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Caspase 3 Activation in the Primary Enamel Knot of Developing Molar Tooth

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Summary

Mammalian teeth develop during embryogenesis as epithelio-mesenchymal organs. The primary enamel knot is considered as a signaling center in tooth morphogenesis. After tooth bell formation, this epithelial structure undergoes apoptosis. Activation of caspase 3 represents a crucial step in the intracellular death machinery. Procaspase 3 and caspase 3 molecules were localized in the primary enamel knot of the field vole using immunohistochemistry. Different fixation procedures in cryopreserved and paraffin-embedded tissues and detection systems based on peroxidase and alkaline phosphatase mediated color reactions were applied. Apoptosis was detected using morphological criteria and the TUNEL assay. Procaspase 3 was found in both the epithelial and mesenchymal part of the tooth germ. Active caspase 3 was localized particularly in the primary enamel knot, its distribution correlated with dental apoptosis and showed a similar pattern in the field vole as in the mouse.

Key words

Apoptosis • Caspase 3 • Primary enamel knot • Tooth development

Introduction

It is now clear that programmed cell death is an essential process in animal development and maintenance of tissue architecture (for review see Meier *et al.* 2000, Doseff 2004). The general system operating during metazoan development leads to the overproduction of cells followed by apoptotic culling during development to match the relative number of cells of different types to achieve proper organ function (Jacobson *et al.* 1997). Apoptosis must also be considered as an active morphogenetic mechanism engaged in embryonal organ shaping, including the teeth (Matalová *et al.* 2004a).

The nature of apoptotic death is characterized by various morphological and biochemical changes that allow the disassembly of cellular structures, decomposition of cellular contents and tagging of apoptotic cells for their engulfment by neighboring phagocytes (for review see Savill and Fadok 2000, Fadeel 2003). Most of the morphologic alterations characteristic for apoptotic cell death (Kerr *et al.* 1972) are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These proteases belong to a large protein family called caspases (Alnemri *et al.* 1996), which are highly preserved during evolution (Earnshaw *et al.* 1999, Boyce *et al.* 2004). Apoptotic cell death execution is mediated by effector caspases, such as caspase 3 and caspase 7, which cleave a limited subset of critical

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cellular polypeptides. These effector caspases can be activated through proteolytic processing by upstream initiator caspases such as caspase 8 (extrinsic pathways) and caspase 9 (intrinsic pathways) (for review see Riedl and Shi 2004).

Activated caspase 3 was found in the interdigital segments of limbs, in the thymus, small intestine epithelium, kidney and neurons where cell death naturally occurs during development (Raff *et al.* 1993, Krajewska *et al.* 1997, Urase *et al.* 1998, Eijnde *et al.*1999, Araki *et al.* 2003, Resendes *et al.* 2004) and was also correlated with TUNEL-positive cells in mouse tooth development (Shigemura *et al.* 2001).

The primary enamel knot has been considered as a signaling center in developing teeth, which is eliminated by apoptosis (Thesleff and Jernvall 1997). Moreover, apoptosis occurring in the primary enamel knot (and later the secondary one) is considered as an important mechanism which may be involved in final tooth crown formation (Matalová *et al.* 2004a). Spatially restricted localization of apoptotic cells has been found in all animal models investigated so far (Lesot *et al.* 1996, Vaahtokari *et al.* 1996, Jernvall *et al.* 1998, Sasaki *et al.* 2001) and recently also in the field vole (Matalová *et al.* 2004b). The field vole represents a useful model for comparative studies with the mouse (Keränen *et al.* 1999) due to the same tooth formula 1003/1003 but different final molar tooth shape with characteristic zig-zag cusp pattern.

The aim of our study was to explore caspase 3 activation during enamel knot formation and elimination in the first molar tooth germ of the field vole using immunohistochemical labeling of procaspase 3 and activated caspase 3 followed by correlation of activated caspase 3 molecules with dental apoptosis.

Methods

Tissue preparation and fixation

Field vole embryos (*Microtus agrestis*, Rodentia), two at each stage, obtained in the frame of embryonic collection (www.iach.cz/lge/sbirkae.htm), embryonic days (ED) 13-16 (according to the vaginal plug) were used in this study. Head parts of embryos were cryoprotected (by immersing into liquid nitrogen) and kept at –80 °C before processing. Cryosections were placed on SuperFrostPlus slides, air-dried and fixed in acetone (2 min/–20 °C) and neutral buffered formalin (20 min at room temperature), respectively. Formalin fixed heads were dehydrated in ethanol and wax embedded after fixation. Decalcification was not applied.

Active caspase 3 labeling

In frozen tissues, a biotin-conjugated, rabbit anti-active caspase 3 polyclonal antibody (Transduction Laboratories) was used following the recommended staining protocol. After fixation, slides were washed three times in PBS and incubated with primary antibody (1:50) for 1 h at the room temperature. Simultaneously, an overnight incubation was tested. When washed in PBS, streptavidin-avidin-alkaline phosphatase system (BD Biosciences, Pharmingen) was applied and followed by substrate reaction using FastRed (Sigma Aldrich) to reach the final color of positive cells (red). Simultaneously, a streptavidin-avidin-peroxidase system with DAB (3,3'-diaminobenzidine tetrahydrochloride; BD Biosciences) the color reaction was tested.

In formalin-fixed, paraffin-embedded tissues, a rabbit anti-active caspase 3 polyclonal antibody (Alexis) was applied using the same incubation procedure as for frozen samples with streptavidin-avidin-peroxidase mediated color reaction.

Procaspase 3 labeling

Rabbit procaspase 3 polyclonal antibody (Ab-4, NeoMarkers, Fremont, USA) was applied using the same procedure as for active caspase 3 in formalin-fixed tissue.

Procaspase 3 and active caspase 3 analysis

Hematoxylin or nuclear red were used as a counterstain of cell nuclei before the final analysis. Samples were photographed after dehydration and mounting. No dehydration but a water-based medium (Vector Laboratories) was used for AP (alkaline phosphatase) samples.

TUNEL assay (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) In Situ Cell Death Detection Kit, POD was used for apoptosis detection in individual cells. The TUNEL procedure was carried out following the manufacturer's directions (Roche Biochemicals). Endogenous peroxidase was blocked by incubation in 3 % H_2O_2 in phosphate-buffer saline (10 min at room temperature) before enzymatic labeling. During the TUNEL procedure samples were washed in phosphate-buffer saline (pH 7.4): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 in distilled water. Fluorescent signal conversion using

anti-fluorescein antibody conjugated with peroxidase and substrate color reaction applying chromogen DAB (3,3'-diaminobenzidine tetrahydrochloride; Roche Biochemicals) were performed after enzymatic labeling and counterstained with alcian blue.

Cell morphology

Tissue sections were stained using haematoxylin-eosin for histological analysis and evaluation of morphological changes characteristic for apoptosis.

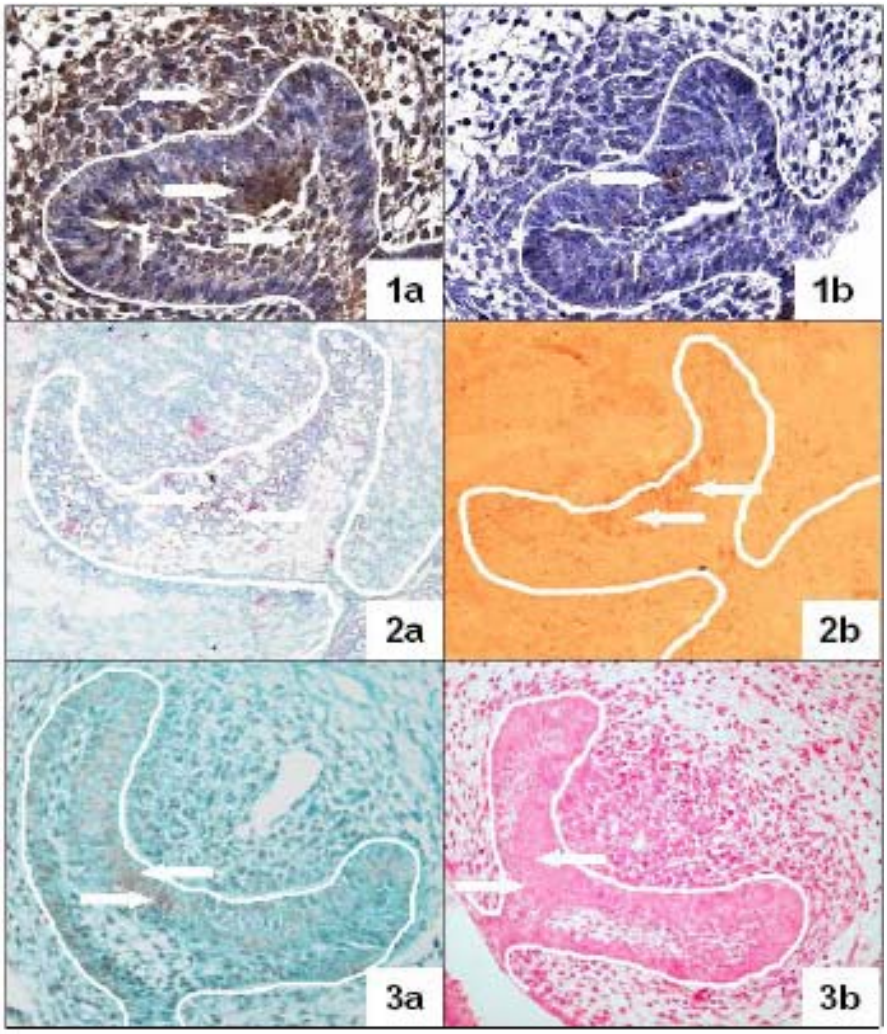

Fig. 1. Immunohistochemical labeling of procaspase 3 and active caspase 3 molecules at the cup stage (ED 14.5). Procaspase 3 (brown) can be found in both, epithelial (lower arrow) and mesenchymal (upper arrow) part of the molar tooth cup (Fig. 1a). The middle arrow is pointing to the primary enamel knot where active caspase 3 (brown, white arrow) can also be found (Fig. 1b). Formalin-fixed, paraffin-embedded samples. Hematoxylin counterstain.

Fig. 2. Immunohistochemical labeling of active caspase 3 at the bell stage (ED 15.5) in cryopreserved tissues, formalin fixation (Fig. 2a) and acetone fixation (Fig. 2b). Both procedures revealed active caspase 3 localized throughout the whole primary enamel knot, which is apoptotically eliminated at this stage. Alcian blue (Fig. 2a) and eosin counterstain (Fig. 2b).

Fig. 3. Detection of apoptotic cells in the tooth germ. TUNEL test (Fig. 3a) showing cells with DNA fragmentation (brown) in the area of the primary enamel knot. Counterstained by alcian blue. Confirmation of apoptosis by altered cell morphology, arrows pointing to apoptotic bodies after hematoxylin-eosin staining (Fig. 3b).

Results

Serial tissue sections were examined using field vole embryos from day 13.5 (ED 13.5) to day 15.5 (ED 15.5) of gestation when enamel knot appears morphologically distinguishable. Immunohistochemistry was used to evaluate procaspase 3 and active caspase 3 distribution in epithelial and mesenchymal cells of the developing tooth organ. Contribution of caspase 3 activation to dental apoptosis occurring in the primary enamel knot was evaluated after correlation with apoptotic cells identified using morphological (hematoxylin-eosin staining) and biochemical (TUNEL assay) criteria.

In the field vole, scattered apoptotic cells and apoptotic bodies can be detected in the first molar tooth germ since ED 13 when the primary enamel knot forms on the tip of the tooth bud. The apoptotic area expands gradually during the next embryonic day when the tooth germ enter its cap stage and massive apoptosis can be found in the primary enamel knot at ED 15.5 as described in detail earlier (Matalová *et al.* 2004b). Caspase 3 activation strongly correlated with the increased apoptosis (Fig. 1). At the cap stage, procaspase 3 labelling showed strong positivity in most epithelial and mesenchymal cells of the growing tooth organ (Fig. 1a), whereas activated caspase 3 was strongly present in epithelial cells of the primary enamel knot (Fig. 1b). At

the bell stage (ED 15.5), activated caspase 3 was spread through the whole primary enamel knot (Fig. 2), which is eliminated by apoptosis at this stage (Fig. 3). The restricted pattern of active caspase 3-positive cells was not influenced by the fixation, although the tissue morphology was remarkably better preserved in the formalin fixed tissue (Figs. 2a, 2b). Apoptotic cells were confirmed both by the appearance of apoptotic bodies (Fig. 3a) and by specific DNA fragmentation (Fig. 3b).

Early molar tooth development in the mouse and the field vole is compared in Fig. 4

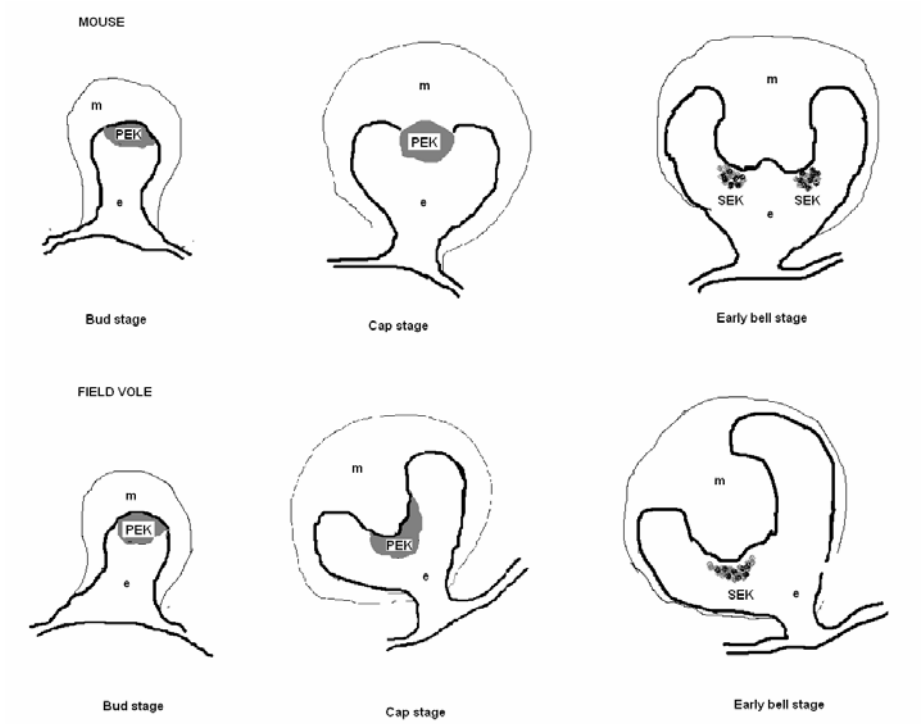

Fig. 4. Early development of the first upper molar in the mouse and in the field vole. Grey areas correspond to structures of primary enamel knot (PEK) where apoptosis as well as caspase 3 activation were demonstrated. Epithelial (e) and mesenchymal (m) parts are displayed from the tooth bud up to early bell stage, period when the primary enamel knot fulfils its signalling function and is gradually eliminated by apoptosis.

Discussion

The teeth are of epithelio-mesenchymal origin and they are often used as organ model systems for molecular studies of development, differentiation and diseases. In general, all teeth pass through identical embryonic stages, however, the final shapes, size and patterns differ in various species. The laboratory mouse is the most common mammalian species engaged in these investigations, due to the availability of mutants, transgenic models and specific probes and antibodies. The mouse tooth also represents an appropriate tissue for studying apoptosis in organ development (Peterková *et al.* 2003). Thus, a comparison of other mammalian species is used to study evolutionary conservation of the gene pool and to understand interspecies differences following general mechanisms. The field vole has often been exploited for such comparative studies due to the same tooth pattern as the mouse but different final molar tooth shape (Chaline *et al.* 1993, Jernvall *et al.* 2000). The taxonomic relationship to the mouse allows exploitation of mouse detection systems in the field vole (Keränen *et al.* 1999).

Little has been known about the functions and mechanisms of apoptosis occurring during tooth development, however, the characteristic distribution pattern of apoptosis in mammalian odontogenesis suggests several essential roles of programmed cell death in tooth morphogenesis (reviewed by Matalová *et al.* 2004a). Clarification of molecular pathways, which underscore tooth embryonic development and their possible modulation, is of considerable importance with emerging molecular dentistry (Ohazama *et al.* 2004).

There are two main pathways leading to apoptosis: positive induction by ligand binding to the corresponding plasma membrane receptor and negative induction by loss of suppressor activity leading to internal breakdown of the cell. According to the information about the signaling network and apoptosis-related molecules involved in tooth development, both pathways might to be involved in dental apoptosis. BMPs (bone morphogenetic proteins) are considered to interplay with FGFs (fibroblast growth factors) and SHH (sonic hedgehog) to maintain balance between proliferation and

cell death with specific apoptotic elimination in the enamel knot area with putative signaling roles (Jernvall *et al.* 1998, Cobourne *et al.* 2001, Peterková *et al.* 2003). Particularly in these cells, apoptosis could be an active process rather than just elimination of unwanted cells.

Since caspase 3 is a central player in apoptotic intracellular machinery, we investigated procaspase 3 molecules and their activation using immunohistochemistry. The results were correlated with the spatiotemporal pattern of apoptotic cell elimination in primary enamel knot of the field vole described recently (Matalová *et al.* 2004b).

The molecule of caspase 3 is present in its inactive form in most cells of the embryo. The driving force for caspase activity is activation of this proenzyme based on a web of interactions (for review see Boatright and Salvesen 2004). However, the non-active epitope of procaspase 3 can also be exposed by the standard procedure commonly used for histochemistry on serial sections (using formalin fixation and paraffin embedding). To avoid this non-specific recognition of the active and inactive forms of caspase 3, both cryopreserved and formalin-fixed tissues were used in this study and two different anti-active caspase 3 antibodies were applied. Furthermore, two different enzyme detection systems were exploited to avoid possible false positive results. The number of active caspase 3-positive cells was not higher in the sections treated with alkaline phosphatase than in those treated with peroxidase.

Studies in caspase-deficient mice revealed a strict requirement for caspase 3 (for review see Zheng *et al.* 1999) in caspase-mediated apoptotic pathways. Caspase 3 deficiency leads to perinatal lethality (depending on the mouse strain) due to neuronal hyperplasia and structural disorders. Observation of caspase 3 activation in molar tooth development of the field vole showed that caspase 3 is strongly activated in the primary enamel knot, and this dental apoptosis seems to be caspase 3-dependent. Spatiotemporal correlation of caspase 3-activated molecules and apoptotic DNA fragmentation revealed a gradual activation of caspase 3 corresponding to a later increasing number of apoptotic cells within the primary enamel knot. Distribution of activated caspase 3 showed correlations with specific areas of the tooth organ where apoptosis can be found (primary enamel knot, stalk or dental lamina). As in the mouse (Shigemura *et al.* 2001), the extent of caspase 3 activation and apoptosis in the field vole correspond to different morphological formation of the primary and secondary enamel knots (Jernvall *et al.* 2000). The engagement of caspase 3 seems to be important for dental apoptosis in both species and it awaits its final functional appraisal in tooth development.

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