Original Article

Mouse molar morphogenesis revisited by three-dimensional reconstruction. III. Spatial distribution of mitoses and apoptoses up to bell-staged first lower molar teeth

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ABSTRACT Computer-assisted 3D reconstructions were used to follow the development of the embryonic mouse first lower molar (M_1). At ED 12.5, the thickening of the oral epithelium, which was thought to correspond to the molar dental lamina, regressed in its anterior part as a result of apoptosis. Only the posterior part later gave rise to molars. The transition to the cap stage entailed medial and lateral extensions of the dental epithelium. The growth and histo-morphogenesis of the enamel organ as well as cervical loop formation proceeded more rapidly in the anterior part of the M_1 during the cap and early bell stages producing significant morphological differences along the antero-posterior axis. Apoptosis was temporarily intensive in the anterior part of the bud- and cap-shaped epithelium and thus pointed domains which do not participate in the formation of the final M_1 enamel organ. In the well-formed cap, apoptoses displayed maximum concentration in the enamel knot (EK). No increase in the number of metaphases could be detected in the vicinity of the EK. Mitoses were distributed throughout the epithelial compartment until cap stage and then mainly concentrated in the inner dental epithelium at the early bell stage. At this later stage, either lateral views or thick virtual sections performed in the reconstruction demonstrated a clear cut distribution of mitoses and apoptoses in the enamel organ. At the early bell stage, mitoses in the mesenchyme demonstrated an increasing postero-anterior gradient.

KEY WORDS: *tooth, morphogenesis, 3D reconstruction, apoptosis, mitosis*

Introduction

The developing first lower molar (M_1) in mouse embryos is widely used as a model for studying the nature of the epithelio-mesenchymal interactions which control morphogenesis (Slavkin, 1990; Cam *et al.*, 1992; Kronmiller, 1995; Ruch, 1995; Sharpe, 1995; Thesleff *et al.*, 1995; Weil *et al.*, 1995; Chen *et al.*, 1996), histogenesis (Osman *et al.*, 1979; Palacios *et al.*, 1995) and cytodifferentiations (Lesot *et al.*, 1992; Ruch *et al.*, 1995; Slavkin, 1995). The mouse M_1 is also currently used for *in vitro* experiments to test the specific roles of different molecules such as matrix constituents (Lesot *et al.*, 1985; Mark *et al.*, 1990), growth factors (Kronmiller *et al.*, 1991; Bègue-Kirn *et al.*, 1992, 1994; Mitsiadis and Luuko, 1995; Young et al, 1995; Tabata *et al.*, 1996), retinoids (Mark *et al.*, 1992; Kronmiller *et al.*, 1994) and transcription factors (Sharpe, 1995; Chen *et al.*, 1996; Kratochwil *et al.*, 1996). Mouse molar model is also utilized to study the cascade of events these factors may trigger during morphogenesis or cytodifferentiations (Ruch *et al.*, 1995; Sharpe, 1995; Thesleff *et al.*, 1995).

Differential mitotic activities have been suspected to play an important role in odontogenesis (Gaunt, 1955, 1956; Cohn, 1957; Butler and Ramadan, 1962; Butler, 1967; Ruch, 1984). Cohn (1957) suggested the existence of specific sites of cell division. A theoretical model has been developed to explain how mitotic activity could interfere with organogenesis and thus specify morphogenesis by generating mechanical forces that might change cell position (Osborn, 1993).

Apoptosis, or programmed cell death, occurs not only in pathological conditions but also during normal embryonic development

Abbreviations used in this paper: ED, embryonic day; EK, enamel knot; M_1, first lower molar; M_2, second lower molar; 3D, three dimensional.

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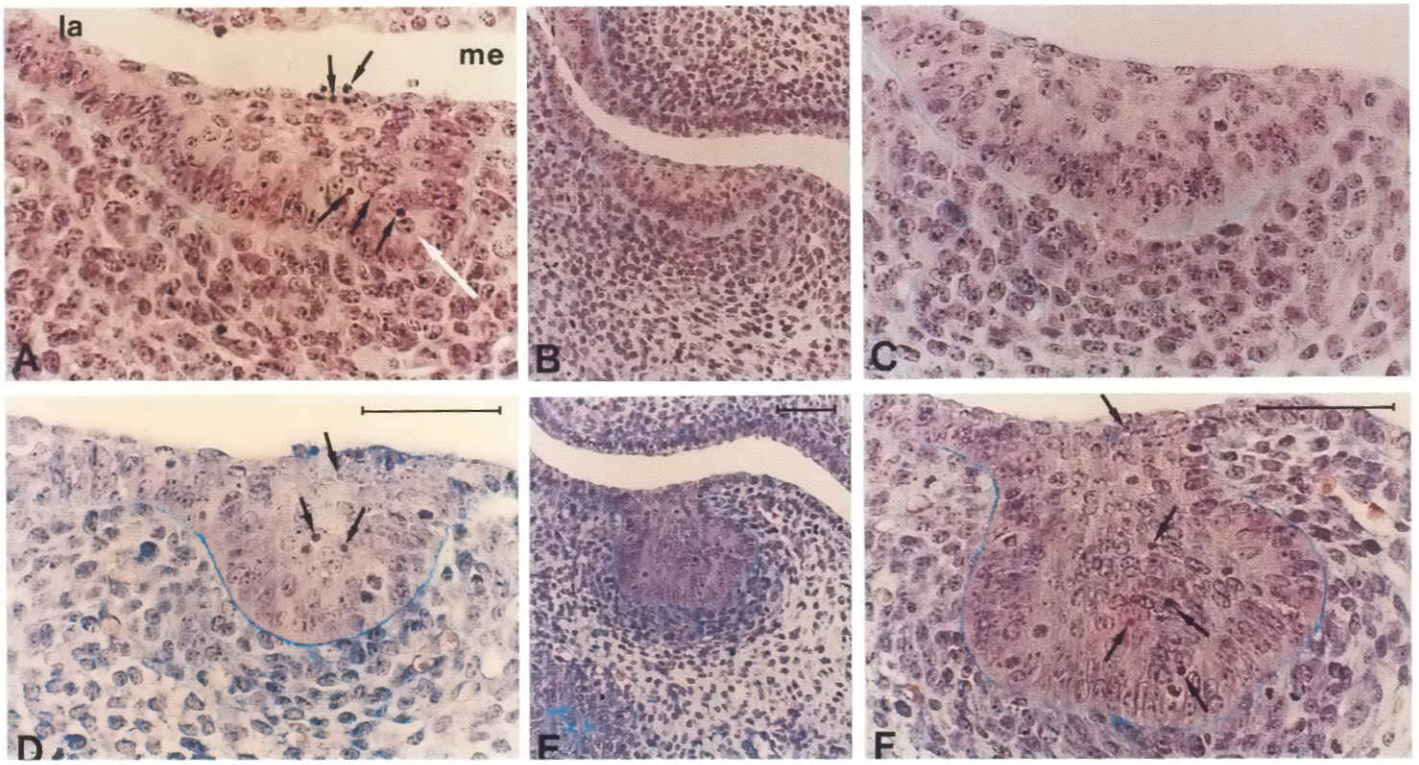

Fig. 1. Frontal histological sections *documenting the anterior* ***(A, D)*** *and the middle part* ***(B-C*** *and* ***E-F)*** *of the dental epithelium in the cheek region of the mandible in the mouse embryo at ED 12.5, wtc. 101-125 mg* ***(A, B, C)****, and ED 13.5, wtc. 151-175 mg* ***(D, E, F)****. C and F are enlargements of B and E respectively. Thin black arrows indicate the apoptotic cells and bodies, white arrow points to the medial projection of the early bud-shaped epithelium. la, lateral; me, medial. Bar, 50 µm.*

(Kimura and Shiota, 1996; Roffler-Tarlof *et al.*, 1996). In the latter case, apoptosis may be involved in embryonic tissue remodeling (Hopkinson-Woolley *et al.*, 1994; Zakeri *et al.*, 1994). Cell death has been observed during dental lamina, bud, cap and bell stages of tooth development (Nozue, 1971; Kindaichi, 1980; Lesot *et al.*, 1996; Turecková *et al.*, 1996; Vaahtokari *et al.*, 1996b; Peterková *et al.*, 1997 and references therein). Cell death has also been reported in the gubernaculum of the enamel organ (Lesot *et al.*, 1996; Vaahtokari *et al.*, 1996b) and during reduction of the ameloblast number (Moe and Jessen, 1972; Bronckers *et al.*, 1996).

Most authors have investigated the development of mouse molars *in vivo* and *in vitro* using histological, histochemical, immunological or ultrastructural approaches as well as in situ hybridization. However, individual sections can hardly be considered representative of an overall situation: three dimensional representations have long appeared necessary for a better understanding of tooth morphogenesis (Röse, 1892; Ahrens 1913; Gaunt, 1955; Ooë, 1956; Radlanski, 1993; Peterková *et al.*, 1993, 1995, 1996; Jernvall *et al.*, 1994; Seipel *et al.*, 1995; Lesot *et al.*, 1996; Turecková *et al.*, 1996). Such an approach using computer technology was employed in this paper to trace the development of the embryonic mouse M_1 up to the early bell stage, including the spatial distribution of mitoses and apoptoses. This work provides a general framework for further analysis of the spatial distribution of antigens and transcripts.

Results

Histological aspects and 3D reconstructions of the dental epithelium

In terms of terminology, anterior has been preferred here to mesial, posterior to distal, medial to lingual or internal, and lateral to vestibular, jugal or external.

ED 12.5

The anterior part of the dental epithelium in the mandibular cheek region exhibited an early bud-shape (Figs. 1A, 4B). The medial side of this structure included an epithelial extension toward the mesenchyme (Figs. 1A, 4B, 6A). Posteriorly, this projection narrowed, conferring on the dental epithelium the shape of a large dental lamina (Figs. 1B-C, 4C). In the most posterior mandibular region, only an epithelial thickening was observed. Anteriorly to the bud-shaped epithelium, a low dental lamina extended over a short distance and progressively decreased in thickness (Figs. 4A, 6A,D).

The cells at the periphery of the mesenchymal condensation surrounding the lamina-shaped part of the dental epithelium tended to be oriented concentrically (Fig. 1B). Many apoptotic cells and bodies were found inside the medial projection and next to the oral surface of the bud-shaped dental epithelium (Fig. 1A).

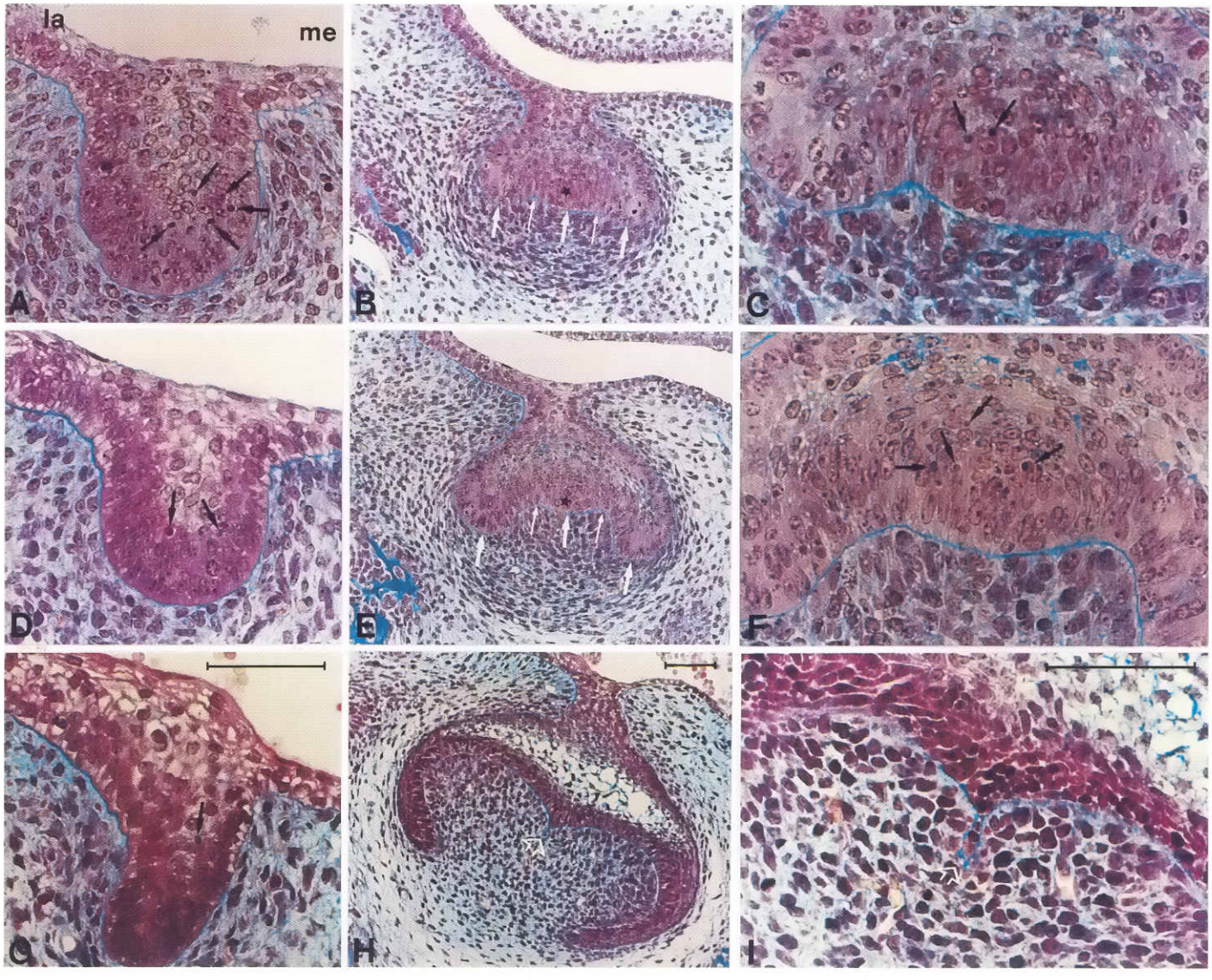

Fig. 2. Frontal histological sections *documenting the anterior part* ***(A, D, G)*** *and the middle part of the dental epithelium in the cheek region of the mandible in the mouse embryo at ED 14, wtc. 276-300 mg* ***(A, B, C)****, ED 15, wtc. 426-450 mg* ***(D, E, F)****, and ED 16, wtc. 751-800 mg* ***(G, H, I)****. C, F and I are enlargements of B, E and H respectively. Thin black arrows indicate the apoptotic cells and bodies. Thin white arrows* ***(B and E)*** *point to the lateral and medial enamel groove. Broad white arrows* ***(B and E)*** *indicate the lateral, central and medial ridge. Notice the duplication of the basement membrane (hollow arrows on H and I) in place of the former central ridge containing the enamel knot (black stars on B and E). la, lateral; me, medial. Bar, 50 μm.*

ED 13.5

A long cylinder-like structure was visible from the 3-D reconstruction of the dental epithelium at ED 13.5 (Figs. 4D-F, 7A). The middle segment of this structure exhibited a well formed bud shape in frontal sections (Figs. 1E, 4F), narrowing gradually toward both the anterior and posterior directions. A small bud-like structure was detected beside the antero-medial part of the main epithelial bud (Fig. 4E). The posterior epithelial thickening extended up to the isthmus faucium. At this stage, the delimitation of both the bud shaped dental epithelium and the condensing dental mesenchyme became much easier to detect in histological sections than at ED 12.5 (Fig. 1E). The mesenchymal cells exhibited a predominantly concentric arrangement in the condensation around the bud-shaped epithelium (Fig. 1E, F).

Accumulation of apoptotic cells and bodies was found in the epithelium of the lamina-shaped anterior part (Figu. 1D) and also in the largest part of the bud-shaped epithelium (Fig. 1F). Phagocytes filled with apoptotic bodies were observed at the oral surface.

ED 14.0

At the early cap stage, the apical surface of the dental epithelium consisted of three longitudinal ridges separated by two grooves (Fig. 2B). This arrangement reflected the initial formation of the cap cavity (Fig. 3A, 8A). Both the medial and lateral ridges extended

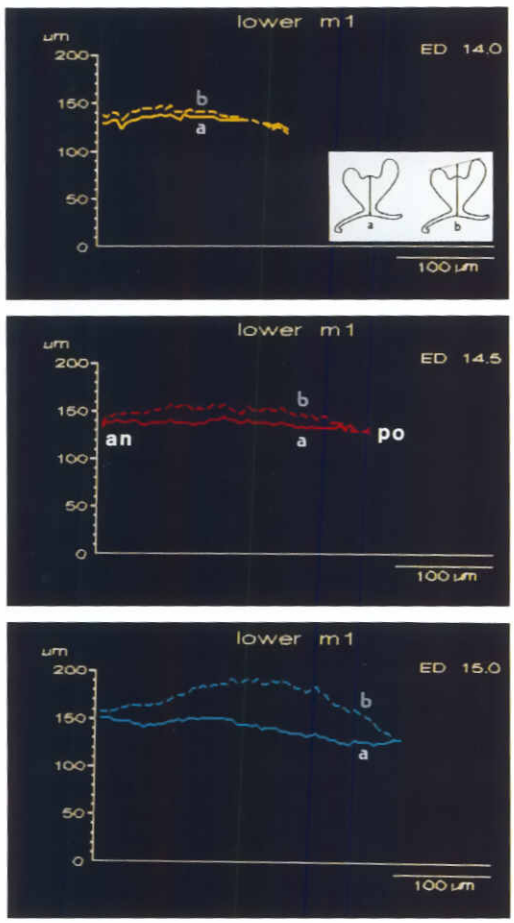

Fig. 3. Morphometry. *Graphs representing the antero-posterior changes in the height of the central part (full line-distance a) and the marginal part (dashed line-distance b) of the dental epithelium during cap formation at ED 14 (wtc. 276-300 mg), ED 14.5 (wtc. 301-325 mg) and ED 15 (wtc. 426-450 mg). an, anterior; po, posterior.*

and protruded toward the mesenchyme (Figs. 2B.C, 5A-C), corresponding to the medial and lateral parts of the prospective cervical loop. The central ridge included the EK area (Figs. 2B-C). In continuity with the posterior part of the forming M_1 cap, a bud-shaped epithelial thickening was observed which may correspond to the initiation of the M_2 development. Both the anterior part of the forming M_1 cap and the posterior part of the forming M_2 bud changed into a lamina-shaped epithelium whose thickening progressively reduced (Fig. 5A-B, 8A,D). In the anterior lamina- and bud-shaped epithelium, apoptotic cells and bodies were detected (Fig. 2A).

At this stage, histodifferentiation of the the inner and outer dental epithelium and the stellate reticulum was initiated (Fig. 2C). The EK started to take on its characteristic cellular arrangement and condensation (for details see Lesot *et al.*, 1996); apoptotic cells and bodies were found there (Figs. 2C, 8C,F). The antero-posterior extension of the distinct EK was about 100 µm in length.

ED 15.0

In the well-formed cap, the lateral and medial ridges continued to extend toward the mesenchyme (Figs. 2E, 3C, 5F). Ridge extension was more advanced in the anterior part of the cap (Fig. 5D-E) so that the cap cavity was bounded anteriorly, medially and laterally but not posteriorly (Figs. 5D-E, 9A,D). The former central ridge constituted the roof of the well formed cap and included the EK (Figs. 2E-F). Apoptotic cells and bodies were still detected in the lamina- and bud-shaped epithelium located anteriorly to the M_1 cap (Fig. 2D).
The histogenesis of the cap epithelium into the inner and outer dental epithelium and stellate reticulum progressed (Fig. 2E). The EK containing frequent apoptotic cells and bodies was well delineated with regard to adjacent inner dental cells and stellate reticulum by concentrically arranged cells at its periphery (Fig. 2F). The antero-posterior extension of the EK was about 250 µm in length.

ED 16.0

At the early bell stage, the cervical loop was well developed except in the posterior part, where its progression was still delayed (Figs. 5G-H, 10D). On the roof of the bell cavity, foldings of the basement membrane detached from the cells of the inner dental epithelium were observed in the anterior two thirds of the tooth germ (Figs. 2H-I, 5G-I, 10A). The two grooves previously observed at cap stage enlarged considerably and were now separated by a long folding of the basement membrane (Figs. 2H, 5I, 10A).

Histogenesis continued, giving rise to the inner and outer dental epithelium (Fig. 2H). The intercellular spaces in the stellate reticulum increased significantly in size (Fig. 2H).

Mitoses and apoptoses

ED 12.5

Dispersed apoptotic bodies were observed over a large area whilst others tended to concentrate antero-medially, in the early bud-shaped epithelium (Figs. 6C,F, 11A-B). Numerous apoptoses were associated with the superficial cells of the dental epithelium, adjacent to the oral cavity (Fig. 6F). At this stage, mitoses were ubiquitously distributed in the epithelium (Fig. 6C,F) and in the adjacent mesenchyme (Fig. 6B,E).

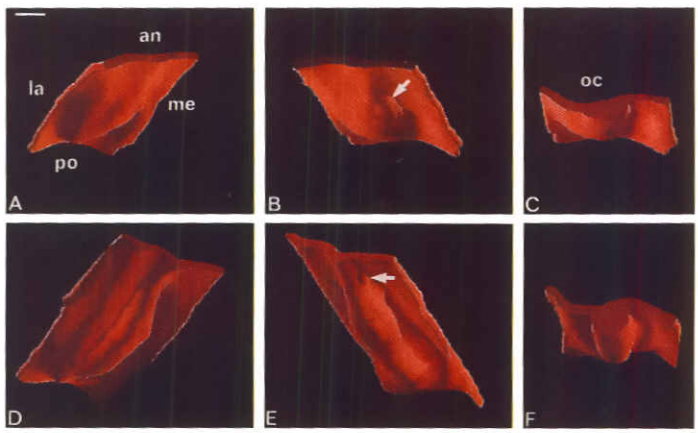

Fig. 4. 3D reconstructions of the dental epithelium of the developing first lower molar at ED 12.5 (A-C) and 13.5 (D-F). *The dental epithelium is oriented along an antero-posterior axis **(A-F)**, and observed antero-laterally **(A, D)**, antero-medially **(B, E)** and frontally **(C, F)**. Frontal sections performed in the central part of the reconstructions demonstrated the lamina-shaped **(C)** and bud-shaped **(F)** dental epithelium. White arrows indicate the medial protrusion of the early bud-shaped epithelium at ED 12.5 **(B)** and the small bud-like structure detected beside the antero-medial part of the main epithelial bud at ED 13.5 **(E)**. an, anterior; po, posterior; la, lateral; me, medial and oc, occlusal. Bar, 100 µm.*

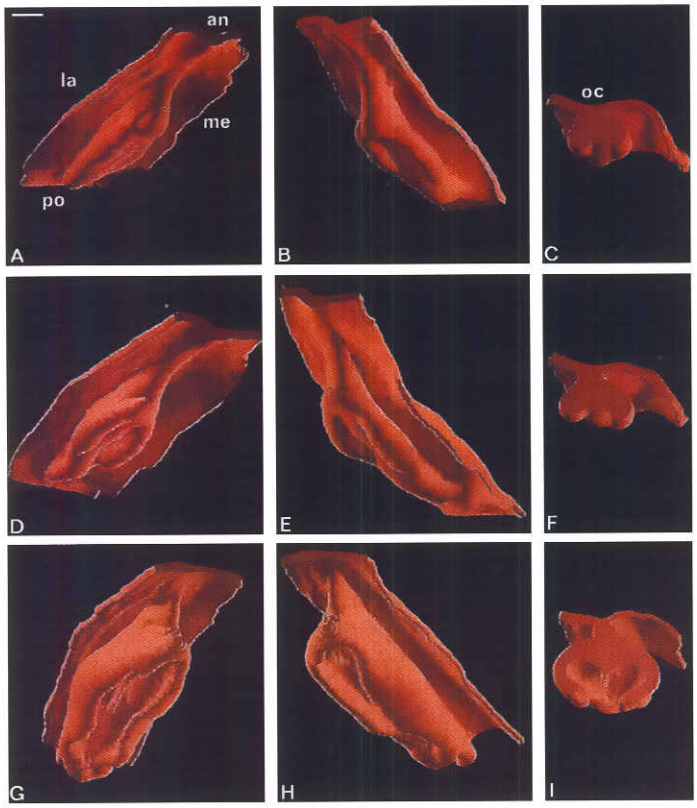

Fig. 5. 3D reconstructions of the dental epithelium of the developing first lower molar at ED 14 (A-C), 15 (D-F) and 16 (G-I). *The dental epithelium is oriented along an antero-posterior axis **(A-I)**, and observed antero-laterally **(A, D, G)**, antero-medially **(B, E, H)** and frontally **(C, F, I)**. Frontal sections performed in the central part of the reconstructions demonstrated early cap **(C)**, well formed cap **(F)** and early bell (I) stages. The lateral and medial ridges grew in the apical direction (**A-C**), then united progressively in the anterior part of the cap to form a U-shaped ridge open posteriorly **(D-F)** and finally developed into the cervical loop which remained unclosed posteriorly at the early bell stage **(G-I)**. an, anterior; po, posterior; la, lateral; me, medial and oc, occlusal. Bar, 100 μm.*

ED 13.5

Apoptotic bodies within the epithelial compartment (Fig. 7C,F) accumulated locally in the anterior part of the bud-shaped epithelium and the anteriorly extending lamina (Fig. 7A,D). At this stage again, most apoptoses were located close to the oral cavity (Fig. 7F). However a mass of apoptotic bodies appeared to proceed towards the epithelio-mesenchymal junction, inside the large bud-shaped epithelium (Figs. 7F, 11C-D, see also Fig. 1F).

Mitoses were observed all along the dental bud epithelium (Fig. 7F). Within the condensing mesenchyme, mitoses maintained their widespread distribution in the antero-posterior direction (Fig. 7B,E), but were predominant in the medial part (Fig. 7B).

ED 14.0

Apoptoses (Fig. 8C,F) concentrated in a region anterior to the forming enamel cap (Fig. 8A,D), affecting the lamina- and bud-shaped epithelium and also cells adjacent to the oral cavity. Apoptoses were also observed in the oral epithelium behind and medially to the epithelial thickening which would later give rise to the M_2 (Fig. 8C). The number of apoptotic bodies in the oral epithelium (Figs. 8F, 11E-F) decreased greatly when compared to earlier stages (Figs. 7F, 11C-D). Apoptoses already started to concentrate in the EK (Figs. 8C,F, 11 E-F).

Mitoses in the epithelium were more frequent in the lateral and medial ridges (prospective cervical loop of the bell) than in the central one (Fig. 8C). Mitoses in the epithelium displayed a postero-anterior increasing gradient (Fig. 8F). A similar pattern was also observed in the mesenchyme (Fig. 8E). However, in the anterior area, mitoses were very rare in the mesenchyme opposite to the regressing epithelium (Fig. 8B,E).

ED 15.0

A large number of apoptotic bodies accumulated in the EK (Figs. 9C,F, 11G-H). A further concentration of apoptotic bodies also occurred on the lateral side of the EK (Figs. 9C, 11H). The frequency of apoptotic bodies in the region anterior to the M_1 cap still decreased (compare Fig. 9C with Fig. 8C; Fig 11G with Fig. 11E). In the oral epithelium, many apoptoses were still present posteriorly on the medial side of the developing M_2 (compare Fig. 9C with Fig. 8C).

Antero-posterior decreasing gradients of mitoses could no longer be detected at this stage, either in the epithelium (Fig. 9C,F), or in the mesenchyme (Fig 9B,E). Mitoses in the mesenchyme were much less abundant than at ED 14 (compare Fig. 9B,E with

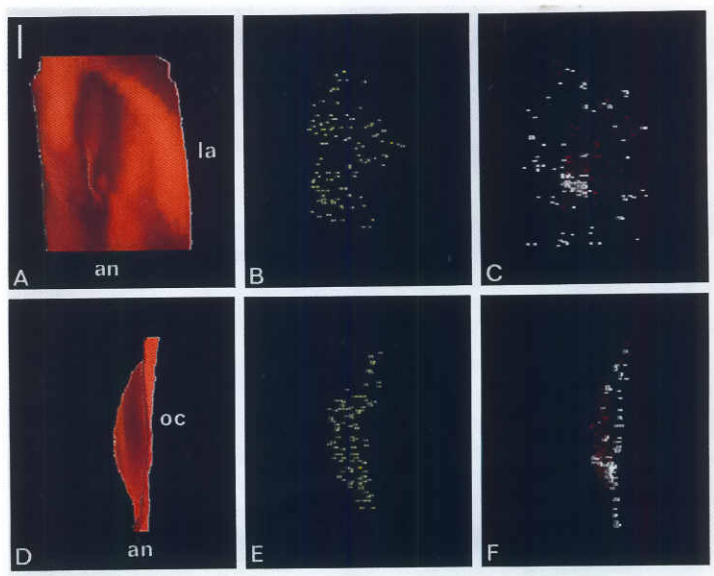

Fig. 6. 3D distribution of mitoses and apoptoses in the mandibular cheek region at ED 12.5. *Spatial distribution of mitoses in the dental mesenchyme (green dots in **B and E**), in the dental epithelium (red dots in **C and F**) and of apoptotic bodies (white dots in **C and F**). Spatial distributions of mitoses and apoptoses **(B, C, E, F)** can be replaced in their context by comparison with the shape of the dental epithelium **(A, D)** as seen in apical **(A-C)** and lateral **(D-F)** views. Apoptotic cells and bodies concentrated mainly in the anterior part of the dental epithelium **(F)**, especially inside the medial protrusion **(C)** and along the surface in contact with the oral cavity **(C, F)**. an, anterior; la, lateral and oc, occlusal. Bar, 100 μm.*

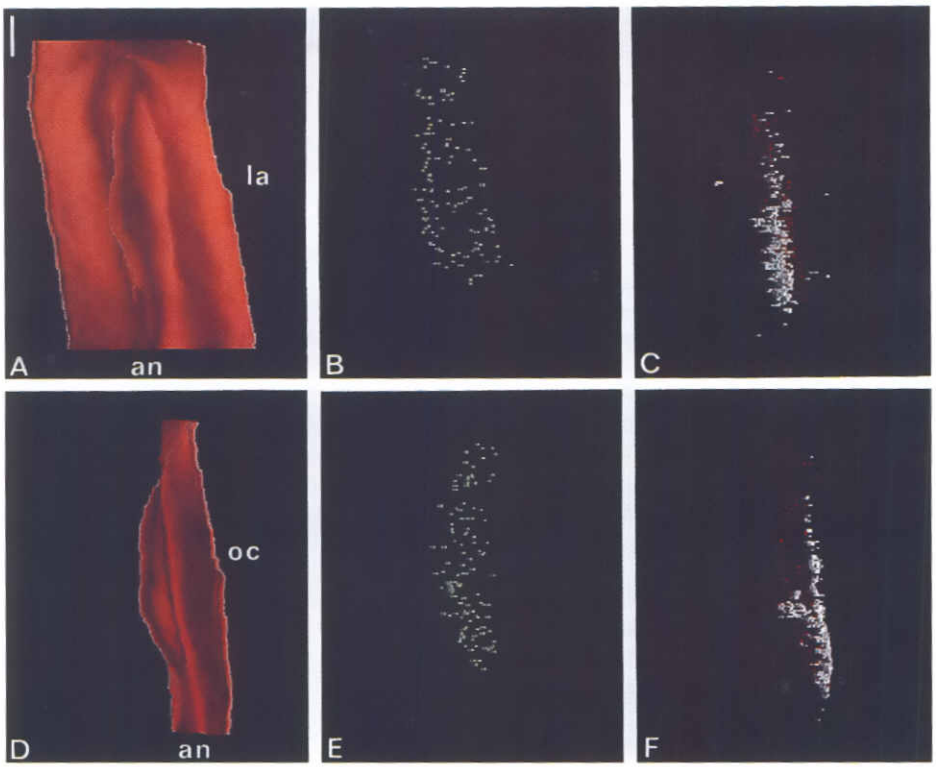

Fig. 7. 3D distribution of mitoses and apoptoses in the mandibular cheek region at ED 13.5. *Spatial distribution of mitoses in the dental mesenchyme (green dots in **B and E**), in the dental epithelium (red dots in **C and F**) and of apoptotic bodies (white dots in **C and F**). Spatial distributions of mitoses and apoptoses **(B, C, E, F)** can be replaced in their context by comparison with the shape of the dental epithelium **(A, D)** as seen in apical **(A-C)** and lateral **(D-F)** views. Apoptotic cells and bodies concentrated mainly in the anterior half part of the bud **(C, F)** and along the surface in contact with the oral cavity **(F)**. an, anterior; la, lateral and oc, occlusal. Bar, 100 μm.*

Fig. 8B,E). During the same period, the dental mesenchyme tended to expand. To better visualize mitoses in the vicinity of the EK, thick sagittal (Fig. 11G) and frontal (Fig. 11H) sections were reconstructed: the mitotic activity showed no increase in the proximity of the EK, either in the epithelium, or in the mesenchyme.

ED 16.0

Apoptoses in the anterior part of the M_1 were associated with the oral epithelium and also with a specific condensed part of the enamel organ located between the gubernaculum of the enamel organ and the stellate reticulum (Fig. 10C,F). A concentration of apoptotic bodies was also detected in the posterior part of the M_1 (Fig 10C,F).

From ED 15 to ED 16, the dental mesenchyme extended by about 30% and the frequency of mitoses in the mesenchyme (including the dental papilla and dental sac) was much higher at ED 16 (compare Fig. 10B,E with Fig. 9B,E).

The lateral view suggested that two distinct areas were actively dividing within the enamel organ: the first area corresponded to the anterior two thirds and the second to the posterior third of the M_1 bell (Fig. 10F). Furthermore, mitoses concentrated in the inner dental epithelium (Fig. 11I-J) although at previous stages they had an ubiquitous distribution in the enamel organ (compare with Fig. 11A-H).

Discussion

Computer-assisted 3D reconstructions have previously been used to study morphogenesis of the developing upper molar in mouse embryos (Lesot *et al.*, 1996; Peterková *et al.*, 1996). A similiar approach, allowing correlations with 2D histological data and reinterpretation of some of them, was undertaken to investigate the M_1 development. Morphogenesis results from coordinated cellular activities whose respective roles are currently being investigated using several different models (i.e. lung, kidney, intestine, or salivary gland). These activities include cell adhesion and migration, mitoses (Osborn, 1993; Ellis et all., 1995), apoptoses (Gumbiner, 1996), differential cell-cell (Cunningham, 1995; Takeichi, 1995) and cell-matrix interactions (Roskelley *et al.*, 1995). In this study, mitoses and apoptoses were considered.

Before the cap stage, growth of the epithelial compartment occurred along the antero-posterior axis. Correlation of histological sections with 3D reconstructions demonstrated the presence of morphologically different developmental stages (i.e. thickening - lamina - bud - lamina - thickening) along the antero-posterior axis of the dental epithelium in the mandibular cheek region (Lesot *et al.*, in press). Transition from the bud to the cap stage, resulting in a downward extension of the medial and lateral margins (prospective cervical loop) of the dental epithelium, was initiated in the anterior part of the M_1 and progressively proceeded antero-posteriorly. The expression of Lef-1, which can exogenously be regulated by BMP-4, has been shown to be involved in the transition from bud to cap stage (Kratochwil *et al.*, 1996). A gradient is to be expected in the expression of either BMP-4 or Lef-1. The accelerated morphogenesis in the anterior part of the M_1 was maintained at the late cap and early bell stages, resulting in the antero-posterior progressive completion of the cervical loop. Nevertheless, the dental papilla was still not entirely delimited posteriorly at the early bell stage.

Tooth development involves cell-kinetic dependent developmental processes (Butler, 1956; Gaunt, 1956; Ruch, 1984). The spatial distribution of metaphases was thus investigated in both epithelial and mesenchymal components of the developing tooth germ. In the epithelium, histogenesis of the enamel organ gave rise to compartments with different cell densities. At a very early stage, when the dental lamina and bud were apparent, no specific concentration of mitoses could be detected in the corresponding epithelium. Previous systematic investigations demonstrated that although differential mitotic activities do exist during odontogenesis (Gaunt, 1956; Butler, 1967; Ruch, 1984), the dental lamina formation did not result from such a process but from a preferential orientation of the spindle in dividing cells of the thickening oral epithelium (Ruch, 1984). Our data agree with Osman and Ruch

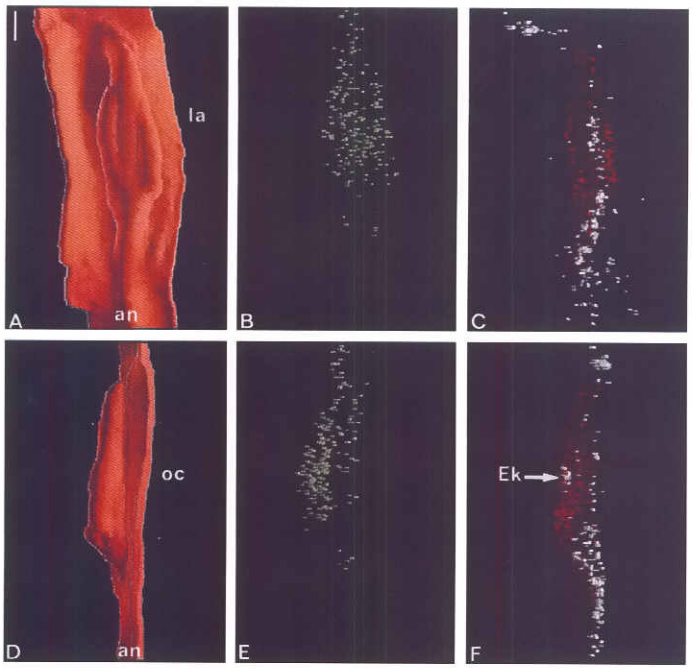

Fig. 8. 3D distribution of mitoses and apoptoses in the first lower molar at ED 14. *Spatial distribution of mitoses in the dental mesenchyme (green dots in **B and E**), in the dental epithelium (red dots in **C and F**) and of apoptotic bodies (white dots in **C and F**). Spatial distributions of mitoses and apoptoses **(B, C, E, F)** can be replaced in their context by comparison with the shape of the dental epithelium **(A, D)** as seen in apical **(A-C)** and lateral **(D-F)** views. Apoptotic cells and bodies concentrated mainly in a region anterior to the developing cap **(C, F)**, inside the median ridge of the cap: in the enamel knot (Ek in **F**) and in a postero-medial area behind the epithelial thickening which will later give rise to the M_2 **(C)**. Very rare mitoses were detected in the dental mesenchyme opposite to the regressing (anterior) epithelial region **(B, E)**. an, anterior; la, lateral and oc, occlusal. Bar, 100 μm.*

(1975), but neither with Mina and Kollar (1987) nor with Kronmiller *et al.* (1992) as previously discussed (Ruch, 1995). Mitoses in the enamel organ maintained their ubiquitous distribution up to the maintained their ubiquitous distribution up to the well-formed cap stage (ED 15). At ED 14, a sligt asymmetry was observed on frontal views of the M_1, resulting mainly from asynchronous development of the lateral and medial margins of the cap: the development and elevation of the lateral margin being somewhat delayed compared with the medial one. The asymmetrical latero-medial development of the cervical loop documented here was also accompanied by an asymmetric distribution of the lamimin-5 subunits (α3, β3 and γ2) (Yoshiba et al., submitted), and of γ catenin (Fausser et al., submitted). Furthermore, differences in cell-matrix interactions, as far as hemidesmosomes containing BP-230 antigen (Fausser *et al.*, submitted) and integrin b4 (Salmivirta *et al.*, 1996) were concerned, have been documented. All of these modifications, which started at the bud stage, suggested a reduced adhesion/cohesion of medial outer dental epithelial cells when compared to lateral ones (Fausser *et al.*, submitted).

At the early bell stage, mitoses were concentrated mainly in the inner dental epithelium. Establishment of mitotic indices previously demonstrated a clear distinction between the inner and outer dental epithelium already at ED 15 (Ruch, 1984). Epithelio-mesenchymal interactions have been shown to control mitotic activity during histogenesis and the role of the basement membrane has been experimentally investigated *in vitro* at the bell stage (Olive and Ruch, 1982). This approach suggested that mesenchyme-dependent changes in the basement membrane could be involved in the control of the differentiation of the inner (vs outer) dental epithelium. Heterotopic reassociations demonstrated that the dental papilla controled the differentiation of the inner dental epithelium which was accompanied by increased mitotic activity (Olive and Ruch, 1982). Since there is a delay between the G_1/S transition and reaching the stage of metaphasis, the putative causal changes related to the basement membrane itself (Yoshiba *et al.*, submitted) or to basement membrane-associated molecules (Vukicevic *et al.* 1992) should be documented at the well formed cap stage. The basement membrane represents a highly integrated structure whose components interact with active molecules such as growth factors. One might thus expect similar changes to occur in the distribution of these active molecules during this critical period. Up to now, data showing the immunolocalization of growth factors are still lacking. Although regional variations in the mitotic activity of the

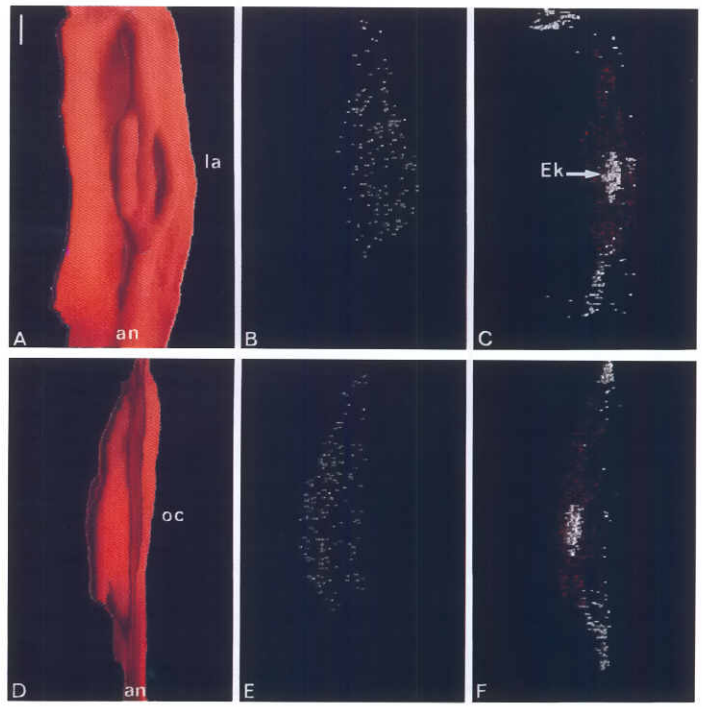

Fig. 9. 3D distribution of mitoses and apoptoses in the first lower molar at ED 15. *Spatial distribution of mitoses in the dental mesenchyme (green dots in **B and E**), in the dental epithelium (red dots in **C and F**) and of apoptotic bodies (white dots in **C and F**). Spatial distributions of mitoses and apoptoses **(B, C, E, F)** can be replaced in their context by comparison with the shape of the dental epithelium **(A, D)** as seen from apical **(A-C)** and lateral **(D-F)** views. Apoptotic cells and bodies strongly accumulated in the enamel knot (Ek in **C**). Apoptotic bodies also concentrated in a region anterior to the well formed cap **(C, F)**, and in a postero-medial area behind the epithelial thickening which will later give rise to the M_2 **(C)**. Rare mitoses were detected in the dental mesenchyme opposite to the regressing (anterior) epithelial region **(B, E)**. an, anterior; la, lateral and oc, occlusal. Bar, 100 μm.*

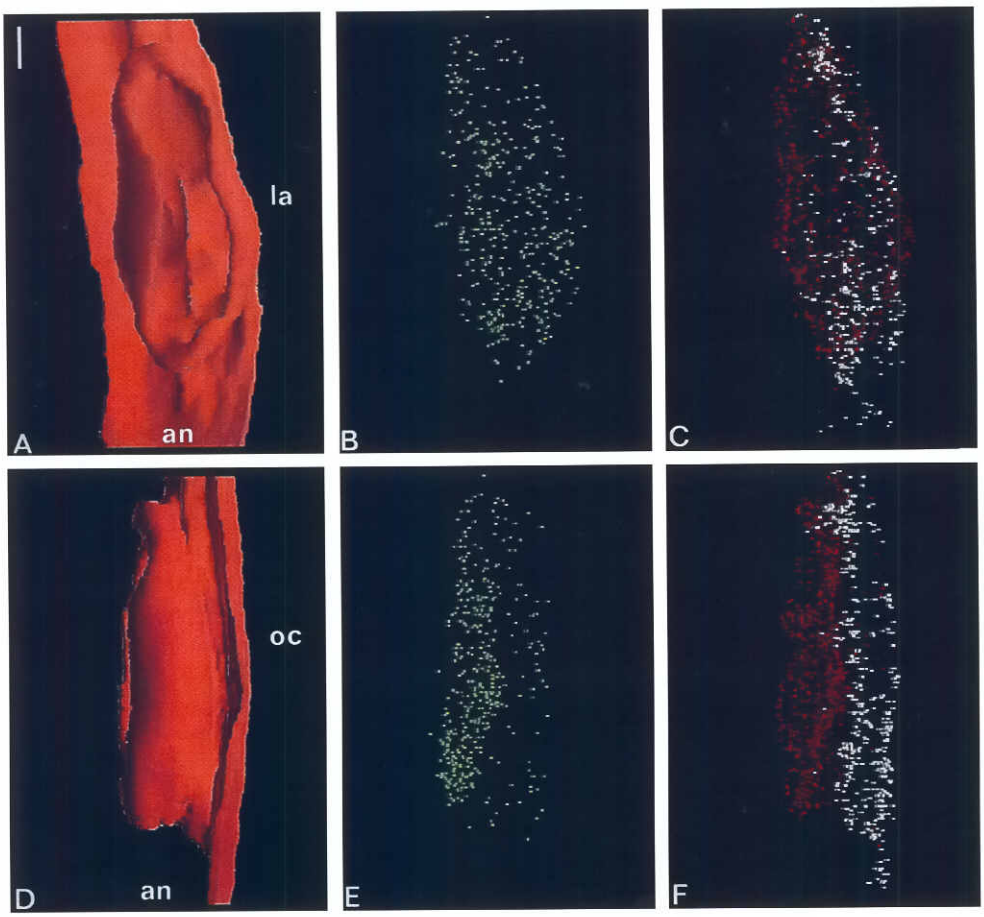

Fig. 10. 3D distribution of mitoses and apoptoses in the first lower molar at ED 16. *Spatial distribution of mitoses in the dental mesenchyme (green dots in **B and E**), in the dental epithelium (red dots in **C and F**) and of apoptotic bodies (white dots in **C and F**). Spatial distributions of mitoses and apoptoses **(B, C, E, F)** can be replaced in their context by comparison with the shape of the dental epithelium **(A, D)** as seen from apical **(A-C)** and lateral **(D-F)** views. Apoptotic cells and bodies were associated with an anterior condensed part located between the gubernaculum of the enamel organ and the developing bell **(C, F)**, and with the posterior part of the M_1 **(C, F)**. an, anterior; la, lateral and oc, occlusal. Bar, 100 μm.*

inner dental epithelium was assumed to correlate with cusp formation (Ruch, 1987), folding of the inner dental epithelium has not yet been directly correlated with differential mitotic activities. Thus changes in cell adhesion could play an important role in the dynamic spatial specification of the very active development of the enamel organ. These changes in cell adhesion might also be involved in the specific folding of the inner dental epithelium. On the other hand, the mesenchyme itself could play an important mechanical role (Gaunt and Miles, 1967) and the concentration of mitoses in the inner dental epithelium would then allow this cell layer to cover the increasing surface of the mesenchyme during cusp formation.

Apoptosis participated in the early tooth morphogenesis in the mandibular cheek region. During lamina, bud and cap stages, an area exhibiting a high density of apoptotic bodies was observed in a region anterior to the prospective M_1 (see also Lesot *et al.*, in press). This phenomenon might be in relation to the regression of unidentified tooth primordia. Such an accumulation of apoptotic bodies could not be observed during the formation of the M_2 (Viriot *et al.*, unpublished observations). Apoptoses also temporarily concentrated in the oral epithelium. Cell death in the bud shaped epithelium has been interpreted as related to the M_1 of mouse embryos until day 13.5 (Kindaichi, 1980; Vaahtokari *et al.*, 1996b). Similarly, expression of BMPs close to the epitheliomesenchymal junction in the tooth bud, at ED 13.5, was attributed to the initiating EK formation in the M_1 (Vaahtokari *et al.*, 1996a). However, it is not clear yet whether or not these processes reflect the regression of a premolar rudiment in the mandible, as illustrated for the maxilla (Peterková *et al.*, 1996; Turecková *et al.*, 1996).

At the well-formed cap stage, changes occurred in the distribution of apoptotic bodies which tended to progressively disappear from the oral epithelium but concentrated in the EK until the disappearance of this structure. Apoptosis thus seems to be a very dynamic process with specific regulation in space and time effecting constant remodeling of the developing M_1. The fact that the EK has been observed in the teeth of various placental mammals (Butler, 1956) suggested that it should have a function although this function is still undetermined. A potential role has been attributed in cusp formation (Orban, 1928; Butler, 1956) and this hypothesis has recently been reactivated (Jernvall *et al.*, 1994; Vaahtokari *et al.*, 1996a). These authors showed that EK cells express transcripts for FGF, BMPs, Shh suggesting that, if proteins are expressed, they might stimulate the mitotic activity of their neighbor cells (Vaahtokari *et al.*, 1996a). Major discrepancies between the expression of transcripts and that of corresponding proteins have recently been documented during tooth development (Yoshiba *et al.*, submitted). Furthermore, we could not detect any specific changes in the distribution of mitoses in the epithelium and mesenchyme, either at the well formed cap or at the early bell stage. It will then be important to demonstrate that these transcripts are translated. It also still remains to be determined how the diffusion of active signal molecules, possibly originating from the EK, could give rise to the two first cusps (protoconid and metaconid) at ED 16 when the next cusps (anteroconids, hypoconid and entoconid) only appear at ED 17.5. Furthermore, developing teeth produce morphogenetic signals which are not restricted to the EK (Koyama *et al.*, 1996). If active molecules issued from the EK do play a causal role in cusp formation, they would require a long half life, whilst conceivable, this would also imply a time-space specific action. Furthermore,

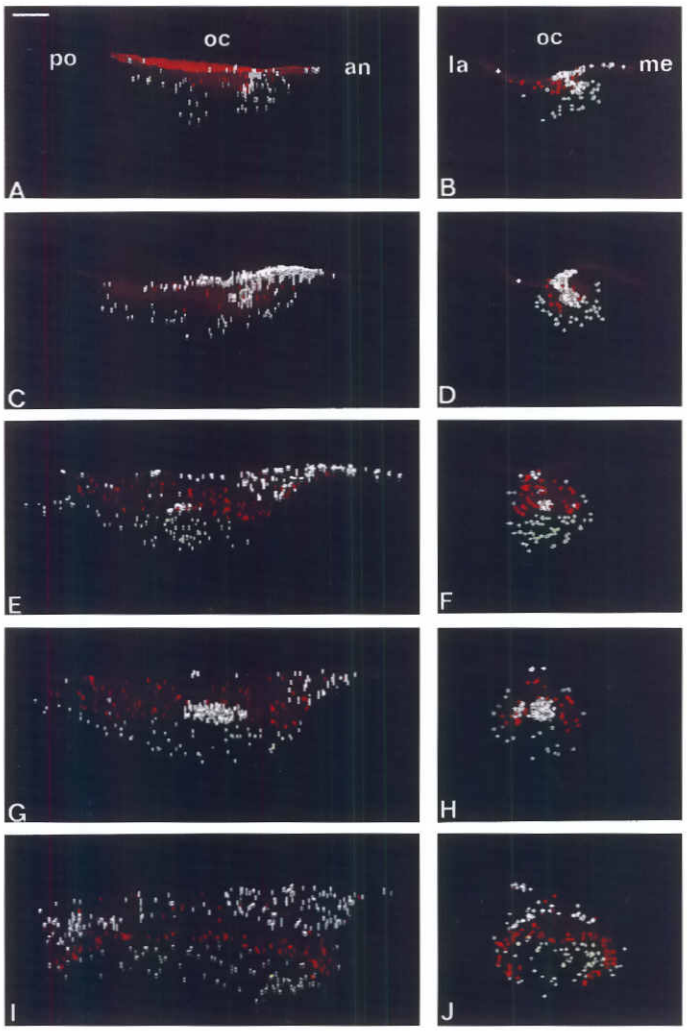

Fig. 11. 3D distribution of mitoses and apoptoses in the dental epithelium and mesenchyme presented as computer made thick sagittal and frontal sections. *Spatial distribution of mitoses in the dental mesenchyme (green dots), in the dental epithelium (red dots) and of apoptotic bodies (white dots). Sagittal views (**A, C, E, G, I**) are 75 μm thick so that they correspond to the maximum latero-medial extension of the enamel knot at ED 15 **(G)**. Frontal views **(B, D, F, H, J)** are 170 μm thick so that they correspond to the maximum antero-posterior extension of the enamel knot at ED 15 **(H)**. From ED 12.5 to 15, mitoses were widespread in the dental epithelium **(A-H)**. At ED 16, mitoses were concentrated mainly in the inner dental epithelium (I-J). an, anterior; po, posterior; la, lateral; me, medial and oc, occlusal. Bar, 100 μm.*

whatever the origin of growth factors, their spatial specification would have to be mediated by an endogenous process involving cell-surface receptors. The EK would then be no more than a group of cells providing signalling molecules in their vicinity. The EK might also comprise of a segregation of cells which are to be eliminated because their potential activity has to be avoided (Lesot *et al.*, 1996).

At the early bell stage *in vivo*, foldings of the basement membrane were observed in the anterior two thirds of the M_1. The longest loop of basement membrane was interposed between two large grooves which are a morphological sign of cusp development (the prospective protoconid originates in the lateral groove and prospective metaconid in the medial groove). This phenomenon was temporarily observed after the disappearance of the EK, and the two events might be connected. The disappearance of the cells from the EK originally in contact with the basement membrane and their replacement by neighboring epithelial cells, depositing a new basement membrane, might account for the occurrence of a «free» basement membrane loop in the mesenchyme. Such extensions of basement membrane in the mesenchyme were much more apparent in cultured molars (Meyer *et al.*, 1995) where cusp formation is accelerated compared with *in vivo*. If this phenomenon had to be related with further cusp development *in vivo*, one would expect this detachment and folding of the basement membrane to extend later to the posterior part of the molar.

Molecular mechanisms mediating the induction of apoptosis in physiological conditions are multiple (Wertz and Hanley, 1996); apoptosis can be induced by an external stimulus or by the lack of a survival factor. During tooth development, apoptosis could be regulated by BMP-4, and possibly by BMP-2, as previously discussed (Lesot *et al.*, 1996; Peterková *et al.*, 1996, 1997; Turecková *et al.*, 1996), or by a lack of EGF (Camp and Martin, 1996). Phenomenological and experimental data suggested putative roles for EGF at different steps of tooth initiation and morphogenesis (Partanen and Thesleff, 1987; Cam *et al.*, 1990; Kronmiller *et al.*, 1991; Hu *et al.*, 1992; Heikinheimo *et al.*, 1993). However this hypothesis could not be confirmed since until now, the apoptotic processes which occur during molar morphogenesis could not be correlated with a decrease in EGF expression. Furthermore, the highly specific spatial evolution of the process implies regulation at the cellular level; this is not supported by the distribution of EGFR from dental lamina to early bell stage although the situation might still be more complex (Kere *et al.* 1996). In most instances, these apoptoses occurred as a «catastrophic» process in specific areas. It is not clear yet whether these large amounts of cell debris are eliminated during tooth development by neighboring cells or macrophages (Camp and Martin, 1996). Large phagocytes have been reported close to the periderm cell layer underlying diastemal rudiments (Turecková *et al.*, 1996). Phagocytes were observed among the most superficial cells of the epithelium, in contact with the oral cavity, but not in the enamel knot.

Materials and Methods

ICR mouse embryos, whose age was determined in embryonic days (Peterková *et al.*, 1996), were harvested at noon and midnight from ED 12.5 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. One specimen from each weight class at each chronological stage was chosen and its head processed for histology.

Histology

5 μm frontal serial sections from paraffin embedded heads were stained with alcian blue-hematoxylin-eosin.

In frontal sections, the epithelial thickening, dental lamina and bud stages of tooth development were identified according to previously defined criteria (Peterková *et al.*, 1996). The dental epithelial thickening differed from the adjacent oral epithelium by its larger number of layers of deeper columnar cells with a prevalent orientation of the long axes of their

nuclei perpendicular to the basement membrane. One or two layers of flat peridermal cells were present at the oral surface. Dental lamina was formed by infolding of the thick stratum. The fold groove was filled by smaller cells. On the mesenchymal aspect, the medial and lateral slopes of the fold formed an angle equal to or greater than 90° with the adjacent epithelium (Fig. 1C). Cells of the stratum in the dental bud were larger and mostly columnar when in contact with the basement membrane and smaller in the center. At least one of its medial and lateral mesenchymal faces exhibited a protrusion showing an angle smaller than 90° with the mesenchymal face of the oral epithelium (Fig. 1A, see also 1F).

The cap and bell stages were identified in frontal sections according to the criteria described by Cohn (1957) and Orban (1928).

Mitoses and apoptoses

When representing mitoses, only metaphases were taken into account. Mitoses in the dental mesenchyme included those present in the dental papilla and the condensed part of the dental sac (Lesot *et al.*, 1996).

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).

3D reconstructions

The contours of the mandibulary oral epithelium were drawn from serial histological sections (5 μm intervals) using a Zeiss microscope equipped with a drawing chamber at a magnification of 320x. Mitoses were recorded in the dental epithelium and mesenchyme. Since very few or no apoptoses 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 previously been 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).

Morphometry

In order to characterize the formation of the cap cavity, measurements were made along an axis running through the middle of the gubernaculum of the enamel organ and the middle of the central ridge of the cap (Fig. 3). Two measurements were plotted on the graphs: (a) distance between the oral epithelium and the point of intersection with the basement membrane of the central ridge; and (b) distance between the oral epithelium and the point of intersection with a line connecting the apexes of the medial and lateral ridges of the cap (see insert in Fig. 3). These measurements were performed using the camera lucida projection (magnification 320x) of frontal histological sections at 5 μm intervals of the antero-posterior course.

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