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Aberrant localization of fusion receptors involved in regulated exocytosis in salivary glands of Sjögren's syndrome patients is linked to ectopic mucin secretion

María-José Barrera [a,1], Marianela Sánchez [a,1], Sergio Aguilera [b,2], Cecilia Alliende [a], Verónica Bahamondes [a], Claudio Molina [d], Andrew F.G. Quest [a,c], Ulises Urzúa [a], Isabel Castro [a], Sergio González [d,e], Hsiao Hsin Sung [a], Amelina Albornoz [a], Marcela Hermoso [a], Cecilia Leyton [a], María-Julieta González [a,*,2]

[a] Faculty of Medicine, Institute of Biomedical Sciences (ICBM), University of Chile, Postal Code 8389100, Santiago, Chile
[b] INDISA Clinic, Chile
[c] Fondo de Investigación Avanzada en Áreas Prioritarias (FONDAP), Center for Molecular Studies of the Cell (CEMC), Chile
[d] Mayor University, Chile
[e] San Sebastián University, Chile

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ABSTRACT

Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disease that mainly affects tear and salivary glands, whereby SS-patients frequently complain of eye and mouth dryness. Salivary acinar cells of SS-patients display alterations in their cell polarity; which may affect the correct localization and function of proteins involved in regulated exocytosis. Here we determined whether the expression and localization of SNARE proteins (membrane fusion receptors) involved in regulated secretion, such as VAMP8, syntaxin 3 (STX3), STX4 and SNAP-23 were altered in salivary glands (SG) from SS-patients. Additionally, we investigated SNARE proteins function, by evaluating their ability to form SNARE complexes under basal conditions. In SG from SS-patients and control subjects mRNA and proteins levels of SNARE complex components were determined by real-time PCR and Western blotting, respectively. SNARE protein distribution and mucin exocytosis were determined by indirect immunofluorescence. In SS-patients, the expression levels of mRNA and protein for VAMP8, STX4 and STX3 were altered. STX4, STX3, SNAP-23 and VAMP8 relocated from the apical to the basal region of acinar cells. Increased formation of SNARE complexes in a manner independent of external stimuli for secretion was detected. Mucins were detected in the extracellular matrix (ECM). Presence of mucins in the ECM, together with the observed alterations in SNARE protein localization is indicative of ectopic exocytosis. In the context of SS, such aberrantly localized mucins are likely to favor a pro-inflammatory response, which may represent an important initial step in the pathogenesis of this disease.

1. Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disease that mainly affects the exocrine glands and usually is detected because of symptoms, such as persistent dryness of the mouth and eyes due to functional impairment of the salivary and lachrymal glands, respectively [1,2]. The sensation of dry mouth in these patients has been attributed to a variety of factors, including atrophy and a reduced number of acini as well as ducts due to augmented apoptosis [1,3], glandular denervation [4], inhibition of neurotransmitter release by cytokines [5], enhanced breakdown of acetylcholine by increased levels of cholinesterase [6], blockade of M3 muscarinic receptor by anti-muscarinic auto-antibodies [6], altered NO production [7], perturbation of calcium-induced calcium release by altered levels of cADPr (possibly as a result of altered NO levels) [7], altered Ca^{2+} tunneling [8] and altered expression or distribution of AQP5 [9]. All these factors are related to water transport from acinar cells to the lumen of salivary glands [8].

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, synaptosome associated protein; STX, syntaxin; VAMP, vesicle associated membrane protein; SS, Sjögren's syndrome; NO, nitric oxide; cADPr, cyclic ADP ribose; LSG, labial salivary gland; TJ, tight junctions; RT, room temperature; ECM, extracellular matrix; CCK, cholecystokinin; PIP2, phosphoinositol-4,5-diphosphate; AA, Araquidonic acid; PM, plasma membrane.

* Corresponding author. Tel.: +56 2 978 6017; fax: +56 2 737 3158.
E-mail address: jgonzale@med.uchile.cl (M.-J. González).

[1] These authors contributed equally to this work.
[2] Share senior authorship.

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Sulfated and sialylated groups present in mucins bind high amounts of water, thus facilitating humectation of the oral mucosa [10,11]. Recently, we reported on a decrease of MUC5B sulfation in labial salivary gland (LSG) of SS-patients [12]. This alteration is relevant, since SS-patients with reduced or normal whole unstimulated flow and approximately 80% decreased levels of sulfation all complained of the mouth and eye dryness sensation [12]. Mucins are synthesized, processed and exocytosed through the regulated secretory pathway. This secretory process requires baso-apical directionality, which again depends on two fundamental elements. First, correct cell polarity and precise localization of the molecular components that participate in exocytosis is required. Tight junctions (TJ) are important structures that define apical and basolateral domains in the plasma membrane. For acinar cells of LSG from SS-patients, dramatic changes in the expression, as well as apical to basolateral redistribution of TJ-proteins have been observed in response to TNF-α and IFN-γ stimulation [13]. Second, another highly relevant aspect is the assembly of the membrane fusion complex which involves several protein families, including SNAREs, Rab GTPases, and Sec1/Munc-18 related proteins [14]. This machinery only becomes active when all individual components are assembled into functional units at the appropriate location. For instance, all t-SNAREs need to be present in the same physical location at the plasma membrane and all v-SNAREs must be assembled onto the secretory granules in the binding competent conformation [15]. Previous findings from our laboratory analyzing salivary glands revealed that RAB3D, a protein required for directing secretory granules towards the apical plasma membrane without being involved in the fusion reaction itself, loses its specific apical localization and becomes distributed throughout the cytoplasm of acinar cells (~50% of salivary acini) [10]. With these aspects in mind we wondered what happens in salivary acinar cells of SS-patients with the fusion receptors (SNARE proteins) involved in regulated secretion, such as VAMP8, syntaxin 3 (STX3), STX4 and SNAP-23. The current study was designed to determine whether the expression and localization of selected v- and t-SNARE proteins in LSG of SS-patients were altered and to evaluate the functionality of these proteins by measuring their ability to form SNARE complexes under basal conditions.

2. Patients and methods

2.1. Patients with primary SS and controls

A total of 67 individuals participated in the present study after signing an informed consent form based on guidelines established by the Ethics Committee of the Faculty of Medicine, University of Chile. Thirty-nine individuals were diagnosed as patients with primary SS according to the American/European consensus group criteria [16] and twenty-eight subjects (age and gender matched) were controls. The controls were selected from individuals who did not fulfill the primary SS classification criteria. All control subjects were negative for rheumatoid factor, antinuclear, Ro and La antibodies. In LSG biopsies from controls, only mild, non-specific, chronic sialadenitis was observed (Chisholm and Mason, grade 1). A detailed description of demographic, serological, and histological characteristics of the SS-patients and controls are summarized in Table 1.

2.2. Biopsies

LSGs were obtained as previously described [17]. Following surgery, samples were split into two portions. One portion was immediately frozen in liquid nitrogen and stored at −80 °C to extract RNA and proteins. The second portion was processed for morphological characterization and immunofluorescence experiments.

Table 1
Demographic, serological, and histological characteristics of the SS-patient and control groups.

Parameters	Controls	SS-Patients
Gender (Female/Male)	22/6	36/3
Age, mean ± SD (range) years	41 ± 12.3 (20–65)	48 ± 11.3 (22–70)
Focus score, foci number/4 mm^2 of tissue	0	1–2 ($n = 23$) 3 ($n = 9$) 4 ($n = 6$)
USF, mean ± SD mL/15 min (range)	3.2 ± 2.2 (0.3–10.5)	0.98 ± 1.4 (0–5.3)*
Ro antibodies	0/28	27/39
Ro/La antibodies	0/28	16/39
Antinuclear antibodies	0/28	30/39
Rheumatoid factor	0/28	17/39

USF: Unstimulated salivary flow, SD: Standar deviation, *$p \leq 0.05$.

2.3. Quantitative RT-PCR

RNA extraction, yield and purity were evaluated essentially as described [18]. Primer sequences for the genes h18S, VAMP8, STX3 and STX4 were designed with the AmplifX 1.4 software (Supplementary Table 1). For qPCR reactions, the Brilliant II SYBR Green QPCR Master Mix kit (Stratagene, CA, USA) was employed. Template cDNA was obtained by reverse transcription of 1 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen, CA, USA). For amplification of VAMP8, STX3 and STX4, thirty five PCR cycles, consisting of denaturation at 95 °C for 15 s, primer annealing for 15 s at 60 °C and elongation at 72° for 15 s were employed. Dissociation curves to determine specificity and Tm of DNA amplicons were obtained using one additional cycle consisting of denaturation at 95 °C for 10 s, hybridizing at 70 °C for 1 s and a gradual increment of 0.02 °C/s up to 95 °C during 10 min. Ct values were obtained for each sample using the MxPro-Mx 3000P software (Stratagene, CA, USA). Efficiency (E) values were obtained from standard curves with a control sample using serial dilutions of the cDNA for all genes to be evaluated and a housekeeping gene (h18S). The relative expression ratio of a target gene was expressed in comparison to a reference gene as previously described [10] and the REST program (V2.0.7). Ratios over 1.0 indicated upregulation and ratios below 1.0 were indicative of downregulation.

2.4. Protein extraction and Western blotting

LSGs were homogenized in RIPA buffer as previously described [18]. The protein concentration of extracts was determined by the Bradford method [19]. 20 μg (STX3) ó 15 μg (STX4 and VAMP8) of total protein were used. Proteins were separated according to their molecular weights by SDS-PAGE on 11% gels under reducing conditions. Separated proteins were transferred to nitrocellulose membranes (BioRad Laboratories Headquarters Hercules, CA, USA) during 15 h at 4 °C. Membranes were blocked for 1 h at room temperature (RT) in 5% skimmed-milk (protease-free) prepared in TBST buffer (10 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20). Blots were then separately incubated with anti-human antibodies to STX3, STX4, VAMP8 and β-actin (Supplementary Table 2) for 2 h at RT. After 5 washes in TBST buffer, membranes were incubated with goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies for 1 h at RT. Target proteins were detected by chemiluminescence (Pierce, IL). Protein bands were quantified by densitometry. Protein levels were normalized to β-actin.