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Primary enamel knot cell death in Apaf-1 and caspase-9 deficient mice

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ABSTRACT

During molar development, apoptosis occurs in a well-characterised pattern suggesting several roles for cell death in odontogenesis. However, molecular mechanisms of dental apoptosis are only poorly understood.

In this study, Apaf-1 and caspase-9 knockouts were used to uncover the engagement of these members of the apoptotic machinery during early tooth development, concentrating primarily on their function in the apoptotic elimination of primary enamel knot cells. Molar tooth germ morphology, proliferation and apoptosis were investigated on frontal histological sections of murine heads at embryonic days (ED) 15.5, the stage when the primary enamel knot is eliminated apoptotically.

In molar tooth germs of both knockouts, no apoptosis was observed according to morphological (haematoxylin-eosin) as well as biochemical criteria (TUNEL). Morphology of the mutant tooth germs, however, was not changed. Additionally, knockout mice showed no changes in proliferation compared to wild type mice.

According to our findings on knockout embryos, Apaf-1 and caspase-9 are involved in apoptosis during tooth development; however, they seem dispensable and not necessary for proper tooth shaping. Compensatory or other mechanisms of cell death may act to eliminate the primary enamel knot cells in the absence of Apaf-1 and caspase-9.

1. Introduction

Normal development of an individual implies regulated proliferation, producing new cells and subsequently liquidation of cells that do not fit or are not needed anymore. The process that allows elimination of cells under physiological conditions without threat of inflammation is called apoptosis.[1]

Apoptosis is well characterised according to specific morphological and biochemical features: plasmatic membrane changes, DNA fragmentation and formation of apoptotic bodies.[1,2] Several signals can trigger apoptosis via different pathways, involving death receptor activation, mitochondria or endoplasmic reticulum.[3] The main mediators of the mitochondrial dependent apoptosis are Apaf-1 and caspase-9. Apaf-1 and procaspase-9 together with cytochrome c released from mitochondria form a multimolecular complex—apoptosome. In this complex procaspase-9 is cleaved into its active form and initiates the typical apoptotic caspase cascade.[4,5]

Odontogenesis is based on epithelial–mesenchymal interactions between stomodeal ectoderm and the surrounding neural crest ectomesenchyme.[6,7] Apoptosis is present mainly in the epithelial compartment from the early morphogenesis stage until eruption. During early morphogenesis of mouse molars, apoptosis is believed to contribute either actively or

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passively to elimination of the diastemal dental lamina together with diastemal and antemolar vestiges, structures that appear in the diastema in particular developmental period but do not give rise to functional teeth and disappear later in development. Apoptosis is also assumed to eliminate cells of the enamel knot, a signalling centre expressing many molecules involved in the bud-cap transition.[8] Although temporospatial pattern of apoptosis in odontogenesis suggests several important roles of apoptosis in tooth formation, the molecular mechanisms of dental apoptosis remain largely elusive.[9,10]

Dental apoptosis seems to be caspase dependent as supported by experiments *ex vivo*.[11] Caspase-3, considered as the central caspase of the intracellular apoptotic machinery, is activated in cells undergoing cell death during tooth development.[12,13] Caspase-3 knockout mice displayed altered tooth phenotype at the bell stage and no apoptotic cells were observed in craniofacial region of these mice.[14] Although it has been suggested that Bmp and Fgf interact in apoptosis during tooth development, nothing is known about molecules upstream of caspase-3 that may be essential for dental apoptosis. Caspase-3 can be activated by both mitochondrial and receptor mediated pathways. To identify the upstream cascades of dental apoptosis we have analysed Apaf-1 and caspase-9 mutants, mice lacking key players of the mitochondrial apoptotic pathway. We have investigated both apoptosis and proliferation in the molar tooth germs of Apaf-1 and caspase-9 deficient mouse embryos, paying particular attention to apoptosis in the primary enamel knot.

2. Material and methods

2.1. Specimens

Apaf-1 deficient mice were obtained by courtesy of Professor H. Yoshida (Kyushu University, Japan) and Professor T.W. Mak (University of Toronto, Canada). Knockout mice were created as described in Yoshida et al.[15] ED 15.5 embryos, according to the vaginal plug, were fixed in 4% paraformaldehyde.

Caspase-9 deficient mice were obtained by courtesy of Professor Kevin Roth (University of Alabama at Birmingham, USA) and generated as described in Kuida et al.[16] ED 15.5 embryos, according to the vaginal plug, were fixed in Bouin's solution.

Corresponding wild types to Apaf-1 and caspase-9 were prepared using the same procedure as in the mutant specimens.

2.2. Samples

Heads of three wild type mouse embryos at ED 15.5, three Apaf-1 deficient mice, and three caspase-9 deficient mice were embedded in paraffin, cut into 5 μm frontal sections, 100–120 sections showing the developing first molar were split over three parallel SuperFrost Plus slides. Haematoxylin-eosin stained sections were used for examination. TUNEL, and PCNA immunohistochemistry were performed on the parallel slides.

2.3. *In situ* cell death detection

Apoptosis was evaluated according to morphological (presence of apoptotic bodies in haematoxylin-eosin stained sections) and biochemical criteria (DNA fragmentation). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling—TUNEL (*In Situ* Cell Death Detection Kit) was used to investigate apoptotic DNA breaks.

Tissue was dewaxed and rehydrated in decreasing concentrations of ethanol. The TUNEL working procedure was carried out following the producer's directions (Chemicon). After proteinase K pre-treatment (20 μg/ml at 37 °C/humidified chamber/30 min), 3% hydrogen peroxide was applied to the slides to avoid endogenous peroxidase reaction. Slides were incubated with terminal deoxyribonucleotide transferase (at 37 °C/humidified chamber/1 h). Antidigoxygenin antibody conjugated with horseradish peroxidase was applied and DAB (3,3′-diaminobenzidine) (Sigma) chromogene was used to visualise apoptotic DNA strand breaks. Sections were counterstained with Gills haematoxylin. A positive control of TUNEL labelling was prepared using Nuclease (R and D Systems) treatment (5 μg/ml at 37 °C/humidified chamber/30 min). A negative control was obtained by omitting the terminal transferase from the labelling procedure (label solution only instead of TUNEL reaction mixture).

2.4. PCNA immunohistochemistry

Proliferation cell nuclear antigen was detected using monoclonal mouse antibody PC-10 (kindly provided by Dr. Nenutil, Masaryk Memorial Cancer Institute). Slides were dewaxed, rehydrated and endogenous peroxidase was blocked using 3% hydrogen peroxide. For 4% paraformaldehyde samples, antigen retrieval was applied (citrate buffer pH 6 in microwave). Slides were incubated with 4× diluted primary antibody at 4 °C overnight. ABC Elite Kit Mouse (Vector) was employed according to the manufactures directions to detect the antibody binding. Visualisation was done after substrate reaction of DAB (DAKO) catalysed by horseradish peroxidase. Samples were counterstained by Gills haematoxylin (blue) to contrast positive cells in brown. Mouse small intestine was used as positive control. Negative control was obtained by omitting the primary antibody.

3. Results

First molar tooth germ morphology, primary enamel knot apoptosis, and proliferation of the dental epithelium and mesenchyme were examined in wild type, Apaf-1−/−, and caspase-9−/− mice at ED 15.5.

At ED 15.5, wild type murine first molar tooth germs had reached the early bell stage, with a concentrically arranged cluster of cells protruding into the dental mesenchyme, forming the enamel knot in the centre of the enamel organ (Fig. 1a).

In the wild type molar tooth germs, apoptotic bodies and TUNEL positive cells were observed in the primary enamel knot, in the stalk and in the vestibular part of the tooth germ adjacent to the outer enamel epithelium on the border