Int. J. Dev. Biol. 44: 389-396 (2000)

Original Article

Inhibition of apoptosis in the primary enamel knot does not affect specific tooth crown morphogenesis in the mouse

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ABSTRACT The enamel knot (EK), located in the center of cap-stage tooth germs, is a transitory cluster of non-dividing epithelial cells, eventually linked to the outer dental epithelium by the enamel septum (ES). It might act as a signaling center providing positional information for tooth morphogenesis and could regulate the growth of tooth cusps through the induction of secondary signaling EKs. The EK undergoes apoptosis, which could constitute a mechanism whereby the signaling functions of this structure are terminated. Recently, we demonstrated the segregation of 5-bromo-2'-deoxyuridine (BrdU) negative inner dental epithelial (IDE) cells of the EK into as many individual groups of cells as cusps will form and suggested a morphogenetic role for these particular IDE cells. Using Z-VAD-fmk, a specific caspase inhibitor, apoptosis in the primary EK of first mouse lower cap-staged molars and lower incisors cultured *in vitro* was abrogated. No obvious histological alterations were observed in the incisors, whereas a prominent EK and an ES connecting the outer dental epithelium (ODE) and the BrdU negative IDE cells capping cusp L2 were observed in the molars. EK specific transcription (*Shh, Msx-2, Bmp-2, Bmp-4*) was down-regulated in the body of these structures with the exception of the associated IDE cells. In these experimental conditions, segregation of non-dividing transcriptionally active IDE cells occurred and a normal cusp pattern was expressed.

KEY WORDS: *apoptosis, caspase inhibitor, enamel knot, enamel septum, non-cycling IDE cells, Shh, Msx-2.*

Introduction

The enamel knot (EK) was first detected in developing cap-stage teeth by Arhens (1913) and represents a transitory cluster of cells located in the central part of the developing enamel organ. These eventually become connected with the outer dental epithelium via a string of cells, the enamel septum (ES). The ubiquitous existence of these structures has been disputed (Butler, 1956) and the septum has rarely been observed in mouse teeth. However, MacKenzie *et al.* (1992) demonstrated both the EK and ES in embryonic mouse molars by means of *in situ* hybridization for *Msx-2* transcripts and suggested a possible morphogenetic role for these structures. Thesleff and co-workers extended these observations and identified the EK as a putative signaling center transcribing particularly *Shh*, *Bmp-2* and *-4* and *Fgf-4* (Jernvall *et al.*, 1994, 1998). These authors suggested that the primary molar EK could induce the formation of secondary EKs located at the tips of forming cusps and endowed with similar signaling activities (Keranen *et al.*, 1998). These signaling centers might regulate cuspal growth.

The EKs are formed by non-dividing cells (BrdU negative) strongly expressing p21 transcripts (Bloch-Zupan *et al.*, 1998; Jernvall *et al.*, 1998), which could be regulated by mesenchymal BMP-4 (Jernvall *et al.*, 1998). The primary EK (and septum) of the molar is partially eliminated through apoptosis about 24 h after its appearance (Lesot *et al.*, 1996, 1999; Jernvall *et al.*, 1998). Apoptosis was also observed in the secondary EKs (Shigemura *et al.*, 1999).

Recently, we demonstrated the subdivision of the initial single group of BrdU negative molar inner dental epithelial (IDE) cells of the primary EK area, into as many distinct groups as cusps will form by means of sequential and continuous BrdU labeling (Coin *et al.*, 1999). Cellular continuity exists between non-dividing IDE cells of the primary and secondary EKs. In the incisor, where only

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

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0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

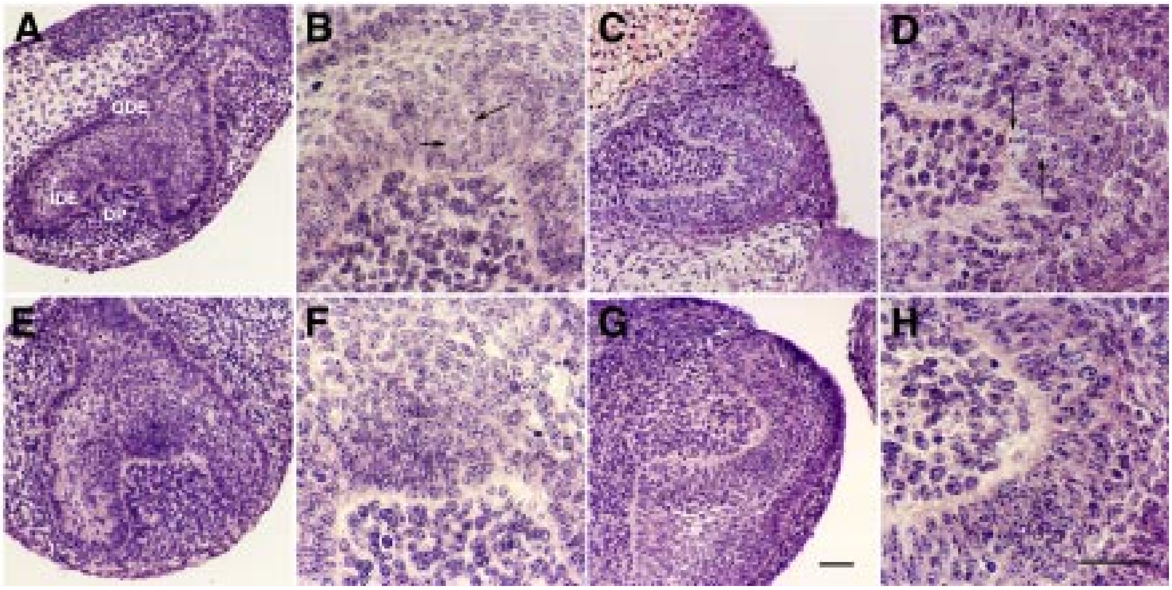

Fig. 1. Histological sections of E-13.5 first lower molars (A, B, E, F) and of E-12 lower incisors (C, D, G, H) cultured for 1 day *in vitro* in control medium (A, B, C, D) or in the presence of 200 μM of Z-VAD-fmk (E, F, G, H). *The control molar* **(A, B)** *and incisor* **(C, D)** *show apoptotic bodies (arrows) in the area of the enamel knot (EK). In the Z-VAD-fmk treated molar* **(E, F)** *and incisor* **(G, H)***, the EKs are devoid of apoptotic bodies. Bar, 100 μm.*

one cusp develops, such a segregation was not observed and the BrdU negative IDE cells were maintained at the tip of the tooth. We suggested that these BrdU negative IDE cells could act as tooth specific organizers of the morphogenetic units (OMU), the cusps. In such an intriguing context, we hypothesized that abrogation of apoptosis in the primary EK and ES, might affect normal molar and incisor, crown morphogenesis.

Inhibition of apoptosis was achieved through treatment *in vitro* of cultured mouse molars and incisors with the specific integral caspase inhibitor Z-VAD-fmk. In these conditions, the molars and the incisors developed a normal, tooth specific, cusp pattern which corresponded with the presence of distinct BrdU negative IDE cells located at the tip of each cusp. In the molars, the primary EK and ES were maintained forming a distinct voluminous histological structure. The non-dividing cells of this structure did not maintain EK specific transcription of *Shh*, *Msx-2*, *Bmp-2* or *Bmp-4,* which were down-regulated in the core of the EK-ES. Survival of the primary molar EK interfered neither with the segregation of BrdU negative IDE cells expressing *Msx-2* and *Shh* nor with normal cusp formation.

Results

Histological observations after 1 or 2 days in vitro

Fig. 1A, B and Fig. 1C, D illustrate the presence of apoptotic bodies in the enamel knot area of E-13.5 molars and E-12 incisors respectively, after culture for one day *in vitro* in control medium. In the molars, apoptosis was localized in the center of the knot (Fig. 1 A, B). In the incisors, the EK appeared to be much more discrete and apoptotic bodies were scarce. E-13.5 molars and E-12 incisors incubated for 2 days in the presence of 1% DMSO (the solvent for Z-VAD-fmk) did not reveal any histological differences to the corresponding controls (not shown).

Careful histological observation of serial sections of E-13.5 molars and of E-12 incisors cultured for one day *in vitro* in the presence of 200 μM of caspase inhibitor demonstrated the absence of apoptotic bodies in the area of the molar and incisor EKs (Fig. 1E, F and Fig. 1G, H respectively). In the molar, the epithelial cells of the EK were more crowded as compared to the controls. In contrast, the density of incisor EK cells was comparable to that of the corresponding controls. In addition, in the molar the outer dental epithelium showed an abnormal thickening near to the gubernaculum.

Histological observations after removal from Z-VAD-fmk

Teeth treated for 2 days with the caspase inhibitor were post-cultured for 1 to 8 days in control medium. In the molars, the EK was maintained. After 3 and 4 days of post-culture, this persistent structure appeared as a densely packed group of epithelial cells connecting the IDE and ODE. Apoptotic bodies were not observed and cusp formation was apparent (Fig. 2A, B, D, E). In the incisors, a much more discrete EK was maintained (Fig. 2G, H).

After longer post-culture of molars (8 days), the persistent EK-ES demonstrated involution with residual, discontinuous domains connected with the IDE and the ODE respectively (Fig. 2C, F). In both the molars and the incisors, terminal differentiation of odontoblasts and ameloblasts was initiated (Fig. 2C, F, I).

Three-dimensional reconstruction

Three E-13.5 first left lower molars cultured for 2 days in the presence of the caspase inhibitor and then post-cultured in control medium for 4 days were analyzed by means of 3D reconstruction and demonstrated normal cusp patterns. One specimen is represented in Fig. 3. Six cusps (L1, L2, L3, B1, B2, B3) separated by a transversal cleft were present. The anterior cusps L1, L2, B1, B2 expressed a typical trefoil like pattern. The two posterior cusps (L3, B3) were also well formed and orientated. The seventh posterior-median cusp (4) was still absent (Fig. 3A). The persistent EK-ES, represented in green (arrow), was located above the median L2 cusp (Fig. 3B).

BrdU labeling of the molars and incisors: distribution patterns of BrdU negative IDE cells

E-13.5 first lower mouse molars and E-12 lower incisors were cultured for 2 days in the presence of the caspase inhibitor, then in control medium for 0, 1, 2, 3 or 4 days and finally labeled for 8

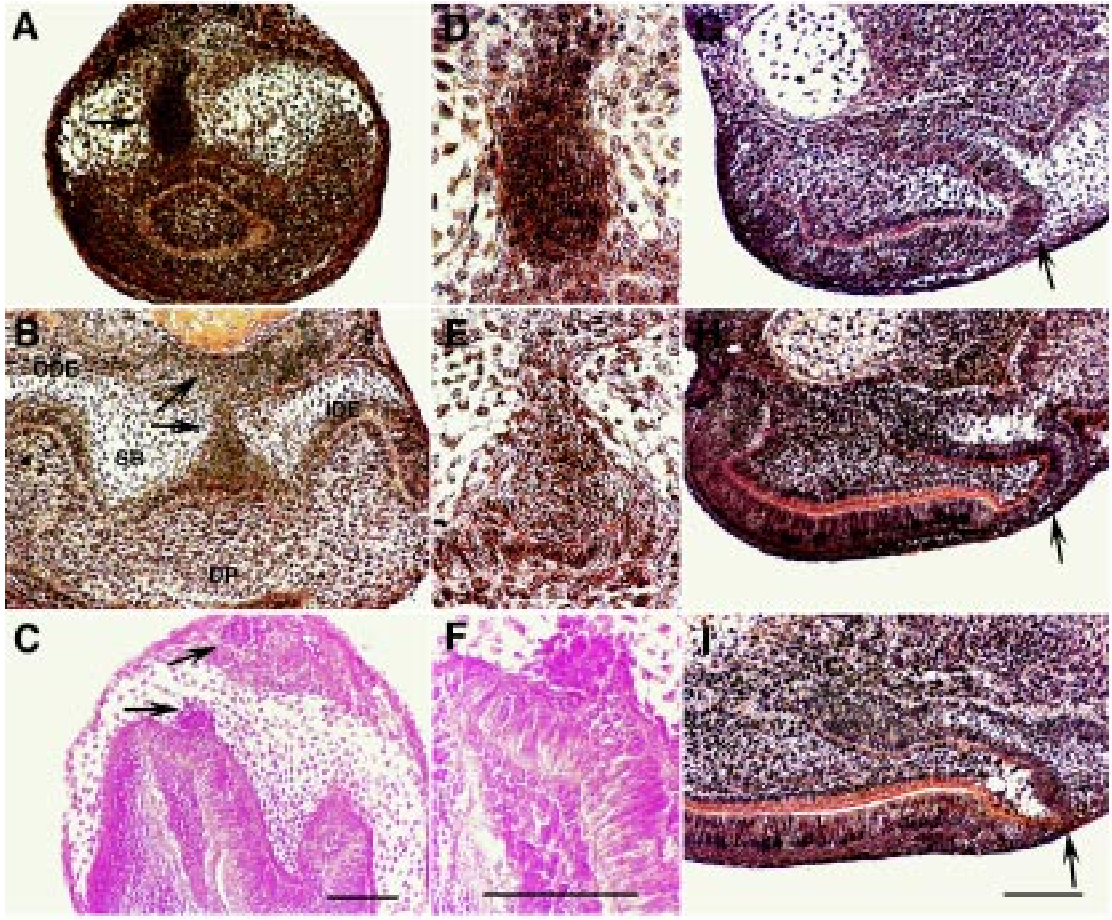

Fig. 2. Histological sections of E-13.5 first lower molars (A-F) and of E-12 lower incisors (G-I) cultured *in vitro* for 2 days in the presence of 200 μM of Z-VAD-fmk and post-cultured in control medium for 3 (A, D, G), 4 (B, E, H) or 8 (C, F, I) days. (A, B) *After 3 or 4 days in control medium, the molars show well formed cusps and persitence of the EK and ES (arrows).* **(D, E)** *Higher magnification of the densly packed EK-ES connecting the IDE and ODE.* **(C, F)** *After 8 days of post-culture in control medium, residues of the EK-ES are still present. Terminal differentiation of odontoblasts and polarization of ameloblasts is initiated.* **(G)** *Afer 3 days of post-culture, an EK-like structure is maintained in the incisor. After 4 days* **(H),** *or 8 days* **(I)** *of post-culture, the gradual terminal differentiation of odontoblasts and progressive polarization of ameloblasts is obvious. Bar, 100 μm.*

hrs with BrdU. The distribution pattern of BrdU negative IDE cells was analyzed in serial sections and for the molars, 3D reconstructions were performed. Immediately after the 2 days of caspase inhibition (Fig. 4A), as well as after 1, 2, 3 or 4 days (Fig. 4B-E) of post-culture in control medium, the 8 h BrdU labeling of the molars indicated that the persistent EK-ES were always formed by non-cycling cells, including the corresponding IDE cells.

For the incisors, the less well defined EK, mainly composed of IDE cells, was always BrdU negative. The negative area progressively extended and corresponded to the physiological withdrawal of labial ameloblasts and lingual IDE cells from the cell cycle (Fig. 4F-O). The distribution pattern of the BrdU negative and positive IDE cells of the molars was analyzed more precisely by means of computer assisted 3D reconstructions from serial histological sections (Fig. 5). Figures 5A and 5E allow comparison of the distribution pattern of BrdU negative IDE cells of an E-13.5 molar cultured for 6 days in control medium (Fig. 5A) with a corresponding E-13.5 molar treated for 2 days with the caspase inhibitor and post-cultured in control medium for 4 days (Fig. 5E). For the caspase inhibitor treated molar, the persistent EK-ES corresponded to cusp L2 (Fig. 5F). Figure 5G visualizes the relationship of the persistent EK-ES and BrdU negative IDE cells. Figure 5B, C, D show the distribution pattern of BrdU negative cells in a molar after 3 days of post-culture. Three negative areas of the IDE probably corresponded to cusps L2, B2 and L3. The persistent EK-ES corresponded to cusp L2 (Fig. 5C, D).

Gene expression in the persistent EK-ES

Using *in situ* hybridization, transcription of *Shh*, *Msx-2*, *Bmp-2* and *Bmp-4* which are expressed in the EK, was investigated. In the absence of Z-VAD-fmk, the EK cells of E-13.5 molars demonstrated strong expression of *Shh*, *Msx-2*, *Bmp-2* and *Bmp-4* as

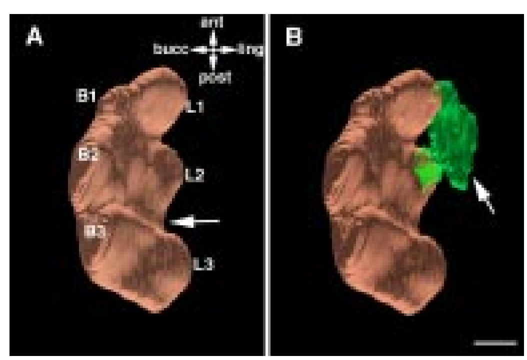

Fig. 3. Computer assisted 3-D reconstructions of an E-13.5 first lower molar cultured *in vitro* for 2 days in the presence of 200 μM of Z-VAD-fmk and post-cultured in control medium for 3 days. (A) *The dental papilla demonstrates the presence of 6 cusps [(B1, B2, B3 and L1, L2, L3) according to the nomenclature of Gaunt (1955, 1961)].* A transversal cleft demarcates the two posterior cusps (arrow). **(B)** *The persistent EK-ES (in green), arrow, is capping cusp L2. Bar, 100 μm.*

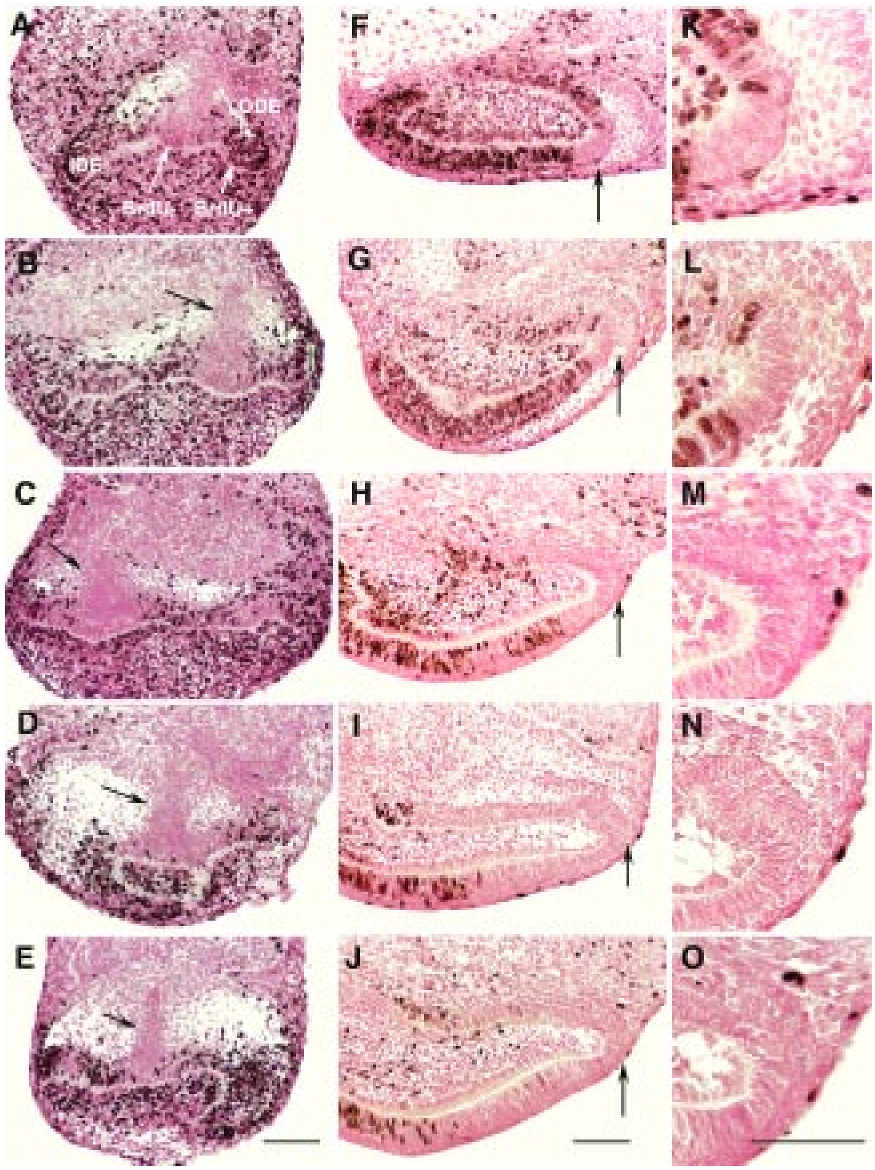

Fig. 4. Histological sections of E-13.5 first lower molars (A-E) and of E-12 lower incisors (F-O) cultured *in vitro* for 2 days in the presence of 200 μM of Z-VAD-fmk and post-cultured in control medium for 0 (A, F, K), 1 (B, G, L), 2 (C, H, M), 3 (D, I, N) or 4 (E, J, O) days. All the teeth were labeled with BrdU for 8 hrs at the end of the culture. *Distinct 5-bromo-2'-deoxyuridine negative (BrdU-) and 5-bromo-2'-deoxyuridine positive (BrdU+) areas are observed in the inner dental epithelium (IDE) of each tooth.* ***(A)*** *After 2 days of culture in the presence of the caspase inhibitor, the EK-ES (BrdU negative) are maintained (white arrows). These BrdU negative structures persist after 1* ***(B)****, 2,* ***(C)****, 3* ***(D)*** *or 4* ***(E)*** *days of post-culture (black arrows). For the incisors, a BrdU negative area of IDE cells exists at the tip of the tooth immediatly after the 2 days of Z-VAD-fmk treatment and corresponds to a rather indistinct EK* ***(F, K)****. During lengthened post-culture* ***(G, L, H, M, I, N, J, O)****, the BrdU negative area of the IDE progressively extends in an anterior-posterior direction as a function of progressive withdrawal of differentiating labial ameloblasts and lingual IDE cells from the cell cycle. Bar, 100 μm.*

previously demonstrated (Keranen *et al.*, 1998 and references therein) (Not shown). Two and four days after removal from Z-VAD-fmk, transcription for the four genes was down-regulated in the body of the persistent EK-ES (Fig. 6). This down-regulation appeared to be somewhat delayed for *Msx-2* and *Shh* (Fig. 6A, B, G, H). In contrast, the EK associated IDE cells capping the cusp L2 expressed *Msx-2* (Fig. 6A-D) and *Shh* (Fig. 6E-J) as well as specific IDE cells located at the tips of other developing cusps (Fig. 6K, L).

Discussion

Programmed cell death (apoptosis) occurs during the development of all animals studied (Jacobson, 1997; Jacobson *et al.*, 1997). There are two central pathways that lead to apoptosis: 1) positive induction by ligand binding to a plasma membrane receptor and 2) negative induction by loss of a suppressor activity involving the mitochondria. Each leads to activation of cysteine proteases showing homology to interleukin-1β converting enzyme (ICE) (Thornberry and Lazebnik, 1998). Specific caspase inhibitors have been shown to inhibit the induction of apoptosis in various tumor cell lines (Schlegel *et al.*, 1996; Martins *et al.*, 1997; Huang *et al.*, 1999; Guo and Kyprianou, 1999; Rincheval *et al.*, 1999; Utaisincharoen *et al.*, 1999) as well as in normal cells (Jacobson *et al.*, 1997; Gastman *et al.*, 1999; Zaks *et al.*, 1999). In particular, the general caspase inhibitor Z-VAD-fmk (Jorquera and Tanguay, 1999) prevents G2/M peak decline as well as the appearance of a sub-G1, apoptotic population showing typical nucleosomal-sized DNA fragmentation and a reduced mitochondrial transmembrane potential (Talanian *et al.*, 1997; Ekert *et al.*, 1999; Stennicke and Salvesen, 1999). Abrogation of programmed cell death *in vivo* adversely affects the organism and generally has devastating consequences on development (White *et al.*, 1994; Steller, 1995; Thompson, 1995; Zheng *et al.*, 1999a,b; Borner and Monney, 1999; Wang and Lenardo, 2000).

During mouse tooth development, bud-cap stage mesenchyme controls formation of the primary EK, a cluster of non-dividing epithelial cells located in the center of the cap-stage tooth germ. The EK expresses *Fgf-4* (Jernvall *et al.*, 1994), *Bmp-2*, *-4*, *-7*, and *Shh* (Vaahtokari *et al.*, 1996b) as well as *Fgf-9* (Kettunen and Thesleff 1998) and has been suggested to act as a signaling or organizing center providing positional information for tooth morphogenesis and regulating the growth of tooth cusps (Jernvall *et al.*, 1994). According to these authors, the mechanism of the emergence of the secondary EKs also expressing signaling molecules could be a consequence of planar genetic signals coming from the primary enamel knot. However, we demonstrated (Coin *et al.*, 1999) cellular continuity between the primary and secondary EKs. About 24 hours after its appearance, most of the molar's EK cells undergo apoptosis (Vaahtokari *et al.*, 1996a; Peterkova *et al.*, 1997; Lesot *et al.*, 1996, 1998, 1999) except for the associated non-dividing IDE cells which segregate according to a cusp specific pattern in the molars (Coin *et al.*, 1999).

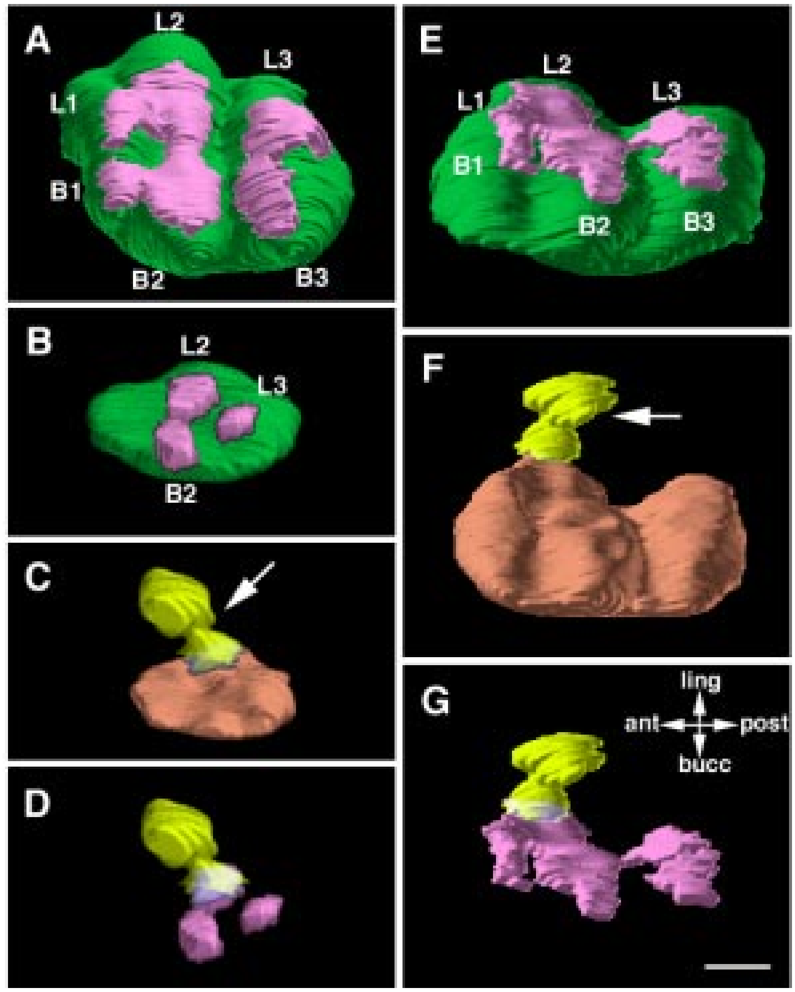

Fig. 5. Computer assisted 3-D reconstruction of an E-13.5 first lower molar cultured for 6 days in control medium (A) and E-13.5 first lower molars cultured *in vitro* for 2 days in the presence of 200 μM of Z-VAD-fmk and post-cultured in control medium for 3 (B, C, D) and 4 days (E, F, G). The teeth were labeled with BrdU for 8 hrs at the end of the culture. *The 5-bromo-2'-deoxyuridine negative (BrdU-) groups of inner dental epithelial cells are represented in pink. The 5-bromo-2'-deoxyuridine positive (BrdU+) domains are stained in green. The distribution pattern of the BrdU negative IDE cells is comparable in the control* **(A)** *and in the corresponding Z-VAD-fmk treated molar* **(E)***: the negative areas correspond to the 6 forming cusps. The persistent EK-ES (also BrdU negative) of the molar of Fig. 5E capped the BrdU negative area of the IDE of the cusp L2* **(F, G)**. **(B)** *After 3 days of post-culture, 3 BrdU negative domains of the IDE correspond to 3 developing cusps (L2, B2, and probably L3).* **(C, D)** *Again, the BrdU negative persistent EK-ES (arrow) capped the BrdU negative area of the IDE corresponding to cusp L2. Bar, 100 μm.*

The possible consequences of apoptosis inhibition during mouse first lower molar and incisor development have been investigated *in vitro* using the general caspase (Cytosolic Aspartate-specific Proteases) inhibitor: Z-VAD-fmk. For the incisors, caspase inhibition had no significant histological effects. A prominent, persistent EK-ES was not observed in the incisors. This correlates well with the physiologically very discrete primary EK demonstrating very little apoptosis (Kieffer *et al.*, 1999). As for the controls, normal crown histo-morphogenesis occurred and BrdU negative IDE cells, initially present at the level of the EK were maintained at the tip of the developing incisors. Normal cytodifferentiation of the odontoblasts and labial ameloblasts were observed in both cases.

After 1 and 2 days of caspase inhibition, the molars demonstrated abrogation of apoptosis and the consequential persistence of a BrdU negative EK and ES. One to four days after removal from Z-VAD-fmk, these structures were always maintained. Partial involution was observed eight days after removal. In the meantime, the BrdU negative IDE cells, initially associated with the EK, partially segregated into distinct aggregates corresponding to the forming cusps whose pattern was normal.

Our *in situ* hybridization data demonstrated a down-regulation of the transcription of EK specific genes, including *Msx-2*, *Shh*, *Bmp-2* and *Bmp-4* in the body of the persistent EK-ES. The signaling activity of the EK appears to be subject to precise temporal control and the physiologically programmed cell death might well correspond to the elimination of cells whose signaling activity has been achieved. One consequence of this signaling could be the segregation of BrdU negative IDE cells, the putative organizers of the cusps (Coin *et al.*, 1999). Interestingly, the IDE cells underlying the persistent EK-ES, and capping the developing cusp L2 maintained transcription of *Shh* and *Msx-2.* The primary EK appears to be located at the tip of the presumptive cusp L2 and during development of this cusp, some of the IDE cells associated with the EK kept their initial localization. Cellular continuity between the primary and L2 specific secondary EK existed. The other developing cusps were capped by non-dividing, transcriptionally active IDE cells also originating from the area of the primary EK (Coin *et al.*, 1999). The present data provide preliminary molecular support to our working hypothesis implying a morphogenetic role for specific IDE cells, the OMU. To understand the molecular and cellular mechanisms involved in cuspidogenesis, further investigations are necessary. The signaling activity of specific IDE cells, the OMU, most probably regulates local cell proliferation, cell adhesion and cell migration.

Since the transitory signaling activities documented by Vaahtokari *et al.*, (1996b) and Keranen *et al.*, (1998) mainly involve the EKs to the detriment of the ES, it appears questionable whether all the cells of the persistent structure (EK and ES) have the same developmental

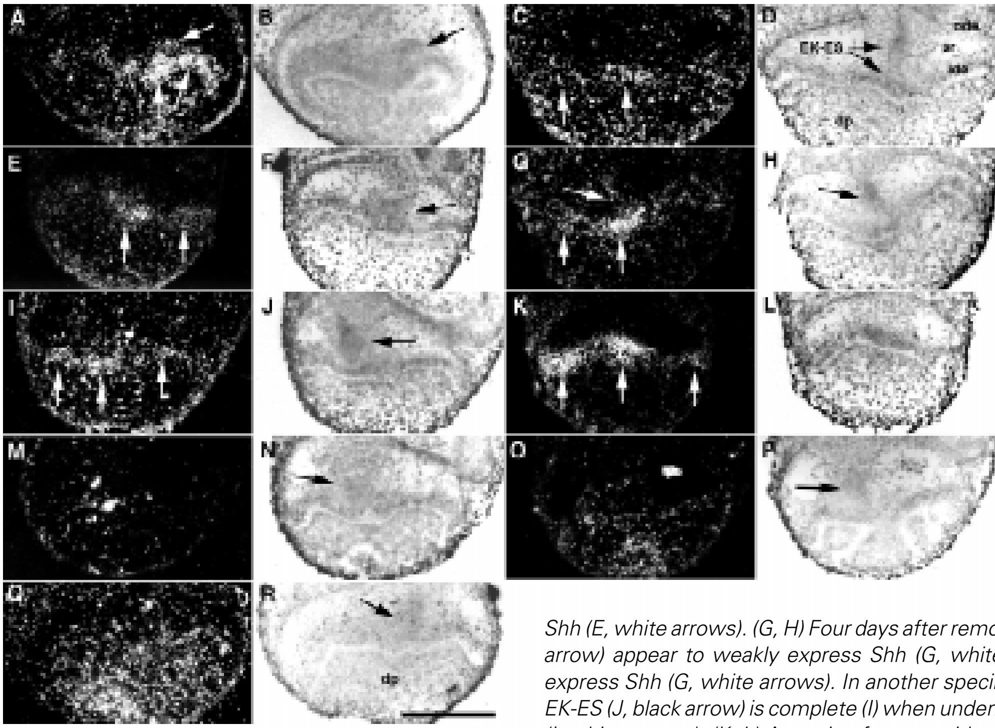

Fig. 6. *Msx-2, Shh, Bmp-2* and *Bmp-4* expression in the persistent enamel knot-enamel septum (EK-ES) and inner dental epithelial cells (IDE) 2 and 4 days after removal from Z-VAD-fmk. Dark and bright field illustrations of the same sections of E-13.5 molars cultured *in vitro*. (A-D) *Msx-2 expression after 2 (A, B) and 4 (C, D) days of removal. After 2 days, some transcripts (A, white arrow) are still present in the persistent EK (B, black arrow). The underlying IDE cells strongly express Msx-2 (A, white arrows). After 4 days, down-regulation of Msx-2 in the persistent EK-ES (C, D) is complete. Underlying IDE cells as well as IDE cells, capping a developing cusp, express Msx-2 (C, white arrows).* **(E-L)** *Shh expression 2 (E, F) and 4 (G, H; I, J; K, L) days after removal. The persistent EK (F, black arrow) is negative (E) while underlying IDE cells as well as IDE cells capping a forming cusp express Shh (E, white arrows). (G, H) Four days after removal, some cells of the persistent ES (H, black arrow) appear to weakly express Shh (G, white arrow). The underlying IDE cells strongly express Shh (G, white arrows). In another specimen (I, J), down-regulation in the persistent EK-ES (J, black arrow) is complete (I) when underlying and cusp capping IDE cells express Shh (I, white arrows). (K, L) A section from outside the persistent EK-ES (L), demonstrating Shh expression in IDE cells capping developing cusps (K, white arrows).* **(M-P)** *Bmp-2 expression 2 (M, N) and 4 (O, P) days after removal. Complete down-regulation of Bmp-2 expression in the persistent EK-ES (N, P, black arrows) and the IDE is obvious (M, O).* **(Q, R)** *Bmp-4 expression 4 days after removal is restricted to the dental papilla (dp). The persistent EK-ES (R, black arrow) as well as the IDE are negative. sr, stellate reticulum. Bar, 200 μm.*

significance. The septum could correspond to a phylogenetic vestige. Comparative anatomic and molecular investigations are necessary to elucidate this point and to understand the real significance of this structure.

Materials and Methods

Tissues

Mouse embryos were obtained by mating laboratory inbred ICR mice. The morning of the appearance of the vaginal plug was designed as day 0 of embryonic development. First lower molars were isolated on day 13,5 of gestation (E-13.5) and cultured *in vitro*. E-12 lower incisor pairs (left and right incisors) were also dissected and cultured.

Organ culture

E-13.5 molars and E-12 pairs of incisors were cultured in 2 ml of semi-solid medium per Petri dish (Nunc, Roskilde, Denmark; 35x10 mm) for 1, 2, 3, 4, 5, 6 and 8 days. The medium consisted of BGJ-B (Gibco, Fitton Jakson modified) supplemented with ascorbic acid (0.18 μg/ml, Merck), L-Glutamine (2 mM, Seromed), fetal calf serum (20%, Boehringer Bioproducts), kanamycin (0.1 μg/ml, Gibco) and Difco agar (0.5%). The teeth were incubated and grown at 37°C in a humidified atmosphere of 5% CO_2 in air and the medium was changed every two days. For each experimental condition, about 10 samples were used.

Apoptosis inhibition

Apoptosis was inhibited using the specific caspase inhibitor (Z-VAD-fmk, R&D Systems Europe Ltd., Abingdon, United Kingdom) at a concentration of 200 μM in DMSO. The inhibitor reagent was present in the culture medium from the beginning of the culture for 2 days, then the teeth were transferred into control culture medium. A solvent control (1% DMSO) was used to monitor any DMSO-related effects.

Bromodeoxyuridine labeling

Cell proliferation was investigated by mapping the distribution of S-phase cells after incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, cell proliferation kit; Amersham Life Science). E-13.5 first lower molars and E-12 lower incisors were cultured in the presence of 0.4 ml of BrdU at a concentration of 3 μg/ml for 8 h at the end of the culture period.

Histology- immunohistochemistry

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

3D reconstructions

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

In situ *hybridization*

Serial sections (5 µm) were collected on slides treated with 2% amino-3-propyltriethoxysilane (Prolabo). The 2.6 kb mouse *Shh* ^{35}S-labeled antisense riboprobe was produced from the cDNA cloned in pBluescript II SK (a gift from Dr A. MacMahon, Cambridge, Massachussets), linearized with *Eco* RI, and transcribed with T7 RNA polymerase. The 240 bp Msx2 probe was made from an *Alu* I / *Eco* RI fragment cloned into pTZ18 (a gift from Dr. B. Robert, Institut Pasteur, Paris), linearized with *Bam* HI and transcribed with T7 RNA polymerase. The 1kb mouse Bmp4 probe was prepared from the cDNA cloned in pTZ18 (a gift from Dr B. Hogan, Nashville), linearized with *Eco* RI and transcribed with Sp6 RNA polymerase. Bmp2 antisense RNA probe was made from an 1.2 kb *Eco* RI fragment cloned into pGEM3 (a gift from Dr B. Hogan, Nashville), linearized with *Eag* I and transcribed with Sp6 RNA polymerase. *In situ* hybridization was performed as described by Niederreither and Dollé (1997).

Acknowledgements

We wish to thank Dr A. J. Smith for critical comments on this manuscript and Mr. A. Ackermann for technical help. We are grateful to Dr F. Perrin-Schmitt for her in situ hybridization assistance. This research was partially financed by the International Human frontier Science Program (grant TG-558/95 M). R. Coin was financed by the Faculty of Odontology-Strasbourg.

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Received: March 2000
Accepted for publication: April 2000