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Localization of FGF-6 and FGFR-4 during prenatal and early postnatal development of the mouse sublingual gland

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Abstract: A number of fibroblast growth factors (FGFs) are involved in regulatory mechanisms of the salivary gland development. However, the role of FGF-6 unique in myogenic cells has not been elucidated in the developing sublingual gland. In the present study, temporo-spatial expression of FGF-6 and its receptor (FGFR)-4, in conjunction with some related histochemical properties, were investigated in the sublingual gland of the prenatal and early postnatal mice. The earliest expression of both FGF-6 and FGFR-4 was detected in immature acinar cells at gestational day 17 (GD17). The staining intensity increased gradually and some acinar cells showed a distinct staining at postnatal day 0 (PD0). The immunopositive cells had a relatively round profile and were assumed to be acinar cells. The positive staining decreased thereafter and disappeared completely by PD11. To confirm the identity of cells positive for FGF-6, double immunolabeling with anti-αsmooth muscle actin (αSMA) and anti-FGF-6 antibodies was performed. The positive staining of αSMA, a marker of myoepithelial cells, was detected in the flattened cells surrounding the acini but not in the cells positive for FGF-6. The staining properties of secretory granules in acinar cells were also examined with periodic acid-Shiff (PAS) and alcian blue (AB). PAS-positive granules abundant in the late gestational stages (GD17 to PD0) began to be replaced with AB-positive mucous granules at early neonatal days (PD0-3), when the FGF-6/FGFR-4 expression was the strongest. These findings suggest that FGF-6/FGFR-4 might be involved in the changes of secretory granule content of acinar cells in the sublingual gland during the late gestational and early neonatal stages. (J. Oral Sci. 48, 9-14, 2006)

Keywords: FGF-6/FGFR-4; sublingual gland development; PAS; immunohistochemistry; αSMA.

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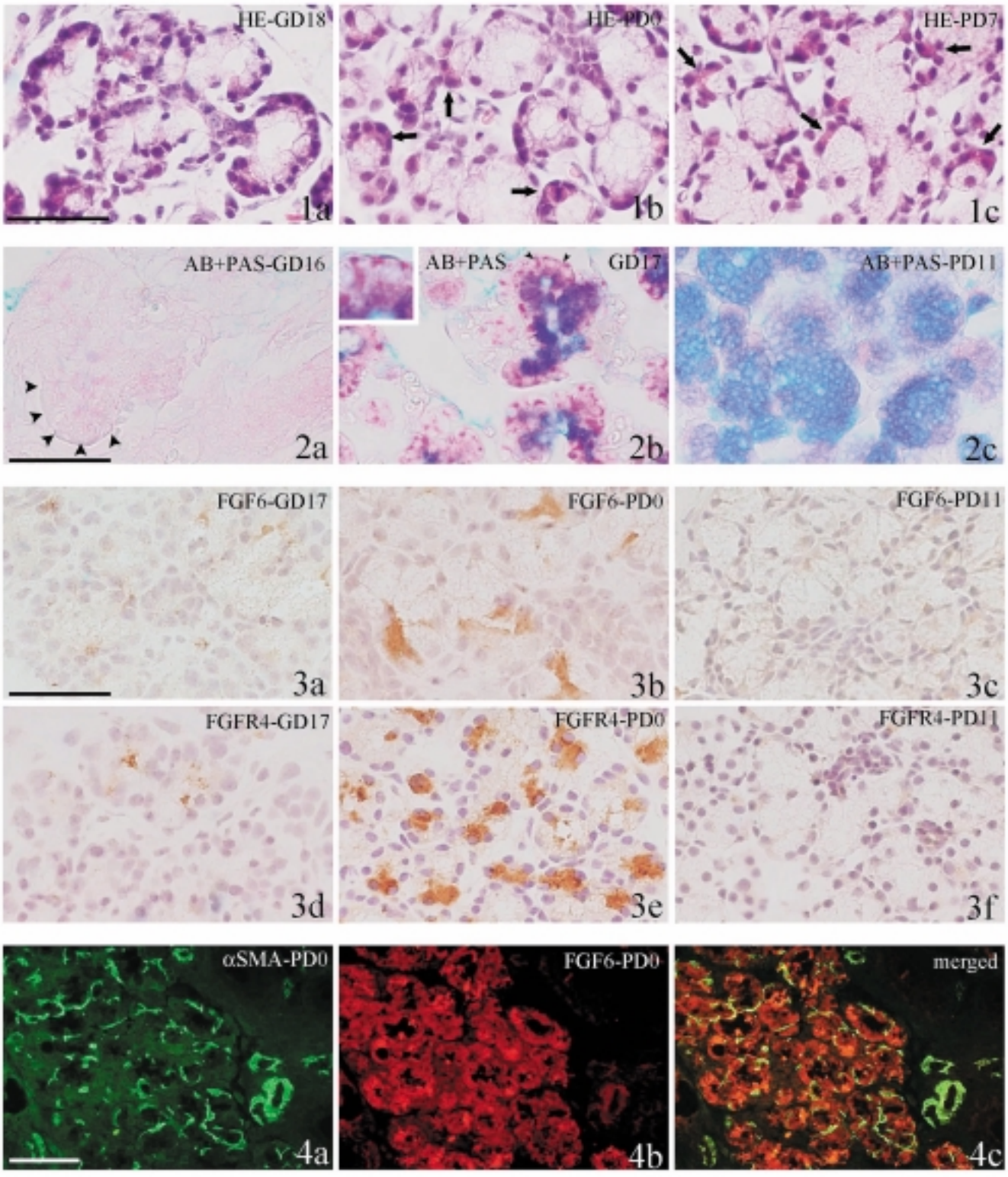
Introduction

The secretory units of the sublingual gland mainly consist of two different cell types; mucous and serous cells. These two cell types share a common lumen initially and then serous cells begin to accumulate in the distal periphery of acini to form serous demilune (1-4). In addition to these two cell types, several other specific cell lineages such as myoepithelial cells and oncocyte are observed, although the biological functions of these cells are not fully elucidated.

The development of the salivary glands is precisely controlled by a number of factors (5-7). Out of these, fibroblast growth factors (FGFs) consisting of 22 family members (8) play crucial roles. For instance, FGF-10/FGFR-2b signaling is important for morphogenesis of embryonic submandibular gland (9). FGF-2/FGFR-1, FGF-8/FGFR-2IIIc or FGF-10/FGFR-2b signalings contribute to the bud formation and branching of embryonic submandibular gland (10,11). FGF-6 has a unique characteristic. The molecular structure of FGF-6 is very similar to that of the other members of FGFs (8), however its expression is found exclusively in myogenic cells and appears to be crucial in the differentiation of fetal muscle masses (12-15). Myoepithelial cells are known to express muscle-specific proteins and are believed to be epithelially-derived cells (16-19).



In this study, the temporo-spatial expression of FGF-6/FGFR-4 is examined in the developing mouse sublingual gland. It has been discussed whether these molecules are potentially involved in the development of sublingual gland and, especially, in the myoepithelial cell differentiation.

Materials and Methods

Animals and tissue preparation

Pregnant ddY mice were purchased from Sankyo Laboratory, Japan, and maintained under pathogen-free conditions in the Animal Facility of Nihon University

Fig. 1 HE stainings of mouse sublingual gland.
(a) The immature acinar cells have large nuclei and cuboidal cytoplasms at GD18. (b) Acini are capped with eosinophilic demilune cells (arrows) at PD0. (c) Demilune cells (arrows) have a flattened shape at PD7. Scale bar = 25 μm. Magnification ratios of a-c are the same.

Fig. 2 PAS and AB double stainings of the mouse sublingual gland.
(a) No positive stainings were detected at GD16 (arrowheads show the outline of acini). (b) At GD17, the basal side cytoplasm of acinar cells was positively stained with PAS (inset shows the cell indicated by arrowheads; oil immersion image). (c) At PD11, acinar cells showed positive reactions only for AB. Scale bar = 25 μm. Magnification ratios of a-c are the same.

Fig. 3 Immunohistochemical stainings of mouse sublingual gland with anti-FGF-6 and anti-FGFR-4.
(a, d) Both FGF-6 and FGFR-4 were detected first at GD17. (b, e) The strong positive reactions were observed at PD0. (c, f) No positive reactions were seen at PD11. Scale bar = 25 μm. Magnification ratios of a-f are the same.

Fig. 4 Immunofluoresence stainings of mouse sublingual gland with anti-αSMA and anti-FGF6.
(a) Anti-αSMA antibody stained the flattened cells surrounding the acini. (b) Anti-FGF-6 antibody stained the acinar cells. (c) No overlapped stainings was observed, when the green fluorescein (a) was superimposed on the red (b). Scale bar = 25 μm. Magnification ratios of a-c are the same.

School of Dentistry. The mice were anesthetized intraperitoneally with pentobarbital sodium (100 mg/kg body weight). Developing sublingual glands were excised from fetuses at 16, 17, 18 days of gestation (GD16, 17, 18; n = 4) and from postnatal animals at 0, 1, 3, 7, 11, 14 days (PD0, 1, 3, 7, 11, 14; n = 3). Specimens fixed in 4% paraformaldehyde for 16 hrs were embedded in paraffin and processed for Hematoxylin-Eosin (HE) staining. Histochemical analysis was performed with periodic acid-Schiff (PAS) and alcian blue (AB) according to the procedures described (20). Specimens embedded immediately in OCT compound (Sakura Finetechnical, Tokyo) and frozen in liquid nitrogen were used to prepare cryosections for immunohistochemical examination.

Antibodies

Rabbit polyclonal antiserum to human FGF-6 and goat polyclonal antiserum to mouse FGFR-4 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used at 1 : 100 dilution. Goat polyclonal antiserum to human FGF-6 from R & D systems (USA) was used at 1 : 75 dilution (14). Mouse monoclonal antibody to human αsmooth muscle actin (αSMA) from Dako Cytomation (clone 1A4, Kyoto, Japan) was used at 1 : 200 dilution (21). Secondary antibodies, horseradish peroxidase (HRP)-conjugated goat polyclonal antiserum to rabbit IgG and HRP-conjugated rabbit polyclonal antiserum to goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated goat polyclonal antiserum to mouse IgG was purchased from Upstate (MA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat polyclonal antiserum to mouse IgG and tetramethyrhodamine isothiocyanate (TRITC)-conjugated goat polyclonal antiserum to rabbit IgG were purchased from Chemicon (USA). Secondary antibodies were used at 1 : 100 dilution except from antiserum to mouse IgG, which was diluted to 1 : 400. These antibodies were diluted with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS; pH 7.35).

Immunohistochemistry

Cryosections, 8 μm in thickness, were fixed with ice-cold acetone for 15 min, and incubated for 30 min with 0.3% H_2O_2 to block endogenous peroxidase activity and then for 30 min with 1% BSA-PBS to block the nonspecific binding. The sections were incubated overnight at 4°C with each primary antibody diluted with 1% BSA-PBS. These sections were washed extensively with PBS and incubated with the HRP-conjugated secondary antibodies for 60 min at room temperature. After washing with PBS, the sections were incubated with 0.42 mg/ml of 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, MO, USA) and 0.001% H_2O_2 solution for 15min at room temperature. The immunostained sections were washed with PBS and counterstained with hematoxylin. Controls for immunostaining were performed by substituting the primary antibodies with 1% BSA-PBS.

For immunofluorescence staining, cryosections were incubated with rabbit polyclonal antiserum to human FGF-6 and mouse monoclonal antibody to human αSMA overnight at 4°C. After washing with PBS, the sections were incubated with FITC- and TRITC-conjugated secondary antibodies for 1 hr at room temperature and washed with PBS. The stained sections embedded in mounting medium (Kirkegaard & Perry Laboratories, WA, USA) were examined with an epifluorescence microscope (ECLIPSE E600; Nikon, Tokyo).

Results

HE staining

HE staining results of the developing sublingual glands are shown in Figure 1. At GD18, acinar cells showed large nuclei and cuboidal cytoplasm and appeared still immature. A few eosinophilic cells were sparsely distributed among those immature cells (Fig. 1a). The number of acini increased as development proceeded, and most acini observed at PD0 were capped with relatively large eosinophilic, demilune cells (Fig. 1b). However, by PD7, serous cells in the form of demilune decreased in their size and became flattened. In contrast, the size of the mucous acinar cells increased (Fig. 1c).

PAS and AB staining

The PAS and AB staining properties of acinar cells showed marked changes during development. At GD16, immature acinar cells were completely negative with PAS and AB staining (Fig. 2a), but only one day later, at GD17, the cells became positive (Fig. 2b). PAS-positive granules were numerous in the basal side of cytoplasm, and AB staining was detected in the apical side. The number of PAS-positive granules in acinar cells decreased gradually and PAS-positive granules became preferentially located in the basal side of the acinar cells at PD0. PAS-positive granules disappeared by PD11 and the cytoplasm of mucous acinar cells were densely occupied with AB-positive granules (Fig. 2c).

Immunohistochemical staining

FGF-6 and FGFR-4 were first detected at GD17 in the cytoplasm of acinar cells (Fig. 3a, d). The staining intensity increased as development proceeded and was strongest during the period from PD0 to PD3 (Fig. 3b, e). Positive staining of FGF-6 was observed in the lumen and cytoplasm of acinar cells (Fig. 3b), while FGFR-4 staining was observed only within the cytoplasm (Fig. 3e). There are some differences in the distribution of FGF-6 and FGFR-4, however their temporal expression pattern was coordinated with each other. The intense staining of FGF-6 and FGFR-4 and the number of positive acinar cells decreased after PD3 and the distinct staining disappeared by PD11 (Fig. 3c, f).

Two different kinds of antibodies to FGF-6 were used in this study and no difference was found in their staining patterns (data not shown). Moreover, control sections incubated with nonimmune serum in place of first antibodies lacked specific staining as described above.

In the double immunolabeling with anti-αSMA and anti-FGF-6, anti-αSMA conjugated with FITC visualized green flattened cells surrounding acini (Fig. 4a). On the other hand, anti-FGF-6 conjugated with TRITC stained reddish acinar cells (Fig. 4b). In the images exposed doubly for FITC and TRITC, no overlapped staining was observed (Fig. 4c), indicating that αSMA-positive, myoepithelial cells were distinct from FGF-6-positive acinar cells.

Discussion

It is believed that the expression of FGF-6 is restricted to myogenic cells (12-15). The only exception reported so far is human prostate gland in which non-muscle cells have been shown to express FGF-6 in a pathological condition (22). Thus, FGF-6 expressed in the developing sublingual gland is likely to be detected in the myoepithelial cells. However, FGF-6-positive cells are morphologically indistinguishable from acinar cells. This notion is confirmed by double immunolabeling with anti-αSMA and anti-FGF-6; namely, αSMA-positive myoepithelial cells and FGF-6-expressing acinar cells were demonstrated as two distinct cell populations.

Another major finding in this study is that both FGF-6 and FGFR-4 have been localized in the developing sublingual gland in a temporo-spatially specific manner. Exact spatial distributions of these two molecules were slightly different. FGF-6 was localized in the lumen and in the cytoplasm of acinar cells and FGFR-4 was shown only within the cytoplasm. However, their temporal expression pattern was coordinated with each other, which suggests a developmental stage-specific functional correlation between FGF-6 and FGFR-4. Their highest expression was observed from PD0 to 3, which is late when compared with the expression of other FGF family members involved in the epithelial budding and/or branching morphogenesis of the salivary gland (9-11).

The functional significance of FGF-6/FGFR-4 expression in the sublingual gland is unknown. However, it is important to note that the timing of the expression of FGF-6/FGFR-4 corresponds to the onset of suckling. Histochemical analysis with PAS and AB staining in this study showed that a transient increase of PAS-positive secretory granules was temporally consistent with the initial expression of FGF-6 and FGFR-4. The PAS-positive granules in the late gestational stages decreased considerably and began to be replaced with AB-positive mucous granules in the early neonatal days (PD0-3), when FGF-6/FGFR-4 is highly expressed in the sublingual gland acini. These changes are likely to be related to the neonate suckling, or dietary intake of milk. The FGF-6/FGFR-4 might be involved, in concert with the other functionally related molecules, in the regulation of cellular changes which occur at birth in the sublingual gland.

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References

1. Leeson CR, Booth WG (1961) Histological, histochemical, and electron-microscopic observations on the postnatal development of the major sublingual gland of the rat. J Dent Res 40, 838-845
2. Taga R, Sesso A (1998) Postnatal development of the rat sublingual glands. A morphometric and radioautographic study. Arch Histol Cytol 61, 417-426
3. Wolff MS, Mirels L, Lagner J, Hand AR (2002) Development of the rat sublingual gland: a light and electron microscopic immunocytochemical study. Anat Rec 266, 30-42
4. Laj M, Borghese E, Di Caterino B (1971) Acinar ultrastructure of the sublingual gland of mus musculus during embryonic development. J Submicr Cytol 3, 139-151
5. Amano O, Yoshitake Y, Nishikawa K, Iseki S (1993) Basic fibroblast growth factor in rat salivary glands. Cell Tissue Res 273, 467-474
6. Hoffman MP, Kidder BL, Steinberg ZL, Lakhani S, Ho S, Kleinman HK, Larsen M (2002) Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis in vitro through BMP- and FGF-dependent mechanisms. Development 129, 5767-5778
7. Jaskoll T, Zhou YM, Chai Y, Makarenkova HP, Collinson JM, West JD, Hajihosseini MK, Lee J, Melnick M (2002) Embryonic submandibular gland morphogenesis: stage-specific protein localization of FGFs, BMPs, Pax6 and Pax9 in normal mice and abnormal SMG phenotypes in FgfR2-IIIc(+/Delta), BMP7(-/-) and Pax6(-/-) mice. Cells Tissues Organs 170, 83-98
8. Katoh M (2002) WNT and FGF gene clusters. Int J Oncol 21, 1269-1273
9. Jaskoll T, Abichaker G, Witcher D, Sala FG, Bellusci S, Hajihosseini MK, MelnickM (2005) FGF10/FGFR2b signaling plays essential roles during in vivo embryonic submandibular salivary gland morphogenesis. BMC Dev Biol 5, 11
10. Madan AK, Kramer B (2005) Immunolocalization of fibroblast growth factor-2 (FGF-2) during embryonic development of the rat submandibular gland. SADJ 58, 60-61
11. Steinberg Z, Myers C, Heim VM, Lathrop CA, Rebustini IT, Stewart JS, Larsen M, Hoffman MP (2005) FGFR2b signaling regulates ex vivo submandibular gland epithelial cell proliferation and branching morphogenesis. Development 132, 1223-1234
12. de Lapeyriere O, Ollendorff V, Planche J, Ott MO, Pizette S, Coulier F, Birnbaum D (1993) Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo. Development 118, 601-611
13. Han JK, Martin GR (1993) Embryonic expression of Fgf-6 is restricted to the skeletal muscle lineage. Dev Biol 158, 549-554
14. Zhao P, Hoffman EP (2004) Embryonic myogenesis pathways in muscle regeneration. Dev Dyn 229, 380-392
15. Coulier F, Pizette S, Ollendorff V, deLapeyriere O, Birnbaum D (1994) The human and mouse fibroblast growth factor 6 (FGF 6) genes and their products: possible implication in muscle development. Prog Growth Factor Res 5, 1-14
16. Franke WW, Schmid E, Freudenstein C, Appelhans B, Osborn M, Weber K, Keenan TW (1980) Intermediate-sized filaments of the prekeratin type in myoepithelial cells. J Cell Biol 84, 633-654
17. Gugliotta P, Sapino A, Macri L, Skalli O, Gabbiani G, Bussolati G (1988) Specific demonstration of myoepithelial cells by anti-alpha smooth muscle actin antibody. J Histochem Cytochem 36, 659-663
18. Lazard D, Sastre X, Frid MG, Glukhova MA, Thiery JP, Koteliansky VE (1993) Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. Proc Natl Acad Sci USA 90, 999-1003
19. Deugnier MA, Moiseyeva EP, Thiery JP, Glukhova M (1995) Myoepithelial cell differentiation in the developing mammary gland: progressive acquisition of smooth muscle phenotype. Dev Dyn 204, 107-117
20. Mowry RW (1963) The special value of methods that color both acidic vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-Schiff reaction. Ann NY Acad Sci 106, 402-423
21. Ogawa Y, Yamauchi S, Ohnishi A, Ito R, Ijuhin N (1999) Immunohistochemistry of myoepithelial cells during development of the rat salivary glands. Anat Embryol (Berl) 200, 215-228

22. Ropiquet F, Giri D, Kwabi-Addo B, Mansukhani A, Ittmann M (2000) Increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. Cancer Res 60, 4245-4250