Phenotypic Changes of Calponin in Salivary Gland Myoepithelial Cells during Postnatal Development of Rats

Miyako Namba¹, Kazuto Yamada^{1,2}, Wataru Kudeken¹, Gho Mizutani¹, Kouji Tsukitani³, Masahiko Mori¹ and Yoshiaki Takai¹

¹Department of Oral and Maxillofacial Surgery, Asahi University School of Dentistry, Gifu 501–0296 and ²Department of Oral and Maxillofacial Surgery, Fukui Red Cross Hospital, Fukui 918–8501 and ³Department of Oral & Maxillofacial Surgery, Kyoto Prefectural University of Medicine, Kyoto 602–0841

Received July 31, 1998; accepted March 17, 2000

Calponin (CALP) is a 34 kDa protein which interacts with actin, tropomyosin and calmodulin, and regulates contractive roles of smooth muscle cells. The present study deals with the expression of CALP and actin in myoepithelial cells (MECs) during postnatal development of rat salivary glands. In the postnatal development of MECs, the specimens obtained from submandibular (SMG) or sublingual glands (SLG) on 0 to 84 days old rats were examined by immunocytochemistry. MECs detected by CALP and actin were observed from day 0 to mature salivary glands, and the expressions of actin immunoreaction in MECs of salivary glands on days 5 or 7 were higher than those of CALP,

while in MECs of salivary glands of rats over 5 weeks old, the expressions of CALP were a little higher than in actin. MECs in SMGs and SLGs during the postnatal development were characterized by existence of CALP immunoreactivity, whereas actin immunostaining was strongly localized in the smooth muscle cells of the blood vessels. No MECs detected with CALP and actin were observed in ductal segments including striated ducts and granular convoluted tubules (GCT), suggesting that the secretion from GCT cells was not related to CALP and actin. The CALP immunoreactivity is a useful marker for MECs in salivary glands in the rat.

Key words: Myoepithelial cells, Calponin, Developmental salivary gland, Rat

I. Introduction

Secretory portions of exocrine glands, such as salivary, mammary or sweat glands, are commonly associated with myoepithelial cells (MEC) showing long cytoplasmic processes around the acini [6, 19]. Functional roles of MECs are considered to be contraction and secretion from terminal acinar segments. Histochemical properties of MECs in salivary glands were described as cells that characteristically include smooth muscle proteins of actin and myosin as contractive proteins suggesting contractive roles [4, 6]. For enzyme histochemical studies, MECs of salivary glands in mice and rats showed alkaline phosphatase activity, and those in human beings were positive for adenosine triphosphate activity [15, 16].

Calponin (CALP) is a 34 kDa protein bound to actin, calmodulin and tropomyosin, and they are purified from

vertebrate smooth muscle tissues. CALP might regulate contractive functions [3, 5, 7]. It has been reported that CALP is a specific marker for identification of human smooth muscle cells and myoepithelial cells of breast or salivary glands [11].

The present study deals with CALP immunoreactivity in MECs of submandibular or sublingual salivary glands at postnatal developing stages in rats, and CALP immunostaining compared to that of actin immunostaining.

II. Materials and Methods

Materials

A total of 130 Sprague-Dawley rats of both sexes were used. Groups of 5 rats were sacrificed on 0, 1, 3, 5 days old and 1, 2, 3, 4, 5, 6, 7, 8 and 12 weeks old. The submandibular glands (SMG) and sublingual glands (SLG) were removed, and fixed in 10% neutral formalin at 20°C for 12 hr. The tissues were embedded in paraffin and 4 µm paraffin sections were made for examining histology with hematoxylin-eosin stain and for detecting immuno-

Correspondence to: Dr. Kazuto Yamada, Department of Oral and Maxillofacial Surgery, Asahi University School of Dentistry, 1851 Hozumi-cho, Motosu-gun, Gifu 501–0296, Japan.

histochemistry of calponin (CALP) and smooth muscle actin.

Deparaffinized sections were immersed in 0.1% proteinase at room temperature for 10 min. After rinsing, they were treated with methanol containing 0.3% hydrogen peroxide for 20 min for blocking endogenous peroxidase. Following immersion in normal rabbit serum (1 : 20, Dako) for 30 min, they were treated with monoclonal anti-calponin antibody and anti-smooth muscle actin antibody. The primary antibodies against calponin (1 : 50, Dako) and smooth muscle actin (1 : 30, Dako) were applied at room temperature for 1 hr. After washing with PBS, biotinylated secondary antibody (1/200, Dako) was applied at room temperature for 30 min. After a further wash and incubation with peroxidase conjugated by streptavidin biotin complex kit (Dako), the reaction product was visualized by 3,3'-diaminobenzidine tetrahydrochloride containing 0.05% hydrogen peroxide. The sections were counter-stained with methyl-green. The sections for negative controls were incubated without the primary antibody.

III. Results

Histological features

The day 0 submandibular gland (SMG) consists of undifferentiated tubules and delicate connective tissue stroma including blood vessels. Nuclei of primary tubular cells were arranged at basal aspect (Fig. 1A). The day 0 sublingual gland (SLG) showed relatively differentiated mucous acini and undifferentiated striated ducts (Fig. 1D).

From day 5 postnatal development, undifferentiated tubule cells in the SMG had begun to mature and differentiate to proacinar cells. They showed nucleus arrangement at the periphery of tubule segments but no differentiation into the interrelated ducts and acinar cells (Fig. 2A). From the days 1 and 3 postnatal development, the histochemical features are similar to those of day 5 SMG. At the same stage, SLG (Fig. 2D) morphology revealed almost no changes as seen in the day 0 specimen.

In the specimens from week 1 to week 4, acinar cells of SMG were developed from proacinar and tubular cells of SMG, and nuclei were distributed in the periphery of acinar cells. SLG revealed well-developed mucous acinar cells, accompanying basal deluminal cells.

In week 5 SMGs, there are differentiating granular convoluted tubule (GCT) cells from immature ductal segments, and acinar cells were matured (Fig. 3A). GCT cells were not well-developed. The week 5 sublingual gland (SLG) showed differentiated mucous acini and ducts, but the size was smaller than adult acini and ducts (Fig. 3D).

In week 8 and later, SMGs composed of well-developed GCTs and acini, and a few striated duct cells (Fig. 4A). The week 8 sublingual gland (SLG) showed differentiated mucous acini and ducts (Fig. 4D).

Immunohistochemical features of calponin and actin

Calponin (CALP) immunoreactivity in the SMG and the SLG was strongly confined to the myoepithelial cells and was weak positive for smooth muscle cells of blood vessels. Actin immunoreactivity in both salivary glands was characteristically expressed strongly in myoepithelial cells and smooth muscle cells of blood vessels.

In day 0 salivary glands (SMG and SLG), immunoreactivities of CALP and actin were positive in myoepithelial cells, showing delicate fibrillar shapes in the submandibular glands (Fig. 1B, C). CALP staining in the developing submandibular glands was very weak (IE).

In the day 5 glands, an immunostaining of myoepithelial cells was strong for CALP and actin (Fig. 2B, C, E, F) in SMGs and SLGs. Myoepithelial cells were developed at the periphery of undifferentiated tubules and proacinar cells in the SMGs showed spindle and triangular shapes (Fig. B, C). Those in SLGs were also well-developed. No immunoreactivity in the peri-ducta area was observed. During postnatal development of SMG, the MEC obtained from days 5–7 showed spindle and dendritic shapes. Immunoreactivity of actin was the highest and that of CALP was much lower (Fig. 2B, C).

In the 2 to 4 week old SMGs and also in the 5 week old SMGs, spindle and fibrillar shaped myoepithelial cells located around the acinar compartments and they showed conspicuous immunoreactions for CALP and actin. No immunoreaction products for CALP and actin were found in striated ducts and undifferentiated GCT segments (Fig. 3B, C). In SLGs of the same stages, CALP and actin, were immunopositive in myoepithelial cells, and showed in the periphery of matured acinar cells (Fig. 3E, F). Strongly immunopositive reaction for actin was expressed in smooth muscle zones of blood vessels in connective tissues of salivary glands (Fig. 3C, F).

In the 6 to 12 week old SMGs, gland structures were fully differentiated into granular convoluted tubule (GCT) segments, myoepithelial cells were immunostained with CALP and actin at the periphery of the acinar compartment. No reaction product for CALP and actin was found in myoepithelial cells in GCTs (Fig. 4B, C). In SLGs of the same stage, well-developed myoepithelial cells were markedly positive for CALP and actin (Fig. 4E, F).

IV. Discussion

Calponin is a 34 kDa protein which interacts with calmodulin in a Ca^{2+} dependent manner and with F-actin and tropomyosin in a Ca^{2+} independent manner, and the protein regulates the contraction of smooth muscle cells. Draeger *et al.* [3] described that the existence of phenotypically specific CALP during the differentiation of smooth muscle cells is not synchronized but temporally separated, whereas actin or myosin isoforms belong to the early differentiation markers, and the CALP is detected only in differentiated smooth muscle cells. Frid *et al.* [5] noted similar features, that actin and myosin (heavy chain)

appeared firstly in the aortic smooth muscle cells with CALP expression, and caldesmon appeared at later stages, and they postulated that actin and myosin play a structural role in smooth muscle cells, whereas CALP and caldesmon were important for the regulation of contraction and might be useful markers of late events in the process of aortic smooth muscle differentiation and maturation.

In the present study, the expression of actin and CALP in developing myoepithelial cells of the salivary glands was described in different postnatal stages of rats.

The present report was the first to evaluate immunostaining intensity for CALP in different stages of postnatal developing myoepithelial cells of SMGs and SLGs. Existence of myoepithelial cells in SMGs and SLGs was detected from the day 0 specimens revealed by CALP and actin immunostaining, although histological features only showed immature types of cells. The MEC detected with CALP and actin was the same in shapes and immunostaining features (Fig. 1B, C). During the postnatal development of rat SMGs, the gland tissue obtained from

Fig. 1. The day 0 of submandibular gland (SMG) (A–C) and sublingual gland (SLG) (D–F). $\times 200$. **A:** Histological feature shows undifferentiated tubule cells and nuclei arranged in the peripheral area of the tubule. **B:** CALP immunostaining. MECs show delicate linear patterns located around the tubular cells. **C:** Actin immunoreactivity. Actin immunostaining is observed as periphery of tubules. **D:** H & E staining. The day 0 sublingual glands. Relatively differentiated acini and undifferentiated ducts are observed. **E:** CALP immunostaining. Very slight immunoreactivity is observed. **F:** Actin immunostaining is limited to myoepithelial cells.

Fig. 2. The day 5 SMG (A–C) and SLG (D–F). $\times 200$. **A:** Histological appearance shows tubular segments with proacinar cells and undifferentiated ducts (arrow). **B:** CALP immunoreactivity. Positive immunostaining of MECs around tubular segments is observed. **C:** Actin immunostaining. The strongest immunoreaction of MECs is found. **D:** SLG morphology shows relatively differentiated acinar cells and striated duct. **E:** CALP immunostaining. Positive immunoreaction is confined to MECs with slight levels. **F:** Actin immunostaining exist MECs.

Fig. 3. The week 5 specimens of SMG (A–C) and SLG (D–F). $\times 200$. **A:** Histological findings of the week 5 SMG show matured acinar cells and undifferentiated GCT cells. **B:** CALP immunoreactivity. The MEC of SMG is immunopositive for CALP immunostaining. **C:** Actin immunoreactivity. MECs and blood vessels immunostain strongly for actin. No immunoreaction product of CALP and actin is found in MECs of the ductal segments. **D:** The week 5 sublingual gland showed differentiated mucous acini and ducts, but the size was smaller than adult acini and ducts. **E:** CALP immunostaining in SLG. **F:** Actin immunostaining in SLG. Expression of CALP reaction is higher in MECs of the SLG than that of actin. Smooth muscle cells of blood vessels are immunostained strongly for actin.

Fig. 4. The week 8 SMG (A, B) and SLG (C, D). $\times 200$. **A:** In week 8 and later, SMGs composed of well-developed GCTs (arrow) and acini, and a few striated duct cells. **B:** CALP immunoreactivity is limited to MECs of SMG. No MECs exist in GCT segments. **C:** Actin immunoreactivity is present in MECs. The SMG duct including GCT shows no expression of CALP and actin. **D:** The week 8 sublingual gland (SLG) showed differentiated mucous acini and ducts. **E:** CALP immunostaining in SLG. MECs show marked CALP reaction. **F:** Actin immunostaining in SLG.

the day 5 and 7 displayed most differentiating MECs which had conspicuous immunoreactions for actin rather than CALP, indicating immature stages of MECs [3]. In the 5 week old specimens, CALP immunostaining of MEC in SLGs was stronger than actin immunostaining, but immunostainings of CALP and actin in SMGs were not different or a little stronger in actin staining.

Actin distribution and phalloidin fluorescence of MECs in fetal human salivary glands have been reported by Lee *et al.* [12]. In their study MECs began to develop 15–16 week gestation stages when acinar cells were still immature, and primitive MECs showed polyhedral shapes and matured MECs showed dendritic shapes. In the present result, immature myoepithelial cells showed polyhedral as spindle and triangular in shapes. It has been reported that MECs could be detected by phalloidin fluorescence and actin protein [20]. The MECs in human fetal SMGs expressed actin before the acinar cell differentiation. In the present study, MECs detected with actin immunostaining were demonstrated in day 0 rat SMGs and SLGs before the acinar cell maturation. Redman *et al.* [17] noted that in fetal rat salivary glands, MECs and secretory glandular cells have developed at the same time during the fetal stage.

In the week 5 to week 8 SMGs of the rats, existence of developing GCT segments was observed that expressed EGF, NGF and biologically active polypeptides [1, 8–10, 14]. No immunoreactivity for CALP and actin was observed in MECs of duct segments including striated ducts and GCT. For salivary gland secretion, MECs associated with acinar compartments were involved in contractive roles as a result of physiological functions for actin and CALP, but secretion from GCT cells, such as discharge of granules containing EGF and NGF, was not mediated by those contractile proteins [14].

The MEC in either postnatal developing or matured salivary glands was characterized by an existence of immunocytochemically detectable CALP, and smooth muscle cells in blood vessels were also weakly immunopositive for CALP. On the contrary, actin immunoreactivity was obvious in both MECs and smooth muscle cells of blood vessels. The CALP immunostaining seems to be a significant biological marker for MECs in tissue sections.

The immunohistochemical approach using CALP has been described in MEC and stromal cells of human breast tissues and tumors [2, 11] and pleomorphic adenoma of salivary gland [16]. Further investigations of biological significance for modified and neoplastic MECs of salivary gland tumors could be explored with the use of CALP staining for the comparison of other immunohistochemical markers.

V. References

1. Barka, T.: Biologically active polypeptides in submandibular glands. *J. Histochem. Cytochem.* 28; 836–859, 1980.
2. Deugnier, M. A., Moiseyeva, E. P., Thiery, J. P. and

- Glukhova, M.: Myoepithelial cell differentiation in the developing mammary gland; Progressive acquisition of smooth muscle phenotype. *Develop. Dynamics* 204; 107–117, 1995.
3. Draeger, A., Gimona, M., Stuckert, A., Celis, J. E. and Small, J. V.: Calponin: Developmental isoforms and a low molecular weight variant. *FEBS Lett.* 291; 24–28, 1991.
4. Drenckhahn, D., Groschel-Stewart, U. and Unisicker, K.: Immunofluorescence microscopic demonstration of myosin and actin in salivary glands and exocrine pancreas of the rat. *Cell Tissue Res.* 183; 273–279, 1977.
5. Frid, M. G., Shekhonin, B. V., Kotliansky, V. E. and Glukhova, M. A.: Phenotypic changes of human smooth muscle cells during development: Late expression of heavy caldesmon and calponin. *Develop. Biol.* 153; 185–193, 1992.
6. Garrett, J. R. and Emmelin, N.: Activities of salivary myoepithelial cells: A review. *Med. Biol.* 57; 1–28, 1979.
7. Gimona, M., Herzog, M., Vandekerckhove, J. and Small, V.: Smooth muscle specific expression of calponin. *FEBS Lett.* 274; 159–162, 1990.
8. Gresik, E. W.: Postnatal development changes in submandibular glands of the rats and mice. *J. Histochem. Cytochem.* 28; 860–870, 1980.
9. Gresik, E. W. and Barka, T.: Immunocytochemical localization of epidermal growth factor in mouse submandibular gland. *J. Histochem. Cytochem.* 25; 1027–1035, 1977.
10. Gresik, E. W. and Barka, T.: Immunocytochemical localization of epidermal growth factor during the postnatal development of the submandibular gland of the mouse. *Am. J. Anat.* 151; 1–10, 1978.
11. Lazard, D., Sastre, X., Frid, M. G., Glukhova, M. A., Thiery, J. P. and Kotliansky, V. E.: Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. *Proc. Natl. Acad. USA* 90; 999–1003, 1993.
12. Lee, S. K., Hwang, J. O., Chi, J. G., Yamada, K. and Mori, M.: Prenatal development of myoepithelial cells of human submandibular gland observed by immunohistochemistry of smooth muscle actin and rhodamine-phalloidin fluorescence. *Path. Res. Pract.* 189; 332–341, 1993.
13. Mori, M., Takai, Y. and Kunikata, M.: Review: Biologically active peptides in the submandibular gland.—Role of the granular convoluted tubule—. *Acta Histochem. Cytochem.* 25; 325–341, 1992.
14. Mori, M., Hamada, K., Naito, R., Tsukitani, K. and Asano, K.: Immunohistochemical localization of epidermal growth factor in rodent submandibular glands. *Acta Histochem. Cytochem.* 16; 546–558, 1983.
15. Palmer, R. M.: The identification of myoepithelial cells in human salivary glands. A review and comparison of light microscopical methods. *J. Oral Pathol.* 15; 221–229, 1986.
16. Palmer, R. M., Lucas, R. B., Knight, J. and Gusterson, B.: Immunocytochemical identification of cell types in pleomorphic adenoma, with particular reference to myoepithelial cells. *J. Pathol.* 146; 213–220, 1985.
17. Redman, R. C. and Ball, W. D.: Differentiation of myoepithelial cells in the developing rat salivary gland. *Am. J. Anat.* 156; 543–566, 1979.
18. Savera, A. T., Gown, A. M. and Zarbo, R. J.: Immunolocalization of three novel smooth muscle-specific proteins in salivary gland pleomorphic adenoma: Assessment of the morphogenetic role of myoepithelium. *Modern Pathol.* 10; 1093–1100, 1997.
19. Tamarin, A.: Myoepithelium of the rat submaxillary gland. *J. Ultrastruct. Res.* 16; 320–338, 1966.
20. Wulf, E., Deboen, A., Bautz, F. A., Faulotich, H. and Wieland, T. H.: Fluorescent phalloidin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA* 76; 4498–4502, 1979.