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Original Article

Initial features of the inner dental epithelium histo-morphogenesis in the first lower molar in mouse

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ABSTRACT First lower molar development in the mouse was investigated from the cap to early bell stage using histology, morphometry, TEM and 3D reconstructions. This period was characterized by the histogenesis of the enamel organ (EO), folding of the epithelio-mesenchymal junction and growth of the tooth. The histogenesis of the EO and appearance of the enamel knot (EK) were initiated at the early cap stage (ED14). From ED14 to ED15, the anterior and posterior extension of the EK was very prominent whilst the length of the enamel organ did not substantially change. The EK appeared as a dynamic and transitory histological structure including dying and replacement cells. At ED16, the folding of the IDE, which extended over the anterior two thirds of the molar, was the first sign of cuspidogenesis. It was accompanied by a local remodeling of the basement membrane (BM): IDE cells involved in this folding transitorily lost contact with the BM which formed a loop in the mesenchyme. During this period, the growth of the lower M1 along the antero-posterior axis was restricted to the posterior part of the molar. Histogenesis occurred in the whole EO, whilst initial cuspidogenesis was limited to the anterior part of the tooth. Distinct cell populations were thus involved in different contemporary processes leading to changes in the cell density in the mesenchyme, in the mitotic activity, in cell-shape, and cell-matrix interactions in the IDE, and remodeling of the BM where both epithelium and mesenchyme might participate.

KEY WORDS: *molar, morphogenesis, cuspidogenesis, basement membrane, apoptosis, mitosis*

Introduction

Characteristic cusp patterns of mammalian teeth are assumed to result from specific folding of the epithelio-mesenchymal junction followed by mineralized matrix deposition by functional odontoblasts and ameloblasts (Butler, 1956; Gaunt and Miles, 1967; Ruch, 1987; Osborn 1993; Lyngstadaas *et al.*, 1998). Differential mitotic activities and spatially specified modulations of cell adhesion mechanisms (cell-cell and matrix-cell interactions) might support the folding of the epithelio-mesenchymal interface during odontogenesis (Fausser *et al.*, 1998; Yoshiba *et al.*, 1998). The enamel knot (EK) is a histological structure formed by a cluster of densely packed epithelial cells adjacent to the forming cavity of the enamel cap and has been suggested to direct the folding of the epithelio-mesenchymal interface (Orban, 1928; Butler, 1956). More recently, cells related to this structure were found to express signalling molecules and the whole EK was suggested to act as an organizing center (MacKenzie *et al.*, 1992; Jernvall *et al.*, 1994; Coin *et al.*, 1999), however, this hypothesis remains questionable (Lesot *et al.*, 1996; Ruch *et al.*, 1997; Peterková *et al.*, 1997, 1998; but see also Coin *et al.*, 1999 in this issue). Previous analysis of the first lower molar development in the mouse embryo, using 3D reconstructions, has shown that the first sign of cuspidogenesis appeared as a folding of the inner dental epithelium (IDE) in the anterior two thirds of the molar (Viriot *et al.*, 1997). The present work investigates the contemporary histodifferentiation of the enamel organ, the appearance of the epithelial folding which separates longitudinally the lateral (buccal) and medial (lingual) rows of forming cusps, and the antero-posterior (mesio-distal) growth of the first lower molar from ED14 to ED16.

Results

Morphogenesis of the enamel organ and initial aspects of the cuspidogenesis

At the early cap stage (ED14), apical views of the epithelio-mesenchymal junction demonstrated the presence of medial and lateral grooves, which delineated the medial, central and lateral

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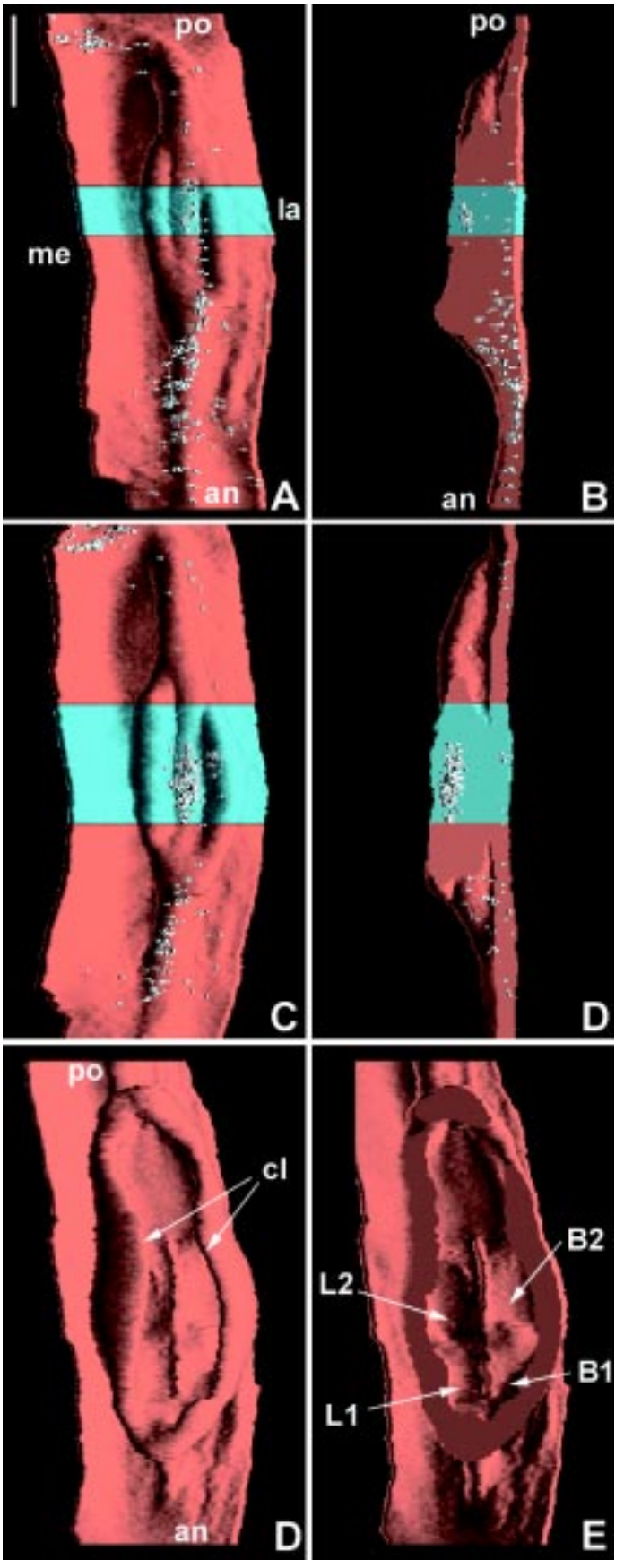

ridges (Figs. 1A and 2A). The lateral groove extended more anteriorly and the medial one more posteriorly (Fig. 1A). At this stage, the EK was localized in the posterior half of the molar cap, in a specific segment of the central ridge (Fig. 1A,B). It extended over 100 μm along the antero-posterior axis of the tooth .

In the well formed cap at ED15, the medial and lateral sides of the cap arose by increases in the respective ridges present before, whilst the central ridge remained as the cap bottom (Fig. 2A,B). Although the cap maintained approximately the same length from ED14 to ED15 (compare Fig. 1A and C), the length of the EK increased to about 250 μm (Fig. 1C,D). Comparison of the two reconstructions showed that during these 24 h the EK had extended beneath the central ridge both in anterior and posterior directions (compare Fig. 1A,B with Fig. 1C,D and see Fig. 7K).

During the cap-bell transition, the enamel organ extended in width (compare Fig. 2B and 2C) and length (compare Fig. 1C with Fig. 1E). The elongation in the posterior direction was the most apparent (Figs. 1C,E and 7B,L-N). The elongating posterior third was narrower, passed rapidly the cap stage, and exhibited a flat bottom during the whole period (Figs. 1E,F, 7).

At the early bell stage (ED16), a major folding in the epithelio-mesenchymal junction separated the forming medial and lateral rows of cusps and extended antero-posteriorly along the anterior two-thirds of the enamel organ (Figs. 1E,F, 2C). Other minor foldings, extending from the former one in the medial and lateral directions, were also observed (Fig. 1E,F), which separated the L1, B1, L2 and B2 forming cusps (Figs. 1F, 7O). These morphological changes in the epithelio-mesenchymal junction were accompanied by histogenesis of the enamel organ.

Histogenesis of the enamel organ

During the initial cap stage, the most anterior and posterior ends of the enamel organ maintained the histological organization of a bud-like epithelium on frontal sections: large columnar cells in contact with the BM and smaller polyhedral cells in the center. However, the main part of the enamel organ exhibited a forming cap configuration on sections (Fig. 3A-C). The internal cells gave rise to the loosely arranged stellate reticulum (SR). The SR was hardly visible at ED14 (Fig. 3A-C). It became apparent on sections where the medial and lateral free margins of the enamel organ elevated, regardless the antero-posterior location. Appearance of the SR was the first sign of histogenesis of the enamel organ and allowed the identification of the next components

Fig. 1. Three-D reconstructions of the dental epithelium of the first lower molar at the early cap stage at ED14 (A-B), well formed cap at ED15 (C-D) and early bell at ED16 (E-F) *as seen in apical views* **(A,C,E)**, *and lateral views of thick section* **(B,D,F)**. *The EK extended antero-posteriorly all along the region represented in blue. The presence of the EK was determined on the basis of histological criteria. Apoptotic bodies in the epithelium are represented as white dots. Some of these were associated with the EK at ED14* **(A,B)** *and their number in the EK increased at ED15* **(C,D)**. *In the early bell stage at ED16,* **(E)** *the EK had disappeared and the inner dental epithelium showed a major longitudinal folding in the anterior region of the enamel organ* **(E,F**, *arrowhead***)**. *Minor transverse foldings also delineated the L1, L2, B1 and B2 posteriorly forming cusps (according to the terminology of Gaunt, 1955). an, anterior; po, posterior; la, lateral and me, medial; cl, cervical loop. Bar, 200 μm.*

arising from the former layer of columnar cells in contact with the basement membrane: the prospective outer dental epithelium (ODE) and the complex of prospective stratum intermedium-IDE (Fig. 3).

On frontal sections, the EK had a round shape and consisted of a group of densely-packed epithelial cells (Fig. 3B,C,E,F). The EK protruded towards the mesenchyme and was delineated medially, and to a lesser degree laterally, by the grooves (Fig. 3B,C,E,F). The aspect of the EK cells has been described earlier (Lesot *et al.,* 1996). At ED14, the EK already demonstrated the presence of apoptotic bodies in its anterior part (Fig. 1A,B). Comparison between an apical and lateral view of a thick section (Fig. 1A,B) showed that, amongst all apoptoses detected at this stage, only a few of them concentrated in the EK: apoptoses were also observed in the antemolar region (Fig. 1B), and in association with the epithelium that connected the forming tooth to the oral epithelium (Fig. 1B). On frontal histological sections, apoptotic bodies present in the EK mainly accumulated in its central part; very few of them were observed close to the epithelio-mesenchymal junction (Figs. 1B,D, 3).

At the stage of well formed cap (ED15), the SR was more developed in the middle part than in the posterior or anterior part of the enamel organ (compare Fig. 3F 3E). However, the progress of SR differentiation was difficult to follow in space since it was locally retarded in various parts of the EO. The number of apoptotic bodies associated with the EK had increased (Fig. 1C,D) except in its posterior part which remained devoid of apoptosis at this stage. On frontal sections, the diameter of the EK was much smaller in its posterior part (Fig. 3F) than in its middle part (Fig. 3E), where many apoptoses concentrated (Fig. 1C,D). Apoptoses also became visible on the lateral side of the molar at ED15 (Fig. 1C), in an internal region of the forming cervical loop. They did not seem to affect the epithelial cells in contact with the basement membrane.

During the cap–bell transition, apoptoses faded gradually in the EK whose delimitation disappeared, initially towards the SR. The small cells of the EK, adjacent to the SR lost their concentric organization although they still remained more densely packed than the SR cells. When the concentric arrangement of cells delineating the EK ceased to be apparent, the IDE became distinct. The morpho- and histodifferentiation in the elongating posterior part of the enamel organ progressed rapidly. At this time, the EK had already started to regress (Fig. 7C-D,L-N) and thus never extended in the newly formed posterior part of the molar (Fig. 7E,N).

At the early bell stage (ED16), the height of the columnar epithelial cells in contact with the basement membrane (IDE, ODE) decreased compared to the former cap (Fig. 3). The extracellular matrix in the SR and papilla mesenchyme (except for the mesenchyme at the anterior end of the tooth germ) became more abundant. At that time, the size of cells decreased in both tissues (Fig. 3). The EK disappeared and the SI became apparent (Fig. 3G-I). The SI was more prominent in the middle area (Fig. 3H). In the anterior part, it was more difficult to distinguish the cells of the IDE from those of the SI because of the orientation of the cells (Fig. 3G). Posterior to this, the specific compartments (IDE, ODE, SR, SI) were well distinct except for the very posterior end of the enamel organ, where a cap configuration was apparent and histodifferentiation was delayed.

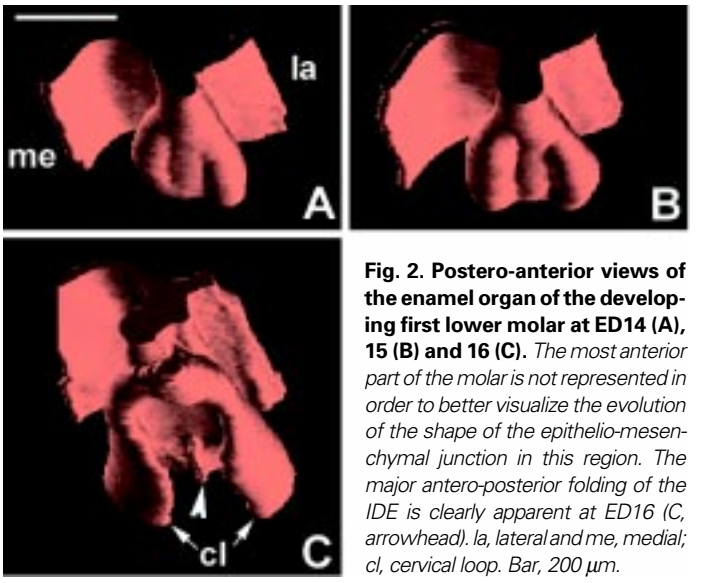

Fig. 2. Postero-anterior views of the enamel organ of the developing first lower molar at ED14 (A), 15 (B) and 16 (C). *The most anterior part of the molar is not represented in order to better visualize the evolution of the shape of the epithelio-mesenchymal junction in this region. The major antero-posterior folding of the IDE is clearly apparent at ED16 (C, arrowhead). la, lateral and me, medial; cl, cervical loop. Bar, 200 μm.*

The progressive morphogenesis led to foldings of the IDE corresponding to the initiation of cusps formation (Fig. 1E,F). The major folding extended along the antero-posterior axis of the tooth germ and was associated with an underlying loop of basement membrane extending into the dental mesenchyme (Viriot *et al.*, 1997). Smaller transversal foldings extended in the medial and lateral directions. However, these did not appear to be accompanied by changes in the interaction between IDE cells and the basement membrane at this stage: observations on sagittal sections demonstrated that the basement membrane remained adherent to cells of the IDE (not shown).

Changes in the epithelio-mesenchymal junction

At the margin of the EK, at ED15, the interface between the elongated-curved cells and the IDE was frequently marked by a long split-like intercellular space (Fig. 4A). Some of these cells were packed with glycogen suggesting cell degeneration (not shown). Apoptotic figures were never observed in epithelial cells directly in contact with the basement membrane (Fig. 4C). Mitochondria frequently exhibited a dense matrix contrasting with their prominent cristae (Fig. 4F). The basal lamina in contact with cells of the EK showed a fluffy texture and was thick (Fig. 4F) when compared to that interposed between IDE cells and dental mesenchyme at a distance from the EK (Fig. 4G). The basal lamina in contact with EK cells appeared "digested" with empty holes in the lamina lucida whilst numerous coated pits and vesicles were observed in the adjacent epithelial cells (Fig. 4F).

When the longitudinal inter-cusp folding of the epithelio-mesenchymal junction formed at ED16, cells of the IDE penetrated into a groove defined by the basal lamina (Fig. 4B). Again, empty spaces were observed in the lamina lucida (Fig. 4E), which might precede local loss of interaction of IDE cells with the basal lamina (Fig. 4D,E). In some places, the basal lamina was duplicated (Fig. 4E). Underlying the folding of the IDE, the basal lamina showed loops (Fig. 4D) and in some cases, a double ringed lamina densa was observed around epithelial cell processes. These features were never observed at a 20-30 μm distance from this specific

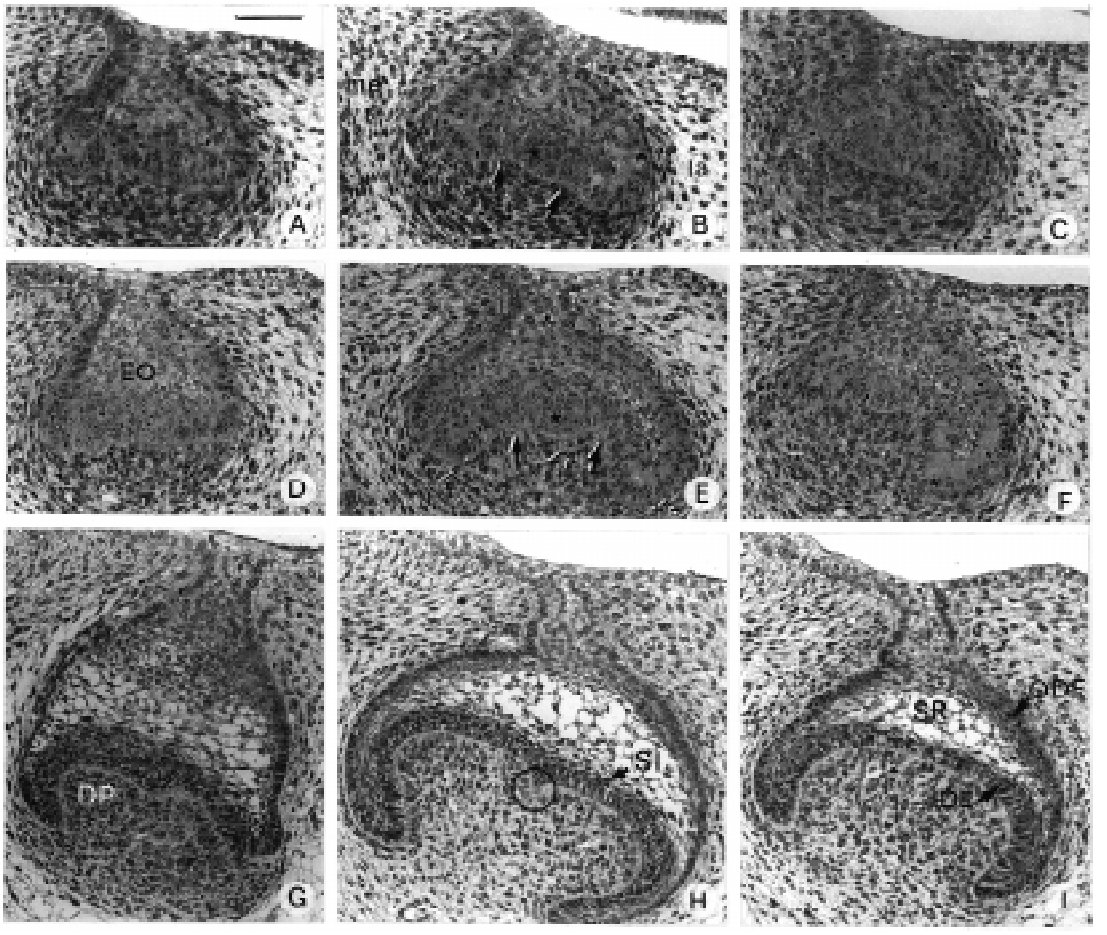

Fig. 3. Frontal histological sections documenting the anterior part (A,D,G), the middle part (B,E,H) and the posterior part (C,F,I) of the developing M1 early cap at ED14, wtc. 276-300 mg (A,B,C), well formed cap at ED15, wtc. 426-450 mg (D,E,F), and early bell at ED16, wtc. 751-800 mg (G,H,I). *Black arrows (B and E) point to the lateral and medial enamel grooves. Open arrows (E) indicate the lateral, central and medial ridges. The antero-posterior folding of the IDE (circled in H) occurred in place of the former central ridge containing the enamel knot (black stars on B and E). The loop of basement membrane associated with this folding was further observed by transmission electron microscopy (see Fig. 4) At the bell stage, note the difference in the cell density in the dental papilla (DP) in the anterior part (G) compared to the median or posterior part (H,I). la, lateral; me, medial; EO, enamel organ; ODE, outer dental epithelium; SI, stratum intermedium; SR stellate reticulum. Bar, 100 μm.*

region (not shown). In the region where the IDE folded, epithelial cells in contact with the basement membrane still showed numerous coated pits and vesicles (Fig. 4E).

Mitotic index and cell density

The cell density in the condensed dental mesenchyme at the early cap stage or in the dental papilla (DP) at later stages, and the mitotic indices in both this condensed dental mesenchyme-DP and prospective IDE-SI cells were measured in three distinct zones (I, II, III) along the antero-posterior axis of the first molar germ (Fig. 5).

The density of mesenchymal cells significantly increased in the most anterior region (zone I) at the early bell stage compared to younger stages (Fig. 6A). This situation was clearly visible on histological sections (compare Fig. 3G with 3H or I).

At ED14, the mesenchyme in the anterior part of the forming cap (zone I) exhibited the lowest mitotic index when compared to the more posterior zones II and III (Fig. 6B), but this was not statistically significant. Within ED14 and 15, the mitotic index of mesenchymal cells in zone II (Fig. 6B) significantly decreased from 6.9% to 4.3%. The changes in zones I and III, were not significant. At ED16, similar mitotic indices were measured in the mesenchyme of all three zones (Fig. 6B).

From ED14 to ED15, no significant change was observed when comparing the mitotic index calculated for the prospective IDE cells in the three distinct zones (Fig. 6C), when the EK cells in contact with the basement membrane were included for calculations. In these conditions, the mitotic index was the same (about 5%) all along the tooth (Fig. 6C). However, the mitotic index calculated for the IDE cells outside the EK area was significantly higher in the zone II compared to other zones at both ED14 and 15 (Fig. 6D).

From ED15 to ED16, the mitotic index of the IDE cells (when the EK cells in contact with the basement membrane were taken into account for calculations) significantly decreased in the anterior part of the molar (Fig. 6C). In these conditions, the apparent changes in zones II and III (Fig. 6C) were not significant. The mitotic index was also calculated without considering the EK cells in contact with the basement membrane at ED14 and ED15; then, the decrease in the mitotic index in zones I and II at ED16 became significant (Fig. 6D).

Discussion

Growth of the tooth germ

During cap formation (ED14-ED15), the length of the first lower molar was almost constant. Growth of the tooth accompanied the transition from the cap to bell stage (Fig. 7). The simultaneous changes in the size of the tooth germ and in the shape of the epithelio-mesenchymal junction were accompanied by modifications in the mitotic activity. The existence of regional variations in the mitotic activity of dental epithelial cells has been documented

after incorporation of either H^3-thymidine or BrdU (Ruch *et al.*, 1978; Jernvall *et al.*, 1998) and by the expression of *p21* (Bloch-Zupan *et al.*, 1998; Jernvall *et al.*, 1998).

Although most of the mitoses in the enamel organ concentrated in the IDE at the early bell stage (Viriot *et al.*, 1997), the mitotic index of IDE cells did not significantly increase during the cap-bell transition from ED15 to ED16 when an apparent decrease in the size of IDE cells was seen. Furthermore, histogenesis in the enamel organ during this period was characterized by an important development of the stellate reticulum where the cell density became very low. In the mesenchyme, the mitotic index which tended to decrease from the early cap to bell stage (ED14 to 16) transiently achieved a minimum value in the middle zone of the tooth at ED15 (i.e., when facing the EK area). The anterior part of the first molar at ED16 was characterized by an increased cell density in the dental mesenchyme and a decrease in the cell size. In the same region, cells of the IDE exhibited a strikingly low mitotic index. From ED14 to ED16, changes in the mitotic activity in the epithelium and mesenchyme did not occur in the same region at the same time. Cell migrations and/or differential production of extracellular matrix should play important roles in harmonizing the development of epithelial and mesenchymal compartments during tooth growth.

The comparison of the 3D reconstructions of the enamel organ during the cap–bell transition (Fig. 7) clearly suggested that the apparent elongation of the first lower molar occurred in its posterior part. The longitudinal folding of the epithelio-mesenchymal junction observed at the bell stage spatially coincided with the central ridge present in the former cap and included the EK region. This structure has been considered contradictorily either as separating the prospective buccal and lingual cusps, or as an indicator of the tip of the first forming (primary) cusp (for review see Butler, 1956). Our data support the former explanation (Figs. 2 and 7). The main antero-posterior folding of the epithelio-mesenchymal junction at the early bell stage separated the prospective lingual (L) and buccal (B) cusp rows. Other transversal foldings also appeared, suggesting the first signs of B2/B1 and L2/L1 cusp separation (according to the terminology of Gaunt, 1955). These data showed that the development of the lower M1 along the antero-posterior axis is not linear but discontinuous. The present data suggested that the cap of the first lower molar corresponded to the anterior two thirds of the prospective tooth crown bearing L1,2 and B1,2 cusps. This fits with the fact that the cervical loop is closed anteriorly, delineating the enamel organ at the onset of the bell stage (Viriot *et al.*, 1997). The posterior segment of the enamel organ started to form later, during the cap-bell transition. In contrast to the former segment, the latter part of the enamel organ exhibited a flat roof and differentiated much later to give rise to the L3 and B3 cusp areas.

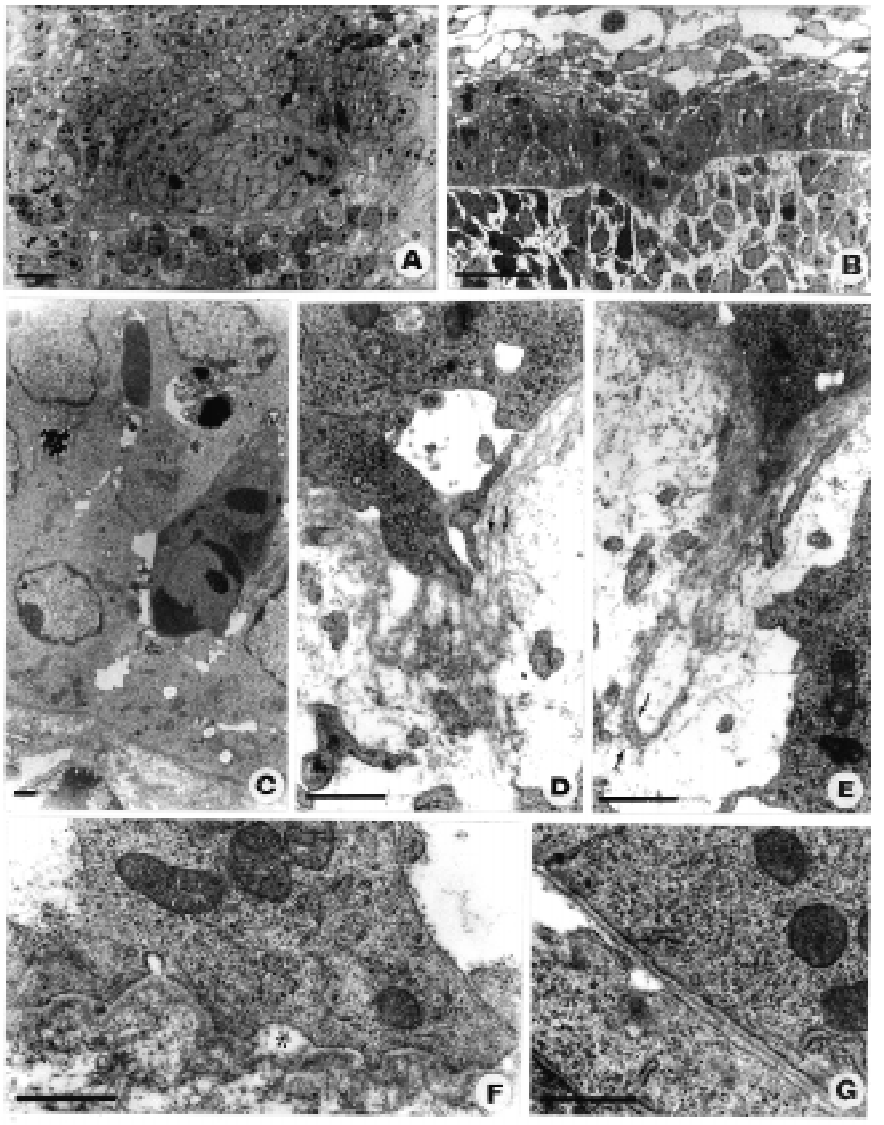

Fig. 4. Epithelio-mesenchymal junction of first lower molars at ED15 (A,C,F,G) and ED16 (B,D,E) from semi-thin (A,B) and ultra-thin sections (C-G). *Frontal semi-thin section through the enamel knot area at ED15* **(A)***: cells of the EK were densely packed and showed several apoptotic bodies (arrows). Apoptotic cells were observed close to, but not in contact with the basal lamina* **(C)***. The basal lamina in contact with cells of the EK showed a fluffy texture* **(F)***, different from that observed at 15-20 μm distance from the EK* **(G)***. Numerous small spaces (asterisk) appeared between the basal lamina and the basal pole of the epithelial cells* **(F)***. The mitochondrial matrix was frequently increased and dense* **(F)***. The major antero-posterior folding of the IDE* **(B)** *was accompanied by local changes in epithelial cell-basement membrane interactions* **(D,E)** *leading to duplication (arrows) of the basal lamina in some places* **(D)** *and to formation of a loop of basement membrane* **(E)** *extending into the*

Histogenesis of the enamel organ

The mouse molar bud consists of two cell types: a large stratum of mostly columnar cells in contact with the basement membrane and smaller cells in its center (Pourtois, 1961; Peterková *et al.*, 1996). From the cap stage, a complex histogenesis of the enamel

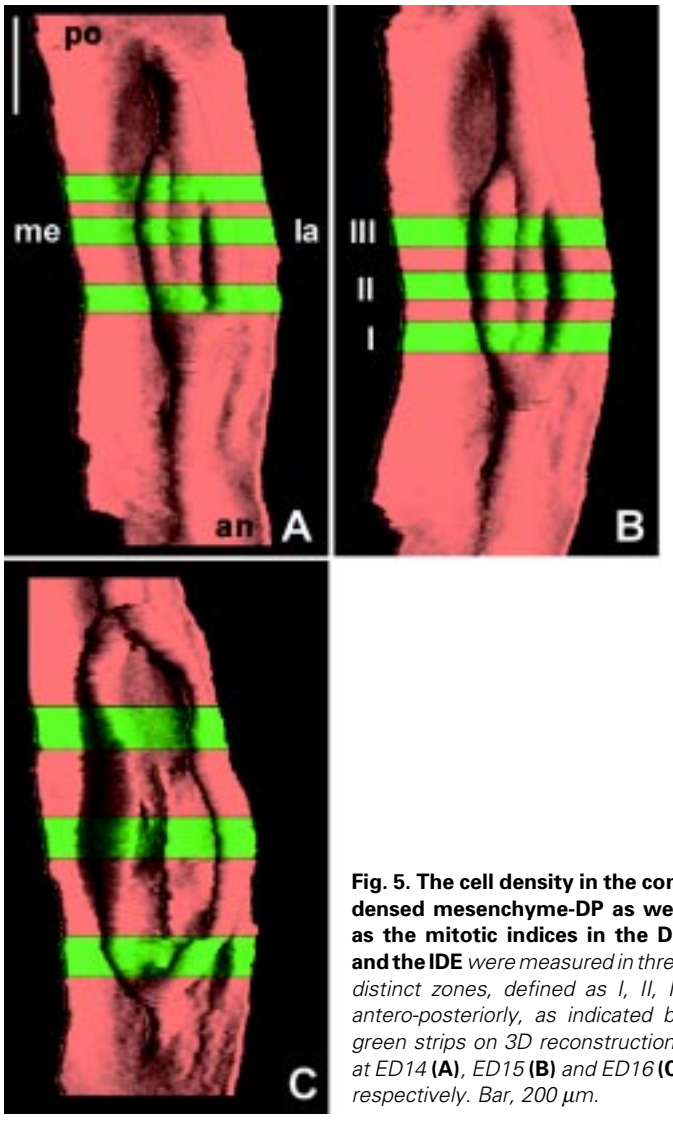

Fig. 5. The cell density in the condensed mesenchyme-DP as well as the mitotic indices in the DP and the IDE *were measured in three distinct zones, defined as I, II, III antero-posteriorly, as indicated by green strips on 3D reconstructions at ED14* **(A)**, *ED15* **(B)** *and ED16* **(C)** *respectively. Bar, 200 μm.*

organ is initiated which ultimately leads to the appearance of the SR, ODE, IDE and SI (Cohn, 1957). This process is associated with the morphogenesis and growth of the enamel organ, and results from several complementary mechanisms including changes in cell-cell and cell-matrix interactions as well as regional changes in mitotic activity (Ruch *et al.*, 1983; Palacios *et al.*, 1995; Salmivirta *et al.*, 1996; Fausser *et al.*, 1998; Yoshiba *et al.*, 1998).

The first clear signs of histogenesis in the enamel organ appeared at the early cap stage (ED14) when the EK and SR started to emerge. Although these two aspects are contemporary, the SR was apparent also in the anterior part of the tooth, at a stage when the EK has not yet extended to this area. The dynamics of the EK is reflected by a simultaneous extension along the antero-posterior axis and disappearance of some of its cells by apoptosis. Whilst the antero-posterior growth of the M1 remained discrete during cap formation (within ED14-15), the apparent extension of the EK along this axis occurred both in posterior and anterior directions (Fig. 7K). Since most if not all of the cells of the EK do not divide (Jernvall, 1994; Coin *et al.*, 1999), the extension of this structure has to result from a histological rearrangement of a specific cell population within the enamel organ. The EK thus appears as a structure where cells segregate from others cells of the EO. The EK was shown to express transcripts that encode for BMPs (Jernvall *et al.*, 1998). If translation indeed occurs, the corresponding BMPs might be involved in tooth cusps formation (Jernvall *et al.*, 1994), but also in the regulation of local apoptosis (Lesot *et al.*, 1995; Jernvall *et al.*, 1998). This latter hypothesis is supported by the increased expression of *BMP*s by epithelial cells of antemolar rudiments in the upper jaw prior to their reduction by apoptosis (Turecková *et al.*, 1995, 1996; Peterková *et al.*, 1998). Except for the most posterior part of the EK, this histological rearrangement, which led to the segregation of a specific cell population within the enamel organ, was rapidly followed by apoptosis. In this respect, it differed from apoptosis observed in other regions of the developing tooth germ or even in the region anterior to the forming M_1 (Viriot *et al.,* 1997). In these latter locations, no sign of segregation of the cells upon to die could be detected.

Transient changes in the distribution of adherens junctions (E-cadherin), in the expression of laminin-5, α6β4 integrin, and the hemidesmosome-associated BP-230 antigen could play a crucial role in the control of enamel organ histo-morphogenesis (Palacios *et al.*, 1995; Salmivirta *et al.*, 1996; Fausser *et al.*, 1998; Yoshiba *et al.*, 1998). At the late cap stage, cells of the IDE and ODE expressed hemidesmosomal constituents such as HD1, BP230 (Fausser *et al.*, 1998). IDE cells also expressed α6β4 integrin (Salmivirta *et al.*, 1996), which could interact with laminin-1 (LN1) and laminin-5 (LN5), present all along the basement membrane delineating the enamel organ at this stage (Yoshiba *et al.*, 1998). Contemporary to the transition from the cap to bell stage, changes in the composition of the basement membrane were illustrated by the disappearance of LN5 from the basement membrane facing the IDE (Yoshiba *et al.*, 1998). A critical component of hemidesmosomes (Stepp *et al.*, 1990; Jones *et al.,* 1991; Sonnenberg *et al.*, 1991), the integrin β4 chain, disappeared from the same region at the same time (Salmivirta *et al.*, 1996). During the cap to bell transition, cell-cell interactions also demonstrated

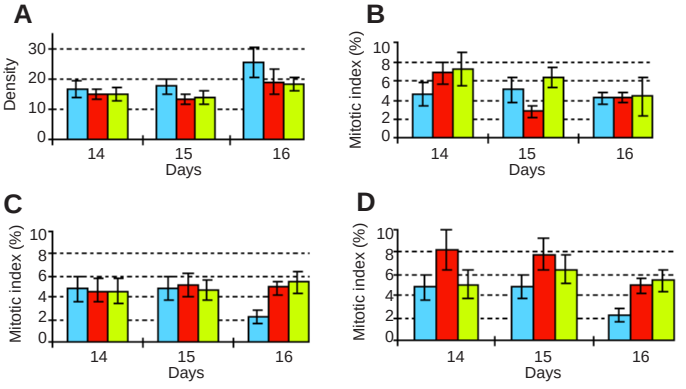

Fig. 6. The cell density in the condensed mesenchyme-DP (A) as well as the mitotic indices of DP cells (B) and of prospective IDE-SI cells (C,D) *were measured in zones, I (blue), II (orange) and III (yellow). The mitotic indices in the prospective IDE-SI cells* **(C,D)** *were calculated either taking into account the cells of the EK in contact with the basement membrane* **(C)**, *or omitting them* **(D)**. *Bars indicate the standard deviation.*

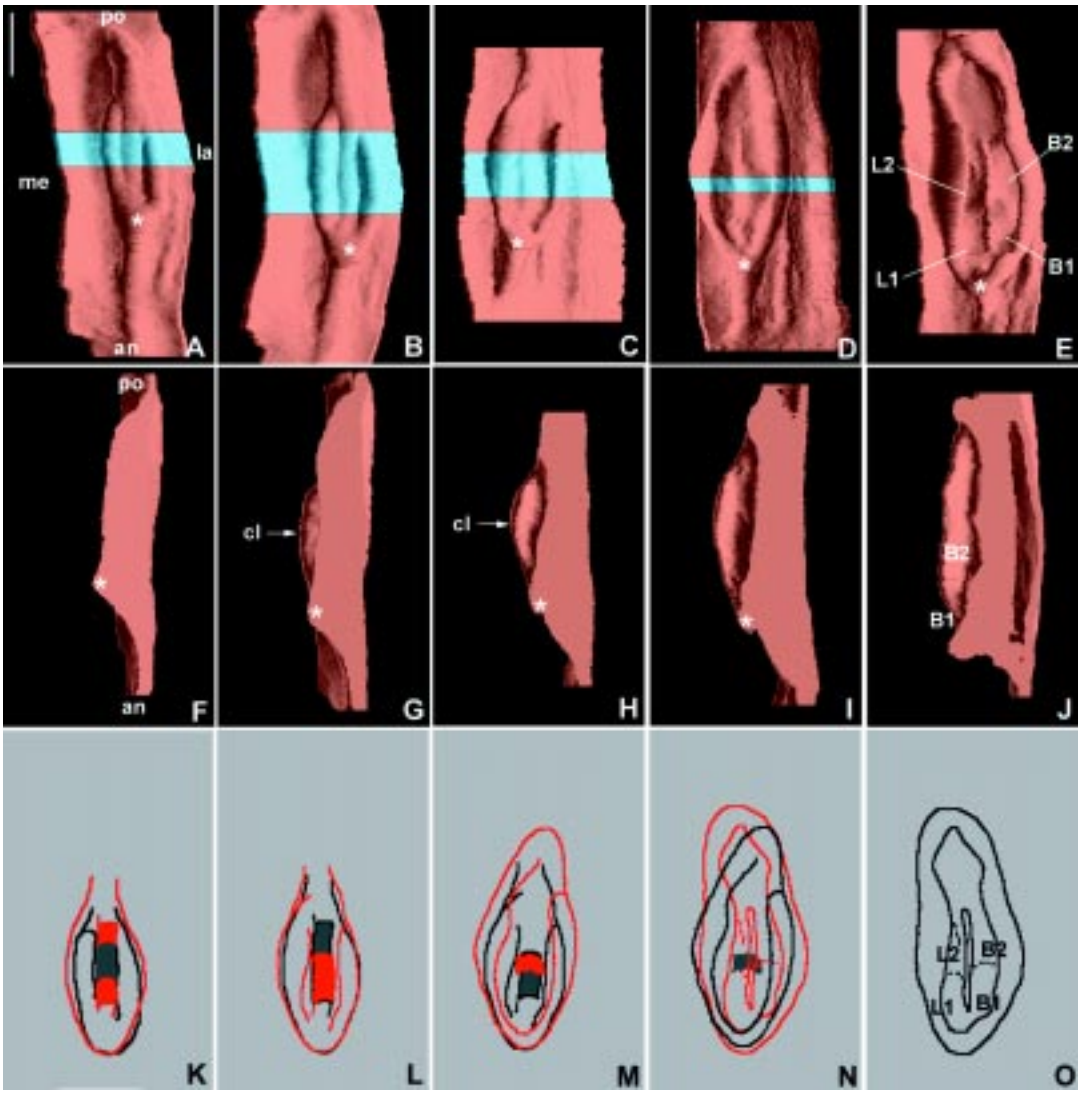

Fig. 7. Three-D reconstructions (A-J) and schematic representations (K-O) of the morphogenesis of the enamel organ from the early cap to the early bell stage. *The antero-posterior extension of the EK is visualized by blue stripping on aerial views* **(A-E)** *of the enamel organ at ED14, weight class 276-300 mg* **(A)**, *ED15, weight class 426-450 mg* **(B)**, *ED15.5, weight class 501-550 mg* **(C)**, *ED16, weight class 601-650 mg* **(D)** *and ED16 weight class 751-800 mg* **(E)**. *Sagittal sections were performed in reconstructions of the enamel organs from the same samples and lateral views* **(F-J)** *document the progressive development of the cervical loop (cl) and show the position of its most anterior part (*). This anterior delimitation (*) is visualized both in aerial and sagittal views except in* **(J)** *where the section was performed in a slightly more lateral position to illustrate the localization of B1 and B2 forming cusps. Schematic representation of molar elongation is illustrated* **(K-N)**. *In each scheme, two successive stages were superposed visually, the younger in red and the older in black, in order to compare the early cap with the well formed cap stage* **(K)**, *two late cap stages* **(L)**, *the late cap with the early bell stage* **(M)** *and two different bell stages* **(N)**. *These superpositions illustrate the elongation of the molar as well as the anterior and posterior extensions of the EK during the cap stage* **(K)**, *followed by the regression of this structure during the cap-bell transition* **(L-N)**. *All along this period* **(K-M)** *and still at the bell stage* **(N)**, *the first molar elongated in its posterior part. During the bell stage, the EK completely disappeared* **(N)**. *The major longitudinal and minor transversal folding of the IDE became visible and the areas of prospective cusps L1, B1, L2 and B2 could be detected* **(O** *– compare with Fig. 1* **E,F**). *an, anterior; po, posterior; la, lateral and me, medial. Bar, 200 μm.*

changes in the IDE. At ED16, a transitory decrease in the immunostaining for constituents of adherens junctions (E-cadherin and γ-catenin) and of desmosomes (desmoglein) has also been observed in cells of the IDE (Fausser *et al*., 1998).

Folding of the inner dental epithelium

At the late cap stage (ED15), a localized remodeling of the basement membrane started in the region where the BM was in contact with cells of the EK. In this region, the BM thickened and presented several electron-lucent areas, whilst the adjacent epithelial cells showed numerous coated pits. However, apoptotic bodies in the EK were never observed in contact with the basement membrane. Similar observations have been reported in the case of granulosa cells in the ovarian follicle, which underwent apoptosis earlier when distal from the basement membrane (Aharoni *et al*., 1997). These authors suggested that, amongst constituents of the basement membrane, laminin could play a role in protecting cells from apoptosis. However, the aspect of mitochondria in our observations demonstrated that cells in contact with the BM also showed initial stages of apoptosis. It must thus be assumed that the cells in contact with the BM could also undergo apoptosis but that then they lost contact with their neighbors (Clarke, 1990) and with the BM itself. The localization of apoptotic bodies does not necessarily correspond to that of the cell where they originated from. Indeed, the conversion of a cell into a cluster of apoptotic bodies is completed within several minutes whilst phagocytosis by neighboring healthy cells and degradation of apoptotic bodies is completed within several hours (Kerr *et al.*, 1995).

During the transition from ED15 to ED16, folding of the IDE along the antero-posterior axis of the tooth extended over the anterior two-thirds of the molar (Viriot *et al*., 1997), which corresponded to the location of the former EK area (Fig. 7K-O). It appeared as a major change associated with initial cuspidogenesis.

In this region, the basement membrane detached from the IDE at the tip of the folding and extended as a loop into the mesenchyme. This local detachment of IDE cells from the basement membrane was specifically restricted to the few cells involved in the folding of the IDE. At 20-30 μm distance on more lateral or medial directions, a single basement membrane was directly in contact with the basal plasma membrane of the IDE cells. Furthermore, such loops of basement membrane were still observed at much later developmental stages (i.e., long after the histogenesis of the IDE had proceeded) and again in regions where folding of the IDE was associated with further cusp formation (Lesot, unpublished observations). This process, which is very transitory *in vivo*, has also been reported *in vitro* (Meyer *et al.,* 1995) and several authors have published similar images in cases of cultured tooth germs (Hu *et al.,* 1992; Mark *et al.,* 1992; Sato *et al.,* 1993). The increased motility of epithelial cells, which supported the folding of the epithelio-mesenchymal junction, was mediated by a loss or strong decrease in the interactions between IDE cells and the adjacent basement membrane.

The major folding of the epithelio-mesenchymal junction along the antero-posterior axis of the molar might be considered to be a consequence of the disappearance of the EK (Fig. 7K-O). However, the two events can be dissociated since 1) minor foldings also occur for example at the posterior part of L2 cusp at ED16, which do not fit in space and 2) free basement membrane in the mesenchyme underlying a folding of the IDE was still observed at ED18.5 (data not shown), which does not fit in time.

Temporal and spatial aspects of cellular events involved in the cap to bell transition

Three phenomena characterized the short period of tooth development that led from the cap to bell stage: histogenesis of the IDE, folding of the epithelio-mesenchymal junction and growth of the molar. Three-D reconstructions showed that tooth elongation occurred in the posterior part of the molar, histogenesis was observed in the whole enamel organ and initial cuspidogenesis was limited to the anterior two thirds of the first molar. Histogenesis of the IDE was accompanied by a change in the composition of the basement membrane illustrated by the transitory disappearance of LN5 (Yoshiba *et al.*, 1998). At the bell stage, folding of the IDE, which extended maximally along the anterior two thirds of the molar, was the first sign of cuspidogenesis. It was accompanied by local remodeling of the basement membrane: the cells involved in this folding transitorily lost contact with the BM which formed a loop extending in the dental mesenchyme. This initial step of cuspidogenesis, which preceded the formation of secondary enamel knots, stressed the dynamics of the cell-matrix interactions. A localized expression or activation of enzymes involved in the remodeling of the basement membrane (Sahlberg *et al.,* 1992) might play a major role.

These observations thus demonstrate that cell populations from different regions of the developing molar were involved in different contemporary processes leading to a) changes in the cell density in the mesenchyme, b) changes in the mitotic activity, in the cell-shape and cell-matrix interactions in the IDE, and c) remodeling of the basement membrane where both the epithelium and mesenchyme might participate. This clear temporo-spatial knowledge of the morphogenetic events that occur during the cap to bell transition is a prerequisite to interpretation of data from immunolocalization or *in situ* hybridization as well as from *in vitro* tooth development.

Materials and Methods

ICR mouse embryos, whose age was determined in embryonic days (Peterková *et al*., 1996), specified by the body weight (Peterková *et al*., 1993) were harvested at noon and midnight from ED14 to 16. The specimens weighing up to 500 mg were distributed in 25 mg weight classes (wtc.) and for larger weights (up to 1000 mg) in 50 mg classes. The embryos were fixed in Bouin-Hollande fluid. At least one specimen from each weight class at each chronological stage was chosen and its head processed for histology.

Histology

Five μm frontal serial sections from paraffin embedded heads were stained with alcian blue-hematoxylin-eosin. The cap and bell stages were identified in frontal sections according to the criteria described by Cohn (1957) and Orban (1928). Specimens, whose first lower molar exhibited the early cap, well formed cap and early bell stages were selected at ED14, 15 and 16 respectively for a more detailed analysis by 3D reconstructions (distribution of apoptoses, length of the EK) and evaluation of cell density and mitotic indices.

Cell density and mitotic index

The density of DP cells of first lower molars at ED14, 15, and 16 was measured by counting the nuclei on each third section in three distinct zones along the antero-posterior axis of the tooth germ (Fig. 5). Cell densities were calculated by counting cell nuclei in three areas from the same sections of 55 μm x 40 μm, using a x40 objective. All counts were repeated three times and confirmed for statistical significance using ANOVA (SigmaStat, Jandel GmbH, Erkrath, Germany), with a significance threshold of 0.05.

The mitotic indices (MI) were measured by counting mitotic figures (late prophases, metaphases, anaphases and early telophases) either in the dental mesenchyme or in the inner dental epithelium (Olive and Ruch, 1982). All counts were repeated three times in the same areas as for determining cell density. The mitotic indices were compared using the exact Fisher test applied to a 2x2 contingency table. Whether two mitotic indices were different was not tested merely, but more precisely if a given mitotic index was smaller than another one. The difference was considered as significant when $p<0.05$.

Three-D reconstructions

The contours of the mandibular dental and adjacent oral epithelium were drawn from serial frontal histological sections (5 μm intervals) using a Zeiss microscope equipped with a drawing chamber at a magnification x320. Apoptotic cells and bodies were identified from histological sections on the basis of morphological criteria (Kerr *et al*., 1995, Turecková *et al*., 1996); their nature has previously been confirmed using the TUNEL method (Turecková *et al*., 1996). Since very few or no apoptosis could be detected in the dental mesenchyme, only epithelial apoptoses were indicated in the drawings.

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).

Transmission electron microscopy

Tooth germs of ED15 and 16 mouse embryos were immersed in a solution containing 2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, rinsed in cacodylate buffer and post-fixed for 1 h in a 1% OsO_4 solution

in the same buffer. After dehydration, the specimens were embedded in Epon 812. Semithin sections were stained with toluidine blue. Ultrathin sections, contrasted with uranyl acetate and lead citrate, were examined in a Siemens Elmiskop 102.

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