

ABNORMAL DISTRIBUTION OF AQUAPORIN-5 AND LYMPHOID INFILTRATION IN SALIVARY GLANDS OF PRIMARY SJÖGREN'S SYNDROME

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Abstract : **Objectives:** Patients with Sjögren's syndrome (SS) suffer from impaired saliva secretion due to an autoimmune destruction of salivary glands. Based upon its abnormal distribution in SS salivary glands, a potential role for the water channel protein aquaporin-5 (AQP5) is proposed in the pathogenesis of SS. **Methods:** The immunohistochemical distribution of AQP5 was compared in minor salivary gland (MSG) biopsies obtained from primary SS with a variety of lymphoid cells infiltration in MSG, and healthy volunteers. **Results:** Biopsies from normal subjects revealed AQP5 primarily at the apical membrane of the salivary gland acinus. In contrast, biopsies from SS patients with both moderate and severe lymphoid infiltration revealed AQP5 expression diffusely at the cytoplasm of the acinus. Computer-assisted microscopy was performed to quantitatively evaluate AQP5 distribution in the acini. Biopsies of SS patients exhibited lower labeling indices (LI) at the apical membrane compared with biopsies from control subjects. The LI of the acini obtained from SS patients with both moderate and severe lymphoid infiltration showed no difference. **Conclusions:** Abnormal distribution of AQP5 in salivary gland acini could be responsible for a loss of saliva secretion, and this abnormal distribution is likely to develop during the stage of moderate lymphoid infiltration in salivary gland.

Key words : aquaporin-5, salivary gland, Sjögren's syndrome, xerostomia

INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune inflammatory disease characterized by lymphocytic infiltration of salivary and lacrimal glands leading to glandular hypofunction and dry mouth and eyes¹⁾. Salivary gland biopsies show lymphocytic infiltrates with a maximal 50% decrease in acini number²⁾, however, in addition to loss of gland elements, gland dysfunction plays an important role in the clinical expression of the disease¹⁾. Several factors may contribute to impaired salivary

flow, including proinflammatory cytokines, such as IL-1 and TNF- α , that can inhibit both basal and stimulated secretion^{3,4}. Decreased protein kinase C levels in salivary gland acinar cells of SS patients⁵, along with the presence of antibodies against muscarinic M3 receptor in primary SS patients could prevent normal gland responses to neuronal stimulation⁶. We recently reported possible involvement of human regeneration gene product and its autoimmunity in the degeneration of salivary gland, leading to impaired saliva secretion⁷.

The aquaporins, a family of water-specific membrane channel proteins, provided insight into the molecular mechanism of membrane water permeability in a variety of tissues⁸⁻¹². Aquaporin-5 (AQP5) was cloned from rat submandibular glands and is also present in the lacrimal gland and lung, suggesting roles in generation of saliva, tears, and pulmonary secretions¹³. Ma *et al.*¹⁴ reported that transgenic mice lacking AQP5 exhibit decreased saliva secretion, confirming the physiologic relevance of AQP5 in salivary gland function.

Because the pathomechanisms responsible for diminished function of the exocrine glands are still obscure, a report that AQP5 had lost its normal apical localization in secretory salivary acinar cells in SS patients raised considerable interest¹⁵. In SS patients AQP5 was found to be diffusely distributed, not only in the apical, but also in the basolateral acinar plasma membrane domain. This finding on abnormal translocation was confirmed by Tsubota *et al.*, who reported similar loss of acinar cell polarization in lacrimal glands¹⁶.

None of the previous reports explain the abnormal distribution of AQP5 during the progress of sialoadenitis and xerostomia. The aims of this study were to characterize the cellular distribution of human aquaporin-5 (hAQP5) in minor salivary glands (MSG) from SS patients with a variety of the stage of lymphoid cell infiltration and normal subjects, and to investigate potential differences in the pattern of expression of hAQP5.

PATIENTS and METHODS

Patients

Minor salivary gland (MSG) biopsy specimens were taken from the patients with primary Sjögren's syndrome (SS) after informed consent. Twenty cases of primary SS patients were obtained from the file of the Pathology Laboratory of the Department of Otorhinolaryngology. The diagnosis of SS was confirmed by the revised diagnostic criteria established by the Committee on Sjögren's Syndrome of the Ministry of Health and Welfare of Japan¹⁷. Biopsy cases were primary SS patients with moderate infiltration with 1 or more focus; corresponding to Greenspan's¹⁸ grades 3 and 4 (1 male and 9 females; mean age 49.1 ± 5.7 years, range 15-69 years), SS patients with severe infiltration enough to form lymph follicles (1 male and 9 females; mean age 58.4 ± 2.0 years, range 50-71 years), and control subjects (9 males and 1 female; mean age 30.9 ± 2.6 years, range 27-34 years). We measured saliva production on the Saxon test as a quantitative test for xerostomia. For the Saxon test, saliva production was measured by weighing a gauze pad before and 2 min after chewing without swallowing; the low-normal value is 2 g. The procedures followed were in compliance with the Declaration of Helsinki, and all subjects signed an informed consent before participation in the study.

Immunohistochemical staining for hAQP5

MSG tissues for immunohistochemical analysis were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo), frozen with liquid nitrogen, and stored at -80°C until use. Cryostat sections were cut into $4\text{-}\mu\text{m}$ slices. After washing in phosphate buffered saline, the sections were fixed in cold acetone for 10min, and when washed. Indirect immunofluorescence staining was performed essentially as described¹⁹. The antigen retrieval step was performed using Bond Epitope Retrieval Solution 1 (citrate-based pH 6.0 solution). The tissue slices were incubated with an affinity-purified antibody to the C-terminus of hAQP5 as a primary antibody overnight at 4°C in a humidified environment. After washing, the antibody was detected by the Bond polymer method [Autoimmunostainer Bond MAX (Mitsubishi Chemical Medience Co., Tokyo, Japan)]. The affinity-purified antibody to the C-terminus of hAQP5 was kindly provided by Dr. N. Ishida, Ophthalmic Research Division, Santen Pharmaceutical, Takayama, Ikoma, Nara, Japan.

Computer-assisted immunoreactive analysis

Computer-assisted immunoreactive analysis of the immunoreactive acinar cell was performed using an Olympus BX50 microscope with an UPlanFI objective, combined with a Polaroid PDMCIe/OL Digital Camera (Tokyo, Japan) and the Win ROOF image processing software (Mitani Corp., Tokyo, Japan) for Windows. This software allowed accurate identification and calculation of the immunostained area. Five digitized, multispectral images for each case were obtained at random and captured at $\times 200$ magnification. The labeling index (LI) obtained from the image analysis was calculated as a percentage of immunoreactive area for hAQP5 in the apical region (the apical side a one-third area of apical side against the whole area) of an acini cell. The immunoreactive acinus area was extracted automatically using two distinct macroinstructions composed chiefly of algorithms for color identification based red-green-blue (RGB) and hue-luminosity-saturation (HLS) parameters.

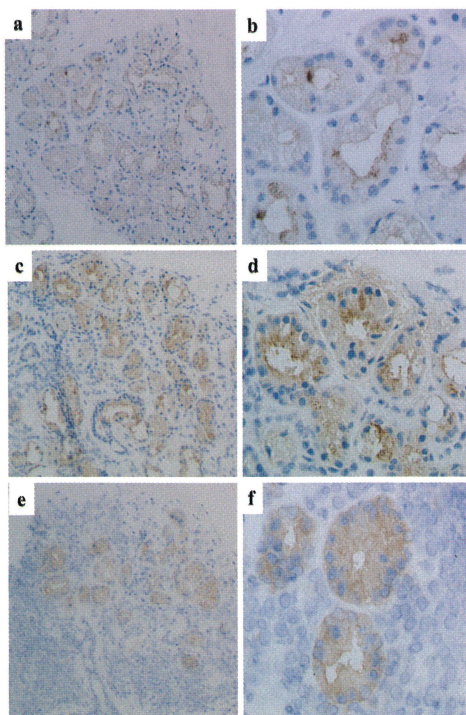
Statistical Analysis

Laboratory data are expressed as the mean \pm SEM and the Mann-Whitney U test was used to evaluate the statistical differences in hAQP5 expression in MSG from control subjects and SS patients. A correlation analysis between the LI of the immunoreactive acini cell and salivary secretion volume was performed using Spearman's rank correlation test. All statistical analyses were carried out using software.

RESULTS

Immunohistochemical localization of hAQP5 in MSG

We surveyed the localization of the hAQP5 immunohistochemically in the MSG from SS patients and control subjects. In MSG biopsy specimens from control subjects, almost of all hAQP5 was detected at the apical membrane of the acini cell (Fig. 1a, 1b). In contrast, in MSG biopsy specimens from SS patients, hAQP5 was not predominantly localized to the apical membrane of the acini cell but diffusely expressed at the cytoplasm (apical, basolateral



and intracellular) of the acini cell. The hAQP5 distribution in MSG biopsy specimens from SS patients with moderate lymphoid infiltration was similar to that from SS patients with severe lymphoid infiltration (Fig. 1c, 1d, 1e, 1f).

Fig. 1 : Immunohistochemical localization of hAQP5 in MSG. hAQP5 was localized on MSG sections from a control subject (a, b), an SS patient with moderate lymphoid infiltration (c, d), an SS patient with severe lymphoid infiltration (e, f) (a, c, and e, original magnification, $\times 40$; b, d, and f, original magnification, $\times 100$). Indirect immunofluorescence staining using affinity-purified hAQP5 antibodies was performed.

The LI of the immunoreactive acini for hAQP5 in MSG

A computer-assisted microscopy procedure was performed to quantitatively evaluate the labeling index (LI) of the immunoreactive acini for hAQP5. The LI corresponds to the percentage of acinus area immunoreactive for hAQP5 in the apical region (a 1/3 area of apical side against the whole area) of an acinar cell. The LI of MSG obtained from SS patients exhibited significantly lower compared with the LI of MSG obtained from control subjects ($40.9 \pm 1.3\%$ vs. $85.3 \pm 1.6\%$, $p < 0.001$, Fig. 2a). The LI of MSG from SS patients with both moderate lymphoid infiltration ($41.1 \pm 2.1\%$) and severe lymphoid infiltration ($40.8 \pm 1.5\%$) were significantly lower than that from control subjects ($p < 0.001$, Fig. 2b). However, there was no difference of the LI of MSG obtained between from SS patients with moderate lymphoid infiltration and from SS patients with severe lymphoid infiltration (Fig. 2b).

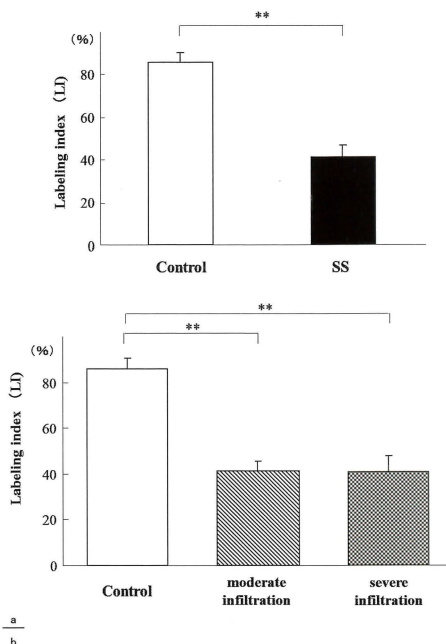


Fig. 2. Quantitative analysis of the LI of the labeled structures was performed using Win ROOF computer-assisted microscope system. LI corresponds to the percentage of the acinus area (a 1/3 area of apical side against the whole area) stained for hAQP5. LI obtained by discriminant analysis performed between control subjects and SS patients (a), and between SS patients with moderate lymphoid infiltration and SS patients with severe lymphoid infiltration (b). Values are expressed as the mean \pm SEM. Mann-Whitney U test was used to evaluate the statistical differences.

The relation of localization of hAQP5 to salivary secretion

Salivary secretion volume from SS patients with moderate lymphoid infiltration (2.6 ± 0.6 g/2 min) and from SS patients with severe lymphoid infiltration (0.8 ± 0.2 g/2 min) were significantly lower than that from control subjects (5.1 ± 0.4 g/2 min, $p < 0.01$ and $p < 0.001$, Fig. 3a). There was significant more salivary secretion volume from SS patients with moderate lymphoid infiltration than that from SS patients with severe lymphoid infiltration ($p < 0.01$, Fig. 3a). The no correlation, on the other hand, was exhibited between the LI of immunoreactive acini for hAQP5 and salivary secretion volume from SS patients (Fig. 3b).

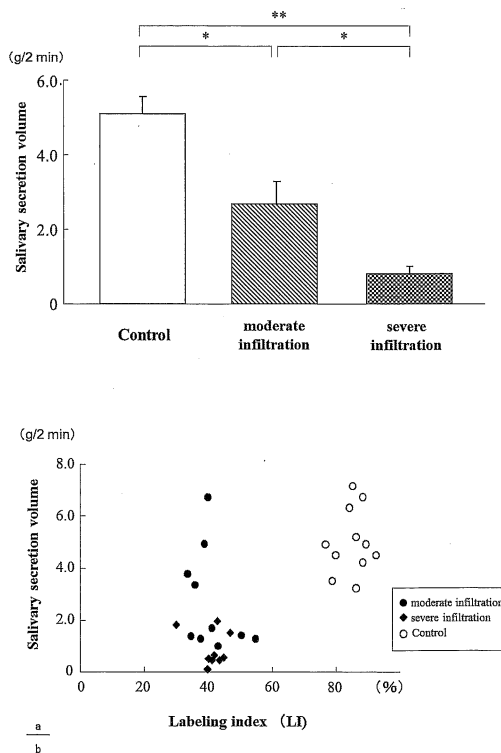


Fig. 3 : Salivary secretion volume performed among control subjects, SS patients with moderate lymphoid infiltration and SS patients with severe lymphoid infiltration (a). Data distributions resulting from analysis of salivary secretion volume and LI distinguishing among control subjects (○), SS patients with moderate lymphoid infiltration (●), and SS patients with severe lymphoid infiltration (◆) (b). Values are expressed as the mean \pm SEM (a). The Mann-Whitney U test and the Spearman rank test were used to evaluate the statistical differences.

Features	Control	SS patients	
		Lymphoid infiltration	
		Moderate	Severe
		<i>n</i> =10	<i>n</i> =10
male/female	9/1	1/9	1/9
Age: mean \pm SEM, y	30.9 \pm 0.8	49.1 \pm 5.7	58.4 \pm 2.0
Salivary secretion volume: mean	5.1 \pm 0.4	2.6 \pm 0.6	0.8 \pm 0.2
\pm SEM, g/2 min			

Table 1 : Demographic and clinical features of control and the SS patients

DISCUSSION

Although the biological mechanisms that lead to impaired saliva secretion in SS are poorly understood, it is increasingly evident that dryness of mouth does not result solely from gland destruction. Cytokines, autoantibodies, or soluble factors may also contribute to gland dysfunction²⁰. Based upon its distribution, a potential role for the water channel protein AQP5 was evaluated in the pathogenesis and the physiological mechanism in saliva secretion of

SS in this study. The subcellular localization of AQP5 in rat and human salivary glands has been demonstrated^{15, 21, 22}. However, to our knowledge, the present study provides the first description of the relation among the localization of AQP5 in human MSG, the volume of salivary secretion, and the grade of lymphoid infiltration in MSG.

We have shown a distinct distribution pattern in MSG from SS patients. The immunoreactivity of hAQP5 was predominantly localized to the apical membrane of acinar cells from control subjects. In contrast, hAQP5 was diffusely expressed at the cytoplasm (apical, basolateral and intracellular) of the acinar cells in MSG biopsies from SS patients. A quantitative analysis using a computer-assisted microscope system further confirmed that the LI, indicating a percentage of acinus area immunoreactive for hAQP5 in the apical region (a 1/3 area of apical side against the whole area) of an acinar cell, was lower in MSG from SS patients as compared with normal subjects.

This AQP5 misdistribution could result from pathophysiological mechanisms occurring in SS. Under diseased conditions, AQP5 trafficking may be altered and intracellular vesicles expressing AQP5 may not be able to translocate to the apical membrane and therefore accumulate in the cytoplasmic compartment. Several mechanisms could be responsible for this alteration. First, defective activation of muscarinic M3 receptor could account for aberrant translocation of AQP5. Whether the abnormal distribution of AQP5 is linked to the presence of autoantibodies to M3 receptor²³ is an interesting question that still remains to be addressed in the mouse models for SS used in the present study. Second, altered expression of proteins involved in the regulation of AQP5, such as prolactin-inducible protein²⁴, could also account for AQP5 misdistribution. Third, inflammation may play a role in AQP5 altered distribution as impaired TGFs signaling has been shown to lead to an inflammatory disorder resembling SS and to a non-polarized and substantial intracellular localization of AQP5²⁵.

The distribution of AQP5 on the apical membrane of acinar cells strongly suggested its physiological importance in water transfer during primary saliva production. To clarify this, Ma *et al.*¹⁴ generated an AQP5-null mouse model in which they examined changes of the amount of saliva and its composition in a pilocarpine-induced state. These analyses revealed a more than 60% reduction in saliva in AQP5-null mice. Together with this report, our data indicated that impaired AQP5 trafficking could be responsible for the decrease in saliva production observed in SS patients.

The difference of hAQP5 localization in acinar cells between MSG with moderate lymphoid infiltration and severe lymphoid infiltration is of interest, because salivary secretion volume from SS patients with moderate lymphoid infiltration was significantly decreased than that from SS patients with severe lymphoid infiltration. The LI of MSG from SS patients with both moderate lymphoid infiltration and severe lymphoid infiltration were significantly lower than that from control subjects, however, there was no difference of the LI of MSG obtained between from SS patients with moderate and severe lymphoid infiltration. In addition, no correlation was exhibited between the LI of acini and salivary secretion volume from SS patients. Soyfo *et al.*²⁶ compared the subcellular distribution of AQP5 in submandibular acinar cells in three different animal models for SS depicting different stages of SS. There was a shift in AQP5 distribution from the apical to the basolateral membrane and intracellular vesicles in all three mouse models for SS investigated, and AQP5 misdistribution appeared

to be concomitant to the presence of inflammatory infiltrates and acinar destruction. Our observations can be explained, in part, by the consideration that acinar cells are affected with impaired AQP5 trafficking in the stage of moderate lymphoid infiltration in MSG. This impaired AQP5 trafficking continues until the stage of severe lymphoid infiltration in MSG, and the salivary gland function declines together with the other factors including the loss of acinar cells during the destruction of salivary gland.

In conclusion, our present study provides the localization of AQP5 in human MSG, and indicates an abnormal distribution of AQP5 in MSG from SS patients, which could be responsible for a loss of saliva secretion in these patients. It should be emphasized that this abnormal distribution of AQP5 is likely to develop during the stage of moderate lymphoid infiltration in MSG.

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