The eternal tooth germ is formed at the apical end of continuously growing teeth*

Hayato Ohshima[1], Naohiro Nakasone[1, 2], Emi Hashimoto[1], Hideo Sakai[1], Kuniko Nakakura-Ohshima[3] and Hidemitsu Harada[4]

[1]Department of Tissue Regeneration and Reconstruction and [2]Department of Biological Science, Niigata University Graduate School of Medical and Dental Sciences, [3]Polyclinic Oral Care Unit, Niigata University Medical and Dental Hospital, and [4]Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry

A short running title: Apical bud in continuously growing teeth

Key words: Rodentia, Regeneration, Stem Cells, Tooth

Address for correspondence:
Hayato Ohshima, DDS, PhD
Division of Anatomy and Cell Biology of the Hard Tissue, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Niigata 951-8514, Japan
Tel: +81-25-227-2812, Fax: +81-25-227-0804
E-mail: histoman@dent.niigata-u.ac.jp
Hidemitsu Harada, DDS, PhD
Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
Tel: +81-6-6879-2872, Fax: +81-6-6879-2875
E-mail: hide-h@dent.osaka-u.ac.jp

*This work was supported by 21st century COE program, Grant-in-Aid to promote 2001-Multidisciplinary Research Projects in 2001-2005, KAKENHI (B) (16390523 to HO and 16390527 to HH), and KAKENHI (C) (15592159 to KN-O) from MEXT, Japan.

Summary

Rodent incisors are known to be continuously growing teeth that are maintained by both the cell-proliferation at the apical end and the attrition of the incisal edge. This type of tooth had a special epithelial structure for the maintenance of stem cells, showing the bulbous epithelial protrusion at the apical end. The morphological transition of the epithelial-mesenchymal compartment by serial transverse sections of the apical end toward the incisal direction is likely to reflect the development of the tooth germ in the prenatal stage. Based on the present histological and previous molecular biological studies, the special structure at the apical end is obviously different from the cervical loop giving rise to Hertwig's epithelial root sheath (HERS), in human, mouse and rat molar tooth germs. Hence, we propose a new concept that the eternal tooth bud producing various dental progeny is formed at the apical end of continuously growing teeth, and a new term "apical bud" for indicating this specialized epithelial structure. Furthermore, BrdU labelling analysis suggested that the guinea-pig molars, which were continuously growing teeth, also possessed plural specific proliferative regions and "apical bud" at the apical end.

Introduction

Rodent incisors are continuously growing teeth, and all stages of odontogenesis including amelogenesis and dentinogenesis can be surveyed if we prepare the sections of the tooth from the apical end to the incisal edge.[1-3] This phenomenon is maintained by both the cell-proliferation at the apical end and the attrition of the incisal edge. Recent molecular biological studies have clearly demonstrated the existence of the niche for the self-renewing adult stem cells in these rodent incisors and the molecular signals regulating the maintenance and cell fate decision of adult stem cells by the epithelial-mesenchymal interaction through fibroblast growth factor (FGF) signalling.[4,5] The term “cervical loop” has been so far used for indicating the epithelial tissue situated at the proliferative end of the rodent incisor.[4-6] However, the “cervical loop” is the term referring to the junctional zone where the inner enamel epithelium meets the external enamel epithelium at the rim of the enamel organ.[7] Thus, there is no suitable term for indicating this specialized epithelial compartment at the apical end of rodent incisors.

The stem cells divide slowly to give rise to one daughter cell that remains in the apical region and another cell enters the zone of rapidly dividing inner enamel

epithelial cells (transit-amplifying cell population) to differentiate into ameloblasts to deposit the enamel matrix. In addition to the previous cell proliferation assays in rodent incisors,[1,2,4,8,9] our recent cell kinetic studies by double staining of 5-bromo-2'-deoxyuridine (BrdU) and Ki67 as the markers of dividing cells have clearly shown the presence of adult stem cells in the apical end of rodent incisors.[10] Thus, the apical region of these teeth is totally different from "cervical loop" in rodent molars or human teeth from the viewpoint of the morphology and the biological significance.

The continuously growing teeth are represented not only by rodent incisors but also molars in certain other species, including rabbits, guinea-pigs, and field voles. In these animals, the structural similarity of the dental epithelium has been detected.[11-13] Basically, these specific structures are composed of a large amount of the stellate reticulum and the basal epithelium. The present study aims to clarify the morphological features of the apical end of continuously growing teeth from both rodent incisors and guinea-pig molars and the structural similarities and differences between these two species.

Materials and Methods

All experiments were performed following the Guidelines of the Niigata University Intramural Animal Use and Care Committee. The incisors were dissected carefully from the mandibles of 2 or 3-day-old mice after the amputation of heads under deep anesthesia. Dissected teeth were fixed in 2% paraformaldehyde + 2.5% glutaraldehyde + 1% acrolein in 0.01M phosphate buffer saline (PBS) (pH 7.2) overnight at 4°C. They were then post-fixed in 1% OsO_4 with 1.5% potassium ferrocyanide for 2 hours, dehydrated through a graded series of ethanol and embedded in Epon 812. Serial semithin sections were cut at a thickness of about 1 μm and stained with toluidine blue at both parallel and right angles to the long axis of the apical end of the epithelial compartments.

For the observation of three-dimensional features of the apical end of the epithelial compartments, the dissected incisors of 2 or 3-day-old mice were incubated in 2% collagenase Dulbecco's minimum essential medium (D-MEM) at 4°C for 8 hours and the epithelium were separated with the mesenchyme carefully using the forceps. The separated tissues were fixed in 2% paraformaldehyde + 2.5% glutaraldehyde + 1% acrolein in 0.01M PBS (pH 7.2) overnight at 4°C. They were then post-fixed in 1%

OsO_4 with 1.5% potassium ferrocyanide for 2 hours, dehydrated through a graded series of ethanol and critical-point-dried with liquid CO_2 (Hitachi, HCP-1). The specimens were sputter-coated with gold in a vacuum evaporator (Eiko, IB-3) and observed by scanning electron microscopy (SEM; Hitachi S-570) using an accelerating voltage of 5-15 kV.

Three or 4-week-old guinea-pigs were injected by an intraperitoneal injection of BrdU 2 hours or 5 days before the fixation and transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg). The maxillae and mandibles including molar teeth were *en block* and immersed in the same fixative for an additional 6 hours. Following decalcification 10% ethylenediaminetetraacetic acid (EDTA) solution for 4 weeks at 4°C, the specimens were dehydrated through a graded series of ethanol and embedded in paraffin. Serial horizontal sections of the molars, 5 μm thick, were stained with hematoxylin and eosin (H&E) or processed for BrdU cell proliferation assay using BrdU immunohistochemistry system (Merck Chemical Ltd, UK).

Results

Morphological features of the stem cell component in continuously growing incisors

Sagittal semithin sections of the rodent incisor tooth germ including the apical end showed that the labial epithelial compartment appeared as an oval-shaped structure. The morphology of serial transverse sections of the apical epithelial compartment showed the morphological features equivalent to bud, cap and bell stages-tooth germs of molar teeth. These structures were composed of the cells of inner and outer enamel epithelium, and stellate reticulum. Cutting more incisally, the mesial and lateral Hertwig's epithelial root sheaths (HERS) elongated toward lingual side and met each other finally to encircle the dental pulp totally (Fig. 1). The SEM clearly demonstrated that three-dimensional views of the apical epithelial compartment appeared as a human head-like structure equipped with his arm corresponding to the cervical loop (Fig. 1).

Morphological features of the stem cell component in continuously growing molars

Serial horizontal paraffin sections from the apical end to the occlusal surface of guinea-pig molars showed plural buds and bell stage-tooth germ (Fig. 2). When cells labeled by BrdU are examined at 2 hours after BrdU injection, they were localized in

the inner enamel epithelium and basal epithelium of the buds. At 5 days after injections, the labeled cells reside only at the border between the stellate reticulum and the basal epithelium. The occlusal view of the molar represented the S-shaped features, and the buccal side of the tooth was covered with enamel: the lingual side – both enamel and cementum (Fig. 2).

Discussion

Adult stem cells are present in many vertebrate regenerative tissues including the hematopoietic system, nervous system, gut, gonads, skin, olfactory epithelium and tooth.[4,14-16] Adult stem cells have been shown to undergo asymmetric cell division resulting in one daughter cell remaining in the stem cell compartment and another undergoing further cell divisions and giving rise to differentiated cells.[4,14] It is reasonable to suppose that a variety of continuously growing teeth possess the dental adult stem cells. The present and previous[10] cell kinetic studies using BrdU and/or Ki67 as the markers of dividing cells clearly demonstrated that the dental epithelium of the rodent incisor and the guinea-pig molar has a special structure for the maintenance of stem cells at the apical end including the cell-proliferative region.

The serial semithin sections represented that the morphological features of the apical epithelial compartments from mouse incisors were equivalent to bud, cap and bell stages-tooth germs at the prenatal stage. These structures were composed of the cells of inner and outer enamel epithelium, and stellate reticulum. These morphological features were also observed in the guinea-pig molar where plural specific proliferative regions existed at the apical end and the serial transverse sections of the apical end reflect the development of the tooth germ with limited growth. These results suggest that the situation of dental stem cell niche in the mouse incisor also applies to the tooth germ of guinea-pig molars. In the latter cases there may be plural stem cell compartments.

Concerning the molecular mechanism of continuously growing teeth, previous studies elucidated that the molecular signals regulating the maintenance and cell fate decision of adult stem cells, such as Notch-1, Lunatic fringe, fibroblast growth factor (FGF)-10, are expressed in the epithelial structure and the surrounding mesenchyme.[4,5,10,13] In the case of the tooth with limited growth, on the other hand, these signals were transiently expressed during cap stage when the tooth grows rapidly and epithelium undergoes folding morphogenesis, and their expressions ceased

gradually according to the progress of the tooth development.[17] Taken together, the tooth bud corresponding to the developing tooth germ at the prenatal stage is eternally maintained at the apical end of continuously growing teeth. Thus, we would like to propose the new term “apical bud” for referring to the epithelial stem cell compartment in continuously growing teeth and to claim that “apical bud” is obviously different from the cervical loop and/or cell population of inner enamel epithelium as transit-amplifying cells in the molar germs with limited growth.

References

1. Smith CE, Warshawsky H. Cellular renewal in the enamel organ and the odontoblast layer of the rat incisor as followed by radioautography using 3H-thymidine. *Anat Rec* 1975;**183**:523-561.
2. Smith CE, Warshawsky H. Movement of entire cell populations during renewal of the rat incisor as shown by radioautography after labelling with ^{3}H-thymidine. The concept of a continuously differentiating cross-sectional segment. (With an appendix on the development of the periodontal ligament). *Am J Anat* 1976;**145**:225-259.
3. Ohshima H, Yoshida S. The relationship between odontoblasts and pulp capillaries in the process of enamel- and cementum-related dentin formation in rat incisors. *Cell Tissue Res* 1992;**268**:51-63.
4. Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signalling. *J Cell Biol* 1999;**147**:105-120.
5. Harada H, Toyono T, Toyoshima K, Yamasaki M, Itoh N, Kato S, Sekine K, Ohuchi H. FGF10 maintains stem cell compartment in developing mouse incisors. *Development* 2002;**129**:1533-1541.

6. Kieffer-Combeau S, Meyer JM, Lesot H. Cell-matrix interactions and cell-cell junctions during epithelial histo-morphogenesis in the developing mouse incisor. *Int J Dev Biol* 2001;**45**:733-742.
7. Ten Cate AR, Sharpe PT, Roy S, Nanci A. Development of the tooth and its supporting tissues. In: Nanci A, editor. *Ten Cate's Oral histology. Development, structure, and formation*, 6th edition. St. Louis: Mosby, 2003, p.79-110.
8. Smith CE, Warshawsky H. Quantitative analysis of cell turnover in the enamel organ of the rat incisor. Evidence for ameloblast death immediately after enamel matrix secretion. *Anat Rec* 1997;**187**:63-98.
9. Smith CE. Cell turnover in the odontogenic organ of the rat incisor as visualized by graphic reconstructions following a single injection of 3H-thymidine. *Am J Anat* 1980;**158**:321-343.
10. Harada H, Ohshima H. New perspectives on tooth development and the dental stem cell niche. *Arch Histol Cytol* 2004;**67**:1-11.
11. Starkey WE. The migration and renewal of tritium labeled cells in the developing enamel organ of rabbits. *Brit Dent J* 1963;**115**:143-153.
12. Harada H, Mitsuyasu T, Toyono T, Toyoshima K. Epithelial stem cells in teeth. *Odontology* 2002;**90**:1-6.
13. Tummers M, Thesleff I. Root or Crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development* 2003;**130**:1049-1057.
14. Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell* 1997;**88**:287-298.
15. Fuchs E, Segre JA. Stem cells: a new lease on life. *Cell* 2000;**100**:143-155.
16. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;**81**:531-535.
17. Kettunen P, Laurikkala J, Itaranta P, Vainio S, Itoh N, Thesleff I. Associations of FGF-3 and FGF-10 with signalling networks regulating tooth morphogenesis. *Dev Dyn* 2000;**219**:322-332.

Figure legend

Fig. 1. A diagram showing three-dimensional transverse views at different position of a

rodent incisor. The morphological transition by the transverse view from the apical to incisal edge is very similar to that of the tooth germ from bud to bell stage, respectively (a–c). Cutting toward the incisal edge, the mesial and lateral Hertwig's epithelial root sheath (HERS) elongate toward lingual side and finally encircle the dental pulp (d, e). The right panel indicates the three-dimensional view of the apical epithelial compartment obtained from a lower incisor of a 3-day-old mice by scanning electron microscopy (SEM). The apical epithelial region (*) appears as a human head-like structure and the cervical loop (arrows) corresponds to its arms.

Fig. 2. A diagram showing transverse views at different positions of a guinea-pig molar. Continuously growing molars show the developmental stages including bud-bell stages, the development and fusion of HERS, and the formation of both crown- and root-analogue dentin at the buccal and lingual sides, respectively. Interestingly, another epithelial compartment (*) appears in the lingual side to result in the S-shaped dentin formation.