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Apoptosis during Rat Tooth Development

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Teeth develop via a reciprocal induction between the ectomesenchyme originating from the neural crest and the ectodermal epithelium. During complete formation of the tooth morphology and structure, many cells proliferate, differentiate, and can be replaced with other structures. Apoptosis is a type of genetically-controlled cell death and a biological process arising at the cellular level during development. To determine if apoptosis is an effective mechanism for eliminating cells during tooth development, this process was examined in the rat mandible including the developing molar teeth using the transferase-mediated dUTP-biotin nick labeling (TUNEL) method. The tooth germ of the mandibular first molar in the postnatal rat showed a variety of morphological appearances from the bell stage to the crown stage. Strong TUNEL-positive reactivity was observed in the ameloblasts and cells of the stellate reticulum. Odontoblasts near the prospective cusp area also showed a TUNEL positive reaction and several cells in the dental papilla, which are the forming pulp, were also stained intensively in this assay. Our results thus show that apoptosis may take place not only in epithelial-derived dental organs but also in the mesenchyme-derived dental papilla. Hence, apoptosis may be an essential biological process in tooth development.

Key words : tooth development, apoptosis, TUNEL

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Introduction

Tooth development is a complex process that involves biological events, such as differentiation, morphological development and mineralization, and is controlled by the interaction between epithelial cells and ectomesenchymal cells. The tooth organ is formed by the ectodermal oral epithelium. The inner dental epithelium, a component of four layered-dental organs, is differentiated into ameloblasts, and the enamel is formed. The dental papilla and dental follicle are formed by ectomesenchymal cells originating from the neural crest. The dentin is formed by odontoblasts differentiated from some cells of the dental papilla, and dental pulp is formed by other mesenchymal cells. In addition, periodontal tissues, such as the periodontal ligament, cementum and alveolar bone are formed by fibroblasts, cementoblasts and osteoblasts differentiated from the cells of dental follicle, respectively (Ten Cate, 1994).

During tooth development, it was reported that complementary cellular events, such as cell division, migration and cell death, occurred continuously and the difference in cell division ability played an important role in tooth development (Ruch, 1995). Many studies also showed that some cells degenerated along with cell proliferation, such as enamel nodules during enamel formation (Lesot *et al*., 1996; Viriot *et al*., 1997), other cells forming dental laminae (Vaahtokari *et al*., 1996), Hertwig's epithelial root sheath related to dentin formation at the apical region (Suzuki *et al*., 2002), and various structures around the crown during tooth eruption (Kaneko *et al*., 1997) that had degenerated and disappeared. Similarly, in the developing tooth bud, the replacement of many cells is accomplished at various times (Nishikawa, Sasaki, 1995; Bronckers *et al*., 1996; Kaneko *et al*., 1997, Jernvall *et al*., 1998).

Many features of apoptosis observed by optical and electron microscopy, and through DNA ladder formation on electrophoresis can be distinguished from necrosis and

pathological cell death (Kerr *et al*., 1972; Wyllie, 1987). The morphological changes that occur during apoptosis include pycnosis, cell shrinkage, ruffled cell membrane, and the formation of apoptotic body and could be phagocytized by adjacent cells or macrophages (Steller, 1995). The TUNEL method is one of most widely used histochemical analytic methods of apoptosis, and several changes occurring during human development can be visualized easily using this method (Gavrieli, 1992; Wijsman *et al*., 1993).

This study examined the morphological changes in a developing tooth bud showing continuously repeated emergence and degeneration of cells at various time points as well as the distribution of apoptosis in a developing tooth bud involved in cell replacement.

Material and Method

All experiments were approved by the Animal Care and Use Committee of Chonnam National University. Postnatal 1 to 15 days rats including developing teeth were used. Each rat was sacrificed by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). After the blood were washed out using PBS (10 mM phosphate, pH 7.4, 0.15M sodium chloride) including heparin, perfusion fixation through the ear was performed using 4% paraformaldehyde-0.1M PBS solution and the lower jaw was extracted.

For optical microscopy, immersion fixation was carried out for 24 hours in the same solution and rinsed in PBS. The specimens were decalcified in a 10% EDTA solution (PH 7.4, 4°C) for 1 to 4 weeks, and then dehydrated in a series of ethanol, followed by clearing in xylene and paraffin embedding. Subsequently, 4 μm-serial sections were obtained from the embedded tissue on Probe On® slide glass (Fisher Scientific, Pittsburgh, PA, USA), dried and stained in hematoxylin-eosin for observation.

The TUNEL method was used to detect apoptosis. The deparaffinized sections were pre-treated with 5 μg/ml proteinase K (Sigma, St Louis, MO, USA) for 15 minutes at room temperature, rinsed several times in distilled water, and incubated in a 0.5% H_2O_2 solution for 30 minutes to eliminate the endogenous peroxidase. The following process was performed using an Apop tag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The sections were incubated in an equilibration buffer for 15 seconds and cultured in the mixed solution of terminal deoxynucleotidyl transferase and digoxygenin-dUTP (TdT reagent) for 1 hour at 37°C. To terminate the reaction, the sections were placed in Stop/wash buffer solution for 10 minutes at 37°C, and reacted in an anti-digoxygenin-peroxidase solution for 30 minutes at room temperature. Finally, the sections were stained in 0.002% 3,3'-diaminobenzidine tetrahydrochloride including 0.005% H_2O_2 in 0.05M Tris-HCl buffer solution to check the reaction. PBS was used instead of the TdT reagent as a negative control and all stained sections were counter-stained with Meyer's hematoxylin.

Results

General histological findings

The tooth germ of the mandibular 1st molar in the postnatal a day rat showed the configuration of the bell stage. The dental organ, which consisted of 4 layers, including the outer dental epithelium, inner dental epithelium, stellate reticulum and stratum intermedium, was markedly separated. The inner dental epithelium and adjacent dental papilla cells showed a short columnar shape, and the partially inner dental epithelium showed the morphological changes into long columnar cells in the portion forming a cusp in the future. A distinct acellular zone was observed between the dental organ and dental papilla (Fig. 1a, b). The crown stage was initiated by the formation of dentin at postnatal 3 days. The cells adjacent to inner dental epithelium of the dental organ differentiated into long columnar shaped odontoblasts and formed dentin. Subsequently, the inner dental epithelium differentiated into long columnar shaped ameloblasts and formed enamel above the dentin. The nuclei of odontoblasts and ameloblasts were located near the basement membrane, and exhibited the typical features of active secretory cells, i.e. polarity changes (Fig. 1c). In the cervical region of the tooth, dentin was formed by odontoblast differentiated from the dental papilla, and the Hertwig's epithelial root sheath consisted of an inner dental epithelial layer, and an outer dental epithelial layer was observed markedly for root formation (Fig. 1d). The dental organ was observed as a few cell layers due to the involution of stellate reticulum together with the progression of enamel formation (Fig. 1e).

TUNEL staining

The TUNEL staining results showed a positive reaction in some cells. TUNEL-positive cells were distributed mostly as either independent or aggregations of a few cells, and the positive cells included a small nucleus or occasionally multiple segments. No staining of the dental organ or dental papilla was observed at the postnatal day before the formation of dentin or enamel. After postnatal 3 days, at the beginning of crown stage, positive reactivity in odontoblasts or ameloblasts began to be observed. In particular, apoptotic changed cells in the stellate reticulum of the dental organ, which covered the early forming enamel could be observed (Fig. 2a, b). Moreover, at postnatal 5 days, a positive reaction was noted in some ameloblasts (Fig. 2c), and a few apoptotic changed-odontoblasts adjacent to the prospective cusp area showed the initial stages of dentin formation (Fig. 2d). A few perivascular cells in the dental papilla forming pulp (Fig. 2e) and dental follicular cells near the tooth germ were positively stained (Fig. 2f). Distinct brown stained cells were observed at the rat thymus used as a positive control (Fig. 2g)

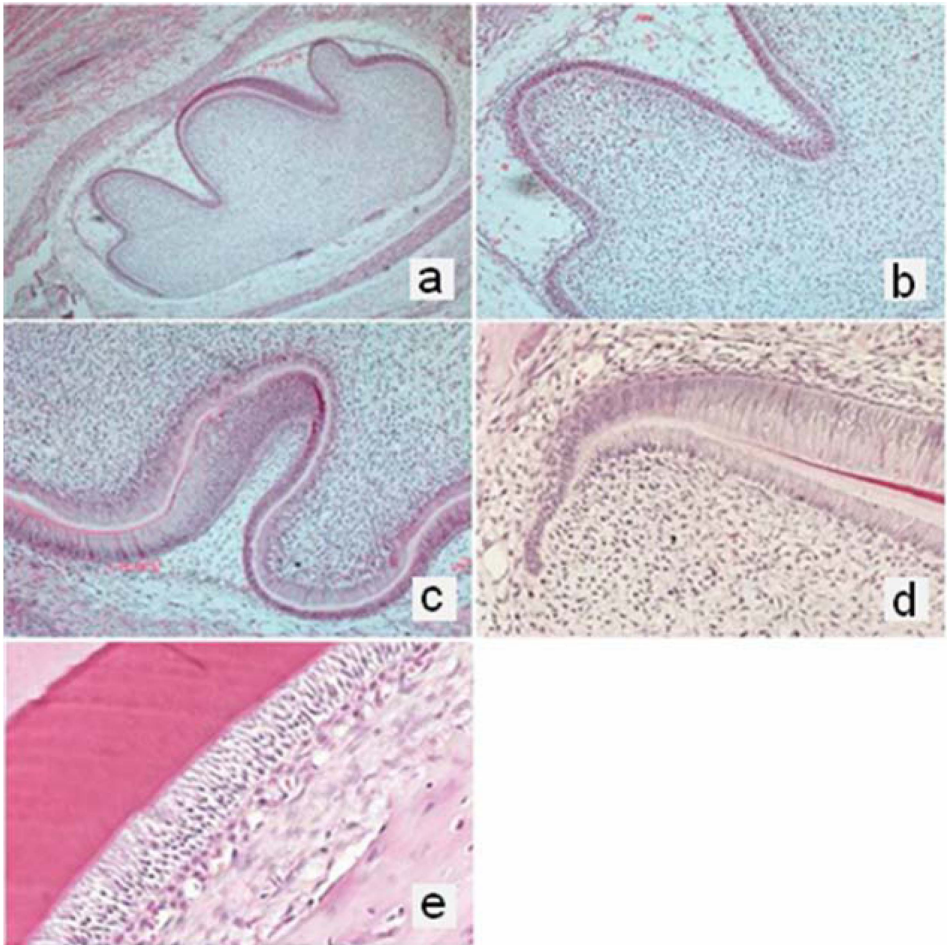

Fig. 1. General histological findings of mandibular 1st molar tooth germ by H-E stain. (a) Tooth germ of mandibular 1st molar in the postnatal a day rat shows the configuration of the bell stage for tooth development. × 40, (b) Higher magnification of Fig. 1a. Dental organ, which have 4 layers including outer dental epithelium, inner dental epithelium, stellate reticulum and stratum intermedium, is separated from dental papilla by acellular zone. × 200, (c) Tooth germ of mandibular 1st molar in the postnatal three-day old rat shows the configuration of crown stage for tooth development. H-E stain, × 200, (d) Hertwig's epithelial root sheath consists of the inner dental epithelium and outer dental epithelium. H-E stain, × 400, (e) Dental organ consists of a few cell layers due to involution of the stellate reticulum with the progression of enamel formation. H-E stain, × 400.

Discussion

The concept of apoptosis began as granular cytoplasm by optical microscopy in the early 1900s. Since Kerr *et al*. (1972) had found the distinct form of cell death from necrosis and called apoptosis many researchers regarded physiological cell death as genetically well-controlled that occurs during processes, such as embryonic development, metamorphosis or turnover of normal cells. Apoptosis can be identified morphologically by the optical and electronic microscopy examinations, and many additional methods for examining apoptosis have been proposed (Kim and Seo, 2006). Among them, the TUNEL method, which is labeled biotinylated dUTP by TdT to the 3′ ends of DNA breaks formed by endonuclease followed by stained avidin-conjugated peroxidase staining, was used in this study to detect apoptosis. This method has been used in a range of tissues (Gavrieli *et al*., 1992; Abe, and Watanabe, 1995; Rink *et al*., 1995). The studies for apoptosis during tooth development through the TUNEL method are extremely rare (Kaneko *et al*., 1997). In this study, the location of apoptotic changing cells in the tooth germ and surrounding tissue cells was identified correctly using TUNEL method.

In the maxillofacial region, there is some discussion on the relation of apoptosis in the resorption of teeth or the periodontal ligament, facial development and malformation (Viriot *et al*., 1997; Jernvall *et al*., 1998). In addition, apoptotic changes in the parenchymal cell of salivary gland were detected after experimental radiation on the salivary gland or ligation of the salivary duct (Choi, 1996). The appearance of apoptotic cells were also reported in the cells of the dental lamina or vestibular lamina (Vaahtokari *et al*., 1996), inner dental epithelial cells or ameloblasts (Shibata *et al*., 1995) and enamel knot in the dental organ, which is be believed to determine the cusp shape (Jernvall *et al*., 1998) during the development process in the oral cavity. In this study, apoptosis

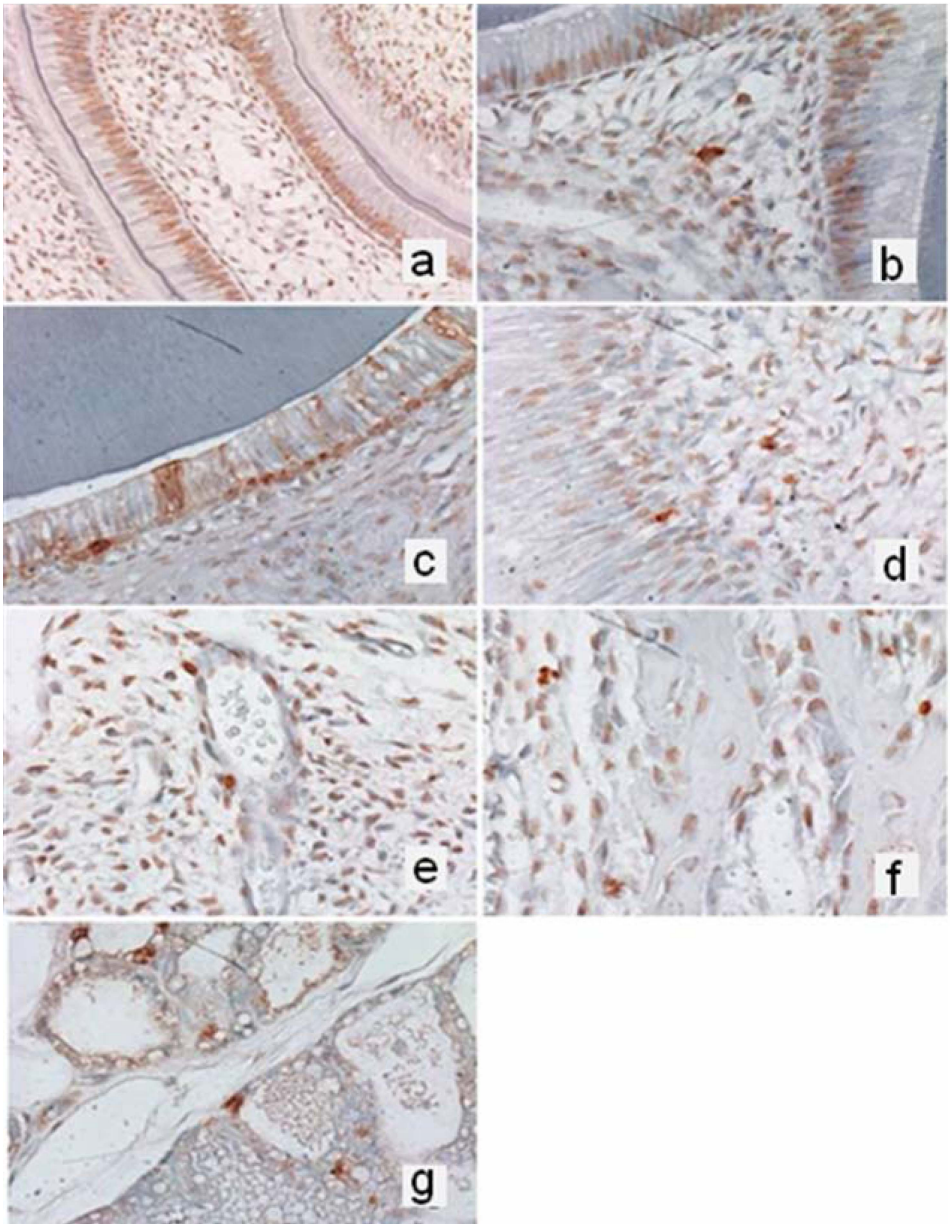

Fig. 2. Histological findings of mandibular 1st molar tooth germ by TUNEL stain. (a) TUNEL-positive nuclei are rare in the inner enamel epithelium and dental papilla cells in postnatal a day. × 200, (b) Strong TUNEL positive reactivity was observed in the cells of the stellate reticulum in a postnatal five day rat. × 400, (c) Strong TUNEL positive reactivity was also observed in the ameloblasts of a postnatal five day rat. × 400, (d) Several odontoblasts near the prospective cusp area include TUNEL positive nuclei. × 400, (e) A few perivascular cells in the dental papilla forming pulp are positively stained. × 400, (f) Dental follicular cells have TUNEL positively stained cells. × 400, (g) TUNEL stain in the rat thymus for the positive control. × 400.

appears to occur not only in the stellate reticulum or inner dental epithelium, which consist of epithelial dental organ, but also the mesenchymal dental papilla cell or dental follicles of the surrounding crown. Therefore, it is believed that the continuous turnover of cells during tooth development can occur through apoptosis regardless of the developmental origin of tissues.

Considering the appearance time of apoptosis during tooth development, it appears to be related to the degree of cell differentiation or functionality. In this study, the tooth germ of the mandibular 1st molar at the postnatal a day did not show any TUNEL positive reactivity, but apoptotic cells appeared at the beginning of the crown stage along with dentin formation after postnatal day 3. Apoptosis increased after the atrophy of ameloblasts and stellate reticulum became morphologically defined. From this point of view, the cells that completed their functional performance or demanded replacement to new types of cells disappear through an apoptotic mechanism. During tooth eruption, apoptosis observed in the resorbing bony crypt or reduced dental epithelium can also be

understood by the same mechanisms (Shibata *et al*., 1995).

Although apoptosis is a type of effective cell death during tooth development, other types of cell death can also occur (Kaneko, 1997). Clarke (1990) reported four types of cell death that could be distinguished from apoptosis. Numerous studies concerning the apoptosis mechanism have been progressed. They focused primarily on examining the signaling pathways occurring with apoptosis, such as the involvement of bcl-2/bax family (Korsmeyer 1992; Kim, 2008) and the caspase cascade (Shigemura *et al*., 2001) using a range of molecular biological tools. p53 and myc gene were reported to be related to apoptosis (Bissonnette *et al*., 1992; Hermeking, Eick, 1994) and have been applied to many biological research fields in relation to determining the cell fate determination. However, the mechanisms for apoptosis during tooth development are unclear. Therefore, further studies using the molecular biological methods will be needed.

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