Cell dynamics in the Growth and Differentiation of Dental Epithelium during Tooth Development: Stratum Intermedium Cells Originated From Inner Enamel Epithelium

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Abstract:The stratum intermedium develops as flattened cell layer on the proximal side of the ameloblast layer during the bell stage of tooth development. Stratum intermedium cells strongly express alkaline phosphatase (ALP) activity and have been considered to play a complementary role in the enamel mineralization, however the origin and/or the role of these cells have not been elucidated. In the present study, we focused on the lineage of stratum intermedium cells in continuously growing rodent incisors and analyzed it by using DiI tracers experiment and using the incisors organ culture. The results indicated that some stratum intermedium cells were originated from the inner enamel epithelium. Immunohistochemical and in situ hybridization studies showed that the stratum intermedium cells expressed the Notch-1, Notch-2, and Hes1, while the inner enamel epithelium and ameloblasts expressed their ligands Jagged-1. Furthermore, we examined th role of Notch signaling in the development of the stratum intermedium cells by use of the dental epithelial cell line, HAT-7. Recombinant Jagged1 protein enhanced the appearance of the stratum intermedium cells in HAT-7.On the other hand, anti-sense Notch1 decreased the number of stratum intermedium cells.Taken together, we propose a hypothesis that the lineage of the stratum intermedium differentiates from the ameloblasts lineage through Notch signaling.

Key words: stratum intermedium, ameloblasts, inner enamel epithelium, Notch, Jagged, DiI, dental epithelial progenitor cells

Introduction

In developing teeth, the dental epithelial progenitor cells differentiate to produce a variety of cells through sequential and reciprocal interactions with the neural crest-derived mesenchyme. However, the molecular mechanisms involved in the cell-differentiation of the dental progenitor cells are not well understood. During the bud stage and cap stages, the dental epithelial progenitor cells differentiate to form the inner enamel epithelium, outer enamel epithelium, and stellate reticulum, and during the bell stage, the stratum intermedium cells appear between the inner enamel epithelium and the stellate reticulum.Because in molar development the cell differentiation is linked to the morphological changes of the germ cells during molar development, it is difficult to study the cell lineage. Rodent incisors are continuously growing teeth and are useful in the study of dental progenitor cells differentiation, and all stages of odontogenesis can be studied if we prepare the serial sections of the tooth from the apical end to the incisal edge[1), 2)]. Here, we examined on the cell lineage of the stratum intermedium by use of rat incisors and the epithelial cell line, HAT-7[3)].

Materials and methods

Dissection and Culture of Tissues and Cells

The incisors were carefully dissected from the lower jaws of a 3-day-old SD rat (SD). The apical ends of the incisors were dissected and cultured in Trowell- type organ cultures[1)]. The culture medium consisted of DMEM and HamF12 (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL). HAT-7 cells were cultured in the same medium supplemented with human recombinant Fgf10 (10 ng/ml,; R&D).

DiI Labeling Analysis

The fluorescent DiI label was microinjected into the dental papilla adjacent to the inner enamel epithelium of the incisors that were subsequently cultured as described above. DiI (Molecular Probes) was diluted in DMSO (0.2% wt/vol). The explants were fixed after 5 d and observed using a fluorescencet microscope.

Immunohistochemistry

The lower incisors were carefully dissected from the mandibles of 3- day-old rats and were immediately frozen and subsequently 9μm sections were cut. The cultured cells were fixed using 4% PFA. The primary antibodies used in this study were anti-Notch1 polyclonal antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Notch2 polyclonal antibody (1:200, Santa Cruz Biotechnology). As secondary antibodies, Alexa fluorTM 488 (1:200, Molecular Probes, Eugene, OR, USA) and Alexa fluorTM 546 (1:200, Molecular Probes) were used as secondary antibodies. We determined the specificity of immunoreactivity by substituting a buffer for the primary antibody.

In situ hybridization

For the in situ hybridization analysis, digoxigenin-labeled antisense riboprobes for Hes1 mRNA were synthesized in accordanceing withto the manufacturer's instructions (Roche Diagnostics). Paraffin sections were preparedmade as described previously[2)]. The chromogen 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP,; Roche Diagnostics) was used for color detection.

Detection of Alkaline Phosphatase Activity

Samples were fixed with 4% PFA. ALP activity was detected using NBT/BCIP / NBT substrate (DAKO, Glostrup, Denmark) or the 2-hydroxy-3-naphtoic acid-2 –phenylanilide phosphate (HNPP) fluorescent detection set (Roche, Mannheim, Germany), in accordance with the manufacturer's instructions[3].

Results and Discussion

To visualize the fate of the inner enamel epithelium, they were labeled with the fluorescent DiI stain at the onset of culture, and the labeled cells were examined after 5 days of culture. Microinjection of DiI into the dental papillshowed that the DiI stain to adhere to the distal side of the inner enamel epithelium in the cultured incisors. After 5 days of culture, fluorescent cells were observedseen to migrate from the inner enamel epithelium toward incisal endally, and interestingly, the DiI label was observed as two lines from the original site in the dental epithelium. These presumably represent the two lineages of ameloblast and stratum intermedium. The proximal line comprised labeled cells that had entered the stratum intermedium from the inner enamel epithelium. Next, to show the evidence in vivo, we observed the serial cross-sections of the rat incisors obtained from the apical end to incisally, and found a stratum intermedium cell, which the nuclei and the proximal half of cell body existed in the stratum intermedium and the distal half remained in the inner enamel epithelium. The situation was seen as if an inner enamel epithelium immigrated into the stratum intermedium. These results suggested that some stratum intermedium cells were derived from the inner enamel epithelium.

In rats, the expression of ALP in the rat lower incisor tooth germ was determined histochemically, so that we could assess the appearance of stratum intermedium duringin HAT-7 differentiation. ALP activity was detected in the stratum intermedium cells of the dental epithelium, odontoblasts, and subodontoblast layers. Using immunohistochemical analysis, Notch1 and Notch2 proteins were detected in the stratum intermedium, stellate reticulum, and outer enamel epithelium immnohistochemically. Hes1 mRNA was detected in the stratum intermedium by in situ hybridization. To investigate the role of Notch signaling during the differentiation of the stratum intermedium, we carried out gain-loss function experiments. When HAT-7 was cultured in the presence of mice recombinant Jagged1 protein, the number of ALP positive cells increased. This effect was negated by anti-Jagged1 antibody. Next, we examined the effect on HAT-7 by the overexpression of the active form of Notch1 active form (internal domain). This induced an increase of ALP positive cells that was similar to the effect of recombinant Jagged 1 protein. Anti-sense of Notch1 mRNA inhibited the appearance of ALP positive cells. Taken together, it was considered that the differentiation of stratum intermedium cells was believed to be regulated through Notch signaling. We fored a hypothesis that the stratum intermedium appears to diverge as a ramification from the ameloblast cell lineage and its the differentiation is regulated by a lateral specification viausing Notch signaling.

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