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Distinct expression of calnexin in major human salivary glands

N. Gassler[1*], J. Bohn[2*], M. Schnölzer[3], J. Scheuerer[1], N. Obermüller[4], H.F. Otto[1] and F. Autschbach[1]

[1]Institute of Pathology, University of Heidelberg, Germany, [2]Department of Plastic and Reconstructive Surgery, Malmö University Hospital, Malmö, Sweden, [3]German Cancer Research Center, Heidelberg, Germany and [4]Division of Nephrology, Medical Clinic IV, University of Frankfurt/ M., Germany

*contributed equally to this work

Summary. Calnexin (Cnx) has been characterized as a membrane-bound protein that transiently interacts in a unique chaperone system with newly synthesized glycoproteins in order to allow the establishment of their proper tertiary and, in most cases, quarternary structures. The aim of the study was to identify and to locate the expression of Cnx in the three major salivary glands of humans by different methods. Strong expression of Cnx protein and mRNA were generally found in serous salivary secretory units. With regard to mucous secretory units, expression of Cnx was only detectable at a low level in mucous acinar cells of sublingual glands, but not of submandibular glands. Expression of Cnx was always preserved in the surface epithelium of intralobar and interlobular duct segments. In addition, expression of Cnx was detected in sebaceous glands of parotid tissues, with a distribution pattern resembling that seen in sebaceous glands of the normal skin. In conclusion, production of saliva is associated with the expression of Cnx. Synthesis of molecules in mucous secretory units is not necessarily associated with a strong Cnx expression, whereas synthesis in serous secretory units apparently is. The tissue-specific Cnx expression is also paralleled by the observation that the secretions produced by the major salivary glands differ in their composition and amount.

Key words: Calnexin, Human, Salivary gland

Introduction

In humans, most of the saliva is produced by the major salivary glands named by their location, the parotid, the submandibular, and the sublingual glands. The salivary glands are compound exocrine tubuloacinar glands characterized by the aggregation of so-called secretory units (Martinez-Madrigal and Micheau, 1989; Martinez-Madrigal et al., 1997). These units consist of acini where the primary saliva formation takes place and a well developed duct system that regulates the final concentration of water and electrolytes before the liquid reaches the oral cavity.

In principal, three major types of salivary secretory units can be distinguished: the serous ones that contain e.g. amylase; the mucous ones, where mucins are secreted; and mixed units, which are composed of mucous and serous cells (Korsrud and Brandtzaeg, 1982). The parotid gland is of the serous type, whereas the submandibular and sublingual glands are of the mixed type. A predominance of serous units is found in submandibular glands and a predominance of mucous units is seen in the sublingual glands. In addition to differences in the salivary secretory units, the histomorphology of the duct system also differs between the three types of the major salivary glands (Martinez-Madrigal et al., 1997). In the parotid gland the intercalated ducts are longer than in the submandibular and sublingual glands, but the striated ducts are longer in the submandibular gland. The intercalated and the striated ducts are located intralobularly and are also known as secretory ducts because of their metabolic activity. The interlobular duct segments are called excretory ducts.

The presence of sebaceous glands in salivary tissues was first described by Hamperl (1931). The occurence of sebaceous glands varies; they are common in parotid tissues, rare in submandibular glands, and probably absent in sublingual glands. The role of sebaceous tissues in the salivary glands has not been satisfactorily explained.

Whereas composition and amount of secretion differ between the major human salivary glands (Quinatrelli, 1963; Otto et al., 1988; Ramasubbu et al., 1991; Reddy et al., 1992; Martinez-Madrigal et al., 1997), the expression of modulating proteins being implicated in secretory pathways has not been well elucidated.

Folding-competent states of newly synthesized

Offprint requests to: Dr. N. Gassler, Universität Heidelberg; Pathologisches Institut, INF 220/ 221, D-69120 Heidelberg, Germany

secretory proteins are guaranteed by several molecules including classical and non-classical lectin-like molecular chaperones located in the endoplasmic reticulum (Chevet et al., 2001). Calnexin (Cnx) has been characterized as a membrane-bound non-classical lectin-like molecule that interacts transiently with newly synthesized glycoproteins in a unique chaperone system found in the endoplasmic reticulum of nearly all eukaryotes, the so-called calnexin-calreticulin cycle (Parodi, 2000; Helenius and Aebi, 2001). The luminal domain of the Cnx molecule contains a lectin domain, as well as a distinct loop structure that might be involved in direct protein-protein interactions (Chevet et al, 2001).

In detail, expression and function of Cnx are associated with cellular secretory activity and occurrence of newly synthesized glycoproteins. Cnx (membrane-bound) and calreticulin (soluble) are homologous lectins of the endoplasmic reticulum that bind transiently to virtually all newly synthesized glycoproteins in order to allow the development of their proper tertiary and, in most cases, quarternary structures (Helenius and Aebi, 2001). Cnx and calreticulin interact with the glycan moieties of substrate glycoproteins in the calnexin-calreticulin cycle for a period ranging from a few minutes to several hours, depending on the rate of folding. Other molecules involved in protein folding include classical molecular chaperones (e.g. glucose-regulated proteins GRP78, GRP94, GRP170), protein disulfide isomerase (ERp59), and other enzymes (ERp72) (Chevet et al., 2001).

The aim of this study was to identify and to locate the expression of Cnx in the three major salivary glands of humans by immunohistochemistry, in situ hybridization, and Western blot analysis. We also wanted to rule out whether Cnx expression differs between the major types of salivary secretory tissues.

Materials and methods

Patients and tissues

Surgical resection samples including the major salivary glands from 10 patients with squamous cell carcinoma of the oral cavity (mean age, 56 years; range, 48 to 68 years) were used. In all cases, the carcinoma diagnosed by conventional clinical and histological criteria did not infiltrate the glandular tissues. Radiation was never performed prior to the surgical resection. Samples of about 0.5 cm^3 in size were taken for analysis from all major salivary glands. One half of the fresh and unfixed material was snap-frozen in liquid nitrogen and stored at -80 °C for molecular experiments, the other half was postfixed in 3% paraformaldehyde, paraffin embedded, and determined for morphological and immunohistological analysis as well as for mRNA in situ hybridization. Paraffin-embedded unaffected skin tissues derived from five patients (mean age, 69 years; range, 62 to 76 years) treated for squamous cell carcinoma of the skin served as controls.

Antibodies

For all experiments the affinity-purified supernatant of a newly generated monoclonal anti-Cnx antibody (IgG2a) from rat was used. Immunization of the rat was performed using a liquid preparation of native Cnx protein in 0.9% NaCl originating from normal purified human small intestinal crypts (Grossmann et al., 1998). Successful establishment of the hybridoma cell line was achieved, when stable clones were produced. The monoclonal antibody (mAb) was purified by antibody affinity chromatography using a HiTrap Protein G HP column following the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, England). It has been further characterized by immunoscreening experiments (see below: Immunoscreening and analysis of inserts) as well as by immunoaffinity chromatography (see below: Immunoaffinity chromatography and matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry). The anti-Cnx antibody was used for immunoblotting analyses (IB) at a dilution of 1:1000, and for immunohistological stainings (IH) 1:200. Further antibodies commercially available were used as follows: anti-ß-actin antibody (IB, 1:1000; Sigma, Deisenhofen, Germany), HRP-conjugated anti-mouse antibody or anti-rat antibody, respectively (IB, 1:10000; Santa Cruz, Santa Cruz, USA).

Immunoscreening and analysis of inserts

Approximately $2.4x10^6$ plaques from a human intestinal mucosa custom library in λTriplEx (Promega, Madison, USA) were plated and transferred onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) treated with IPTG (Sigma, Deisenhofen, Germany). Filters were incubated with the purified mAb overnight (dilution 1:2500 in 5% skimmed milk TBST), the secondary HRP-labeled antibody against rat IgG (1:5000), and subsequently incubated with the ECL substrate (Amersham Pharmacia Biotech, Little Chalfont, England).

After conversion of λTriplEx in pTriplEx following the manufacturer's protocol (Clontech, Palo Alto, USA), the inserts were amplified by PCR (4 min of denaturation at 94 °C, 30 cycles at 94 °C denaturation for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s) using a set of λTriplEx screening amplimers (Clontech, Palo Alt, USA). The PCR products were sequenced by an automated sequencing system (ABI 3700 capillary sequencer; ABI, Weiterstadt, Germany). Sequences were assembled using the program SeqMan (Lasergene, Madison, WI, USA) and GenBank searches were performed.

Immunoaffinity chromatography and matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry

In order to characterize the protein recognized by the

newly established anti-Cnx antibody, immunoaffinity chromatography and subsequently MALDI mass spectrometry were performed. Briefly, total protein isolated from human normal intestine (Gassler et al., 2001) was incubated with the protein G sepharose (Amersham Pharmacia Biotech, Little Chalfont, England) coupled mAb. After an incubation period of 24 h elution of the antigen was achieved by application of one ml 0.1 M glycine, pH 2.6. The samples were purified by 7.5% SDS-PAGE and further analyzed by MALDI mass spectrometry as previously described (Regula et al., 2000). Briefly, gel pieces were washed repeatedly and tryptic digestion was performed at 37 °C overnight in 40 mM ammonium bicarbonate. All MALDI mass spectra were recorded in the positive ion reflector mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser (ion acceleration voltage 20.0 kV; reflector voltage 21.5 kV; first extraction plate 15.4 kV). Mass spectra were obtained by averaging 50 to 200 individual laser shots. Database searching was performed against the NCBInr database using the ProFound search algorithm (http://129.85.19.192/prowl-cgi/ProFound.exe) and the protein prospector software developed at the University of California, San Francisco (http://prospector.ucsf.edu). Mass tolerance for the monoisotopic peptide masses was set to ±100 ppm or ±0.1 Da.

mRNA in situ hybridization

To generate antisense and sense RNA probes for in situ hybridization experiments the pBluescript II KS (±) phagemid containing the Cnx insert of pTriplEx (see above) was restricted with *EcoRI* or *SalI*, and than transcribed with T3 or T7 RNA polymerase (Roche, Mannheim, Germany), respectively. The mRNA in situ hybridization procedure was carried out as previously described (Obermüller et al., 1997). Briefly, deparaffinized tissue sections were postfixed in 4% paraformaldehyde/ PBS for 10 min and treated with proteinase K (8 μg/ml in PBS) (Roche, Mannheim, Germany) for 30 min at 37 °C. To improve the penetration of riboprobes, the transcripts were shortened to a calculated average length of 250 bases by alkaline hydrolysis. Hybridization was carried out overnight at a temperature of 46 °C with a probe concentration of 2 to 4 ng probe per μl hybridization mixture. To demonstrate the specificity of the riboprobe control experiments were performed with sense probes on alternate serial tissue sections. Further negative controls included tissue sections where the antisense/ sense probe or the anti-digoxigenin antibody were replaced by PBS.

SDS-PAGE and Western blot analysis

Samples were homogenized in TRI reagent (Sigma, Deisenhofen, Germany) using the Ultra Turrax equipment (IKA Labortechnik, Staufen, Germany). Proteins solubilized in a solution of 9 M urea/ 50 mM DTT and resolved by one-dimensional SDS-PAGE (7.5%; about 15 μg total protein per lane) were transferred to PVDF Immobilon-P membrane (Millipore Corporation, Bedford, USA) using a semi-dry blot system. Molecular weight markers as well as the ECL substrate were purchased from Amersham (Amersham Pharmacia Biotech, Little Chalfont, England). The amount of each sample analyzed was additionally adjusted to the intensity of ß-actin staining. Therefore, blots were stripped using a mixture of 2% SDS, 100 mM ß-mercaptoethanol, and 62.5 mM Tris-HCl (pH7.5). Negative controls included similarly processed blots in which the primary antibody was totally omitted.

Immunohistochemistry

Sections from paraffin-embedded, formalin-fixed tissues were dewaxed and immunostained as previously described (Gassler et al., 2001). Briefly, sections were treated by microwave in citrate buffer at 500 W for 10 min and subsequently incubated with the anti-Cnx antibody (1:200 in PBS; see above) for 1 h at room temperature in a moist chamber. For ABC immunostaining, the ABC detection kit with DAB as the chromogen was used in accordance to the manufacturer's suggested protocols (DAKO, Glostrup, Denmark). After the staining procedures, sections were counterstained with hematoxylin and mounted. Negative controls included similarly processed sections in which the primary antibody had been completely omitted as well as sections where the appropriate normal serum was used.

Results

Two specifically recognized plaques were initially

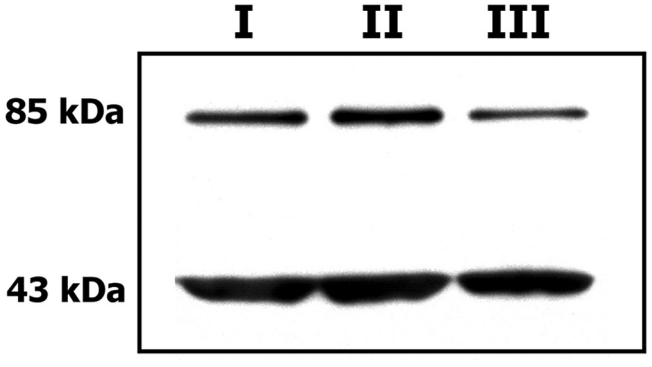

Fig. 1. Western blot analysis of calnexin protein (85 kDa) expression in human parotid (I), submandibular (II), and sublingual salivary gland (III). Calnexin expression appears to be stronger in tissue homogenates of submandibular glands (II) than in homogenates of parotid (I) and sublingual salivary glands (III). ß-actin (43 kDa) labeling is shown as loading control.

identified by immunoscreening experiments and further isolated. Full length sequencing revealed human Cnx in both cases (accession number BC003552). The data were corroborated by MALDI mass spectrometry of the 85 kDa protein isolated by immunoaffinity chromatography and subsequently purified by SDS-PAGE.

In Western blot analysis, expression of the Cnx protein was regularly found as a single 85 kDa band in all salivary glands (Fig. 1). The amount of immunoreactive Cnx was higher in tissue homogenates of the submandibular gland when compared with tissue homogenates of the parotid and the sublingual gland.

In salivary glands, strong expression of Cnx protein as well as Cnx mRNA were generally found in serous salivary secretory units (Fig. 2). Interestingly, the strongest homogeneous expression of Cnx was seen in the serous secretory units of the submandibular gland. Strong or moderate Cnx expression was additionally found in secretory units of the parotid, whereas in the sublingual gland expression of Cnx protein and Cnx mRNA was always moderate. In serous acinar epithelial cells, the Cnx immunosignaling was regularly found in a reticular pattern with strongest immunosignaling at the basolateral cell side. In contrast, cells of the mucous secretory units of submandibular glands did not reveal

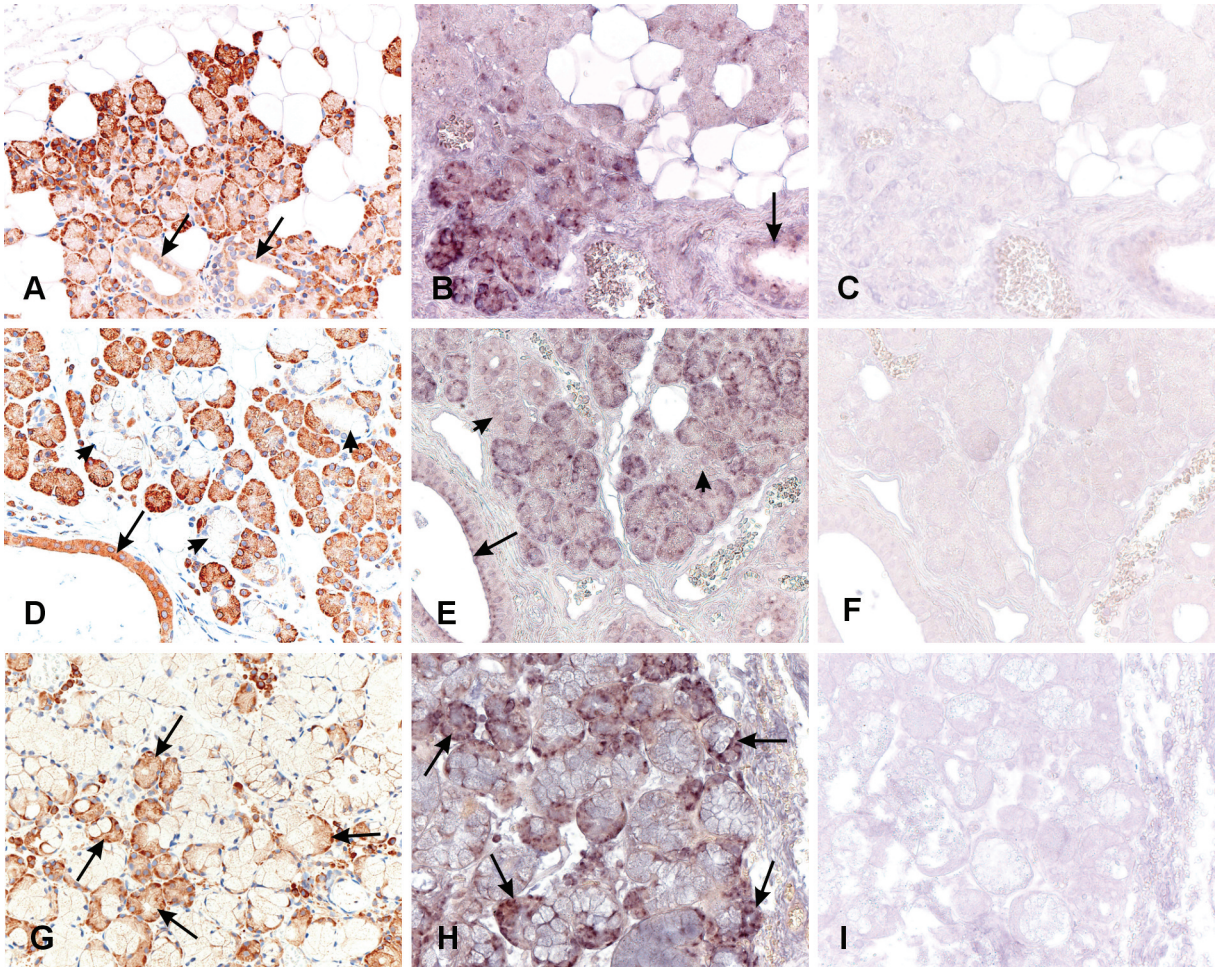

Fig. 2. Deparaffinized sections of normal human parotid **(A-C)**, submandibular **(D-F)**, and sublingual **(G-I)** salivary gland after immunostaining against Cnx **(A, D, G)** as well as after mRNA in situ hybridization **(B, C, E, F, H, I). A.** In parotid tissue, DAB immunstaining against Cnx is found in serous acinar cells as well as in the epithelium lining intralobular ducts (arrows). **B.** Parotid tissue after in situ hybridization for mRNA using digoxigenin-labeled antisense riboprobes to Cnx. A strong signal is found in serous acinar cells and in the surface epithelium of excretory ducts (arrow). **C.** Serial tissue section from **(B)** after mRNA in situ hybridization using digoxigenin-labeled sense riboprobes to Cnx (control). **D.** Submandibular tissue after immunostaining against Cnx. Strong Cnx expression is found in serous acinar cells, whereas mucous acinar cells are not significantly stained (short arrows). Expression of Cnx is found in the surface lining epithelium of ducts (arrow). **E.** Cnx mRNA is especially found in serous acinar cells, whereas Cnx mRNA expression by mucous acinar cells is not really detectable (short arrows). The excretory duct segment is marked by an arrow. **F.** Serial section of **(E)** after incubation with sense riboprobes to Cnx; control. **G.** In sublingual glands, strong expression of Cnx is found in serous acinar cells (arrows), whereas the immunostaining of mucous acinar cells is always moderate. **H.** The distribution of Cnx mRNA is identical to that found for the Cnx protein. Serous acinar cells are marked by arrows. **I.** Serial section of **H** after incubation with sense riboprobes to Cnx; control. A-I x 40

significantly detectable Cnx protein expression by immunohistochemistry, a finding which was corroborated by the results of the mRNA in situ hybridization experiments (Fig. 2). In mucous secretory units of sublingual glands, a slight immunostaining for Cnx was occasionally found contrasting to the immunostainings of mucous secretory units in submandibular tissues.

In intralobar secretory ducts, expression of Cnx was regularly expressed in the surface lining epithelium. The immunostaining for Cnx protein was occasionally very strong in the apical cytoplasm of these epithelial cells. The expression of Cnx in the surface epithelium of interlobular duct segments (excretory ducts) was not different from that found in intralobar ducts (secretory ducts).

In addition, sebaceous glands were found in some parotid tissues. Morphologically, these glands were identical to intradermal sebaceous glands including features of differently shaped sebocytes (Fig. 3). From basal to apical site of the gland, sebocytes showed morphological changes in size and shape suggestive for the holocrine secretion modus. In sebaceous glands, strong expression of the Cnx protein was especially found in the basal cellular layers, whereas sebocytes in the upper cellular layers were devoid of immunostainings against Cnx. The staining intensity of sebocytes was inversely correlated with their content of secretory granules. The distribution of Cnx expression in sebocytes did not vary between individual sebaceous glands of skin and parotid gland, respectively.

Discussion

Although a high number of chemically different molecules are secreted by salivary glands (Quinatrelli, 1963; Otto et al., 1988; Ramasubbu et al., 1991; Reddy et al., 1992; Martinez-Madrigal et al., 1997), there is no systematic analysis of the pathways by which these cells regulate folding of the subsequently secreted molecules. Calnexin (Cnx), a multifunctional molecule, is known to interact in a unique chaperone system of secretory pathways (Parodi, 2000; Helenius and Aebi, 2001).

Our results reveal that Cnx protein and mRNA were strongly expressed by epithelial cells of serous salivary secretory units, especially in submandibular glands.

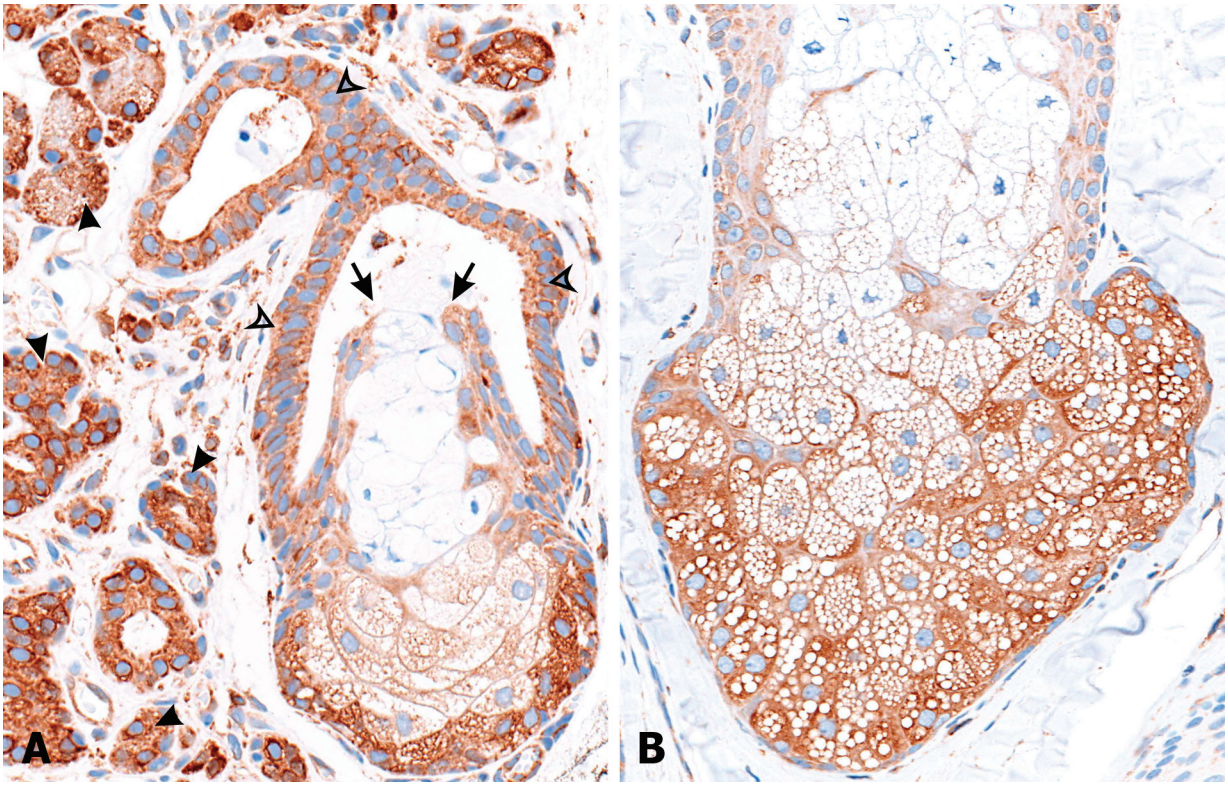

Fig. 3. Deparaffinized sections of human parotid tissue with sebaceous gland **(A)** and dermal tissue with sebaceous gland **(B)** after immunostaining against Cnx. **A.** From basal to apical location, sebocytes showed morphological changes in size and shape suggestive for the holocrine secretion modus. Cnx protein is especially found in the basal cellular layers, whereas sebocytes in the upper cellular layers are free of immunostainings against Cnx. The sebaceous gland is drained into an intralobular duct (arrows). The duct-lining epithelium is marked by open arrowheads. Serous acinar cells of the parotid gland with strong Cnx expression are shown on the left hand of the figure (arrowheads). **B.** Intradermal sebaceous gland with morphological and immunohistological features similar to that shown in **(A)**. x 60

With regard to mucous secretory units, expression of Cnx was only moderate in sublingual glands but not detectable in submandibular glands. This discrepancy in total Cnx expression between the three major salivary glands was additionally documented at the protein level in the Western blot analysis. Highest amounts of Cnx protein were observed in tissue homogenates of the submandibular gland when compared with the parotid and the sublingual gland.

The discrepancy in total Cnx expression is paralleled by the observation that the secretions produced by salivary glands differ in their composition and amount (Quinatrelli, 1963; Otto et al., 1988; Ramasubbu et al., 1991; Reddy et al., 1992; Martinez-Madrigal et al., 1997). Mucins are one important group of secretions produced by salivary glands (Perez-Vilar and Hill, 1999). These major glycoproteins, highly variable in molecular size, coat the surfaces of cells lining the digestive, respiratory, and urogenital tracts in order to protect epithelial cells from infection, dehydration and injury, as well as to aid the passage of materials through a tract. The functions of mucins are dependent on their ability to form viscous solutions or gels. Although expression of mucin species by serous cells has been recorded (Nielsen et al., 1996; Li et al., 2001), in salivary glands expression of mucins is preferentially found in mucous acinar cells (Khan et al., 1998). Our results give evidence that a discrepancy exists in the tissue distribution of Cnx related to the glycoproteins of mucin type. Cnx is preferentially found in serous acinar cells, whereas the overwhelming number of mucins is produced by mucous acinar cells. In conclusion, the secretory pathways of salivary glands are obviously adapted to the quality of molecules synthezised. Synthesis of serous liquids is highly dependent on Cnx expression, whereas production of mucine-rich secretions is not. Membrane and gel-forming mucins are probably processed by different pathways involving other proteins, apart from Cnx, in molecular folding.

In contrast to interlobular duct segments (excretory ducts), high metabolic activity of intralobular ducts (intercalated and the striated ducts) has been recorded (Martinez-Madrigal et al., 1997). Numerous mitochondria and various enzymes are present in the cytoplasm of the striated ducts and provide them with a metabolic and energy system capable of concentrating some of the elements present in the saliva. One principal function of interlobular ducts is to transport secretions. Expression of Cnx was not different in the surface lining epithelium when comparing intralobular and intralobar duct segments.

The occurence of sebaceous glands is common in parotid tissues, rare in submandibular glands, and probably absent in sublingual glands (Martinez-Madrigal et al., 1997). Presence and function of sebaceous tissues in the salivary glands has not been satisfactorily explained. The expression of Cnx in sebaceous glands in parotid tissues is not different from that found in normal sebaceous glands. Therefore immunohistochemical and morphological evidence is given that differentiation and function of normal and metaplastic sebocytes are quite similar.

In summary, we found that the expression of calnexin shows a tissue-specific distribution in salivary glands. Strong Cnx expression is seen in serous acinar cells, whereas expression of Cnx is only detectable at a low level in mucous acinar cells of the sublingual gland, but not found in mucous acinar cells of the submandibular gland. The tissue-specific Cnx expression probably relates to the observation that the secretions produced by the major salivary glands differ in their composition and amount.

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