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Diversity of mucins in labial glands of infants

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Summary. Mucins as highly glycosylated proteins comprise multiple functions like protection, homeostasis, immune defense, cell signaling. Various epithelial tissues including glandular structures express different specific mucin types. We investigated labial salivary glands in infants for the occurrence of MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC7 by immunohistochemistry. MUC1 and MUC4 were detected in serous and ductal glandular cells, partially intensified at the apical plasma membrane. MUC3 was found in ductal glandular cells and in myoepithelial cells. MUC5B exhibited a mosaic expression pattern in mucous glandular endpieces. MUC2 and MUC7 were abundant in serous acini. Glandular structures were negative for MUC5AC. A comprehensive study of specific mucins in labial salivary glands of infants was presented for the first time. As a representative of the minor salivary glands, labial glands are, due to their localization, directly exposed to environmental influences. The distribution of a broad spectrum of mucins in infantile labial glands indicates their importance early in human development to sustain oral health.

Key words: Labial gland, Mucins, MUC, Salivary glycoproteins, Immunohistochemistry

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Introduction

The mucin family comprises a heterogeneous group of at least 20 high-molecular weight glycoproteins. Mucins are categorized in two groups according to their structural and functional characteristics, secreted and membrane bound (cell surface) mucins (Mahomed, 2011). Transmembrane glycoproteins comprise, for instance, MUC1, MUC3 and MUC4. Typical secretory mucins are, e.g., MUC2, MUC5AC, MUC5B, MUC7 (Offner and Troxler, 2000; Liu et al., 2002). In general, mucins protect mucosal surfaces against pathogens and provide a barrier against dehydration (Tabak, 1995). Transmembrane mucins at the plasma membrane show also signaling functions (vanPutten and Strijbis, 2017). Salivary mucins include 20% of the total salivary proteins and are produced by sublingual, submandibular, and minor salivary glands (Takehara et al., 2013). By forming complexes with antimicrobial proteins of the saliva, mucins help to enhance antimicrobial activity (Soares et al., 2004). Mucins of the saliva reduce the adhesion of bacteria in the oral cavity, e.g., MUC7 seems to be involved in interactions between neutrophils and bacteria (Prakobphol et al., 1999). Major salivary mucins are represented by MUC5B and MUC7. MUC5B prevails in mucous acinar cells, while MUC7 predominates in serous acinar cells (Nielsen et al., 1996; Piras et al., 2010). MUC1 and MUC4 are membrane-associated mucins in parotid and submandibular glands and might play a role in signaling pathways for a normal cellular function (Liu et al., 2002). The secretory MUC2 (gel forming mucin) is characteristic for goblet cells in the gastrointestinal and respiratory tract (Mannweiler et

al., 2003). Salivary glandular cells are weakly positive for MUC2 in ductal cells only (Ho et al., 1993; Alos et al., 2005). MUC5AC is a typical gastric mucin and is highly expressed in bronchial epithelium and submucosal glands (Ermund et al., 2017; Giraldi et al., 2018). Alos et al. (2005) found a focally positive staining for MUC5AC-immunoreactivity in ductal cells of salivary glands. Moreover, MUC3, predominantly expressed in the gastrointestinal tract (Cao et al., 1997), was existent in the duct system and in myoepithelial cells of minor salivary glands (Teshima et al., 2011).

Mucins are involved in cancer development and invasion. Cancer cells make use of mucins for their differentiation and proliferation. Aberrant mucin overexpression and glycosylation in cancer cells support invasive and metastatic events (Bhatia et al., 2019). The expression of various mucins in salivary gland mucoepidermoid carcinoma and other tumor-types offers valuable clues for diagnostic and prognostic implications (Mahomed, 2011; Siyi et al., 2014; Ma et al., 2019).

Labial salivary glands located in the mucosa of the upper and lower lip belong to the minor salivary glands. Predominantly of mucous character, these glands are structured like the major salivary glands with some small differences in the ductal system. Mucous and serous endpieces secrete into intralobular ducts passing over into the main excretory duct (Hand et al., 1999). In the absence of local stimuli, minor salivary glands account for the permanent flow of saliva, necessary for the maintenance of oral health (Sonesson et al., 2011; Stoeckelhuber et al., 2016).

Mucin studies of minor salivary glands, especially labial glands, are rare. Comprehensive immunohistological investigations on the occurrence of mucins in infantile labial glands were reported for the first time. The purpose of this study is to provide new insights in mucin distribution of labial infantile glands and therefore increase the knowledge of mucin diversity at this location.

Material and methods

Tissue

All collected specimens are derived from infants born with cleft lips. Excess tissue of the upper lip was obtained during cleft lip repair surgery and fixed immediately in 4% buffered formalin. The material was dehydrated and embedded in paraffin. We received tissue from 29 patients, 17 males and 12 females. The age range was 2-9 months, one child was 3 years old. The study was performed according to the guidelines of the local ethics committee and the Helsinki Declaration.

PAS-reaction and Alcian-blue staining

For differentiation of serous and mucous endpieces, sections were treated with Periodic acid-Schiff (PAS) reagent for neutral carbohydrates (counterstain hematoxylin) and with Alcian-blue (pH 2.5) for polyanions (counterstain nuclear fast red) according to Romeis (2010).

Immunohistochemical staining

Sections (5 μm) were prepared from paraffin-embedded specimens and dewaxed. The avidin-biotin-horseradish peroxidase method was applied using the following mucin antibodies: MUC1 (1:200, Abcam, Cambridge, UK), MUC2 (1:800, Abcam, Cambridge, UK), MUC3 (1:100, Invitrogen, Karlsruhe, Germany), MUC4 (1:400, Proteintech, Manchester, UK), MUC5AC (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), MUC5B (1:300, Abcam, Cambridge, UK), MUC7 (1:500, Abcam, Cambridge, UK). For antigen retrieval, sections were treated with microwave irradiation in citrate buffer at pH 6.0 or by water-bath heating in Tris/EDTA-buffer, pH 9.0 (Dako, Glostrup, Denmark). Endogeneous peroxidase activity was quenched by incubation with 3% hydrogen peroxide (10 min). For unspecific blocking, sections were incubated with 3% normal goat serum (Vector Laboratories, Burlingame, USA) for 30 min. Subsequently, the primary antibody was applied for 1h at room temperature and overnight at 4°C. The second biotin-labeled antibody was used in a 1:200-dilution (Vector Laboratories, Burlingame, USA). Then, slides were incubated with peroxidase-labeled streptavidin (45 min) and the enzyme reaction was visualized by diaminobenzidine (Vector Laboratories, Burlingame, USA). A brief nuclear counterstaining followed in hematoxylin. Controls were performed by replacement of the primary antibody with buffer. An overview of antibodies and staining procedures is listed in Table 1. Sections were studied with a Nikon

Table 1. Primary antibodies.

antibody	source	clone	dilution	antigen retrieval
MUC1	Abcam (ab 109185)	EPR1023	1:200	Tris/EDTA buffer, pH 9.0
MUC2	Abcam (ab134119)	EPR6145	1:800	citrate buffer, pH 6.0
MUC3	Invitrogen	1143/B7	1:100	citrate buffer, pH 6.0
MUC4	Proteintech	polyclonal	1:400	Tris/EDTA buffer, pH 9.0
MUC5AC	Santa Cruz Biotechn.	CLH2	1:200	citrate buffer, pH 6.0
MUC5B	Abcam (ab77995)	19,4E	1:300	citrate buffer, pH 6.0
MUC7	Abcam (ab224342)	polyclonal	1:500	citrate buffer, pH 6.0

microscope and images were recorded by a digital Nikon camera (Nikon, Duesseldorf, Germany). Labeling intensity was categorized as weak, medium, and strong.

Results

PAS and Alcian blue

In labial glands of the mucosal lamina propria, the mucous character predominates visualized by Alcian blue-staining and positive PAS-reaction. Serous acinar cells frequently form demilunes capping mucous endpieces. Occasionally, the secretory product of serous cells in the lumen appeared positive with PAS- and Alcian-blue. Serous and mucous glandular cells secret in a duct-system consisting of intralobular ducts passing over into a main excretory duct (Fig. 1A,B).

MUC1

MUC1-antibody-incubation resulted in a strong reaction at the apical plasma membrane in intralobular and main excretory ducts. The Golgi-apparatus in ductal cells produced MUC1-signals in all cell layers. The cytoplasm of ductal cells showed a weak to medium intense staining. Serous and mucous endpieces exhibited different staining results. While in serous glandular cells MUC1 was expressed in the apical plasma membrane in a medium to strong intensity, mucous endpieces remained unstained (Fig. 2A).

MUC2

Staining appearance with the anti-MUC2 antibody was heterogeneous (Fig. 2B). The staining pattern of serous cells ranged from medium to strong intensity. Some mucous cells were MUC2-positive. The duct system, both intralobular and main excretory ducts, was characterized by an inconsistent staining. In most cases, the apical ductal cell layer was stained positive at the plasma membrane, the cytoplasm was either unstained or weakly stained, just like the lower ductal cell layer. Occasionally, ducts reveiled an overall medium to strong staining in all cell layers with an accentuation at the apical plasma membrane.

MUC3

MUC3-expression was evident in myoepithelial cells lining serous and mucous endpieces. The cytoplasm of ductal cells was stained positive. Staining intensity ranged from weak to medium in all positive structures (Fig. 2C). All blood vessels including capillaries were marked with the antibody to MUC3.

MUC4

MUC4-protein was found in serous cells, both in the cytoplasm and partially in the plasma membrane with a medium to strong staining intensity. Mucous cells showed a negative antibody reaction. Ducts were positive for MUC4 in all cell layers, occasionally apically intensified (Fig. 2D).

MUC5AC

MUC5AC was negative in all glandular structures (Fig. 2E).

MUC5B

Antibody to MUC5B revealed inconsistent protein presence in mucous cells. 6 specimens were positive, 23 were negative. The percentage of positively stained

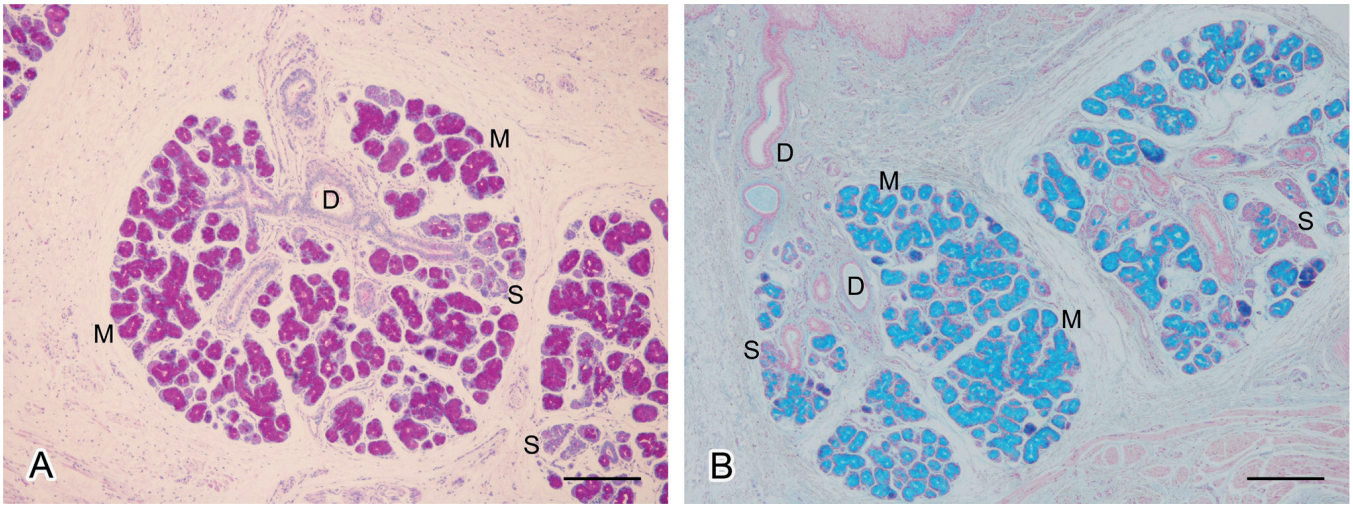

Fig. 1. A. Periodic acid-Schiff (PAS) reaction. **B.** Alcian blue stain. S, serous cells; M mucous cell; D, duct. Sacle bars: 250 μm.

mucous cells varied from 5 to 50 % of the sectioned glandular tissue. The mosaic staining pattern showed uniformly stained mucous endpieces alternating with mucous tubules where positive and negative cells are located side by side. Serous cells were devoid of staining, although sporadically a faint staining at the apical membrane could be observed. Ducts exhibited a weak staining at the apical plasma membrane of the upper cell layer (Fig. 2F).

MUC7

Serous cells displayed a uniform strong staining of MUC7 in a granular form. Mucous cells were unstained. Ductal staining occurred at the apical plasma membrane of the upper cell layer, the intensity of staining ranged from weak to strong (Fig. 2G).

Discussion

We investigated different mucin-types in infantile labial glands. To date, a comprehensive study of mucin-distribution in infantile labial glands is lacking. Investigations examined minor salivary glands from other oral locations (Teshima et al., 2011) or no further specification of the glandular type (Alos et al., 2005) was given. So far, research on minor or specific labial glands mostly describes few specimens, sometimes as controls in the context of pathological glandular structures (Piras et al., 2010; Alos et al., 2005).

The major salivary mucins are MUC5B and MUC7, also designated as high-molecular-mass or mucous glycoprotein 1 (MG1) and lower-molecular-mass mucous glycoprotein 2 (MG2) (Mahomed, 2011). MUC5B is relevant for homeostasis regulation of the oral environment and serves as a gel-forming mucin for retention of anti-microbial proteins to protect the oral mucosa (Wickström et al., 2000). MUC5B occurs in different glycoforms inside the same salivary gland (Veerman et al., 1992; Thornton et al., 1999). This might be the reason for the absence or mosaic staining pattern in mucous endpieces where positive as well as negative-stained mucous cells appeared within the same tubular endpiece. The antibody to MUC5B recognizes an unglycosylated synthetic peptide which might be shielded by the highly glycosylated MUC5B regions. For this reason, various antibody reactions to glycosylated mucins were discussed in other studies (Wickström et al., 1998; Biemer-Hüttmann et al., 1999). There are also discrepancies in the literature about MUC5B expression in glandular location. In a study of Teshima et al. (2011), developing prenatal minor salivary glands and adult glands exhibit no MUC5B in acinar cells, but do so in ductal cells. On the other hand, using the same methodological approach, an intensive MUC5B-cytoplasm staining could be viewed in mucous cells of minor salivary glands, but not in ductal cells (Alos et al., 2005). In our study, MUC7 was expressed in serous acinar cells as was confirmed by Nielsen et al. (1996, 1997) using immunohistochemistry and in situ hybridization. Other authors found MUC7-staining additionally in mucous cells of human submandibular and sublingual glands with immunogold labeled antibodies (Piludu et al., 2003). The fact that MUC7 is able to self-associate leads to the formation of large constructs that helps to agglutinate harmful substances in the oral cavity (Mehrotra et al., 1998). MUC1 is a monomeric membrane-associated mucin present in buccal epithelial cells, major and minor salivary glands (Liu et al., 2002; Hori et al., 2007), but also in the membrane of many epithelial lined organs (Gipson and Inatomi, 1998). We are in accordance with Mannweiler et al. (2003) who identified MUC1 in the apical plasma membrane of serous acini and in the excretory ducts of major salivary glands. Ductal staining in minor salivary glands was demonstrated in a further study (Sengupta et al., 2001), even though no serous acini were stained therein. MUC2 belongs to the secretory mucins and is specifically expressed in intestinal goblet cells, whereas MUC5AC was typical for gastric goblet cells (Pelaseyed et al., 2014). In salivary glands, MUC2 was detected in the excretory duct system (Mannweiler et al., 2003; Alos et al., 2005), another study showed no reactivity to MUC2 (Teshima et al., 2011). We observed MUC2-expression in ducts and serous acini, occasionally in mucous endpieces of the labial glands. Discrepancies might be due to the fact that in previous investigations, minor salivary glands are not specified further or do not include labial glands. So, MUC2 in labial glands may have a similar function as in the intestinal tract by representing the first front of defense at this exposed position. MUC2 stimulates ß-defensin-2 expression in the colonic mucosa (Cobo et al., 2015). ß-Defensin-2 was detected in infantile labial glands in a previous study (Stoeckelhuber et al., 2016). MUC5AC showed negative staining in all glandular structures. An antibody to MUC4 demonstrated immunoreactive protein in serous cells enhanced at the plasma membrane and in ductal cells as reported for major salivary glands and minor salivary glands (Liu et al., 2002; Alos et al., 2005). MUC4 acts as an intramembrane ligand for ERBB2 inducing intracellular processes that result in repression of apoptosis and stimulation of proliferation (Karg et al.,

Table 2. Mucin-expression in infantile labial glands.

Mucin	serous cells	mucous cells	ductal cells
MUC1	++ / +++ apical	-	+++ apical
MUC2	++/+++	- ~ ++	+++ apical
MUC3	-/myoepithelial cells ++	-/myoepithelial cells ++	+/++
MUC4	++/+++ apical	-	+++
MUC5A	-	-	-
MUC5B	+ apical	mosaic +++	+ apical
MUC7	+++	-	+~+++ apical

grading: - negative, + weak, ++ medium, +++ strong staining.

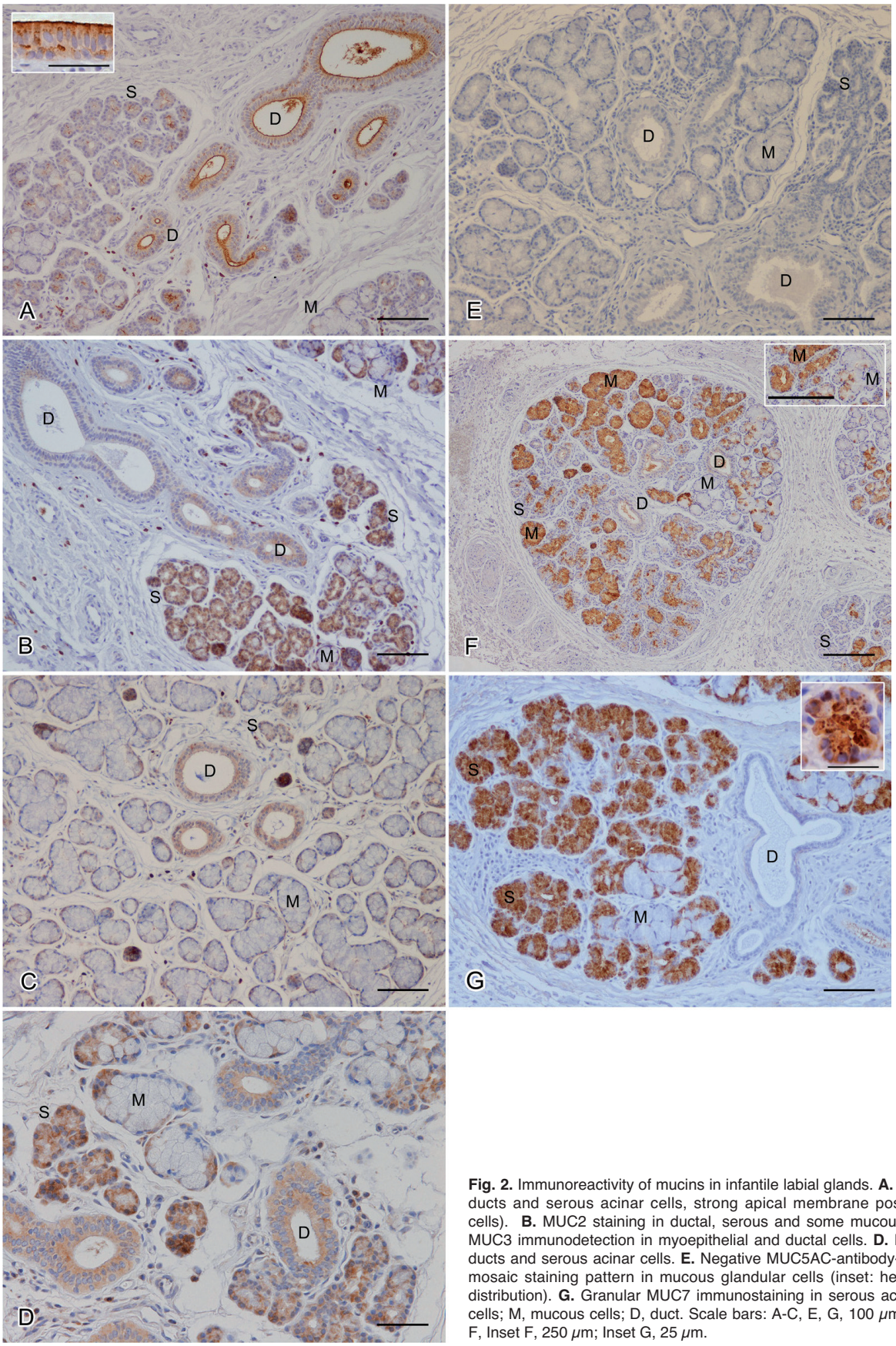

Fig. 2. Immunoreactivity of mucins in infantile labial glands. **A.** MUC1 expression in ducts and serous acinar cells, strong apical membrane positivity (inset: ductal cells). **B.** MUC2 staining in ductal, serous and some mucous glandular cells. **C.** MUC3 immunodetection in myoepithelial and ductal cells. **D.** MUC4-localization in ducts and serous acinar cells. **E.** Negative MUC5AC-antibody-reaction. **F.** MUC5B mosaic staining pattern in mucous glandular cells (inset: heterogenous MUC5B distribution). **G.** Granular MUC7 immunostaining in serous acinar cells. S, serous cells; M, mucous cells; D, duct. Scale bars: A-C, E, G, 100 μm; D, Inset A, 50 μm; F, Inset F, 250 μm; Inset G, 25 μm.

2006). This is the reason for serving as a prognostic factor for carcinomas of extrahepatic bile duct or ductal carcinoma of the pancreas (Saitou et al., 2005; Tamada et al., 2006). Mature minor salivary glands demonstrated an immunoreactive MUC3 glycoprotein in ductal cells and myoepithelial cells (Teshima et al., 2011). Identical results were found in our study.

The existence of mucous material in serous glandular cells seems to be contradictory. Tandler et al. (1969) came up with the hypothesis that the "serous" looking acini in labial glands are in fact mucous cells. In electron microscopy studies, he found only mucous droplets in serous acini showing variable electron density. He speculated that the differences may illustrate various stages of a cellular secretory cycle leading to more or less mature mucous cells. Putative serous acini represent, therefore, mucous depleted cells at the beginning of their secretory cycle. Looking at the PAS and Alcian-blue stained serous endpieces in our study, there seems to be a transition of serous and mucous cells within one endpiece supporting this hypothesis. Eversole (1972) and Harrison (1974) investigated the nature of mucous granules in minor salivary glands and discussed a coherence of maturation of granules with high sulphation. It is evident that sulphation of molecules can effect the immunoreactivity (Rehfeld et al., 1981). Thus, leading to the speculation that different mature degrees of mucous granules might result in a variable antibody reaction.

Age and gender displayed no influence on the secretory volume of unstimulated labial glands (Shern et al., 1993). MUC7 was decreased in labial glands of children, but MUC5B was unchanged (Sonesson et al., 2008; Sonesson, 2011). In stimulated whole saliva, a decrease in MUC1 expression was noted in elderly test persons compared to younger probands (Chang et al., 2011).

The distribution of a broad spectrum of mucins in infantile labial glands indicates their importance early in human development to sustain oral health.

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