

Cementocyte cell death occurs in rat cellular cementum during orthodontic tooth movement

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ABSTRACT

Objective: To clarify the mechanism of root resorption during orthodontic treatment, we examined cementocyte cell death and root resorption in the cellular cementum on the pressure side during experimental tooth movement.

Materials and Methods: Using 8-week-old male Wistar rats, the right first molar was pushed mesiobuccally with a force of 40 g by a Ni-Ti alloy wire while the contralateral first molar was used as a control. Localization and number of cleaved caspase-3-positive and single-stranded DNA (ssDNA) - positive cells were evaluated using dual-label immunohistochemistry with anticlaved caspase-3 and anti-ssDNA antibodies. In addition, tartrate-resistant acid phosphatase (TRAP)-positive cells in the cellular cementum were evaluated using TRAP histochemical staining.

Results: Caspase-3- and ssDNA-positive cells appeared at 12 hours, but were restricted to the compressed periodontal ligament (PDL) and not the cellular cementum. Cleaved caspase-3-positive cementocytes were observed in the cellular cementum adjacent to the compressed PDL on day 1. From days 2 to 4, the number of caspase-3- and ssDNA-positive cementocytes increased. TRAP-positive cells appeared on the cellular cementum at the periphery of the hyalinized tissue on day 7, and resorption progressed into the broad surface of the cementum by day 14.

Conclusion: Cementocytes adjacent to the hyalinized tissue underwent apoptotic cell death during orthodontic tooth movement, which might have been associated with subsequent root resorption. (*Angle Orthod.* 2017;87:416–422)

KEY WORDS: Root resorption; Orthodontic tooth movement; Cell death; Apoptosis; Cementocyte

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INTRODUCTION

Root resorption is one of most common side effects of orthodontic treatment.^{1–7} Root resorption most frequently occurs in the maxillary incisors,^{2,3} with a prevalence of 73% and an average decrease in root length of 1.421 mm after orthodontic treatment.^{2,3} Resorbed root ends and surfaces are repaired by cementoblasts after the discontinuation of exerted force.⁴ However, there have been cases of severe root resorption with strong influences on the function and stability of the affected teeth.⁵ Although various possible factors for root resorption have been suggested,^{5,6} it is difficult to predict root resorption prior to orthodontic treatment.⁶

Orthodontic tooth movement involves complicated biological processes characterized by sequential reactions of periodontal tissue in response to biomechanical forces. Several processes are induced on the pressure side, including the disturbance of blood flow (ischemia/hypoxia) as a result of compression of the periodontal ligament (PDL), cell death and matrix

degradation (hyalinization) of the PDL, and resorption of the hyalinized tissue and bone.^{1,7} Root resorption is spatially associated with hyalinization on the pressure side. Although studies of orthodontic tooth movement have mainly examined the response of PDL cells, several studies have examined the role of osteocytes in alveolar bone remodeling.^{8–12} For example, mRNA expression of cytokines and matrix molecules in osteocytes were elevated in response to orthodontic forces.^{9–10} In addition, osteocytes adjacent to hyalinized PDL underwent cell death via apoptosis, which might have been associated with subsequent bone resorption.^{11,12} However, there is little information on the response of cementocytes during orthodontic tooth movement.

Cementum is a specialized calcified tissue that covers tooth roots^{13–15} and can be classified into the following two types: acellular cementum that contains no cell components and is restricted to the coronal half of the root, and cellular cementum that contains cementocytes in lacunae and is localized to the apical half of the root.¹³ Cementocytes have numerous cell processes throughout the canaliculi that are used to communicate with neighboring cementocytes and cells lining the cementum surface, which are similar to those of osteocytes.^{13,14} In addition, cementocytes share their matrix proteins with osteocytes, such as type I collagen, osteopontin, and osteocalcin.¹⁵ Considering these similarities, we hypothesized that similar apoptotic changes might occur in cementocytes during hyalinization on the pressure side of the PDL.

In this study, we examined cementocyte cell death and subsequent resorption of the cellular cementum on the pressure side during experimental tooth movement using dual-label immunohistochemistry with anti-cleaved caspase-3 and anti-single-stranded DNA (ssDNA) antibodies as well as histochemistry using tartrate-resistant acid phosphatase (TRAP).

MATERIALS AND METHODS

Male 8-week-old Wistar rats were used in this study. Experimental tooth movement was performed using an apparatus that consisted of a stainless steel tube for support and a straight nickel-titanium alloy wire that delivered continuous heavy force (Figure 1A). In this system, the right maxillary first molar was pushed mesiobuccally with an initial force of 40 g. The initial force magnitude was calibrated using a tension gauge (DTG-10NP; Mitutoyo, Kawasaki, Japan) on the plaster model used to fabricate the apparatus. The left maxillary first molars were used as the controls in each rat. The experimental periods were 6 and 12 hours and 1, 2, 4, 7, and 14 days after the application of force; 5 animals were used for each time point. The

experimental protocols were approved by the Animal Care and Use Committee of the Health Sciences University of Hokkaido.

The maxilla was dissected, fixed at 4°C for 24 hours in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), decalcified in 10% ethylenediaminetetraacetic acid at 4°C for 6 weeks, and embedded in paraffin. The paraffin-embedded specimens were cut horizontally to a thickness of 5 µm. The sections were either stained with hematoxylin and eosin or used for immunohistochemistry and TRAP histochemistry.

For the quantitative analysis, five paraffin sections in the apical region, to a depth of 100–250 µm from the apex of the mesial root of the maxillary first molar, were used per animal (Figure 1B). Immunohistochemical staining was performed as described previously.¹⁶ After deparaffinization, the sections were treated with proteinase K, rinsed with distilled water, and then incubated in 50% formamide diluted in distilled water at 56°C for 20 minutes. The sections were rinsed with cold phosphate buffered saline (PBS) for 5 minutes and incubated with 3% Block Ace (Dainippon Sumitomo Pharma, Tokyo, Japan) at room temperature for 20 minutes. The sections were incubated with the anti-ssDNA antibody (Enzo Life Sciences, Farmingdale, N.Y.) overnight at 4°C, rinsed with PBS, and then incubated with a goat anti-mouse immunoglobulin M (IgM) antibody conjugated to Alexa 568 (Cell Signaling Technology, Danvers, Mass.) at room temperature for 15 minutes. After extensive rinsing with PBS, the sections were incubated with an anti-cleaved caspase-3 antibody (Cell Signaling Technology) followed by goat anti-rabbit IgG conjugated to Alexa 488 (Cell Signaling Technology) as described previously. The sections were rinsed with PBS, mounted with Pharma Fluor (Thermo Fisher Scientific, Waltham, Mass.), and observed under a confocal laser scanning microscope (TCS-NT; Leica, Heidelberg, Germany). For the quantitative analysis, cementocytes positive for caspase-3 and ssDNA antibodies were counted on the distopalatal side of the mesial root. The distopalatal side in the apical region appeared to be a pressure area because the applied force tipped the crown of the tooth mesiobuccally and the root apex in the opposite direction. To define the observation area, the line bisecting the distopalatal angle created by the mesiodistal and buccopalatal long axes of the mesial root was used as a reference line (Figure 1C). The area extended 100 µm from the reference bisection in both directions in width.

Five paraffin sections per animal were used for the quantitative analysis of cementoclasts. After deparaffinization, the sections were treated with an Acid Phosphatase, Leukocyte Kit (Sigma-Aldrich, St. Louis, Mo) and counterstained with an acidic hematoxylin

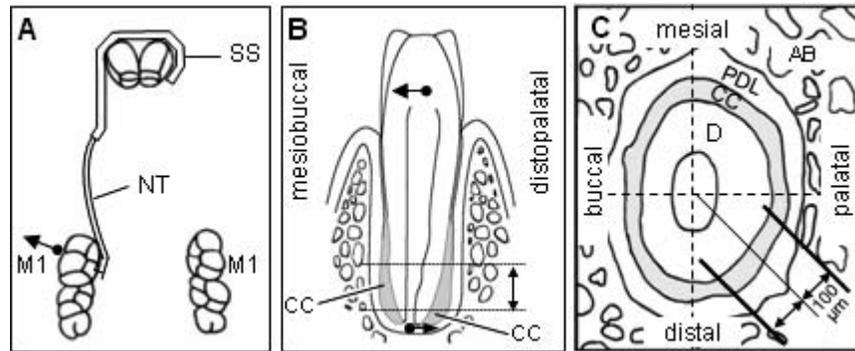


Figure 1. Schematic drawings of (A) the experimental apparatus used for tooth movement and (B, C) the observation area. (A) Using a cantilever Ni-Ti wire (NT), the first molar was forced in the mesiobuccal direction (arrow). (B) Horizontal paraffin sections were made in the apical region of the mesial root where the applied force produced the pressure area on the distopalatal side. Arrows indicate the direction of tooth movement. Double-headed arrow indicates the observation range. (C) The observation area on the horizontal section extended 100 μm from the bisection (thin line) to either side (thick lines) in width. The dashed lines indicate the mesiodistal and buccopalatal long axes of the mesial root. AB, alveolar bone; CC, cellular cementum; D, dentin; M1, maxillary first molar; PDL, periodontal ligament; SS, stainless steel tube.

solution. TRAP-positive cells in contact with the cementum surface within the observation area were counted.

The statistical analysis was performed using SPSS 20.0 (IBM Corp, Armonk, NY). The data from the experimental and control sides were compared using Wilcoxon signed-rank tests, with $P < .05$ considered significant. Data are presented as means \pm standard deviations.

RESULTS

At 6 hours, the width of the PDL was narrower on the experimental side when compared with the control side (Figure 2A,B), and hyalinized tissue appeared broadly in the compressed PDL on day 2 (Figure 2C). On day

4, several empty lacunae appeared in the cellular cementum immediately adjacent to the hyalinized tissue (Figure 2D). Numerous large multinucleated cells were observed between the hyalinized tissue and bone surface. Small resorbed pits appeared on the cementum surface corresponding to the periphery of the hyalinized tissue on day 7 (Figure 2E). Resorption of cementum progressed by day 14 (Figure 2F).

Cells positive for cleaved caspase-3 and ssDNA antibodies were rarely observed after 6 hours in the experimental (Figure 3A) and control sides (data not shown). After 12 hours, caspase-3- and ssDNA-positive cells appeared, but were restricted to the compressed PDL. On day 1, a few cementocytes positive only for caspase-3 were observed in the

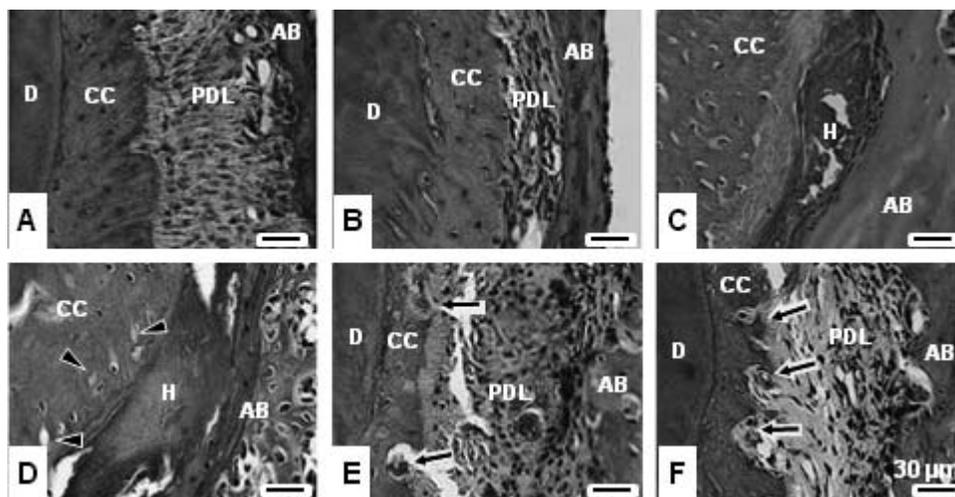


Figure 2. Photographs of horizontal sections of the first molar stained with hematoxylin and eosin. (A) Control side at 6 hours. (B) Experimental side at 6 hours. (C) Day 2. Hyalinized tissue appeared in the compressed PDL. (D) Day 4. Empty lacunae (arrowheads) appeared in the cementum. (E) Day 7. The cementum was resorbed by multinucleated cells (arrows) at the periphery of its surface, which had been in contact with the hyalinized tissue. (F) Day 14. Resorption of the cementum progressed. AB, alveolar bone; CC, cellular cementum; D, dentin; H, hyalinized tissue; PDL, periodontal ligament.

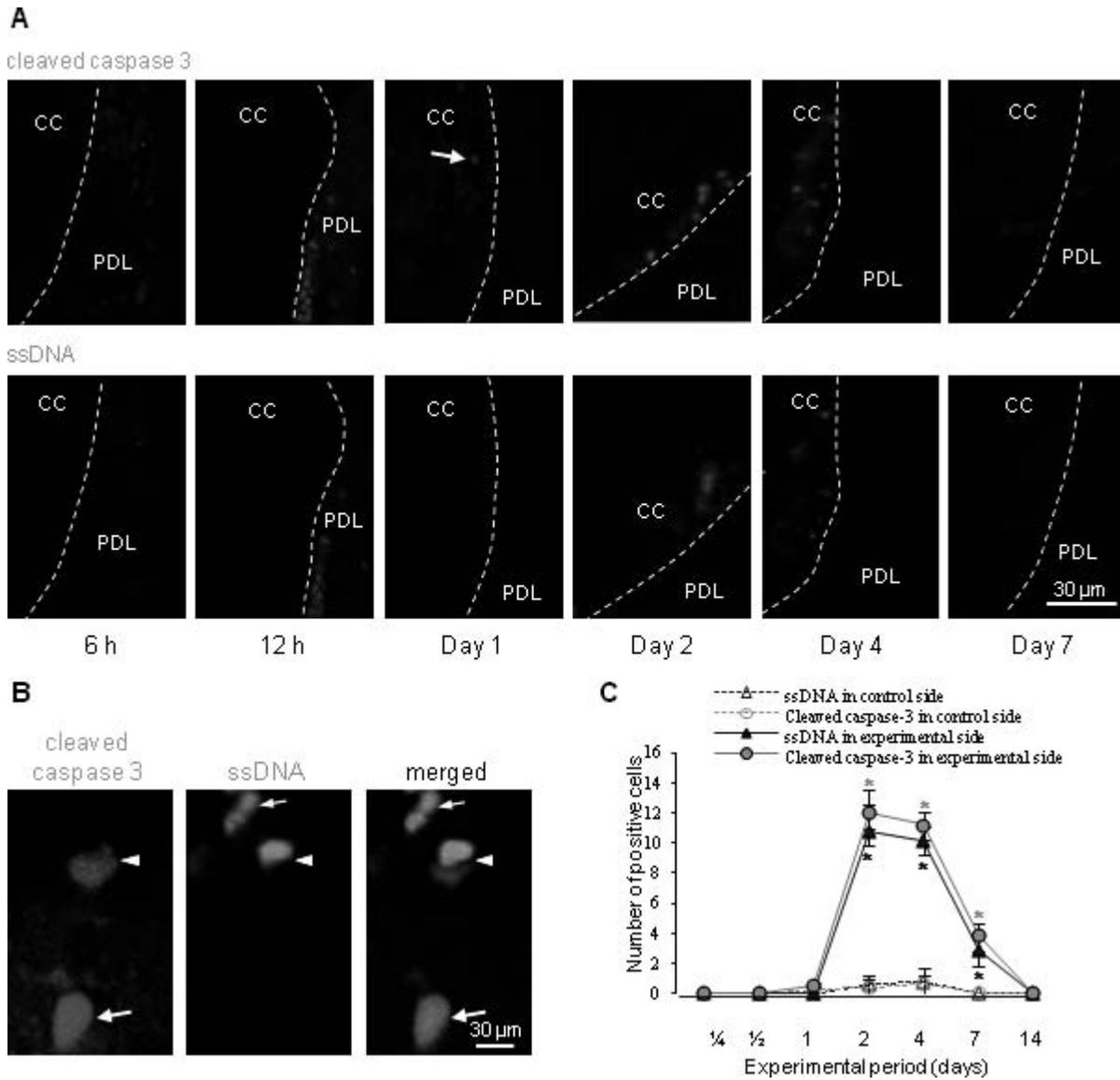


Figure 3. Photographs of horizontal sections of the first molar stained with the anticlaved caspase-3 antibody and single-stranded DNA (ssDNA) antibody. (A) At 12 hours, cells positive for caspase-3 and ssDNA appeared only in the compressed PDL. On day 1, cementocytes stained with only caspase-3 (arrow) were observed. On days 2 and 4, the number of cementocytes positive for caspase-3 and ssDNA increased. On day 7, most caspase-3 and ssDNA-positive cementocytes disappeared. Dashed lines indicate the boundary between the PDL and cementum. (B) Magnification of cementocytes on day 4. Caspase-3 single-positive cells (large arrow), ssDNA single-positive cells (small arrow), and double-positive cells (arrowhead). (C) Changes in the number of caspase-3 positive and ssDNA-positive cells in the cellular cementum. *Represents a significant difference from the control side ($P < .05$). CC, cellular cementum; PDL, periodontal ligament.

cementum adjacent to the compressed PDL. On days 2 and 4, several caspase-3- and ssDNA-positive cementocytes were observed in the cementum adjacent to the hyalinized tissue. Immunostaining of caspase-3 was localized in the cytoplasm and nuclei of cementocytes and was not observed in the cell processes. Immunostaining of ssDNA was localized to the shrunken round nuclei of cementocytes (Figure 3B). Cementocytes positive for only caspase-3 and ssDNA as well as double-positive cementocytes were observed. By day 7, the staining intensity and number

of cementocytes positive for both antibodies had decreased. The quantitative analysis showed that the number of caspase-3- and ssDNA-positive cells peaked from days 2 to 4 and then decreased (Figure 3C).

On day 4, many large multinucleated TRAP-positive cells were observed on the bone surface, but not on the cellular cementum (Figure 4A). On day 7, small TRAP-positive cells appeared on the cementum corresponding to the periphery of the hyalinized tissue that had previously been in contact with the hyalinized

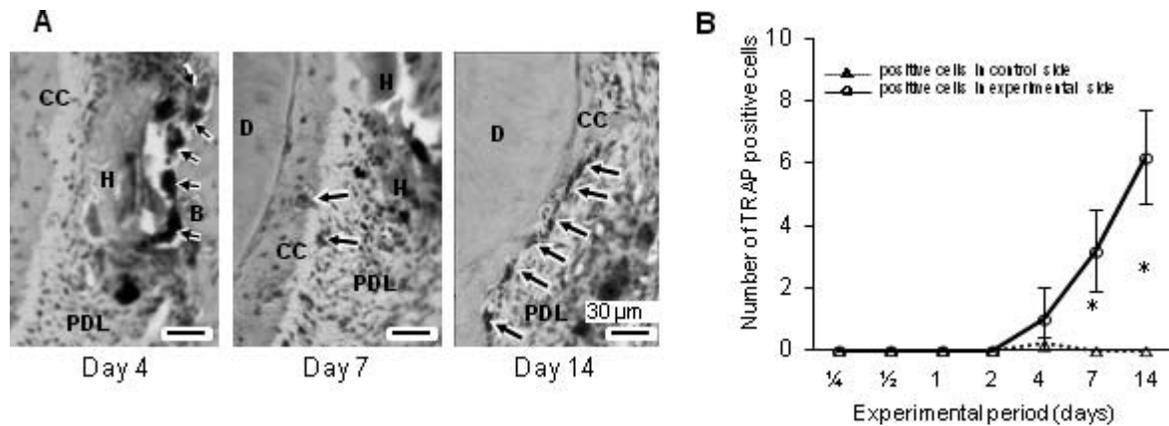


Figure 4. (A) Photographs of the horizontal sections of the first molar stained with tartrate-resistant acid phosphatase (TRAP). On day 4, numerous TRAP-positive cells (osteoclasts; small arrows) appeared. On day 7, the cementum was resorbed by TRAP-positive cells (cementoclasts; large arrows) at the periphery of the hyalinized tissue. On day 14, many cementoclasts appeared on the resorbed surface of the cementum. (B) Changes in the number of TRAP-positive cells. *Represents a significant difference from the control side ($P < .05$). AB, alveolar bone; CC, cellular cementum; D, dentin; H, hyalinized tissue; PDL, periodontal ligament.

tissue. There were many small TRAP-positive cells on the cementum surface on days 7 and 14. The quantitative analysis showed that the number of TRAP-positive cells on the cellular cementum were prominent on day 7, and increased in number until day 14 (Figure 4B).

DISCUSSION

Several studies have indicated that compressive force induces cell death of periodontal cells, such as PDL fibroblasts,¹⁷ osteocytes,^{9,11,12} and cementoblasts.⁵ Diercke et al.⁵ revealed that compressive force induced cementoblast apoptosis in vitro. In this study, we showed that heavy orthodontic force induced apoptosis of cementocytes on the pressure side. Cell death occurs mainly through the following three distinct pathways, depending on morphological criteria: apoptosis, necrosis, and autophagy.¹⁸ Apoptosis is the biological process of nuclear DNA fragmentation and involves chromatin condensation, nuclear fragmentation, and breakup into membrane-bound fragments while maintaining relative integrity of other cytoplasmic organelles.¹⁹ At the molecular level, various cell signaling pathways are involved in these morphological changes; caspases are key effector molecules in executing apoptosis. Caspases lead to the degradation of a variety of cellular proteins and the activation of endonucleases, which ultimately lead to the specific fragmentation of DNA. Currently, except when using electron microscopy, assessing both caspase activation and DNA fragmentation is recommended to evaluate apoptotic cell death in vivo.^{18,19} Therefore, we employed dual-label immunohistochemistry using anticlaved caspase-3 and anti-ssDNA antibodies.

Cementocytes positive only for cleaved caspase-3 first appeared on day 1, which indicated that caspase-3 was activated, but the DNA structure remained intact. This is consistent with the sequence of apoptosis, in which the activation of caspase-3 precedes DNA fragmentation in apoptotic cell death.^{16,19} Although most cementocytes were double positive, there were a few cementocytes positive only for ssDNA. The following are two plausible explanations: (1) caspase-3-negative, ssDNA-positive cells might reach the final stage of apoptosis, and (2) they might undergo caspase-independent cell death because recent studies have indicated a cell death pathway without the involvement of caspases (i.e., the caspase-independent pathway).²⁰

In this study, cementocyte cell death was spatially restricted to the cementum corresponding to hyalinized tissue, which also underwent cell death. Orthodontic force occludes PDL blood vessels on the pressure side of the PDL when it exceeds normal physiological conditions.¹⁷ This is accompanied by ischemia/hypoxia and disturbance of the nutrient-waste exchange process required to support PDL metabolism, which ultimately induces cell death.¹⁷ Cellular cementum has a lacuna-canalicular network similar to bone, which functions as a transport system of cellular molecules that support communication between the cementum and PDL.¹⁴ Therefore, it is plausible that PDL hyalinization elicits ischemia/hypoxia in the adjacent cementum and induces cementocyte cell death. In addition, the lacuna-canalicular network in bone regulates osteocyte survival. When bone is subjected to stress exerted via orofacial function, interstitial fluid flows throughout the network and transmits shear stress to osteocytes, which has an inhibitory effect on osteocyte apoptosis.⁸

Therefore, it is also plausible that PDL hyalinization induces cementocyte cell death by disturbing functional fluid flow in the canalicular network.

In this study, TRAP-positive cells were localized on the cementum surface immediately adjacent to the hyalinized tissue. This correlation is consistent with previous studies.⁷ In addition, the occurrence of hyalinization was linked to cementocyte cell death. Several explanations have been proposed regarding the biological significance of cell death in bone remodeling.^{8–12,21,22} For example, Bronckers et al.²¹ indicated that apoptotic osteocytes occurred near active resorption areas in rat alveolar bone and suggested the following two explanations: (1) dying osteocytes might interrupt the secretion of factors that inhibit osteoclastic activity, and (2) they might transmit signals through their canalicular networks to the bone surface to activate osteoclastogenesis. Recent studies found that dying osteocytes released immunostimulatory molecules through their lacuna-canalicular network toward the bone surface, which promoted proinflammatory cytokine secretion from macrophages and activated osteoclastogenesis.²² Considering the biological similarities between cementocytes and osteocytes, cementocyte cell death might induce root resorption via the lacuna-canalicular network during orthodontic treatment. Because the expression of regulatory factors of osteoclastogenesis, such as the receptor activators of nuclear factor- κ B and osteoprotegerin, have been found in an immortalized cementocyte cell line,²³ further studies should examine their expression in the cellular cementum during orthodontic tooth movement.

CONCLUSION

- Cementocytes in the cellular cementum adjacent to hyalinized tissue underwent apoptotic cell death during orthodontic tooth movement, which might have been associated with the subsequent cementum resorption.

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