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Expression Analysis of Human Salivary Glands by Laser Microdissection: Differences Between Submandibular and Labial Glands

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Key Words

Saliva • Salivary glands • Submandibular • TFF peptides • Mucins • FCGBP • Lysozyme • Histatin • Amylase • Statherin • Laser microdissection

Abstract

Both the major and minor salivary glands are the sources of saliva, a fluid vital for the maintenance of a healthy oral cavity. Here, the expression profiles of human submandibular (SMG) and labial glands (LG) were compared by RT-PCR analysis of laser microdissected mucous and serous cells, respectively. The focus was on trefoil factor family (TFF) genes, but also other genes encoding secretory proteins (mucins, lysozyme, amylase, statherin, and histatins) or aquaporin 5 were included. Immunofluorescence studies concerning TFF1-3, FCGBP, amylase, and lysozyme are also presented. It was shown that LGs clearly contain serous cells and that these cells differ in their expression profiles from serous SMG cells. Furthermore, all three TFF peptides, together with MUC5B, MUC7, MUC19, and FCGBP, were clearly detectable in mucous acini of both LGs and SMGs. In contrast, lysozyme was differentially expressed in LGs and SMGs. It can be expected that labial saliva may play a particularly important role for protecting the teeth.

Introduction

Saliva is a highly dilute, but complex body fluid vital for the maintenance of a healthy oral cavity [1]. Although mostly water (> 99%) it also contains electrolytes and other low-molecular-mass components, as well as a huge variety of proteins and peptides including mucins. Currently, >1600 different proteins have been identified in saliva, with fewer than 100 high abundance proteins [2-4]. The function of salivary proteins is manifold, including oral lubrication (influencing the rheology), oral predigestion and enhancement of taste, immunological barrier against microorganisms, and enhancement of wound healing. Whole saliva is a complex mixture of microbial products, mucosal exudate, gingival crevicular fluid, desquamated epithelial cells, and exocrine secretions of all the salivary glands [major glands with relatively long excretory ducts: sublingual (SLG), submandibular (SMG), parotid (PG); minor glands with short ducts: lingual, labial (LG), palatinal, buccal and molar] and probably also oral keratinocytes [1, 5]. Each day, about 1000-1500 mL sa-

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liva is secreted from the salivary glands. Percentage contributions of the different salivary glands during unstimulated flow are as follows: 20% from PGs, 65% from SMGs, about 8% from SLGs, and about 7% from the numerous minor glands [1].

The SLGs, SMGs and minor salivary glands are mixed glands, containing both mucous and serous cells, with SLGs and minor glands are considered to be predominantly mucous; whereas the PGs contains only serous secretory cells in their acini [6-9]. Minor salivary glands are found in the oral submucosa of the tongue, lower lip, palate, cheeks, and pharynx and are mixed with predominantly mucous appearance. The minor population of „seromucous" cells is progressively more frequent in palatine, posterior lingual, labial, anterior lingual, and buccal glands in that order [7, 8, 10]. Consequently, mucins are most abundant in minor and sublingual, less so in submandibular, and absent from parotid secretions.

The oral epithelium has a particularly high wound-healing capacity, i.e., rapid repair by cell migration [11]. Various motogenic peptides present in saliva have been shown to enhance this process, such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), trefoil factor family (TFF) peptides, and histatins [12-14]. Interestingly, EGF/TGF-α and TFF peptides even synergistically enhance cell migration processes (for review, see ref. [15]). Thus far, only TFF3, but not TFF1 or TFF2, was detectable in human saliva [5, 16]. Using immunohistochemitry, TFF3 peptide has been localized to serous and mucous cells of the SMGs, but with remarkable individual differences [5, 16], as well as mucous cells of SLGs [5, 17], ducts of PGs [5, 18], and in mucous cells of minor salivary glands [5]. Minor salivary glands are a particularly rich source for TFF3 [5]. On the mRNA level, TFF3 expression was detectable in SMGs, SLGs, PGs, and minor salivary glands [16, 19] and in situ hybridization localized TFF3 in mucous acini [19]. Low level TFF1 expression was detectable on the mRNA level in SMGs, SLGs, PGs, and minor salivary glands [16, 19] and low amounts of TFF1 peptide were localized in mucous acini of SLGs [17]. Occasionally, TFF2 expression was detectable in trace amounts on mRNA level only [16, 19].

It was the major aim of the study presented here to clarify finally the complex situation for TFF1-3 expression in SMGs and LGs and to compare the expression profiles of human SMGs and LGs, particularly by RT-PCR analysis of laser microdissected mucous and serous cells, respectively. Thus, the expression of TFF genes but also of other genes encoding typical secretory proteins of salivary glands [such as mucins (MUC), IgG Fc binding protein (FCGBP), amylase (AMY), lysozyme (LYZ), histatins (HTN), and statherin (STATH)] was investigated. Furthermore, a few genes encoding membrane proteins were also included in this study [such as aquaporin 5 (AQP5), the cystic fibrosis transmembrane conductance regulator (CFTR), and a selected claudin (CLDN)]. Generally, these genes were selected on the basis of their potential to act as differentiation markers for mucous and serous cells, respectively.

Materials and Methods

Human Tissue

All investigations followed the tenets of the Declaration of Helsinki and were approved by the Ethic Committee of the Medical Faculty at the University of Magdeburg. Informed consent was obtained from all individuals. Salivary gland specimens from non-pathological SMGs and inferior LGs were investigated from 17 patients (10 LG and 7 SMG specimens; age: 18-75 years).

Laser Microdissection, RT-PCR Analysis

OCT-embedded 7 µm serial cryosections of human SMG and LG specimens were microdissected with the SmartCut Microdissection System (Olympus Deutschland GmbH, Hamburg, Germany) as described in detail previously [20]. Parallel sections were used as "navigation slides" after PAS/AB staining in order to differentiate between mucous and serous cells. RNA was isolated using the Absolutely RNA Nanoprep Kit (Stratagene, Amsterdam, The Netherlands). The concentration and the purity of the RNA was estimated with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Peqlab Biotechnologie GmbH, Erlangen, Germany). cDNA synthesis was performed with 40 ng RNA primed with oligo$(dT)_{12\text{-}18}$ using SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. "Hot" Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) was used for PCR analysis. The relative expression level of 11 selected genes was monitored using the specific primers listed in Table 1.

Furthermore, RNA was also extracted from human biopsy specimens. RNA purification via CsCl ultracentrifugation and RT-PCR analysis using Hot Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) was as described previously [21]. The relative expression level of 16 selected genes was monitored using specific primers as listed in Table 1.

As a control for the integrity of the various cDNA preparations, transcripts for ß-actin were amplified in parallel reactions. The cDNA was also checked for contaminating chromosomal DNA by the use of intron spanning oligonucleotides.

Antisera

The various polyclonal anti-TFF antisera (anti-hTFF1-2, anti-hTFF2-1, anti-rTFF3-1) from rabbits employed in this study

Table 1. Oligonucleotides used for RT-PCR analysis and calculated size of the products. ACTB, β-actin; AMY, amylase; AQP, aquaporin; CFTR, cystic fibrosis transmembrane conductance regulator; CLDN, claudin; FCGBP, IgG Fc binding protein; HTN, histatin (HTN2 is also known as HIS2); LYZ, lysozyme; MUC, mucin; STATH, statherin; TFF, trefoil factor family; n.a. data not available. Cycles used for amplification shown in Fig. 1 (biopsy specimens)/Fig. 2 (laser microdissection).

Gene Acc.No. NCBI Data Base	Primer pairs (nucleotide position)	RT-PCR	Size (bp)	Intron spanning
TFF1 (NM_003225.2)	MD11 (21)TTTGGAGCAGAGAGGAGG (38) MD12 (458) TTGAGTAGTCAAAGTCAGAGCAG (436)	57°C, 35 cycles	438	yes
TFF2 (NM_005423.4)	MD13 (209)AGTGAGAAACCCTCCCCC(226) MD14 (574)AACACCCGGTGAGCCAG(558)	57°C, 35 cycles	366	yes
TFF3 (NM_003226.3)	MD9 (322)GTGCCAGCCAAGGACAG(338) MD10 (623)CGTTAAGACATCAGGCTCCAG(603)	57°C, 30/35 cycles	302	yes
MUC5B (XM_039877.6)	MB327 (2412)CTGCGAGACCGAGGTCAACATC(2433) MB328 (2826)TGGGCAGCAGGAGCACGGAG(2807)	57°C, 30 cycles	415	yes
MUC7 (NM_152291.1)	MB330 (136)CTTCTCGTTCAGTGAAGGTCG(156) MB331 (313)TGGAAGCTTAGGCCTACAGC(294)	57°C, 30 cycles	178	yes
MUC19 (AY236870.1)	MB473 (1878)GAGTTCAGATGGCAAAATGCACA(1900) MB440 (2021)TGCCATCAGGACAGTCAAGTACA(1999)	57°C, 30 cycles	144	n.a.
FCGBP (NM_003890.2)	MB1514a (15384)CACTACTCCATCTGCACTCG(15403) MB1515a (15908)ATCCTGGAAGAAGATGTGGA(15889)	57°C, 30 cycles	525	yes
LYZ (NM_000239.1)	MB916a (115)GAAAAGATTGGGAATGGATG(134) MB917 (447)TGACGGACATCTCTGTTTTG(428)	57°C, 30/35 cycles	333	yes
AMY1A (NM_004038.3)	MB922 (328)GTTCTGCTGGGCTCAGTATT(347) MB923 (724)TGCTGGAAAGTCCCTACTTC(705)	57°C, 30 cycles	397	yes
AQP5 (NM_001651.1)	MB920 (922)AGCTGATTCTGACCTTCCAG(941) MB921 (1263)AGTCCTCGTCAGGCTCATAC(1244)	57°C, 30 cycles	342	yes
CFTR (NM_000492.3)	MB559 (5147)GACTGCACATCAAAATATGCC(5167) MB560 (5491)ATCTACTTACACACCCTCTACC(5470)	57°C, 30 cycles	345	no
STATH (NM_003154.2)	MB918 (119)CCTTGTCTTTGCCTTCATCT(138) MB919 (440)TGTTTTTCATTTGCATTGCT(421)	57°C, 30/35 cycles	322	yes
HTN1 (NM_002159.2)	MB926 (197)CCATTTTATGGGGACTATGG(216) MB927 (523)ATTGCTTTTGGAGAGGAATG(504)	57°C, 30/35 cycles	327	yes
HTN2 (M26665.1)	MB924 (125)AGCTGATTCACATGCAAAGA(144) MB925 (508)ATTGCTTTTGGAGAGGAATG(489)	57°C, 30/35 cycles	384	n.a.
HTN3 (NM_000200.1)	MB928 (124)AGCTGATTCACATGCAAAGA(144) MB929 (500)ATTGCTTTTGGAGAGGAATG(481)	57°C, 30/35 cycles	384	yes
CLDN4 (NM_001305.3)	MB1536 (196)TCAGGACTGGCTTTATCTCC(215) MB1537 (506)ACCACGCAGTTCATCCATA(488)	57°C, 30/35 cycles	311	no
ACTB (NM_001101.3)	MB344 (880)TTCCTGGGCATGGAGTCCT(898) MB345 (1080)AGGAGGAGCAATGATCTTGATC(1062)	57°C, 30 cycles	204	yes

were described previously [21-23]. Furthermore, commercial polyclonal rabbit antisera against human lysozyme (A 0099, DakoCytomation, Glostrup, Denmark), human amylase (A-8273, Sigma-Aldrich Chemie, Taufkirchen, Germany), or human FCGBP (HPA003564, Sigma-Aldrich Chemie, Taufkirchen, Germany) were used. The secondary antibody was Cy3-labeled anti-rabbit IgG/F(ab)$_2$ fragment (C-2306, Sigma-Aldrich Chemie, Taufkirchen, Germany).

General Histology and Immunofluorescence

The preparation of human tissue samples, which were embedded in Technovit 7100, and immunofluorescence on 2-µm-thick sections were performed as described previously in detail [21]. Mucins were stained with a combination of Alcian blue 8GX at pH 2.5 and the PAS reaction as already described [24]. Nuclei were counterstained with Meyer's hematoxylin.

The specificity of the TFF2 and the TFF3 staining was tested by competition with the corresponding peptides, i.e., 1 mL of anti-hTFF2-1 or anti-rTFF3-1 (1:500 dilution), respectively, was pre-adsorbed with 4 µg synthetic peptide FFPNSVEDCHY (representing the C-terminus of human TFF2) or FKPLQETECTF (representing the C-terminus of human TFF3), respectively, at 4°C overnight and then used for immunofluorescence. Photographs were taken on an Axiophot microscope (Carl Zeiss Jena, Jena, Germany) as described previously [21].

Results

RT-PCR Analysis of Salivary Glands

Specimens of the LGs and SMGs, respectively, were analyzed for their expression profiles concerning the following genes: TFF1-3, FCGBP, MUC5B, MUC7, MUC19, LYZ, AMY1a, AQP5, CFTR, STATH, HTN1-3, and CLDN4 (Fig. 1). Most of the genes were expressed in comparable amounts in both the LGs and SMGs with exception of CFTR, STATH, HTN1-3 which were enriched in SMGs.

Laser Microdissection and RT-PCR Analysis

As the next step, cells from mucous and serous acini, respectively, were isolated by laser microdissection from both LGs and SMGs. Mucous acini were recognized by their opalescent appearance (see also Fig. 3) and strongly stained with PAS and/or Alcian blue. Mucous and serous cell populations were analyzed for their expression of the following genes: TFF3, MUC5B, MUC7, MUC19, AMY1a, AQP5, STATH, and HTN1-3 (Fig. 2). The expression pattern of these genes was very different. In Fig. 2, the genes analyzed were arranged according to their distinct expression profiles. TFF3, MUC5B, MUC7, and MUC19 were mainly expressed in mucous cells; whereas AMY1a, AQP5, STATH, and HTN1-3 transcripts were enriched in serous cells. In contrast, lysozyme expression was observed in both mucous and serous cells. Furthermore, the expression profiles of some genes differ when LGs and SMGs were compared. These differences were most pronounced for lysozyme (which was mainly expressed in mucous SMG cells and serous LG cells), but were also detectable for HTN1, HTN3, and to lower extent also for STATH and HTN2 (which were more expressed in serous SMG cells when compared with serous LG cells).

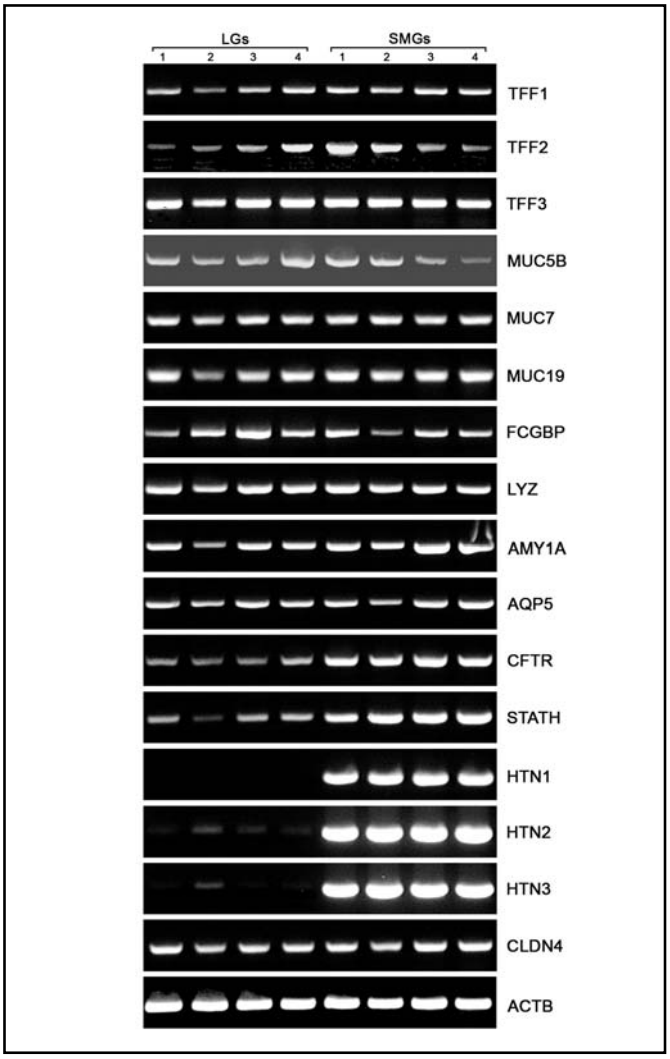

Fig. 1. RT-PCR analysis. Gene expression concerning TFF1-3, MUB5B, MUC7, MUC19, FCGBP, LYZ, AMY1a, AQP5, CFTR, STATH, HTN1-3, and CLDN4 was monitored in surgical specimens from the labial glands (LGs) and the submandibular glands (SMGs). Each set of specimens was investigated from four different individuals. The integrity of the cDNAs was tested by monitoring the transcripts for β-actin (ACTB). See the legend of Table 1 for a list of gene abbreviations.

Immunofluorescence Studies

The cellular localization of selected gene products in LGs and SMGs was determined by the use of immunohistochemistry. Focus was on TFF peptides, but FCGBP, amylase, and lysozyme were also included in this study (Fig. 3). TFF1-3, and FCGBP were present in mucous acini of both LGs and SMGs, which can easily be recognized on phase contrast pictures by their opalescent appearance. TFF3 was additionally localized in serous cells of SMGs. In contrast, amylase was localized in serous acini. Lysozyme showed a complex expression pattern. In LGs it was detected mainly in

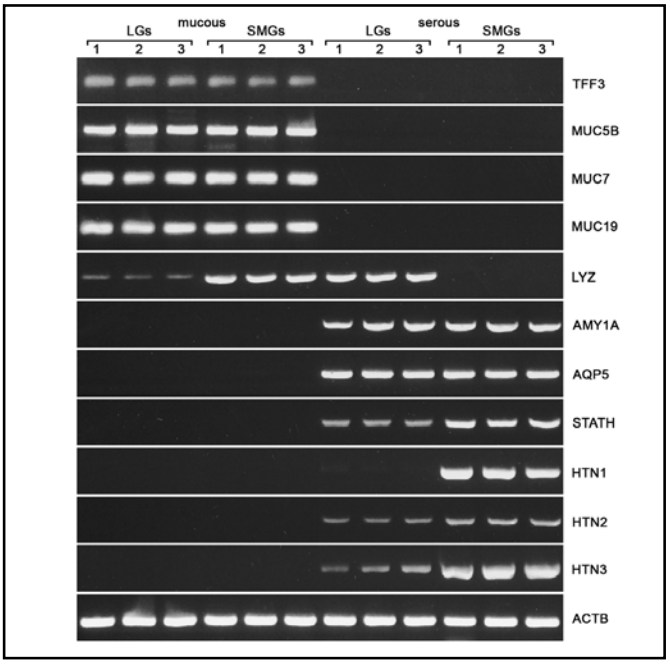

Fig. 2. RT-PCR analysis. Gene expression concerning TFF3, MUB5B, MUC7, MUC19, LYZ, AMY1a, AQP5, STATH, and HTN1-3 was monitored in mucous and serous cells isolated by laser microdissection from the labial glands (LGs) and the submandibular glands (SMGs), respectively. Each set of specimens was investigated from three different individuals. The integrity of the cDNAs was tested by monitoring the transcripts for β-actin (ACTB). See the legend of Table 1 for a list of gene abbreviations.

serous cells and only little in mucous acini; whereas in SMGs lysozyme was present only in mucous acini.

The specificity of the TFF2 and TFF3 immunostaining was confirmed by competition with corresponding synthetic peptides representing the C-terminus of human TFF2 or TFF3, respectively.

Discussion

Laser Microdissection is Very Reliable

Here, specific isolation of serous and mucous cells from LGs and SMGs with the help of laser microdissection is described for the first time. Amylase is a well known marker typical of serous acinar cells [25] (see also Fig. 3); whereas expression of the mucin MUC5B was used as a marker for mucous cells of the salivary glands [26]. As documented in Fig. 2, mucous cells, but not the serous cells, isolated from LGs and SMGs by laser microdissection clearly express MUC5B transcripts. In contrast, AMY1 transcripts were detectable in LGs and SMGs in serous cells only (Fig. 2). These results clearly demonstrate that the protocol

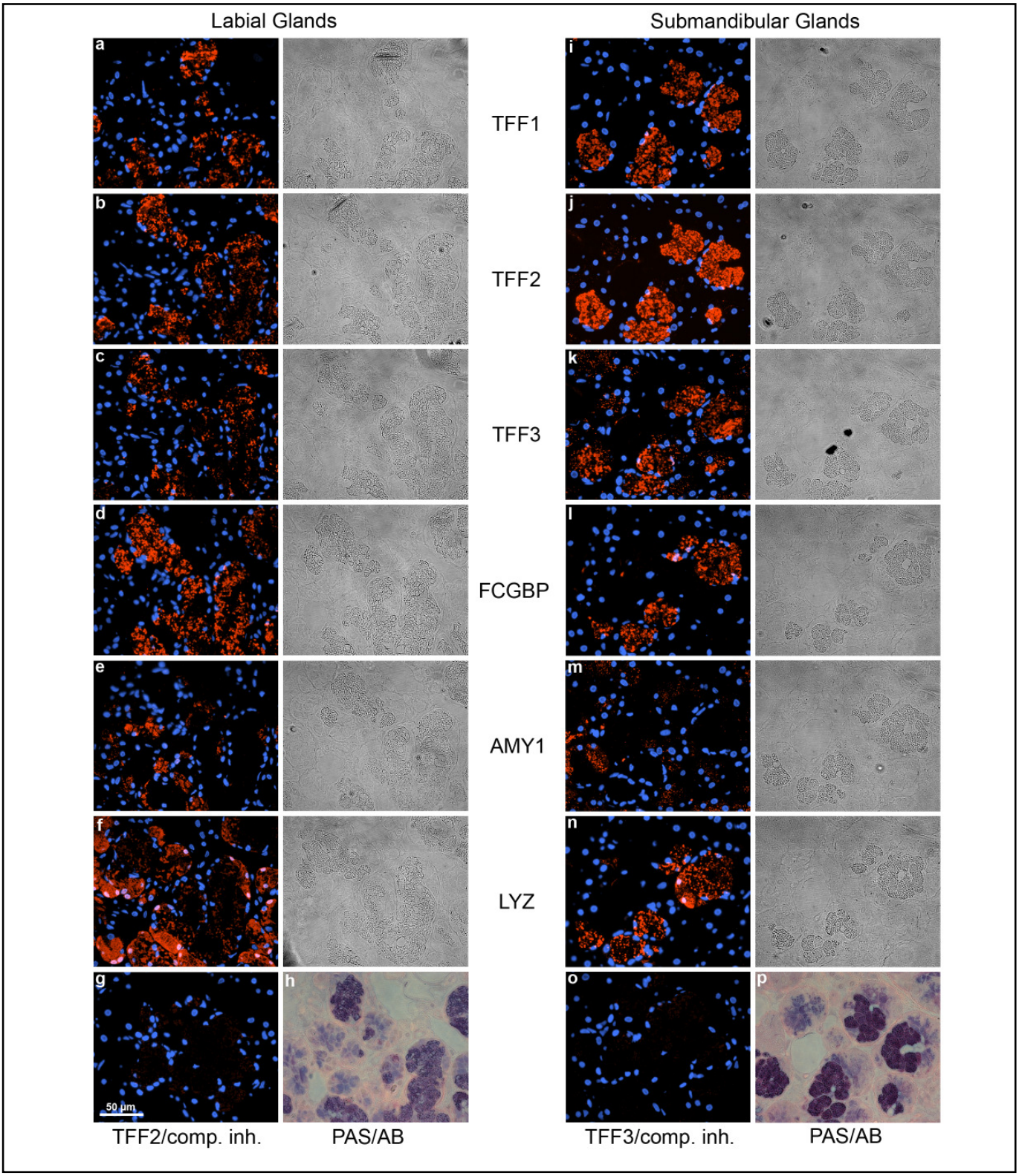

Fig. 3. Localization of TFF1, TFF2, TFF3, FCGBP, amylase (AMY), and lysozyme (LYZ) in human labial and submandibular glands. (a-f, i-n) Immunofluorescence of parallel sections with the corresponding polyclonal antisera; secondary antibody was anti-rabbit IgG/F(ab)$_2$ fragment-Cy3 (*red*); nuclei counterstained with 4,6-diamidino-2-phenylindole (DAPI, *blue*). Shown are also the corresponding phase contrast pictures (on the right). (g) Immunofluorescence of parallel section with the anti-hTFF2-1 antiserum after competition with the synthetic peptide FFPNSVEDCHY. (o) Immunofluorescence of parallel section with the anti-rTFF3-1 antiserum after competition with the synthetic peptide FKPLQETECTF. (h, p) Staining of parallel sections with PAS/Alcian blue. Bar: 50 µm. See the legend of Table 1 for a list of gene abbreviations.

used for laser microdissection resulted in pure serous and mucous cell populations, respectively. This is further confirmed by the clear serous expression pattern of AQP5, STATH, HTN1-3, which is in agreement with previous reports [27-29].

The protocol used here for laser microdissection and RT-PCR analysis was nicely applicable for the study of genes which are expressed relatively strongly. Unfortunately, we could not demonstrate expression of the somewhat weaker expressed genes, such as TFF1, TFF2, FCGBP, CFTR, and CLDN 4 in laser microdissected material. In contrast, expression of these genes was clearly detectable by RT-PCR analysis in extracts of LG and SMG specimens, respectively (see Fig. 1). However, manifold interesting results were obtained with laser microdissection in spite of its somewhat limited sensitivity.

Serous Cells from Labial and Submandibular Glands Differ in Their Expression Profiles

When extracts of LGs and SMGs were compared (Fig. 1), the expression level of genes encoding serous cell products (such as STATH, HTN1-3, and CFTR) was clearly higher in SMGs, with the most pronounced difference found for histatins. One reason for that is certainly, that LGs are considered to be predominantly mucous glands with only little serous content; whereas SMGs are typically mixed glands with much higher content of serous cells [8, 10]. However, when serous cells from LGs and SMGs were compared directly after laser microdissection (Fig. 2) it became clear that these populations differed considerably. Particularly HTN1 and HTN3 expression was highly enhanced in SMGs when compared with LGs; whereas HTN2, AMY, AQP5 expression was roughly comparable in both LGs and SMGs.

Furthermore, serous cells of LGs show high lysozyme expression, which was also confirmed by immunohistochemistry; whereas lysozyme expression was not detectable in serous cells of SMGs (Figs. 2 and 3).

Serous cells from LGs and SMGs also differed in their TFF3 expression pattern. We could detect TFF3 peptide in serous cells from SMGs only but not those of LGs (Fig. 3). The specificity of the TFF3 staining was shown by competitive inhibition with the corresponding peptide (Fig. 3). However, we were not able to confirm this result by RT-PCR analysis of laser microdissected material because of the limited sensitivity of this method, i.e., TFF3 transcripts were not detectable in serous cells of SMGs (Fig. 2). The expression of TFF3 in serous cells of SMGs is in complete agreement with a previous publication [16]. However, there seem to be individual differences. The synthesis of TFF3 in serous cells is remarkable, because TFF3 is mostly known for its co-localization with various mucins depending of the cell type (for review, see ref. [30]). However, there are also a few reports demonstrating TFF3 synthesis in non-mucous cells, such as hypothalamic neurons [22], oral keratinocytes [5], and articular cartilage from patients with osteoarthritis [31].

Taken together, it is shown here that LGs clearly contain a population of non-mucous cells that contribute significantly to the secretion of LGs, e.g., by expressing amylase, lysozyme, AQP5, statherin, HTN2, and HTN3 (Figs. 2 and 3). Thus, this cell population shows typical serous characteristics. In the past, this has often been neglected and there are only scarce reports on such products [32-34]. LGs secrete saliva in close proximity to the tooth surfaces and the flow rate has been associated with the incidence of dental caries [35]. Consequently, is has been stated that minor salivary glands are the most important because of their protective components [1].

TFF1 and TFF2 are Secretory Products of Both Labial and Submandibular Salivary Glands

Here, TFF1 and TFF2 peptide synthesis has been demonstrated for the first time to occur in mucous acini of LGs and SMGs (Fig. 3). This completes the picture showing TFF1 peptide localized in mucous acini of SLGs [17] and is in agreement with previous studies demonstrating TFF1 and rarely also TFF2 transcripts in SMGs and minor salivary glands [16, 19]. Thus, both TFF1 and TFF2 have to be considered as low abundant constituents of human saliva and they are expected to play an important role in the high regenerative capacity of the oral epithelium.

Lysozyme is Differentially Expressed in Labial and Submandibular Salivary Glands

One of the major differences between LGs and SMGs concerns the expression of lysozyme, which is confined to mucous acini in SMGs. In contrast, in LGs lysozyme expression was detectable mainly in serous cells and only in low amounts in mucous cells. This has been shown on both the RNA and protein level (Figs. 2 and 3). Thus, lysozyme expression changes from a mucous localization in SMGs (which is comparable with lysozyme expression in gastric gland mucous cells, i.e., mucous neck cells and antral gland cells; [36]) to mainly a serous localization in

LGs (which could be compared to lysozyme expression in Paneth cells; [36]). The reason for this switch is not currently understood, but it is expected to be correlated with the well known antibacterial property of lysozyme, which could be of special importance for protection of the teeth by the LG saliva.

MUC7, MUC19, and FCGBP are Predominantly Expressed in Mucous Acini of Both Labial and Submandibular Salivary Glands

RT-PCR analysis of laser microdissected material clearly revealed that both MUC7 and MUC19 are typically expressed in mucous acini (Fig. 2), which is expected for mucins. However, both MUC7 and MUC19 are somewhat unusual. MUC19 encodes probably a gel-forming mucin highly expressed in SMGs [37] which could not be detected in human saliva thus far [38]. MUC7 represents a low molecular weight mucin with antimicrobial activity, which interacts with many saliva components, such as amylase, statherin, and histatin-1 [39]. The site of MUC7 synthesis is controversial. One group localized MUC7 to serous acini in SMGs and LGs using immunohistochemistry and *in situ* hybridization [40, 41]; whereas two other groups clearly defined mucous acini as the expression sites using the same techniques [42, 43]. Furthermore, electron microscopic immunogold localization detected MUC7 in both serous and mucous cells with varying intensities [44]. Thus, the results presented here (Fig. 2) are in agreement at least with the results from two different groups [42, 43].

Furthermore, the protein FCGBP was also localized by the use of immunohistochemistry to mucous acini of both LGs and SMGs (Fig. 3). FCGBP is a cysteine-rich, repetitive, high molecular weight protein known as an integral constituent of intestinal mucus [45], which has previously also been detected in the SMGs and the saliva [4, 46]. Of special note, human intestinal FCGBP has recently been shown to form disulfide-linked heteromers with TFF3 [47].

Conclusions

Taken together, the results presented indicate that all three TFF peptides can be expected to contribute to the health of the oral cavity. Surprisingly, serous cells from LGs and SMGs differ in their expression profiles. Future investigations particularly of the labial glands seem promising in order to gain more insights into the mechanisms as to how the saliva is protecting the teeth.

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