Apoptosis in developmental and repair-related human tooth remodeling: A view from the inside

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Abstract

Apoptosis is a key phenomenon in the regulation of the life span of odontoblasts, which are responsible for dentin matrix production of the teeth. The mechanism controlling odontoblasts loss in developing, normal, and injured human teeth is largely unknown. A possible correlation between apoptosis and dental pulp volume reduction was examined. Histomorphometric analysis was performed on intact 10 to 14 year-old premolars to follow dentin deposition and evaluate the total number of odontoblasts. Apoptosis in growing healthy teeth as well as in mature irritated human teeth was determined using a modified TUNEL technique and an anti-caspase-3 antibody. In intact growing teeth, the sequential rearrangement of odontoblasts into a multi-layer structure during tooth crown formation was correlated with an apoptotic wave that leads to the massive elimination of odontoblasts. These data suggest that apoptosis, coincident with dentin deposition changes, plays a role in tooth maturation and homeostasis. Massive apoptotic events were observed after dentin irritation. In carious and injured teeth, apoptosis was detected in cells surrounding the lesion sites, as well as in mono-nucleated cells nearby the injury. These results indicate that apoptosis is a part of the mechanism that regulate human dental pulp chamber remodeling during tooth development and pathology.

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Introduction

Apoptosis is a genetically regulated form of cell death that is implicated in biological processes ranging from embryonic development to aging, from normal tissue homeostasis to various human diseases \cite{1,2}. Genetic evidence has identified both positive and negative regulators of apoptosis \cite{3,4}. Initiators of apoptosis include, between others, ultraviolet ir-

radiation and deprivation of survival factors such as cytokines. These stimuli in turn generate a characteristic pattern of gene expression. The molecular machinery of apoptosis is evolutionarily conserved and intrinsic to all metazoan organisms. The principal effectors are a family of proteases termed caspases \cite{5}. Apoptosis is characterized by morphological and ultrastructural changes that include chromatin condensation, nuclear fragmentation, cell rounding and shrinkage,
leading to cell breakage into apoptotic bodies that are rapidly phagocytosed and digested by macrophages or by neighboring cells [6].

During development, apoptosis eliminates individual unnecessary cells as well as entire vestigial structures. It has been shown that apoptosis plays a key role in tooth shape and size [7–10]. Apoptosis is also involved in tooth anomalies associated with cleft lip and palate [11]. In a dynamic interplay of cell proliferation and cell death, the developing tooth retains precisely the type and number of cells needed to proceed into maturity. It is admitted that odontoblasts are terminally differentiated cells that survive as long as the integrity of the tooth is preserved [12,13]. However, the dental pulp volume decreases gradually on ageing due to the continuous production of dentin matrix by odontoblasts. This age-related pulp chamber reduction is associated with the elimination of a certain number of odontoblasts by apoptosis [14,15]. In pathological conditions involving mild carious lesions, odontoblast activity is stimulated to elaborate apoptotic DNA fragmentation that is related to pathological conditions involving violent stresses (i.e. cavity preparations) lead to odontoblast disintegration and newly formed odontoblast-like cells elaborate a reparative dentin. Reparative dentinogenesis involves either necrosis or apoptosis of odontoblasts. Reactionary dentinogenesis results from a stimulation of existing odontoblasts and this process takes place in the absence of cell death. However, the deposition of reactionary dentin decreases the pulp chamber volume and could thereafter favor apoptosis of odontoblasts in a comparable way to the age-related apoptosis.

The study of apoptosis in growing and injured human teeth is important for at least two reasons. First, as odontoblasts must survive for the entire life span of the animal in spite of the continuous decrease of the dental pulp chamber volume [12,13], an understanding of the regulatory mechanisms that control cell death in dental pulp may provide new insights into the process of tooth homeostasis. Second, conditions or agents that initiate apoptosis (i.e. bacterial infection, pulp ischemia, mechanical stimuli, dental restorative products) trigger dental disorders accompanied by dentin remodeling [19,20]. Using an in situ end-labeling assay to detect cell death, we define a precise time period during tooth maturation when apoptosis is stimulated in the odontoblastic layer. The location and extent of this process helps to explain the differences in size and appearance of the adult dental pulp chamber. Furthermore, concentrated cell death at the injury and caries sites suggests an additional role for apoptosis in dentin remodeling that is related to pathological conditions. These findings improve our understanding on the mechanisms involved in tooth maturation and tooth-related diseases in humans.

Materials and methods

Preparation of teeth

For a first set of experiments, 20 premolars of 10 to 14 year-old patients were used. The premolars were composed of 4 groups of 5 teeth each according to their age (10, 11, 12.5 and 14 years old). These teeth were used to determine the number of odontoblasts per 100 μm and examine apoptosis. In another experimental set, cavity preparations were performed as previously described in 17 premolars of 11 to 12 year-old patients [15], while 4 intact premolars were used as a control. A dental product (IRM) was used to restore the cavities, in conjunction with a calcium hydroxide lining material (Dycal, Dentsply, Milford, DE, USA). The teeth were extracted after a post-operative interval of 8 to 9 weeks. Finally, 10 carious third molars of 30 to 40 year-old patients were used. No radiographic or clinical indication of irreversible pulp reaction was seen in these teeth.

Immediately after extraction, the roots of the teeth were sectioned in order to obtain a quicker fixation. Since fixation plays a major role in triggering anoxic apoptosis [21], 4% neutral formol was used for 5 days to minimize the risk of false results induced by a long-term fixation. After demineralization with 10% formic acid, 5 μm serial sections were performed. Two intact teeth were fixed with 4% paraformaldehyde at 4 °C for 3 days and then were sliced into 50 μm sections without a previous demineralization. Hematoxylin-eosin staining was performed every other section of decalcified normal human teeth.

Histomorphometric and statistical analysis

Hematoxylin-eosin staining was performed on alternate sections of decalcified normal human teeth. The external and internal dentin perimeters were measured as described previously [15]. On the same sections, nuclei of the odontoblasts per 100 μm were recorded in 12 automatically pre-selected fields at the cement-enamel junction area. Statistic analysis was performed as described previously [15].

TUNEL assay

A method for identifying and quantifying apoptosis is the detection of fragmented DNA by terminal transferase mediated dUTP-digoxigenin nick end labeling (TUNEL). Apop Tag® detection kits with either fluorescein- or peroxidase-conjugated antibodies were used (Oncor, Ind., Gaithersburg, MD, USA). The TUNEL assay was performed as previously described [22]. Briefly, the sections were treated with the terminal deoxynucleotidyl transferase (TdT, Gibco-BRL) for 1 h at 37 °C. The demineralized sections were incubated with the peroxidase-conjugated anti-digoxigenin antibodies for 30 min at room temperature, treated with diaminobenzidine (DAB, Sigma) for 6 min, and then were counterstained with methyl green. The 50 μm thick sections were incubated with fluorescein-conjugated anti-digoxigenin antibodies (Vector Laboratories, Burlingame, CA) for 12 h at 4 °C. The slides were then incubated with 5 μg/ml propidium iodide (Molecular Probes, Eugene, OR) and 100 μg/ml RNase A (Sigma) for 30 min at 37 °C. Slides were washed, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and observed by epifluorescence. Sections of rat mammary glands were included in each experiment as positive controls, and negative controls
consisted of sections incubated in reaction buffer without TdT enzyme.

**Immunohistochemistry**

Apoptosis was also detected using a mouse anti-caspase-3 antibody (Transduction Laboratories, Interchim, Montluçon, France). Immunohistochemistry was performed as described previously [16]. Briefly, the anti-caspase-3 antibody (1 μg/ml) was applied on the demineralized sections for 30 min at 37 °C. After incubation with the biotinylated secondary antibody, the sections were incubated with peroxidase-conjugated streptavidine for 10 min. Peroxidase was visualized by incubation with 3-amino-9-ethylcarbazole (AEC) reaction solution. In control sections the primary antibody was omitted.

**Results**

**Organization of odontoblasts within the pulp chamber in intact human teeth**

A close correlation exists between the volume of the dental pulp chamber and the age of the teeth (Fig. 1A). Continuous dentin deposition progressively decreases both the volume occupied by the pulp fibroblasts and the dentin/odontoblast...
The decrease of the dentin/odontoblast interface, without a proportional decrease in odontoblast number, implies a reorganization of the odontoblasts within the pulp chamber, that could be seen either as an alignment of the odontoblast bodies in more than one cell layer or as a decrease in odontoblast size. Histological examination revealed that odontoblasts were aligned in a single layer in the 10 and 14 year-old premolars (Fig. 1C, F and H). However, odontoblasts in 11 and 12.5 year-old premolars were organized in a multi-layer structure (Fig. 1D, E and G). The size of the odontoblasts remained constant during all the periods of odontoblast reorganization. Dentin deposition and alignment of odontoblasts into a single layer resulted to a decrease of the initial number of odontoblasts in the 14 year-old premolars.

**Apoptosis in intact human teeth**

DNA fragmentation and chromatin condensation are early events in the apoptotic process [6]. By combining TUNEL and propidium iodide counter-staining with confocal microscopy, both of these characteristics were assessed in the same section. After fixation of non-demineralized thick sections, DNA of cells was stained with propidium iodide. Apoptotic nuclei appear condensed and intensely stained with fluorescein. Optical sections of both the propidium iodide and the fluorescein-labeled nuclei were then obtained on the confocal microscope. After merging these images, the green fluorescein (apoptotic nuclei) colocalized with the red propidium iodide (all nuclei) gave a yellow signal, representing fragmented nuclear DNA (Fig. 2A and B). Although apoptosis was detected in very few cells at the central core of the pulp, apoptosis was much more evident in the odontoblastic (Fig. 2A) and subodontoblastic (Fig. 2A and B) layers. The same results were obtained after the immunochromical detection of the caspase-3 protein (Fig. 2C). All controls gave negative results.

**Apoptosis in injured and carious human teeth**

Cavity preparation has as an effect on the sucking of odontoblasts into the dentinal tubules (Fig. 3A; red arrowheads), where the odontoblastic nuclei can be easily detected (Fig. 3B and C; red arrowheads). The aspiration of odontoblasts in the tubes will result to their degeneration. Eight weeks after injury due to dentin cavity preparation, degenerated odontoblasts were replaced by newly formed odontoblasts, which elaborate a reparative dentin (Fig. 3D–F). The newly formed odontoblasts were aligned into a single cell layer (Fig. 3D; red arrowheads). A massive apoptosis was seen in odontoblasts that were sucked into the dentinal tubules during cavity preparation (Fig. 3E). Apoptotic events were not observed in odontoblasts at a distance from the cavity preparation (Fig. 3E). Apoptosis is also observed in mono-nucleated cells and endothelial cells of blood vessels (Fig. 3G and H) that are located near the injury site (Fig. 3D and E; asterisks).

In teeth with carious lesions, the secretory activity of the odontoblasts is stimulated to produce either reparative (Fig. 4A, B and D) or reactionary (hypercalcified) (Fig. 4C) dentin. Apoptosis was observed in odontoblasts facing the carious lesion (Fig. 4E–G). The staining was detected in the nuclei of odontoblasts, cells of the sub-odontoblastic layer and several pulp fibroblasts (Fig. 4F and G). Apoptotic cells were present only in areas beneath the bacterially infected dentin (Fig. 4A, C–E; asterisks). Odontoblasts and cells of the sub-odontoblastic layer far away from the site of the decay were not stained (data not shown).

**Discussion**

Apoptosis is a genetically regulated process of cell elimination and plays fundamental roles in both morphogenesis and pathogenesis of all multicellular organisms [23]. During odontogenesis, apoptosis regulates the cell number by eliminating cells that have already achieved their genetic program, thus controlling the pattern, shape and size of the teeth [7,8,24]. This control of patterns and shapes is manifested in the evolutionary diversity of mammalian tooth shapes. Molar shapes are created by different combinations of cusps, which...
are produced by unequal growth of the dental epithelium accompanied by growth of the whole tooth germ [24]. Most of the studies on apoptosis during rodent tooth development have been focused on the epithelium, and more precisely on the enamel knot. The enamel knot is a transient epithelial structure that has a central function in the control and patterning of the cusps [7,24]. The enamel knot acts as a signaling center since it expresses several genes encoding for signaling molecules such as BMP2, BMP4, BMP7, FGF4, and Shh [7,24]. Extensive apoptosis has been detected in the enamel knot [9,10,24] and blocking experiments have shown that apoptosis is a mechanism for silencing this signaling center.

Fig. 3 – Detection of apoptosis in human teeth after cavity preparation. (A) Hematoxylin–eosin staining showing odontoblasts sucked into the dentinal tubules after cavity preparation. Red arrowheads indicate the nuclei of the aspirated odontoblast, green arrows indicate the location of odontoblasts (od) before they were sucked into the tubules. (B, C) Higher magnifications of the dentin (d) showing the nuclei of odontoblasts (red arrowheads) that are aspirated into the dentinal tubules (dt). (D) Hematoxylin–eosin staining showing reparative dentin (rd) production, 8 weeks after cavity (ca) preparation. Red arrowheads indicate newly formed odontoblasts (o), blue arrowheads indicate original (resting) odontoblasts (od). The red dotted line indicates the limits between reparative and secondary dentin (d), and the asterisk an inflammatory area. (E) In similar section stained with methyl green, apoptosis (brown color) is detected in odontoblasts aspirated into the dentinal tubules. Apoptosis is also observed in the inflammatory area (asterisk). (F) Higher magnification of Fig. 2B showing that the apoptotic staining is localized in the nuclei of odontoblasts. Note that the reparative dentin has not a structural continuity with the secondary dentin. (G) Higher magnification of Fig. 2D showing an inflammatory area. Note the presence of mono-nucleated cells (deep violet color) located near blood vessels (v). (H) Higher magnification of Fig. 2E, showing apoptotic staining (brown color) in mono-nucleated cells and some endothelial cells. Additional abbreviation: p, pulp. Scale bar: 100 μm (A, B), 30 μm (C), 150 μm (D, E), and 50 μm (F–H).
Despite the significance of these findings in rodents, there is no information on apoptosis in the enamel knot of the developing human teeth. Furthermore, apoptosis in the pulp mesenchyme of the developing human teeth has been reported in only two studies [14,15].

Apoptosis in intact human teeth at the beginning of secondary dentin deposition

A structural remodeling of the pulp chamber takes place during the successive periods of dentin deposition. In humans, two distinct functional periods have been evidenced: a primary dentin is initially formed by odontoblasts during their active-secretory stage, followed by the deposition of a secondary dentin at their slow secretory period [26]. The time of primary dentin formation is estimated as about two years, with a rate of 4 μm of dentin deposition per day. The pulp chamber progressively decreases during primary dentin deposition and is reduced to a half at the beginning of the secondary dentin deposition. This decrease is accompanied by successive rearrangements of the odontoblasts within the dental pulp chamber: odontoblasts form a single layer at the beginning of primary dentin secretion, thereafter they exhibit a multi-layer organization, and at the beginning of secondary dentin deposition they acquire again a single-layer disposal. These changes contribute to the elimination of an important percentage of odontoblasts by apoptosis at the beginning of secondary dentin formation. This is in accordance with numerous studies showing that aging is accompanied by a general decline of physiological function and significant increase of apoptosis [3,23,27].

Apoptosis is much more significant in the odontoblastic layer than in the rest of the pulp. Apoptosis is however observed in cells of the sub-odontoblastic layer and pulp fibroblasts. Adjacent scavenger cells will phagocytose all these populations of apoptotic cells. A close relation between odontoblasts and sub-odontoblastic cells exists. It has been suggested that sub-odontoblastic cells are arrested progenitor cells having the ability to differentiate into odontoblasts [17,19]. It is possible that the elimination of a given odontoblast by apoptosis may...

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**Fig. 4** - Localization of apoptosis in carious human teeth. (A–C) Hematoxylin–eosin staining showing the production of either reparative dentin (rd) (A,B) or reactionary dentin (rad) (C) beneath the decay sites. Asterisks represent areas of dentin (d) demineralization and infected tubules. (D) Gram staining for tissues (yellow color) showing the bacterial infiltration (asterisks, violet color) of dentin in carious human teeth. The red dotted line indicates the limits between reparative and secondary dentin (d). (E) Methyl green staining. Apoptotic staining (brown color) is detected in odontoblasts (o; arrowheads). Asterisks represent areas of dentin demineralization. (F, G) Higher magnifications showing the apoptotic staining in the nuclei of odontoblasts (o), sub-odontoblastic cells (soc), and some pulp (p) fibroblasts located beneath the carious lesion. Additional abbreviations: nfo, newly formed odontoblasts; v, vessel. Scale bar: 180 μm (A,C,E), 80 μm (B), 150 μm (D), 50 μm (F), and 35 μm (G).
Mechanisms that contribute to dental injury include induction of cell death, activation of immune responses, and alterations in dental tissue physiology [19,20]. Human teeth have several peculiarities when compared with teeth from other species: odontoblastic processes are far away from the enamel–dentin junction and in some cases the processes are absent from large areas of dentin. This is confirmed by immunohistochemical studies using the intermediate filament protein Nestin, which is an excellent marker for the odontoblastic processes [16]. It is thus unlikely that normal (not very deep) cavity preparations will damage the odontoblastic processes, possibly leading to odontoblast necrosis. In human teeth, odontoblasts are sucked into the dentinal tubules during cavity preparations [36], and thereafter undergo apoptosis. Odontoblasts sucked into the tubules are isolated from the rest of the pulp soon after deposition of reparative dentin, which may act as a barrier to the pulp-derived and/or neuron-derived survival factors, thus leading to apoptotic events. The finding of significant apoptosis in the human teeth after cavity preparation confirms similar observations made in rodent teeth [28,37]. In rodent teeth, the deposition of the primary dentin (d) is correlated with increased apoptosis (apo; red arrows and cells) of active-secretory odontoblasts (po). Apoptosis could be originated by either the lack or overproduction of growth factors such as nerve growth factor (NGF) and bone morphogenetic protein (BMP), expressed by these cells or by neighboring cells. External and internal forces could also play a role in apoptotic events. Secondary dentin (sd) production by the remaining slow-secreting odontoblasts (o) progressively decreases the pulp chamber volume (p). In carious teeth (left side), bacterial infiltration (bact) and BMP released from the dentin during the demineralization process will induce apoptosis of odontoblasts located at the carious lesion site (red cells). Apoptosis will stimulate expression of TGFβ. At a second time, BMP and TGFβ will play a role in the formation of reparative dentin (rd) by promoting differentiation of pulp cells into newly formed odontoblasts (no).

The angle and thickness of the sectioning of human teeth are very important for the interpretation of the results: depending on the plan and the thickness of the section one single cell layer could be seen as a multi-layer. To eliminate this possible error we used confocal microscopy that allows a detailed evaluation of different parameters at different levels of the same section. With this technique the number of odontoblasts located in a section of a given thickness is very precise. In addition, histological serial sections of the entire tooth have demonstrated that the homogeneity of the odontoblast layer is maintained in all sections and levels. It can be then safely concluded that a possible artifact due to the sectioning plan was eliminated and the present results are correct and valid.

Both the nervous system and the immune system arise through overproduction of cells followed by the apoptotic death of those that fail to establish functional synaptic connections or auto-antigen specificities [29,30]. A similar phenomenon could be hypothesized for the human teeth: overproduction of odontoblasts will be followed by apoptosis of those that are not in close contact with sources of survival factors (i.e. nerve fibers, sub-odontoblastic cells). Nerve growth factor (NGF) constitutes a trophic signal for many cell types, and it has been well documented that its low affinity receptor p75 is involved in apoptotic events [30]. Previous studies have shown that NGF is expressed in odontoblasts, whereas the p75 receptor is firstly expressed in odontoblasts and thereafter expression was localized in cells of the sub-odontoblastic layer [31,32]. The p75 receptor is re-expressed in odontoblasts at the anterior part of the rodent incisor [31]. These odontoblasts are eliminated by apoptosis, thus keeping the total number of odontoblasts constant in the continuously growing incisor [31,33]. Taken together these findings suggest an important role of NGF for either the survival or apoptosis of odontoblasts (Fig. 5). Several findings also indicate that compressive forces induce apoptosis [34,35], thus suggesting that external forces play a role in pulp volume reduction.

**Apoptosis in adult human teeth exposed to a lesion**

Mechanisms that contribute to dental injury include induction of cell death, activation of immune responses, and alterations in dental tissue physiology [19,20]. Human teeth have several peculiarities when compared with teeth from other species: odontoblastic processes are far away from the enamel–dentin junction and in some cases the processes are absent from large areas of dentin. This is confirmed by immunohistochemical studies using the intermediate filament protein Nestin, which is an excellent marker for the odontoblastic processes [16]. It is thus unlikely that normal (not very deep) cavity preparations will damage the odontoblastic processes, possibly leading to odontoblast necrosis. In human teeth, odontoblasts are sucked into the dentinal tubules during cavity preparations [36], and thereafter undergo apoptosis. Odontoblasts sucked into the tubules are isolated from the rest of the pulp soon after deposition of reparative dentin, which may act as a barrier to the pulp-derived and/or neuron-derived survival factors, thus leading to apoptotic events. The finding of significant apoptosis in the human teeth after cavity preparation confirms similar observations made in rodent teeth [28,37]. In rodent teeth, the deposition of the primary dentin (d) is correlated with increased apoptosis (apo; red arrows and cells) of active-secretory odontoblasts (po). Apoptosis could be originated by either the lack or overproduction of growth factors such as nerve growth factor (NGF) and bone morphogenetic protein (BMP), expressed by these cells or by neighboring cells. External and internal forces could also play a role in apoptotic events. Secondary dentin (sd) production by the remaining slow-secreting odontoblasts (o) progressively decreases the pulp chamber volume (p). In carious teeth (left side), bacterial infiltration (bact) and BMP released from the dentin during the demineralization process will induce apoptosis of odontoblasts located at the carious lesion site (red cells). Apoptosis will stimulate expression of TGFβ. At a second time, BMP and TGFβ will play a role in the formation of reparative dentin (rd) by promoting differentiation of pulp cells into newly formed odontoblasts (no).
teeth, apoptosis occurs rapidly after cavity preparation and the apoptotic phenomena were absent two weeks post-surgery [37]. A novel finding of the present study is that cessation of apoptosis within the injury site does not occur so rapidly. Under most circumstances, clearance of apoptotic cells occurs with remarkable rapidity, without eliciting an inflammatory response [4]. However, clearance of the cellular debris of apoptotic odontoblasts was not possible, most probably because of their aspiration into the dentinal tubules and their subsequent isolation from neighboring cells. It is also possible that the duration and/or pathway of apoptosis may vary according to the species.

A number of significant physiologic changes (i.e. levels of signaling molecules, arterial oxygen content) normally accompany tooth pathology. In mild and medium diffused carious lesions, the underlying carious lesion field odontoblasts, as well as cells of the sub-odontoblastic layer, are eliminated by apoptosis, which is a form of cell death characterized by the activation of specific proteases, known as caspases. The involvement of apoptosis in the process of caries development has been demonstrated through the use of molecular and cellular techniques. For instance, the expression of transforming growth factor beta (TGF-beta) has been shown to be upregulated in the early stages of caries development, suggesting a potential role for this cytokine in the progression of the disease. Additionally, the involvement of various signaling molecules such as BMPs (Bone Morphogenetic Proteins) has been implicated in the regulation of cell apoptosis in the context of tooth development and repair.

In conclusion, the present study establishes a conceptual framework within which the mechanisms that regulate dental pulp chamber remodeling in physiological and pathological conditions can be elucidated (Fig. 5). If addressing apoptosis in tooth pathogenesis is to succeed as a therapeutic strategy, the first challenge will be to identify drugs capable to act by altering the levels of apoptosis.

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