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IMMUNOHISTOCHEMICAL IDENTIFICATION OF CYTOKERATINS IN THE RAT SUBMANDIBULAR SALIVARY GLANDS DURING ONTOGENESIS

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Cytokeratins (CK) are a group of proteins comprised of at least 20 different skeletal polypeptides specific for simple and complex epithelia of almost all tissues. Until recently only a few antibodies for cytoskeletal proteins were available and thus it was very difficult to visualize all the components of postnatal cytodifferentiation of salivary glands.

Therefore, morphologic and immunophenotypic features of the CK polypeptides, have been analized within the rat submandibular salivary glands (SSG) during postnatal development.

SSG were obtained from 1, 30 and 60 days old, male, AO rats. Streptavidin-biotin immunoperoxidase method was used for immunohistochemistry. Cryostat sections were fixed in acetone and incubated with monoclonal antibodies (mAbs) which specifically react with CK polypeptides (CK7, CK8, CK18, CK kidney, K8.12, K8.13) and rat epithelium (PT13D11).

Immunohistochemical analysis of the rat SSG showed phenotypic heterogeneity of particular components of this gland during postnatal development. Regarding CK profiles it was shown that epithelial cells of the acini expressed CK18, whereas epithelial cells of the duct expressed K8.12 and K8.13 mAbs. Epithelial cells of the acini were not stained by K8.12 and PT13D11 mAbs. Intercalated ducts were strongly CK kidney+ and PT13D11+. Myoepithelial cells of the acini were stained by K8.13 mAbs, whereas these cells were not stained by mAbs specific for simple epithelia.

This immunohistological study showed that the heterogeneity expression of CK polypeptides might also be useful to further understand the origin of epithelial SSG celss, as well as their development and function.

Key words: submandibular salivary glands, rat, cytokeratins, monoclonal antibodies, postnatal development

INTRODUCTION

The model of cell differentiation in the SSG is the most complex one in relation to other glands and it provides the basis for understanding the development of salivary glands. The first visible sign of salivary glands in the embryo (day 13 in rats) involves interactions between the epithelial layer and the mesenchyme. During the postanatal development this gland is composed of highly differentiated epithelial cells that produce many essential proteins involved in different biological processes. Many of the SSG products are secreted via ducts into the saliva. The most important function of salivary components is to protect the soft and hard tissues of the mouth. The presence of an epithelium at different stages of proliferation and differentiation raises various questions concerning the histogenesis and differentiation of the salivary gland tissue (Gresik, 1980; Pinkstaff, 1980; Barka, 1980; Dardick *et al.*, 1990; Hand *et al.*, 1996; Denny *et al.*, 1997; Hieda and Nakanashi, 1997).

The presence of different subtypes of CKs in epithelial cells depends on the epithelium type (simple or stratified), as well as on the level of their histological differentiation and embryonal development. Polypeptides CK belong to the family of intermediary filaments which have an important role in the organization of the cytoskeleton. They are composed of at least 20 different polypeptides, molecular mass of 40-68 kDa. On the basis of the number and nature of amino acids they are divided in two types: acidic (type I) and basic (type II) CKs (Moll *et al.*, 1982; Moll, 1998).

Imunohistochemical labelling with antibodies specific for cytoskeletal proteins is an important technique for better understanding of the histogenesis of epithelial tissues, including salivary glands. There is a great number of monoclonal antibodies today that can identify subunits of CK, pairs of CK or common antigenic determinants of CK polypeptides. Many studies described the expression of CK in epithelial cells of salivary glands in human tissues (Dardick *et al.*, 1988; Burns *et al.*, 1988; Born *et al.*, 1987; Draeger *et al.*, 1991; Gustafsson *et al.*, 1988; Li *et al.*, 1996; Martins *et al.*, 2002; Ogawa, 2003), in rats (Takahashi *et al.*, 1994), in dogs (Sozmen *et al.*, 1998), in rabbits (Farina, 1992; Ogawa *et al.*, 2001).

In this study we examined the immunohistochemical profiles of epithelial components in the rat SSG during postnatal development. Specific mAbs for single CK polypeptides or CK pairs were used in the investigation. We demonstrated the heterogeneity of the CK subunit in SSG epithelial cells suggesting their different origin or stage of development.

MATERIAL AND METHODS

Animals

Submandibular salivary glands were obtained from male AO rats, aged 1, 30 and 60 days, from the Farm for Experimental Animals, Military Medical Academy, Belgrade. The last term also indicated the age at which the animal reached sexual maturity. Animals were sacrificed by aether anesthesia. Gland

tissue cryostat sections (5-6 μm), were air dried for 2h and fixed in acetone for 10 min.

Antibodies and reagents

In this study we used mAbs which identify cytokeratin polypeptides (CK7, CK8, CK18, K8.12, K8.13,CK-kidney), rat epithelial PT13D11 in SSG. Their specificity, isotype, dilution and origin are given in Table 1. Secondary antibodies and reagents, goat antimouse IgG subclass specific biotinylated antibodies and streptavidin coupled with peroxidase, were purchased from Amersham International, United Kingdom.

Table 1. Monoclonal antibodies used for immunohistochemistry

Antibody	Specificity	Isotype	Dilution	Manufacturer
CK7	CK7	IgG1	1:100	Sigma
CK8	CK8	IgG1	1:100	Sigma
CK18	CK18	IgG1	1:100	Sigma
K8.12	CK13,16	IgG1	1:50	ICN
K8.13	CK1,5-8,10,11,18	IgG2a	1:50	ICN
PT13D11	epithelial rat	IgM	1:150	*
CK-kidney	CK-kidney (CK7?)	IgG2a	1:10	Amersham

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Immunohistochemistry

For immunohistochemistry streptavidin biotin immunoperoxidase staining was applied. Cryostat sections were incubated with mAbs for 60 min. After incubation with the primary antibody, the sections were washed in TBS (tris-bufferd saline), pH 7.6 for 10 min, and were immersed in absolute methanol containing 3% H_2O_2 for 20 min at room temperature (block endogenous peroxidase activity). Followed, incubation with secondary biotinylated antibodies diluted 1:50 in TBS for 30 min. After washing in TBS, sections were incubated with streptavidin-peroxidase (1:50) for 30 min. Determination of the peroxidase activity was performed by incubation (10 min) of the sections in 0.06% DAB (Serva, FRG) in 0.01% H_2O_2. Finally, slides were counterstained with haematoxylin and mounted in gelatin/glycerol medium. Negative controls for immunostaining were performed by substituting the primary antibodies with tris-buffered saline.

Stained sections were examined using a light microscope and each reaction was described as either negative, weak, moderate, strong or very strong.

RESULTS

The use of anti-CK mAbs demonstrated different expressions of particular CK polypeptides in the rat SSG during postnatal development, as well as phenotypic similarity between some components (Table 2).

Table 2. Expression of CK polypeptides analysis in the rat submandibular salivary glands during postnatal development

Monoclonal antibody	CK7 (days)			CK8 (days)			CK18 (days)		
	1	30	60	1	30	60	1	30	60
Acinar cells									
mucous	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)
serous	2+(c)	3+(c)∫2+(c)	3+(c)∫2+(c)	3+(c)	3+(c)	3+(c)	1+(c)	1+(c)	1+(c)
myoepithelial cells									
(mucous)	–	–	–	–	–	–	–	–	–
(serous)	–	–	–	–	–	–	–	–	–
Intercalated duct									
epithelial cells	2+(c)	3+(c)∫2+(c)	3+(c)∫2+(c)	3+(c)*	3+(c)*	3+(c)*	4+(c)	4+(c)	4+(c)
Striated duct									
epithelial cells	4+(c)	3+(c)*	3+(c)*	3+(c)*	3+(c)*	3+(c)*	4+(c) 4+(c)	3+(b)∫	3+(b)
myoepithelial cells	–	–	–	–	–	–	–	–	–
Excretory duct									
epithelial cells	3+(c)*	3+(c)*	3+(c)*	3+(c)*	3+(c)*	3+(c)*	4+(c)	4+(c)	4+(c)
myoepithelial cells	–	–	–	–	–	–	–	–	–
basal cells	–	–	–	–	–	–	–	–	–

Cont. Table 2.

Monoclonal antibody	CK kidney (days)			K8.13 (days)			K8.12 (days)			PT13D11 (days)		
	1	30	60	1	30	60	1	30	60	1	30	60
Acinar cells												
mucous	1+(c)	1+(c)	1+(c)	–	1+(c)	1+(c)	–	–	–	–	–	–
serous	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	1+(c)	–	–	–	–	–	–
myoepithelial cells												
(mucous)	–	–	–	4+(b)	4+(c)	4+(c)	–	–	–	4+(b)	4+(c)	4+(c)
(serous)	–	–	–	4+(a)	4+(b)	4+(c)	–	–	–	4+(a)	4+(b)	4+(b)
Intercalated duct												
epithelial cells	4+(c)	4+(c)	4+(c)	4+(c)	4+(c)	4+(c)	1+(c)	2+(c)	2+(c)	4+(b)	4+(b)	4+(b)
Striated duct												
epithelial cells	4+(c)	2+(b)2+(b)	4+(b)∫	4+(c)	4+(c)	4+(c)	1+(c)	2+(c)	2+(c)	2+(b)•	2+(b)•	1+(a)•
myoepithelial cells	–	–	–	–	–	–	–	–	–	–	–	–
Excretory duct												
epithelial cells	4+(c)•	4+(c)•	4+(c)•	4+(c)	4+(c)	4+(c)	2+(c)	2+(c)	2+(c)	3+(c)•	4+(c)3+(c)•	4+(c)
myoepithelial cells	–	–	–	4+(c)	4+(c)	4+(c)	4+(c)	4+(c)	4+(c)	–	–	–
basal cells	–	–	–	4+(c)	4+(c)	4+(c)	4+(a)	4+(b)	4+(b)	–	–	–

Legend: – = negative staining; 1+ = weak staining; 2+ = moderate staining; 3+ = strong staining; 4+ = very strong staining; a = 10-20% cells positive; b = 30-50% cells positive; c = majority cells positive; • = staining apical parts of cells; * = staining basal parts of cells; ♦ = staining single cells; ∫ = different staining single cells inside investigated field all investigated structures

Acinar cells

In the present study different patterns of CK expression were observed in acinar cells during postnatal development rats SSG. We found that the serous acinar cells showed an increased positive reaction for CK7 and CK8 in relation to mucous acinar cells. However, serous acinar cells showed a weak expression for mAbs specific for CK18 and kidney CK. Antibodies K8.12 did not react with acinar cells during ontogenesis, as well as and K8.13 with mucous acinar in the neonatal gland. A similar reaction was noted for PT13D11 mAbs.

Epithelial cells of the duct

The use of anti-CK mAbs demonstrated different expressions of particular CK polypeptides in the duct epithelial cells. Duct epithelial cells (intercalated, striated and excretory ductus) were labelled more intensive with K8.13 mAb during different morphological stages of SSG postnatal development (Figure 1). In contrast, K8.12 mAb, raised to CK pair 13/16, had a different distribution during ontogeny. Our data showed that K8.12 mAb weakly stained the intercalated and striated ducts of the neonatal animals, compared to increased intesitivity during postnatal development. In adult rats, these cells exhibited moderate staining for this mAb. Different immunoreactivity of K8.12 mAb was shown in epithelial cells of the excretory duct (Figure 2). This reactivity was intensive in epithelial cells near the basal membrane and their number increased during ontogenesis. In addition, CK8 mAb weakly stained the duct epithelial cells in relation to K8.13 mAb (Figure 3). Namely, this CK subunit was mainly a marker of basal parts of these cells and there were not changes in expression during the postnatal period. In contrast to CK8 mAb, CK18 mAb showed a strong and diffused reaction within the duct epithelium (Figure 4). During postnatal development there was a decrease of CK18 reactivity in single streated epithelial cells. CK7 mAb showed similar immunoreactivity with epithelial cells of this duct during ontogenesis. Kidney CK

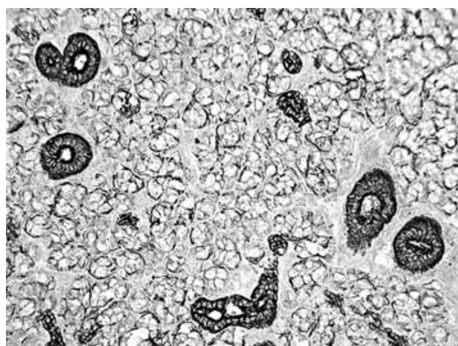

Figure 1. K8.13 mAb reacted with ductal cells and in the myoepithelial cells around acini (60 days), Streptavidin-biotin immunoperoxidase staining. x 20

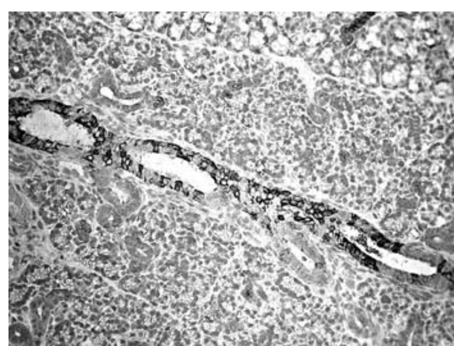

Figure 2. K8.12 mAb is expressed in single cells duct epithelium (30 days), Streptavidin-biotin immunoperoxidase staining. x 20

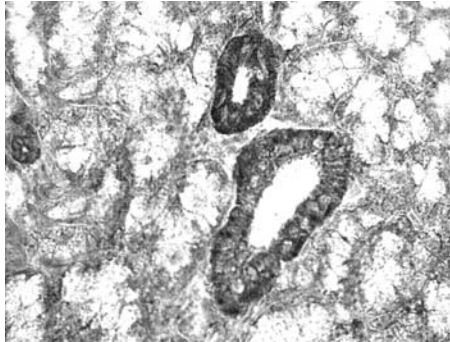

Figure 3. Note that CK8 mAb stains the ductal epithelium (30 days), Streptavidin-biotin immunoperoxidase staining. x 40

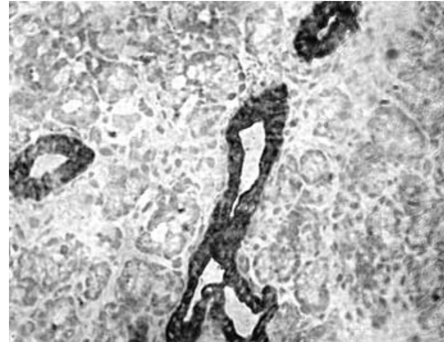

Figure 4. CK18 mAb immunoreaction within duct epithelial cells (60 days), Streptavidin-biotin immunoperoxidase staining. x 20

and PT13D11 mAbs showed similar immunorectivity in duct epithelial cells. Monoclonal antibodies specific for kidney CK that labelled strongly most of the epithelial cells of the intercalated duct, were also expressed in the apical parts of cells of the exrectory duct in adult rats (60 days) (Figure 5). Antigens recognized by PT13D11 mAb, very strong stained the intercalated duct and apical parts of epitehelial cells of the excretory duct.

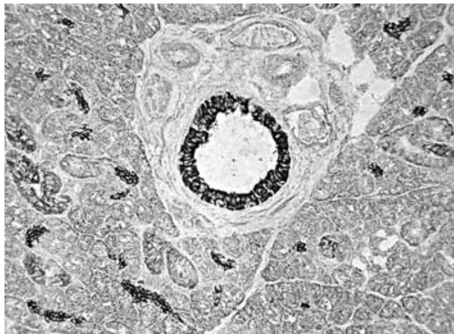

Figure 5. Immunostaining with CK-kidney mAb is expressed by intercalated duct cells and excretory duct (60 days), Streptavidin-biotin immunoperoxidase staining. x 10

Myoepithelial cells

The myoepithelial cells were located around the acinar cells and some of the duct cells. This immunohistological study showed the different expression of CK polypeptides in these cells.

In adult rats, myoepithelial cells of acini (serous and mucous) were stained by K8.13 mAb compared to neonatal patterns (Figure 1). The number of positive cells increased during ontogenesis. After 30 days we detected 30-50% myoepithelial positive cells in relation to adult animals. However, during postnatal

development, myoepithelial cells of the excretory duct showed a very strong rectivity with K8.12 and K8.13 mAbs. Myoepithelial cells of the acini were stained by PT13D11 mAb (Figure 6), whereas these cells were not stained by mAbs CK7, CK8, CK18 i CK kidney.

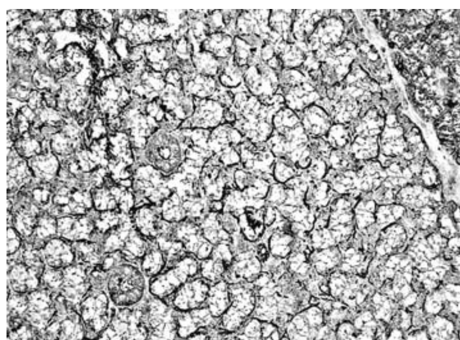

Figure 6. PT13D11 mAb reacts with myoepithelial cells of the mucous acini (30 days), Streptavidin-biotin immunoperoxidase staining. x 40

DISCUSSION

In this study, we applied a large panel of anti-CK mAbs and demonstrated that CK are differently expressed in postnatal development within rat SSG epithelium.

The data presented in this work showed different expressions of CK polypetides in acinar cells from the SSG. However, the reactivity of these antibodies did not differ during ontogenesis. In addition, weak labelled acinar cells were seen with mAbs CK18, K8.13 and kidney CK. In contrast, CK7 and CK8 mAbs showed different immunoreactivities. Their reaction in serous cells was more distinct than that in mucous cells. It can be assumed that weak staining of mucous acinar cells is probably a cosequence of the mucin present. In human SSG, acinar cell expressed CK7 and CK18 (Born *et al.*, 1987; Geiger *et al.*, 1987), while CK8 was not expressed (Born *et al.*, 1987). In distincion from these results, CK8 and CK18 are detected in epithelial cells of human glands (Draeger *et al.*, 1991; Gustafsson *et al.*, 1988; Ogawa, 2003), but CK7 was not reactive with these cells (Draeger *et al.*, 1991). Similar results on rabbit acinar cells were published (Farina and Zeda, 1992). Antibodies that recognize CK7 were not reactive with salivary gland acinar cells (Takahashi, 1994), but in this work we identified the presence of CK7 in acinar cells. We observed that K8.12 mAb raised to CK pair 13/16, and was not expressed in acinar cells. Similar results were published on rabbit (Farina and Zelda, 1992), human (Burns *et al.*, 1988; Born *et al.*, 1987; Geiger *et al.*, 1987), and rat SSG (Sumitomo *et al.*, 1996). These findings indicated that acinar cells (especial serous) possess CK 7,8 and 18 that are characteristic for simple epithelia. Serous and mucous acinar cells were labelled by K8.13 mAb which recognises CK 1, 5,6,7,8,10,11,18. Based on these results, it can be assumed that K8.13mAb could react with CK pair 8/18 and CK7 in acinar cells of rat SSG.

During postnatal development of rat SSG, a more complex CK organization was found in the duct epithelial cells. Our data showed that, CK subunit was present in simple epithelia, and is more expressed in the ductal epithelial than in the acinar cells. Immunoreactivity of mAbs for the selective CK polypeptides 7, 8, 18 was detected in all parts of the duct system in human salivary glands (Draeger *et al.*, 1991; Li *et al.*, 1996; Martins *et al.*, 2002). It was shown that CK18 mAb was strongly labelled in the duct epithelium, however, reactivity of this mAb decreased in the epithelial streated duct in adult rats (day 60). The specific expression of CK18 within the duct epithelium was confirmed by reserches on adult rabbit SSG (Farina and Zelda, 1992; Ogawa *et al.*, 2001), and human salivary glands (Ogawa, 2003; Kusama *et al.*, 2000; Fukuda *et al.*, 2001). In relation to CK18, we have demonstrated a weak immunoreactivity of CK8 mAt in duct epithelial cells. These findings have been confirmed by Ogawa *et al*. (2003) on human salivary glands. Reactivity of CK8 mAb was not changed during postnatal development, but we have shown that this antibody was mainly expressed in the basal part of cells. We found CK7 mAb expression in the epithelial duct, but there were differences in distribution during ontogeny. Namely, in the neonatal period, CK7 is more expressed in the streated duct, whereas in adult rats, its expression in streated and excretory epithelal cells is similar with CK8 mAb. On the contrary, Takahashi *et al.* (1994) has found that CK7 is present only in the epithelial duct of SSG in rats, but was not present in acinar cells. Similar to our results, Li *et al*. (1996) detected CK7 in basal cells present in the duct of human SSG. We have found that K8.13 mAb is a panepithelial marker. Immunoreactvity of this mAb, which recognizes the CK polypeptides 1, 5, 6, 7, 8, 10, 11 was postive in all epithelial cells in rat SSG, including myopithelial and basal cells. Similar results were published on human SSG (Geiger *et al.*, 1987). K8.13 mAb lebelled strongly most of the ductal epithelium in the adult and neonatal rat. In contrast, K8.12 mAb (recognizing CK pairs 13/16) strongly stained basal cells of the rat excertory duct. However, a number of postive basal cells was increased during the postanatal period. This CK pair is also detected in the basal cells of human salivary glands (Burns *et al.*, 1988; Born, 1987; Geiger *et al.*, 1987). A weak positive reactivity of K8.12 mAb was found in luminal duct cells and has been identified by other investigators (Burns *et al.*, 1988; Sumitomo *et al.*,1996). The present results may indicate that CK18 and K8.13 mAbs are the immunohistochemical markers of duct epithelial cells in rat SSG, while CK7 and CK8 mAbs is mainly expressed in adult rats on the basal portion of cells. Basal cells represent a special cell type of the duct system and they are absent from the acinar compartment. These cells were detected by K8.12 mAb, but thay also contain CK polypeptides specific for simple epithelia. Based on these findings, it can be assumed that basal cells contain CK polypeptides specific for simple and stratified epithelia.

The important component of the histophysiological structure of salivary glands are myoepithelial cells, which contain intermediary keratin filaments (Franke *et al*., 1980; Ogawa *et al*., 2003) and numerous myofilaments (Ogawa *et al*., 1999; 2003). In our immunohistochemical analysis we showed phenotypic heterogenity of myoepithelial cells. The CK profile of these cells is different in relation to CK composition of other cells in mammals. By virtue of our results we

concluded that myoepithelial cells of rat SSG do not contain CK specific for simple epitehlia. Similar results are documented by other studies (Ogawa, 2003). In this work we identified CK pair 13/16 in myopethelial cells, but also CK 4, 5, 6 as markers for stratified squamous epithelium.

PT13D11 mAb, specific for rat epithelia, was produced in our laboratory. We did not find literature data which describes similar results of this mAb in the rat SSG. PT13D11 mAb stained the myoepithelial cells around acinar and epithelial cells of the intercalated duct.

CONCLUSION

1. The acinar cels showed a positive reaction with mAbs specific for CKs of simple epithelia (CK8 i CK18) and K8.13 mAb which recognize a group of CKs.

2. Epithelial cells of intercalated, streated and excretory duct of rat SSG contain CKs (CK 7, 8, 18, CK kidney and CKs reacted with K8.13 i K8.12 mAb) that are characteristic for simple and stratified epithelia. These cells express antigens that were detected by PT13D11 mAb.

3. The myoepythelial cells were located around the acinar cells and some duct cells, and have similar characteristics to duct epithelial cells.

4. Antigen characteristics of rat SSG have been changed during ontogenesis. The most important changes occur during postnatal development in a number of positive myoepithelial cells as the expression of their antigens is increasing.

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IMUNOHISTOHEMIJSKA IDENTIFIKACIJA CITOKERATINA U SUBMANDIBULARNOJ PLJUVAČNOJ ŽLEZDI PACOVA U TOKU ONTOGENEZE

DOŽIĆ I, TODOROVIĆ TATJANA, DOŽIĆ B i ČOLIĆ M

SADRŽAJ

Citokeratini (CK) su grupa složenih proteina koji se sastoje od najmanje 20 različitih polipeptida citoskeleta, specifični za običan i složeni epitel skoro svih tkiva. Do skoro je bilo dostupno samo nekoliko antitela specifičnih za proteine citoskeleta i zbog toga je bila otežana vizualizacija komponenti postnatalne citodiferencijacije pljuvačnih žlezda.

U ovoj studiji su, pomoću panela monoklonskih antitela (mAt), analizirane morfološke i imunofenotipske osobine CK polipeptida, u submandibularnoj pljuvačnoj žlezdi (SPŽ) pacova tokom ontogeneze.

Submandiblarne pljuvačne žlezde uzimane su od soja AO pacova, muškog pola, starosti 1, 30 i 60 dana. U ovom radu korišćena je imunohistohemijska metoda streptavidin-biotin peroksidaznog bojenja. Tkivni preseci fiksirani su u acetonu i inkubirani sa mAt specifičnim za CK polipeptide (CK7, CK 8, CK 18, CK bubrega, K8.12, K8.13) i epitel pacova (PT13D11).

U ovoj imunohistohemijskoj analizi, ekspresija anti -CK antitela na SPŽ pacova, pokazuje fenotipsku heterogenost određenih komponenti ove žlezde tokom postnatalnog perioda. Epitelne ćelije acinusa su CK18+, dok epitelne ćelije kanalića imaju izražene CK definisane sa K8.12 i K8.13 mAt. Epitelne ćelije acinusa nisu bile obojene sa K8.12 i PT13D11 mAt. Umetnuti kanali su bili izrazito CK bubrega+ i PT13D11+. Mioepitelne ćelije acinusa su bile obojene sa K8.13 i PT13D11 mAt, dok su ove ćelije imale negativnu reakciju sa mAt specifičnim za obični epitel.

Ova imunohistohemijska studija pokazuje heterogenost u ekspresiji CK polipeptida i može poslužiti za razumevanje porekla, razvoja i funkcije epitelnih ćelija SPŽ pacova.