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Mechanisms of Salivary Gland Secretory Dysfunction in Sjögren's Syndrome

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1. Introduction

Sjögren's syndrome (SS) is a systemic chronic autoimmune disorder affecting exocrine organs such as the salivary and lacrimal glands. SS is characterized by severe dryness of the mouth and eyes due to inflammatory reactions against salivary and lacrimal glands, respectively. Dryness of other mucosal surfaces such as skin, gastrointestinal tract, lungs, and vagina, has also been observed. SS patients also exhibit systemic symptoms such as Raynaud's phenomenon, arthritis, fatigue, peripheral neuropathies, and cognitive impairment. SS exists in two forms: primary SS, unassociated with other autoimmune disesases; and secondary SS, accompanied by another autoimmune disease such as scleroderma, rheumatoid arthritis, or systemic lupus erythematosus (Fox and Kang 1992). SS is the second most common autoimmune rheumatic disease, with a prevalence in the United States estimated at 2-4 million people (Kassan and Moutsopoulos 2004), with a female to male ratio of 9:1. Although SS affects men and children as well, it is most commonly seen if peri- or postmenopausal women.

Diagnostic criteria for SS have been defined most recently by the modified American-European Consensus Group (Vitali et al. 2002). These criteria include histological evaluation of a minor salivary gland for lymphocytic infiltration, serological presence of autoantibodies against SSA or SSB antigens, and assessment of ocular and oral symptoms. Oral involvement is assessed by the patient's subjective symptoms of oral dryness, parotid sialography showing the presence of diffuse sialectasis, salivary scintigraphy showing delayed uptake, reduced concentration, and/or delayed excretion of tracer, and/or the evaluation of unstimulated saliva production. Ocular involvement is assessed by the patient's subjective symptoms of ocular dryness, a Schirmer's test to measure tear secretion or a Rose Bengal test to measure the ocular surface abrasion in patients.

Originally, it was thought that loss of secretory function, a clinical hallmark of SS, was due to apoptotic destruction of acinar cells mediated by $CD8^+$ T lymphocytes in the lymphocytic infiltration in the salivary and lacrimal glands. However, research has demonstrated that transfer of human SS patient IgG to the B-cell deficient SS-prone NOD mouse resulted in altered saliva production in the absence of immune cell infiltration in the glands, indicating a role for autoantibodies in the functional impairment of secretory processes in SS (Robinson, Brayer et al. 1998). This resulted in a paradigm shift in the field, leading to the

belief that lymphocytic infiltration was not the only contributing factor to secretory dysfunction in SS. In addition to autoantibodies targeting muscarinic receptors, pro-inflammatory cytokines have been shown to play a role in SS pathogenesis by contributing to damage of glandular tissue and secretory dysfunction. Nitric oxide production has also been implicated as a potential cause of loss of secretion, as loss of nitric oxide synthase activity in salivary glands paralleled the decline in salivary secretion. A role for apoptotic cell death of acinar cells still remains in SS pathogenesis, however; our group has demonstrated that increased apoptosis is detectable in the salivary glands of SS-prone mice prior to disease onset or lymphocytic infiltration (Bulosan et al. 2008). In this review, these mechanisms and other possibilities that can contribute to loss of secretory function in SS will be discussed in detail.

2. Mechanisms

The clinical hallmark of SS is dryness due to loss of secretory function in the salivary and lacrimal glands. However, the etiology of SS is still not understood. There are numerous underlying mechanisms thought to contribute to this loss of secretory function in salivary glands, though no single mechanism has been identified as the primary cause. Lymphocytic infiltration, autoantibodies targeting muscarinic receptors, pro-inflammatory cytokines, nitric oxide, and apoptotic cell death of acinar cells have all been implicated as potential causes of secretory dysfunction in the salivary glands.

2.1 Lymphocytic infiltration and proinflammatory cytokines in the salivary glands

SS is characterized by lymphocytic infiltration and aberrant activation of epithelial tissues, which appear in salivary and lacrimal glands. It has been reported that this lymphocytic infiltration within the salivary and lacrimal glands consists mostly of CD4+ T cells, B cells, and lesser numbers of CD8+ T cells (Robinson, Cornelius et al. 1998, ; Tapinos et al. 1998). Balance between T and B cells in the lymphocyte infiltrates varies according to disease progression in the mouse model of SS (Robinson, Cornelius et al. 1998, ; Tapinos et al. 1998). It has been shown that leukocytes expressing pro-inflammatory cytokines infiltrate the exocrine glands, and T cells are recruited first to the site of infiltration followed by B cells, establishing lymphocytic infiltrates (Kong et al. 1998). CD8+ T cells, which have been shown to have increased expression of adhesion molecules and Fas/FasL, can also directly kill acinar cells in the salivary glands. Salivary gland dryness and/or formation of lymphocytic infiltration may be the result of glandular destruction mediated by effector cytokines/chemokines from T and B cells, as well as cytotoxic effects of CD8+ T cells.

2.1.1 Cytokines contributing to salivary gland dysfunction

Tumor necrosis factor- α (TNF-α) is one of the proinflammatory cytokines produced in response to infection, tissue damage, and environmental challenges and also known as an Interferon-γ (IFNγ)-inducing cytokine along with IL-12 and IL-18 (Locksley 1993, ; Billiau 1996). TNF-α production has been implicated in many human diseases including autoimmune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE). TNF-α activated the extrinsic apoptotic pathway and induced upregulation of intercellular adhesion molecule-1 (ICAM-1) and CCL20 in human

salivary gland (HSG) cells *in vitro* (Wang et al. 2009). In addition, TNF-α can disrupt tight junction structure in salivary glands from SS patients, potentially resulting in secretory dysfunction (Ewert et al. 2010, ; Baker 2010).

IL-18 and its inducer IL-12 are cytokines that play an important role in T_H1 driven autoimmune responses and inflammatory tissue disease by activating IFNγ secretion. The elevation of these cytokines triggered the inflammatory response in SLE and RA patients (Mosaad et al. 2003). Increased circulating levels and salivary gland expression of IL-18 was observed in SS patients, and IL-18 was detected in periductal inflammatory foci and saliva as well (Bombardieri et al. 2004, ; Bulosan et al. 2009). In addition, increased labial salivary gland IL-18 levels in primary SS patients correlated with increased disease activity parameters (Bikker et al. 2010, ; Bombardieri et al. 2004). Moreover, salivary gland infiltration by macrophages and dendritic cells (DCs), along with the expression of IL-18 and IL-12, appear to play active roles in the expansion and organization of infiltrative injuries and have a correlation with the lymphoma development in the patients with primary SS (Manoussakis et al. 2007). IL-12 transgenic mice showed decreased stimulated salivary flow by pilocarpine than in wild type controls. Also, IL-12 transgenic mice exhibited increased number and size of lymphocytic foci with increased anti-SSB/La antibodies, compared to glands from age-matched controls (Vosters et al. 2009).

IFNγ is the major cytokine which is released from T_H1 cells and regulates cell-mediated immune responses through activation of natural killer (NK) cells, macrophages, and CD8+ T cells. IFN-γ or receptor knockout mouse models (NOD. IFNγ$^{-/-}$ and NOD. IFNγR$^{-/-}$) showed normal development of salivary glands, maintained secretory function, and failed to develop any SS-like phenotypes (Cha et al. 2004). However, its parental strain NOD and a recently developed SS mouse model C57BL/6.NOD-*Aec1Aec2* showed retarded salivary gland growth and acinar cell apoptosis prior to disease onset and proceed to developing full-blown disease phenotype including loss of secretory function (Lee, Tudares, and Nguyen 2009). This indicates that IFN-γ plays an important role in loss of secretory function in SS. In addition, it has been found that IFNγ-induced T cells can produce chemokines (IFN-inducible protein 10 (IP-10)/CXCL9, CXCL10), which can attract NK cell and T cells in SS ductal epithelial cells (Ogawa et al. 2002, ; Ogawa et al. 2004).

There are also cytokines released from T_H2 cells that can play an important role in SS. Elevated levels of IL-4 were found in the serum of primary SS patients who have lymphocytic infiltration and ectopic germinal center formation in their minor salivary glands (Reksten et al. 2009). Studies using the NOD.IL4$^{-/-}$ and NOD.B10-H2b.IL4$^{-/-}$ mice indicated that IL-4 gene knockout mice have pathophysiological abnormalities and leukocyte infiltration in the salivary glands but salivary gland secretion was normal in the absence of IL-4 (Gao et al. 2006). Considering that IL-4 knockout mice fail to produce IgG1 isotypic autoantibodies against cell surface receptor muscarinic type 3 receptor (M3R), isotypic anti-M3R autoantibody is critical in the development of secretory dysfunction (Gao et al. 2004). Moreover, purified IgG fractions isolated from the sera of Stat6 (downstream signal transduction factor of IL-4) knockout mice, which are unable to produce IgG1, were not able to inhibit saliva flow rates when infused to wild type control mice (C57BL/6) (Nguyen et al. 2007). Therefore, IL-4 can affect saliva secretion via antibody production and its isotype switching.

Recently, not only T_H1 and T_H2 effector cells but also T_H17 cells, which mainly release pro-inflammatory cytokine IL-17, are being investigated for their role in disease pathogenesis of many autoimmune diseases including SS. The presence of T_H17 cells and T_H17-associated cytokines, IL-6, IL-23, IL-17, and IL-1β were reported in the serum and minor salivary glands of primary SS patients (Nguyen et al. 2008, ; Sakai et al. 2008, ; Reksten et al. 2009, ; Katsifis et al. 2009). It is also known that IL-18 synergizes with IL-17 to induce secretion of pro-inflammatory cytokines IL-6 and IL-8 in human parotid gland cells (Sakai et al. 2008). Serum levels of IL-17, IL-6, and IL-23 were significantly elevated in primary SS. A recent study in which an adenovirus vector expressing IL-17 was infused into the salivary glands of wild type mice (C57BL/6J) demonstrated the appearance of lymphocytic infiltrates, increased proinflammatory cytokine levels, changes in antinuclear antibody profiles, and temporal loss of saliva flow after infusion (Nguyen et al. 2010). In the reverse approach, infusion of IL-17R:Fc-blocking factor into the SS mouse model to block IL-17 binding to IL-17 receptor showed decreased lymphocytic infiltration in salivary glands, normalization of the antinuclear antibody repertoire, and increased saliva secretion (Nguyen et al. 2011). Therefore, these studies indicate that IL-17 is critical in inducing SS-phenotype in wild type mice. However, the mechanism by which IL-17 functions in altering secretory function in SS needs to be defined.

2.1.2 B cell involvement in SS

In addition to pathogenic T cells and cytokines, loss of B cell tolerance is critical in autoimmune diseases including SS. Levels of the B cell activating factor belonging to the TNF family (BAFF) in serum were higher in patients with SLE, RA and pSS than in normal individuals (Cheema et al. 2001, ; Groom et al. 2002, ; Zhang et al. 2001). BAFF overexpression caused self-reactive B cells at the transitional B cell stage and is responsible for B cell hyperactivity. It is known that over-expressing BAFF in BAFF-transgenic mice resulted in SLE-like disease with increased number of marginal-zone (MZ) like B cells, and at 16-18 months of age, these mice exhibited a SS-like disease with MZ like B cells in the salivary glands (Mackay et al. 1999, ; Groom et al. 2002). However, recent findings indicated that BAFF expression alone is not correlated with disease activity (Cheema et al. 2001, ; Zhang et al. 2001, ; Stohl et al. 2003). Nonetheless, BAFF influences the survival, proliferation, and differentiation of B cells in combination with IL-17 in patients with SLE and its combination can promote the persistence of self-reactive B cells (Doreau et al. 2009).

As described above, T cells and B cells clearly contribute to SS onset and progression. However, activation of T cells and B cells are required for normal immune function, and the trigger for autoimmune reactivity in SS has yet to be identified. Also, studies using immune-deficient mice revealed that secretory dysfunction and other salivary gland abnormalities can still occur in the absence of infiltrating immune cells and their cytokines.

2.2 Autoantibodies targeting muscarinic receptors

The assumption that secretory dysfunction in SS was a direct consequence of acinar tissue loss after lymphocytic infiltration was deeply ingrained in the SS research community for more than 60 years. However, more recently, our understanding of the pathogenesis of secretory dysfunction in SS has undergone a dramatic change. Questions arose concerning

SS patients with viable acinar tissue in their salivary glands but who still suffer from xerostomia. These observations suggest that the salivary gland secretory dysfunction in many SS patients is the result of a disruption of acinar cell function rather than acinar tissue destruction.

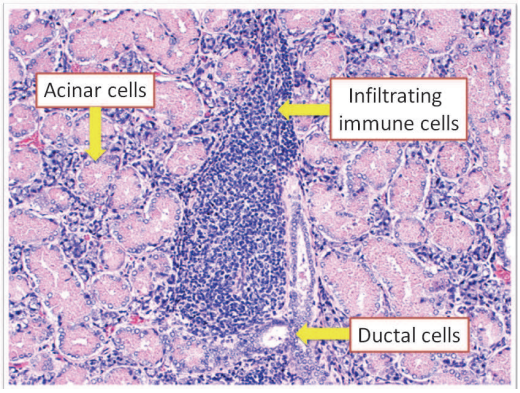

Fig. 1. Immune cell infiltration in the salivary gland of 34 week old SS-prone C57BL/6.NOD-*Aec1Aec2* male mouse.

In addition to salivary gland lymphocytic infiltration, SS patients exhibit hypergammaglobulinemia with a range of autoanitbodies targeting cell surface, cytoplasmic, and nuclear proteins of exocrine tissue (Chan et al. 1991, ; Fox and Kang 1992, ; Haneji et al. 1997). Approximately 90% of patients are positive for antinuclear antibodies (ANA), the most common of which are directed against two ribonucleoprotein antigens known as Ro or SSA and La or SSB. These autoantibodies are included in the modified European–American Diagnostic Criteria for Sjögren's Syndrome (Vitali et al. 2002), but are also found in other autoimmune diseases, particularly systemic lupus erythematosus (SLE). Autoantibodies to other immunoglobulins (known as rheumatoid factors) are also frequently found in SS. Primary SS (pSS) sera can also contain many different autoantibodies against organ or tissue specific autoantigens, including acetylcholine receptors, the carbonic anhydrase and thyroid peroxidase. Finally, autoantibodies directed against the cytoskeletal protein β-fodrin, and the muscarinic receptors M3, have also been described in primary SS, the latter of which we will focus on here.

2.2.1 Muscarinic receptor function in salivary glands

Acetylcholine (ACh) control of fluid secretion in salivary acinar cells is mediated through the G protien-linked muscarinic M3 receptor (M3R). ACh binds to M3R, which causes phospholipase C to generate inositol 1,4,5-trisphosphate (IP3). IP3 binds to and opens the IP3 receptor on the endoplasmic reticulum, which releases Ca^{2+}. The increased concentration of intracellular Ca^{2+} activates the apical membrane Cl^- channel and the basolateral K^+ channel. Efflux of Cl^- into the acinar lumen draws Na^+ across the cells, and the osmotic gradient generates fluid secretion (Tobin, Giglio, and Lundgren 2009). Therefore, blocking or desensitizing muscarinic receptors is detrimental to this signaling pathway and ultimately results in loss of secretory function.

2.2.2 Initial characterization of anti-muscarinic antibodies

In 1994, it was observed that in the NOD mouse model, which exhibits an autoimmune-associated lymphocytic attack on the salivary glands and loss of secretory function, decreased response to beta-adrenergic receptor stimulation was related to a decrease in receptor density and changes in the level of intracellular second messenger signalling (Hu et al. 1994). It was hypothesized that these changes could be due to an autoantibody targeting the β1-adrenergic receptor present in the sera of NOD mice.

In 1996, futher study of the NOD mouse model revealed a reduction in muscarinic receptor density on the salivary glands of prediabetic and diabetic NOD mice compared to BALB/c mice corresponding to reduced secretory function in the NOD (Yamamoto et al. 1996). Additionally, sera from the diabetic NOD but not the BALB/c immunoprecipitated radiolabeled muscarinic receptor, indicating the presence of autoantibody to the receptor in NOD mice (Yamamoto et al. 1996).

Autoantibodies against M3R were first described in human SS patients in 1996 (Bacman et al. 1996). It was demonstrated that IgG present in the sera of primary SS patients could bind and activate muscarinic receptors of rat parotid glands (Bacman et al. 1996). They also demonstrated that the IgG fraction from the sera of pSS patients mimicked the biological effects of muscarinic cholingergic agonists by modifying intracellular events associated with specific receptor activation, such as decreasing cAMP and increasing phosphoinositide turnover (Bacman et al. 1996). These findings suggested that autoantibodies targeting M3R could potentially play a role in SS pathogenesis.

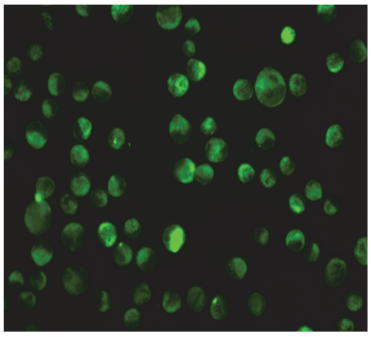

Fig. 2. pSS IgG staining of hM3R-transfected Flp-In CHO cells. Flp-In CHO cells were transfected with hM3R and then incubated with pSS sera (1:50 dilution) containing anti-M3R autoantibodies. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody at 1:250 dilution was used for detection.

2.2.3 Roles for anti-muscarinic receptor antibodies in SS

In 1998, Robinson, et al. were the first to demonstrate that transferring human SS patient IgG to NOD.Igμ^{null} mice resulted in secretory gland dysfunction (Robinson, Brayer et al. 1998). NOD.Igμ^{null} mice lack functional B lymphocytes, and therefore lack the IgG autoantibodies that are produced by their NOD counterparts and human SS patients. NOD.Igμ^{null} mice do exhibit lymphocytic infiltration of the salivary and lacrimal glands, but fail to lose secretory

function. However, when treated with IgG from SS patient sera, a 54% reduction in saliva production was observed, while treatment with IgG from healthy control mice and healthy humans had no significant effect on secretory function (Robinson, Brayer et al. 1998). Furthermore, after prolonged treatment with SS IgG fractions, there was an increase in apoptotic cell death of salivary acinar cells (Robinson, Brayer et al. 1998). These data indicate that anti-M3R autoantibodies play a critical role in the clinical presentation of dryness in SS.

Further evidence for anti-M3R autoantibody-mediated secretory dysfunction in NOD mice was presented in 2000. Infusion of monoclonal antibodies to mouse M3R into NOD-*scid* mice resulted in significantly reduced saliva secretion within 72 hours, while infusion with antibodies to Ro (SSA), La (SSB), or parotid secretory protein (PSP) had no effect on secretory function (Nguyen et al. 2000). Mechanistic studies revealed that translocation of aquaporin-5 to the plasma membrane was inhibited by anti-M3R antibodies, but not the other antibodies again showing a role for anti-M3R autoantibodies in SS pathogenesis (Nguyen et al. 2000).

In 2004, Li, et al. demonstrated the inhibitory effects of autoantibodies from SS patients on muscarinic receptors by showing that carbachol-induced intracellular calcium release was inhibited by SS IgG treatment of HSG cells (Li et al. 2004). Aquaporin-5 trafficking to the apical membrane of rat parotid acinar cells was also inhibited by SS IgG (Li et al. 2004). Additionally, other groups found abnormal translocation of aquaporin-5 in the NOD mouse model of SS and in salivary glands of SS patients (Konttinen et al. 2005, ; Steinfeld et al. 2001). However, these findings are somewhat controversial since others have shown no differences in the subcellular distribution of aquaporin-5 in salivary glands of primary SS patients (Beroukas et al. 2001, ; Tsubota et al. 2001). Our unpublished findings show a definite alteration in GFP-tagged aquaporin-5 trafficking in human salivary gland cells that were pre-treated with SS plasma compared to healthy control plasma. Taken together, these data further support a role for anti-muscarinic receptor autoantibodies in loss of secretory function in SS.

The chronic effects of anti-M3R autoantibodies were examined in 2006 by analyzing the contraction of bladder smooth muscle strips from diseased NOD mice (Cha et al. 2006). The results indicated that the presence of anti-M3R autoantibodies in NOD mice resulted in a desensitization of M3R as measured by direct carbachol-induced responses and an accelerated loss of responses to repeated pilocarpine injections (Cha et al. 2006). This data supports the hypothesis that frequent use of pilocarpine by SS patients who have already progressed to M3R desensitization induced by anti-M3R autoantibodies will be less effective due to a desensitizing synergy between pilocarpine and anti-M3R autoantibodies.

Anti-muscarinic receptor antibodies have also been shown to affect the autonomic nervous system. In 2000, it was demonstrated that sera from primary and secondary SS patients inhibited parasympathetic neurotransmission as measured by carbachol-stimulated bladder contraction using bladder and colon smooth muscle strips in vitro, while sera from healthy controls or SLE patients had no effect (Waterman, Gordon, and Rischmueller 2000). These findings suggest that autoantibodies targeting M3R may contribute to sicca symptoms as well as autonomic dysfunction such as bladder symptoms in some patients (Waterman, Gordon, and Rischmueller 2000).

In vivo evidence was presented in 2004, when passive transfer of SS IgG with anti-M3R activity to BALB/c mice resulted in an increased response to cholinergic stimulation of bladder smooth muscle (Wang et al. 2004). This cholinergic hyperresponsiveness was found to be specifically induced by anti-M3R antibodies following passive transfer (Wang et al. 2004). These findings are consistent with the overactive bladder symptoms experienced by many SS patients, indicating that overactive bladder in SS is an autoantibody-mediated disorder of the autonomic nervous system that could also account for a broad range of cholinergic hyperresponsiveness.

Most recently, it has been demonstrated that primary SS IgG with anti-M3R activity inhibited contraction of the smooth muscle of the GI tract and disrupted contractile motility in the colon (Park et al. 2011). These data may explain the widespread impairment of the GI tract in SS patients including delayed gastric emptying and abnormalities in colonic motility (Cai et al. 2008, ; Kovacs et al. 2003).

2.2.4 Conclusions

Overall, these findings strongly support a role for anti-M3R autoantibodies in the pathogenesis of SS. The data suggest that a number of primary and secondary SS patients have serum IgG capable of binding to and inhibiting muscarinic receptors on salivary acinar cells in vitro. However, due to the lack of a reliable screening assay, relatively few subjects have been tested, and the percentage of SS patients estimated to be positive for anti-muscarinic antibodies varies wildly from 0 to almost 100%. Future studies in this field should focus on the development of a screening assay for anti-muscarinic antibodies to confirm the number of SS patients positive for these autoantibodies and establish or rule-out anti-M3R antibodies as a diagnostic marker for SS.

2.3 Nitric oxide and nitric oxide synthase

In 1986 nitric oxide (NO) was first described as endothelially derived relaxing factor (EDRF) (Palmer, Ferrige, and Moncada 1987). Subsequently, it has been shown to be involved in a multitude of diverse physiological and pathophsiological processes, including potential functions in the regulation of salivary gland secretion and in the development of secretory hypofunction. *In vivo*, NO is found to be synthesized in a wide variety of cell types by the enzyme NO synthase (NOS). There are three known isoforms of NOS, each produced from a distinct set of genes. The two constitutive isoforms are neuronal NOS (nNOS, NOS-1) and endothelial NOS (eNOS, NOS-3), whereby their names reflect the original tissues from which they were discovered. The functional activity of these two isoforms is dependent on a rise in Ca^{2+} and therefore generate low, transient, concentrations of NO. The other isoform, inducible NOS (iNOS, NOS-2), is mainly found in inflammatory cell types including: macrophages, neutrophils, and fibroblasts (Knowles and Moncada 1994). Expression of iNOS can be induced by bacterial lipopolysaccharides (LPS) and inflammatory cytokines. The concentrations of NO produced by iNOS are much greater than either eNOS or nNOS, and at levels that are typically cytotoxic and bactericidal (Kimura-Shimmyo et al. 2002).

2.3.1 Sources of NO in human salivary glands

The increased presence of nitrite (NO_2^-, the oxygenation product of NO) in the saliva of healthy individuals in response to stimulated secretion (Bodis and Haregewoin 1993)

implies a system by which endogenous, constitutively expressed, NO may be produced in glandular cells and in turn alter saliva secretion. Surprisingly, immunohistochemical analyses of human minor and major salivary glands revealed that nNOS is strongly restricted to the non-neuronal duct epithelium and only a minority of the major salivary gland nerve fibers (surrounding acini, tubuli, ducts and blood vessels) expressed nNOS (Soinila, Nuorva, and Soinila 2006). In addition, salivary gland acinar cells have been demonstrated to express NOS (Looms et al. 2002, ; Looms et al. 2000, ; Soinila, Nuorva, and Soinila 2006). Human labial salivary gland acinar cells possess NOS activity and exhibit a very low level of NO production without stimulation *in vitro* (Looms et al. 2000). Stimulation of NO production, with a concomitant rise in Ca^{2+}, in human labial acinar cells was shown to be directly mediated through activation of β-adreneric receptors, which could not be mimicked by a rise in Ca^{2+} alone (Looms et al. 2000). As expected, the expression of eNOS in human minor and major salivary glands is restricted mostly to the vascular endothelium (Soinila, Nuorva, and Soinila 2006). The constitutive expression of NOS and NO in human salivary gland acini and ducts suggests a potential contribution to secretion. However, their exact roles in healthy salivary glands are still undetermined.

2.3.2 Potential function of NO in secretion

Saliva secretion signaling pathways and mechanisms have been studied closely, where the involvement of NO in these pathways is still of great interest. The classical signaling pathway involves autonomic receptor stimulation of acinar cells, which leads to increased IP3-mediated intracellular Ca^{2+} release from the endoplasmic reticulum and cAMP activation of protein phosphorylation. An additional receptor/channel involved in the release of Ca^{2+} from intracellular stores is the ryanodine receptor (RyR), of which, cyclic ADP-ribose (cADPR) has been suggested as an endogenous ligand (Galione, Lee, and Busa 1991, ; Looms et al. 2001). One potential means by which endogenous NO exerts an effect in the salivary gland acinar cells is by binding to the heme moiety of soluble guanylyl cyclases (Denninger and Marletta 1999) thus activating the synthesis of cyclic guanosine monophosphate (cGMP), which can promote the synthesis of the Ca^{2+}-mobilizing cADPR (Galione et al. 1993, ; Looms et al. 2001, ; Willmott et al. 1996). This NO-induced intracellular Ca^{2+} release is proposed to coordinate cellular activation and to have a role in determining the magnitude and time course of the secretory response (Caulfield et al. 2009, ; Harmer, Gallacher, and Smith 2001). Alterations in this response could play a role in salivary gland hypofunction via a disruption in the normal Ca^{2+} signaling pathways.

2.3.3 Potential roles of NO and iNOS in exocrine hypofunction

Evidence suggests that the loss of secretory function associated with SS may occur due to factors which alter Ca^{2+} signaling and not only through direct tissue destruction by infiltrating lymphocytes. One hypothesis for salivary gland exocrine hypofunction is based from the observation that the sera from NOD mice, prone to developing SS, were found to contain autoantibodies against β-adrenergic and muscarinic receptors (Hu et al. 1994, ; Yamamoto et al. 1996). The blockage of the β-adrenergic agonist binding down-regulated receptor density due to the chronic stimulation (Hu et al. 1994). Therefore, according to the previously described model for NO-induced Ca^{2+} release, saliva secretion could be diminished due to blockage of these receptors (Looms et al. 2002).

On the contrary, elevated nitrite is present at increased concentrations in SS patient saliva and serum compared to healthy controls (Konttinen et al. 1997, ; Wanchu et al. 2000). The effects of this possible elevation in NO concentration has been explored in recent experiments where the acute exposure of NO to human submandibular gland acinar cells were able to transiently (20-30 minutes) enhance Ca^{2+} signaling, but a more chronic exposure to NO eventually desensitized these cells to stimulation (Caulfield et al. 2009). The mechanism by which NO exerts its inhibitory effects on the stimulation of secretion is still not understood, but it is most likely not mediated through cGMP nor due to a depletion of the Ca^{2+} stores. It is hypothesized that the inhibition of activity could be due to the NO-mediated nitrosylation of receptors or other proteins involved in the secretion signal transduction pathways (Caulfield et al. 2009). However, this relationship between increased nitrite concentrations and salivary gland hypofunction is more complicated, since other oral inflammatory disorders exhibit increased nitrite concentrations in saliva as well (Kendall et al. 2000, ; Kendall, Marshall, and Bartold 2001, ; Ohashi, Iwase, and Nagumo 1999).

The role of iNOS in the loss of secretory function has also been investigated due to the pro-inflammatory environment present in the salivary glands of SjS patients. As expected, iNOS expression is increased in resident cells of the labial salivary glands of patients with SS when compared to healthy controls (Konttinen et al. 1997). Cytokines (for example: IFN-γ, IL-18, IL-1β and TNF-α) or LPS induction of iNOS leads to a significant increase in NO production (Dinarello 1997, ; Kimura-Shimmyo et al. 2002, ; Liew 1994). NO production from iNOS is long-lasting and at relatively high concentrations when compared to the other two Ca^{2+}-dependent isotypes (Nathan and Xie 1994). This increased NO has been hypothesized to directly nitrosylate functional proteins and thus could induce cell death by potentially disrupting essential cellular processes (Kimura-Shimmyo et al. 2002, ; Sarih, Souvannavong, and Adam 1993). In another course of altering cellular functioning, the product of the reaction of NO with superoxide, peroxynitrite, has also been suggested to promote modulations of cell signaling and even produce oxidative injury (Pacher, Beckman, and Liaudet 2007). It has been shown in several cases how the upregulation of iNOS expression may ultimately lead to secretory hypofunction due to the accumulated damage from NO (Dawson, Fox, and Smith 2006, ; Kimura-Shimmyo et al. 2002, ; Konttinen et al. 1997, ; Takeda et al. 2003).

2.4 Altered glandular homeostasis

In addition to the immune cell-mediated mechanisms that contribute to secretory gland dysfunction, there is also evidence for altered glandular homeostasis in SS glands that appears even prior to disease onset. Specifically, aberrant expression and proteolytic cleavage of PSP, increased serine and cysteine protease enzyme activity, elevated numbers of apoptotic cells, enhanced matrix metalloproteinase activities in salivary gland lysates and decreased amylase activity and epidermal growth factor gene expression are all observed irrespective of the presence of lymphocytic infiltration or detectable autoimmune phenotype. Additionally, submandibular glands of NOD neonates revealed some genetically programmed glandular defects such as retarded salivary gland development. How these early defects influence the onset and development of SS is still under active investigation.

2.4.1 Defects in salivary glands of NOD mouse models after disease onset

NOD mice develop chronic lymphocytic infiltration in the salivary glands that correlate with decreased saliva production concurrently with infiltration in the pancreas that results in phenotypes similar to insulin-dependent diabetes mellitus and SS. To differentiate between immune cell-mediated and non-immune cell-mediated mechanisms in the SS phenotype, salivary glands were characterized in NOD-*scid* mice and other NOD derivatives.

In the absence of a functional immune system, the salivary flow rate of >20 week old NOD-*scid* is similar to 10-12 week old mice (Robinson et al. 1996). However, saliva analysis revealed that epidermal growth factor (EGF, a product of submandibular gland ductal cells) and amylase (a product of salivary acinar cells) were significantly decreased in the saliva of >20 week old NOD-*scid* compared to 10-12 week old mice (Robinson et al. 1996). Additionally, PSP was detected in submandibular gland lysates of 10 week old NOD-*scid* and increased in quantity by 20 weeks of age, while PSP was not detected in control BALB/c glands, and (Robinson et al. 1996). Histological examination of NOD-*scid* submandibular glands revealed a progressive loss of acinar tissue and a decline in the acinar to ductal cell ratio in the absence of lymphocytic infiltrates (Robinson et al. 1996). These differences in salivary protein composition and glandular histology in the absence of lymphocytic infiltration indicate that glandular defects in the NOD genetic background may contribute to the onset of the autoimmune reaction in the salivary glands.

Further analysis of these findings revealed increased cysteine protease activity in the saliva and gland lysates of 20 week old NOD and NOD-*scid* mice compared to age matched BALB/c or 8 week old NOD mice (Robinson et al. 1997). This increased activity was highest in the NOD-*scid* mice indicating that infiltrating immune cells are not responsible for these changes. Additional protease activity in the saliva and gland lysates of older NOD and NOD-*scid* mice generated an enzymatically cleaved PSP (Robinson et al. 1997). These findings suggest that proteolytic enzyme activity contributes to loss of exocrine gland tolerance by generating abnormally processed protein constituents.

2.4.2 Defects in salivary glands of NOD mice prior to disease onset

The changes in the protein composition of saliva, PSP expression, and protease activity in the absence of lymphocytic infiltration or functional immune cells indicate that innate genetic differences in the NOD salivary glands exist and may contribute to the SS phenotype. To further support this theory, salivary gland organogenesis was examined in neonatal NOD mice and compared to wild type mice (Cha et al. 2001). Histomorphological analyses of submandibular glands at 1 day postpartum revealed delayed morphological differentiation during organogenesis in NOD mice compared to wild type mice, acinar cell proliferation was reduced, and expression of Fas, FasL and bcl-2 were increased (Cha et al. 2001). Prior to weaning (up to 21 days) the NOD strains showed increased matrix metalloprotease (MMP)-2 and MMP-9 activity (Cha et al. 2001). This altered glandular development may contribute to an environment capable of triggering autoimmunity.

As mentioned previously, a role for interferon-γ (IFN-γ) in these pre-disease aberrations was discovered when neither NOD.IFNγ $^{-/-}$ and NOD.IFNγR$^{-/-}$ mice exhibited increased acinar

cell apoptosis or abnormal salivary protein expression prior to disease (Cha et al. 2004). Strikingly, without these abnormalities, the NOD.IFNγ $^{-/-}$ and NOD.IFNγR$^{-/-}$ mice showed no autoimmune attack of the salivary glands at 20 weeks old (Cha et al. 2004). Also, NOD-*scid.IFNγ* $^{-/-}$ mice, unlike NOD-*scid* and NOD, showed normal glandular morphogenesis at birth (Cha et al. 2004). These data suggest that IFN-γ has a critical role during the pre-immune phase disease independent of effector functions of immune cells.

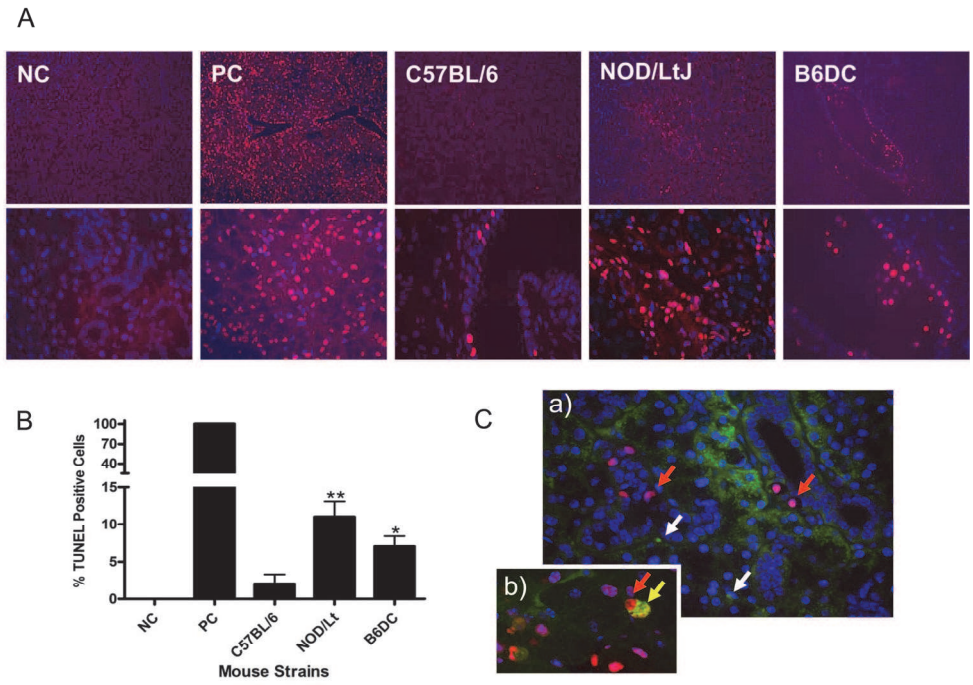

Fig. 3. Increased epithelial cell death in the glands of disease-prone mice at 8 weeks and lack of direct colocalization of caspase-11 with TUNEL-positive cells. (**A**) TUNEL staining was performed on the prediseased salivary glands; upper panel at × 10 and lower panel at × 40 magnifications. (**B**) Percentages of TUNEL-positive cells are shown as a bar graph. For each mouse, three slides were evaluated for TUNEL-positive cells, which were counted using a cell counter. (**C**) Caspase-3-positive cells (yellow arrows in **b**) were colocalized with TUNEL-positive cells (red arrows). White arrows indicate caspase-11-positive cell. Magnification, × 40. NC, negative control; PC, positive control treated with nuclease; TUNEL, transferase-mediated dUTP-biotin nick end labeling. Figure previously published in (Bulosan et al. 2009).

The NOD mouse model was/is used extensively to study SS pathogenesis; however, this model is genetically predisposed to develop at least three autoimmune diseases. To create a primary SS mouse model that only exhibits SS-like phenotype, two chromosomal intervals from the NOD mouse that conferred sialadenitis were bred to non-autoimmune C57BL/6 mice (Cha et al. 2002). These mice, designated C57BL/6.NOD-*Aec1Aec2,* enabled the study

of disease-associated genes alone, and were used to characterize early pathogenic events associated with SS-like disease through microarray analysis of gene expression in the salivary glands during the pre-disease stage (Killedar et al. 2006). Interestingly, C57BL/6.NOD-*Aec1Aec2* exhibited upregulated genes encoding proteins associated with IFN-γ signal transduction pathway (*Jak/Stat*), TLR-3 (*Irf3 and Traf6*), and apoptosis (*casp11 and casp3*) compared to C57BL/6 (Killedar et al. 2006).

The upregulation of caspase-11 in 8 week old C57BL/6.NOD-*Aec1Aec2* mice was detected in our study. Concomitantly, apoptotic cells were more readily detected in this mouse model compared to wild type mice. Further studies were then conducted to determine whether upregulated caspase-11 is responsible for this phenomenon. In these studies it was shown that the upregulated caspase-11 expression from the salivary glands activated caspase-1, but not caspase-3. In effect, apoptotic cells were not positive for caspase-11 staining, suggesting that caspase-11 plays an indirect role in increased apoptotic acinar cell death in the salivary glands before disease onset (Bulosan et al. 2009). This finding led to the hypothesis that inflammatory caspases, such as caspase-11 indirectly functions in apoptosis by activating caspase-1 and resulting in the subsequent release of proinflammatory cytokines into the glandular environment. This hypothesis was tested by co-culturing human salivary gland cells with a human monocyte cell line, THP-1, stimulated with LPS in the presence or absence of IFN-γ. In the presence of IFN-γ, there was an increased rate of HSG cell apoptosis, but when caspase-1 was knocked down by small interfering RNA in the THP-1 cells, the rate of apoptosis in HSG cells was reversed back to normal (Bulosan et al. 2009). These data indicate that the increased caspase-11 expression in macrophages and dendritic cells present in the salivary glands of 8 week old C57BL/6.NOD-*Aec1Aec2* mice may increase apoptotic cell death of surrounding acinar cells by activating capase-1, resulting in the secretion of pro-inflammatory cytokines IL-1β and IL-18. In other words, inflammatory caspases are essential in promoting a pro-inflammatory microenvironment and influencing salivary gland cell death prior to disease onset.

2.4.3 Altered microRNA expression in SS

MicroRNAs (miRNAs) are small non-coding RNA molecules that post-transcriptionally regulate gene expression by binding to the 3′ untranslated regions of specifc mRNAs and blocking translation or causing degradation. Recently, miRNAs have been implicated in a number of diseases including autoimmune disorders. In 2011, it is becoming clear that miRNAs may also play a role in SS, although that specific role has yet to be determined. Alevizos, et al. demonstrated that miRNA expression patterns can accurately distinguish salivary glands from control subjects and SS patients, and that comparing miRNA from patients with preserved or low saliva flow identified a set of differentially expressed miRNAs, indicating a potential role for miRNAs in secretory dysfunction of the salivary glands (Alevizos et al. 2011). Later in 2011, we reported that miR-146a is significantly overexpressed in the PBMCs of SS patients compared to healthy controls and in the salivary glands and PBMCs of 8 week old C57BL/6.NOD-*Aec1Aec2* female mice compared to wild-type mice (Pauley et al. 2011). It is particularly interesting that miR-146a is upregulated in the target tissues (salivary glands) at 8 weeks of age since this is prior to disease onset in this mouse model. These data suggest that miR-146a could play a role in early disease

pathogenesis in SS or could be a result of altered glandular homeostasis prior to disease onset.

Taken together, it is becoming increasingly clear that innate differences in the salivary glands of SS contribute to disease onset and/or loss of secretory function. Developmental defects, altered glandular homeostasis in the absence of immune cell infiltrates, and a tendency towards a proinflammatory environment are all evident in the salivary glands of SS mouse models, sometimes even prior to disease onset. It remains to be seen how these changes develop, but one hypothesis is that chronic stimulation by pathogens can lead to subclinical changes in the glands. In this case, it will be critical to identify the signatures left behind by these pathogens, such as viral or bacterial footprints, in order to use them as early disease markers to detect individuals susceptible to developing SS.

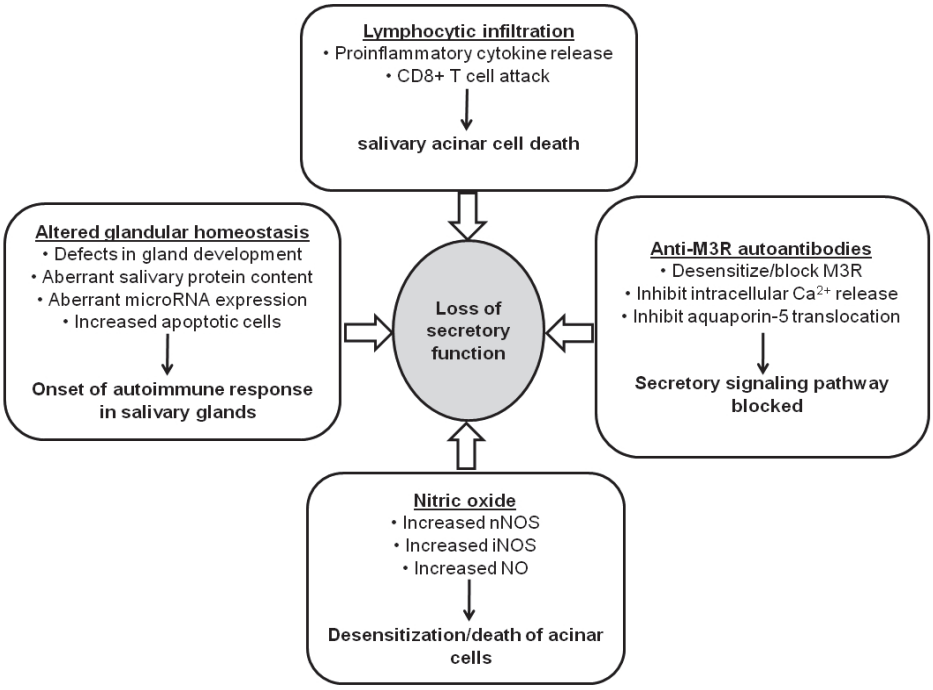

Fig. 4. Mechanisms contributing to secretory dysfunction in SS salivary glands.

3. Conclusion

In conclusion, it is evident that numerous mechanisms contribute to salivary gland dysfunction in SS. The initial trigger of autoimmune reactivity and which of these mechanisms, if any, are more important in SS pathogenesis remains to be seen. Also, it is unclear whether pre-existing genetic factors predetermine certain individuals to develop SS, or if there is a specific environmental or immunological trigger. It would be interesting and very informative to transplant the salivary glands of a pre-disease SS-prone mouse to a

wild-type mouse to see if the recipient would still develop SS. This would identify whether a systemic environment or a glandular environment is critical for the onset of SS. Hopefully, ongoing research in the field of SS will lead to a better understanding of how the different mechanisms of secretory hypofunction discussed here can be prevented or circumvented to improve the quality of life of SS patients. There is a great need for potential new therapeutic strategies that can either turn off the autoimmune reaction in the exocrine tissue or preserve/replace the glandular tissue to restore secretory function.

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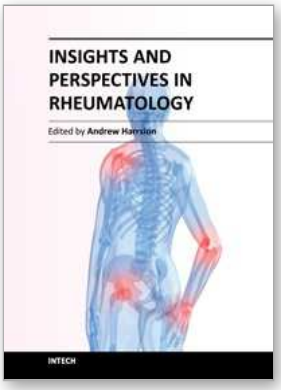

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This book offers a range of perspectives on pathogenesis, clinical features and treatment of different rheumatic diseases, with a particular focus on some of the interesting aspects of Sjögren's syndrome. It contains detailed and thorough reviews by international experts, with a diverse range of academic backgrounds. It will also serve as a useful source of information for anyone with a passive interest in rheumatology, from the genetic and molecular level, through to the psychological impact of pain and disability.

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