ORIGINAL ARTICLE

Reduced statherin reactivity of human submandibular gland in diabetes

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BACKGROUND AND OBJECTIVES: Statherin is a salivary protein involved in the formation of enamel pellicle and in regulation of calcium homeostasis. Diabetes and other pathologies affect both salivary flow and protein secretion by salivary glands, causing increased susceptibility to mucosal infections, tooth demineralization, and caries. The purpose of this study was to compare the statherin expression in submandibular glands of healthy and diabetic subjects.

MATERIALS AND METHODS: Fragments of submandibular glands obtained from diabetic and non diabetic patients were fixed, dehydrated, embedded in Epon Resin and processed for the immunogold histochemistry. The results were statistically evaluated.

RESULTS: Specific statherin labeling was demonstrated in secretory granules of acinar cells in both diabetic and normal samples. The staining was much more intense in the latter compared to those of diabetics. The labeling density was quantified by evaluating the number and spatial distribution of gold particles within the granules. The number of gold particles was significantly lower in glands from diabetics than in control glands.

CONCLUSIONS: The results obtained suggest that a reduced statherin secretion by salivary glands might be partly responsible for a less effective protection of the oral tissues, resulting in an higher incidence of caries and oral infections associated with diabetes.

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Introduction

Statherin is a small salivary protein (43 aa) rich in negatively charged aminoacids, whose highly polar N-terminus contains two phosphorylated serine residues which confer to the molecule a high affinity for hydroxyapatite (Schlesinger and Hay, 1977). Due to this distinctive property, it binds tooth enamel and form, with proline-rich proteins, the acquired enamel pellicle, an acellular layer which covers the entire surface of the dental crown (Raj *et al*, 1992; Yao *et al*, 2003). Adsorbed on enamel, it plays multiple activities related to the maintenance of the dental health. The inhibitory activity of statherin on spontaneous precipitation of calcium phosphate has fundamental importance in promoting salivary supersaturation that allows optimal regulation of tooth demineralization and remineralization (Raj *et al*, 1992; García-Godoy and Hicks, 2008). Moreover, binding on enamel causes changes in the spatial conformation of statherin, so that the C-terminus became available to bind microorganisms forming the dental plaque (Gibbons and Hay, 1988). Therefore, statherin is generally included among the numerous salivary proteins that participate in the protection of the mouth by means of antimicrobial, anti-inflammatory or other regulatory activities, such as lysozyme, lactoferrin, immunoglobulins, histatin and defensins (Humphrey and Williamson, 2001; Edgerton *et al*, 2000; Van Nieuw Amerongen and Veerman, 2002; Dodds *et al*, 2005).

Recently we investigated the ultrastructural localization of this peptide in human normal salivary glands by a post-embedding immunogold staining technique (Isola *et al*, 2008). A strong statherin reactivity was demonstrated over the serous granules of parotid and submandibular glands, confirming that these glands are an important source of statherin in human saliva.

Considering the high incidence of caries, tooth loss, periodontal disease and candidiasis in patients affected by diabetes (Lin *et al*, 1999; Javed *et al*, 2007; Patiño Marín *et al*, 2008; Siudikiene *et al*, 2008) and given that no literature data document statherin concentration in diabetics, we undertook the present study in order to verify, using submandibular glands, if salivary glands of diabetic subjects have abnormal statherin expression, which could be in part responsible for oral complications.

Materials and methods

Transmission electron microscopy tissue preparation

Fragments of submandibular glands were obtained from eight consenting adult patients, aged 40–60 years, four affected by type 2 diabetes and 4 non-diabetic, undergoing surgery for the removal of tumors from the oral region at the Otorhinolaryngology Clinic, University of Cagliari. All samples investigated were taken avoiding the tissues adjacent to the tumors, and appeared normal by the histological analysis. The samples from non diabetics were the same examined in our previous paper (Isola *et al*, 2008). All procedures were approved by the local Institutional Committee for human experimentation at the ASL 8 (Azienda Sanitaria Locale 8), Cagliari. Each sample was cut into small pieces and fixed for 2 h in 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, Darmstadt, Germany). 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 Inc., Santa Cruz, CA, USA), diluted 1:50 in 1% BSA and 5% NRS. After rinsing, the grids were incubated for 1 h at room temperature with the secondary antiserum, a rabbit anti-goat IgG conjugated to 10 nm gold particles (Sigma-Aldrich, Schnelldorf, Germany) diluted 1:50 in BSA-PBS. Controls were incubated with a non immune goat serum or omitting the primary antibody. 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. Two grids from each patient were examined, and secretory cells rich in granules were randomly selected and photographed at low magnification.

Statistical analysis

Quantitative analysis of randomly chosen micrographs was done with Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA). The gold particles were counted inside 150 serous granules randomly selected from 40 electron micrographs (10 from each diabetic patient) and the labeling density was expressed as number of gold particles/μm^2. The data values were expressed as mean ± standard deviation (SD). To determine if the differences were significant, statistical analysis was done using the Student's *t*-test. The values $P < 0.05$ were considered significant. The proportions of granules with no, weak and intense labeling were also calculated and expressed as percent of the 150 granules.

All the results were then compared with those obtained in micrographs of normal samples following the same procedure. In addition, the statherin labeling in diabetics was estimated as % of statherin labeling in controls.

Results

Statherin reactivity was found in all the submandibular gland samples from both normal and diabetic subjects. The labeling was confined to the serous cells, whose secretory granules appeared the specific site of gold deposition (Figures 1 and 2)

Within the positive granules, gold particles decorated uniformly the dense component in both diabetic and normal samples. However, a weaker staining was easily appreciated in diabetic samples with respect to the controls (Figures 1 and 2).

Statistical analysis showed significant reduction of statherin expression in diabetes ($P < 0.001$), since immunoreactivity was decreased approximately 75% in the diabetic (mean 6.67/μm^2 ± SD) versus normal glands (mean 24.25/μm^2 ± SD) (Figure 3). The relative amounts of positive and negative secretory granules also changed: statherin labeling was absent in 3% in normal samples, versus 22% in diabetics. Moreover, granules with intense labeling (10–49 gold particles/μm^2), were not found in diabetic cells, while represented the majority in normal samples (Figure 4).
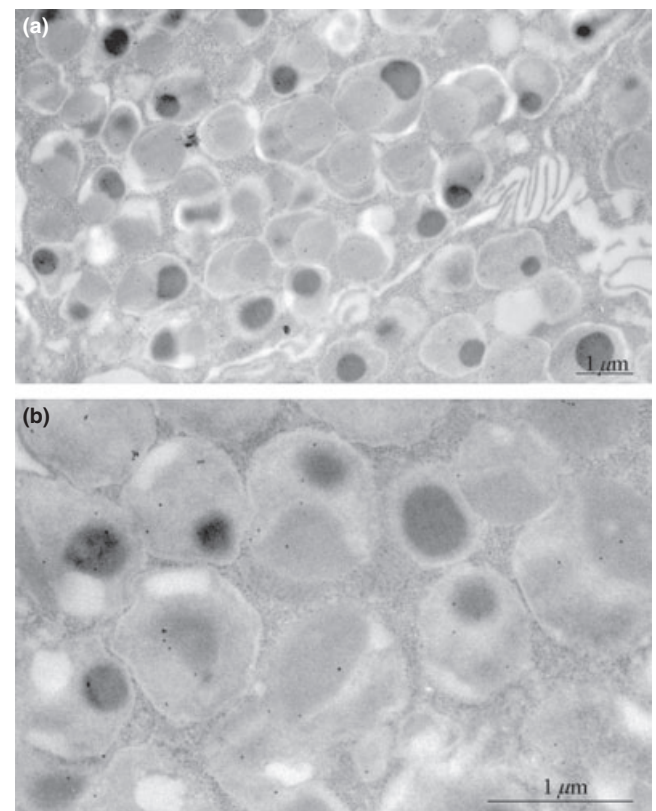

Figure 1 Serous cells of submandibular gland of a diabetic patient, low (**a**) and high (**b**) magnification. Gold particles appear exclusively confined within secretory granules. The majority of them express a weak reactivity for statherin. Moreover, a large number of granules are completely negative
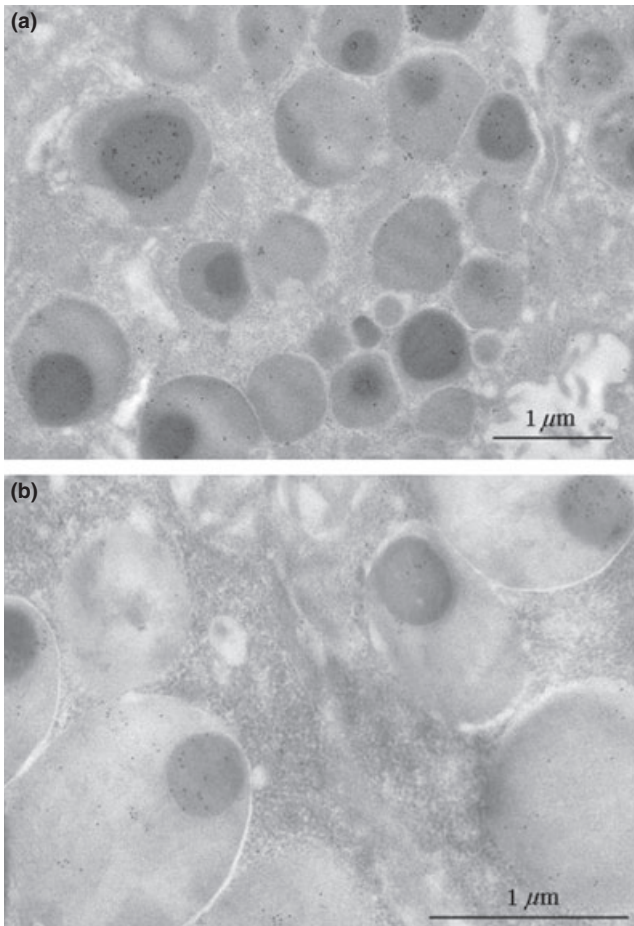
Figure 2 Serous cell of submandibular gland of a non diabetic patient, low (**a**) and high (**b**) magnification. Most of secretory granules exhibit a strong reactivity for statherin, often associated with the dense core
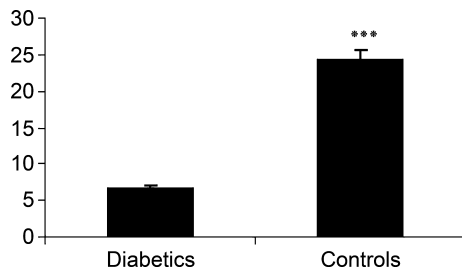

Figure 3 Statherin labeling densities of submandibular secretory granules in diabetics and in controls (gold particles/μm^2). ***$P < 0.001$

Discussion

This study first demonstrates that the statherin reactivity of submandibular glands is significantly decreased in diabetic status. This reduction is clearly demonstrated by the statistical analysis, but it is easily appreciable by the simple observation of the electron micrographs. On the other hand, our samples showed none of the morphological alterations, specific for parotid glands, such as lipid accumulation, sialosis, reduction of acinar
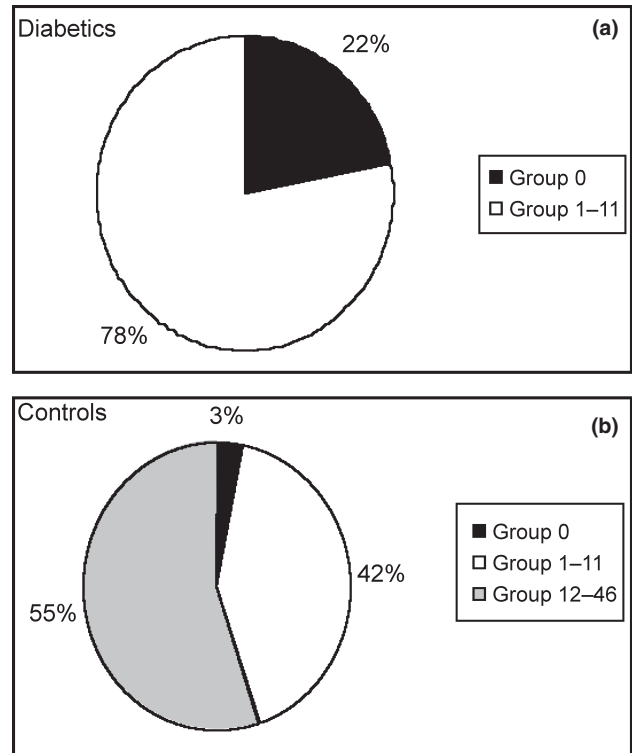

Figure 4 Proportions of submandibular secretory granules with no labeling (group 0), with 1–11 (group 1–11), and with 12–46 (group 12–46) gold particles. (**a**) Diabetic subjects; (**b**) control subjects

size (Baumann et al, 1985; Carda et al, 2005), probably reflecting the absence of xerostomia and other oral complications in the patients.

The reduced statherin immunoreactivity provides new evidence that salivary glands are affected by diabetes and is thus a further example of altered parameters in diabetic subjects (Hirsch, 2004; Venza et al, 2006; Rao et al, 2009; Rudney et al, 2009). The dry mouth symptoms are undoubtedly effects of a reduction of the salivary flow (Dodds et al, 2005; Sabino-Silva et al, 2009). Great attention has been devoted to the protein composition of diabetic saliva, since protein alterations are generally believed to allow bacteria, bacterial plaque and caries to progress rapidly, significantly hampering the quality of life. Although literature data are often conflicting, the total protein content seems to remain unvaried, while the relative amounts of the different proteins appear significantly changed (Dodds et al, 2000; Rao et al, 2009). For example, lactoferrin and sIgA were increased, while a decrease is noticed for ghrelin (Aydin, 2007), salivatin (Kimura et al, 2001), EGF and prolin-rich proteins (Kasayama et al, 1989; Szczepanski et al, 1998). Salivary amylase may also be affected (Baumann et al, 1985; Aydin, 2007; Mednieks et al, 2009). The decreased statherin reactivity shown here suggests that diabetes causes reduced statherin synthesis or changes in its intracellular processing and storage in submandibular glands. Unfortunately, at present the biochemical measurement of salivary statherin in diabetics has not been performed to support this

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assumption. When confirmed, statherin decrease will represent a further evidence that vulnerability of oral tissues may be partly dependent on a defective enamel pellicle, which poorly protect the teeth, and by an unbalanced calcium metabolism. Moreover, although a direct bactericidal activity of statherin has been still not demonstrated, its decrease could be partly responsible for the increased susceptibility to oral infections, since its ability to bind some bacterial species also would be reduced. Besides, the importance of this peptide in the maintenance of tooth integrity was clearly signified by the strong correlation between its high amounts and absence of dental caries (Vitorino et al, 2005).

Therefore, the present results could serve as a starting point for researches that will explain which metabolic signals determine salivary changes in the diabetic status and the relationship between the systemic disorders and the reduced salivary statherin.

Acknowledgments

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