Osteoprotegerin-Knockout Mice Developed Early Onset Root Resorption

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Abstract

Introduction: Recent studies indicate that the osteoprotegerin (OPG)/RANKL/RANK pathway takes part in root resorption. However, the relationship between OPG and root resorption is vague. The purpose of our study was to investigate the role of OPG in root resorption. **Methods:** The first molars of the mandibles of *osteoprotegerin*-knockout (*Opg*-KO) mice and wild-type (WT) mice were evaluated by micro–computed tomography, histology, and immunohistochemistry at 4, 6, 26, and 52 weeks. To detect the activity of the osteoclasts, we induced bone marrow macrophages into osteoclast-like cells from *Opg*-KO mice and wild-type mice *in vitro* and then compared their osteoclast activities. To evaluate the cementum quality, an osteoclast-cementum co-culture model was established *in vitro*. **Results:** In *Opg*-KO mice, root resorption began at the age of 4 weeks. At 6 weeks the cementum damage extended to the coronal and apical regions, and at 52 weeks the damage reached the predentin. At all observed stages, more tartrate-resistant acid phosphatase (TRAP)–positive cells were found on the surface of cementum in *Opg*-KO mice. *In vitro*, the mRNA levels of cathepsin K, TRAP, matrix metalloproteinase-9, and matrix metalloproteinase-1, as well as the protein expression of nuclear factor of activated T cell 1 and TRAP, increased significantly in osteoclast-like cells from *Opg*-KO mice. In addition, the cementum resorption pits of *Opg*-KO mice were larger when co-cultured with osteoclast-like cells. **Conclusions:** Our study demonstrated that loss of OPG led to root resorption via increasing activation of osteoclasts and reducing mineralization of cementum. *(J Endod 2016;42:1516–1522)*

Key Words

Cementum, OPG, osteoclast, root resorption

Significance

Root resorption is defined as a clinical phenomenon of physiological or pathologic defects in cementum. There are many causes of root resorption, such as trauma, inappropriate orthodontia force, and periapical periodontitis. Root resorption has raised extensive attention from dentists.

Root resorption is defined as a clinical phenomenon of physiological or pathologic defects in cementum or dentin. In clinical practice, there are many causes of root resorption, such as trauma, inappropriate orthodontia force, and periapical periodontitis (1), and root resorption has raised extensive attention from dentists. Under the conditions of inflammation or irritation, the root surface undergoes a series of physiological or pathologic changes that disturb the balance between cementum formation and resorption (2). The detailed mechanism of root resorption still remains unclear; this causes difficulties in prevention and treatment of root resorption. Previous studies indicate that overactivation of osteoclasts might be the main reason for root resorption (3–5). Moreover, the osteoprotegerin (OPG)/RANKL/RANK pathway is important for osteoclast differentiation and maturation (6, 7).

OPG is a soluble member of the tumor necrosis factor receptor family and a key inhibitory factor of osteoclastogenesis (8, 9). As a secreted extracellular receptor, OPG combines with different ligands and plays diversified biological roles in cell survival, proliferation, migration, and apoptosis (10–12). The *Opg*-knockout (KO) mouse model is a high-turnover osteoporosis model in which long bone and alveolar bone are affected and in which both osteogenesis and osteoclastogenesis are activated (13).

The relationship between OPG and osteoclasts has been extensively studied (11). However, little is known about how OPG affects cementum mineralization and root resorption. Our study supports the hypothesis that loss of OPG would strengthen the activity of osteoclasts and that osteoclasts are direct factors that initiate root resorption. In addition, our study demonstrates that loss of OPG would affect the mineralization of cementum and that malmineralization of cementum may accelerate the progress of root resorption. Loss of OPG affects both osteoclast activity and mineralization of cementum, and these effects jointly lead to the formation of root resorption.

Materials and Methods

Animals

Opg-KO mice and wild-type (WT) mice (C57BL/6 background) were provided by the Shanghai Research Center for Biomodel Organisms (Shanghai, China). They were genotyped by polymerase chain reaction (PCR) following the methods described previously (14). Experimental time points for radiographic, histologic, and cell culture analysis were 4, 6, 26, and 52 weeks ($n = 4$).

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Micro–Computed Tomography

The first molars of mandibles from *Opg*-KO mice were compared with those of WT mice. At 4, 6, 26, and 52 weeks of age, the animals were killed and perfused. The mandibles were dissected and further fixed in 4% paraformaldehyde for 48 hours. After fixation, the mandibles were digested alternately with 0.2% collagenase I (Invitrogen, Carlsbad, CA) and 0.25% trypsin (HyClone, Logan, UT) for 96 hours. Then the first molars were obtained under a stereoscopic microscope (Stemi 508; Carl Zeiss, Jena, Germany) with tweezers and needles. The density of each tooth was detected by micro–computed tomography-50 (Scanco Medical, Bassersdorf, Switzerland). The micro–computed tomography analysis included a high-resolution scan of the whole tooth (10-μm slice increment, $n = 4$).

Histology Analysis and Immunohistochemistry Staining

After demineralization in 10% EDTA (pH = 7.4; Biotech Well, Shanghai, China) at 4°C for 4 weeks, mandibles were embedded in paraffin and cut into 4-μm sections. Histologic characterizations of mice molars ($n = 4$) at 4, 6, 26, and 52 weeks were stained by hematoxylin-eosin staining (Biotech Well), tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich, St Louis, MO), Masson staining (KeyGEN, Shanghai, China), picrosirius red staining (Bogoo, Shanghai, China), and immunohistochemistry staining (IHC). IHC was performed with an UltraSitive S-P detection kit (MXB, Fuzhou, China). Primary antibodies included mouse anti–bone sialoprotein (BSP) (Abcam, Cambridge, MA; dilution, 1:400), mouse anti-osteopontin (OPN) (Abcam; dilution, 1:400), and mouse anti–dentin matrix acidic phosphoprotein (DMP1) (Abcam; dilution, 1:400). Control sections were incubated without any primary antibody, and the IHC procedures were performed in the same manner. Quantitative evaluation of IHC images was analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

Culture of Osteoclast-like Cells and Osteoclast-Cementum Co-cultured Model

Bone marrow cells from 6-week-old *Opg*-KO mice and WT mice were cultured for 24 hours with α-minimum essential medium (HyClone) containing 10% fetal bovine serum (Gibco, Rockville, MD) and 1% penicillin (10,000 IU)-streptomycin (10,000 μg/mL) (HyClone). Non-adherent cells named bone marrow macrophages (BMMs) were harvested as osteoclast precursors and were co-cultured with the first molars from the mandibles of 3-week-old *Opg*-KO mice and WT mice for 1 day in the presence of mouse macrophage colony-stimulating factor (50 ng/mL; PeproTech, Rocky Hill, NJ). Precursors of osteoclasts were then cultured in the presence of mouse RANKL (100 ng/mL; PeproTech) and macrophage colony-stimulating factor (50 ng/mL) for 6 days. These cells were then fixed in 4% paraformaldehyde and stained to detect TRAP activity. TRAP-positive cells with more than 3 nuclei were counted as osteoclasts. The molars were demineralized in 10% EDTA at 4°C for 1 week, embedded in paraffin, and cut into 10-μm sections. The sections were stained with hematoxylin.

Real-time Quantitative PCR

Total RNA of the molars ($n = 8$) and osteoclast-like cells was extracted by using Trizol (Invitrogen). The concentration of total RNA was quantified by spectrophotometric absorbance at a wavelength of 260 nm. cDNA was obtained by reverse transcription of total RNA with a Transcription First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The primer sets used in real-time PCR were designed by Primer Premier 5.0 software (Palo Alto, CA) and were commercially synthesized (Table 1). The efficiency of the newly designed primers was confirmed by conventional sequencing of the PCR products. The expression level of each gene was normalized to that of *GAPDH*, and all experiments were repeated 3 times.

Western Blot

Osteoclast-like cells were lysed in lysis buffer (1% Nonidet P-40; 150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0) containing protease and phosphatase inhibitors. Protein (20 μg per lane) was electrophoresed in a 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) at 4°C. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline (20 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl) containing 0.1% Tween-20 and 10% non-fat dry milk (blocking buffer). Subsequently, the membranes were incubated at 4°C overnight with rabbit anti–nuclear factor of activated T-cell 1 (NAFT1) (CST, Trask Lane Danvers, MA; dilution, 1:1000) and rabbit anti-TRAP (Santa Cruz Technology, Santa Cruz, CA; dilution, 1:1000). After the membranes were rinsed in phosphate-buffered saline 3 times, they were incubated with a peroxidase conjugated secondary antibody (Biotech Well; 1:5000 dilution), and the target proteins were developed with the ECL system (Millipore). Last, the target proteins were visualized by the Smart Chem TM Image Analysis System (Sage Creation Science, Beijing, China), and the grey levels were expressed as the percentage of GAPDH (Biotech Well; dilution, 1:2000) level. All experiments were repeated 3 times.

Statistical Analysis

The data are presented as the mean ± standard deviation (SD). All statistical analyses were performed by using the Student t test, and $P < .05$ was considered statistically significant.

Study Approval

The animal care and experimental protocols were approved by the Animal Use and Care Committee of TongJi University. The animal experiment approval number of the research was TJmed-013-31.

TABLE 1. Primers Used in Real-time Quantitative PCR

Genes	Forward primer 5′-3′	Reverse primer 5′-3′
Gapdh	GGGAAGCCCATCACCATCTT	GCCTCACCCCATTTGATGTT
Ctsk	CAGCAGAACGGAGGCATTGA	CTTTGCCGTGGCGTTATACATAC
Trap	CACCCTGAGATTTGTGGCTGT	CGGTTCTGGCGATCTCTTTG
Mmp-9	CCATGCACTGGGCTTAGATCA	GGCCTTGGGTCAGGCTTAGA
Mmp-1	TGAAGAATGATGGGAGGCAAGT	GGGTTTCAGCATCTGGTTTCC
Bsp	AGGACTGCCGAAAGGAAGGTTA	AGTAGCGTGGCCGGTACTTAA
Dmp1	AGTGAGTCATCAGAAGAAAGTCAAGC	CTATACTGGCCTCTGTCGTAGCC
Opn	GATCAGGACAACAACGGAAAGG	GCTGGCTTTGGAACTTGCTT

Ctsk, cathepsin k; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Mmp-9, matrix metalloproteinase-9; Mmp-1, matrix metalloproteinase-1.