Regeneration of halved embryonic lower first mouse molars: correlation with the distribution pattern of non dividing IDE cells, the putative organizers of morphogenetic units, the cusps

ROMUALD COIN1#, RÉGINE SCHMITT2#, HERVÉ LESOT1, JEAN-LUC VONESCH3 and JEAN-VICTOR RUCH1*

1INSERM U-424, Institut de Biologie Médicale, Faculté de Médecine, Strasbourg, 2INSERM U-424, U.F.R d’Odontologie de Strasbourg, and 3Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France

ABSTRACT Recently we demonstrated that non-cycling, cap-stage, mouse molar inner dental epithelial (IDE) cells corresponding to the primary enamel knot (EK) area underwent a coordinated temporo-spatial patterning leading to their patchy irregular segregation at the tips of the forming cusps. These non-cycling cells were suggested to perhaps represent the organizers of the morphogenetic units (OMU), the cusps. The present study has analyzed the regenerative capacity of halved cap-stage first lower mouse molars through three dimensional (3D) reconstructions. Partial regeneration of the anterior half and possible complete regeneration of the posterior half were documented. Using BrdU (5-bromo-2'-deoxyuridine) labeling and 3D reconstructions of the IDE, we have correlated the patterns of cusp regeneration with the distribution of BrdU negative IDE cells. These data support a morphogenetic role for the non-cycling IDE cells.

KEY WORDS: mouse molar, regeneration, enamel knot, non-cycling IDE cells

Introduction

Tooth crown morphogenesis is controlled by the bud-cap staged dental mesenchyme (Kollar and Baird, 1969, 1970a,b; Schmitt et al., 1999). It has been hypothesized that the mesenchyme induces the formation of the enamel knot (EK), a transitory epithelial structure, located at the tip of the cap, expressing signaling molecules including sonic hedgehog (SHH), bone morphogenetic proteins (BMP), fibroblast growth factor (FGF) (Keranen et al., 1998). According to Jernvall et al. (1994), this primary EK might represent a signaling center for tooth morphogenesis causing unequal growth of the enamel epithelium and inducing the formation of secondary EKs located at the tips of the forming cusps.

Recently, we demonstrated using BrdU labeling (Coin et al., 1999) that Go cells of the inner dental epithelium (IDE) corresponding to the EK area of the first lower mouse molar underwent sequential segregation and that this led to the formation of distinct groups of cells, each one corresponding to a particular developing cusp. These observations suggested that within cells of the EK that act as the organizers of morphogenetic units (OMU), the activity could specifically lie in these Go cells of the IDE, giving rise to the ameloblasts of the enamel free area (EFA) (Coin et al., 1999).

To experimentally test the possible correlation between the distribution of these OMU and cusp formation, we analyzed both the cusp pattern and the distribution of the OMU in regenerating, halved cap-stage first lower mouse molars using three dimensional (3D) reconstructions. As a matter of fact, Fisher (1971) analyzed the morphological development in vitro of halved lower mouse molar germs and she demonstrated convincingly partial regeneration of the anterior halves of E-13/E-14 first molars and complete regulation of 20% of corresponding posterior halves.

Our results confirmed: 1) the limited regulation (i.e. a reduced number of developing cusps) in cultured anterior halves; 2) the possible complete regeneration (formation of seven cusps) of the posterior halves and 3) demonstrated the factual correlation between the patterned distribution of the OMU and the pattern of cusp formation.

Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; IDE, Inner Dental Epithelium; EK, Enamel Knot; EFA, Enamel Free Area; OMU, Organizer of Morphogenetic Unit; SHH, Sonic Hedgehog; BMP, Bone Morphogenetic Protein; FGF, Fibroblast Growth Factor; 3D, three dimensional.

*Address for reprints: Institut de Biologie Médicale, INSERM U-424, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg cedex France. FAX: 03 88 25 78 17. e-mail: Ruch@odont3.u-strasbg.fr

#These authors contributed equally to this work.
The physiological cusp patterns of the first and second lower molars are shown in Figure 1. The cusps are named according to the terminology of Gaunt (1955, 1961). The M₁ crown comprises seven cusps which have a marked biserial antero-posterior arrangement: two series of three buccal and three lingual cusps, essentially of a paired nature and one single posterior cusp. The crown surface is broken up by a longitudinal cleft, which separates the lingual and buccal ridges with each ridge showing a three-fold subdivision due to an anterior and median transverse valley. All cusps slope in an anterior direction (Fig. 1A).

The second lower molar (M₂) does not show an anterior area of the crown equivalent to that of M₁, but there is a narrow anterior cingulum present. Except for this difference, the median and the posterior part of the M₂ shows a striking resemblance to the M₁ (Fig. 1B).

**Morphological observations throughout in vitro development**

Fifty-eight intact left E-14 molars were cultured in vitro and fifty-five survived. Sixty-seven right E-14 molars were halved and eight anterior parts and three posterior parts demonstrated rapid involution in vitro.

Crown morphogenesis of the intact molars was evident. The anterior parts of the halved molars demonstrated always limited crown morphogenesis. The posterior parts of the M₁ developed systematically several cusps.

The posterior parts of the halved molar rudiments included the primordium of M₂ in some cases, which also developed in vitro and this was observed in about 50% of the specimens.

**Preliminary 3D reconstructions**

Sections from an intact left E-14 molar and the corresponding anterior and posterior parts of the halved right molar, cultured for 6 days were reconstructed. The posterior part developed an M₂. Figure 2 shows the results for the halved molar and for the M₂. The anterior part developed 4 cusps (Fig. 2A), two anterior (tentatively named L₁,B₁) and two posterior (L₂ and B₂). The corresponding posterior half developed 6 cusps (Fig. 2B). The tooth was subdivided by a deep transverse fissure into a larger anterior portion containing four cusps (L₁,B₁,B₂,L₂) and a smaller posterior domain containing two cusps (L₃ and B₃). The M₂ demonstrated a normal cusp pattern: two anterior (L₂,B₂), two posterior (L₃,B₃) and a single postero-median cusp 4 (Fig. 2C).

**3D reconstructions after BrdU labeling**

To assess possible overlap between the dynamic pattern of distribution of BrdU negative IDE cells (Coin et al., 1999) and the pattern of cusp formation, we pulse labeled the intact and the halved teeth after various preincubation periods and performed 3D reconstructions.

**Eight hour pulse labeling after 2 h of preincubation**

The reconstructed intact left molar showed the presence of one distinct area of non-labeled IDE cells located at the center of the IDE with an antero-posterior orientation (Fig. 3A,D).

The corresponding anterior (Fig. 3B,E) and posterior (Fig. 3C,F) halves of the right molar showed respectively 1) a negative...
area still adjacent to the performed section and 2) a rather central negative domain with an antero-posterior orientation.

**Eight hour pulse labeling after 96 h or 108 h of preincubation**

Two intact teeth and the contralateral halves were reconstructed (Fig. 4). After 96 h of preincubation the intact left M, showed 6 cusps with corresponding BrdU negative areas of the IDE (Fig. 4A,D). The anterior half developed three cusps with three corresponding BrdU negative domains (Fig. 4B,E). The posterior half displayed a complete cusp pattern with seven cusps and seven, partially connected BrdU negative areas (Fig. 4C,F). The crown displayed crests rather than tubercles.

After 108 h of preincubation, the crown pattern of the intact left M, showed six cusps: two anterior (L1,B1), which were probably fused, two median (L2,B2) and two posterior (L3,B3) cusps. A deep median transverse fissure subdivided the tooth into a large anterior portion and a smaller posterior one. Five partially fused BrdU negative domains of the IDE coincided with this cusp pattern (Fig. 4G,J). The corresponding anterior half showed the presence of only two cusps, probably L1 and B1 and two fused Brdu negative areas were observed (Fig. 4H,K). The crown of the corresponding posterior half comprised four cusps: two anterior (probably L2 and B2) and the two posterior (probably L3 and B3) and four corresponding BrdU negative domains were observed (Fig. 4L).

**Twenty four hour continuous labeling after 84 h or 108 h of preincubation**

After 84 h of preincubation, one intact left M, and the contralateral halves were reconstructed (Fig. 5A-F). The intact molar developed a normal cusp pattern of a left M, cultured in vitro: six cusps (L1,L2,L3,B1,B2,B3) were observed. The crest connecting L1,B1,B2 and L2 had a lingual discontinuity. Corresponding BrdU negative areas of the IDE were observed (Fig. 5A,D). The anterior half displayed three cusps and three corresponding negative BrdU areas (Fig. 5B,E). The posterior half demonstrated a physiological cusp pattern of a left first lower molar. The crest connecting the four anterior cusps had a lingual discontinuity orientated towards the right side and a supernumerary cusp was observed on the lingual side between L1 and L2. Corresponding BrdU negative areas of the IDE were superposed on the cusps (Fig. 5C,F).

After 108 h of preincubation, two intact left M, and the contralateral halves were reconstructed (Fig. 5G-R). The two intact teeth developed six cusps: two anterior (L1,B1), two median (L2,B2) and two posterior (L3,B3) cusps. BrdU negative areas corresponded precisely to the localization of these six cusps (Fig. 5G,J,M,P). The two corresponding anterior halves demonstrated four cusps, tentatively: L1,B1,B2 and L2. Corresponding BrdU negative areas were observed (Fig. 5H,K,N,Q). The posterior halves contained five cusps: two lingual (L1,L2) and three buccal (B1,B2,B3) cusps. Five corresponding negative BrdU domains were present (Fig. 5I,L,O,R).

Table one summarizes the number of cusps which developed in the corresponding anterior and posterior parts of halved right lower molars.

**Histological observations**

After 2 h of preincubation and 8 h BrdU incorporation, the intact E-14 molars demonstrated obvious BrdU negative primary EKs (Fig. 6A). In contrast, both the anterior and posterior parts of the halved teeth only showed disorganized EKs in which BrdU negative IDE cells were present (Fig. 6E,I respectively).

After 24 h of preincubation and 8 h BrdU labeling, the intact molars revealed the presence of small, BrdU negative, primary EKs (Fig. 6B). The anterior parts of halved molars showed an apparent reorganization of the BrdU negative primary EKs (Fig. 6F). The corresponding posterior parts demonstrated the presence of typical primary EKs (Fig. 6J).

After 84, 96 or 108 h of preincubation and 8 or 24 h BrdU incorporation, BrdU negative IDE cells were located in all the specimens at the tips of the forming cusps (Fig. 6C,G,K).

After 6 days in vitro, terminal differentiation of the odontoblasts was initiated in both intact and halved molars (Fig. 6D,H,L).

**Discussion**

Morphogenesis in general terms may be defined as a set of developmental processes leading to organ specific spatial distribution of functional cells. These processes include cell division, cell death, cell migration, dynamic cell adhesion and cell-matrix interactions. The organ specific control mechanisms share common denominators: epigenetic, sequential and cyclical exchange of information between interacting cells as a result of paracrine factors and their receptors, substrate adhesion molecules and their ligands, cell adhesion molecules and their signaling. These inductive processes regulate the transcription of selected genes and morphoregulatory genes according to Edelman (1992) or of «genomorphens» according to Krasnow (1997). During the initial
stages of organogenesis most organ rudiments are endowed with regenerative capacity.

As far as tooth morphogenesis is concerned, the bud-cap stage dental ecto-mesenchyme controls the tooth specific cusp pattern, eventually through BMP-4 regulated formation of the primary EK (Jernvall et al., 1998). The latter may control tooth crown morphogenesis through induction of secondary signaling EKs (Keranen et al., 1999). Recently, we demonstrated (Coin et al., 1999), using BrdU labeling, the progressive subdivision of the initial group of BrdU negative IDE cells of the primary EK area into as many distinct subgroups as cusps will develop. A cellular continuity exists between components of the primary and secondary EKs and we suggested that these particular IDE cells might act as the organizer of morphogenetic units (OMU), each unit representing a cusp. The signaling OMU’s could regulate the cuspidogenesis controlling local cell proliferation, cell death, cell adhesion and cell matrix interactions.

The possible regeneration of molars has been previously examined. Glasstone (1952) observed that rabbit molars when halved at initial stages of development and grown in vitro displayed a capacity to regenerate and to form two miniature molar teeth. For the halved mouse molar (displaying limited growth), Fisher (1971) demonstrated a limited potential for regulation, since recovery and regeneration were never displayed by both parts of divided tooth germs.

At the histo-morphological level, the present data confirm Fisher’s observations. The anterior and posterior halves of E-14 first lower mouse molars demonstrated differential regulatory potential. The anterior parts never displayed complete regulation: 2,3 or 4 cusps developed. In contrast, the posterior halves developed 4 to 7 cusps. The delayed appearance of cusp 4 (after 6 days of culture) has been previously described (Schmitt et al., 1999). Normal terminal differentiation of the odontoblasts was observed. Terminal differentiation of ameloblasts would be initiated after longer periods of in vitro cultures (Schmitt et al., 1999).

The regenerative capacity of the E-14 dental tissues appears to be irregular since the total number of developing cusps in corresponding halves fluctuated from 6 to 10 (Table 1). All the E-14 embryos are not exactly at the same developmental stage and the molars we used varied from the bud-cap transition to well formed cap. Apparently, during this period the potential for regulation decreased. Furthermore, this potential appears to switch off more rapidly in the anterior part. There exists a transitory antero-posterior increasing gradient of regenerative capacity. Butler (1982) stated that the molar cusps can be traced back to a specific arrangement of presumptive cusps in the cap-stage. Bringing together this possibility and our own observations, we suggest that at the initial cap-stage, the anterior (L1,B1) and median (L2,B2) cusps are determined while the presumptive posterior

<table>
<thead>
<tr>
<th>Number of teeth</th>
<th>Number of cusps</th>
<th>Anterior halves</th>
<th>Posterior halves</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
The main goal of our observations was to assess whether the distribution of BrdU negative IDE cells, the hypothetical organizer of morphogenetic units (OMU), can be correlated with the pattern of regenerative cusps. Coin et al. (1999) have shown that after 8 h BrdU labeling the negative IDE cells include both slow cycling cells and Go cells. The Go cells were identified after continuous (72 h) BrdU labeling, which led, however, to partial inhibition of cusp formation. To avoid such a negative effect of BrdU, in the present work we restricted the incubation with BrdU to 8 or 24 h. The comparison of these two modalities (compare Fig. 4 and Fig. 5) shows that the areas of BrdU negative IDE cells are reduced after 24 h of labeling and include mainly (albeit not exclusively) Go cells. Different periods of pre-incubation were chosen to both follow the initial behavior of BrdU negative cells and their distribution during cusp formation. According to Coin et al. (1999), even the longest preincubation time used in this work, followed by 24 h of BrdU labeling allowed to exclusion of the later presence of physiological post-mitotic ameloblasts arising from the IDE.

After 2 h of preincubation and 8 h BrdU labeling, a distinct BrdU negative EK was only present in the intact molars. In both parts of the halved molars, only BrdU negative IDE cells were observed. Apparently, the division led to disorganization of the EK, which was either partially or completely restored after 24 h of preincubation and 8 h BrdU incorporation in the anterior and posterior halves respectively. This dissimilar EK restoration might be correlated with unequal regenerative capacities. After the longer preincubations, allowing for the expression of the cusp pattern, and 8 or 24 h BrdU labeling a systematic coincidence between the cusp pattern and the patterned distribution of BrdU negative IDE cells (the putative OMU’s) was observed.

We postulated (Coin et al., 1999) that the primary EK controls the tooth specific temporo-spatial patterning of the OMU and consequently the tooth specific cusp pattern. According to this hypothesis, the limited reorganization of the primary EK in the anterior halves could explain the limited regulation. The Tabby tooth phenotype, i.e. reduced cusps number, has also been explained through reduced primary EK formation and hence limited signaling (Pispa et al., 1999).

The present observations demonstrate a correlation between the regenerative cusp pattern and the distribution of the putative OMU’s. Demonstration that this correspondence has causal implications awaits further investigation.

Materials and Methods

Tissues
Laboratory inbred ICR mice were time mated and the day of finding a plug designated day zero. First left and right lower molars were dissected carefully on day 14 of gestation (E-14). The first right lower mouse molars were halved in the bucco-lingual plane using surgical blades (Feather n°11; PTM Köln) leaving anterior and posterior explants which were as nearly as possible of equal size. Corresponding intact first left lower molars were used as controls.
The halved (posterior and anterior parts) and intact teeth were cultured on 2 ml of semi-solid control medium per Petri dish (Nunc, Roskilde, Denmark; 35x10 mm) for 2, 8, 4, 96, 108, and 144 h. The medium consisted of BGJ-B (Gibco, Fitton Jakson modified) supplemented with ascorbic acid (0.18 mg/ml, Merck), L-Glutamine (2 mM, Seromed), foetal calf serum (20%, Boehringer Bioproducts), kanamycin (0.1 mg/ml, Gibco) and Difco agar (0.5%). The teeth were incubated and grown at 37°C in a humidified atmosphere of 5% CO$_2$ in air and the medium was changed every two days.

**Bromodeoxyuridine labeling**

Cell proliferation was analyzed by culturing the tissues in the presence of the thymidine analog 5-bromo-2’-deoxy-uridine (BrdU, cell proliferation kit; Amersham Life Science). The halved and intact teeth were cultured in the presence of 0.4 ml of BrdU at a concentration of 3 μg/ml for 8 or 24 h respectively after 2, 8, 4, 96 or 108 h of culture in control medium.

**Histology-immunohistochemistry**

All the halved and intact teeth were fixed in Bouin-Holland’s fluid, embedded in paraffin and serial 5 μm sections were performed. BrdU incorporated into DNA was identified on the 5 μm thick de-waxed sections with a specific mouse monoclonal antibody and immunoperoxidase labeling following the manufacturer’s instructions (Amersham Life Science). After immunostaining, sections were counterstained with eosin. The other sections were stained with Mallory’s stain.

**Three-D reconstructions**

Drawings of the contours of the mesenchyme and of the inner dental epithelium of the halved right teeth, of intact left teeth and in one case the second molar were made at 5 μm intervals from the serial histological sections, at a magnification of x250, using a Zeiss Jeneval microscope equipped with a drawing chamber. The inner dental epithelium was subdivided into different zones corresponding to the positive and negative cells. The digitalization of the serial drawings was achieved using a Hamamatsu C2400 camera connected to a digital imaging system. The digitalization of the serial drawings and correlation of successive images (Olivo et al., 1993) have been previously described (Lesot et al., 1996). Software packages allowing image acquisition and treatment were developed and adapted to this work. Three-dimensional images were generated using a volume rendering program (Sun Voxel, Sun Microsystems).

**Acknowledgments**

We wish to thank Dr. A.J. Smith for critical reading of this manuscript and Mr. A. Ackermann for technical help. This research was partially financed by the International Human frontier Science Program (grant TG-558/95 M), by the Faculty of Odontology-Strasbourg and by the Fondation Dentaire de France (UB/SS 500144-98002598).

**References**

Regeneration of halved mouse molar in vitro


Received: February 2000

Accepted for publication: February 2000