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ORIGINAL ARTICLE

Diabetes affects statherin expression in human labial glands

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BACKGROUND AND OBJECTIVE: Salivary statherin, which plays a special role in the defense of tooth integrity, is secreted by both major and minor salivary glands. A significantly reduced expression of this was recently found in human major salivary glands removed from diabetic subjects and was correlated with the high incidence of dental diseases occurring in patients with diabetes. In this study, we measured the density of gold particles indicating statherin immunoreactivity in labial glands to reveal a significant difference between diabetic and non-diabetic patients.

MATERIALS AND METHODS: Surgical samples of labial glands obtained from both diabetic and non-diabetic patients were fixed with a glutaraldehyde and paraformaldehyde mixture, embedded in Epon, and treated for immunogold histochemistry using a polyclonal antibody specific for statherin.

RESULTS: Statherin immunoreactivity was detected onto small vesicles diffused throughout the cytoplasm of serous cells. Statistical analysis revealed that the number of stained particles was significantly lower in the samples from diabetic subjects than from non-diabetic subjects.

CONCLUSIONS: The results indicate that diabetes affects statherin secretion in labial glands and support the hypothesis that the increased susceptibility to oral diseases associated with diabetes could be related with a reduced statherin secretion.

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Keywords: statherin; diabetes; labial gland

Introduction

Human saliva participates in the maintenance of the oral homeostasis through washing and lubricating buccal tissues and tooth surfaces and through preventing viral and bacterial attacks (Dodds *et al*, 2005). Salivary production varies in terms of flow rate and composition with age, gender, life habits, medications, and oral or systemic diseases, so that the use of saliva as diagnostic non-invasive fluid alternative to blood progressively gains reliability (Farnaud *et al*, 2010).

Minor salivary glands, which include labial, lingual, buccal, palatine, and von Ebner glands, supply only about 7% of the total saliva volume, but despite their small contribution, they play a pivotal role in the protection of the mouth (Hand *et al*, 1999; Riva *et al*, 1999; Humphrey and Williamson, 2001; Eliasson and Carlén, 2010). Their disseminated localization close to the mucosal surface let the entire oral epithelium be covered with their dense secretions that prevent desiccation. Because of their content in antimicrobial and anti-inflammatory proteins, the minor salivary gland secretions are considered an immunological barrier against pathogens that commonly colonize oral cavity (Ferguson, 1999; Siqueira *et al*, 2008; Eliasson and Carlén, 2010), and the recent finding that their secretory cells produce statherin (Isola *et al*, 2010) represents a further demonstration of their importance. Statherin is a small phosphoprotein that plays a key role in the formation of the acquired enamel pellicle (Yao *et al*, 2003) in that it binds directly to hydroxyapatite and mediates the attachment of other proteins. Moreover, through inhibitory effects on primary and secondary calcium salt precipitation, statherin controls the processes of demineralization and remineralization of tooth surface (Raj *et al*, 1992; Garcia-Godoy and Hicks, 2008). Additionally, its C-terminus was demonstrated to bind some microbial species (Gibbons and Hay, 1988). Therefore, statherin concentration in saliva is considered as a possible risk indicator for the development of caries (Vitorino *et al*, 2005; Rudney *et al*, 2009).

An increased susceptibility to bacterial infections, inflammation, caries, xerostomia, and periodontal disease often associated with diabetes (Lin *et al*, 1999; Hirsch, 2004; Javed *et al*, 2007; Patiño Marín *et al*, 2008; Siudikiene *et al*, 2008) was related with a malfunctioning of salivary glands resulting in insufficient salivary flow and altered protein secretion (Kasayama *et al*, 1989; Kimura *et al*, 2001; Aydin, 2007). In agreement with this statement, we recently found a strongly reduced statherin reactivity in submandibular glands of patients with diabetes with respect to normal

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ones (Isola *et al*, 2011). By the measurement of statherin immunoreactivity in labial glands, this study is the first to report that type 2 diabetes induces effects on labial glands.

Materials and methods

Fragments of labial glands were obtained from 10 consenting patients, aged 45–65 years, undergoing surgery for the removal of tongue carcinoma at the Otorhinolaryngology Clinic, University of Cagliari. Those patients were not habitual smokers, alcohol consumers or obese. Neither were affected by autoimmune diseases nor subjected to chemotherapy or radiotherapy before the surgery. Moreover, none of the subjects reported dry mouth sensation. Five of the subjects under study were affected by medically diagnosed type 2 diabetes: three males, aged 45, 51 and 56 (representing 3, 6 and 7 years of diagnosed diabetes, respectively), and two females, aged 48 and 65 (4 and 8 years of diagnosed diabetes, respectively). In all patients, blood glucose level was efficaciously controlled either by diet or by oral hypoglycaemic medications. Of the five non-diabetic subjects, two were males, aged 48 and 58, and three were females, aged 47, 52 and 64. All procedures were approved by the local Institutional Committee for human experimentation at the ASL 8 (Azienda Sanitaria Locale 8), Cagliari.

Transmission electron microscopy tissue preparation
Samples were cut into small pieces and fixed for 2 h with a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Then, they were rinsed in cacodylate buffer added with 3.5% sucrose, dehydrated and embedded in Epon Resin (Glycide Ether 100; Merk, Germany, Darmstadt). Semithin sections stained with methylene blue were examined to check the histological appearance. Ultrathin sections were collected on nickel grids and processed for the immunohistochemical analysis.

Immunohistochemical analysis
The grids were treated with 1% bovine serum albumin (BSA) and 5% normal rabbit serum (NRS) in phosphate-buffered saline (PBS) solution to block non-specific binding. Then, they were incubated overnight at 4°C with a goat polyclonal antibody specific for statherin (Santa Cruz Biotechnology) and diluted 1:50 in 1% BSA and 5% NRS. The grids were then incubated for 1 h at room temperature with the secondary antiserum, a rabbit anti-goat IgG conjugated to 10-nm gold particles (Sigma) diluted 1:50 in BSA-PBS. After washing with PBS and distilled water, they were stained with uranyl acetate and bismuth subnitrate and finally observed and photographed in a JEOL 100S transmission electron microscope. Controls were incubated with a non-immune goat serum or omitting the primary antibody.

Statistical analysis
Two grids from each patient were examined, and serous cells were randomly selected and photographed at low magnification. Quantitative analysis of the micrographs was performed with Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA). Labeling density was estimated by counting the gold particles inside 40 cytoplasmatic areas (20 in diabetic and 20 in normal samples) randomly selected. The areas occupied by secretory granules, mitochondria and nuclei were excluded for the labeling quantification. The labeling density was expressed as number of gold particles per μm^2. Quantitative data obtained in diabetic samples were compared with those in non-diabetic ones, and statistical analysis was carried out using the Student's *t*-test to determine whether the differences were significant. The data values were expressed as mean ± standard deviation (s.d.). The values $P < 0.001$ were considered significant. In addition, the statherin labeling in patients with diabetes was estimated as % of statherin labeling in non-diabetic patients.

Results

From a morphological point of view, no appreciable differences were seen between diabetic and non-diabetic samples at both histological and ultrastructural levels (Figure 1a,b).

Statherin labeling also showed identical sites of localization, restricted to serous cells. Golgi cisternae, small vesicles and tubules isolated or in groups throughout the cytoplasm appeared reactive (Figure 2a–c), while secretory granules, other organelles, nuclei and membranes were always unstained. Labeled vesicles appeared frequently crowded near the cell surfaces

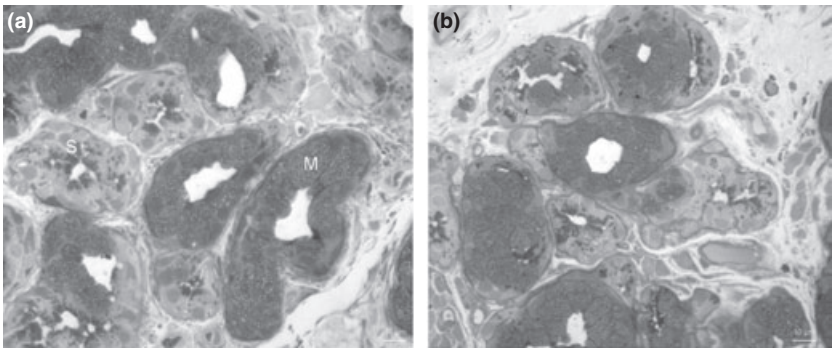

Figure 1 Semithin sections of labial glands: (**a**) Diabetic subject (**b**) Non-diabetic subject. The parenchyma of diabetic samples did not show signs of morphological alterations. S, serous cells; M, mucous cells

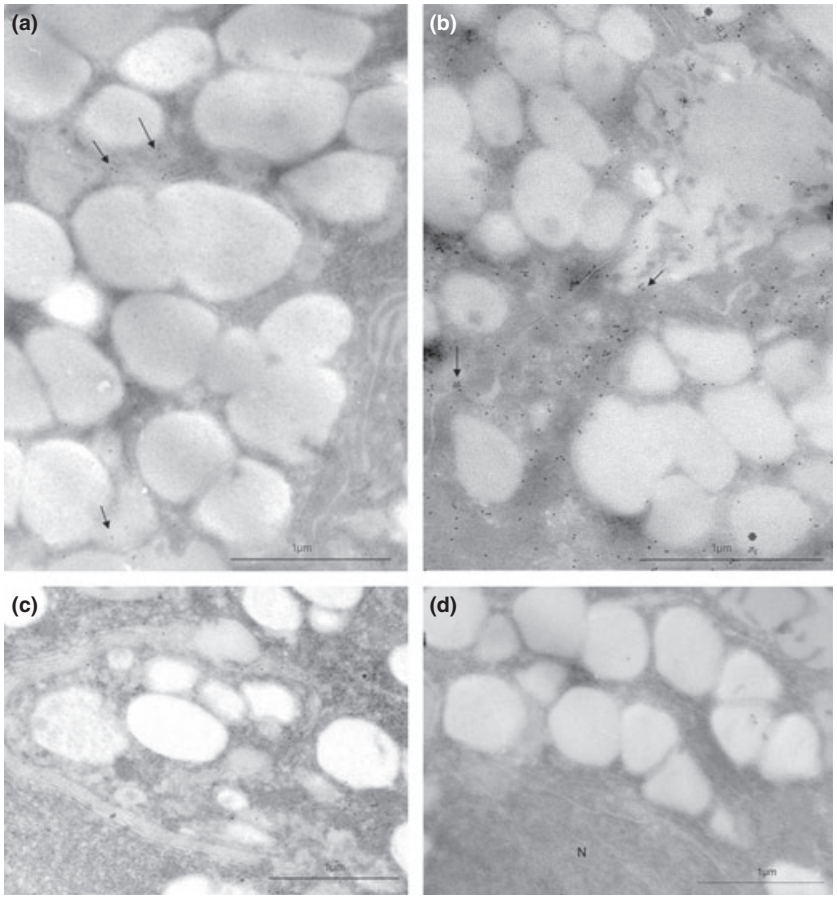

Figure 2 (**a**) Portion of serous cell of labial gland (diabetic). Gold particles were found on small vesicles and tubules surrounding secretory granules (arrows). Serous granules were completely negative. (**b**) Portion of serous cell of labial gland (non-diabetic) showing an intense reactivity for statherin. Labeled vesicles often appeared near the cell surfaces (arrows), among the granules, and occasionally fused with them (*). (**c**) Positive Golgi apparatus of labial serous cell (diabetic). (**d**) Portion of a serous cell of labial gland (non-diabetic). The sample, used as control, was incubated with a non-immune goat serum and no staining appeared

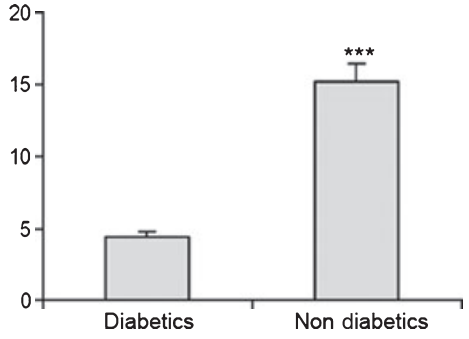

Figure 3 Statherin labeling densities of labial glands in diabetic and non-diabetic patients (gold particles per μm^2). ***$P < 0.001$

(Figure 2b) and among the secretory granules, with which they occasionally seemed partially fused (Figure 2a,b). Control samples were completely negative (Figure 2d).

Strong differences were easily appreciable in staining intensity when diabetic and non-diabetic glands were compared, in that the former exhibited a greatly reduced gold particle deposition (Figure2a,b). Quantification and statistical analysis (Figure 3) demonstrated a significant difference in staining intensity ($P < 0.001$), in that the number of gold particles in diabetic glands (mean 4.47/μm^2 ± 0.35 s.d.) was reduced of approximately 67% with respect to non-diabetic samples (mean 15.14/μm^2 ± 1.33 s.d.).

Discussion

In this investigation, we demonstrated that statherin immunoreactivity in labial glands of diabetic subjects is significantly reduced with respect to non-diabetic ones. The difference was immediately appreciated at the mere observation of the micrographs, because only a few cytoplasmic vesicles were stained in diabetic samples, while the majority was labeled in non-diabetic ones. The statistical analysis showed that statherin immunoreactivity was decreased by 67%, suggesting that less protein is produced in labial glands of diabetic subjects.

Several studies report conspicuous changes in salivary flow rate and protein concentration in patients affected by type 1 and type 2 diabetes (Mata *et al*, 2004; Venza *et al*, 2006; Piras *et al*, 2010). In addition, morphological alterations are described in salivary glands of subjects affected by type 1 and type 2 diabetes, but they are referred principally to the major salivary glands (Carda *et al*, 2005; Piras *et al*, 2010). Only one report deals with some histological alterations of labial glands, such as lymphocyte infiltration and parenchymal destruction as consequences of type 1 diabetes (Markopoulos and Belazi, 1998). In our study, no morphological alterations

were observed in labial samples of patients with diabetes.

The present results resemble those obtained recently with submandibular glands, where a marked reduction in statherin labeling (approximately of 75%) was observed in the type 2 diabetic *vs* non-diabetic samples (Isola *et al*, 2011). All these data suggest that scarce statherin amounts are to be expected in diabetic whole saliva. To date, no biochemical measurement of statherin concentration has been performed in saliva of patients with type 2 diabetes. However, with respect to type 1 diabetes, a profound reduction in salivary statherin concentration was found in the saliva of children (Cabras *et al*, 2010). Given the importance of this protein in the formation of the acquired enamel pellicle (Raj *et al*, 1992; Yao *et al*, 2003; Vitorino *et al*, 2005), structural defects in this protective layer because of insufficient statherin amounts predictably have drastic consequences, such as altered calcium homeostasis and increased susceptibility to oral diseases. Moreover, statherin effectiveness to bind bacteria could also be compromised, thus explaining the higher numbers of oral pathogens in the saliva of patients affected by type 2 diabetes (Khovidhunkit *et al*, 2009).

Saliva produced by labial glands, similarly to that produced by all the minor salivary glands, is very important in the defense of the mouth not only because of its richness of mucins and antimicrobial and anti-inflammatory substances, but also because its secretion occurs continuously, even during night and rest (Humphrey and Williamson, 2001; Eliasson and Carlén, 2010). Interestingly, there is a conspicuous difference between the labial glands and the submandibular glands with respect to the location of statherin. Whereas in the labial glands, statherin was located to the small vesicles (Isola *et al*, 2010), statherin is located to the secretory granules in the submandibular glands (Isola *et al*, 2008; Isola *et al*, 2011). This difference may be interpreted as morphological signs of 'constitutive-like secretion' (Castle and Castle, 1993; Huang *et al*, 2001; Gorr *et al*, 2005) and 'major regulated secretion' (Castle and Castle, 1993; Gorr *et al*, 2005), respectively. The constitutive pathway (minor glands) may provide the oral cavity with a steady continuous supply of protective constituencies such as statherin (Castagnola *et al*, 2002), whereas the contribution via the regulated pathway (major glands) occurs mainly during the meal. Thus, in diabetes, a failure of the minor glands to continuously secrete sufficient amounts of statherin day and night may be particularly deleterious for the oral health.

As judged from the literature, salivary gland proteins as well as their concentrations in the saliva do not show a uniform pattern in response to diabetes, for instance increased levels of amylase and IgA (Piras *et al*, 2010; Aydin, 2007) but reduced ghrelin, salivatin, epidermal growth factor and proline-rich proteins (Kasayama *et al*, 1989; Szczepanski *et al*, 1998; Kimura *et al*, 2001; Aydin, 2007). The cause of changed protein expression in diabetes is unknown. By a general point of view, the vascular defects that often accompany the diabetic condition could result in scarce tissue oxygenation, leading to decreased metabolism and protein synthesis. Moreover, multiple effects of hyperglycemia on protein synthesis have been ascertained, such as post-transcriptional modifications, which create abnormal protein glycosylation (Nicolau *et al*, 2009), intracellular storage and release, and altered cell response to endoplasmic reticulum stress (Yoshida, 2007).

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Author contributions

The doctors Isola M, Solinas P, Diana M, prepared the samples for light microscopy and transmission electron microscopy. Dr. Isola M, Isola R, Loy F, performed the immunohistochemistry investigation and morphometric analysis of images. The Professors Cossu M, Lantini MS designed study and drafted paper.

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