

# Cell Dynamics in Tooth Root Formation: Hertwig's Epithelial Root Sheath and Cellular Cementogenesis

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**Abstract:** Tooth root formation is initiated by the elongation of Hertwig's epithelial root sheath (HERS). HERS cells are widely accepted to control root formation, but the mechanisms regulating HERS growth have not been elucidated. To solve this problem, we have established a new culture method for postnatal mouse mandibular molars and, using it, examined the role of insulin-like growth factor-I (IGF-I) during tooth root formation. Our immunohistochemical study revealed the specific localization of the IGF-I receptor in HERS in the tooth root. In control cultures of molars prepared from postnatal 5- and 20-day-old mice, normal development of both HERS and periodontium proceeded as seen *in vivo*. However, the presence of 100 ng/ml IGF-I in the culture medium resulted in further elongation of HERS and increased cell proliferation in its outer layer of 5-day-old mice molars, and in promotion of cellular cementogenesis at the root surface of 20-day-old mouse molars. Thus, our results show that IGF-I played different roles depending on the stage of root formation

**Key words:** Cementogenesis, Hertwig's epithelial root sheath (HERS), insulin-like growth factor I (IGF-I), organ culture, tooth root formation

## Introduction

Tooth root formation is initiated by the elongation of Hertwig's epithelial root sheath (HERS), the epithelial bilayer derived from the inner and outer enamel epithelia fused below the level of the cervical margin of the crown. HERS cells are widely accepted to play an important role in root formation during tooth development. Recent reports have shown that various regulatory factors are associated with the secretion of enamel-related proteins and dentinogenesis in rodent teeth<sup>1)</sup>. HERS cells also are known to secrete enamel-related proteins, such as ameloblastin, during root formation<sup>2)</sup>. As insulin-like growth factor-I (IGF-I) aids crown formation and enlargement of the size of tooth germs<sup>3)</sup>, as well as stimulates the differentiation, matrix secretion by, and the maturation of cells of the enamel organ in embryonic teeth<sup>4)</sup>, we hypothesized that IGF-I might also be involved in tooth root formation. However, the relationship between IGF-I and root formation has not been elucidated in detail. To examine the mechanisms regulating the HERS growth and root development, therefore, we used a new method for culturing tooth germs in the stage of root formation<sup>5)</sup> to examine the effects of IGF-I on tooth germs *in vitro*.

## Materials and Methods

The procedures for organ culturing were carried out as described previously<sup>5)</sup>, and 100 ng/ml of recombinant human IGF-I (Chemicon International, Temecula, CA, USA) was added to the culture medium for experiments. The specimens for light or electron microscopy were fixed and decalcified by conventional methods prior to embedment in paraffin or Epon mixture for preparing sections. To determine the length of HERS and formation

of cementum, we traced the outline of the inner layer of HERS and that of the tooth root and cellular cementum, respectively, and then measured them by using a digitizer. For proliferation assays, explants were incubated in the presence of 0.5 mg/ml BrdU for 4 h. BrdU on the sections was detected by using a BrdU staining kit (Calbiochem, Oncogene Research Products, Cambridge, MA, USA). To compare the mitotic activity in both inner and outer layers of HERS, we counted the number of BrdU-immunopositive cells and all cells in both layers and then estimated the mitotic index in each. For evaluating changes in HERS elongation and the length of newly developed cellular cementum during the culture period, we measured total lengths of the molar teeth and HERS, and total lengths of the molar root and cellular cementum. The results were reported as the mean  $\pm$  standard deviation (SD), and the significance of differences between control and treatment groups were evaluated statistically by one-way ANOVA followed by Student's *t*-test.

## Results and Discussion

### *Cell dynamics of HERS in molars during root elongation stage*

Mandibular first molar tooth germs of 5-day-old mice were in the initial stage of root formation. The morphology of inner and outer enamel epithelia was a bilayered epithelial sheath termed HERS. The tooth germs were cultured for 6 days (n=5), and we observed the morphology of the dental root including HERS and surrounding tissues to be the same as seen *in vivo*. Under our culture conditions, the roots elongated to approx. 1.5 times their original length (time 0) when grown for 6 days in normal medium (Fig. 1A) and to approx. 2 times it in the IGF-I-containing medium (Fig. 1B). Furthermore, the addition of antibodies specific for IGF-I to cultures treated with IGF-I resulted in less elongation (Fig. C). These data indicate that IGF-I is effective for promoting tooth root formation. The appearance of IGF receptors coincided with the start of tooth root formation (data not shown), suggesting that IGF-I and its receptor are involved in tooth root formation.

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As the effect of IGF-I seemed to differ between the inner and outer layers of HERS, we counted the number of BrdU-positive cells in each layer and estimated the mitotic index for each. The results given in Fig. 1D show that BrdU-positive cells were located in both the inner and outer layer of HERS in the control and experimental cultures but that the mitotic index of the outer layer was slightly higher than that of the inner layer in the control cultures. This result is consistent with the report of Heritier and Fernandez<sup>6</sup>, who used 16-day-old mice. In the IGF-I group, the outer layer showed significantly higher activity; i.e., the mitotic indexes of the outer and the inner layers were 0.0745 and 0.0502, respectively. In the presence of the anti-IGF-I, the mitotic indices of the outer and inner layers were lower than those in the control experiment.

*Cell dynamics of HERS in the molar after root elongation*

Mandibular first molar teeth of 20-day-old mice were in the stage after root elongation and just before the onset of cellular cementogenesis. We counted the number of BrdU-positive cells in each layer of HERS and estimated the mitotic index. BrdU-positive cells were located in cells of HERS in both the control and experimental cultures, but their numbers were fewer than those in the 5-day-old molars. The mitotic index of the outer layer was slightly higher than that of the inner layer for the control cultures. In the IGF-I group (presence of 100 ng/ml IGF in the culture medium), the outer layer showed significantly lower activity. These results from the late stage of root formation seemed to differ from those from the initial stage, which showed that IGF-I had a positive effect on root elongation; therefore, we observed the morphology in each layer by electron microscopy. The length of HERS in the IGF-I group was shorter than that of the control group, and the basement membranes at areas on the occlusal side of HERS were disrupted (data not shown). During a culture period of 14 days, on the other hand, the cementum in the roots in the IGF-I-containing medium was deposited over a wide range that covered from the root apex to almost half of the root, in contrast to one-third of the root in the control group (data not shown). There were some cementoblast-like cells and cell clusters like epithelial rests of Malassez in the developing periodontal ligament of the apical root area. From these results, we propose that IGF-I is involved in regulating cellular cementogenesis, not in that of the mitotic activity in HERS cells, in root formation after elongation.

Taken together, our results show that IGF-I plays different roles in 2 stages of root development, promoting the proliferation of HERS cells in an early stage and cellular cementogenesis later.

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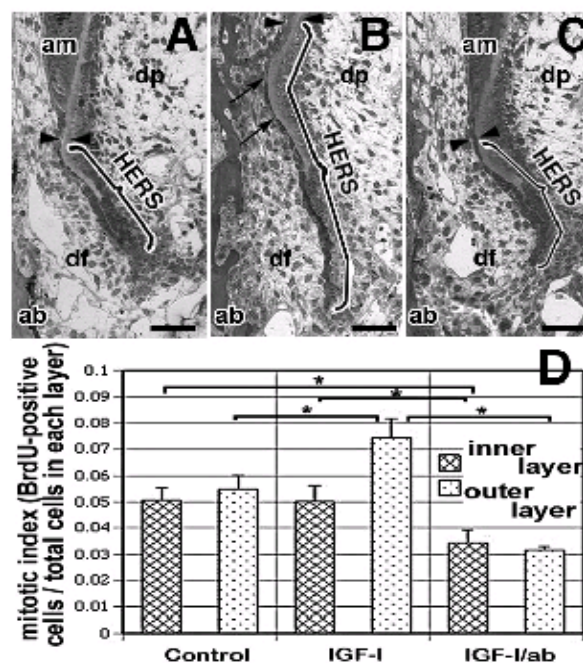


Fig.1 Histology of root formation in the first molar *in vitro*. The specimens were prepared from 5-day-old mice and cultured for 6 days (A) in control medium, for 6 days (B) in IGF-I medium, or for 6 days (C) in medium containing both IGF-I and anti-IGF-I antibodies (IGF-I/ab). Arrowheads indicate the cervical region. am: ameloblast layer, ab: alveolar bone, dp: dental pulp, df: dental follicle. bar = 50  $\mu$ m. (D) The mitotic index for BrdU-labeled explants. The number of BrdU-labeled cells in the outer layer of the IGF-I group was statistically significantly ( $p < 0.01$ ) increased compared with that in the same layer of the control and IGF-I/ab groups. The significance of difference in the mitotic index between 2 groups (with error bars) was calculated by using Student's t-test. \* $P < 0.01$ .

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