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Original Article

Mouse molar morphogenesis revisited by three-dimensional reconstruction. II. Spatial distribution of mitoses and apoptosis in cap to bell staged first and second upper molar teeth

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ABSTRACT Tooth morphogenesis is a complex multifactorial process in which differential mitotic activities and cell death play important roles. Upper first (m1) and second (m2) molars from mouse embryos were investigated from early cap to bell stage. m2 differed from m1 by delayed origin of the enamel grooves delimiting the protrusion of the cap bottom towards the dental papilla, and retardation of the enamel knot formation. The width of the m2 enamel organ was conspicuously smaller during cap formation and length remained smaller throughout the period of observation. Formation of the cap depression was comparable in m1 and m2, however margins delimiting the enamel organ cavity arose in m1 and m2 as mirror images. Attempts were made to correlate changes in the distribution of apoptotic cells and bodies and/or mitoses with morphogenesis. These cellular activities were recorded from histological sections and represented in space using computer-assisted three-dimensional reconstructions. Mitoses in the epithelial compartment were associated with the development of the cervical loop. In the mesenchyme of m1 at early bell stage, a postero-anterior increasing gradient of mitoses was observed which might be correlated with the anterior growth of the molar. Cells in the enamel knot demonstrated a high level of apoptosis, retarded in m2, but absolutely no division. Apoptotic processes were also involved in the anterior delimitation of the m1 epithelium. Apoptosis might correspond to the programmed destruction of cells whose function had to be suppressed or whose potential activity had to be avoided.

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

Introduction

Tooth morphogenesis includes condensation in the mesenchyme (Kollar, 1972; Ruch, 1984), thickening and infolding of the epithelium, histogenesis, mainly in the epithelial compartment (Gaunt and Miles, 1967; Ruch, 1995; Sharpe, 1995) and changes in the morphology of the epithelio-mesenchymal junction ultimately leading to cusps formation (Butler, 1956; Gaunt and Miles, 1967; Radlanski *et al.*, 1988). Morphogenetic processes specify the class of tooth by supporting the dynamic establishment of a specific epithelio-mesenchymal junction that anticipates the shape of the future dentin-enamel interface. All these steps involve complementary cellular events such as cell division, migration and death, demonstrate overlap in time and are controlled by epithelio-mesenchymal interactions (Kollar, 1983; Slavkin, 1990; Ruch, 1995; Thesleff *et al.*, 1995). Differential mitotic activities (Butler, 1967; Ruch, 1984; Kronmiller *et al.*, 1992) have been reported to play an important role during tooth morphogenesis although no general agreement exists (for review see Ruch, 1995). Besides proliferation, cell death has been reported during odontogenesis (Nozue, 1971; Moe and Jessen, 1972; Kindaichi, 1980; Nishikawa and Sasaki, 1995; Shibata *et al.*, 1995; Vaahtokari *et al.*, 1996a) and was suggested to be a critical process in tooth development. Apoptosis is also involved in the disappearance of the rudimental tooth anlagen in front of the bud and cap of the mouse upper first molar (Turecková *et al.*, 1996; Peterková *et al.* in this issue).

Until very recently, most information about tooth development was obtained from histological, immunological or *in situ* hybridiza-

Abbreviations used in this paper: m1, first molar; m2, second molar; 3D, three dimensional; BMP, bone morphogenetic protein; ED, embryonic day; FGF, fibroblast growth factor; wtc, weight class.

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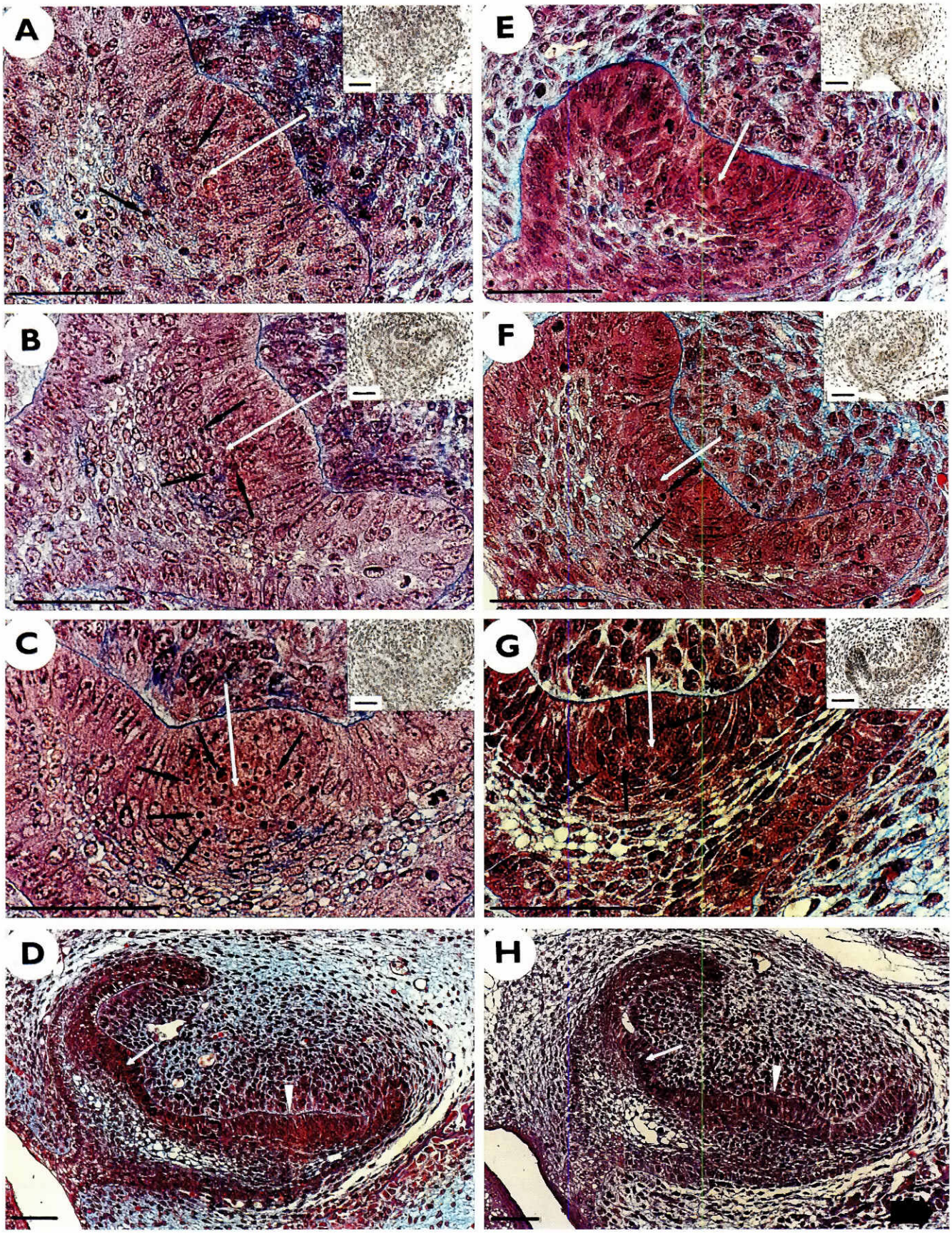
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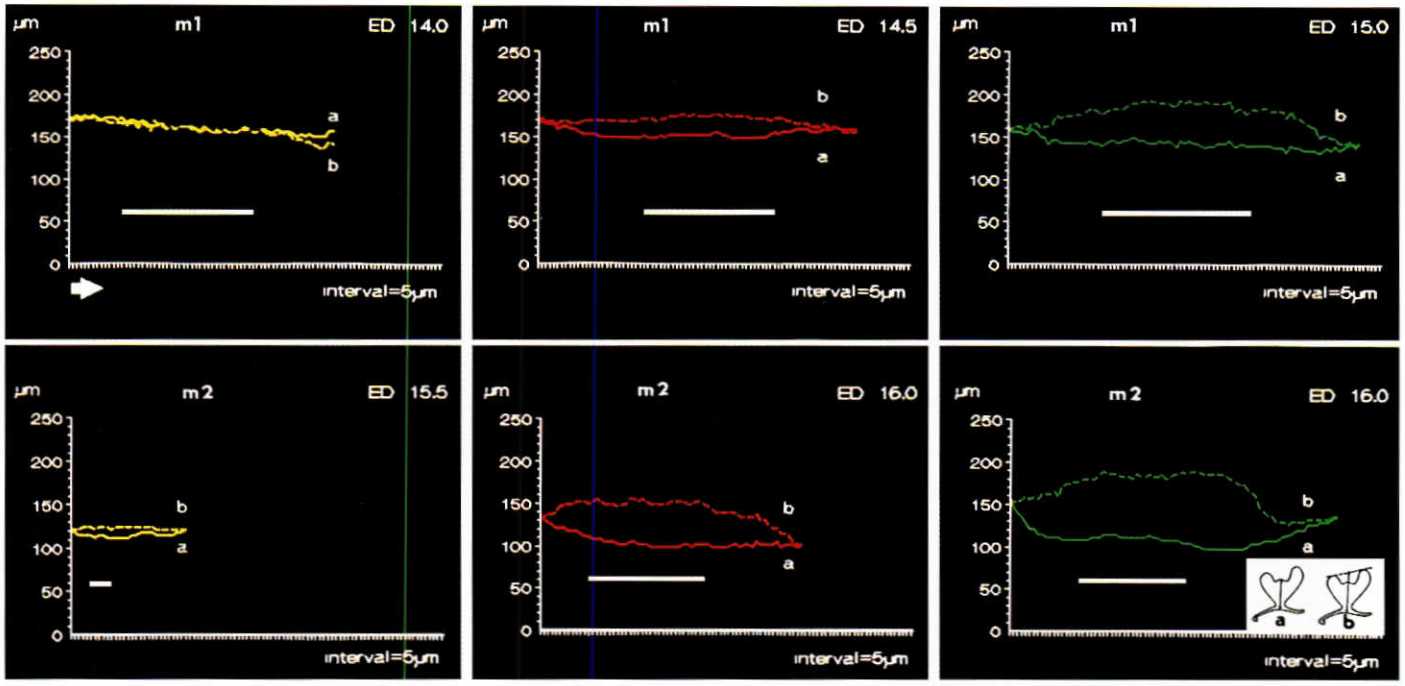

Fig. 2. Morphometry. *Graphs represent the antero-posterior changes of distance a (full line) and b (dashed line) in micrometers (µm) which represent the height of the middle (a) and marginal (b) part of the dental epithelium, respectively, during cap formation. The curves are documented for the first molar (m1) at ED 14.0 (wtc. 276-300 mg), ED 14.5 (wtc. 301-325 mg) and ED 15.0 (wtc. 426-450 mg), for the second molar (m2) at ED 15.5 (wtc. 651-700 mg), ED 16.0 (wtc. 601-650 mg) and ED 16.0 (wtc. 751-800 mg). The large arrow points distally. White bar indicates the antero-posterior extent of the enamel knot (compare with Fig. 1).*

tion approaches on sections (Peterková *et al.* in this issue and references therein). Where possible, serial sections were investigated but data were documented as images from single sections considered to be representative. More recently, computer-assisted methods have allowed 3D representations which appear essential for correct morphological interpretation (Peterková *et al.*, 1993; Radlanski, 1993). The use of a computer allowed to increase effectiveness of hand-made 3D reconstruction methods, applied sporadically in odontogenesis studies for more than a hundred years (Röse 1892; Ahrens, 1913; Gaunt, 1955; Ooë, 1956). The needs for such representations have been discussed recently (Radlanski, 1995). Different approaches were adapted to solve specific questions such as precise 3D morphogenetic dynamics, 3D patterns of specific cellular activities including gene expression or mineralization (Radlanski, 1993, 1995; Peterková *et al.*, 1993, 1995; Jernvall *et al.*, 1994; Diekwisch *et al.*, 1995; Seipel *et al.*, 1995; Turecková *et al.*, 1996; Vaahtokari *et al.*, 1996b).

The spatial development of the upper first molar in mouse has been analyzed at the dental lamina and bud stages (Peterková *et al.* in this issue). In the present study, investigation of the upper first molar (m1) morphogenesis was extended to the early bell stage and compared to the second molar (m2). Attempts were made to correlate 3D morphological data with the distribution of apoptotic bodies and mitoses.

Results

First molar

Histological aspects

During cap formation, histogenesis was initiated in the enamel organ leading to the formation of the inner and outer dental epithelium and of the stellate reticulum. At the same time, the dental papilla arose (Fig. 1A,B,C). The enamel knot, a transitory epithelial structure characterized by condensation and specific arrangement of cells, was apparent at the early cap stage (Fig. 1A) along the middle part of the antero-posterior axis in the enamel organ. The enamel knot had a round appearance on frontal sections (i.e. a cylinder-like shape in space) and protruded towards the mesenchyme. The protrusion was delimited laterally and medially by respective grooves. The enamel knot was formed by two types of cells: 1) cells in contact with the basement membrane, in continuity with the inner dental epithelium, and 2) smaller inner

Fig. 1. Histological sections of the developing first and second upper molars. *Frontal sections documented various stages of cap formation in the first* **(A,B,C)** *and second* **(E,F,G)** *molars. Initial stage at ED 14.0, wtc. 276-300 mg* **(A)** *and at ED 15.5, wtc. 651-700 mg* **(E),** *after establishment of the cap cavity at ED 14.5, wtc. 301-325 mg* **(B)** *and at ED 16.0, wtc. 601-650 mg* **(F),** *well-formed cavity at ED 15.0, wtc. 426-450 mg* **(C)** *and at ED 16.0, wtc. 751-800 mg* **(G)**. *The early bell stage is documented for the first molar at ED 16.0, wtc. 751-800 mg* **(D)** *and for the second molar at ED 18.0, wtc. 1401-1500 mg* **(H)**. *Slim white arrow shows the well-formed* **(A,B,C,E,F,G)** *or former* **(D,H)** *enamel knot area. White triangle indicates the secondary, medial protrusion of the enamel organ. Slim black arrows show the apoptotic cells and bodies. Stars indicate the first appearance of the medial and lateral enamel grooves. Large arrow points medially. Bar, 50 µm.*

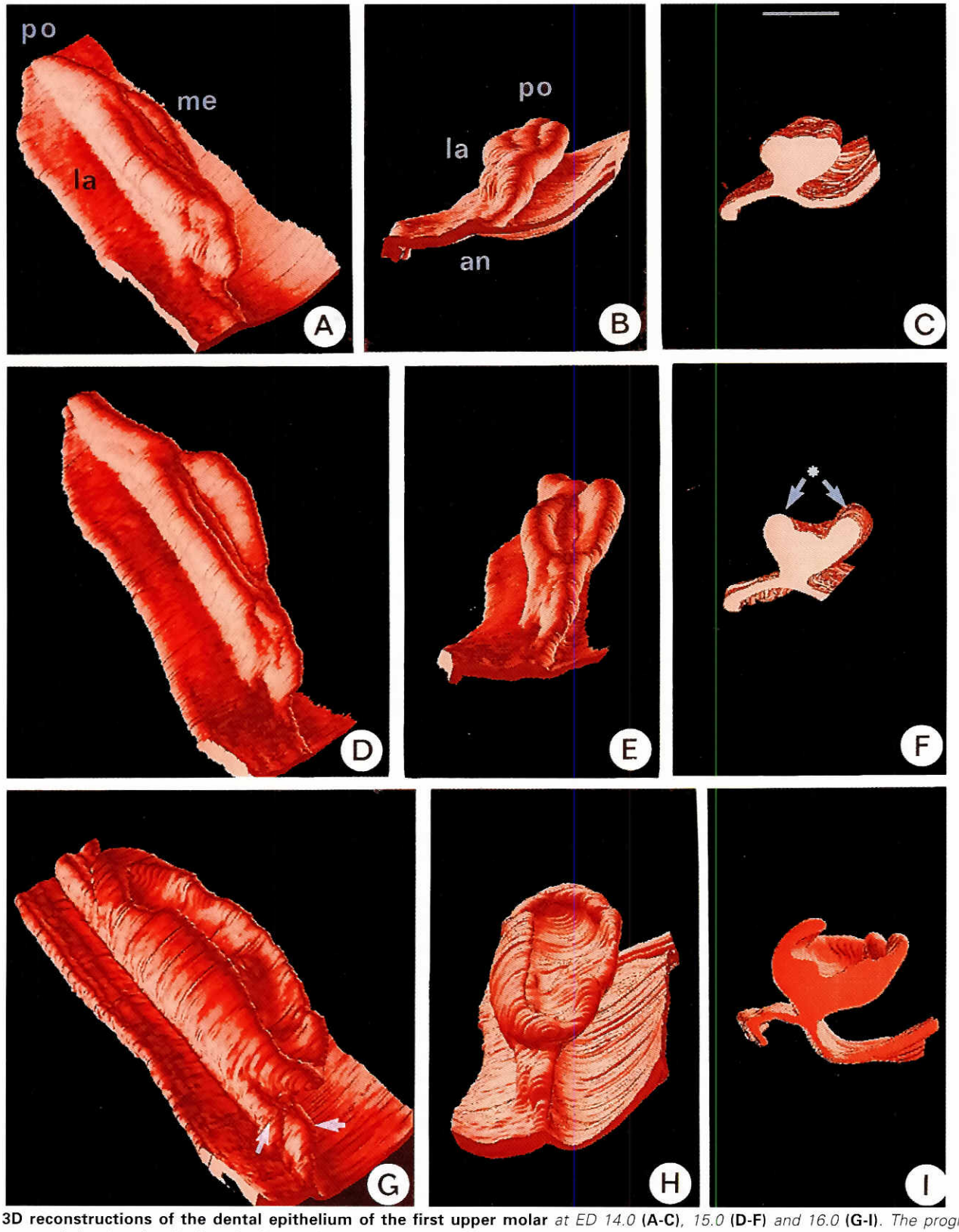

Fig. 3. 3D reconstructions of the dental epithelium of the first upper molar *at ED 14.0* **(A-C)**, *15.0* **(D-F)** *and 16.0* **(G-I)**. *The progressive development of the dental epithelium is observed as antero-posterior views* **(A-I)**, *from antero-lateral* **(A,D,G)** *and frontal* **(B,E,F)** *views. Frontal sections were performed in the central part of the reconstructions* **(C,F,I)**. *Arrows* **(G)** *indicate the medial and lateral ridges, in continuity with the m1 enamel organ (Peterková et al., in this issue). an, anterior; la, lateral; me, medial and po, posterior.* ***, *elevation of the lateral and medial bulges leading to the formation of the cervical loop. Bar, 100 μm.*

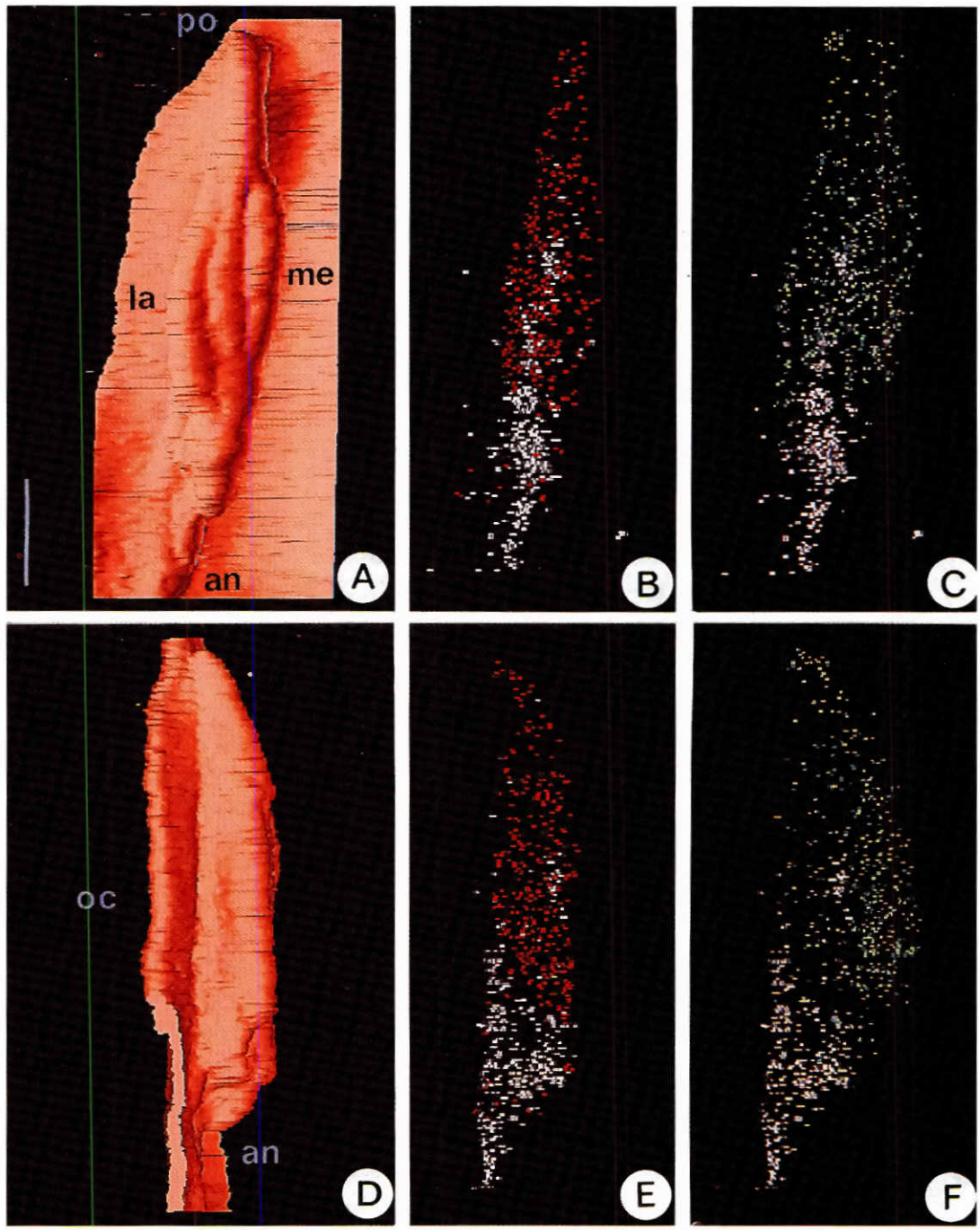

Fig. 4. 3D distribution of mitoses and apoptosis in the first upper molar at ED 14.0. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)**, *in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)** *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. an, anterior; la, lateral; me, medial; po, posterior and oc, occlusal. Bar, 100 μm.*

cells which did not participate in the formation of the stellate reticulum. The cells at the periphery of the enamel knot were concentrically arranged (Fig. 1A,B,C). The antero-posterior extension of the enamel knot was from 160 μm at the initial cap stage to 185 μm in the well formed cap. During cap cavity formation, the enamel knot could be detected in 44-46% of frontal sections in the forming cap cavity (Fig. 2).

Apoptotic cells and bodies were apparent in the enamel knot as soon as this structure could be identified, at the early cap stage, and seemed to involve predominantly the smaller inner cells. Later, apoptotic cells and bodies extended to the whole enamel knot and their frequency increased during cap formation (Fig. 1A,B,C). Morphometric measurements (Fig. 2) demonstrated that the massive destruction of cells in the enamel knot did not result in a conspicuous loss of height in the middle part of the developing enamel organ. Mitoses were never observed within the enamel knot and the number of mitoses was not increased around this structure. This observation was further confirmed by 3D reconstructions.

At the early bell stage, the morphological complexity of the enamel organ still increased in sections (Fig. 1D) and the stratum intermedium started to be distinct. The enamel organ enlarged.

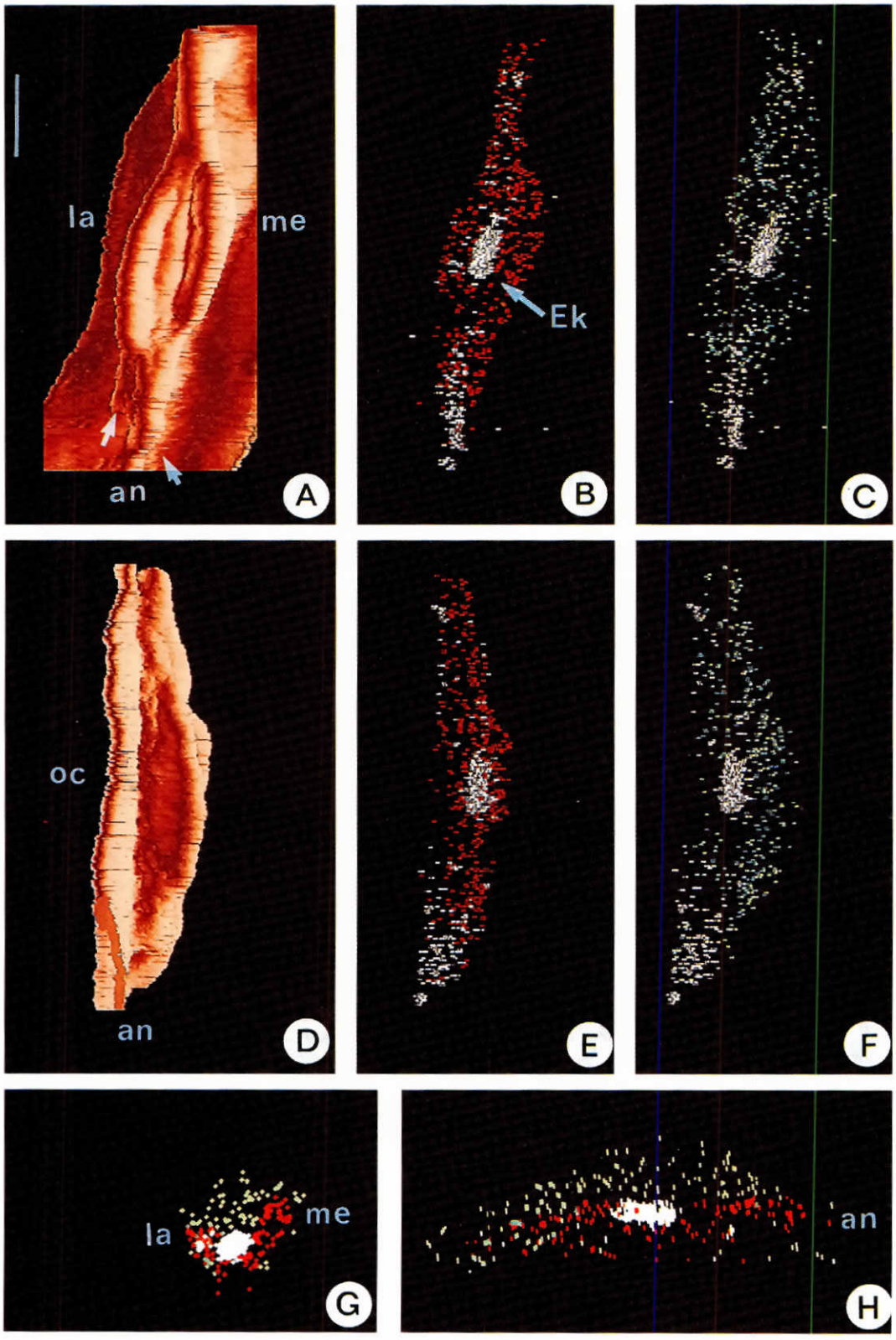

Fig. 5. 3D distribution of mitoses and apoptosis in the first upper molar at ED 15.0. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)**, *in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)**, *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. Apoptotic cells and bodies* **(B,C,E,F)** *concentrate in the enamel knot (Ek). Computer-made frontal section whose thickness corresponds to the antero-posterior extension of the enamel knot* **(G)**, *and sagittal section whose thickness corresponds to the diameter of the enamel knot* **(H)**, *did not show any preferential increase in mitotic activity, either in the epithelial or in the mesenchymal constituent of the tooth germ. Arrows* **(A)** *indicate the medial and lateral ridges, in continuity with the m1 enamel organ (Peterková et al., in this issue). an, anterior; la, lateral; me, medial and oc, occlusal. Bar, 100 µm.*

The medial side of the enamel organ grew more rapidly than the lateral one, leading to a conspicuous asymmetry of the tooth germ on frontal sections. As a result, the former enamel knot area went in the lateral part of the enamel organ (compare Fig. 1C and 1D).

In the middle segment of the antero-posterior course of the bell, a further epithelial protrusion was found towards the medial half of the dental papilla. This protrusion was separated from the former enamel knot area by the medial groove (Fig. 1D). The former

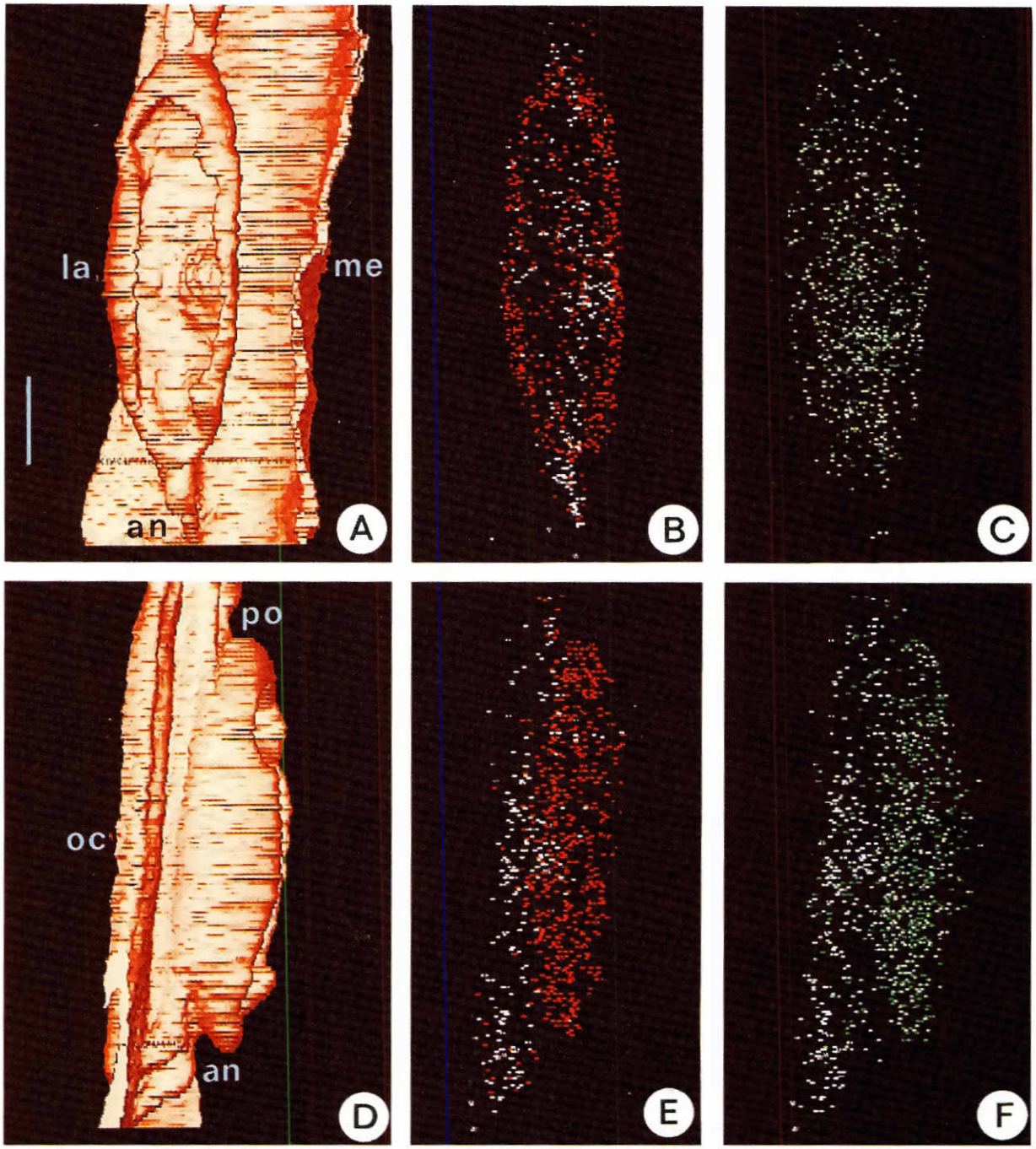

Fig. 6. 3D distribution of mitoses and apoptosis in the first upper molar at ED 16.0. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)** *or in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)**, *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. an, anterior; la, lateral; me, medial; po, posterior and oc, occlusal. Bar, 100 μm.*

enamel knot area could be detected by the presence of condensed epithelial cells as well as the conspicuous protrusion of this area towards the dental papilla (Fig. 1D). However, the former accumulation of apoptotic cells and bodies could no longer be detected there.

Cusps formation was initiated at ED 16.0 and corresponded to the increased complexity of the epithelio-mesenchymal junction as seen on frontal sections (Fig. 1D).

3D reconstructions

At ED 14.0, the upper surface of the dental epithelium appeared as three intertwined cords: lateral, central and medial, separated by the lateral and medial enamel grooves respectively. The enamel knot area was included in the central cord (Fig. 3A,B,C).

At ED 15.0, an elevation of the medial and lateral cords occurred along the antero-posterior axis of the tooth germ giving rise to the medial and lateral margin of the cap (Fig. 3D,E,F). The medial and

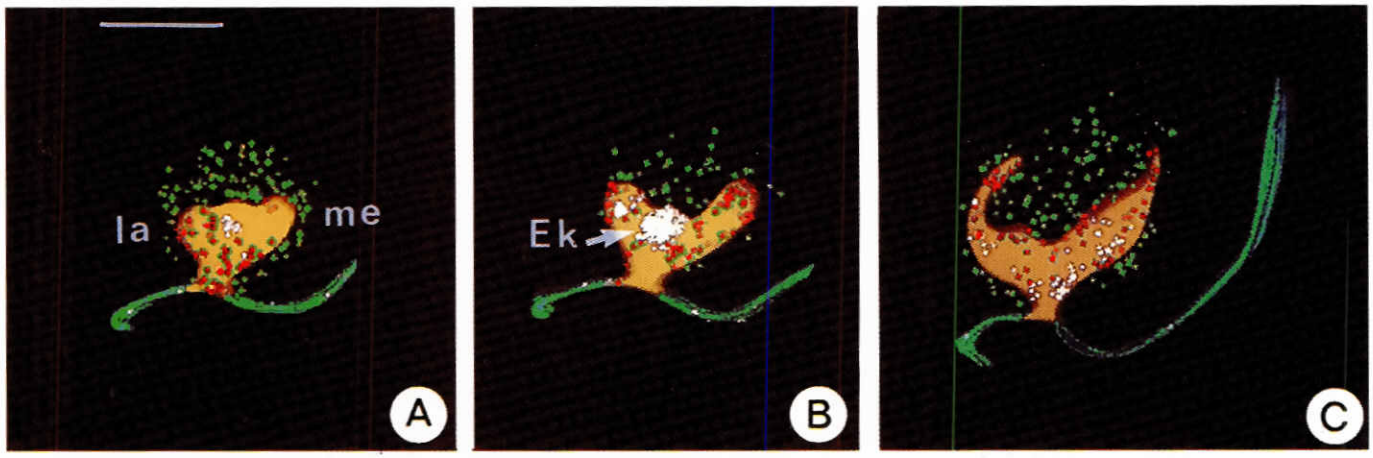

Fig. 7. 3D distribution of mitoses and apoptosis in the first upper molar. *100 μm thick frontal sections from reconstructions illustrate the distribution of the different cellular activities in the central part of the developing tooth at ED 14.0* **(A)**, *15.0* **(B)** *and 16.0* **(C)**. *Mitoses in the epithelium were represented as red spots, in the mesenchyme as green spots and apoptosis as white spots. Maintaining the same orientation, the dental epithelium was visualized in yellow and the buccal epithelium in pine green. la, lateral and me, medial. Bar, 100 μm.*

lateral epithelial ridges were observed in continuity with the m1 cap (Fig. 5A, arrows).

Early bell stage, at ED 16.0, was characterized by a further growth of the enamel organ and an elevation of its lateral and medial margins, corresponding to the cervical loop development on histological sections. Together with the elevation of the posterior margin of the enamel organ, the bell cavity was progressively enclosed by a continuous cervical loop, whose development however was retarded in the anterior part (Fig. 3G,H,I). Formation of the anterior margin was initiated later as a consequence of the anterior growth of the enamel organ. During this process, the forming bell extended towards the anterior direction, partially overlapping the medial and lateral epithelial ridges (Fig. 3G).

Mitoses and apoptosis

At ED 14.0, mitoses in the epithelium were uniformly distributed in the area of the three epithelial cords up to an anterior region where they were markedly diminished (Figs. 4B,E, 7A). At ED 15.0, the mitotic activity was concentrated in the lateral and medial margins of the cap as they elevated. No mitosis could be detected

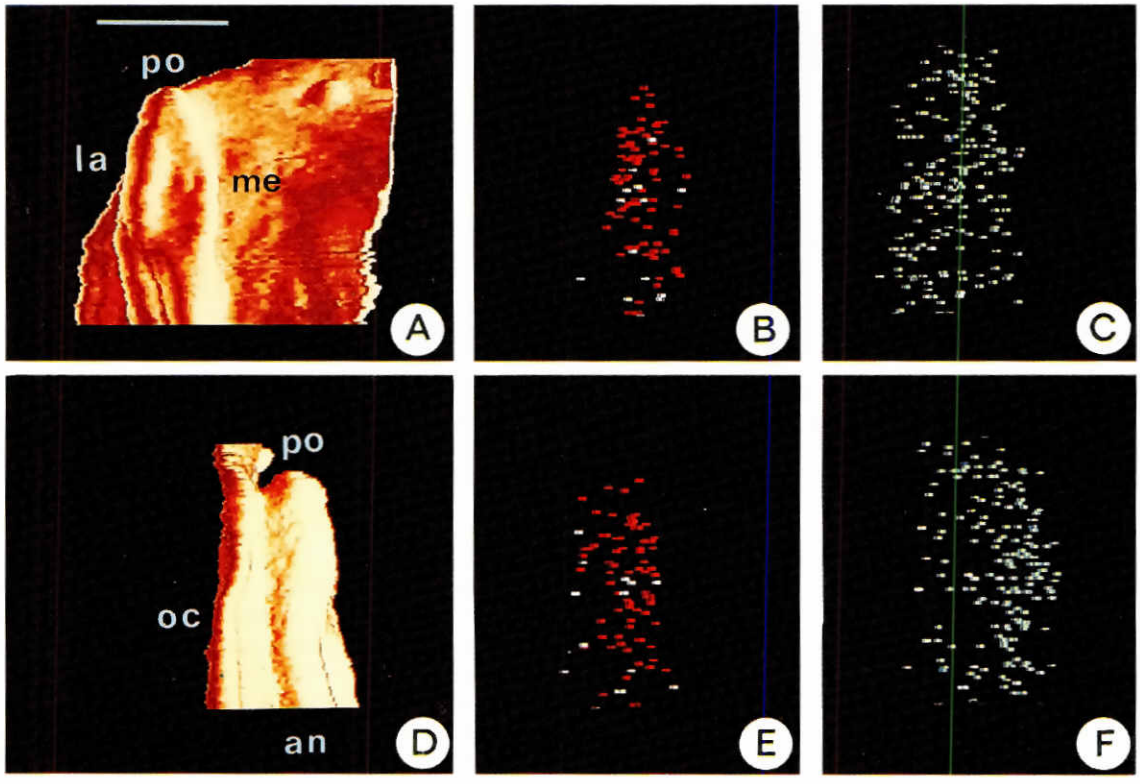

Fig. 8. 3D distribution of mitoses and apoptosis in the second upper molar at ED 15.5. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)**, *in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)** *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. an, anterior; la, lateral; me, medial; po, posterior and oc, occlusal. Bar, 100 μm.*

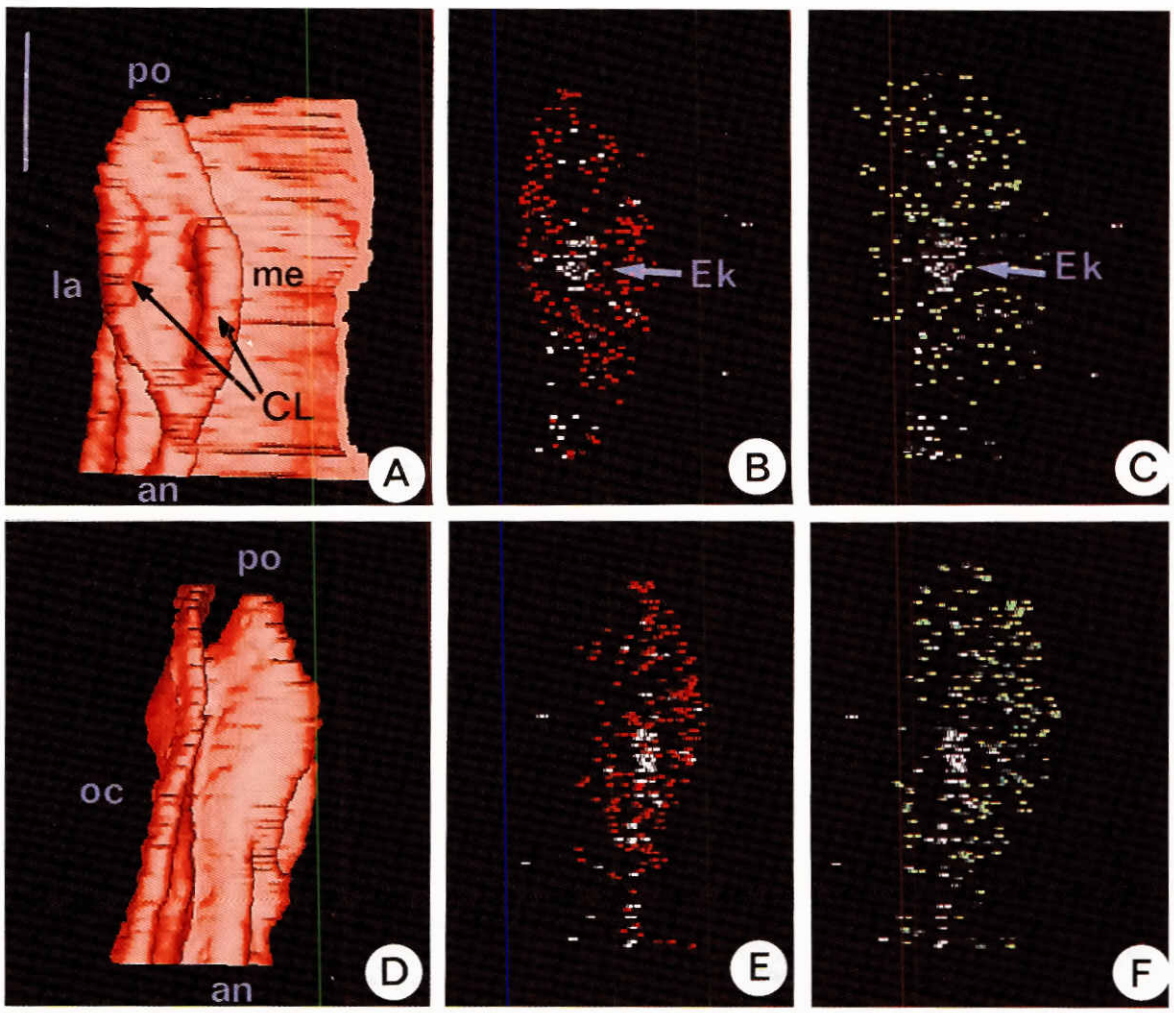

Fig. 9. 3D distribution of mitoses and apoptosis in the second upper molar at ED 16.0. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)**, *in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)** *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. High number of apoptotic bodies* **(B,C,E,F)** *are concentrated in the enamel knot (Ek). an, anterior; la, lateral; me, medial and oc, occlusal. CL, cervical loop. Bar, 100 μm.*

in the enamel knot area (Figs. 5B,E, 7B). At the early bell stage, ubiquitous distribution of mitoses was apparent antero-posteriorly in the medial and lateral parts of the cervical loop enclosing the bell cavity (Figs. 6B,E, 7C).

Mitoses were frequent in the mesenchyme surrounding the early cap at ED 14.0 and very low in the mesenchyme associated with the regressing epithelium in the anterior area (Fig. 4C,F). At ED 15.0, in the computer made frontal section whose thickness corresponded to the antero-posterior extension of the enamel knot (Fig. 5G) or in the sagittal section whose thickness corresponded to the diameter of the enamel knot (Fig. 5H), no increase in the number of mitoses was observed: neither in the epithelial nor in the mesenchymal cells close to the enamel knot. In contrast, the number of mitoses in the mesenchyme tended to decrease at ED 15.0, especially above the anterior part of the enamel knot region (Figs. 5C,D, 7B). At the early bell stage, mitoses in the mesenchyme showed an antero-posterior decreasing gradient (Fig. 6C,F).

Apoptotic activity was very high in the epithelium anterior to the forming cap at ED 14.0 (Fig. 4B,E). The number of apoptotic cells and bodies progressively decreased in this area at ED 15.0 (Fig. 5B,E) and 16.0 (Fig. 6B,E). Since apoptosis are very sparse in the mesenchyme, only the epithelial ones were represented in the reconstructions.

Apoptotic cells and bodies were observed in the enamel knot from the beginning of the cap stage (Figs. 4B,E, 7A). Their accumulation dramatically increased during cap formation (Figs. 5B,E, 7B). At the early bell stage, disappearance of the characteristic cellular arrangement of the enamel knot (Fig. 1D) was associated with the disappearance of apoptosis from this area (Figs. 6B,E, 7C). At this stage, most apoptosis was restricted to the gubernaculum, i.e. epithelium joining the enamel organ to the buccal epithelium (Figs. 6B,E, 7C).

Second molar

Histological aspects

In comparison to the first molar, the histodifferentiation of the enamel organ progressed faster in the second molar (Fig. 1). In

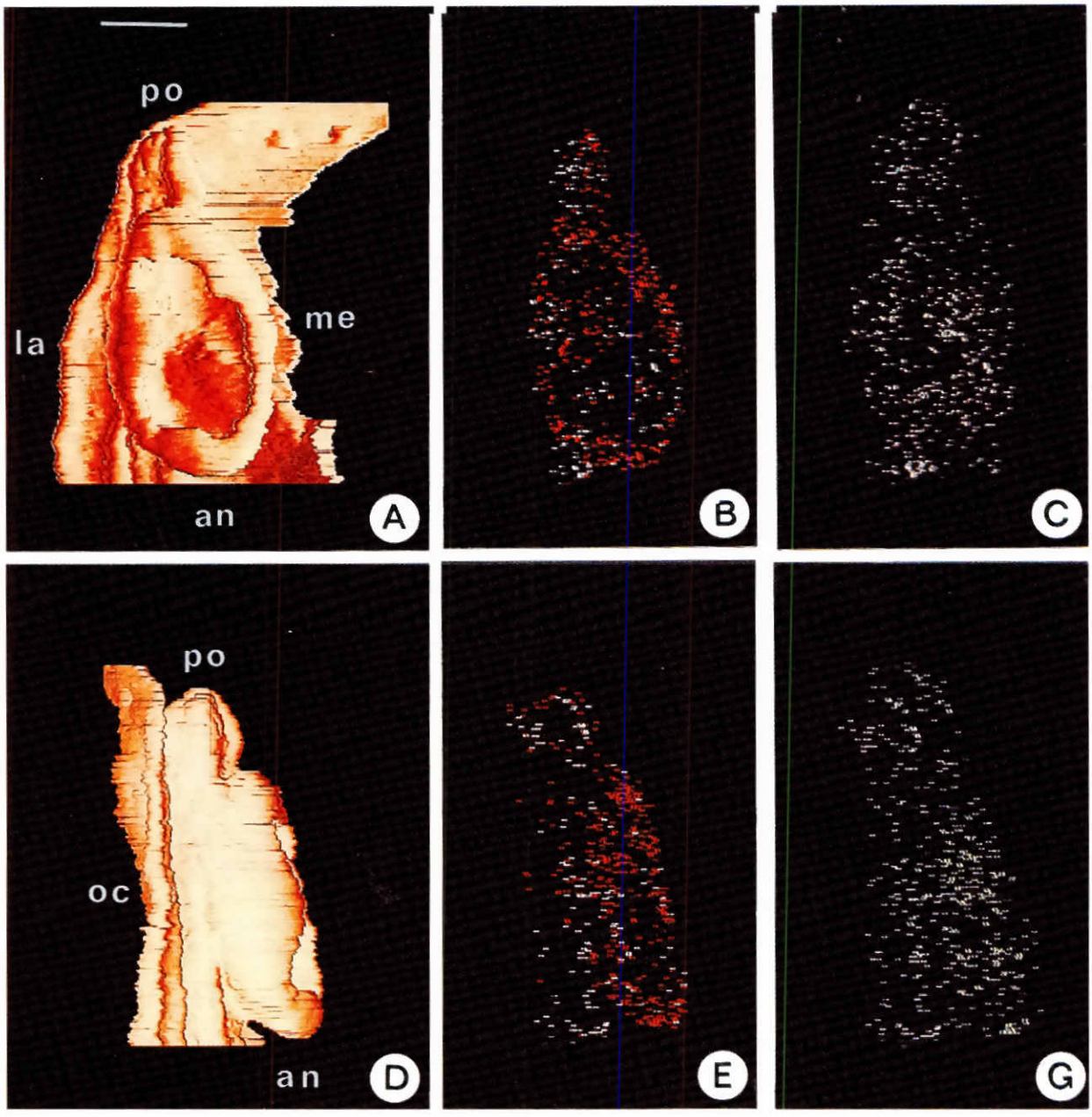

Fig. 10. 3D distribution of mitoses and apoptosis in the second upper molar at ED 18.0. *Spatial distribution of mitoses in the epithelium, represented as red spots,* **(B,E)**, *in the dental mesenchyme, represented as green spots* **(C,F)** *and of apoptosis, represented as white spots* **(B,C,E,F)** *can be compared to the shape of the dental epithelium* **(A,D)** *as seen from aerial* **(A-C)** *or lateral* **(D-F)** *views. an, anterior; la, lateral; me, medial; po, posterior and oc, occlusal. Bar, 100 μm.*

contrast, the enamel knot formation and the appearance of the enamel grooves delimiting its protrusion towards the dental papilla were delayed (Fig. 1). At the initial cap stage, only a small group of cells suggested the beginning of enamel knot formation (Fig. 1E) in 22% of sections where a cap depression was apparent (Fig. 2). After the cap concavity was established, the enamel knot had a demi-lune (half circle) aspect in frontal sections (Fig. 1F), half cylinder shape in space. Protrusion of the enamel knot towards the dental papilla and significant increase in apoptosis could not be detected as late as in the well formed cap (Fig. 1G). Apoptotic bodies, however, were far from being as frequent as during cap formation in the first molar (Fig. 1). The cross-sectional area of the enamel knot was smaller in the m2 cap than in m1. This difference mainly resulted from the fact that in m2, fewer inner cells participated in the enamel knot formation (compare Fig. 1B,C with 1F,G). The elongation of the cap margins as well as the depth of the cap cavity in m2 and m1 were comparable (Figs. 1, 2). The enamel knot extended over 135-145 μm and could be detected in 34-46% of sections in the formed cap cavity (Fig. 2).

Compared to the first molar, the size of the second molar during cap formation was conspicuously smaller in frontal sections (Fig. 1). Regardless of these differences, first and second molars at the

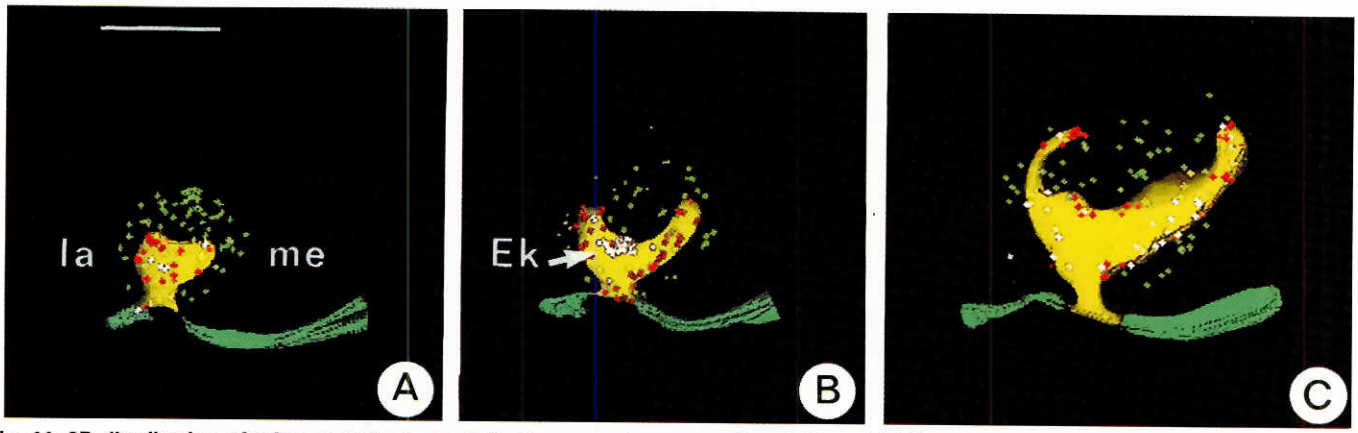

Fig. 11. 3D distribution of mitoses and apoptosis in the second upper molar. *100 μm thick frontal sections from reconstructions illustrate the distribution of the different cellular activities in the central part of the developing tooth at ED 15.5* **(A)**, *16.0* **(B)** *and 18.0* **(C)**. *Mitoses in the epithelium were represented as red spots, in the mesenchyme as green spots and apoptosis as white spots. Maintaining the same orientation, the dental epithelium was visualized in yellow and the buccal epithelium in pine green. la, lateral and me, medial. Bar, 100 μm.*

early bell stage exhibited similar cross-sectional area and morphological features in frontal sections passing through the middle part of their antero-posterior course (compare Fig. 1D with 1H).

3D reconstructions

At ED 15.0, the bud of the second molar could be detected in the posterior part of the upper jaw dental epithelium, close to the isthmus faucium (Fig. 5A). Initiation of the cap stage was observed twelve hours later (Fig. 8A,D). In the central part, a shallow depression was formed along the antero-posterior axis. Neither longitudinal enamel grooves nor epithelial cords were apparent (FIG. 8A).

Before the bell stage existed at ED 18.0 (Fig. 10A,D), a well formed cap stage was observed at ED 16.0 (Fig. 9). The central cord separated by enamel grooves from the lateral and medial sides of the cap, as seen in m1, could not be detected in m2 (Fig. 9A,D). In contrast with m1 (Fig. 5A), the margin of the cap in m2 was well formed anteriorly (Fig. 9A,D): the margin elevation was retarded in the posterior part of m2 (Figs. 9A,D, 12). Formation of the margin of the enamel organ in the first and second molar occurred as mirror images (Fig. 12). When compared to m1, m2 demonstrated a conspicuously smaller antero-posterior length during all cap and early bell stages (Fig. 12) and a smaller medio-lateral width during the cap stage (compare Figs. 7 and 11).

Mitoses and apoptosis

At ED 15.5 mitoses in the dental epithelium were prevalent on the lateral side (Fig. 8D) and associated with a thickening of this region of the epithelium (Fig. 8A). At ED 16.0, mitoses coincided with the further development of the epithelial margin of the cap (Figs. 9, 11B). At ED 18.0, a high mitotic activity was still associated with the developing margin (Figs. 10, 11C) and the posterior half of the enamel organ appeared to be the most active area (Fig. 10E).

The mitotic activity within the condensing dental mesenchyme at ED 15.5 was higher on the lateral side of the second molar (Fig. 8F). At ED 16.0, mitoses showed an ubiquitous distribution (Figs. 9C,F, 11B) whilst at ED 18.0, the highest activity was observed in the posterior half of the second molar (Fig. 10F).

At ED 15.5, very few apoptotic bodies were observed (Figs. 7B,E, 11A). The majority of apoptotic bodies at ED 16.0 were associated with the enamel knot (Fig. 9B,E), few of them were present in the anterior part of the tooth (Fig. 9B,E). At ED 18.0, apoptotic cells and bodies were mainly observed along the antero-posterior axis of the tooth (Fig. 10B) in the epithelium interconnecting the enamel organ to the buccal epithelium (Fig. 10E). Some were also associated with the developing margin (Fig. 10B,E).

Discussion

This study of spatial distribution of mitoses and apoptosis during early stages of upper molar tooth morphogenesis in the mouse is complementary to the results presented in the accompanying paper (Peterková *et al.* in this issue). Our 3D reconstructions illustrated changes in shape of the dental epithelium that accompany the transition of first and second upper molars from early cap to early bell stages, and demonstrated apoptotic processes associated with the enamel knot, the gubernaculum and the epithelium anterior to the first molar cap. Mitoses in the epithelial and mesenchymal constituents have been evaluated and shown to be involved in the development of the cervical loop in both molars, together with the anterior elongation of m1. These data allow discussion of complementary processes involved in spatial tooth morphodifferentiation leading to early bell stage formation in the upper first and second molar.

Comparison of m1 versus m2

At the initial cap stage, the presence of three cords separated by enamel grooves as seen in the first molar could not be observed in m2: the enamel grooves delimitating the medial bulging of the epithelium towards the dental mesenchyme were not detected in the well formed m2 cap. For other aspects, morphogenesis in the first and second upper molars followed a similar scheme to reach the bell stage, although by mirror image: the delimitation of the enamel organ cavity by a continuous cervical loop was retarded anteriorly for the first molar and posteriorly for the second one. Later, the width handicap of the m2 anlage at initial stages of cap formation was partially compensated by intensive growth: on frontal sections, the epithelio-mesenchymal interface of both m1 and m2 exhibited similar shapes and comparable widths at the early bell stage although no major change could be detected in the

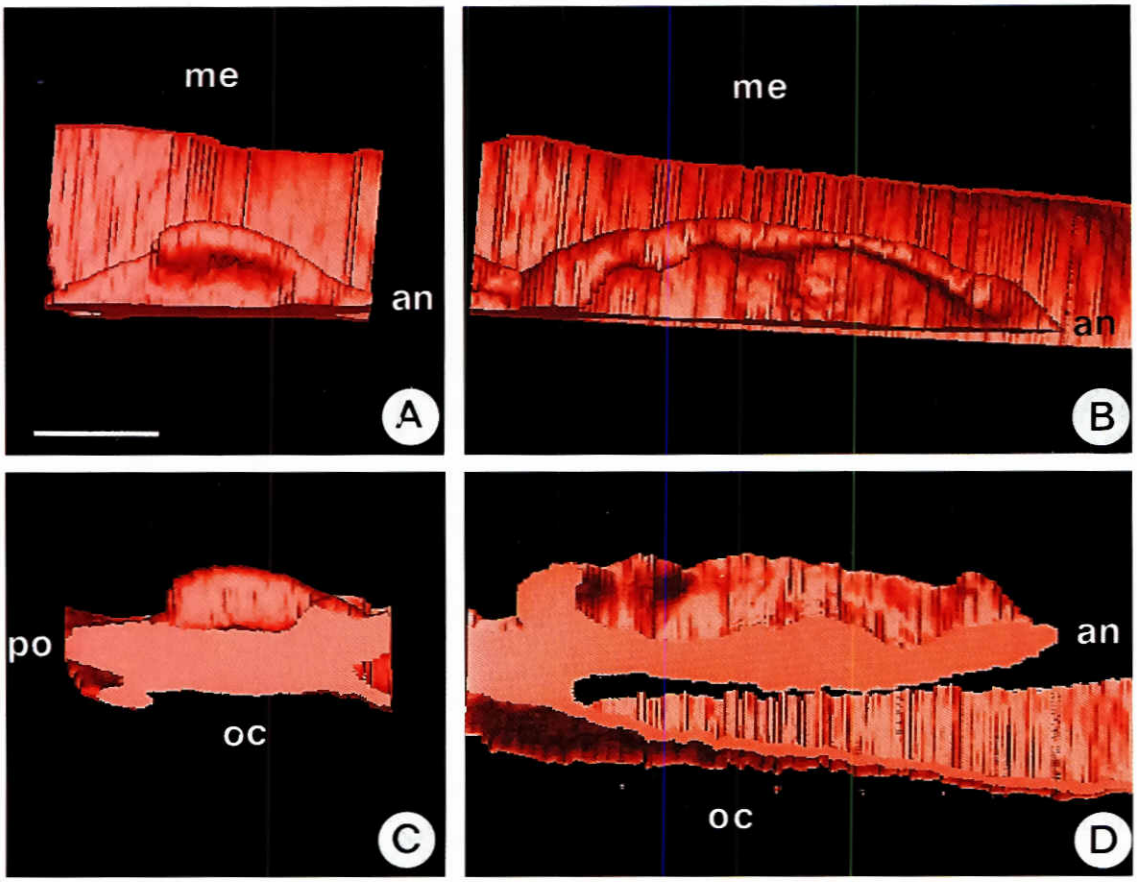

Fig. 12. 3D-reconstructions of the dental epithelium of first and second upper molars at ED 16.0. *Aerial* **(A,B)** *and lateral* **(C,D)** *views showing the shape of the dental epithelium after sagittal section has been performed in reconstructions of the second* **(A,C)** *and first* **(B,E)** *molars. Closure of the cervical loop is retarded in the anterior (an) part of the first molar* **(B,D)** *and in the posterior (po) part of the second one* **(A,C)**. *me, medial; oc, occlusal. Bar, 100 μm.*

number of mitoses. The rapid size increase which occurs in m2 might thus result either from subtle change in the number of mitoses or from a shortening in the duration of the cell cycle in m2. Despite this change in width, the antero-posterior extension of m2 remained much lower than that of m1. The conspicuous medial enlargement of the upper m1 and m2 early bell, led to asymmetry of the enamel organ on histological sections. This asymmetrical enlargement seems to be implicated in development of that part of the enamel organ devoted to the formation of the third medial row of cusps arranged in the antero-posterior direction. This asymmetrical growth appeared more pronounced in the upper molars than in the lower ones, where only two longitudinal rows of cusps are present (Gaunt, 1955). The morphodifferentiation of the molar epithelium at the early bell stage results from complementary processes involving specific temporo-spatial patterns of different mitotic and apoptotic activities.

Mitoses

It is generally accepted that differential mitotic activities are a prerequisite for successful prenatal formation and postnatal renewal in various organs. 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). The results presented in this paper document spatial changes in the distribution of mitoses during molar cap and early bell development. At the earliest cap stage, epithelial cells actively divided although the distribution of mitoses was uniform: neither in m1 nor in m2, the existence of specific sites where cells divided, as suggested by Cohn (1957), could be confirmed with the 3D representations. When the epithelial margins started to elevate, mitoses in the epithelial compartment were associated with extension of the cap margins, progressively delimiting the dental papilla. The lateral margins of the dental epithelium, as observed from 3D reconstructions, will give rise to the cervical loop defined as when histogenesis allows the distinction of inner and outer enamel epithelium. At ED 16.0, the spatial distribution of mitoses in the enamel organ of m1 was much more difficult to correlate with the morphogenetic changes ultimately leading to cusps formation; the same was observed for m2 at ED 18.0. On the basis of the present data, however, the localization of the prospective cusps could not be determined in the early bell stage molar epithelium. Cusps formation involves complex mechanisms which, independently of unequal growth of the dental epithelium, might include cell migration and/or physical participation of the dental papilla (Gaunt and Miles, 1967). Osborn (1993) developed a theoretical model to explain how mechanical forces, for example generated by mitotic activity, could interfere with the position of cells during organogenesis and thus specify morphogenesis. Differential mitotic activities

have been reported here, particularly in the developing dental epithelium, which might affect the final distribution of cell progeny although no cell migration *stricto sensu* is directly involved. This latter process might represent one more level of modulation in tooth morphogenesis in the mesenchyme while folding or epiboly might intervene in the epithelium. A postero-anterior increasing gradient of mitotic activity exists in the dental mesenchyme at the early bell stage in the first molar and might be correlated with the anterior growth of the developing tooth germ.

Apoptosis

Apoptosis or programmed cell death is a physiological and widespread phenomenon allowing the deletion of useless, unwanted, or crippled cells in every tissue, during organ remodeling and in embryonic development (Clarke, 1990; Oppenheim, 1991; Prindull, 1994; Vermes and Haanen, 1994; Wride *et al.*, 1994; Tilly *et al.*, 1995). Several groups are currently investigating the molecular control of this process (Hoffman and Liebermann, 1994; Fotedar *et al.*, 1995; Kroemer *et al.*, 1995). According to Graham *et al.* (1994), BMP-4 induces apoptosis in the rhombomeres. BMP-4 has been suggested to play a similar role during regression of the diastemal dental rudiments in the mouse (Turecková *et al.*, 1996).

Accumulation of apoptotic cells and bodies was documented in the epithelium anterior to the prospective or well-formed m1 cap. This apoptotic process was associated with reduction of the transitory tooth rudiments, generally but wrongly considered as the bud of the upper first molar at day 12 and 13 (Peterková *et al.* in this issue). From ED 15.0 and during further development leading to the early bell stage, the reduced epithelium anterior to the developing m1 seemed to be implicated during a secondary anterior extension of the m1 enamel organ proper. In this context, it is important to stress that BMP-4 expression in the epithelium anterior to the first upper molar in the mouse within day 11(20)-13(8) has been interpreted as being related to the bud of the upper first molar (Turecková *et al.*, 1995). This expression seems to be implicated in the regulation of apoptosis during regression of epithelium anterior to the first molar bud and cap (see also Peterková *et al.* in this issue).

The enamel knot, whose function remains speculative, has been observed in various species of placental mammals and is present both in molars and incisors in the mouse. The enamel knot has been suggested to be involved in cusps formation (Orban, 1928; Butler, 1956). More recently, the possible role of the enamel knot in cusps formation was reconsidered: production of FGF-4 mRNA by non-dividing cells of the primary and secondary enamel knots was assumed to correlate with stimulation of the mitotic activity within surrounding cells (Jernvall *et al.*, 1994). The enamel knot was reported to express several other signaling molecules such as BMPs and was considered as an organizing center during tooth morphogenesis (Vaahtokari *et al.*, 1996b) involved in the control of epithelio-mesenchymal interface folding and prospective cusp pattern determination. However, no increase in the number of mitoses could be observed in the present study and regardless of the similarity in morphology and FGF-4 expression between the primary and secondary enamel knots (Jernvall *et al.*, 1994), their potential role in cusps formation appeared to be opposite: the primary enamel knot is located in between prospective cusps, whereas the secondary enamel knots are located at their tips. Furthermore, in the second molar, the cap concavity was initiated nearly in the absence of an enamel knot. In both m1 and m2, the cap margins elongated and the cap cavity was well formed in regions where the enamel knot was not present. Cell death has been reported in the enamel knot in man as well as in the enamel knot in the mouse first molar (Nozue, 1971; Kindaichi, 1980). These data have been confirmed for the first lower molar in the mouse and cell death has been identified as apoptosis (Vaahtokari *et al.*, 1996b). Apoptosis was detected in the enamel knot of the first and second upper molar caps. In m2 however, the apoptotic process started later, associated with delay in enamel knot formation, and its intensity was lower compared to that in m1 at similar stages. It is not yet known if the several transcripts detected by *in situ* hybridization in the enamel knot are indeed translated. On the other hand, developing teeth produce morphogenetic signals (Koyama *et al.*, 1996) which are not restricted to the enamel knot. Furthermore, Olive and Ruch (1982) demonstrated that the mitotic activity of the inner dental epithelium is controlled by the dental papilla. Finally, till yet no convincing data demonstrate that the differential mitotic activity of the inner dental epithelium is really involved in cusps formation. Apoptosis in the enamel knot might correspond to the programmed destruction of cells whose function had been accomplished or whose potential activity (i.e. potential cervical loop cells of fused tooth primordia) has to be avoided.

At the bell stage, apoptosis was concentrated in the gubernaculum, a structure which progressively disappeared at later stages. Apoptosis thus intervened in the elimination of different epithelial constituents with a highly specific temporo-spatial pattern. Further developmental studies including experimental blocking of mitoses or apoptosis should elucidate their morphogenetic role during tooth development. The results presented here are being extended to analyze cellular activities at later stages of tooth development when histogenesis proceeds and cytodifferentiations are initiated.

Materials and Methods

Upper first and second molar morphogenesis was investigated in ICR mouse embryos whose age was determined in embryonic days (ED) (Peterková *et al.* in this issue). Embryos were harvested at noon and midnight within ED 14.0-18.0. Since the chronological staging specified by external morphological criteria appeared too crude for this study, the weight of embryos was used as an additional criterion (Peterková *et al.*, 1993, and this issue). The specimens weighing up to 500 mg were distributed in 25 mg weight classes (wtc.), for larger weights up to 1000 mg in 50 mg classes and for heavier specimens, in 100 mg classes. The embryos were fixed in Bouin-Hollande fluid. The head of one specimen from each weight class at each chronological stage was processed for histology.

Histology

5 μm frontal serial sections from paraffin-embedded heads were stained with alcian blue-hematoxylin-eosin. In this study, tooth staging was determined at the histological level according to Cohn (1957).

Mitoses and apoptosis

For 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.

Apoptotic cells and bodies were identified from histological sections according to morphological criteria (Kerr *et al.*, 1995; Turecková *et al.*, 1996); their nature was confirmed using the TUNEL method (Mori *et al.*, 1994; Turecková *et al.*, 1996).

3D reconstructions

Drawings of the contours of the maxillary oral epithelium were made in 5 μm intervals from serial histological sections using a Zeiss Jenaval microscope equipped with a drawing chamber at a magnification of 320x. The area under investigation extended from the level of the second palatal ruga until the isthmus faucium. Mitoses were recorded in the dental epithelium and mesenchyme. Since very few or no apoptosis could be detected in the dental mesenchyme, only epithelial apoptosis were indicated in the drawings. The digitalization of the serial drawings was achieved by means of a Hamamatsu C2400 camera connected to a digital imaging system. Correlation of successive images was performed using a real time superimposition method (Olivo *et al.*, 1993). Softwares 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 in the first and second molar, measurements were performed on the axis determined by the middle of the gubernaculum of the enamel organ and the top of the epithelial protrusion towards the dental papilla, delimited by the medial and lateral enamel grooves (in the second molar, before the protrusion was formed, the axis passed through the middle of the gubernaculum and middle of the cap excavation). Two dimensions were plotted in the graphs: distance «a» between the oral epithelium and the top of the protrusion (or center of the cap excavation), and distance «b» between the oral epithelium and the point of intersection of the axis with the interconnection between the medial and lateral margin of the enamel organ (see insert in Fig. 2). Measurements were performed using the camera lucida projection (magnification 320x) of frontal histological sections in 5 μm intervals of the antero-posterior course.

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