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ORIGINAL ARTICLE

Expression of two drug-metabolizing cytochrome P450-enzymes in human salivary glands

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OBJECTIVE: The oral cavity is constantly lubricated by saliva and even small amounts of xenobiotics and/or their metabolites in the saliva may affect the oral mucosa. Our aim was therefore to clarify if xenobiotic metabolizing enzymes CYP1A2 and CYP3A4 are expressed in salivary glands.

METHODS: Formalin-fixed paraffin-embedded specimens from parotid (10), submandibular (7) and labial (10) salivary glands were examined immunohistochemically and by *in situ* hybridization for expression of CYP1A2 and CYP3A4 protein and mRNA.

RESULTS: CYP1A2 and CYP3A4 protein and mRNA were detected in ductal and seromucous/serous acinar cells in all gland types although to a varying degree and intensity. Mucous acinar cells were positive to a lesser extent.

CONCLUSION: The results indicate a xenobiotic metabolizing capability of salivary glands. This may have implications for development of oral mucosal disease as a result of mucosal exposure to metabolites originating from internal sources (blood) as well as from saliva.
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Keywords: cytochrome P-450; salivary glands; *in situ* hybridization; immunohistochemistry

Introduction

The oral cavity is covered and lubricated by saliva providing an important protection through its cleansing, moistening, buffering and antimicrobial properties. Whole mouth saliva predominantly comprises secretions from three paired major glands – the parotid (PG), the submandibular (SG), and the sublingual glands. Secretions from a large number of minor mucosal salivary glands, e.g. labial glands (LG), also contribute to a smaller extent. The major salivary glands account for about 90% of the fluid production, while the protein-rich secretion from the minor salivary glands have an important function in constantly lubricating the oral mucosa. The structure and function of each of the salivary gland types differ. PG is a purely serous gland producing a watery secretion, SG is mixed and predominantly serous producing a more viscous and mucin-rich saliva, and in general the minor glands are mixed and predominantly mucous (Pedersen *et al*, 2002). There has been some controversy on whether the morphologically different acinar cells in LG were reflecting two different cell types (mucous and seromucous) or various stages of the cell cycle of mucous cells (Tandler *et al*, 1969; Vered *et al*, 2000). Thus, in this study we differentiate between mucous and seromucous acinar cells in LG.

Many drugs can be monitored in the saliva. This is used therapeutically in establishment of the therapeutic drug dose and in screening for narcotics (Jusko and Milsap, 1993). The transcellular routes by which the drug and/or its metabolites are transported from the blood to the saliva may occur by passive diffusion or active transport of plasma substances into the salivary gland cells (Tandler *et al*, 1969; Tandler and Erlandson, 1972; Riva and Riva-Testa, 1973). Drugs may also enter the saliva by paracellular transport through the intercellular spaces and tight junctions (Choo and Huestis, 2004). Even small amounts of drugs and/or their metabolites in the saliva may affect the oral mucosa which under such conditions is exposed to the drug and/or its metabolite both from the saliva and the

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blood. It is however an open question if the salivary glands – be they major or minor gland types – contribute to the organism's metabolism of xenobiotics, e.g. drugs and other exogenous substances.

The cytochrome P450-enzyme (CYP) family, in particular the CYP1-3 subfamilies, are the most important initial phase drug-metabolizing enzymes, but they are also very important in activation or inactivation of other exogenous substances. The majority of the marketed drugs are metabolized by CYP3A4. Some of the CYPs, i.e. CYP2D6, CYP2C9, and CYP2C19, have frequent clinical relevant polymorphisms, which result in absent, reduced, or increased enzyme activity in affected individuals (Hasler *et al*, 1999). CYPs can be induced or inhibited by environmental factors such as drugs and pollutants; for example, CYP1A2 is induced by tobacco smoke (Rasmussen *et al*, 2002).

While the liver is still believed to be the predominant drug metabolizing organ, the impact of drug metabolism in the small intestine is also recognized as very important for several drugs, such as CYP3A4 substrates, thereby contributing substantially to the first pass effect of these drugs (Damkier *et al*, 1999; Kato *et al*, 2003). As the CYPs are involved in activation and inactivation of xenobiotics, the CYP expression of the main entrances of the body, e.g. the gastrointestinal tract and the respiratory system, has drawn some attention (Ding and Kaminsky, 2003). Thus, extra-hepatic CYP expression has been demonstrated in several human tissues, e.g. skin, lungs, brain, and pancreas (Murray and Burke, 1995; Kivisto *et al*, 1997; Yokose *et al*, 1999; Baron *et al*, 2001; Siegle *et al*, 2001; Standop *et al*, 2002b). In favor of the salivary glands being part of the extra-hepatic drug metabolism a few studies have shown CYP expression in human or animal salivary glands. By immunohistochemistry (IH), CYP2C expression has been demonstrated in the intercalated ducts and the serous acinar cells of normal human salivary gland tissue (type not specified) but not in mucous acinar cells or in the epithelium of the larger ducts. CYP3A expression was not found in normal human salivary gland (Yokose *et al*, 1999). An IH study on the cellular distribution of CYP4A in rabbit PG reported intense immunostaining in the epithelial components of the intercalated, striated, and interlobular ducts but not in the acinar cells (Kikuta *et al*, 2001).

Our hypothesis is that the salivary glands have the capacity to metabolize drugs and other xenobiotics and here we explore this by investigating the protein and mRNA expression of the inducible CYP1A2 and the widely metabolizing CYP3A4 in the PG, SG, and LG.

Subjects and methods

Labial glands were obtained from 10 healthy volunteers. The glands were removed under local analgesia at the School of Dentistry, University of Copenhagen as described previously (Pedersen *et al*, 1999). Macroscopically and histomorphologically normal-appearing PG tissue was obtained from 10 patients surgically treated for benign parotid salivary gland tumors. Likewise, normal-appearing SG tissue was obtained from seven patients having the SG resected because of sialolithiasis (1) or as part of a head-neck gland resection during oral cancer management (6). A further eight PG and three SG were excluded because of extensive lipid degenerative changes or massive inflammation. All major salivary gland tissues were obtained from the patients under general anesthesia at the Department of Otolaryngology/Head and Neck Surgery at the University Hospital of Copenhagen (Rigshospitalet). The study was approved by the Ethical Committee of Copenhagen and Frederiksberg Municipality, Denmark (KF13 288 267), and written informed consent was obtained from each of the participants.

Lifestyle and health data

For each person, data on demographic, lifestyle, and health features were obtained from hospital files or dental records except for three patients where only gender and age were known. All smokers were cigarette smokers and one cigarette was set as equal to 1 g of tobacco. Smoking habits were categorized as: never smokers, former smokers, light smokers (1–14 g day^{-1}), moderate smokers (15–24 g day^{-1}), and heavy smokers (≥25 g day^{-1}) (Godtfredsen *et al*, 2004). Alcohol intake was categorized as: less than 1 drink (12 g alcohol) per week, equal or less the maximum recommendation by the Danish Health Authorities (females ≤14 drinks and males ≤21 drinks per week), and more than the maximum recommendation (Sundhedsstyrelsen, 2005).

Tissue preparation

Specimens were fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin, cut into 4 μm sections, and mounted on coated glass slides (Superfrost plus, Menzel, Germany). Hematoxylin- and eosin-stained sections were evaluated and all tissues were normal according to the anatomical location from where they were obtained.

Immunohistochemistry

For IH, the sections were deparaffinized in xylene, rehydrated in ethanol, and heated in a microwave oven at 105°C for antigen retrieval in Target Retrieval Solution, pH 9 (Dako code S2368, Copenhagen, Denmark) for 15 min. After blockage of endogenous peroxidase activity the sections were incubated with polyclonal antibodies for 30 min; CYP1A2 (Chemicon code AB1248; 1:300, Chemicon, Temucula, CA, USA) and CYP3A4 (Chemicon code AB1254, 1:300). Substitution of primary antibodies with buffer and Negative Control Rabbit Immunoglobulin Fraction (Dako code X0936) served as negative controls and human liver sections as positive controls. Powervision + ™ (ImmunoVision Technologies, Daly City, CA, USA) was used as detection system in accordance with the manufacturer's protocol. The slides were examined under a light microscope by two investigators (J.R., C.K.) for absence or presence of immunostaining in the tissue components. The immunostainings were furthermore scored as: − negative, + weak/moderate, and ++ strong

staining within each specimen. The specificity of the antibodies was tested by the manufacturer (Chemicon).

In situ *hybridization (ISH)*
For ISH, the sections were deparaffinized in xylene, rehydrated in ethanol, and treated with proteinase K for removal of masking proteins. Hybridization was carried out overnight in a humid chamber (Dako Hybridizer) where the sections were incubated with enzyme-specific, fluorescein-labeled probes dissolved in hybridization buffer (50% formamide, 4X SSC, 1X Denhart, 800 μg ml^{-1} yeast tRNA, 500 μg ml^{-1} sonicated salmon sperm DNA). After hybridization the sections were stringently washed in three baths of 1X TBST succeeded by one bath of 1% TBST wash buffer. The Genpoint™ Fluorescein system (Dako code K0618) was used for signal amplification in accordance with the manufacturer's protocol. Sense probes provided as control for probe specificity and hybridization without probe in the hybridization buffer served as negative control (Table 1). Human small intestine sections served as positive control. The slides were examined under a fluorescence microscope by two investigators (J.R., C.K.) for absence or presence of signal. The ISHs were furthermore scored as: − negative, + weak/moderate, and + + strong signal within each specimen. Because of high homology between the DNA sequences of the CYPs the sequences were aligned using the software available on http://bibiserv.techfak.uni-bielefeld.de/dca/submission.html before making allele-specific hybridization probes. The software OLIGO 6 was used to design suitable probes with regard to melting temperature and Guanine-Cytosine (G-C) composition. The hybridization probes designed were blatted to ensure allele-specificity using the software available on http://genome.cse.ucsc.edu.

Statistics
Statistical analysis was performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). The data were analyzed using descriptive analyses as frequency counts and summary statistics only.

Results

Table 2 shows detailed demographic, lifestyle, and health features related to the type of salivary gland tissue.

The immunostainings of the negative control sections were negative as were the ISH reactions with sense probes and with hybridization buffer. Both immunostaining and ISH reactions were positive in positive control sections. In the epithelial salivary gland cells the positive immunostainings and ISH signal were seen as diffuse cytoplasmic signals (Table 3).

Human submandibular salivary gland
Immunostaining for CYP1A2 was generally negative in the cytoplasm of ductal cells, however, a few segments showed a weak reaction (Figures 1–4). Immunostaining in serous acinar cells varied considerably between adjacent cells within the same lobulus; from strong to negative. In general five cases were predominantly positive while two were predominantly negative.

Table 1 *In situ* hybridization probe sequences, concentration, hybridization, and stringency wash temperature

Enzyme		*Probe sequence*	*Probe concentration (μM)*	*Hybridization temperature*	*Stringency wash temperature*
CYP1A2	Anti-sense	5′-GGA GAA GCT CTG TGG CCG AGA AGG GAA CAG ACT GGG ACA A-3′	1	54	59
	Sense	5′-TTG TCC CAG TCT GTT CCC TTC TCG GCC ACA GAG CTT CTC C-3′	1		
CYP3A4	Anti-sense	5′-GGA AAT CCA CTC GGT GCT TTT GTG TAT CTT CGA GGC GA-3′	0.51	48	55
	Sense	5′-TTG GCT CCT TTG CTT CTC ACG GGA CTA TTT CCA CCA CC-3′	0.51		

Table 2 Demographic, lifestyle, and health features

		Age (years)	*Smoking habits (no. smokers)*						*Alcohol intake drinks per week*				*Daily medication*			*Systemic diseases*		
Salivary gland	*Gender (n)*	*mean (range)*	*Never*	*Former*	*Light*	*Moderate*	*Heavy*	*n.k.*	*< 1*	*1–14/21*[a]	*≥14/21*[a]	*n.k.*	*No*	*Yes*	*n.k.*	*No*	*Yes*	*n.k.*
Parotid gland	♀(5)	70.8 (54–82)	4	1	0	0	0	0	2	0	0	3	3	2	0	1	4	0
	♂(5)	49.2 (27–68)	0	1	3	0	1	0	2	1	2	0	1	4	0	2	2	1
Submandibular gland	♀(4)	49.8 (32–59)	0	1	0	2	0	1	1	0	2	1	2	1	1	1	2	1
	♂(3)	55.3 (36–71)	0	0	0	1	0	2	0	0	1	2	1	0	2	0	1	2
Labial glands	♀(5)	25.4 (22–30)	5	0	0	0	0	0	1	3	1	0	5	0	0	5	0	0
	♂(5)	22.2 (21–30)	5	0	0	0	0	0	1	3	0	1	5	0	0	5	0	0

n.k., not known.
[a]The Danish Health Authority's recommendation for maximum alcohol intake is for females ≤14 drinks and males ≤21 drinks per week.

Table 3 Immunostaining and *in situ* hybridization signal pattern in the cellular components of the labial (LG), submandibular (SG), and parotid glands (PG)

Immunostaining	*LG*		*SG*		*PG*	
Tissue component	*CYP1A2*	*CYP3A4*	*CYP1A2*	*CYP3A4*	*CYP1A2*	*CYP3A4*
Ductal cells	+	+	–/+	+	+	+
Seromucous/serous acinar cells	+/–	+	+/–	+/–	+/–	–/+
Mucous acinar cells	–	+/–	–	–	+	

ISH signal	*LG*		*SG*		*PG*			
Tissue component	*CYP1A2*	*CYP3A4*	*CYP1A2*	*CYP3A4*	*CYP1A2*			*CYP3A4*
Ductal cells	+	+	+	+	+	–	+/–	+/–
Seromucous/serous acinar cells	+	+	+	+	–	+	+/–	+/–
Mucous acinar cells	+	+	–	–				

–, negative; +, positive immunostaining/ISH signal; ISH, *in situ* hybridization.
Ductal cells include the cells of intercalated, striated, excretory ducts.

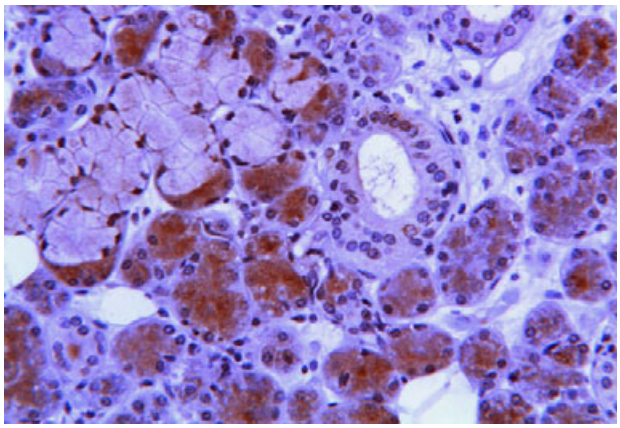

Figure 1 CYP1A2 protein in submandibular gland. Serous acinar cells are positive whereas mucous cells are negative. In this specimen duct cells are negative as well (immunohistochemistry × 200)

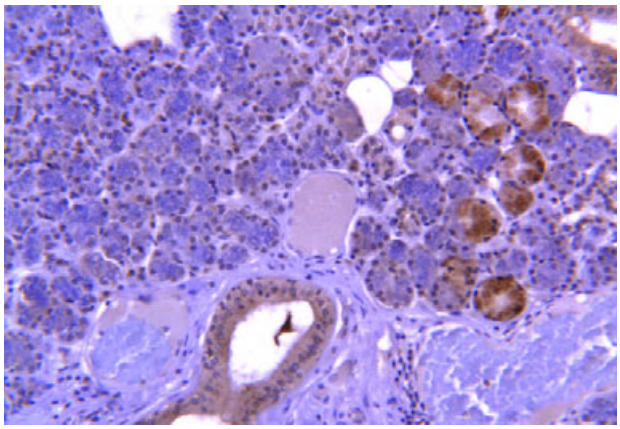

Figure 3 CYP3A4 protein in submandibular gland. Mucous acinar cells and duct cells are positive whereas serous cells are negative (immunohistochemistry × 150)

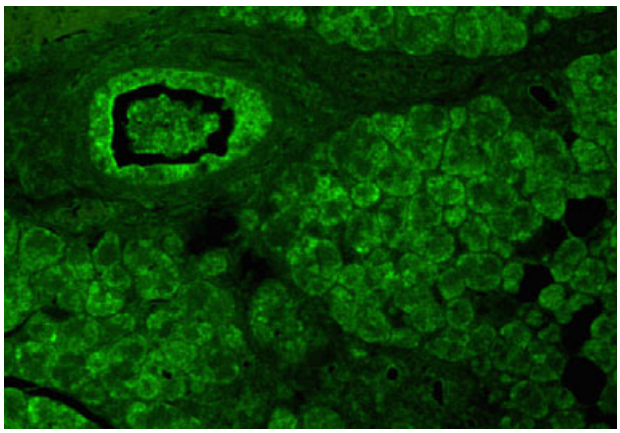

Figure 2 CYP1A2mRNA in submandibular gland. Positive signal in serous acinar cells and duct cells (*in situ* hybridization × 120)

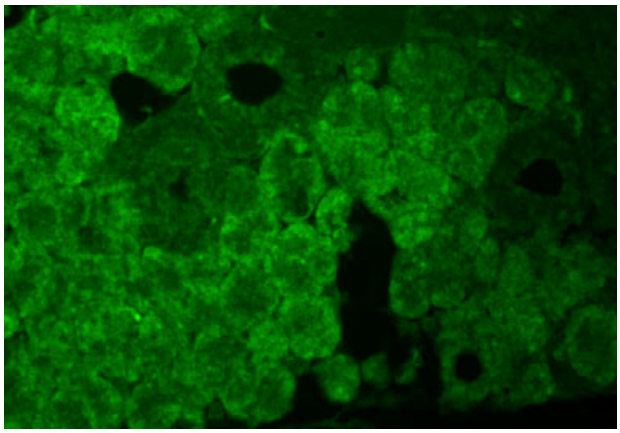

Figure 4 CYP3A4 mRNA in submandibular gland. Positive signal in serous acinar cells and weak signal in duct cells (*in situ* hybridization × 150)

Mucous acinar cells were negative. CYP3A4 immunostaining was strong in the cytoplasm of ductal cells. In the cytoplasm of the serous acinar cells CYP3A4 staining was similar to that seen with CYP1A2 showing great diversity between adjacent cells; the pattern was predominantly positive and negative in one case each, negative in four cases, and about 50% positive/50% negative in one case. The periphery of the cytoplasm of mucous acinar cells was negative in four cases and moderately positive in three cases.

The ISH signals for CYP1A2 varied from strong to moderate intensity in both acinar and ductal cells. Among the acinar cells there was great diversity between adjacent cells. The ISH signals of CYP3A4 were more homogeneous and strong intensity was scored in serous acinar cells while moderate intensity was detected in ductal cells. Mucous acinar cells were negative for CYP1A2 and CYP3A4 signals.

Human parotid salivary gland

In general, immunostaining for CYP1A2 was moderate in the cytoplasm of ductal cells, but the overall staining of serous acinar cells was positive but more diverse as adjacent cells varied from strong to negative within the lobules as in the PG (Figures 5–8). CYP3A4 immunostaining in the cytoplasm of the ductal cells was strong, whereas serous acinar cells were negative in seven cases or predominantly negative with sparse weakly positive cells in three cases.

The ISH signals for CYP1A2 showed a high diversity between the different samples. Three samples showed strong signal intensity in ductal cells and nil or sparse reaction in the serous acinar cells, however, four samples showed negative reaction in the ductal cells and very strong (two cases) and varying from strong to negative (two cases) in the serous cells. The last three samples varied from strong to negative reaction within both ductal and serous acinar cells. Generally, the CYP3A4 ISH signals were positive in both the ductal and serous acinar cells but considerable variation was seen between adjacent serous acinar cells and different ductal segments varying from strong to negative signal.

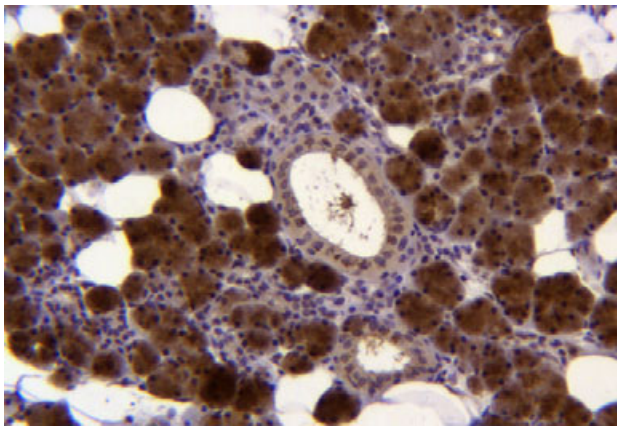

Figure 5 CYP1A2 protein in parotid gland. Serous acinar cells are strongly positive and duct cells are weakly positive (immunohistochemistry × 150)

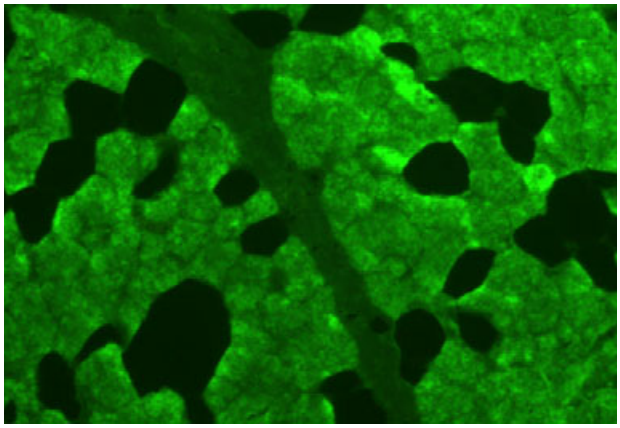

Figure 6 CYP1A2 mRNA in parotid gland. Positive signal in serous glands. Black areas are fat cells (*in situ* hybridization × 120)

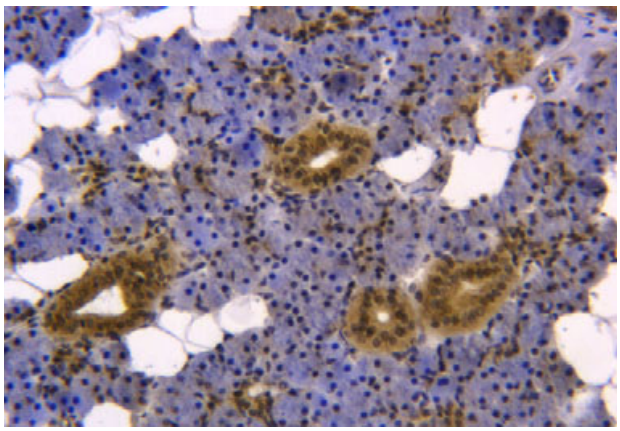

Figure 7 CYP3A4 protein in parotid gland. Duct cells are positive whereas serous acinar cells are negative (immunohistochemistry × 150)

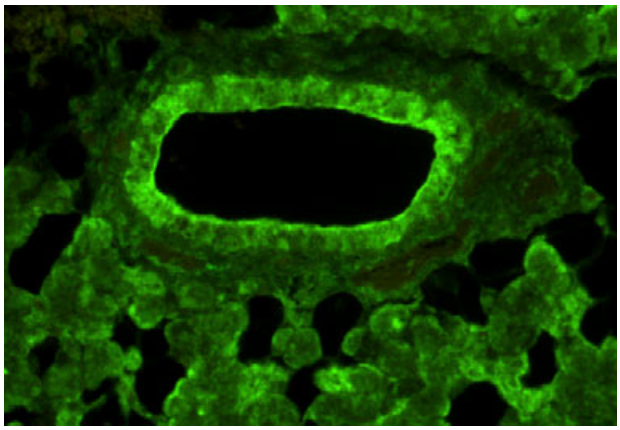

Figure 8 CYP3A4 mRNA in parotid gland. Duct cells are strongly positive whereas serous acinar cells are weakly positive and negative (*in situ* hybridization × 120)

Human labial salivary glands

Immunostainings for CYP1A2 were quite consistent in the LG as the cytoplasm of the ductal cells was positive varying from strong to moderate in different parts of the

glands (Figures 9–12). In general, the cytoplasm of seromucous acinar cells showed moderate immunostaining but there was variation between adjacent cells as some were positive and others were negative. There was no immunostaining in the mucous acinar cells. CYP3A4 immunostainings were strong in the cytoplasm of both the ductal and the seromucous acinar cells and moderate to strong staining was seen in the mucous acinar cells as a staining in the cell periphery. Some mucous acinar cells, however, were negative.

The ISH signals were detected with moderate intensity in the cytoplasm of the ductal cells and with strong intensity in the seromucous acinar cells for both CYP1A2 and CYP3A4. Mucous acinar cells were moderately positive at the cell periphery for both enzymes.

It was not possible to distinguish immunostaining or ISH patterns between smokers and non-smokers, medicated and non-medicated individuals, or alcoholics and non-alcoholics in any of the salivary gland samples.

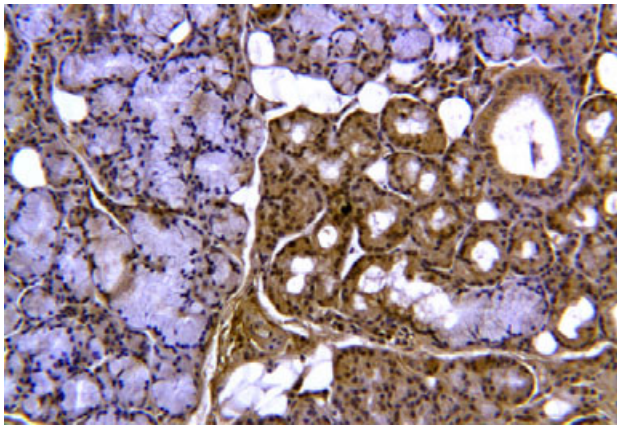

Figure 9 CYP1A2 in labial gland. Seromucous acinar cells and duct cells occupying right part of the illustration are positive. Mucous acinar cells negative (immunohistochemistry × 150)

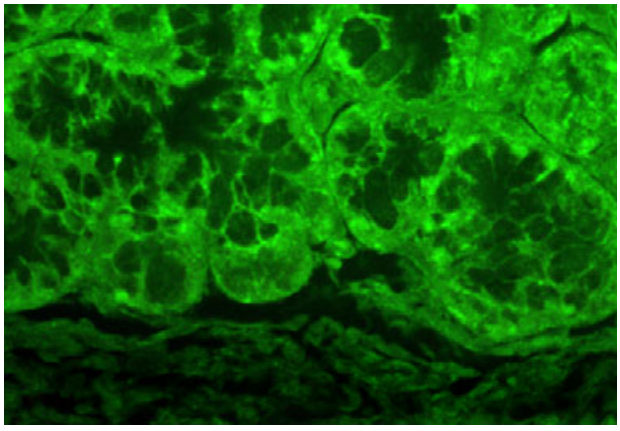

Figure 10 CYP1A2 mRNA in labial gland. Seromucous acinar cells are positive. Mucous cells are positive in the peripheral part of the cytoplasm (*in situ* hybridization × 250)

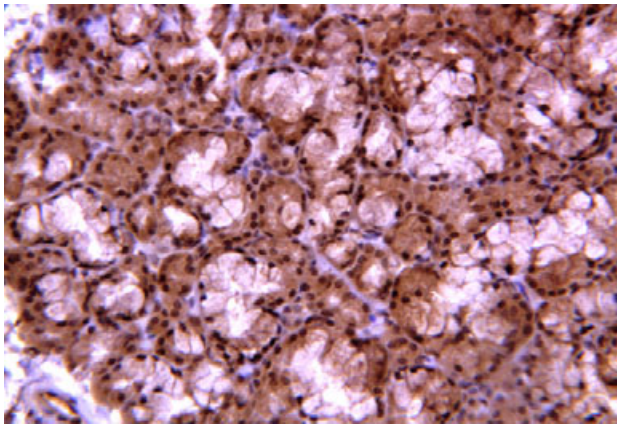

Figure 11 CYP3A4 protein in labial gland. Seromucous cells are positive whereas mucous acinar cells are negative (immunohistochemistry × 200)

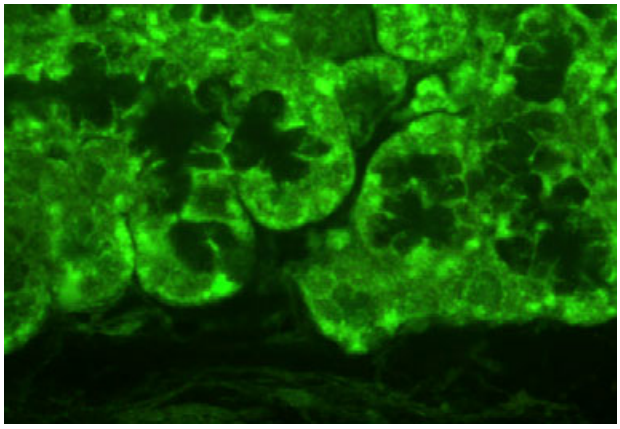

Figure 12 CYP3A4 mRNA in labial gland. Seromucous cells are positive and the mucous acinar cells are positive in the cell periphery (immunohistochemistry × 250)

Discussion

We have demonstrated that CYP1A2 and CYP3A4 mRNA and protein are expressed in the parenchymal cells of human labial, parotid and submandibular salivary glands. In LG both ductal and seromucous acinar cells showed intense staining of the CYP proteins whereas the mRNA signals were highest in the seromucous acinar cells. Surprisingly, mRNA signal for both CYPs was seen in the mucous acinar cells although only CYP3A4 proteins were detected. In SG the serous acinar cells had more intense staining compared with ductal cells, which was reflected in the mRNA expression. There was no ISH signal in the mucous acinar cells although CYP3A4 protein was detected in some of the samples. The lack of protein in cells with mRNA signal may be explained by the lack of translation or a fast rate of degradation of the protein. In PG, CYP1A2 protein staining was higher in serous acinar cells than in ductal cells. The reverse result was obtained concerning CYP3A4 expression. The mRNA signal did vary considerably in the PG. The serous acinar cells were

negative or predominantly negative with sparse positive cells with regard to CYP3A4 protein, which is in general agreement with the result from rabbit PG (Kikuta *et al*, 2001). On the contrary, Yokose *et al* (1999) did not find CYP3A4 proteins in human normal salivary gland tissue or neoplastic PG tissue in their multi-tissue study. These discrepancies may be attributed to the use of different antibodies and methodology.

Differences in the immunostaining and ISH signal may reflect variations in the distribution of organelles in the tissue components. In the hepatocytes the CYPs are synthesized by the ribosomes in the rough endoplasmatic reticulum (RER) and anchored into the membrane of the smooth endoplasmatic reticulum (SER) where they extrude from the cytosolic side of the organelle (Bar-Nun *et al*, 1980). In a study on CYP enzymes in the pancreas there was a remarkable inter-individual difference in the cellular distribution of the immunostaining that varied from a diffuse cytoplasmic, Golgi-related, and nuclear pattern in the different cells (Standop *et al*, 2002a). In our study only diffuse cytoplasmic staining was seen implying that the CYPs in the salivary parenchyma are localized in the endoplasmatic reticulum. The seromucous/serous acinar cells of the LG, SG, and PG have the potential of substantial CYP-protein synthesis with a well developed RER, numerous free ribosomes, and energy-producing mitochondria (Tandler *et al*, 1969; Tandler and Erlandson, 1972; Riva and Riva-Testa, 1973), which supports the intense ISH signal found in our study. The interlobular, striated, excretory, and main excretory duct cells contain a few cisternae of RER and a varying amount of SER where CYP-proteins most likely are located (Tandler *et al*, 1970; Riva *et al*, 1976; Lantini *et al*, 1990). The immunostaining of the ductal cells reflects the abundance of the SER in these cells. The ultrastructural features of mucous acinar cells are less investigated than the other salivary gland components but the mucous acinar cells of the LG have sparse RER and Golgi complex which are displaced to the periphery of the cell by the mucous granules filling almost the entire cytoplasm (Tandler *et al*, 1969). This is reflected by the ISH signal in mucous acinar cells of the LG in our study.

Our finding of great diversity in CYP expression in adjacent cells correlates with a study on the expression pattern of xenobiotic metabolizing enzymes in the pancreas (Standop *et al*, 2002a) in which significant inter-individual differences and differences within the different cellular components of the pancreas were found. Also, it was found that diabetic individuals, males, and old individuals had a lower expression than non-diabetics, females, and young individuals. We did not succeed in differentiating between different health and lifestyle profiles in our study, possibly because of the small sample size.

A variety of oral mucosal diseases, e.g. oral lichen planus, erythema multiforme, and discoid lupus erythematosus, have been considered in some cases to represent adverse drug eruptions (Torpet *et al*, 2004). Furthermore, polymorphisms in *CYP1A1* have been reported to increase the risk of oral cancer (Sato *et al*, 2000) although other studies did not find this association (Hahn *et al*, 2002). The presence of CYPs in salivary gland parenchyma indicates a xenobiotic-metabolizing capacity of the salivary glands. Thus, oral tissues may be exposed to drug metabolites by double exposure from internal (blood-borne) and external (saliva) sources. An additional metabolizing capacity of the surface epithelia of the oral mucosa has been suggested from immunoblotting studies on gingival tissues and from studies on isolated buccal epithelial cells and cell cultures (Zhou *et al*, 1996; Vondracek *et al*, 2001). The mere accumulation of metabolites in the oral mucous membranes may explain the appearance of disease processes; also accumulation of non-metabolized drugs caused by polymorphism of CYPs may lead to mucosal disease. Probably, the regions in the oral cavity with the highest drug and/or metabolite concentration or longest residence time would be the most vulnerable. The areas in the vicinity of the orifices of the major salivary glands (buccal mucosa, floor of the mouth) would most likely be more exposed to xenobiotics in the saliva and because of gravity and head position the saliva pools in the inferior regions of the mouth where the longest exposure time is to be expected. The buccal mucosa is the most frequent site for oral lichen planus lesions (Axell and Rundquist, 1987) and the tongue and the floor of the mouth are the areas with the highest incidence of cancer (Batsakis, 2003), lending support to the hypothesis of an association between xenobiotics in saliva and development of mucosal diseases. In this respect the floor of the mouth has been reported to be the most permeable region of the oral mucosa (Lesch *et al*, 1989).

In conclusion we have shown that CYP1A2 and CYP3A4 proteins and mRNA are expressed in human salivary gland parenchyma indicating a xenobiotic-metabolizing capability of salivary glands. This may have implications for development and progression of mucosal disease as a result of mucosal exposure to metabolites not only originating from internal sources (blood) but also from saliva. Further studies are needed to elucidate the importance of these findings for progression of oral mucosal disease.

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