Root resorption of primary molars without successor teeth. An experimental study in the beagle dog

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Tooth agenesis is a common craniofacial congenital malformation in humans, but little is known about the mechanisms of root resorption in this condition. The purpose of this study was to investigate the mechanisms of root resorption in primary molars without successors. An animal model without permanent tooth germs was established by surgery in beagles. The times of onset of primary molar root resorption, with and without successors, were compared. The distribution of immune cells, odontoclasts, and their activating factors were determined by histochemistry and immunohistochemistry. Root resorption of primary mandibular molars without successors began later than physiological resorption. In primary molars without permanent germs, odontoclasts and immune cells were present mainly in the apical pulp at the start of root resorption, whereas in control teeth receptor activator of nuclear factor-κB ligand (RANKL)-positive cells were found mainly in the region of the periodontal ligament. $CD14^+$ and $CD3^+$ cells were found in both the pulp and the periodontal ligament region. These results suggest that the dental pulp of primary molars, as well as immune cells, may play an important role in root resorption in primary molars without permanent tooth germs.

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Root resorption is a physiological phenomenon of deciduous teeth. It is known that odontoclasts (which are smaller and have fewer nuclei than osteoclasts, and produce smaller resorption lacunae) are responsible for the resorptive process. Many factors are thought to be involved in root resorption, including the dental follicle of the underlying permanent teeth, the pulp, the periodontal ligament (PDL), and mechanical stress.

During the eruption of permanent teeth, the dental follicle and the stellate reticulum seem to be important in the resorption of the deciduous root (1, 2). The role of the dental follicle was suggested by KRONFELD (3). MARKS & CAHILL (1, 2) showed that the dental follicle of the permanent tooth controls the eruption process and regulates resorption events in the overlying bone and presumably also in the roots of the primary predecessor tooth. It was found that the dental follicle is necessary for tooth eruption in dogs and that specific cellular changes occur in the follicle at the onset of tooth eruption (4). Monocytes enter the follicle from the microvasculature and then migrate to the walls of the bony crypt to participate in the formation of the eruption pathway (4, 5).

Research on time-related histological changes in root resorption (6) has shown that the odontoclastic resorption of coronal dentin occurs just before shedding. From this, it may be assumed that the pulp of deciduous teeth plays an active role in the physiological root-resorption process. The dental pulp cells of deciduous teeth could regulate this by secreting cytokines and transcription factors, which mediate the formation of osteoclasts/odontoclasts from the monocyte–macrophage lineage (7, 8). ANGELOVA et al. (9) showed that the number of immunocompetent cells within the pulp of the primary tooth increases as physiological root resorption progresses and suggested that the immunocompetence of deciduous teeth is altered by this process.

In all the studies described above, root resorption was initiated and coordinated by the dental follicle of the permanent tooth germ. However, the roots of primary teeth that do not have permanent successors will also eventually be resorbed. How does this occur? What are the major factors triggering physiological root resorption in a deciduous tooth without a successor permanent tooth? The present study was designed to explore the cellular events that occur at the onset of root resorption in primary molars without permanent tooth germs, and to investigate the mechanisms involved.

Material and methods

Animal model

All dogs used in this study were purebred male beagles (Beijing Marshall Biotechnology, Beijing, China). They were housed in light- and temperature-controlled rooms and

allowed access to food and water *ad libitum*. Their care and the experimental procedures employed in this study were in accordance with the guidelines of the US National Institutes of Health regarding the care and use of animals for experimental procedures, and the recommendations of the *Beijing Administration Rules of Laboratory Animals*. The study was formally reviewed and approved by the Biomedical Institutional Review Board of Peking University (no. LA2010-018).

We first established an animal model without permanent tooth germs by surgically removing the permanent mandibular premolar germs from 10-wk-old male beagles weighing 3.5 kg, as described previously (1, 2). The dogs were anaesthetized with intravenous pentobarbital sodium (30 mg/kg; Sigma-Aldrich, St Louis, MO, USA). The intra-oral surgical approach to the mandibular premolar germs was via full-thickness mucoperiosteal flaps on the lateral aspect of the respective jaw (Fig. 1A, B). After flap reflection, the locations of the premolar germs were determined from preoperative periapical radiographs. The overlying cortical bone was removed gently with a water-cooled fissure bur at high speed. Special care was taken to protect the primary molar roots. The dental follicles were revealed and excavated after pieces of bone had been gently removed (Fig. 1B). Periapical radiographs confirmed that the tooth buds and dental follicles had been completely excavated (Fig. 1C). After washing of the wound with normal saline, the flap was closed and the resorption of the mandibular molar roots was observed periodically. All surgical procedures were performed by the first three authors.

Determination of root resorption

All periapical films were taken by the same technician using the bisecting angle technique with a projection angle of −37° and an exposure time of 0.1 s. Periapical films were taken every week after surgery until all of the primary molars had exfoliated. Blurring of the root and the periapical tissue was considered to indicate early resorption, and exfoliation constituted the end of root resorption. The periapical films were interpreted by four independent, experienced clinicians (the authors), and the time of onset of root resorption was determined for each primary tooth. In the event of disagreement, a joint decision was made by all of the authors.

Eight beagles were studied. The permanent premolar germs of the primary mandibular right 2nd and 4th molars and the left 3rd molars were removed as described in the section 'Animal model'. The contralateral permanent premolar germs were left untouched, and the corresponding primary molars were used as controls. The time of onset of root resorption was recorded for all of these teeth.

Specimens

Specimens of primary molars with and without permanent successors were collected before root resorption had begun and at the onset of root resorption. An intact primary root was defined as the absence of blurring of the root and the periapical tissue on periapical films (both groups, $n = 6$); the onset of root resorption was defined as minute blurring (both groups, $n = 6$).

The specimens were fixed with 4% paraformaldehyde solution (4% paraformaldehyde in 0.01 M PBS, pH 7.4) for 24 h at 4°C, rinsed in Tris-buffered saline (TBS) (pH 7.4), and demineralized with 10% ethylenediaminetetraacetic acid (EDTA) at 4°C. After decalcification, the specimens were rinsed in TBS, dehydrated in a graded series of ethanol, and embedded in paraffin wax. Serial 5-μm sections were cut and stained with haematoxylin and eosin or used for histochemistry and immunohistochemistry.

Tartrate-resistant acid phosphatase staining

Histochemical demonstration of tartrate-resistant acid phosphatase (TRAP) activity was performed according to BURSTONE (10), as described previously (11). In brief, sections were incubated for 30–60 min at 37°C in 0.1 mM acetate-buffered medium (pH 5.4) containing naphtha AS-BI and Fast Red Violet (Sigma Chemical Co., St Louis, MO, USA), along with 50 mM sodium tartrate (Sigma). After incubation, the sections were washed in running water and stained with haematoxylin.

Immunohistochemistry

Antibodies to receptor activator of nuclear factor-κB ligand (RANKL) (1:400 dilution) and CD14 (1:50 dilution) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while antibodies to CD3 (1:100 dilution) were purchased from Abcam (Cambridge, UK). The antibodies were diluted with TBS, as indicated. Sections were first incubated in 0.3% H_2O_2 for 30 min to reduce endogenous peroxidase activity, then incubated in trypsin at 37°C for 10 min to retrieve antibody activity, and, finally, incubated with one of the primary antibodies overnight at 4°C, followed by consecutive incubations with Polymer Helper and Poly Peroxidase-anti-Goat/Rabbit/Mouse IgG (Polink-2 Plus HRP System Kits; Zhongshan Golden Bridge Biotechnology, Beijing, China). All incubations were followed by at least three washes in TBS. The sections were developed with a 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zhongshan Golden Bridge Biotechnology), counterstained with haematoxylin, and examined under a light microscope.

Fig. 1. Establishment of an animal model without permanent tooth germs in a 10-wk-old male beagle. (A) Intra-oral photograph taken before surgery. (B) The tooth buds and dental follicles of the mandibular 2nd, 3rd, and 4th permanent premolars were surgically removed. (C) Periapical radiograph taken after the procedure to confirm complete removal of the tooth buds and dental follicles (arrows).

Negative-control staining was always conducted in parallel by incubating sections with TBS instead of primary antibody.

Statistical analysis

The Mann–Whitney U-test was used to compare the time of onset of root resorption between primary molars and primary molars without permanent successors, using SPSS software (version 17.0; SPSS, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Time of onset of root resorption

Root resorption in primary mandibular 2nd and 3rd molars began at a mean of 122 d and 110 d after birth, respectively, whereas in the absence of permanent tooth germs it began at a mean of 148 d and 145 d after birth, respectively (Table 1). Root resorption took 7–14 d in both the presence and absence of permanent premolar germs; the primary molars then exfoliated. Most of the primary mandibular 4th molars were lost prematurely owing to the ectopic eruption of the 1st permanent molar; therefore, the onset of root resorption could not be determined for these teeth.

Table 1

Comparison of the time of onset of root resorption in primary molars from Beagle dogs with and without permanent tooth germs

	Primary mandibular 2nd molars ($n = 8$)			Primary mandibular 3rd molars ($n = 8$)		
	Mean	Median	SD	Mean	Median	SD
Primary molars	122.50*	122.50	6.48	110.25†	112.00	4.95
Without permanent teeth	147.88*	147.00	6.94	145.25†	147.00	4.95

The values represent the number of days after birth.
*,†Mann–Whitney U-test ($P < 0.05$).

TRAP staining and immunohistochemistry

Before the onset of root resorption, no TRAP-positive multinucleated cells were found in the experimental teeth (Fig. 2A) or in the control teeth.

At the onset of root resorption, TRAP-positive multinucleated cells were mainly found along the PDL side, close to the permanent tooth germs (Fig. 2B). In primary molars without permanent tooth germs, many TRAP-positive multinucleated cells were found along the odontoblast layer in the apical pulp (Fig. 2C); no TRAP-positive cells were found along the PDL side at the onset of root resorption.

Immunohistochemistry showed that, before the onset of root resorption, RANKL$^+$, CD14$^+$, and CD3$^+$ cells were sparse within the pulp and the odontoblast layer, as well as in the PDL (Fig. 3A–F). There were no differences between the experimental teeth and the control teeth.

At the onset of physiological root resorption, RANKL$^+$ cells were found mainly in the region of the PDL (Fig. 4A, D), and few CD14$^+$ or CD3$^+$ cells were found in either the pulp or the PDL (Fig. 4B, C, E, F). At the onset of root resorption of primary molars without permanent successors, large numbers of RANKL$^+$, CD14$^+$, and CD3$^+$ cells were concentrated in the odontoblast layer (Fig. 4G–I), especially in the apical part, but were sparse in the central part of the pulp. RANKL$^+$, CD14$^+$, and CD3$^+$ cells were also sparse in the region of the PDL (Fig. 4J–L); this distribution was similar to that observed before root resorption began.

Discussion

Tooth agenesis is a common congenital craniofacial malformation in humans. The incidence of agenesis of permanent teeth, excluding 3rd molars, ranges from 3.4% to 10.1% (12), depending on the population studied; the mandibular 2nd premolar, the maxillary lateral incisor, and the maxillary 2nd premolar are the most susceptible. Most primary molars with missing permanent successors show delayed root resorption; however,
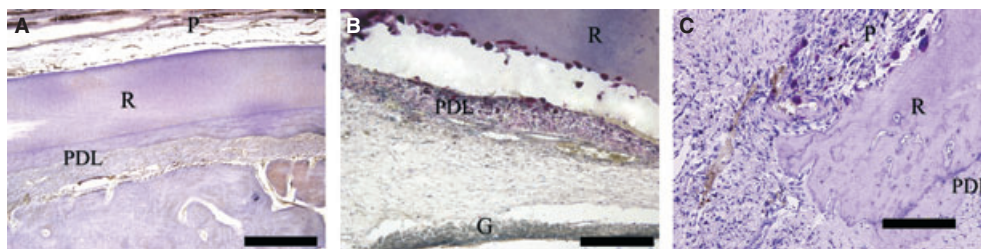

Fig. 2. Tartrate-resistant acid phosphate (TRAP) staining of mandibular 3rd primary molars, with and without permanent tooth germs at the onset of root resorption. Before the onset of root resorption, no TRAP-positive multinucleated cells were found in the experimental teeth. In primary molars without permanent tooth germs, TRAP-positive cells were found mainly along the odontoblast layer in the apical pulp, whereas in physiological root resorption, these cells were found mainly along the PDL side close to the permanent tooth germ. (A) Mandibular 2nd primary molar before the onset of root resorption. (B) Mandibular 2nd primary molar at the onset of root resorption. (C) Mandibular 2nd primary molar without permanent tooth germ at the onset of root resorption. G, permanent tooth germ; P, dental pulp; PDL, periodontal ligament; R, primary root. Bar = 200 μm.
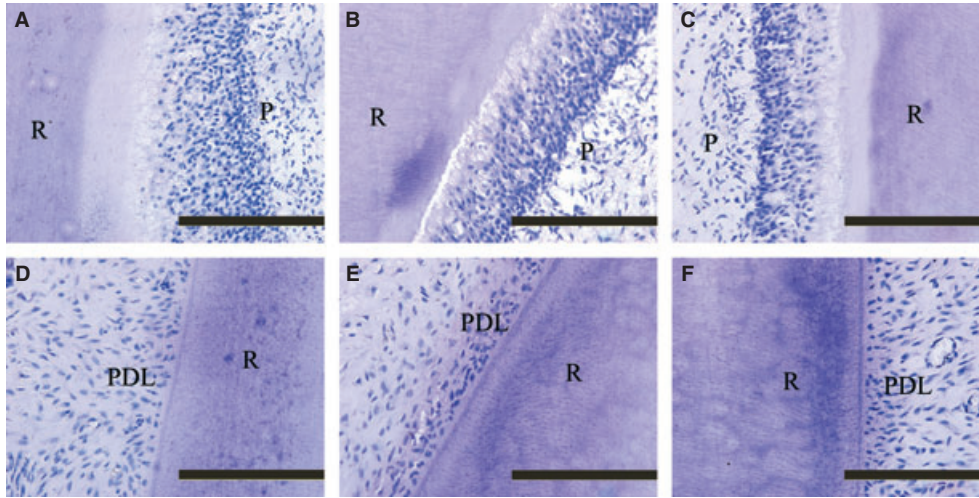
Fig. 3. Immunohistochemical staining of mandibular 2nd primary molars without permanent tooth germs before the onset of root resorption. Receptor activator of nuclear factor-κB ligand (RANKL)$^+$, CD14$^+$, and CD3$^+$ cells were sparse in the pulp and the odontoblast layer, as well as in the periodontal ligament (PDL). (A and D) RANKL staining; (B and E) CD14 staining; (C and F) CD3 staining. P, dental pulp; PDL, periodontal ligament; R, primary root. Bar = 200 μm.

they are eventually lost, especially those with pulpitis or periapical inflammation. These primary molars may play an important role in the development of occlusion and maxillofacial growth and their retention is essential for treatment planning in clinical practice. Many mechanisms for physiological root resorption in primary teeth have been suggested, but it has never been clearly explained how primary root resorption without a permanent successor occurs. Given the lack of a transgenic animal model with congenital absence of permanent tooth germs, and the difficulty of conducting this type of research in humans, we established an animal model, without permanent tooth germs, by surgery. The absence of permanent tooth germs in this model differs from congenital absence in humans and the procedure may have modified the histological environment of the primary roots, with a risk of inducing root resorption. To avoid these problems, surgery was performed during the 10th postnatal week. This is much earlier than the onset of physiological root resorption in the primary molars in beagles and thus allowed sufficient time for mandibular bone healing and minimized the effect of the dental follicle.

After surgery, root resorption began later in primary molars without permanent premolar germs than in normal primary molars. This result is similar to the findings of previous research (2) and suggests that the contribution of the dental follicle of the permanent tooth germ to root resorption had been reduced. Before the onset of root resorption, no CD3$^+$ or CD14$^+$ cells were found in the pulp or the PDL of primary molars without permanent germs, indicating that their presence at the onset of root resorption was not a consequence of surgery or of infection following surgery.

Staining for TRAP showed that, at the onset of root resorption in primary molars without permanent germs, odontoclasts were located mostly in the apical pulp and the odontoblast layer, but not in the PDL.

This suggests that, in the absence of permanent tooth germs, TRAP-positive mononuclear cells might enter the dental pulp before the onset of root resorption. Here they fused to form odontoclasts, and inner rather than outer resorption was dominant at the onset of resorption in these primary teeth. This differs from physiological root resorption, in which resorption starts at the part of the root that is closest to the permanent successor (13). It also further confirms that, in our animal model, the effect of the dental follicle of permanent tooth germs on the resorption of primary predecessors was minimized.

Receptor activator of nuclear factor-κB ligand is a member of the tumour necrosis factor superfamily and is expressed by marrow stromal cells, osteoclast precursors, and activated T cells. It plays a critical role in osteoclast migration, differentiation, activation, and survival (14, 15). Our RANKL staining results suggest that, at the onset of root resorption in primary molars without permanent tooth germs, the pulp may be more important for odontoclastic resorption than the PDL. Osteoclasts are multinucleated members of the monocyte/macrophage family (16), and CD14 is a marker of monocytes. The concentration of CD14$^+$ cells in the odontoblast layer and the apical pulp indicates that pre-odontoclasts may be initially recruited and activated in these areas.

T lymphocytes could play an important role in the resorption of dental hard tissues through the generation of cytokines that activate macrophages. Furthermore, activated T lymphocytes generate RANKL, which may contribute to root resorption in primary teeth (17). Our immunohistochemical staining results show that the number of T lymphocytes (CD3$^+$ cells) was markedly increased in the apical pulp and odontoblast layer at the onset of root resorption in primary molars without permanent tooth germs. This suggests that the immune system may have been involved in triggering the degradation of these primary teeth. The underlying mechanism
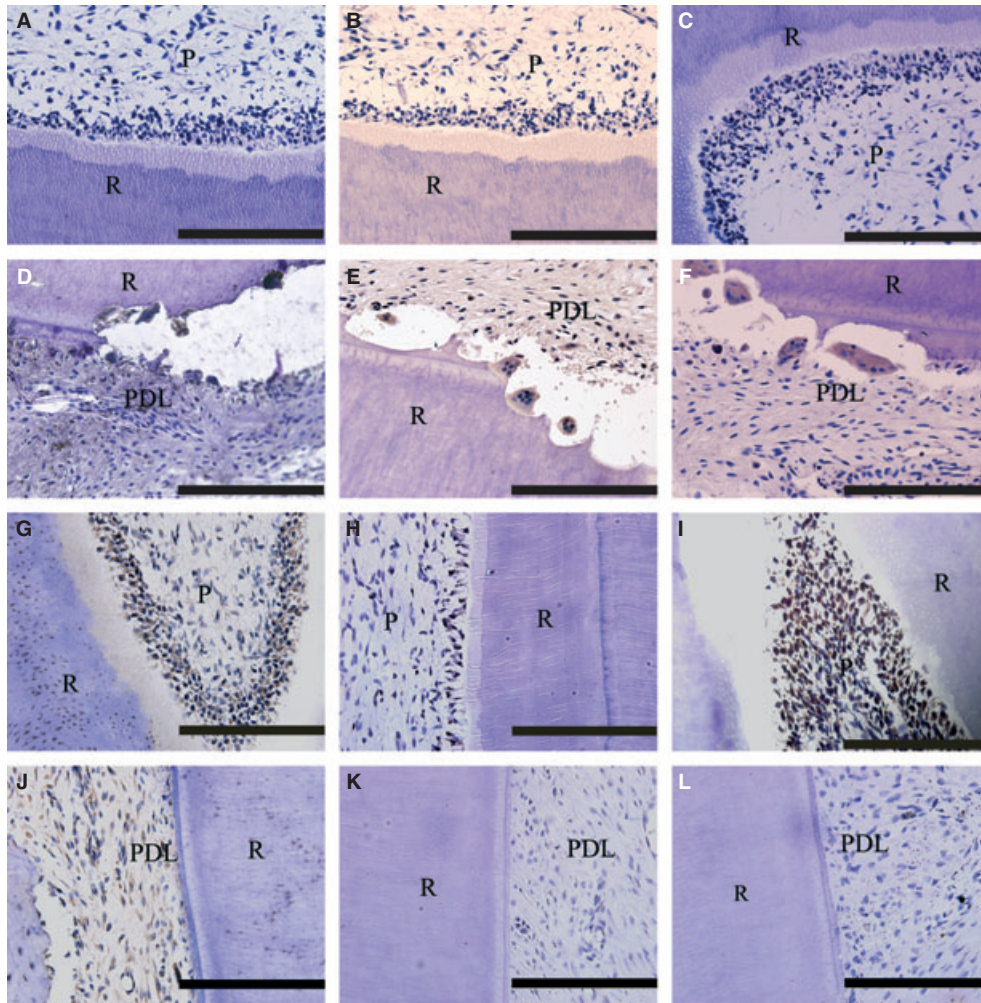
Fig. 4. Immunohistochemical staining of mandibular 2nd primary molar without permanent tooth germ and of control mandibular 2nd primary molar at the onset of root resorption. In primary molars without permanent tooth germs, receptor activator of nuclear factor-κB ligand (RANKL)$^+$, CD14$^+$, and CD3$^+$ cells were concentrated in the odontoblast layer, especially in the apical part, but were sparse in the periodontal ligament (PDL). At the onset of physiological root resorption, RANKL$^+$ cells were found mainly in the region of the PDL, and CD14$^+$ and CD3$^+$ cells were found in both the pulp and the PDL. (A, D, G, and J) RANKL staining; (B, E, H, and K) CD14 staining; and (C, F, I, and L) CD3 staining. P, dental pulp; R, primary root. Bar = 200 μm.

could be an increase in the number of opportunities for local antigen presentation. This would lead to the activation of T lymphocytes and thereby contribute to the immunodefence of pulp tissue against stimuli (e.g. exposure of dentinal tubules) that may occur from the degeneration of odontoblasts in primary teeth. The fact that only a few CD14$^+$ and CD3$^+$ cells were found in the pulp and the PDL at the onset of physiological root resorption suggests that the immune system was less involved in the activation of odontoclasts than it was in the absence of permanent successors. In the presence of the dental follicles of permanent teeth, other mechanisms may be involved in the initiation of root resorption, as previously reported (5, 18).

The present study shows that, during root resorption in primary molars of beagle dogs without permanent tooth germs, odontoclast and odontoclastogenic factors/cells are initially concentrated in the odontoblast layer and apical pulp. Further research is needed to clarify the underlying mechanisms. Our results suggest that the dental pulp of primary molars, as well as immune cells, may play important roles in root resorption in primary molars without permanent tooth germs.

Conflicts of interest – The authors report no conflicts of interest.

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