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ABSTRACT

Objectives: MUC5B contains sulfated and sialylated oligosaccharides that sequester water required for moisturising the oral mucosa. Xerostomia, in patients with Sjögren syndrome, is generally associated with reduced quantities, rather than altered properties, of saliva. Here, we determined the amount of MUC5B (mRNA and protein) as well as sulfation levels in salivary glands of patients with normal or altered unstimulated salivary flow.

Methods and results: Localisation of MUC5B and sulfated MUC5B, as well as total levels sulfated groups were determined and compared with acini basal lamina disorganisation.

Patients and methods: In all, 18 patients with normal or altered unstimulated salivary flow and 16 controls were studied. MUC5B mRNA and protein were evaluated in salivary glands by semiquantitative RT-PCR and Western blot analysis. MUC5B sulfation was determined by Western blotting. MUC5B and sulfo-Lewis antigens localisation were assessed by immunohistochemistry. The total amount of sulfated oligosaccharides was determined microdensitometrically.

Results: No significant differences were detected in MUC5B mRNA and protein levels between controls and patients, while sulfo-Lewis positive mucous acini was reduced in patients but no correlation was observed between lower levels of sulfation and unstimulated salivary flow. Microdensitometric data confirmed the presence of reduced sulfated oligosaccharides levels in mucous acini from patients with highly disorganised basal lamina.

Conclusion: Disorganisation of the basal lamina observed in patients with Sjögren syndrome may lead to dedifferentiation of acinar mucous cells and, as a consequence, alter sulfation of MUC5B. These changes are suggested to represent a novel mechanism that may explain xerostomia in these patients.

Primary Sjögren syndrome is an autoimmune exocrinopathy. Secretory activity of salivary and lacrimal glands is highly compromised, and severe dryness of the mouth and the eyes are the consequence. Autoantibodies against muscarinic M3 receptors, variations in cytokine levels, glandular derervation, acinar atrophy, redistribution of aquaporin-5 in the acinar cells and increased levels of cholinesterase are the most frequent molecular and morphological changes associated with symptoms of dryness. However, only some of these changes are observed in these patients. Thus, correlating the observed changes with specific symptoms, such as dryness, remains as a controversial issue in the field.

Morphological changes in the secretory granules have been consistently observed in all patients evaluated and could be considered one of the most reliable indicators for this disease. In non-obese diabetic (NOD) mice, an animal model for Sjögren syndrome, changes in secretory granule morphology and distribution of key effectors of exocytosis are apparent at 4 weeks after birth, which may be taken to indicate that disease onset occurs prior to lymphocyte infiltration. A better understanding of events specifically associated with early alterations in secretory granule function should be insightful.

MUC5B, the predominant mucin in salivary gland high molecular weight mucous glycoprotein 1 preparations, is synthesised by mucous acini and glycosylated to varying extents. The associated carbohydrates are heterogeneous and include neutral, sulfated and sialylated oligosaccharides. Sulfated and sialylated mucins retain large amounts of water and contribute thereby to generating the hydrophilic gel essential for lubrication of the oral epithelium.

Previous studies revealed high levels of high molecular weight mucous glycoprotein 1 in resting saliva of patients with Sjögren syndrome. These authors attributed the changes to either the reduced presence of water or, alternatively, to a limitation in the capacity to retain water and suggested that this might explain xerostomia. The objective of this study was to evaluate whether symptoms of xerostomia were linked to reduced salivary flow or qualitative/quantitative changes (eg, post-translational modifications) in the MUC5B produced.

PATIENTS AND METHODS

A total of 18 patients (females) with primary Sjögren syndrome (mean (SD) age = 50.5 (12.3) years) diagnosed according to the American–European Consensus Group criteria, were selected for these studies. Several tests were performed to identify ocular, oral and serological involvement. The focus score was 1 in patients with 50–80% of remnant parenchyma. Dry eye and mouth symptoms were observed in all patients. Unstimulated salivary flow was lower than 1.5 ml per 15 min in 11/18 patients. Additionally, scintigraphy and Schirmer test results were altered. Patients had neither been treated with corticosteroids, hydroxychloroquine, or immunosuppressive drugs for at least 1 month before biopsies were obtained.

Supplementary table 1 is published online only at http://ard.bmj.com/content/vol67/issue10

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The control group was composed of 16 subjects (females) (mean (SD) age = 45.09 (12.18) years) who had consulted their doctors because of oral and ocular dryness symptoms over more than 3 months, but who did not fulfil the criteria for Sjögren syndrome. Serological, scintigraphy and unstimulated whole salivary flow data were normal in these patients. Biopsied salivary glands were normal, with scarce and scattered distribution of mononuclear cells, well-preserved parenchyma, and lack of fibrous or adipose tissue. Control individuals were free of systemic diseases and did not use drugs that could affect the function of the exocrine glands. Biopsies were obtained following informed consent of the control group and patients. No significant differences (p = 0.12) between controls and patients according to age were detectable. This study was conducted according to the guidelines of the Ethics Committee of the Faculty of Medicine, University of Chile.

Biopsies
Labial salivary gland biopsies were obtained in the morning (after at least 2 h of fasting), using the technique described by Daniels.17 Glands were fixed for immunohistochemistry using two different conditions. One half of the gland was treated with alcoholic Bouin fixative (MUC5B and sulfo-Lewis a antigen) and the other half was fixed in 1% paraformaldehyde (laminin) and then embedded in paraffin. For RNA and protein preparations, the glands were snap-frozen in liquid nitrogen and stored at −70°C.

RNA extraction/semiquantitative RT-PCR/Preparation of protein extracts
Experiments were performed essentially as previously described18–20 (see also Supplementary material).

Western blotting
Gland protein extracts were prepared as previously described.19–21 Aliquots of 25 µg of proteins were separated on 6% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose over 15 h at 60 mA and 4°C. Membranes were incubated with PANH2 (a mouse antibody, recognises partially deglycosylated MUC5B) or F2 (a mouse antibody, recognises the SO3Galβ1-3GlcNAc moiety of sulfo-Lewis a antigen, both obtained from the European Consortium of Mucins), diluted 1:10 or 1:50 in Tris buffered saline/Tween (TBST), respectively, for 2 h at room temperature. Alternatively, membranes were incubated with the anti-actin antibody (MP Biomedicals, Aurora, Ohio, USA) diluted 1:5000 in blocking solution. Then, blots were incubated with anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) (dilution 1:5000) for 1 h at room temperature, and horseradish peroxidase (HRP) was detected with ECL as described.18–20 MUC5B and Sulfo-MUC5B bands were analysed densitometrically (UN-Scan-IT, Silk Scientific Corporation, Orem, Utah, USA) and values obtained were normalised to those of β-actin. An amount of 10–70 µg of total protein yielded a densitometric β-actin signal within the linear range.

Immunohistochemistry analysis
Sections used for MUC5B and sulfo-Lewis a antigen detections were blocked to prevent non-specific antibody binding with 0.25% casein in phosphate buffered saline (PBS) and incubated for 20 h at 4°C with non-diluted PANH2 or F2 diluted 1:50 in 1% bovine serum albumin/PBS, followed by a biotinylated secondary antibody and a streptavidin–peroxidase conjugate (Dako, Carpinteria, California, USA). The reaction was developed with 3′,5′-diaminobenzidine. Mayer haematoxylin was used for counterstaining. As a negative control, a mouse IgG1 (PANH2) or IgM (F2) recognising an epitope not present in mammalian tissues was employed. For additional information concerning PANH2 and F2 antibodies, see Supplementary material. Laminin was detected as previously described and these sections were also stained with Alcian blue pH 1.0 for 15 min at room temperature, (for details see Supplementary material).

Quantification of the histochemistry reaction of sulfated mucins
Gland sections were stained with Alcian blue pH 1.0 to identify sulfated mucins. Staining intensity was measured with a MPM-400 microdensitometer (Carl Zeiss, Jena, Germany). For each biopsy, 50 random fields containing parenchyma were analysed and the mean value was expressed in arbitrary units of absorbance (AU). The data were evaluated using the Systat 9.0 program (SPSS, Chicago, Illinois, USA).

Statistical analysis
Normalised data of mRNA, proteins and sulfo-Lewis a antigen were averaged to calculate mean values and the standard deviation. A Mann–Whitney U test and non-parametric Spearman correlation were used. Microdensitometric measurements of sulfated mucins were compared by unpaired Student t test analysis. p Values less than 0.05 were considered significant.

RESULTS
mRNA and protein levels of MUC5B
As shown in fig 1A,B, no significant changes in relative mRNA expression levels were found when comparing samples from patients (n = 10) with control individuals (n = 9) (p = 0.07); however, a tendency towards decreased mRNA levels was detected in patients. The PANH2 antibody, which recognises partially glycosylated MUC5B, was employed for MUC5B protein determination.21 Two broad and diffuse bands were observed, one migrating with an apparent molecular weight somewhat larger than 200 kDa and the second being so large that it was retained in the stacking gel. The electrophoretic pattern was similar between patients and controls. No significant differences (p = 0.33) were found when bands were analysed by scanning densitometry either separately (not shown) or together (fig 1C,D).

Localisation of MUC5B
MUC5B was detected in mucous acini from controls and patients (fig 2A–F). MUC5B levels were similar in all mucous acinar cells present in the normal gland sections (fig 2A) and localised to the basal region of these cells (fig 2B). Double staining with PANH2/Alcian blue pH 1.0 revealed sulfated secretion products in the apical region of mucous acinar cells in controls (fig 2C). In patients, MUC5B was detectable in the basal and apical zones of mucous acinar cells (fig 2D,E). Stronger immunoreactivity was detected in mucous acini adjacent to foci of inflammatory cells (fig 2D,E), while in regions without foci; the intensity was similar to those observed in controls (fig 2F).

In patients, increased immunoreactivity for MUC5B and particularly presence in apical suggests that MUC5B glycosylation was decreased.
Levels of sulfo-MUC5B

The degree of sulfation in a specific and well characterised oligosaccharide, the sulfo-Lewis a antigen, was analysed using the monoclonal antibody F2 that recognises the antigen (SO3Galβ1-3GlcNAc). Sulfo-Lewis a levels (fig 3A,B) were notably decreased in gland extracts from patients (n = 10) as compared with controls (n = 9) (p < 0.001). The electrophoretic pattern for sulfo-Lewis a antigen was similar to the pattern obtained for MUC5B; however, the upper band of sulfo-Lewis a antigen (located in the stacking gel) was broader and of greater intensity than the one detected for MUC5B, particularly in controls. This is probably indicative of a heterogenic population of sulfo-Lewis a glycoforms.

Localisation of sulfo-Lewis a antigen

The substantial decrease in sulfo-Lewis a antigen observed in salivary gland extracts from patients was also evaluated in gland sections. As shown in fig 4, similar staining patterns were observed with the F2 antibodies in controls (A–D) and patients (E–G): for one group of mucous acini generalised cytoplasmic staining was apparent (fig 4A,E), in another group a mosaic-like staining pattern was detected (fig 4B,F), and in a third group no staining of cells could be detected (fig 4C,G). In fig 4C, gland sections were sequentially stained for MUC5B (dark brown signal) and sulfo-Lewis a antigen (light brown signal). In some mucus acini only MUC5B staining was detected in basal regions of acinar cells, while in neighbouring mucus acini MUC5B and sulfo-Lewis a antigen were apparent. These results suggest that sulfo-Lewis a antigen-negative acini expressed MUC5B lacking sulfation in (SO3Galβ1-3GlcNAc).

To test whether other sulfated groups were present in sulfo-Lewis a negative/MUC5B positive mucous acini, these sections were also stained with Alcian blue pH 1.0 (fig 4D). Indeed, some acini were triple positive for MUC5B/sulfo-Lewis a/Alcian blue while in others only MUC5B and Alcian blue were detected (fig 4D). Signal intensity was similar in sulfo-Lewis a positive acini of patients and controls (fig 4A–C, E–G). However, the number of sulfo-Lewis a positive mucus acini was dramatically reduced in patients (compare fig 4E,F,G). No signal was detected with the IgM used as negative control for the F2 antibody (data not shown).

Relationship between sulfo-Lewis a positive mucus acini and salivary flow

The number of sulfo-Lewis a positive mucus acini was expressed as a percentage of the total number of mucus acini evaluated in three gland sections from either patient or control individuals. Each section contains at least 500 mucus acini. The

Figure 1  MUC5B mRNA and protein levels in labial salivary glands from patients with Sjögren syndrome and healthy control individuals. A. MUC5B mRNA was detected by semi-quantitative RT-PCR, as a single band of 400 bp and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a band of 650 bp. C. Gland extracts were tested by immunoblotting with monoclonal anti-human MUC5B antibody (PANH2) followed by anti-mouse IgG conjugated to horseradish peroxidase (HRP). β-actin was used as a loading control. B and D. Quantification of amplicons and protein bands normalised to GAPDH and β-actin levels, respectively; p values of patients group compared with healthy controls are indicated. Values shown were averaged from nine control individuals for mRNA and five for protein data. Alternatively, material from 10 patients was used for mRNA and from 6 patients for protein studies. Data are representative of three independent experiments. p Values <0.05 were considered statistically significant.
unstimulated salivary flow data indicate that 7/18 patients had a flow rate greater than 1.5 ml/15 min, while for 11/18 patients the flow rate was less than 1.5 ml/15 min (fig 5A). Interestingly, in both patient groups, unlike controls, 80% of mucous acini were negative for the sulfo-Lewis a antigen, \(p < 0.001; \text{fig 5B}\). The percentage of sulfo-Lewis a positive mucous acini was associated with the unstimulated salivary flow, but no correlation was found in either controls \((r = 0.346)\) or patients \((r = 0.168)\) (see fig 5C,D).

Microdensitometric analysis of total sulfate in mucous acini
The basal lamina has previously been shown to be altered in acini and ducts of salivary glands from patients with Sjögren syndrome.9 The aforementioned results showed information concerning a single type of sulfated oligosaccharide (SO\(_3\)Gal\(\beta_1-3\)GlcNAc) were showed. Here, we consider correlating total levels of sulfated oligosaccharides with basal lamina integrity by double staining using Alcian blue pH 1.0 and an antibody directed against laminin. Microdensitometric analysis of Alcian blue pH 1.0 staining was significantly lower in patients than in controls \((p = 0.04; \text{fig 6A})\). This reaction was associated with a uniform and strong laminin signal in control individuals (fig 6B). Conversely, weak positive staining for Alcian blue pH 1.0 in patients correlated with decreased laminin immunoreactivity (fig 6C).

DISCUSSION
Messenger RNA, protein and sulfation levels of MUC5B as well as the localisation of MUC5B protein and sulfated moieties in labial salivary glands from patients with Sjögren syndrome were evaluated here. Significant differences were not detectable for mRNA or protein levels when comparing patients with controls...
Importantly, levels of sulfation were substantially reduced in gland extracts obtained from patients (fig 3). Additionally, decreased staining for SO$_3$Gal$eta$_1-3GlcNAc moieties and total sulfate oligosaccharides present in mucous acini was observed (figs 5 and 6).

Post-translational processing of mucins, including O/N-glycosylation and sulfation, occur in the Golgi complex. Since our current understanding of the regulation of glycosyltransferases/sulfotransferases is limited, pin-pointing the defect in sulfation pathways that could explain the results of this study is difficult. Lower expression levels and/or lower enzymatic activity of sulfotransferases, reduced amounts of substrates (eg, oligosaccharide moieties, sulfate groups, etc.) and/or an increased activity of sulfatases could explain the results of this study. Although sulfatases are thought to be located in lysosomes, more recent evidence favours the existence of a novel non-lysosomal sulfatase pool in the Golgi complex, as well as at the cell surface. Thus, the Golgi complex could be the organelle responsible for the changes reported in this study; however, further work is necessary to provide a conclusive explanation for decreased sulfation observed in patients with Sjögren syndrome.

In human myeloid cells, tumour necrosis factor (TNF)$\alpha$ stimulates sulfation of GlcNAc present in the cell adhesion glycoprotein CD44, possibly by activating a GlcNAc6sulfotransferase that modulates the degree of sulfation of N- and O-linked glycans. By contrast, treatment of cultured...
bovine articular synoviocytes with TNFα caused a decrease in Galβsulfotransferase activity, suggesting a cell type- and sulfotransferase-specific regulation of sulfation. Additionally, these data indicate that the stimulation of an inflammatory response could alter the ratio of specific sulfated glycan structures present in a cell. Since TNFα levels are elevated in patients with Sjögren syndrome, inhibition of some sulfotransferase may provide an explanation for the low levels of glycoprotein sulfation detected in these patients. However, no correlation was observed between reduced sulfation and focus score (data not shown).

A striking correlation was detected between reduced levels of total sulfated oligosaccharides and the degree of basal lamina disorganisation observed (fig 6). Receptors (integrins) located in the basolateral plasma membrane interact with basal lamina proteins and thereby trigger a variety of cellular responses, including secretory processes. Thus, if these membrane receptors are surrounded by a modified environment, changes in the interaction between proteins of both compartments could alter receptor functionality, even under circumstances where ligands or agonists are still present. Previous studies have reported variations in the organisation and expression of several laminins and nidogens in acini of patients with Sjögren syndrome. Such changes may affect acinar cell differentiation and polarisation. Altered signalling events potentially contribute to differential engagement of, for instance, the secretory

Figure 5  Relationship between sulfo-Lewisα positive mucous acini and salivary flow. A. Values for control individuals and patients with high (↑) and low (↓) unstimulated salivary flow (USF) are shown. B. Percentage of sulfo-Lewisα mucous acini in controls and patients. C. Spearman correlation between sulfo-Lewisα positive mucous acini and salivary flow in control individuals and patients. D. USF: unstimulated salivary flow, SS: patients with Sjögren syndrome.

Figure 6  Microdensitometric analysis of total sulfate in mucous acini of labial salivary glands from control individuals and patients with Sjögren syndrome. A. Box plot indicating that absorbance expressed in AU for Alcian blue positive areas was higher in control individuals than in patients. B and C. Double staining with Alcian blue pH 1.0 and laminin in microphotographs of salivary gland samples from controls (B) and patients (C). Bars in B and C: 50 μm. AU: arbitrary units.
machinery. Thus, alterations in the input to signal relationship due to basal lamina disorganisation may represent a causative factor leading to acinar mucous cell dedifferentiation and alterations in post-translational processing of glycoproteins like MUC5B. The factors contributing to such changes in basal lamina surrounding acini in patients with Sjo¨gren syndrome are currently under investigation.

No correlation between unstimulated salivary flow and the percentage of mucous acini with sulfo-Lewis™ antigen was found (fig 5C,D). The mucins, in particular MUC5B, play an important role in lubrication, since they maintain hydration via interactions between water molecules and hydrophilic moieties, such as sulfate groups, sialyl acid and hydroxyl groups. Our data indicate that the extent of MUC5B sialylation was low and no statistically significant differences were observed between patients and control individuals (data not shown). Thus, independent of the unstimulated salivary flow measured, the dry mouth sensation observed in all patients is currently best explained by reduced sulfation of MUC5B and other mucins present in these glands.

It is important to note that all patients analysed in this study fulfilled the criteria to be diagnosed with this disease, with the exception of the unstimulated salivary flow values detected in 7/18 patients (fig 5). This strengthens the aforementioned conclusion that the oral sensation does not depend exclusively on the amount of water or quantity of saliva, but rather on the quality of saliva and the presence of specific components, such as sulfation.

In mucins, sulfated and sialic acid residues interact with Ca™ and H™ generating interstrand crosslinks that displace water molecules and compact the mucin granule. Thus, low sulfation of mucins will impact unfavourably on secretory granule assembly. During exocytosis, these ions are replaced by Na™ and water; however, under conditions of low sulfation of mucins, such exchange does not occur and as a consequence insufficiently hydrated mucins are secreted.

Interestingly, Saari et al. reported high concentrations of mucous glycoprotein 1 in resting whole saliva of patients with Sjo¨gren syndrome, supporting the hypothesis that low water retention capacity could explain xerostomia. In conclusion, loss of mucin and particularly MUC5B sulfation was observed in the mucous acini from labial salivary glands of patients with Sjo¨gren syndrome. This molecular change did not appear to relate to alterations in saliva volume. Instead, reduced water content of mucins with low sulfation may provide an explanation for the dry mouth sensation. Thus, we propose that post-translational modifications of MUC5B, rather than changes in mucin levels per se, play a role in salivary gland malfunction observed in patients with Sjo¨gren syndrome and could contribute significantly to xerostomia. An important corollary of this study is that future treatments for patients with Sjo¨gren syndrome should include not only enhanced production/intake of water but also, more importantly, an increased capacity to retain it, for example, by modulating the synthesis of mucins with appropriate post-translational processing.

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Ethics approval: This study was conducted according to the guidelines of the Ethics Committee of the Faculty of Medicine, University of Chile.

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