

Apoptosis is involved in the disappearance of the diastemal dental primordia in mouse embryo

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ABSTRACT Three transient dental primordia (D1, D2 and D3) exist in the upper diastema in mouse embryos and their regression is associated with the presence of cell death. In order to specify the type of cell death and its temporo-spatial distribution, staining with hematoxylin, supravital staining with Nile Blue, TUNEL method, electron microscopic analysis and computer assisted 3-D reconstructions were performed. These data demonstrated that apoptosis is involved in the disappearance of the diastemal dental rudiments. Apoptosis occurred first with prevalence in the buccal part of the epithelium of the diastemal dental primordia and extended later to the whole epithelium of the dental rudiments and the dental lamina interconnecting them with the incisor and molar epithelia. Cell death occurred only sporadically in the adjacent mesenchyme. The prospective upper diastema in mouse embryos may provide a model for studies of developmental determination of toothless areas in the jaw as well as a tool for analyses of regulatory mechanisms of programmed cell death in morphogenesis.

KEY WORDS: *apoptosis, diastema, mouse, odontogenesis, three dimensional*

Introduction

Cell death occurring during normal morphogenesis has been recognized for decades (Glücksman, 1965; Saunders, 1966). Two types of cell death have been distinguished, necrosis and apoptosis (Wyllie, 1981), whose morphological and biochemical characteristics are different (Koseki *et al.*, 1992; Buja *et al.*, 1993; Martin, 1993; Schwartz and Osborne, 1993).

In necrosis, early changes in mitochondrial shape and function occur and the plasma membrane loses its integrity. As a result, dysregulation of osmotic pressure leads to cellular swelling and rupture. DNA degradation producing a smear pattern is a later event resulting from the activity of proteases and DNases. Disintegrated necrotic cells can induce an inflammatory response in adjacent tissue (Wyllie, 1981; Martin, 1993; Ueda and Shah, 1994).

The apoptotic process is characterized by cell shrinkage, loss of intercellular junctions and microvilli when present as well as early changes in the nucleus, which do not exist during necrosis. Chromatin becomes dense and lines the nuclear membrane. Later, the nucleus and cytoplasm with partly destroyed mitochondria and intact ribosomes are segmented to give rise to the membrane-bound apoptotic bodies phagocytosed by living cells in their vicinity. The presence and removal of apoptotic cells does not induce an inflammatory response (Wyllie, 1981;

Gerschenson and Rotello, 1992; Lockshin and Zakeri, 1992; Cohen, 1993; Kerr *et al.*, 1995).

Cell death plays an important role in morphogenesis of various organs. Cell death (specified now as apoptosis) is involved for example in neural and thymic development (Clarke, 1990; Raff, 1992), formation of digits (Schweichel and Merker, 1973; Milaire, 1992), kidney organogenesis (Koseki *et al.*, 1992), heart development (Krstic and Pexieder, 1973) and secondary palate formation (Mori *et al.*, 1994) in vertebrates. Cell death has also been reported during development of functional teeth (Nozue, 1971; Moe and Jessen, 1972; Kindaichi, 1980).

In mouse embryos, a dental lamina and later three distinct transient epithelial primordia originate in the prospective upper diastema region between the incisor and molar anlagen. The diastemal rudiments reach maximally the bud stage before the regress, which is associated with cell degeneration (Peterková, 1983; Peterková *et al.*, 1993, 1995). Phylogenetic and molecular arguments may support a dental nature for the rudiments (Peterková *et al.*, 1993, 1995; Turečková *et al.*, 1995). In the mandible, only an inconspicuous epithelial thickening (without

Abbreviations used in this paper: 3-D, three dimensional; E, embryonic day; TdT, terminal deoxynucleotidyl transferase; DAB, diaminobenzidine; PBS, phosphate buffer saline; TUNEL, TdT-mediated dUTP-biotin nick and labeling; wt.c., weight class.

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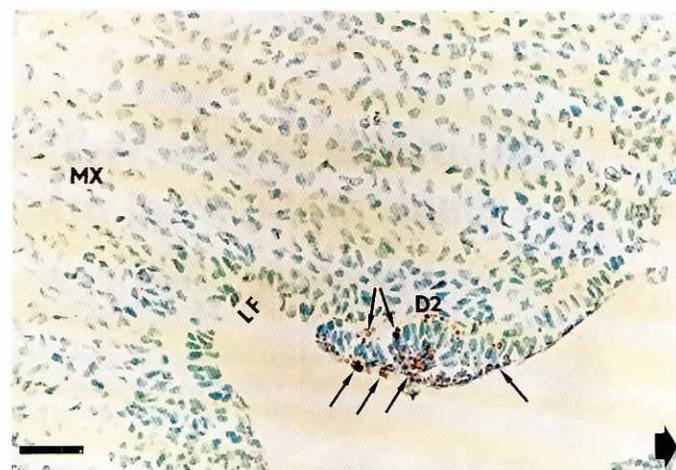
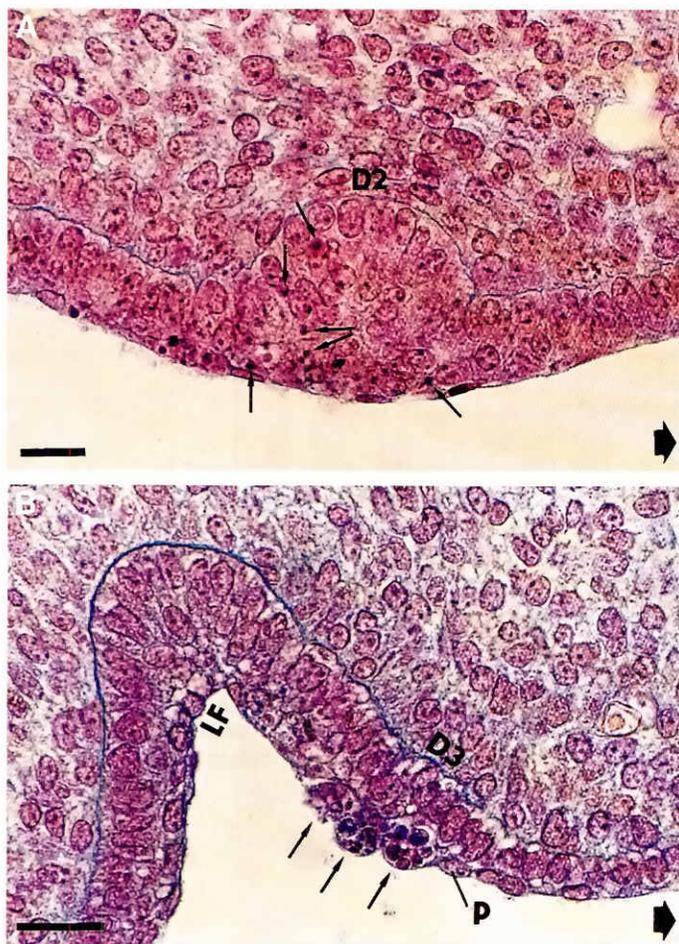


Fig. 1. The diastemal dental anlage in frontal paraffin section of head of E13.5 mouse embryo, wt.c. 126-150 mg (A,C) and wt.c. 201-225 mg (B) stained with alcian blue-hematoxylin-eosin (A,B) and TUNEL method (C). Narrow arrows point to the apoptotic cells or bodies (A), phagocytes (B) or the marked DNA fragments (C). Large arrow points medially. MX, maxilla; D2, the second diastemal dental rudiment; D3, a remnant of the third diastemal dental rudiment; P, periderm cell layer; LF, lip furrow. Scale bar, 20 μ m (A,B), 50 μ m (C).

further differentiation and signs of cell death) extends mesially from the molar anlage (Peterková *et al.*, 1995).

In the present study, the type of cell death was identified and its temporo-spatial distribution analyzed during regression of the diastemal primordia in the mouse embryonic upper jaw.

Results

Histology

On histological sections, various morphological signs of cell degeneration (Fig. 1A) started to be apparent in the diastemal dental epithelium from embryonic day (E) 12.5, weight class (wt.c.) 101-125 mg. The affected cells differed from the viable cells in their smaller size, shrunken appearance, more eosinophilic cytoplasm and smaller and dark nucleus containing condensed chromatin. In the area of cell death, a large number of condensed bodies of various size (always smaller than viable cells) was present. These contained one or more dark particles in a small amount of cytoplasm. Some bodies seemed to be comprised solely of a dark condensed substance. Occasionally, a translucent area was apparent around them. Large cells crowded with the bodies were found at the peridermal cell layer, but were not apparent among the deeper epithelial cells. Their frequency increased as the regression proceeded leading to reduction of the diastemal dental epithelium (Fig. 1B).

During the early phase of regression of the diastemal dental rudiments, cell degeneration was present predominantly in the buccal part of the dental bud and affected especially the central and superficial epithelial cells (Fig. 1A). Finally, the area of cell death extended to the whole diastemal dental epithelium.

TUNEL method

The labeled cells and bodies (Fig. 1C) were similar in their shape and localization to the affected cells and condensed bodies observed in histological sections (Fig. 1A).

Dying cells were detected only sporadically in the mesenchyme adjacent to the diastemal dental epithelium in both histological sections and sections labeled by the TUNEL method.

Electron microscopy

Various stages of degeneration of the epithelial cells were observed (Fig. 2): the chromatin of affected cells rapidly formed dense aggregates lining the nuclear membrane. Complex invaginations developed in the nuclear membrane resulting in shrunken and lobed nuclei (Fig. 2A) and later, in nuclear segmentation. Mitochondria were irregular in shape and sometimes swollen with their cristae and membranes partly destroyed. Cisternae of the endoplasmic reticulum were dilated. Free ribosomes and polysomes were visible until the late stages of apoptosis. The segmented nucleus and cytoplasm of

the dying cell formed membrane-bound apoptotic bodies (Wyllie, 1981). Desmosomes were visible until organelles were disrupted and the cytoplasm began to be more dense. The apoptotic bodies contained either a dense homogeneous area and/or a heterogeneous area of moderate density surrounded by a limiting membrane. The apoptotic bodies were more often found engulfed in the adjacent living epithelial cells than free in the intercellular spaces. Cytoplasmic extensions from neighboring cells were found to outgrow the free apoptotic bodies (Fig. 2B).

On the basis of the analysis of paraffin and ultrathin sections, the affected cells and condensed bodies apparent in histological sections could be identified as apoptotic cells and apoptotic bodies, respectively.

3-D reconstructions

In the diastemal epithelium, numerous apoptotic cells/bodies were present predominantly buccally inside the areas corresponding to the D2 and D3 primordia at E12.5 and E13.0. In non-dental oral epithelium, the apoptotic cells/bodies occurred only sporadically (Fig. 3B,C,F,G).

At E13.5, the diastema region elongated as well as the distance between the D2 and D3 rudiments themselves (Fig. 3I,M). In the developmentally least advanced specimens, the most mesial D1 rudiment was distinct and the buccal prevalence of cell death was apparent in it. Distally, a continuous zone of apoptosis extended to the whole diastemal dental epithelium (Fig. 3J,K). Later, the diastemal dental lamina disrupted between the D2 and D3 primordia (Fig. 3M). The dying cells could still be detected buccally in the D1 primordium. Distally, a continuous zone of degenerating cells extended through the regressing D2 and D3 rudiments. At this stage, dying cells were present also inside the epithelium of the rugae palatinae and lip furrow (Fig. 3N,O).

Nile blue staining

Supravital staining with Nile blue (Fig. 3D,H,L,P) confirmed the pattern of degenerating cells visualized after 3-D reconstructions at each of the observed embryonic stages.

Discussion

The complementary approaches we applied allowed us to identify the type of cell death involved during extinction of the transient anlage of the dentition in the prospective upper diastema in mouse and monitor the temporo-spatial distribution of dying cells.

The histological and electron microscopic data were in agreement with the morphological changes characteristic of apoptotic cell death (Wyllie, 1981; Gerschenson and Rotello, 1992; Koseki *et al.*, 1992; Lockshin and Zakeri, 1992; Cohen, 1993; Kerr *et al.*, 1995). Our electron microscopic data suggested that the apoptotic bodies may be engulfed by the epithelial cells in their vicinity (Moe and Jessen, 1972; Wyllie, 1981; Kerr *et al.*, 1995). Occasionally, large phagocytes crowded with apoptotic bodies (Fig. 1B) seemed to be extruded from the surface of the affected epithelium into the oral cavity. Phagocytosis has been reported also in the peridermal cells during the process of programmed cell death (Hinrichsen *et al.*, 1984). With regard to the

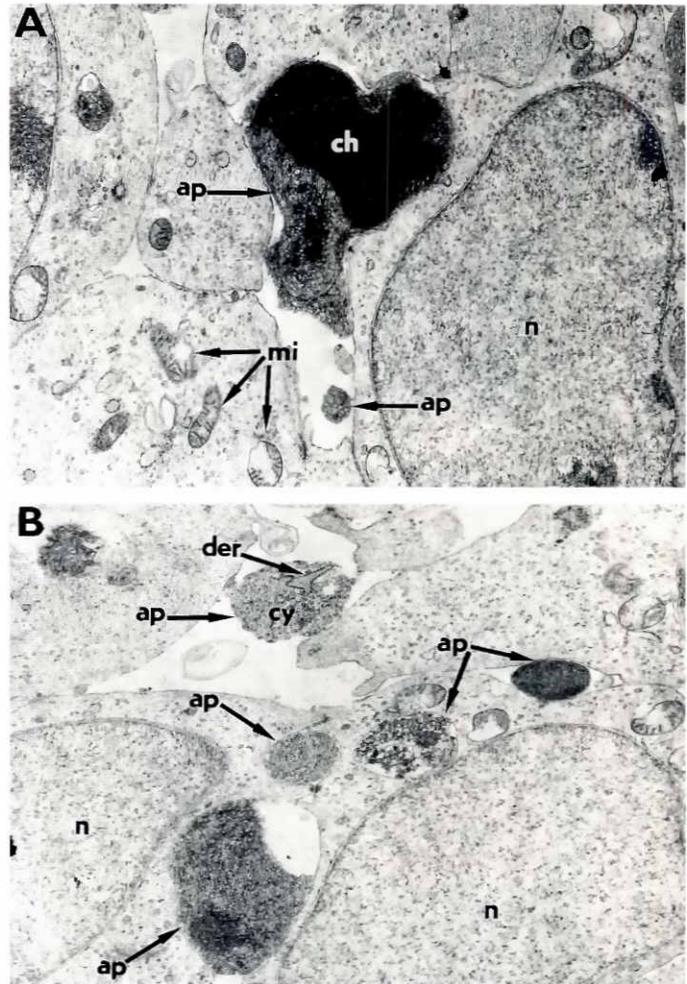


Fig. 2. Electron microscopic views (A,B) of the epithelial cells of D3 primordium at E13.5 (wt.c. 126-150 mg). *ap*, apoptotic body; *ch*, condensed chromatin; *cy*, condensed cytoplasm; *der*, dilated endoplasmic reticulum; *mi*, partly destroyed mitochondria; *n*, nucleus of living cell. Magnification $\times 5650$.

localization of the phagocytes (Fig. 1B), their peridermal origin cannot be excluded. Determination of the origin of these cells, however, would need a more detailed study.

It has been suggested that the internucleosomal fragmentation of DNA by an endogenous endonuclease should not be considered as a hallmark of apoptosis (Gerschenson and Rotello, 1992; Ueda and Shah, 1994). However, Oberhammer *et al.* (1993) have reported that in several epithelial cell types undergoing apoptosis, DNA is always cleaved to large 50kbp or 300kbp fragments, independently of the subsequent "laddering" of DNA. The TUNEL method has proved to be reliable for specific *in situ* detection of fragmented DNA in nuclei of various cell types during apoptosis (Gavrieli *et al.*, 1992; Mori *et al.*, 1994). Our positive results for the TUNEL method confirmed the histological and electron microscopic findings and allowed classification of the cell death in the diastemal dental epithelium as apoptosis including the DNA fragmentation.

The Nile blue supravital staining of the upper jaw, including both epithelium and mesenchyme, showed a temporo-spatial

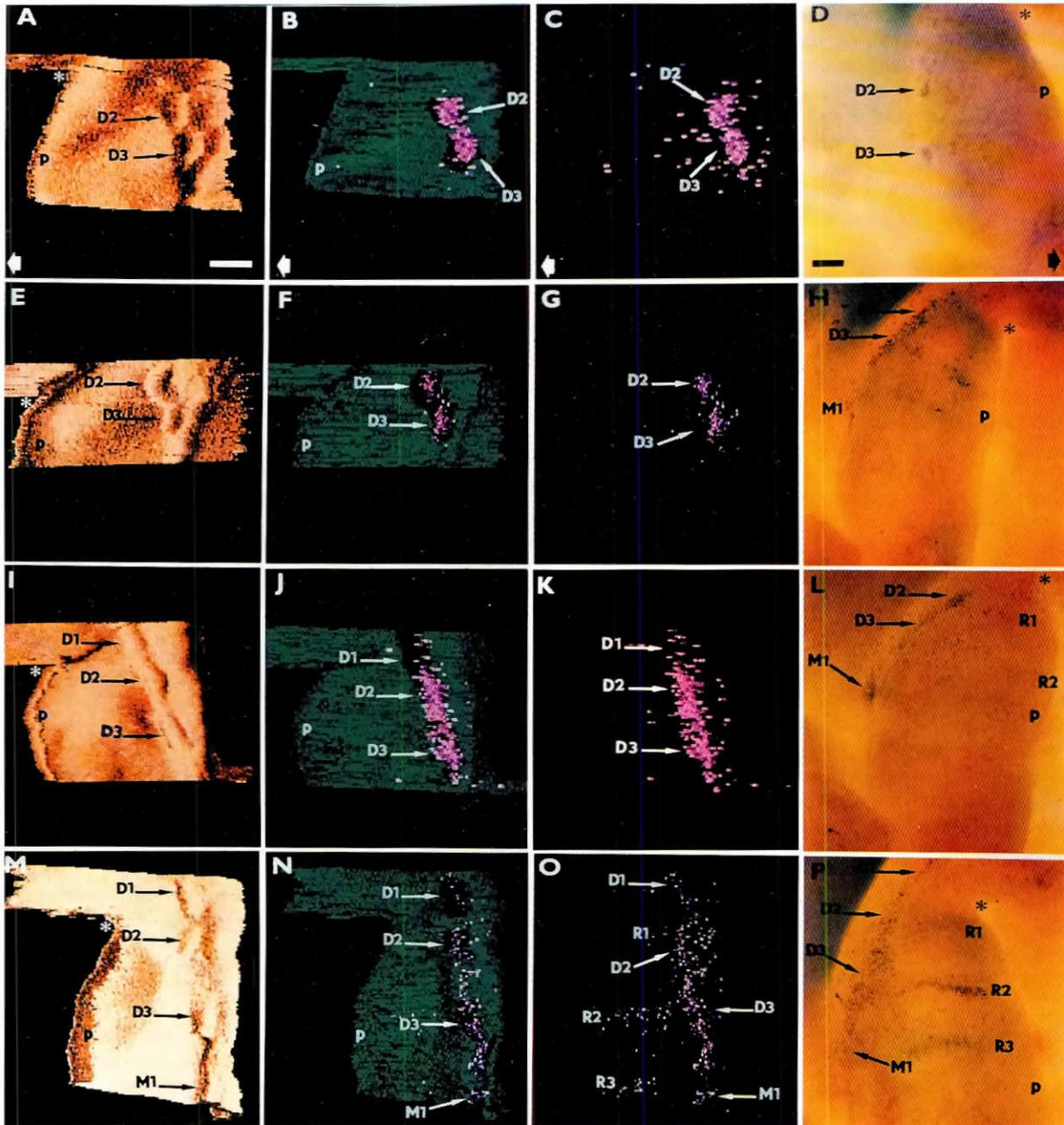


Fig. 3. 3-D reconstructions and distribution of apoptoses. The upper right prospective diastema region in mouse embryos at E12.5 (wt.c. 126-150 mg; **A-D**), E13.0 (wt.c. 101-125 mg; **E-H**), E13.5 (wt.c. 126-150 mg; **I-L**, and wt.c. 151-175 mg; **M-P**). 3-D reconstructions of the epithelium representing view of its mesenchymal surface (**A,E,I,M**), supplemented by distribution of apoptotic cells/bodies (violet dots) either in the oral epithelium (green) and diastema dental epithelium represented by black areas (**B,F,J,N**), or after suppression of a structural background (**C,G,K,O**). (**D,H,L,P**) demonstrate the distribution of cell death in an oral view of the right upper jaw stained supravitaly with Nile blue. Note the presence of cell death also in the epithelium of rugae palatinae and in the epithelium lining of the lip furrow situated buccally to the dental primordia (**N,O,P**). D1, D2, D3, transient diastemal dental primordia; M1, the first molar anlage; R1, R2, R3, rugae palatinae; P, margin of palatal shelf. Asterisk indicates the primary choana opening. The anterior/posterior direction corresponds to the top/bottom in all figures. Large arrow points medially. Scale bar, 100 μ m (3-D reconstructions), 120 μ m (Nile blue).

pattern of degenerating cells very similar to that documented by 3-D representations of apoptosis in the epithelium solely. The 3-D reconstructions further documented precise co-localization of apoptosis with respect to the spatial arrangement of the upper jaw epithelium. Presence of apoptosis in the epithelia of the

palatal rugae and the upper lip furrow in the diastema region was synchronous with culmination of the apoptotic process in the diastemal dental epithelium. This finding might be explained by the developmental relationship of all these structures originating from the same thickening of the maxillary oral epithelium

(Peterková, 1985). We suggest that the apoptotic process is initiated in the whole epithelial thickening in the diastema region of E10-11 embryos. After segmentation of the epithelial thickening into anlagen of the oral vestibule, dentition and palatal rugae (Peterková, 1985; Peterková *et al.*, 1995), these structures follow their own developmental processes. Accordingly, further progress and extension of apoptosis is modulated in relation to its specific localization. The apoptosis reaches its full development in the diastemal anlage of the dentition resulting in its extinction.

The programmed cell death by apoptosis is a normal developmental process. It is associated with exposure to or withdrawal of specific physiological signals (hormones, growth factors, etc.) and leads to the loss of specific cells in a temporally and spatially predictable manner (Raff, 1992; Cohen, 1993; Schwartz and Osborne, 1993). Our previous study demonstrated that in the diastemal dental epithelium, *msx-2* is downregulated from the stage of epithelial thickening, whereas the *BMP-4* gene is expressed until the rudiments start to regress (Turecková *et al.*, 1995). The *BMP-4* protein has been reported to induce apoptosis and upregulate *msx-2* expression in the neural crest cells of the third and fifth rhombomeres (Graham *et al.*, 1994). Therefore, the *BMP-4* protein might be involved in the regulation of apoptosis in the diastemal dental rudiments, which takes place in the absence of *msx-2* expression.

The programmed cell death occurring by apoptosis plays a key role in normal ontogenesis resulting in the selective elimination of unnecessary cells in tissues (Martin, 1993; Mori *et al.*, 1994). This type of cell death also appears to be involved in prenatal reduction of the diastemal dental anlagen in the upper jaw in mouse. In the lower diastema region, only a low epithelial lamina extends anteriorly from the molar epithelium at comparable stages: neither differentiation of tooth buds nor signs of cell death appear there (Peterková *et al.*, 1995). The functional specialization of the rodent dentition during evolution was associated with modification of the antemolar teeth. Consequently, only one continuously-growing incisor separated by the toothless diastema from maximally two upper and one lower premolar is present in the antemolar region of recent and fossil rodents (Grassé and Dekeyser, 1955; Viret, 1955; Wood, 1962). The reduction of premolars has been attributed to hypertrophy of the gnawing incisor (Hershkovitz, 1967). The difference in the rodent dentition is associated with changes in the jaw bones, the entire skull and the masticatory musculature (Peyer, 1968). The advantage of the absence of teeth in the diastema of rodents is that the sides of the lips can be drawn in during gnawing, preventing retained food from flying back into the mouth (Kershaw, 1983).

Existence of a vestigial tooth may be considered as a manifestation of an advanced stage of tooth reduction (Moss-Salentine, 1978). From this viewpoint, the diastemal rudiments in the mouse might represent the last traces of the existence of the maxillary antemolar dentition (present in ancestors) during its phyletic reduction (Peterková *et al.*, 1995). The difference between prenatal development of the upper and lower diastema in mouse, reflecting various degrees of tooth involution, is in accordance with the trend towards rodent antemolar teeth reduction, which is always more advanced in the lower jaw (Luckett, 1985).

The upper jaw in mouse embryos, whose prospective diastema includes transitory dental anlagen, will represent an interesting tool to study the molecular mechanisms regulating development of tooth bearing and toothless areas in dentition.

Materials and Methods

Pregnant female mice (ICR strain) were killed by cervical dislocation at embryonic days (E) 12.5, 13.0 and 13.5. The midnight before the morning detection of vaginal plug was considered as E0.0. Embryos were transferred in phosphate buffer and weighed. As morphological staging (Grüneberg, 1943; Theiler, 1972; Kaufman, 1994) has proved to be too crude for detailed study of early odontogenesis, embryos at each chronological stage were distributed into 25-mg weight classes (Peterková *et al.*, 1993). For further processing, representative specimens were selected.

Histology

The embryos were fixed in Bouin-Hollande fluid. Their heads were then dissected and dehydrated prior to embedding in paraffin. Series of 5 µm thick frontal sections were prepared and stained with alcian blue-hematoxylin-eosin. The hematoxylin staining appeared to be suitable for visualization of dying cells in histological sections (Pexieder, 1972; Koseki *et al.*, 1992; Kerr *et al.*, 1995).

3-D reconstructions

From the series of 5 µm histological sections, drawings of the right upper jaw oral epithelium situated between the incisor and molar anlagen were made using a Wild-Leitz Orthoplan microscope equipped with a drawing chamber under magnification 240x. The position of the apoptotic cells and bodies (Kerr *et al.*, 1995) inside the epithelia was indicated in the drawings. It was not always possible to precisely determine how many apoptotic bodies did represent a cluster of dark particles. In order to standardize the method, a condensed substance larger than nucleoli and smaller than interphase nuclei was indicated as the apoptotic body. Where possible, margins of the diastemal dental rudiments were also marked as the lowest point of the lingual and buccal feet of the diastemal dental lamina or bud on frontal sections.

For digitalization of the serial drawings, a Hamamatsu C2400 camera with a digital imaging system (series 151 Imaging Technology) was used. Correlation of successive images was performed by real time superimposition method (Olivo *et al.*, 1993). Softwares allowing image acquisition and treatment were developed in Strasbourg. 3-D images were generated using a volume rendering program (Sun Voxel, Sun Microsystems).

Supravital staining

Heads of embryos were stained with Nile blue sulphate solution (Merck) for 30 min at room temperature (1:40,000 in physiological Locke's buffer; Milaire, 1992). After a 4 h wash in Locke's solution the upper jaws were dissected, put on hollowed slides and observed under a Docuval (Zeiss) microscope.

Electron microscopy

The upper jaws of representative embryos were dissected after glutaraldehyde fixation (2.5% glutaraldehyde in phosphate buffer, pH 7.4, for 1.5 h). After washing in phosphate buffer (pH 7.4), the jaws were post-fixed in 1% OsO₄ in buffer for 2 h. The tissues were passed through graded series of ethanol with uranyl acetate and through propylene oxide, embedded in Durcupan-Epon medium (Fluka), and polymerized at 60°C for 3 days.

Ultrathin sections made by Reichert microtome were put on copper grids and stained with uranyl-acetate and lead citrate. An Opton EM109 transmission electron microscope was used for observation.

TUNEL method

Embryonic heads were fixed for up to 24 h at 4°C in freshly prepared 4% formaldehyde buffered with 0.05 M sodium phosphate and 0.2 M NaCl (pH 7.4). The heads were dehydrated and embedded in paraffin according to standard procedures. Serial frontal sections (7 µm) were placed on silanized slides and stored at +4°C until further processing. *In situ* detection of DNA fragmentation was performed according to a protocol provided by Oncor Inc. (Gaithersburg, MD, USA) for TUNEL method using the Apoptosis detection kit designated for visualization with peroxidase label. Deparaffinized sections were treated with Proteinase K (20 µg/ml) for 15 min at room temperature (RT), and with 0.2% hydrogen peroxide in PBS for 5 min at RT. Equilibration buffer (Oncor) was applied to sections under a plastic coverslip for 15 sec at RT. After this pretreatment, the sections were incubated with TdT enzyme (Oncor) in a humidified chamber at 37°C for 1 h. The reaction was stopped with Stop/wash buffer (Oncor) applied for 30 min at 37°C. For color detection of the DNA fragments, anti-digoxigenin-peroxidase (Oncor) and 0.05% DAB were used (for 30 min and 20 min at RT, respectively). After counterstaining with methyl green, the sections were dehydrated and mounted in xylene containing medium.

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