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Expression of Uchl1 in the Dental Epithelium during Mouse Tooth Development

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Abstract : Tooth is formed by the reciprocal interactions between the ectoderm and ectomesenchyme derived from neural crest. It has not been clear that neuronal factors involved in the morphogenesis and differentiation of tooth.

To identify the roles of neuronal factors during the tooth development, the expression patterns and localization of Uchl1 were investigated in the developing mouse tooth germ by *in situ* hybridization and immunohistochemistry.

Uchl1 transcripts were weakly expressed in the oral epithelium and dental lamina at bud stage. However, expression of *Uchl1* was not found in the oral epithelium from cap stage and observed in the inner enamel epithelium, stellate reticulum and dental papilla. From the bell stage, *Uchl1* was expressed in the inner enamel epithelium and ameloblasts. Uchl1, was appeared to be localized in the inner enamel epithelium and differentiating ameloblasts of molar and incisors at neonates. Uchl1 was localized strongly in the fully differentiated ameloblasts and adjacent papillary layer whereas localized weakly in the odontoblasts of the molar at postnatal day 5. From these results, Uchl1 was expressed and localized in the differentiating dental epithelium and ameloblasts during tooth development.

The results suggest that neuronal protein, Uchl1 may play roles in the histo- and cyto-differentiation of non-neuronal dental epithelium.

Key words : Uchl1, Dental epithelium, Tooth, Development, Mouse

Introduction

Ubiquitin carboxyl-terminal hydrolase L1 (Uchl1) is a member of family of UCH isozymes (Wilkinson et al. 1989). The C-terminal hydrolase is an isopeptidase that releases ubiquitin from the ubiquitin-protein conjugates or after degradation of protein conjugated with ubiquitin in the ubiquitin-proteasome proteolytic pathway (Ciechanover 1994). This enzyme has been reported to be involved in cell fate (Huang et al. 1995), cell cycle (King et al. 1996), cell growth control (Zhu et al. 1996), or transcription activation (Verma et al. 1995). Since it has been isolated as a protein gene product 9.5 (PGP9.5) from the human brain extracts by means of two dimensional polyacrylamide gel electrophoresis (Jackson and Thompson 1981), Uchl1 represents a major component of the neuronal cytoplasm and was widely used as a general neuronal marker to define the pattern of innervation in brain (Wilson et al. 1988), respiratory epithelium (Iwanaga et al. 1992), teeth (Chri-

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stensen et al. 1993), oral mucosa (Hilliges et al. 1996), tongue (Wakisaka et al. 1996), gingiva (Ramieri et al. 1990) and skin (Karanth et al. 1991). Although it has been well known in the neuronal tissues, the localization of Uchl1 is not understood well in the non-neuronal tissues and their development. Previously, we reported that Uchl1 is distributed not only in all neural components of the head of neonatal mouse but also in the non-neural tissues, such as dental epithelium, bone forming cells together with epidermal growth factor receptor (Kim et al. 2003).

The mammalian tooth is formed by the reciprocal interactions between the oral epithelium and the neural crest-derived ectomesenchyme during development (Thesleff and Sharpe 1997). It has been well known that lots of transcription factors and signaling molecules such as BMP, Shh, FGFs mediate the morphogenesis and cytodifferentiation during tooth development (Thesleff 2003). Although epithelial organs except the specialized sensory organs, such as taste buds and eyes, developed free of neural components, it has been reported that some neuronal proteins are also identified in the dental epithelium during the morphogenesis and differentiation (Mitsiadis et al. 1993). In addition, the early reports suggest the roles of neurotrophins in tooth morphogenesis (Mitsiadis and Luukko 1995, Luukko 1998). However, it is not clear whether the neuronal proteins are locally synthesized in the dental epithelium and involved in the morphogenesis and differentiation of tooth.

Therefore, this study was aimed to investigate the expression of Uchl1 in the developing mouse tooth germs at the protein and transcripts level in order to define its roles in tooth morphogenesis and cytodifferentiation.

Materials and Methods

1. Tissue preparation

C57BL/6N mice were mated overnight, and the appearance of a vaginal plug was taken as day 0.5 of embryonic development (E0.5). The day of birth was designated as P0 (postnatal day 0).

The pregnant mice were killed by cervical dislocation or decapitation and E12.5～E18.5 embryos were carefully dissected in cold phosphate buffered saline (PBS). Whole embryos (E12.5～E13.5) or dissected heads were fixed overnight at 4°C in 4% paraformaldehyde/PBS (PFA, w/v). For whole-mount in situ hybridization, the tissues were washed three times with PBS containing 0.1% Tween-20 (PBT), dehydrated into 100% methanol and stored in methanol at −20°C until use. For frozen section, tissues were equilibrated with 30% sucrose/PBS (w/v) and embedded in OCT compound (Sakura, Torrance, USA). Cryostat sections (15～20 μm) were mounted on Superfrost slides (Fisher Scientific, Pittsburgh, USA) and stored in airtight boxes at −80°C.

For immunohistochemistry, embryos and neonatal mice (P0, P8 and P15) were immersed or perfused with a cold fixative composed of 4% PFA. After fixation, the tissues were decalcified with 10% ethylene diamine tetra-acetic acid (EDTA) for 7～10 days at 4°C. Then the tissues were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin. Five-micron thick sections were serially cut and then mounted onto precleaned slides.

2. Cloning and probe preparation

Uchl1 gene fragment was amplified by RT-PCR with total RNA isolated from E11.5 mouse head and designed oligonucleotide primer set (forward 5′-AAA GAG ATG CAG CTG AAG C-3′ and reverse 5′-CTG TAG AAC GCA AGA AGA CAG -3′). The *Uchl1* DNA amplicon was a 792-bp fragment spanning nucleotides between the 3 and 794 (Genbank NM_011670.1) and subcloned into the pBluescript II KS+ vector (Stratagene, La Jolla, USA). The plasmid was linearized with *Xba*I or *Hind*III for antisense or sense probe production, respectively. The RNA probes labelled with digoxigenin-11-UTP were then generated by *in vitro* transcription using T3 or T7 RNA polymerases (Roche Diagnostics GmbH, Mannheim, Germany). No specific signal was detected in the sections hybridized with control sense probes.

3. Whole mount and section *in situ* hybridization

In situ hybridization was carried out as described previously (Bok et al. 2005). Prepared whole embryos or dissected organs were treated with 5 : 1 methanol/30% hydrogen peroxide for 3～5 hrs at room temperature. After washes with methanol, the tissues were rehydrated through a methanol-PBT series and washed three times in PBT. The tissues were treated with 10 μg/mL proteinase K in PBT for 2～20 min at room temperature depending on the age of embryos, followed by two washes for 5 min each with PBT containing 2 mg/mL glycine. The tissues were refixed in 4% PFA/0.2% glutaraldehyde for 20 min at room temperature and then washed three times with PBT. The tissues were prehybridized for 1 hr at 70°C in hybridization buffer containing 50% formamide; 5× SSC, pH 4.5; 50 μg/mL tRNA; 50 μg/mL heparin; 1% SDS. The hybridization buffer was replaced, and single stranded RNA probes labelled with digoxigenin were added to 1 μg/mL, and the tissues were hybridized overnight at 70°C. The tissues were washed through Wash I (0.3 M NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 1% SDS) and Wash II (50 mM NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 0.1% SDS) for 30 min at 65°C, respectively. The tissues were blocked for 1 hr in 5% normal goat serum in TBST. Then tissues were incubated for 2 hrs with anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1 : 250 in blocking solution. After rinsing with TBST, tissues were equilibrated with NTMT (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween-20). Antibody was visualized by using the 4-nitroblue tetrazolinium chloride / 5-bromo-4-chloro-3-indolyl- phosphate (NBT/BCIP) blue color reaction. Prior to photography, tissues were refixed in 4% PFA and cleared in 75% glycerol.

Frozen sections were washed in PBT and treated for 10 min with 0.2N HCl and for 15 min with proteinase K (1 μg/mL in PBT). Sections were then washed in PBS, re-fixed for 15 min in 4% PFA/2% glutaraldehyde, and treated twice for 15 min with glycine (2 mg/mL in PBT). Sections were prehybridized for 1 hr at room temperature in hybridization buffer. Hybridization was performed at 70°C for 16 hrs in hybridization buffer containing 1.0 μg/mL riboprobes. Excess probe was removed by sequential washes in 2×SSC (30 min at 70°C), 0.1× SSC (1 hr at 65°C), and TBST (1.4 N NaCl, 27 mM KCl, 250 mM Tris, pH 8.0 and 0.1% Tween-20) twice at room temperature. Detection of hybridization was performed as the same procedures described in above. After detection, slides were quickly dehydrated through a graded series of ethanol, cleared in xylene and mounted with Permount (Fisher Scientific).

4. Immunohistochemistry

Immunostaining was performed by the labeled streptavidin-biotin (LSAB) method with a Histostain-plus kit (Zymed Laboratories, San Francisco, USA) as described previously (Kim et al. 2003). After deparaffinization and hydration, the sections were incubated with 3% hydrogen peroxide in methanol for 20 min to remove endogenous peroxidase activity. Possible background staining was blocked by incubation with normal goat serum, diluted 1 : 10 in 50 mM phosphate buffered saline (PBS), pH 7.5 for 30 min at room temperature. The sections were incubated for 16 hrs at 4°C in a humidified atmosphere with rabbit polyclonal antibodies against PGP9.5 (Quartett Immunodiagnostika und Biotechnologie GmbH, Berlin, Germany), which was diluted 1 : 100 in 50 mM PBS, respectively. Then, they were rinsed several times with PBS and incubated with a biotinylated secondary antibody for 30 min at room temperature. After rinsing three times with PBS, they were incubated with streptavidin-horseradish peroxidase conjugate for 30 min at room temperature. After the sections were rinsed three times with PBS, they were incubated in PBS containing 0.03% 3,3′-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide for 10 min. Then they were rinsed with PBS and lightly counterstained with hematoxylin. Immunohistochemical controls were performed either by preincubation with normal rabbit serum or by omission of the primary antisera. Embryos or sections were photographed by using a Nikon SNZ-U stereo-microscope (Nikon, Japan) or Zeiss Axiophot microscope (Carl Zeiss Jena GmbH, Jena, Germany) and a Colorview II digital camera (Soft Imaging System, Munster, Germany). The figures were prepared with Adobe Photoshop 6.0 program (Adobe Systems, San Jose, USA).

Results

1. Expression patterns of *Uchl1* in embryo

In E12.5 mouse embryo, *Uchl1* transcripts showed restricted expression in the some parts of developing central nervous system and ganglia such as telencephalic vesicles, mesencephalic vesicles, trigeminal ganglia, dorsal root ganglia (Fig. 1A, B). At E13.5, the periods of initiation of mouse tooth development, *Uchl1* was specifically expressed in the regions of incisors and molar both of maxilla and mandible (Fig. 1C, D).

2. Expression of *Uchl1* in the tooth development

At E13.5, *Uchl1* was weakly expressed in the oral epithelium and dental lamina (Fig. 2A). At E14.5,

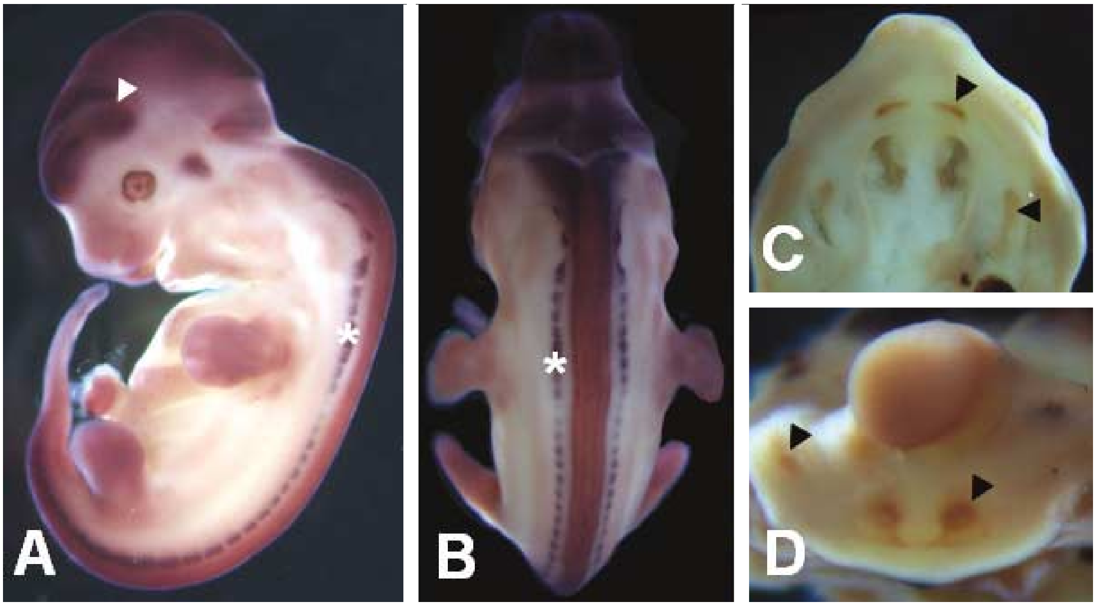

Fig. 1. Expression patterns of Uchl1 in the mouse embryo (A-B) *Uchl1* transcripts are expressed in the developing central nervous system and ganglia such as telencephalic vesicles (white arrowhead), mesencephalic vesicles, trigeminal ganglia, dorsal root ganglia (asterisks) of mouse embryos at E12.5. (C-D) At E13.5, *Uchl1* is specifically expressed in the tooth buds of incisor and molar (black arrowheads) both of maxilla and mandible.

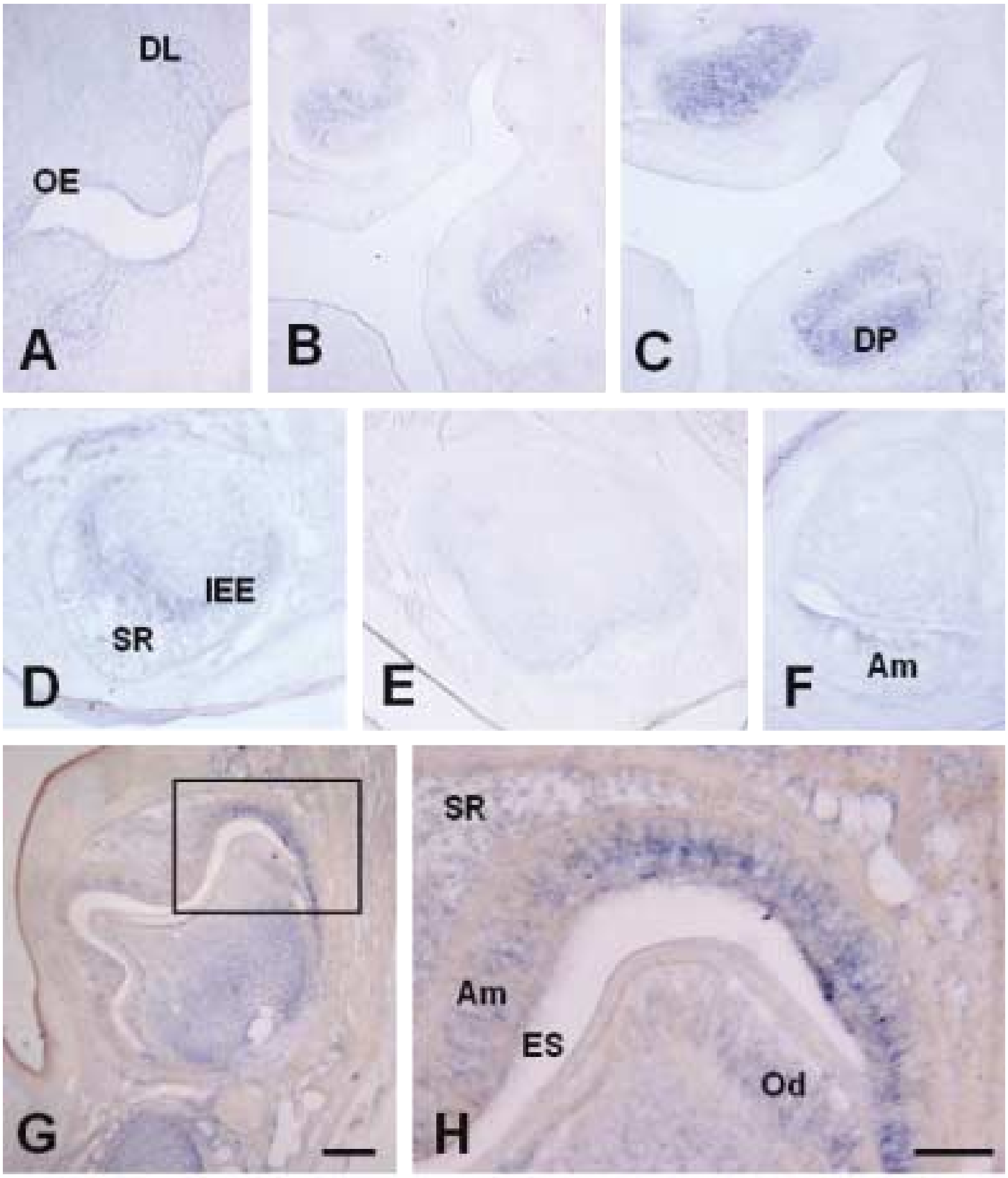

Fig. 2. Expression of *Uchl1* in the mouse tooth development. (A) *Uchl1* is weakly expressed in the oral epithelium and dental lamina at E13.5. (B-C) *Uchl1* is expressed in the inner enamel epithelium at E14.5 and its expression is expanded to the dental papilla at E15.5. (D) At E17.5, expression of *Uchl1* is observed in the inner enamel epithelium. (E-F) At E18.5, expression of *Uchl1* is observed in the inner enamel epithelium of molar and ameloblasts of incisor. (G-H) At P8, *Uchl1* transcripts are more widely expressed in the ameloblasts, odontoblasts, and the cells of the stellate reticulum and dental pulp. Expression of *Uchl1* is strong in the ameloblasts around cusp regions but gradually wanes along with the cervical-loop epithelium. DL: dental lamina, OE: oral epithelium, SR: stellate reticulum, Am: ameloblast, ES: enamel space, Od: odontoblast, IEE: inner enamel epithelium, SR: stellate reticulum, Am: ameloblast, DP: dental papilla. Scale bars=200 μm (G), 50 μm (H)

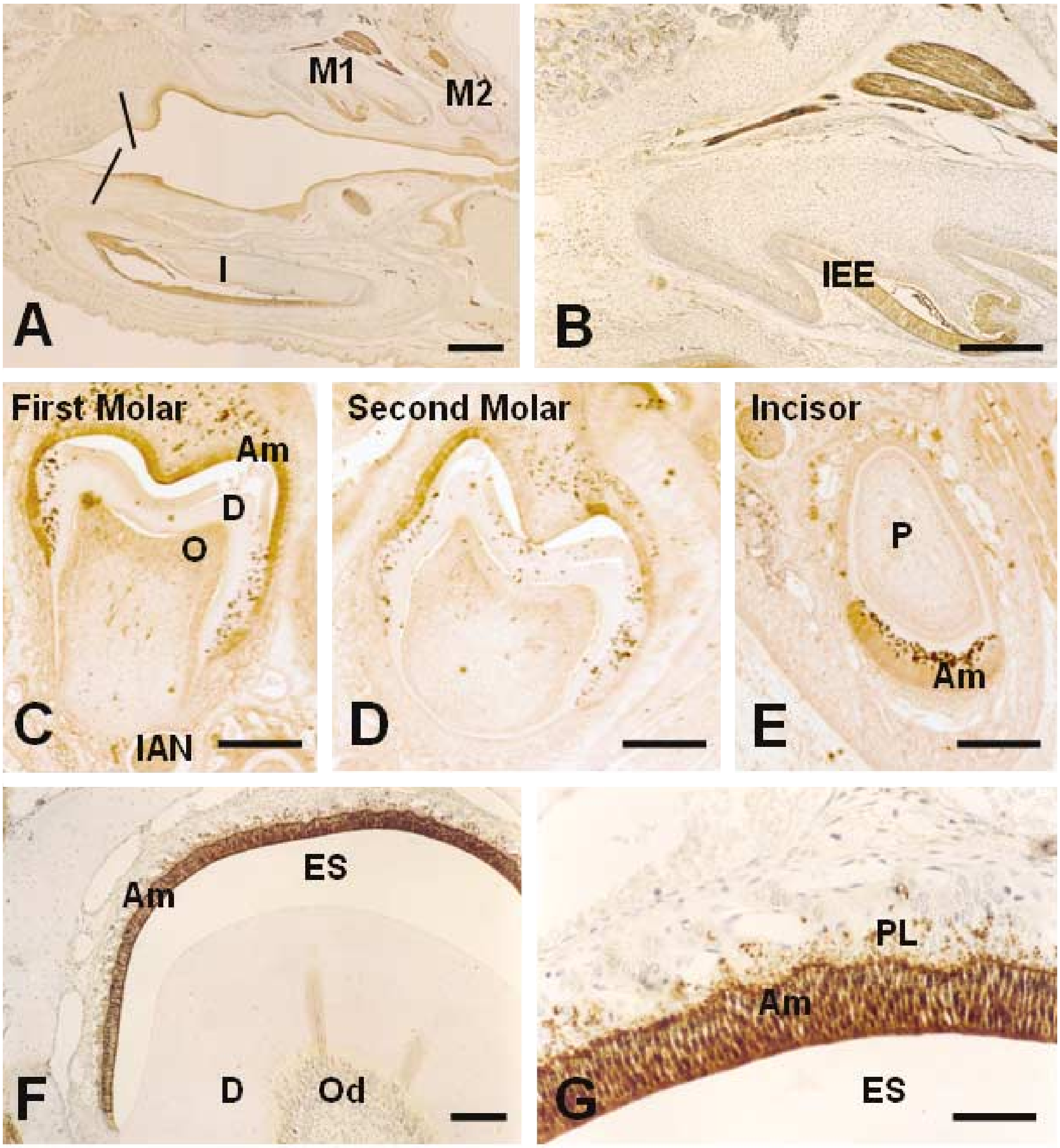

Fig. 3. Immunohistochemical localization of Uchl1 in the developing mouse tooth. (A-B) In the sagittal section of mouse at P0, Uchl1 immunoreactivities are specifically localized in the oral epithelium, differentiating inner enamel epithelium of maxillary molar and ameloblasts of mandibular incisor. (C-E) At P8, strong Uchl1 immunoreactivities are localized in the ameloblasts of the first molar (C) but are observed in the ameloblasts around cusp of the second molar (D). Ameloblasts of the mandibular incisor shows strong expression of Uchl1 immunoreactivities in the ameloblasts (E). (F-G) At P15, strong Uchl1 immunoreactivities are specifically distributed in the differentiated ameloblasts and some cells of papillary layer of mandibular first molar. Am: ameloblast, D: dentin, ES: enamel space, I: incisor, IAN: inferior alveolar nerve, IEE: inner enamel epithelium, M1: first molar, M2: second molar, Od: odontoblast, P: pulp, PL: papillary layer. Scale bars=100 μm (A-B), 200 μm (C-E), 50 μm (F), 20 μm (G)

Uchl1 was expressed in the inner enamel epithelium and adjacent cells of dental papilla (Fig. 2B). From these stages, *Uchl1* transcripts were not observed in the oral epithelium. *Uchl1* was strongly expressed in the stellate reticulum and dental papilla at E15.5 (Fig. 2C). However, strong expression of *Uchl1* was observed in the inner enamel epithelium of molar at E17.5 (Fig. 2D). At E18.5, expression of Uchl1 was observed in the inner enamel epithelium of molar and ameloblasts of incisor (Fig. 2E, F).

In the mouse molar at P8, *Uchl1* was widely expressed in the ameloblasts, the odontoblasts, and the cells of the stellate reticulum and dental pulp (Fig. 2G). Expression of *Uchl1* was strong in the ameloblasts around buccal cusp but gradually waned along with the cervical-loop epithelium (Fig. 2G, H).

3. Localization of Uchl1 in tooth development

In the para-sagittal section of mouse head at P0, Uchl1 immunoreactivities were specifically localized in the oral epithelium, the inner enamel epithelium of the maxillary first molar and the ameloblasts of the mandibular incisor (Fig. 3A). Uchl1 immunoreactivities were strongly observed in the most stages of ameloblasts except the cervical-loop region of the mandibular incisor (Fig. 3A). In higher magnification of the maxillary first molar, Uchl1 immunoreactivities were strong in the inner enamel epithelium around the cusp but waned along with cusp slope (Fig. 3B).

In the frontal section of mandible at P8, Uchl1 immunoreactivities were localized in the ameloblasts of the first molar (Fig. 3C) but were observed in the ameloblasts around cusp of the second molar (Fig. 3D). In incisor, Uchl1 immunoreactivities were strongly localized in the ameloblasts (Fig. 3E).

At P15, strong Uchl1 immunoreactivities were observed in the secretory ameloblasts (Fig. 3F, G). Uchl1 immunoreactivities were also found in the odontoblasts in the pulp chamber but were weaker than those in ameloblasts. In higher magnification, Uchl1 immunoreactivities were found in the cells of papillary layer of molar as well as ameloblasts (Fig. 3G).

Discussion

Currently it is not clear that neuronal factors are involved in the morphogenesis of the non-neuronal tissues. Present study was performed to identify whether the neuronal factors are involved in the non-neuronal morphogenesis of dental epithelium. We demonstrates that Uchl1 is expressed and localized in the dental epithelium during tooth development using immunohistochemistry and *in situ* hybridization. The result suggests that neuronal factors may play roles in the morphogenesis and cytodifferentiation of dental epithelium regardless of neuronal development during tooth development.

It has been well known that tooth is formed by the reciprocal interactions between the oral epithelium and dental mesenchyme. Most of epithelial organs develop regardless of neural factors. However, Christensen et al. (1993) identified that nerve growth factor receptor (NGF-R) was expressed in the developing dental epithelium as well as in the neural components and they postulated that neural factors can mediate cell growth and differentiation of non-neuronal cells. In fact, NGF-R has been demonstrated in a variety of rodent non-neuronal tissues such as limb buds, somites, meninges, hair follicles, salivary glands and blood vessels at specific developmental stages (Yan and Johnson 1988). Recently, McKee et al. (2005) has been revealed that RNA-binding proteins (RBPs) exhibit neural specific expression as well as novel RBPs that show expression in non-neural tissues. In their report, almost of RBPs show specific expression in the inner enamel epithelium and ameloblasts. However, the origin and role of the neuronal factor as well as RBPs have not known well in the developing dental epithelium. In this study, a representative marker for neuronal factor, Uchl1 and its transcripts were expressed and localized in the dental epithelium and ameloblasts. In addition, Uchl1 was also expressed in the odontoblasts. These results indicate that Uchl1 was not come from the surrounding tissues but was locally synthesized in the dental epithelium and odontoblasts. Therefore, the neuronal factors produced in dental epithelium might be involved in the differentiation of dental epithelium into the inner enamel epithelium, stellate reticulum and ameloblasts.

In the other hands, Uchl1 has been also identified in the non-neuronal organs such as kidney, testis and ovary (Wilson et al. 1988). Although it has been initially identified as a neuronal cytoplasmic proteins, Uchl1 may have more diverse roles in the ubiquitination and proteolysis in the respect to isopeptidase (Ciechanover 1994). These suggestions are supported by the previous reports that this enzyme is involved in cell fate (Huang et al. 1995), cell cycle (King et al. 1996), cell growth control (Zhu et al. 1996), or transcription activation (Verma et al. 1995). In enamel formation, the roles of proteolytic enzymes are very important to maintain the structural integrity through the cleavage of secreted enamel proteins and the accumulation of minerals. To date, matrix metalloproteinase 20 (MMP 20, enamelysin) and kallikrein-4 are known to primary proteases in the enamel maturation (Hu et al. 2002). In point of proteolytic enzyme, Uchl1 may be participated in the enamel maturation. It is consistent with present results that secretory ameloblasts showed highest expression of Uchl1 than the differentiating inner enamel epithelium. However, Uchl1 and its transcripts were also detected in the epithelial buds of the early tooth development. This result indicates that Uchl1 may be involved not only in the epithelial proliferation and differentiation during dental epithelial morphogenesis but also in the maturation of enamel.

Several enamel proteins have been identified in the neoplasms. Enamelysin has been detected in the odontogenic tumors (Takata et al. 2000) and Apin, a recently discovered secretory enamel protein (Solomon et al. 2003), has also been found as a gastric cancer specific gene products (Aung et al. 2006). In addition, carbonic anhydrase 6 (CA6) also has identified in the rat incisor enamel organs (Smith et al. 2006). The presence of Uchl1 in the neoplasms has been reported early (Wirnsberger et al. 1992). In this context, Uchl1 may share the functional similarities with the other enamel proteins. However, the precise roles of these enamel proteins are need to be elucidated in the neoplasms.

In conclusion, Uchl1 and its transcripts are expressed in the non-neuronal dental epithelium and ameloblasts during mouse tooth development. The results suggest that neuronal protein, Uchl1 may play roles in the histo- and cyto-differentiation of non-neuronal dental epithelium and particularly in amelogenesis.

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생쥐 치아 발생과정 중 치아상피에서 Uchl1의 발현

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간추림 : 치아는 발생과정에서 외배엽과 신경능으로부터 유래되는 중간엽의 상호유도작용에 의하여 형성되는 것으로 알려져 있다. 현재까지 치아의 형태형성과 분화과정에서 신경인자가 관여하는 지에 대해서는 분명하지 않다. 본 연구에서는 치아 발생과정에서 신경인자의 역할을 구명하기 위하여, 생쥐 치배를 대상으로 *in situ* hybridization과 면역조직화학법을 이용하여 Uchl1의 발현 및 분포양상을 확인하였다.

싹시기의 치배에서 *Uchl1*의 전사체는 치판과 구강상피에서 약하게 발현되었으나 모자시기의 치배에서는 구강상피에서는 발현되지 않았으며 안쪽치아상피와 별모양그믈, 치유두에서 제한적으로 발현되었다. 종시기부터 *Uchl1*은 안쪽치아상피와 법랑모세포에서 발현되었다. Uchl1는 신생 생쥐의 앞니와 어금니의 안쪽 치아상피와 분화하는 법랑모세포에서 분포하고 있었다. 생후 5일된 생쥐의 어금니에서 Uchl1은 완전히 분화된 법랑모세포와 유두층에서 매우 강하게 발현되는 반면에 상아모세포에서는 약하게 발현되었다. 이상의 결과로 보아 Uchl1은 치아 발생과정에서 분화하는 치아상피와 법랑모세포에서 발현되고 분포함이 확인되었다.

따라서 신경유래 단백질인 Uchl1은 비신경성 치아상피의 조직분화와 세포분화 과정에서 중요한 역할을 수행할 것임을 제시한다.

찾아보기 낱말 : Uchl1, 치아상피, 치아, 발생, 생쥐

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