conducted by the disease process at its sub-clinical or early stages.

Herein should be mentioned that Kwon and co-workers from the University of Chile have published somewhat divergent LM-α1 and -α4 expression results (Kwon et al., 2006). They reported that LM α1-chain protein levels were significantly increased in extracts of LSGs from patients with SS having low interacinar fibrosis but not from patients with high interacinar fibrosis. Kwon and coworkers used a commercial polyclonal rabbit anti-human LM α1-chain antibody (H-300) raised
against an epitope corresponding to amino acids 1856–2099 of LM α1 chain of human origin and Western blotting. We used monoclonal mouse anti-human LM α1 chain antibody (MAb161EB7) raised against a recombinant LM α1 polypeptide corresponding to the carboxyterminal globular domains G4-G5 of E3 region of human LM α1 chain and immunofluorescence staining (Virtanen et al., 2000). In the Swedish Human Protein Atlas H-300 stains weakly over 75% of the glandular cells (cellular staining), whereas the MAb161EB7 clearly stains the acinar BMs (basement membrane staining). The specificity of MAb161EB7 was confirmed by using immunoprecipitation of culture medium of metabolically labeled JAR human choriocarcinoma cells (Virtanen et al., 2000). It may be that the difference in the reactivity of the LM α1 chain antibodies used in these two reports explain their contradictory results.

Also the results of LM α4-chain were apparently somewhat divergent compared to the present study. Densitometric results of reverse transcriptase-PCR showed no differences in LM α4-chain levels between controls and SS (Kwon et al., 2006). However, LM α4-chain is also synthesized by fibroblasts, adipocytes and endothelial cells, which acts as a confounding factor in PCR analysis. Somewhat contrary to the PCR results, the intensity of the LM α4 chain protein bands in densitometry was increased in SS patients with low interacinar fibrosis (p = 0.01), but decreased in SS patients with high interacinar fibrosis (p = 0.03) (Kwon et al., 2006). Maybe the decreased LM α4 levels in Western blotting in high interacinar fibrosis group reflect the lack of inflammation and repair in such advanced cases.

Another conclusion in the present study drawn from the LM chain labelling was that LSGs also contain LMs-311, -332 and -511, but both in acinar and ductal compartments. It seems that these LMs function as structural laminins defining and maintaining the normal architecture of the tubuloacinar LSGs whereas LMs-111, -211 and -411 have more specific acinus-related functions described above. Altogether it is clear that the laminin distribution in LSGs is highly complex. Based on the literature, disease pathomechanisms and present results, it is concluded that a diminished expression of LMs-111 and -211 and an increased expression of LM-411 in SS are early changes. Later even more drastic inflammation and proteolysis related local BM changes develop during the autoimmune inflammatory stage. Laminin expression in general is then mainly decreased at the site of lymphocyte infiltrates as the disease process proceeds, except for LM-411, which is increased possibly already at an early stage of the disease process as a repair attempt. These involvements can contribute to some of the main clinical features of SS, namely dry mouth and diminished salivary flow.
9.5. DISTRIBUTION OF POTENTIAL INTEGRIN AND NON-INTEGRIN RECEPTORS OF LAMININS IN LABIAL SALIVARY GLANDS

The laminin receptors are important during the development and maintenance of the salivary glands because they deliver messages from the laminins in the underlying BMs to the epithelial cells (Loureño and Kapas, 2005). These receptors have overlapping and somewhat redundant functions as they may recognize several different laminins, which was one of the reasons for the relatively wide laminin receptor profiling in this study. Similarly to laminins, the combinatorial rules of integrin heterodimers are also known (Hynes, 2002; Patarroyo et al., 2002). This allowed conclusions to be drawn of the eventual presence of various heterodimeric integrin receptors from the staining results of the individual Int α and β subunits. The complex expression of laminins in LSGs suggests that also integrins have variable and BM laminin-related roles in LSGs. Strong uninterrupted linear staining of the basal domains of the acinar and ductal epithelial cell membranes was observed for α3, α6, β1 and β4 Int subunits, suggesting that Ints α3β1, α6β1 and α6β4 face the corresponding BMs. The morphology of the salivary gland is maintained by the firm attachment of epithelial cells to the underlying BM laminins by laminin-receptors. The Ints α3β1, α6β1 and α6β4 have been reported to be receptors mainly for the LM-332 and LM-511 (Patarroyo et al., 2002). These LMs were shown above to be present and form uninterrupted firm lines around ductal and epithelial cells (except for heavily infiltrated areas in SS; I). These findings were similar in healthy control subjects and in patients with SS, which suggests that these integrins play important structure maintaining roles. In early stages of SS the structure of the glands is maintained even though glandular function is diminished or lost. These integrins might as well prevent the shedding of the LSG epithelial cells to saliva as result of myoepithelial cell contractions and shear forces exerted by the salivary flow. In the pattern recognition analysis these Ints and LMs had reciprocal profiles, which would be in accordance with their mutual interactions. This expression pattern strongly suggests structure maintaining and dynamic supporting function as suggested above (I).

Recently the role of Int α6β4 in attachment to BM has been studied in detail. As stated above, the maintenance of equilibrium between cell-cell and cell-basement membrane attachment is required to sustain acinar cell survival. No significant differences in Int α6 or Int β4 subunit mRNA levels were detected. Int α6 subunit protein levels did not differ between patients and controls, but Int β4 subunit protein levels were lower in patients than in controls (p = 0.02). In addition, in patients with SS having only mild alterations in their BMs, redistribution of Int β4 subunit also to the lateral surface of the mucous acinar cells was observed and it was
hypothesised to promote formation of cell–cell contacts via Int α6β4, which would help to maintain acinar organization and cell viability. When already severe BM alterations were present, cytoplasmic and basolateral staining of Int β4 of serous and mucous acinar cells increased but it was concluded that this was no longer sufficient to maintain acinar cell survival (Velozo et al., 2009).

Most integrin receptors have multiple ligands, e.g. Int α6β4 binds to LM-111, -211, -332, -411 and -511 (Aumailley and Smyth, 1998). This suggests that Ints may have extremely dynamic and versatile roles in LSGs. In addition to the above mentioned structural functions Int α6β4 might also have regulatory functions in interactions with LM-111 and -211. Normal epithelial cell growth and their differentiation to breast and prostate cells are promoted by Int α6β4 (Giancotti et al., 1996; Mercurio et al., 2001). Because Lm-111 and -211 have weak expression in SS, the eventual regulatory roles of Int α6β4 might be lost or impaired in SS.

Lutheran blood group antigen formed continuous and uninterrupted lines along the basal aspects of the acinar and ductal epithelial cell membranes. A recent study has revealed that Lutheran blood group antigen is a specific receptor for LM-511 into which it binds with great affinity (Kikkawa and Miner, 2005). This additional receptor for LM-511 in LSGs might help the Ints α3β1, α6β1 and α6β4 in executing their tasks because the expression pattern of the Lutheran blood group antigen was highly similar to that of these integrins. Whether it also has some other specific functional tasks in LSGs remains currently unknown.

The expression of Ints α1β1 and α2β1 differed clearly from other Ints in that they were found almost exclusively in the acinar cells, although Ints α1 and α2 subunits were also found in human HSG cells with an intercalated duct cell phenotype. This suggests some type of acinar cell compartment-specific or –dominant function. Indeed, these integrin receptors are the major receptors for LM-111 and -211 (Tulla et al., 2008). In healthy control glands Ints α1β1 and α2β1 were expressed in an interrupted and irregular pattern mimicking a similar pattern seen for LM-111 and -211 in report I. It is therefore concluded that also in salivary glands Ints α1β1 and α2β1 bind to their LM-111 and -211 ligands. Furthermore, a very interesting finding was that also the expression of Ints α1β1 and α2β1 was very weak in the SS salivary glands corresponding very well to the LM-111 and -211 results. This leads to a conclusion that the LM-111 and -211-to-Int α1β1 and α2β1 interactions are defective in SS and, because the salivary gland function and structure become impaired in SS, it is also concluded that these specific molecular interactions are crucial to the normal function and maintenance of the healthy LSG acini.

The main pathologic functional feature of LSGs in SS is the diminished resting salivary flow. The impaired function has earlier been suggested to relate to the acetylcholine muscarinic 3 receptor blocking autoantibodies or the abnormal, non-polarised location of the aquaporin-5 water channels (Steinfeld et al., 2001; Gao et al., 2004). Also some studies have concentrated on the defective coupling between
the postganglionic parasympathetic nerve terminals and acinar cells in heavily inflamed salivary gland tissues in patients with SS. However, these changes were focal and topologically limited to the glandular areas infiltrated by lymphocytes (Konttinen et al., 1992). On the contrary, the reduced expression of LM-111 and -211 as well as Ints α1β1 and α2β1 was seen in all acini, not only in lymphocyte infiltrated areas. LM-111 and -211 together with their integrin receptors have been shown to be important for target recognition, differentiation or stabilization of synapses (Fox and Umemori, 2006). Impaired organization of the acetylcholine containing parasympathetic nerve terminals and the muscarinic receptor containing post-synaptic acinar cell membranes could lead to inadequate stimulus-response coupling and/or abnormal muscarinic receptor exposure, which could contribute to disturbed secretory function of the LSGs.

The other interesting possible function for the LM-111 and -211-to-Int α1β1 and α2β1 interaction might relate to the maintenance of the acinar cell pool. Acini are subjected to continuous and fairly rapid remodelling, at least in rodents (Denny and Denny, 1993). During this continuous remodelling, acinar cells supposedly die by means of apoptosis and are detached from the BM, leaving an empty lot on it and leading to loss of cellular confluence in salivon. A progenitor cell from the close-by intercalated duct migrates then onto this empty lot using integrin-BM interactions and once there, specializes into a secretory acinar cell. As mentioned above, the progenitor cell pool is located in the short tubular segment of the ductal system known as the intercalated duct, located between the secretory acinus and the saliva modulating striated duct (Figure 1). The phenotype of the intercalated duct cells is simple and undifferentiated and they have no specialized secretory function. It is thus concluded that these cells form a progenitor cell pool able to undergo asymmetric divisions. One of the two daughter cells, often called the parent cell, maintains the stemness and progenitor pool but the other one receives a migratory and differentiation signal leading to migration into the acinus and a terminal differentiation into acinar cell, at least in part as a result of local stimuli originating from the BM. This local signal induces in situ maturation of the undifferentiated intercalated duct progenitor cell into a mature secretory acinar cell. This view is supported by the findings that cloned intercalated duct cells have this differentiation potential (Kishi et al., 2006). Also cells belonging to the immortalized human salivary HSG cell line displaying an intercalated duct epithelial cell phenotype are induced into acinar cells upon culture on LM-111 containing mouse Matrigel substrate (Royce et al., 1993). Accordingly, the lack of LM-111 and -211-to-Int α1β1 and α2β1 signalling could lead to an inability to maintain the acinar cells and secretory acinar cell function. Ultimately this might lead to acinar cell atrophy and loss coupled with a reciprocal ductal cell hyperplasia. This is exactly a typical finding in LSGs in advanced SS, a combination of acinar atrophy and ductal cell hyperplasia. Thus, based on this three-fold line of evidence, LM-111 and -211-to-Int α1β1 and α2β1 signalling
may well be crucial for the maintenance of normal structure and function of the healthy human LSGs and for its failure in SS.

9.6. DHEA AND CRISP-3 CONCENTRATION IN SALIVA AND THEIR DISTRIBUTION IN LABIAL SALIVARY GLANDS

In the report III CRISP-3 protein was used as a biomarker for local androgen effects. Analyses of the crisp-3 gene revealed tentative androgen responsive elements (ARE) in the gene promoter region. DHEA-androgen receptor complex is known to bind AREs (Roche et al., 1992; Schwidetzky et al., 1995). Crisp-3 gene activity has earlier been shown to be strongly androgen dependent in the mouse salivary glands (Haendler et al., 1993). A gene bank search performed in this study disclosed several direct or indirect putative AREs also in the human crisp-3 gene complex simultaneously suggesting that also the human crisp-3 gene is under androgen control.

In this study the effect of DHEA on the cultured human submandibular gland cell line was tested. SS is strongly a sex and age related disorder, in which androgen deficiency may play an important part. Indeed, HSG cells stimulated with DHEA increased their CRISP-3 mRNA copy numbers showing that androgens have stimulating effect on the HSG cells. This finding was further extended by use of explants of LSGs from healthy individuals in two parallel experiments, which also resulted in a clear-cut increase of the CRISP-3 mRNA copy numbers reinforcing the hypothesis of CRISP-3 being regulated by DHEA.

CRISP-3 was used as a biomarker for local androgen effects in human salivary glands in healthy controls and in SS patients. In normal LSGs the staining of the acinar crescent-like (demilune) structures was very strong, whereas mature mucous cells that had entered the resting phase did not stain. In patients with SS this type of segregated staining pattern was found to be distorted suggesting that there are structural disturbances in LSGs in SS. These findings were confirmed by in situ hybridization, which also showed labelling of demilunes with autoradiography grains in healthy labial salivary glands whereas in patients with SS CRISP-3 labeling intensity was weak and diffuse. The output of the CRISP-3 into saliva was higher in healthy control subjects than in patients with SS (III).

An interesting feature of the findings presented here is that the changes described above were generalized over the entire area of the LSGs. In contrast to the earlier studies most of the pathological changes described in SS in salivary glands have been strongly related to the local inflammatory cell infiltrates. These areas contain locally produced cytokines, which may locally cause inflammatory functio laesa via their paracrine mode of action (Konttinen et al., 1997). However, results in this study strongly suggest that the presence of some systemic factor or lack of it cause
the generalized findings just discussed. This systemic factor is strongly suspected to be DHEA or its metabolites and this study shows that at least DHEA is able to regulate acinar cell CRISP-3 production. Low CRISP-3 production in patients with SS suggests low concentrations of DHEA systemically, locally or both. Furthermore, the level of DHEA-S in the serum of the patients with SS has been reported to be low (Valtsdottir et al., 2001). Therefore, the level of the DHEA-S was measured in serum also in this study and found to be low. The present study shows that also the salivary concentrations of DHEA are clearly diminished in patients with SS compared to healthy control subjects (III).

To further settle the relationship between low serum levels of DHEA and low CRISP-3 production in patients with SS, experiments with cultured HSG cells were performed. HSG cells were exposed to DHEA and according to the results at least in vitro, DHEA is able to up-regulate the CRISP-3 expression. In this study the laminin α1-chain containing Matrigel alone clearly increased the number of CRISP-3 mRNA copies, but addition of DHEA further increased the number up to nearly five-fold (256 vs. 1274). This confirms the presence of AREs in the gene promoter areas and show a dominant up-regulatory effect of DHEA on CRISP-3 levels.

In this respect one earlier study published in 2002 concentrating on the CRISP-3 mRNA levels in the LSGs of patients with SS and healthy controls should be discussed. This work reported that CRISP-3 is found mainly in B lymphocytes in the LSGs of patients with SS, while CRISP-3 was not observed at all in B lymphocytes or in acinar or any other epithelial cells in normal LSGs (Tapinos et al., 2002). The primers used in that study for qRTP-PCR and for production of in situ hybridization probes recognized something that was absent in healthy control LSGs and only found in SS. However, a detailed analysis of the primers used in that study revealed that they do not recognize human or any other known member of the human CRISP protein family. In the present study CRISP-3 was found in the normal healthy controls subjects both at the mRNA and protein level, and its expression was decreased in SS.

Studies of lacrimal glands in the experimental mouse model of SS have shown that androgens effectively increase the expression of their own receptors in epithelial cells and have an immunosuppressive impact on lymphocyte infiltrates (Sato and Sullivan, 1994; Ono et al., 1995). Some of these effects reflect direct effects of androgens on lacrimal gland acinar cells and their function (Sullivan and Edwards, 1997). There are also reports that androgens have regulatory effect on CRISP-3 in mouse lacrimal glands (Haendler et al., 1993). All of these earlier findings are in accordance with the findings of the present study.

In conclusion, the low level of CRISP-3 in saliva and in the LSGs in SS might be caused by low levels of circulating and/or salivary DHEA or by its inefficient intracrine processing in LSGs. Ultimately this may influence the fate of the acinar cells by impairing their remodelling and function. These findings also suggest that
systemic factors contribute to the clinically evident pathologic findings and in future these factors might provide targets to treatment with e.g. systemically administered drugs.

9.7. ANDROGEN EFFECT ON LAMININ CHAINS AND INTEGRIN SUBUNITS IN SALIVARY GLANDS IN SJÖGREN’S SYNDROME

As discussed in detail above, SS has two interesting characteristic features: 1) low levels of androgens both in the systemic circulation as well as locally in the salivary glands (III; Porola et al., 2008) and 2) defects in those signalling chains from BMs to acinar epithelial cells which use the LM-111 signalling path via Int α1β1 and α2β1 receptors (I, II). The report IV aimed to assess whether these two characteristic features are interconnected. The results of this study imply that androgens effectively increase the expression of Int α1β1 and α2β1 receptors for LM-111 both in ductal and acinar cells of the LSGs. Several androgens (DHEA, DHT and testosterone) were used in these experiments. Interestingly, it was the relatively weak androgen DHEA, which showed to be the most effective upregulator of Ints α1β1 and α2β1. DHEA showed a dose-dependent behaviour in upregulating these integrins (excluding the integrin α2 subunit in acinar HSG cells). The effect of LM-111 was assessed using the Matrigel matrix. Integrin receptor expression was initially relatively low in intercalated duct cells, but without any hormone stimulation increased approximately two-fold upon differentiation to acinar cells on LM-111 containing Matrigel. This suggests that the mere contact with laminin α1-chain increases the expression of LM-111 binding integrins (II), but DHEA and its intracellularly produced metabolites are able to even further increase the expression of these integrin receptors.

The up-regulation of integrins by DHEA was seen in healthy salivary glands, whereas in SS salivary glands this androgen effect on integrin expression was not seen. Thus, it may be that in SS salivary glands the function of the DHEA metabolizing intracrine machinery is impaired (Porola et al., 2008; Spaan et al., 2009). However, the levels of mRNAs of both Ints were to start with higher in patients with SS than in healthy individuals. This might be due to the fact that also the CD4 and CD8 positive T lymphocytes contain Ints α1β1 and α2β1 (Richter et al., 2007). These inflammatory cells are abundant in SS salivary glands, where they form infiltrates. This increased expression of lymphocyte Ints α1β1 and α2β1 has been suggested to be important for generation of inflammatory tissue responses (Andreasen et al., 2003).

The observations presented here provide a link between two features of SS; androgen depletion and extracellular matrix to cell signalling. The locally balanced intracrine processing and stimulatory effects of DHEA seem to play an important role
for the normal remodelling and in the long term for the function of salivary gland cells. In the LSGs of patients with SS DHEA is unable to increase the expression of Ints. Defects in this mechanism could lead to acinar cell atrophy and ductal cell hyperplasia and thus impaired formation of primary saliva in SS.
10. SUMMARY AND CONCLUSIONS

Sjögren’s syndrome is a common autoimmune disease, which affects predominantly elderly women. The main reasons behind this fact have not yet been unveiled. This study suggests that the basement membrane components and their receptors are important for the normal remodelling function of the salivary glands. The maintenance of these functions seems to be androgen dependent. In addition to the systemic circulating concentrations of androgens, also the local intracrine processing of androgens at the target tissue level is important and seems to be impaired in Sjögren’s syndrome.

MAJOR CONCLUSIONS:

1. Laminins have complex and highly regulated distribution in LSGs. Laminins have specific tasks in the dynamic regulation of acinar cell function. LM-111 is important for the normal acinar cell differentiation and its expression is diminished in SS. Also LM-211 and -411 seem to have some acinar specific but somewhat less clear functional tasks in LSGs. Other laminins such as LM-311, -332 and -511 seem to have more general structure maintaining and supporting roles in LSGs and were found to be relatively intact also in SS.

2. Also the distribution of integrin and non-integrin laminin receptors appears to be highly complex in LSGs. Ints α3β1, α6β1 and α6β4 as well as the Lutheran blood group antigen seem to maintain the morphology of the LSGs supplying structural basis for the firm attachment of epithelial cells to the BM. The expression of Ints α1β1 and α2β1 differed clearly from that of the other Ints in that they were found almost exclusively around the acini and probably also intercalated duct cells in saliva suggest some type of acinar cell compartment-specific or –dominant function. Expression of these integrins was lower in SS compared to healthy controls suggesting that the LM-111 and -211-to-Int α1β1 and α2β1 interactions are defective in SS and are crucial to the maintenance of the acini in LSGs. However, one must bear in mind that nearly all integrins have multiple ligands, which substantially widens the possible signalling routes.

3. Low DHEA/DHEA-S concentration in serum and locally in saliva of patients with SS seems to have effects on the salivary glands. This effect on salivary glands was first detected using the androgen-dependent CRISP-3 protein, the production and
secretion of which were clearly diminished in SS. This might be due to the impaired function of the intracrine DHEA prohormone metabolizing machinery, which fails to successfully convert DHEA into its active metabolites.

4. It seems that the progenitor epithelial cells from the intercalated ductal area of the labial salivary glands migrate to the acinar compartment and then undergo a phenotype change from progenitor cells into secretory acinar cells. This migration and subsequent phenotype change seem to be regulated by the LM-111-to-Int α1β1/Int α2β1 interactions. Lack of appropriate LM-111-to-Int α1β1/Int α2β1 interaction seems to be one factor limiting the remodelling process. Androgens are effective stimulators of Int α1β1 and α2β1 expression in physiologic concentrations. Addition of DHEA to the culture medium had the most effective stimulating effect of the androgens tested and its effect may be deficient in the LSGs of patients with SS, perhaps partly due to the lack of properly functioning intracrine metabolizing machinery.
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