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Apoptosis in the developing tooth: association with an embryonic signaling center and suppression by EGF and FGF-4

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SUMMARY

Apoptosis was localized in developing mouse teeth from initiation of morphogenesis to completion of cusp formation by using modified TUNEL method for serial sections and Nile Blue staining for whole mounts. Apoptosis was first detected at bud stage (E12-E13) in the central cells of the invaginating dental epithelium suggesting involvement of cell death in epithelial budding morphogenesis. During cusp development, apoptotic cells were located in the enamel knots, which are transient clusters of dental epithelial cells proposed to act as signaling centers directing the morphogenesis of tooth cusps. Apoptosis was also detected in other restricted epithelial cell populations including the dental lamina, ameloblasts, as well as stratum intermedium and stellate reticulum cells suggesting that the removal of these epithelial cells occurs by apoptosis. Apoptotic cells, presumably osteoclasts, were also located on the surfaces of the developing alveolar bone.

When dissected E13 dental epithelium or mesenchyme were cultured in isolation, apoptotic cells were abundant throughout the tissues, whereas when cultured together, apoptosis was inhibited in both tissues close to their interface indicating that epithelial-mesenchymal tissue interactions prevent apoptosis. Epidermal growth factor (EGF) and fibroblast growth factor-4 (FGF-4) inhibited apoptosis in the dental mesenchyme when applied locally using agarose or heparin-coated acrylic beads, suggesting involvement of these or related growth factors in the prevention of apoptosis in dental tissues in vivo.

The spatially and temporally restricted distribution patterns of apoptotic cells suggest multiple roles for programmed cell death in dental development. Of particular interest is the removal of the enamel knots by apoptosis which may terminate their tasks as regulators of the patterning of the tooth cusps. The apical ectodermal ridge (AER) of the limb bud has similar signaling characteristics as the enamel knot, and it also undergoes apoptosis. Hence, apoptosis may be a general mechanism for the silencing of embryonic signaling centers.

Key words: Tunel, Nile Blue, enamel knot, tooth cusp, ameloblasts, epithelial-mesenchymal interactions, osteoclasts, organ development, programmed cell death, mouse

INTRODUCTION

The developing molar tooth is a good model for studying the regulation of organ morphogenesis (Fig. 1). The main stages of tooth development are presented in Fig. 1. At the site of future tooth anlage, the oral epithelium thickens indicating the initiation of tooth development (E10-E11 for mouse lower first molars). At bud stage (E12-E13), the epithelium invades the underlying neural crest-derived mesenchyme and forms an epithelial bud, around which the mesenchymal cells condense. As a result of epithelial folding, cap stage is reached (E14-E15) and a transient structure called the primary enamel knot appears in the middle of the enamel epithelium. At bell stage (E16-P7), secondary enamel knots appear at the tips of the developing cusps. The enamel knots have been suggested to regulate the shape of the tooth germ and, in particular, cusp morphogenesis (Jernvall et al., 1994). The epithelial dental lamina, which connects the tooth to the oral epithelium, becomes disrupted during advancing morphogenesis. Around birth, the mesenchymal-derived odontoblasts and epithelial-derived ameloblasts undergo terminal differentiation and start the secretion of dentine and enamel, respectively. The epithelial cells of the enamel organ, including ameloblasts, stratum intermedium and stellate reticulum cells disappear by the time the tooth erupts to the oral cavity (about 2-3 weeks after birth).

Programmed cell death (PCD) probably constitutes an important mechanism of embryonic development. In PCD, death of a cell is triggered by the appearance or loss of an external signal, leading to the activation of an internal cell death program (Schwartz and Osborne, 1993). One form of PCD is apoptosis (Kerr et al., 1972; Wyllie, 1987), and its characteristic morphological features include fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation, and endonucleolytic cleavage of DNA (Steller, 1995).

Although it is often stated in the literature that apoptosis controls cell number and tissue shape, during organ morphogenesis these processes have been rather poorly studied. For example, apoptosis in the developing kidney was reported relatively recently despite intensive research on kidney mor-

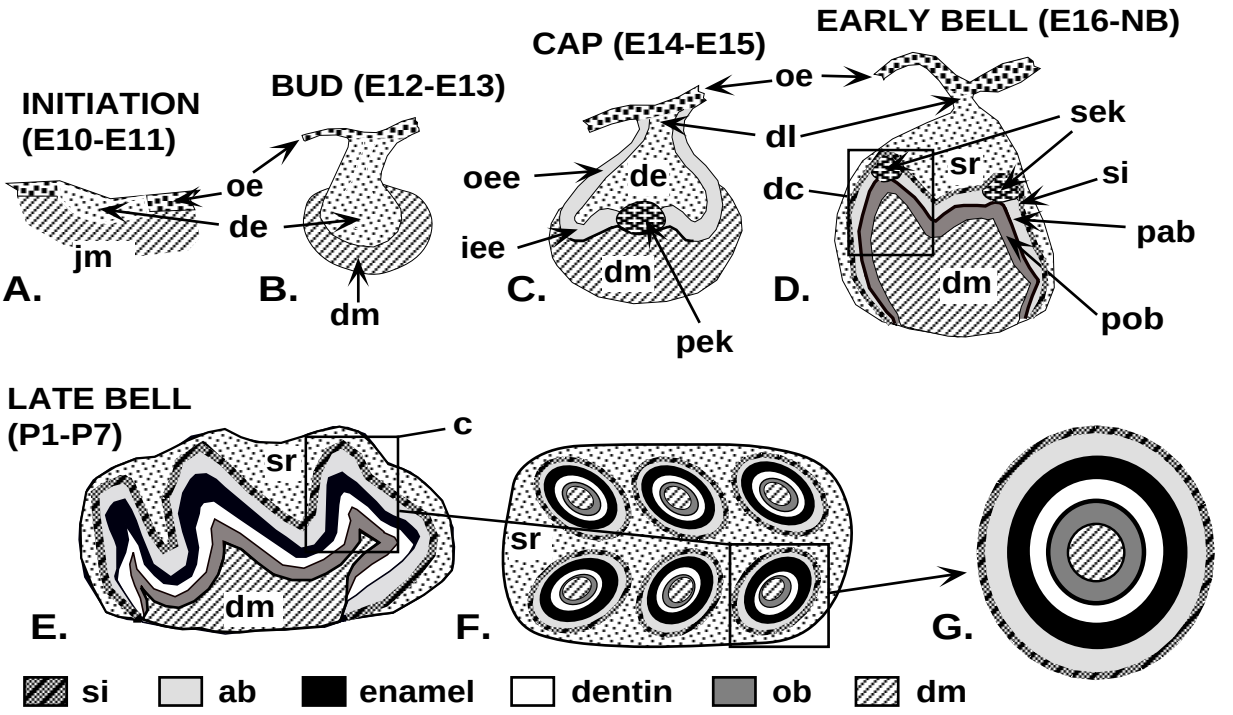

Fig. 1. Schematic representation of different stages of mouse tooth development until completion of cuspal morphogenesis. Dental follicle, which surrounds the tooth germ from cap stage until the crown morphogenesis is completed, is omitted for the sake of clarity. Drawings of (A,E) sagittal sections, (B-D) frontal sections, (F) a transversal section, (G) a higher magnification of a cusp. ab, ameloblasts; c, cusp; dc, developing cusp; de, dental epithelium; dm, dental mesenchyme; dl, dental lamina; iee, inner enamel epithelium; jm, jaw mesenchyme; ob, odontoblasts; oe, oral epithelium; oee, outer enamel epithelium; pek, primary enamel knot; sek, secondary enamel knot; si, stratum intermedium; sr, stellate reticulum.

phogenesis (Koseki et al., 1992; Coles et al., 1993). In the kidney, apoptosis is speculated to be associated with cell differentiation and to match the numbers of epithelial and mesenchymal cells (Coles et al., 1993).

Apoptosis has been proposed to have a role in the production of tooth anomalies associated with cleft lip and palate (Sharma and Kharbanda, 1991). However, as there is little information concerning apoptosis in tooth development, and the existing studies have focused on apoptosis in postnatal ameloblasts (Joseph et al., 1994 and references therein), we have now analyzed apoptosis in detail during mouse tooth development.

There is an increasing amount of data suggesting that growth factors acting through tyrosine kinase receptors are important survival factors suppressing apoptosis (Collins et al., 1994). There is even some evidence indicating that as a default, all cells undergo apoptosis unless they are rescued by survival factors (Raff, 1992; Steller, 1995). As epidermal growth factor (EGF) and fibroblast growth factor-4 (FGF-4) are expressed in tooth germs, we investigated their possible involvement in the regulation of apoptosis in cultured dental tissues (Snead et al., 1989; Niswander and Martin, 1992; Heikinheimo et al., 1993).

MATERIALS AND METHODS

Preparation of tissues for TUNEL and localization of TRAP

Heads, jaws, lower first molar regions and limb buds from embryonic day 10 (E10) to postnatal day 7 (P7) mice (CBA × NMRI) were dissected (vaginal plug = day 0) and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline, pH 7.3 (PBS). Tissues from postnatal mice were decalcified in 0.3 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 for about 3 weeks at 4°C. After the tissues were embedded in paraffin and sectioned, they were either used for terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) or for localization of tartrate resistant acid phosphatase (TRAP) as described by Reponen et al. (1994).

TUNEL staining

To detect apoptotic cells from tissue sections, we used digoxigenin-based modification of the original TUNEL method introduced by Gavrieli et al. (1992). TUNEL is based on the enzymatic incorporation of labeled nucleotides into the free 3′ ends of DNA. The apoptotic cells due to their DNA fragmentation get heavily labeled and can be specifically detected with antibody staining. Several steps in our procedure were applied from the in situ hybridization method described by Wilkinson (1992).

The paraffin sections were heated at 60°C for 30 minutes whereafter they were deparaffinized in xylene twice for 10 minutes. They were dehydrated in 100% ethanol for 5 minutes, then transferred to ethanol/acetic acid (2:3) for 20 minutes, incubated in 0.5% H_2O_2 in MeOH for 30 minutes, and rehydrated with 94%, 70%, 50% and 30% MeOH in PBS for 5 minutes each. After washing the sections in PBS containing 0.1% Triton X-100 (PBT) twice for 5 minutes each, they were incubated in 50 mM Tris-HCl, 5 mM EDTA, pH 8.0, containing 7 μg/ml proteinase K (Sigma P-4914) for 10 minutes and washed twice in PBT for 5 minutes each.

The sections were fixed in fresh 4% PFA in PBS for 20 minutes and rinsed twice with PBT for 5 minutes each. The sections were

labeled for 1 hour at 37°C in a labeling mix (TdT buffer (100 mM cacodylate, pH 6.8, 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 100 μg/ml BSA) containing 1 nM dig-11-dUTP (Boehringer Mannheim, 1093 088), 1 nM dATP, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma C-3023), and 27 U/100 μl terminal transferase (TdT) (Promega)). The labeling mix used for the negative controls did not contain any TdT. The reaction was stopped by incubating the slides in 300 mM NaCl, 30 mM sodium citrate, 0.1% CHAPS for 15 minutes with gentle rocking.

To confirm that the staining method was working properly, DNA was deliberately degraded in some sections after proteinase K treatment. Onto these sections, 0.2 U DNAse I (Promega M6101) in TdT buffer containing additional 4 mM $MgCl_2$, 0.1 mM dithiothreitol, was pipetted. The sections were incubated for 15 minutes at 37°C in a humid box and washed with PBT twice for 10 minutes each before the postfixation.

The sections were preblocked with 10% sheep or goat serum, 2% BSA in TBT for at least 3 hours in a humid box. Embryo powder and preabsorbed Fab fragments from an anti-digoxigenin antibody conjugated with alkaline phosphatase (anti-dig-AP) were done as reported earlier (Wilkinson, 1992). The preabsorbed anti-dig-AP was pipetted onto the sections, which were incubated overnight in a humid box at 4°C. After incubation, the sections were washed three times with TBT for 5 minutes each, then three times for 30 minutes each with rocking.

The slides were washed with 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$ (NTM) once for 5 minutes and then twice with NTM, 2 mM levamisole (Sigma L-9756) for 5 minutes each. NTM containing 338 μg/ml 4-Nitro blue tetrazolium chloride (NBT, Boehringer Mannheim 1087479, solubilized in 70% dimethylformamide) and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer Mannheim 760994, solubilized in 100% dimethylformamide) was pipetted onto the sections and development of the color reaction was monitored under a microscope. The reaction was stopped by washing the sections in PBT four times for 5 minutes each and then the slides were left overnight in PBT.

On the next day, the slides were rinsed with deionized water for 5 minutes and counterstained with Delafield's hematoxylin. The slides were mounted with Aquamount (BDH) and either photographed using Leitz Orthoplan microscope or digitized using Cohu 4912 CCD camera (Cohu, Inc.) and Scion LG-3 Frame Grabber card (Scion Corporation) with NIH Image 1.56 software (public domain software, Wayne Rasband, National Institutes of Health).

Nile Blue staining

For Nile Blue staining, heads, jaws or limbs from E10-E15 mice (CBA × NMRI) were dissected and stained with watersaturated Nile Blue (Gurr's) diluted to 1:1000 in PBS and filtered. The tissues were kept in Nile Blue solution until no more specific stain was evident, and then washed with PBS five times and either photographed immediately using Olympus SZH stereomicroscope or first left in PBS overnight at 4°C.

Organ culture experiments

The mandibular first molar regions were dissected from E13-E13.5 mice. This stage was selected for experiments because the expression of *Egf* and *Fgf-4* is not detected in dental tissues at E13 but has been reported at E14 (Snead et al., 1989; Heikinheimo et al., 1993; Jernvall et al., 1994). Thus, the dental mesenchyme before E14 has not been exposed either to EGF or FGF-4. Epithelial and mesenchymal tissues were separated by trypsin treatment and mechanical manipulation (Vainio et al., 1993).

25-1000 ng EGF (mouse, Boehringer mannheim), 1-250 ng FGF-4 (human sequence, recombinant, British Bio-technology Products Ltd), or 1-125 ng BSA diluted in 0.1% BSA in PBS was incubated with ca. 100 Affi-Gel Blue agarose beads (100-200 mesh, 75-150 μm, Bio-Rad) or, for FGF-4 and BSA, acrylic beads containing 1000 μg heparin per ml packed gel (Sigma) at 37°C for 30 minutes. Human and murine FGF-4 are 82% homologous in amino acid sequence. The beads were rinsed in PBS and placed in intimate contact with the separated dental mesenchyme or epithelium. Sometimes more than one mesenchyme or epithelium were cultured together because then the tissues grow better. We also cultured separated mesenchymes and epithelia either together or in isolation.

The explants were cultured on 0.1 μm pore-size Nuclepore filters (General Electron) placed on metal grids in Eagle's Minimum Essential Medium (GibcoBRL) supplemented with 10% fetal calf serum (GibcoBRL) for 16-24 hours at 37°C, in 5% CO_2. After incubation, the tissues were either stained with Nile Blue or fixed in 4% PFA in PBS. The fixed tissues were analyzed with TUNEL as paraffin sections.

RESULTS

Initiation of tooth development and bud stage (E11-E13.5)

TUNEL and Nile Blue stainings were performed to analyze apoptosis during the initiation of first molar development. In serial sections of the head of E11 mouse embryo, no apoptotic cells were evident in the epithelial thickenings or in nearby tissues (Fig. 2A). At early bud stage (E12-E13), apoptotic cells were detected at the oral surface and, in the budding dental epithelium, beneath the oral ectoderm (Fig. 2B). At late bud stage (E13.5), the streak of apoptotic cells extended to the tip of the epithelial bud (Fig. 2C). No apoptosis was detected in the mesenchyme around the dental epithelium (Fig. 2A-C).

Cap stage (E14-E15)

Because of the rapid progress of tooth morphogenesis during cap stage, serial sections from developing molars staged as E14, E14.5 and E15 were studied. At E14, when the epithelial bud starts to acquire a cap shape, apoptotic cells were observed within the epithelial enamel knot (Fig. 2D) and, at E14.5, their number increased (Fig. 2E). At late cap stage (E15), when the enamel knot has largely disappeared, almost no apoptotic cells were detected (data not shown). At E14, apoptosis was detected in the epithelium of the vestibular (data not shown) and dental lamina (Fig. 2D), where it continued at E15 (data not shown). Some dying cells could be detected on the periphery of the condensed dental mesenchyme throughout the cap stage (Fig. 2F).

Early bell stage (E16-E18)

During bell stage, cuspal morphogenesis continues and odontoblasts as well as ameloblasts undergo terminal differentiation. As at earlier stages, the distribution of apoptotic cells was temporally and spatially regulated.

At E16 and E18, apoptosis was located in the epithelial cells of the dental lamina and outer enamel epithelium (data not shown; Fig. 2G,H). At E17, no cell death was visible in first molars (data not shown), whereas, at E18, apoptosis was evident in the secondary enamel knots (Jernvall et al., 1994) as well as in stratum intermedium cells next to the enamel knots (Fig. 2G-I). In the dental mesenchyme, some scattered apoptotic cells were detected but they did not seem to be concentrated in specific areas except that some of them were adjacent to the dental follicle, where apoptosis was also detected (Fig. 2G).

Late bell stage (P0-P7)

In newborn mice, apoptosis continued in the dental lamina (data not shown) and in the adjacent outer enamel epithelium (Fig. 3A). In addition, apoptotic cells were evident in the presumed bud of the secondary tooth and in the disappearing secondary enamel knots (Fig. 3A). In the dental follicle, apoptotic cells were also detected (data not shown). At P2, apoptosis was only visible in the outer enamel epithelium and dental follicle (Fig. 3B). At P4, in addition to some ameloblasts, high numbers of dying cells were seen in the epithelial cells of the stratum intermedium and in stellate reticulum (Fig. 3C). Apoptotic cells were also located on the surfaces of the forming bone between the tooth germ and the oral epithelium (Fig. 3D). The distribution of these cells resembled that of osteoclasts as detected by TRAP staining (Fig. 3E) (Reponen et al., 1994).

At P7, cell death was most prominent in stratum intermedium cells and, in the vicinity of these cells, some apoptotic ameloblasts were detected (Fig. 3F,G). The apoptotic stratum intermedium cells formed stretches in which the apoptotic cells were immediate neighbors or only a few cells apart from each other (Fig. 3F,G). Outside these stretches of apoptotic cells, no dying stratum intermedium cells were detected (Fig. 3F,G). In postnatal mice, no apoptosis was detected in the dental mesenchyme (Fig. 3A-G).

Nile Blue whole-mount stainings

In whole-mount stainings of mandibles, apoptosis was evident in the epithelium overlying the molar buds and incisors and in the epithelium between the incisors (E12-E15) (Fig. 4A,C). In contrast, in the maxilla, also the toothless diastema region contained apoptotic cells at E13 (Fig. 4B). In addition, in the palatal ridges (rugae), apoptosis was evident at E13-E15 and the apoptotic regions in the dental arch and in the rugae seemed to make contact (Fig. 4B).

Control stainings

Serial sections were necessary to find out the complete distributions of apoptotic cells during tooth development. In general, apoptosis was not evident in all sections and usually a single section did not show all locations of apoptosis. In addition, because often in individual sections only a few stained cells were detected in a certain area, repeated stainings in serial sections were performed to confirm the results.

In TUNEL, no staining was detected in the negative controls, whereas the sections treated with DNAse I were stained all over (data not shown). For both TUNEL and Nile Blue staining, limb buds were used as positive controls. At E10-E12.5, apoptosis was detected in the apical ectodermal ridge (AER) of the limb buds (Fig. 4D,E). When the digits started to separate, apoptosis was seen between the digits (data not shown). These results are

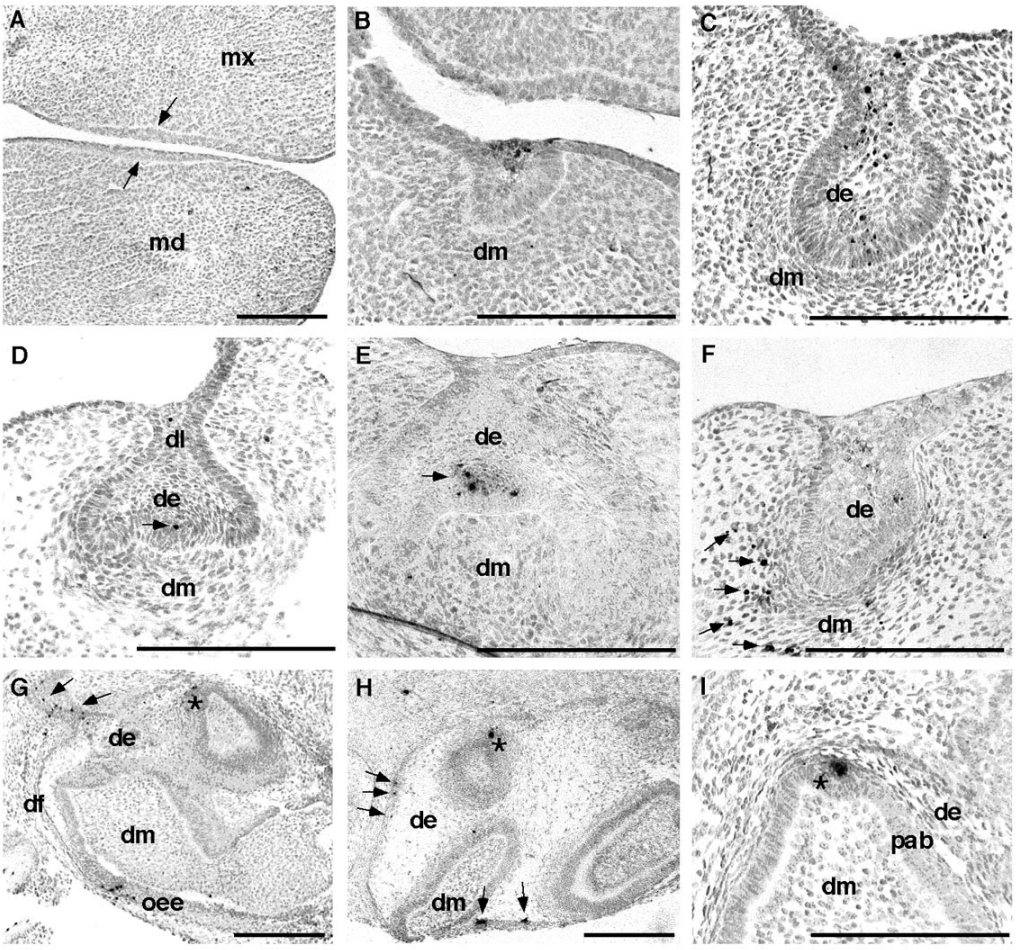

Fig. 2. Apoptosis during bud, cap and bell stages of tooth development as detected in serial paraffin sections by TUNEL. Digitized computer images. (A-F) Frontal sections, (G-I) transversal sections. (A) E11 mandible (md) and maxilla (mx). Thickening of the oral epithelium indicates initiation of tooth development (arrows). No apoptosis is detected either in the epithelial thickenings or in the surrounding tissues. (B) Early bud stage (E12). Apoptotic cells extend from the oral surface epithelium to the central part of the budding dental epithelium. (C) At late bud stage (E13), apoptotic cells extend from the surface epithelium to the tip of the dental epithelial bud. (D) Early cap stage (E14). Apoptosis is detected in the dental lamina (dl) and in the primary enamel knot (arrow). (E) Cap stage (E14.5). Apoptosis is prominent in the enamel knot (arrow). (F) Late cap stage (E15). Apoptotic cells are located at the periphery of the condensed dental mesenchyme (arrows). (G-I) Bell stage (E18). (G) Apoptosis is seen in the dental lamina (arrows), dental follicle (df), outer enamel epithelium (oee) and in stratum intermedium cells adjacent to the secondary enamel knot (asterisk). (H) Outer enamel epithelial cells undergo apoptosis (arrows) as well as the secondary enamel knot cells (asterisk). (I) In the secondary enamel knot and adjacent stratum intermedium cells, apoptosis is detected (asterisk). pab, preameloblasts; de, dental epithelium; dm, dental mesenchyme. Scale bar, 200 μm.

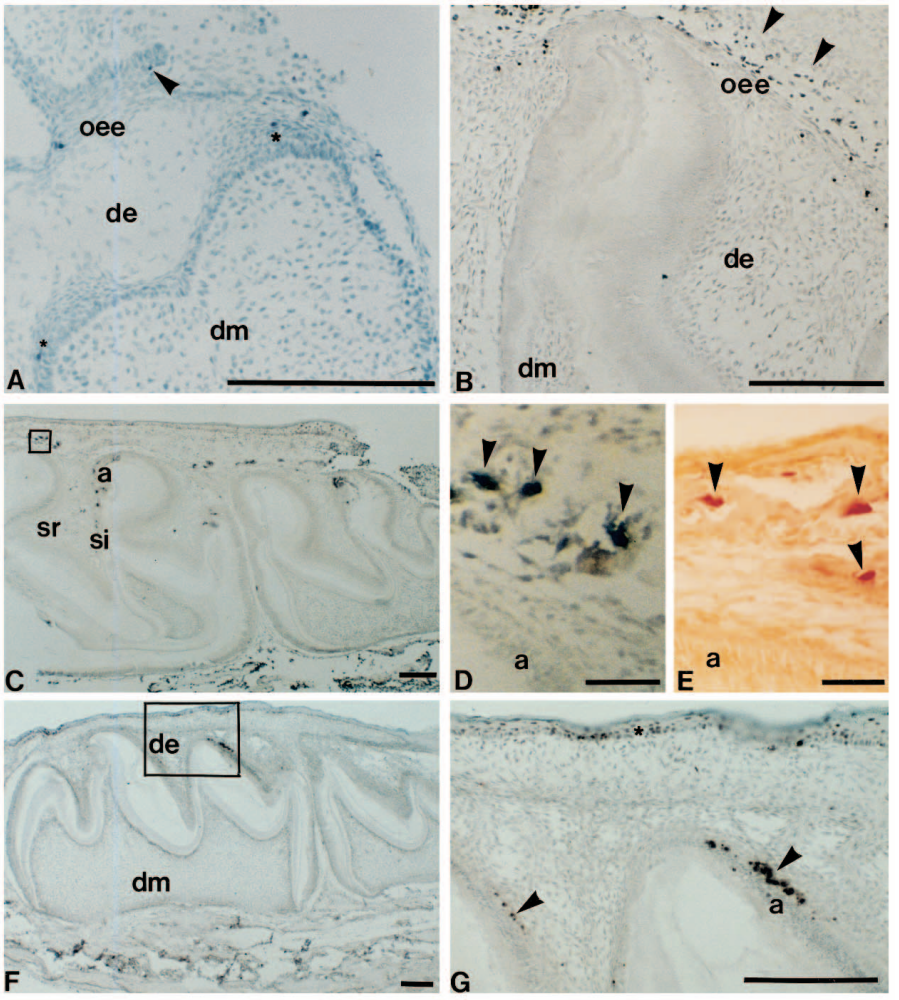

Fig. 3. Apoptosis in sections of postnatal molars as detected by TUNEL. Molars of a (A) P0, (B,E) P2, (C,D) P4 (F,G) P7 mouse. (A), a frontal section, (B-G), sagittal sections. (A) Apoptosis is seen in the presumptive secondary tooth bud (arrow), in outer enamel epithelial cells and in the disappearing secondary enamel knots (asterisks). (B) Apoptotic cells are located in the dental follicle (arrows) and in the outer enamel epithelium. (C) Apoptosis is detected in ameloblasts (a), stratum intermedium (si) and stellate reticulum cells (sr). (D) A higher magnification of the box marked in C. Dying cells are also evident on the surface of the alveolar bone (arrows). (E) Localization of TRAP in a similar section as in (D) shows the distribution of osteoclasts (arrows) which closely resembles that of the apoptotic cells in (D). (F) Some stratum intermedium cells and ameloblasts undergo apoptosis. Apoptosis is not seen in other dental cell populations. (G) A higher magnification of F. Apoptotic stratum intermedium cells (arrows) form stretches where the dying cells are next to each other. Apoptosis is also detected in the epithelial cells of the oral mucosa (asterisk). a, ameloblasts; de, dental epithelium; dm, dental papilla mesenchyme; oee, outer enamel epithelium. Scale bar, 200 μm for A-C, F,G; and 50 μm for D,E.

in agreement with previous descriptions of apoptosis during limb development (Hinchliffe, 1982; Lee et al., 1993).

Effects of EGF and FGF-4 on apoptosis in vitro

When epithelial and mesenchymal components of E13-E13.5 tooth germs were separated and cultured in isolation for 16-24 hours, apoptosis was abundant in the explants (data not shown). When mesenchyme was cultured in contact with the epithelium, stainings with TUNEL and Nile Blue indicated that apoptosis was prevented both in the epithelium and mesenchyme at the tissue interface whereas apoptotic cells were abundant elsewhere in the explants (Fig. 5A). As shown earlier, a translucent zone was induced in the mesenchyme next to the epithelium (Vainio et al., 1993; data not shown).

Beads soaked in EGF and FGF-4 prevented apoptosis on dental mesenchyme. After 16-24 hours of culture, apoptosis was detected by TUNEL and Nile Blue staining all over the tissue except around the EGF- and FGF-4-releasing beads, and a translucent zone similar to that induced by the epithelium was detected around the beads in approximately the same area

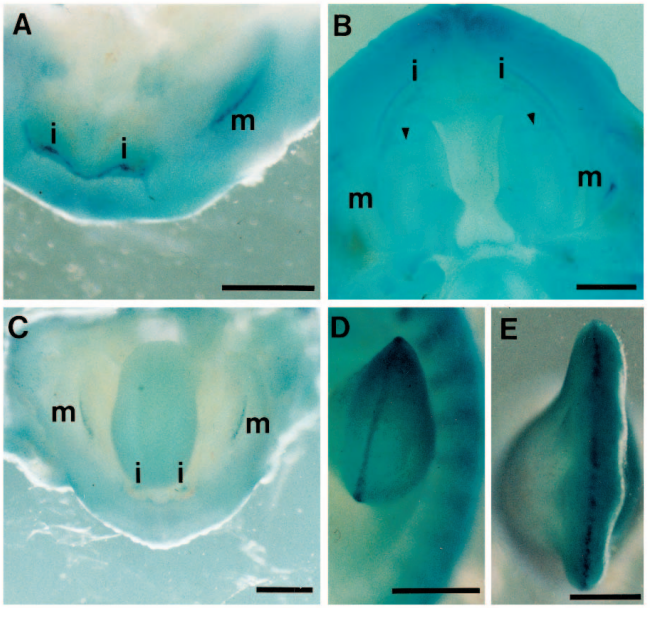

Fig. 4. Apoptosis in jaws and limbs as detected by Nile Blue staining of whole tissues. (A) E13 mandible. Apoptosis is located in the medial surface of the dental epithelium in molars and incisors and in the epithelium between the two incisors. (B) E13 maxilla. Cell death is seen in the odontogenic epithelium from the incisors to the molars and in palatal rugae (arrows). (C) E14 mandible. Apoptosis persists in the epithelia of molars and incisors. (D) E11 limb bud. Apoptotic cells are abundant in the AER. (E) E13 limb bud. Apoptosis is still seen in the AER. i, incisor; m, molar. Scale bar, 500 μm.

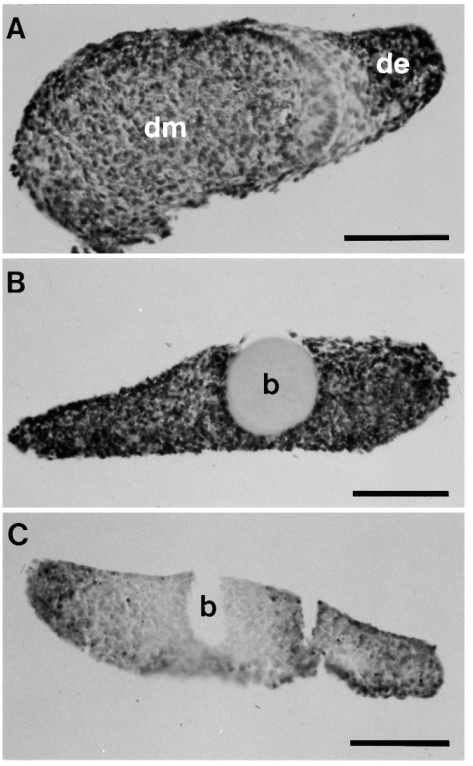

Fig. 5. Effect of FGF-4 on apoptosis as detected in serial sections of dental tissue explants by TUNEL. E13 dental mesenchyme (dm) cultured together for 16-24 h before staining (A) with dental epithelium (de), (B) with BSA-releasing agarose bead (b) and (C) with FGF-4-releasing agarose bead. (A) Apoptosis is not detected in the epithelial-mesenchymal interface although in other areas of the explant a high number of apoptotic cells is seen. (B) The whole explant undergoes apoptosis. (C) The tissue around the FGF-4-releasing bead is devoid of apoptosis but the apoptotic cells are located in regions further away from the bead. The bead has detached from the tissue during sectioning. b, a hole indicating the location of the bead during the culture. Scale bar, 100 μm.

(Figs 5C, 6E,F,I-L,M,N,Q,R) (Jernvall et al., 1994). The effects of EGF and FGF-4 were dependent on the concentrations of the growth factors in which the beads were incubated. EGF prevented apoptosis when the beads were soaked in 125 ng EGF per 100 beads (5 out of 8 explants) (Fig. 6K,L). The effect of EGF was dramatic: the translucent zone and the lack of apoptosis extended throughout the explants in most cases (Fig. 6K,L). Surprisingly, all lower (25 and 75 ng) and higher (250, 600 and 1000 ng) amounts of EGF tested had no effects (0 out of 22 explants and 0 out of 15 explants, respectively) (Fig. 6C,D,G,H,O,P; data not shown).

25-250 ng of FGF-4 were effective (41 out of 59 explants) but not lower amounts (1 and 5 ng) (0 out of 19 explants) (Fig. 6A,B; data not shown). With 25 ng of FGF-4 per 100 beads, the translucent zone and the area where apoptosis was inhibited were substantially smaller than with 75-250 ng of FGF-4 per 100 beads (Fig. 6E,F,I,J,M,N,Q,R). Beads soaked in BSA did not prevent apoptosis in dental mesenchyme, and no translucent zone was detected (Fig. 5B).

We also cultured E13 dental epithelia with FGF-4- and BSA-releasing beads but the epithelia did not grow well in isolation. Additionally, the small size of the epithelium made it difficult to place the bead on top of it. Although we sometimes detected that FGF-4 prevents apoptosis also in the dental epithelium (data not shown), we cannot draw definite conclusions about the effects of FGF-4 on apoptosis in dental epithelium due to technical difficulties.

DISCUSSION

Although apoptosis as a phenomenon has been known for a long time, its analysis has been problematic. The recently developed TUNEL method allows specific localization of cell death both in tissue sections and whole mounts. However, even large-scale cell death can easily be ignored in sections because usually only few apoptotic cells are detected in any single section. For example, it has been estimated that more than 97% of the newly formed thymocytes die during normal thymus development but only 0.2% of these cells were found to have typical morphology for dying cells in tissue sections studied (Coles et al., 1993). Our observations indicated that, in order to reliably localize apoptosis, it was necessary to analyze serial sections and to perform repeated experiments.

Apoptosis is apparently not a random event which only controls tissue size of the tooth germ. Instead, apoptosis clearly correlates with changes in morphogenesis and with the removal of specific cells. In the tooth, apoptosis was mostly detected in epithelial cells. During early development, cell death was associated with epithelial budding morphogenesis and in advanced teeth, apoptosis was detected in several epithelial cell types, most of which will disappear by the time of tooth eruption.

Our finding that apoptosis was associated with a variety of developmental processes in tooth germs was unexpected. Since there are few detailed studies on apoptosis in other organ systems available, it is possible that, in organogenesis in general, the role of apoptosis may have been underestimated.

We also located apoptotic cells on the surface of the alveolar bone around the tooth germ. Growth and eruption of the developing tooth is associated with resorption of the surrounding alveolar bone and, during stages examined here, osteoclasts are abundant around the tooth germ (Irie and Ozawa, 1990; Reponen et al., 1994). Based on the correlation of distribution of apoptotic cells and TRAP-positive cells, we consider it probable that the apoptotic cells detected in the bone are osteoclasts. Recently, apoptosis has been demonstrated in osteoclasts in vitro but, to our knowledge, it has not been earlier reported in vivo (Kameda et al., 1995).

Apoptosis has been associated with the expression of a number of different molecules, specifically transcription factors (Buttyan et al., 1988; Liu et al., 1994). Of these, *Egr-1, N-myc, c-fos, Msx-2* and Bcl-2 have been localized during tooth development (Hirning et al., 1991; Yamada et al., 1992; Karavanova et al., 1992; MacKenzie et al., 1992; Smeyne et al., 1993; Slootweg and de Weger, 1994). Our results do not indicate any association between apoptosis and the published expression data of these transcription factors except for *Msx-2*, which shows a partial correlation with cell death in the enamel knot (MacKenzie et al., 1992).

Apoptosis in the budding morphogenesis of the dental epithelium

The first appearance of apoptotic cells in the tooth germ was associated with invagination of the epithelium forming the

dental bud. In addition to the oral surface of the epithelium, apoptotic cells were apparent inside the tooth bud, suggesting that apoptosis may have a role in the budding morphogenesis of the dental epithelium. As epithelial invagination is a common theme in organogenesis, the selective removal of the central cells of the growing epithelium may contribute to the achievement of correct shape also in other developing organs.

Interestingly, the overall patterns of apoptosis in the early epithelium differed between the mandible and maxilla at E13-E15; in the mandible, the toothless diastema region between the incisors and molars was devoid of apoptosis, whereas in the maxilla, apoptosis was detected also in the diastema region. This correlates with the observation that, in the upper jaw, there are transitory tooth buds in the diastema region, whereas, in the lower jaw, such rudiments have not been reported (Peterková et al., 1993).

The apoptotic stripes in the palatal rugae, which appeared to be connected with the apoptotic streaks in the oral epithelium containing odontogenic potential, may indicate the potential for invagination in the head mesenchyme. It is known that, in some fish, the primary palate contains teeth (Kershaw, 1994). The capacity for tooth formation may be present also in the embryonic secondary palatal epithelium of the rodents.

Apoptosis in the removal of the enamel knots

We suggest that the striking localization of apoptosis in the cells of the enamel knots reflects a significant role of apoptosis in the regulation of tooth form. Enamel knots are morphologically distinct, transient thickenings of the enamel epithelium, which have recently been suggested to be important signaling centers regulating tooth morphogenesis (Jernvall et al., 1994). The primary enamel knot appears during the transition from bud to cap stage, and it presumably determines and regulates the formation of the tooth cusps. Secondary enamel knots are associated with the formation of additional cusps during bell stage. Interestingly, apoptosis was seen both in the primary enamel knot at cap stage (E14-15) and in the secondary enamel knots at bell stage (E18). Thus, apoptosis may be the mechanism whereby the enamel knot cells are removed after they have fulfilled their task in initiating cusp formation.

We have recently localized in the enamel knots the expression of several signal molecules which are also present in such well-known signaling centers as the node, the notochord, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) (MacKenzie et al., 1992; Jowett et al., 1993; Vainio et al., 1993; Vaahtokari et al., 1996).

The enamel knot resembles in many aspects the AER in the developing limb: both express *Fgf-4*, *Bmp-2* and *Msx-2* (Lyons et al., 1990; Niswander and Martin, 1992, 1993; Liu et al., 1994). FGF-4 upregulates proliferation in the mesenchyme adjacent to the AER and enamel knot (Niswander and Martin, 1993; Jernvall et al., 1994), and, as we have demonstrated, both epithelial structures undergo apoptosis. In earlier reports, apoptosis in AER has not been associated with the inductive properties of the AER but rather has been linked to the later morphogenesis and the separation of the digits (Lee et al., 1993; Coelho et al., 1993). We propose that apoptosis could be the mechanism whereby the signaling function of both AER and enamel knot is terminated. Apoptosis may be an even more general mechanism for the silencing of embryonic signaling and organizing centers.

It is also tempting to speculate that differences in the timing of apoptosis in these centers may influence the morphology of cusp and limb patterns. Hence, apoptosis may be an important mechanism causing heterochrony in vertebrate evolution.

Apoptosis in the disruption of dental lamina and secondary tooth bud

Disruption of the dental lamina results in loss of connection between tooth germ and oral epithelium during late bell stage. Based on studies on rat tooth development, it has been proposed that this event does not occur via apoptosis (Khaejornbut et al., 1991). However, at bell stage, we located apoptosis in the dental lamina and adjacent outer enamel epithelial cells. This suggests that apoptosis is involved in the removal of dental lamina. The proper disruption of dental lamina has clinical significance because if clusters of epithelial cells from the dental lamina persist, they may form cysts over the developing tooth and delay eruption (Ten Cate, 1994).

On the lingual aspect of the dental lamina, the transient epithelial bud is thought to represent a rudiment of the secondary tooth, which does not develop further in mice, although many other vertebrates have secondary dentitions (Khaejornbut et al., 1991; Kershaw, 1994). The bud is visible at bell stage, and it disappears with the dental lamina in postnatal mice. As we detected apoptosis in this bud, we suggest that apoptosis may be involved in the disruption of the secondary tooth germ.

Apoptosis in the reduction of dental epithelium during enamel formation

The epithelial cells of stratum intermedium and stellate reticulum become significantly reduced during advancing enamel formation. Our results demonstrate that a significant number of stellate reticulum cells, and a moderate number of stratum intermedium cells and ameloblasts undergo apoptosis. The apoptotic stratum intermedium cells and ameloblasts appeared to be very near each other, sometimes even immediate neighbors. Thus, their pattern of cell death differed from that in the other dental epithelial cells. Our observations on apoptosis in ameloblasts are in agreement with a previous study that suggested, based on cell turnover kinetics, that about 50% of ameloblasts may die during maturation (Smith and Warshawsky, 1977).

Apoptosis is prevented in cultured dental tissues by epithelial-mesenchymal interactions and by EGF and FGF-4

When we cultured either E13 dental mesenchyme or epithelium alone for one day, the tissue underwent apoptosis but when the tissues were cultured in combination, apoptosis was prevented in the cells near the interface of the tissues. This is not surprising because it is well known that growth and differentiation in embryonic organs are stimulated by epithelial-mesenchymal interactions, and that these interactions are necessary also for tooth development (Thesleff et al., 1995). Apparently, the isolated mesenchymal and epithelial tissues do not get the proper signals for survival when they are deprived of the other tissue component.

It has been proposed that cells are programmed to commit suicide and continuously require signals, such as peptide growth factors, from other cells in order to survive (Raff, 1992;

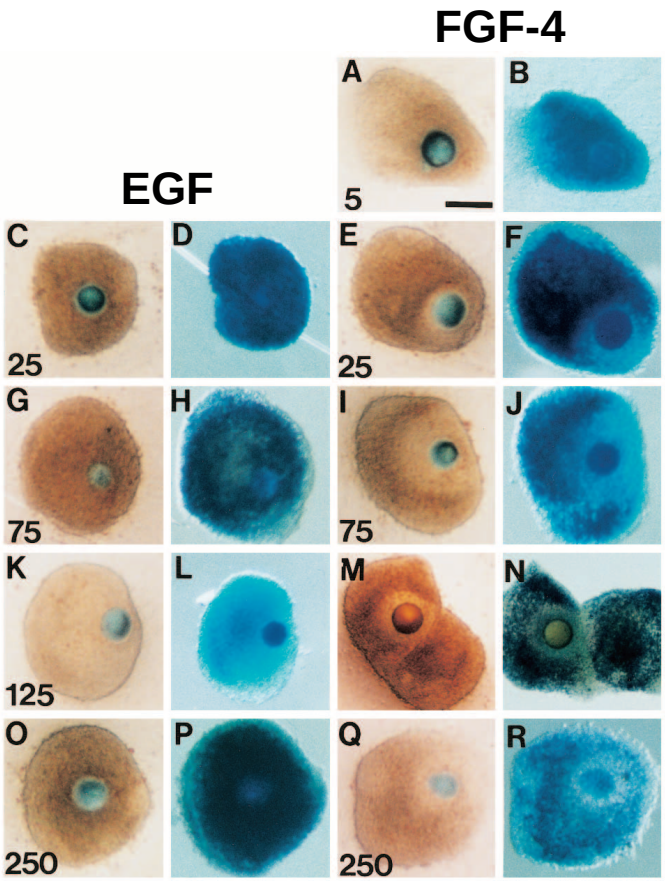

Fig. 6. Analysis of the effects of EGF and FGF-4 on apoptosis by whole-mount Nile Blue stainings. E13-E13.5 dental mesenchymes (dm) were cultured for 16-24 h with an EGF-releasing agarose bead (D,H,L,P) or with an FGF-4-releasing agarose (B,F,J,R) or heparin (N) bead. (C,G,K,O,A,D,I,Q,M) The same tissues before the stainings, respectively. The amount of EGF per 100 beads is (C,D) 25 ng, (G,H) 75 ng, (K,L) 125 ng, (O,P) 250 ng and the amount of FGF-4 per 100 beads is (A,B) 5 ng, (E,F) 25 ng, (I,J) 75 ng, (M,N) 125 ng, (Q,R) 250 ng. (C,G,O) 25, 75, or 250 ng EGF have not caused any apparent morphological changes, and (D,H,P) the whole explant has undergone apoptosis. With 125 ng EGF, (K) the whole mesenchyme is translucent and (L) apoptosis is suppressed throughout the explant. With 5 ng FGF-4, (A) no translucent zone is detected around the bead and (B) apoptosis is seen throughout the explant, also around the bead. (E,I,M,Q) 25-250 ng FGF-4 have induced a translucent area around the bead and (F,J,N,R) apoptosis is prevented around the bead in an area approximately corresponding to the translucent zone. Scale bar, (A) 180 μm, (B,C,D,E,G,I,K,L,O,Q) 200 μm, (F,H,J,M,N,P,R) 265 μm.

Raff et al., 1993; McConkey and Orrenius, 1994; Collins et al., 1994; Steller, 1995). For example, chick embryo limb mesoderm cells require FGF-2 for survival (MacCabe, 1993) and, in the developing rat kidney, EGF inhibits apoptosis (Koseki et al., 1992; Coles et al., 1993). We have now shown that in the cultured dental tissues, EGF and FGF-4 prevent apoptosis.

EGF was a very powerful inhibitor of apoptosis but it prevented apoptosis and induced a translucent zone around the beads only when the beads were soaked in 125 ng of EGF per 100 beads, whereas lower and higher amounts had no effects. In contrast, FGF-4 prevented apoptosis when beads were soaked in 25-250 ng of FGF-4. This indicates that there are differences in the effective doses of EGF and FGF-4 preventing apoptosis.

Our findings may explain the observations reported earlier about the effects of EGF on tooth morphogenesis in vitro (Partanen et al., 1985). When tooth germs are cultured in vitro, the dental follicle largely disappears. However, in the presence of EGF, the dental follicle is maintained (Partanen et al., 1985). The dental mesenchyme, in which EGF was shown to prevent apoptosis in our study, also includes the progenitors of dental follicle cells. In addition, we localized high amounts of apoptotic cells in the dental follicle cells in vivo. In conclusion, we propose that the effects of EGF in cultured dental tissues may have been at least partly due to prevention of apoptosis, and not only due to enhanced proliferation as suggested earlier (Partanen et al., 1985).

FGF-4-releasing beads rescued the surrounding cells from apoptosis in the dental mesenchyme suggesting that FGF-4 may act as a survival factor in this tissue. During tooth development, *Fgf-4* transcripts are first detected at E14 and they are only produced in epithelial enamel knot cells. Although FGF-4 stimulates proliferation and inhibits apoptosis in neighboring dental cells, the enamel knot cells do not divide (Jernvall et al., 1994). Instead, our data suggests that they are unresponsive to the effects of FGF-4 and undergo apoptosis even in its presence. We do not know if this is a result of the lack of proper FGF receptors in these cells, or whether these cells differ in some other way from their neighboring cells. The unresponsiveness of enamel knot cells to FGF-4 may be associated with acquisition of the apoptotic pathway. Apoptosis of these cells may constitute an important mechanism whereby the activity of the signaling center is terminated and which thereby determines the pattering of the tooth cusps.

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