Dual Role of 5-Lipoxygenase in Osteoclastogenesis in Bacterial-induced Apical Periodontitis

Francisco Wanderley Garcia Paula-Silva, DDS, MSc, PhD,† Igor Bassi Ferreira Petean, DDS,* Léa Assed Bezerra da Silva, DDS, MSc, PhD,* and Lúcia Helena Faccioli, PhD†*

Abstract

Introduction: The aim of this study was to evaluate the role of 5-lipoxigenase (5-LO) in the signaling for osteoclast formation and bone resorption in apical periodontitis (AP) after root canal contamination with oral bacteria. **Methods:** AP was experimentally induced in C57BL/6 mice because of contamination of the root canals left open to the oral environment. MK886 was used as a systemic inhibitor of 5-LO (5 mg/kg, daily). After 7, 14, 21, and 28 days, the animals were euthanized, and tissues were removed for gene evaluation by quantitative reverse transcriptase polymerase chain reaction, histologic analysis, and tartrate-resistant acid phosphatase staining. **Results:** Root canal contamination induced the expression of messenger RNA for 5-LO and leukotriene B4 receptors BLT1 and BLT2. The administration of the 5-LO inhibitor reduced early receptor activator of nuclear factor kappa-B and receptor activator of nuclear factor kappa-B ligand synthesis but augmented late receptor activator of nuclear factor kappa-B ligand and osteoprotegerin expression during the course of AP development. Interestingly, long-term inhibition of 5-LO resulted in increased bone resorption and induced tartrate-resistant acid phosphatase–positive osteoclast formation. The divergent findings related to 5-LO inhibition in osteoclastogenesis signaling, osteoclast formation, and bone resorption were accompanied by differently regulated inflammatory gene expression. *Il1b*, *Il11*, *Ccl3*, *Ccl7*, and *Spp* were down-regulated by the 5-LO inhibitor in early AP, but later on *Il11*, *Ccl3*, *Cxcl9*, *Cxcl15*, and *Spp* were up-regulated. **Conclusions:** 5-LO presented a dual role in osteoclastogenesis during the course of AP development. Early on, osteoclastogenesis signaling was down-regulated by the inhibition of 5-LO, but long-term inhibition failed to prevent synthesis of catabolic mediators that resulted in increased bone loss. *(J Endod 2016;42:447–454)*

Key Words

5-lipoxygenase, apical periodontitis, bone resorption, cytokines, osteoclast, osteoclastogenesis

Apical periodontitis (AP) represents a localized immune response against the microorganisms inside the dental root canal (1, 2) and is characterized by the presence of a mixed inflammatory infiltrate composed of lymphocytes, neutrophils, macrophages, and plasma cells depending on the stage of the disease (2, 3). An uncontrolled inflammatory response results in periapical bone resorption (2), albeit the mechanisms involved in the recruitment of hematopoietic lineage cells to differentiate into osteoclasts during development of the disease are not fully understood (4, 5).

Autocrine and paracrine signaling of cytokines and chemokines are important for osteoclast maturation and activity (6, 7). Receptor activator of nuclear factor kappa-B ligand (RANKL) is a soluble mediator; is a member of the tumor necrosis factor superfamily; is synthesized by osteoblasts, T lymphocytes, and endothelial cells; and binds to receptor activator of nuclear factor kappa-B in osteoclastic cells to promote tartrate-resistant acid phosphatase (TRAP) enzyme expression and bone resorption (4, 8). Osteoclastogenic signaling mediated by RANKL is blocked by the soluble decoy receptor osteoprotegerin (OPG) produced by osteoblasts under anabolic stimuli (8).

In an AP scenario, several inflammatory mediators are produced locally to orchestrate an immune response. Eicosanoids are among those molecules and represent a class of lipid mediators synthesized from arachidonic acid through the action of cyclooxygenases or lipoxygenases to generate prostaglandins and thromboxanes or leukotrienes and lipoxins, respectively (9). Lipoxygenase metabolites have been found in rat inflamed dental pulp and human AP (10–13), but only leukotriene B4 (LTB_4) is positively correlated to polymorphonuclear cell recruitment and pain (10, 11). LTB_4 binds G-coupled receptors (leukotriene B4 receptor 1 [BLT1] and leukotriene B4 receptor 2 [BLT2]), resulting in an increase in intracellular calcium and a reduction of cyclic adenosine monophosphate (cAMP) to mediate kinase activation, genic transcription, and, ultimately, cell recruitment (14, 15). Despite the fact that lipid mediators are produced in response to an infection in AP, the role of the 5-lipoxygenase pathway in disease severity and associated bone loss is not known.

Therefore, the aim of this study was to evaluate the role of 5-lipoxygenase in the signaling for osteoclast formation and bone resorption in AP after root canal contamination with oral bacteria.

Materials and Methods

Animals

C57BL/6 6-week-old male mice (*Mus musculus*, $n = 96$) were used for experimentation after institutional review board approval (#12.1.62.53.0). Animals were

From the *Department of Pediatric Clinics, School of Dentistry of Ribeirão Preto and †Laboratório de Inflamação e Imunologia das Parasitoses, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

Address requests for reprints to Dr Francisco Wanderley Garcia Paula-Silva, Avenida do Café, s/n 14040-904, Ribeirão Preto, SP, Brazil. E-mail address: franciscogarcia@usp.br

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anesthetized intramuscularly with ketamine hydrochloride (150 mg/kg [Ketamine 10%; National Pharmaceutical Chemistry Union Agener S/A, Embu-Guaçu, Brazil]) and xylazine (7.5 mg/kg [Dopaser, Labs Calier S/A, Barcelona, Spain]).

Operative Procedures

Animals were placed on a surgical table with a device for mandibular retraction. The upper and lower first molars of each animal were used; the right side was used for root canal contamination, and the left side was used as the control.

Occlusal root canal accesses were gained with 1011 spherical diamond burs (KG Sorensen IND. com Ltda, Barueri, SP, Brazil), root canals were located with a #06 K-file (Les Fils d' Auguste Maillefer S/A, Ballaigues, Switzerland), and the radicular pulp tissue was removed. Then, the root canals were left open to the oral environment for 7, 14, 21, and 28 days (n = 6 teeth per group per period). Experiments were repeated twice.

5-Lipoxygenase Pharmacologic Inhibition

MK886, a 5-lipoxygenase activator protein inhibitor (Merck Frosst Inc, Kirkland, QC, Canada), was dissolved in alcohol (100 μL), diluted in distilled water (400 μL), and administered by gavage (0.5 mL, 5 mg/kg body weight) 1 hour before root canal contamination and daily throughout the experimental period. Control animals received the vehicle only.

Total RNA Extraction

Animals were euthanized by intramuscular anesthetic overdose; then, tissues containing bone and tooth from the periapical area were dissected, collected, and snap frozen. RNA was extracted from a pool of 3 teeth using the RNeasy Mini kit (RNeasy Mini; Qiagen Inc, Valencia, CA) and samples treated with DNAse I (RNase-Free DNase Set, Qiagen Inc) according to manufacturer protocol. RNA integrity was analyzed using 1% agarose electrophoresis, and quantity was estimated using the NanoDrop 1000 (Thermo Fisher Scientific Inc, Wilmington, DE) at 260-ηm wavelength.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Complimentary DNA (cDNA) was synthesized from 700 ng total RNA using random primers (High Quality cDNA Reverse Transcriptase Kits; Applied Biosystems, Foster City, CA). Aliquots of 2 μL of the total cDNA were amplified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using primers for *Alox5* (Mm01182747), *Ltb4r1* (Mm02619879), *Ltb4r2* (Mm01321172), *Tnfrsf11a* (Mm00437135), *Tnfsf11* (Mm00441906), and *Tnfrsf11b* (Mm01205928) (TaqMan Gene Expression Assay, Applied Biosystems) in an Eppendorf Mastercycler ep Realplex (Eppendorf AG, Hamburg, Germany). *Gapdh* (Mm99999915) was used as reference gene. qRT-PCR reactions were performed in duplicate, and amplification was done under the following conditions: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative quantification was performed using the ΔΔCt method.

Morphometric Analysis of AP Size under Light Microscopy

Longitudinal tooth cuts were obtained, and analysis was performed in hematoxylin-eosin–stained sections using the microscope at 10× magnification in the bright field. In each specimen, the size of the periapical lesion was delineated in the section representing the most central part of the lesion, and the area was determined in μm^2 using Leica Application Suite V3.8 software and the Leica DM 5000B microscope (Leica Microsystems, Wetzlar, Germany). Delineation was performed excluding the intact tooth and bone structures (periodontal ligament, cementum, and alveolar bone).

TRAP Histoenzymology

Analysis of TRAP activity was performed as an indicator of active osteoclasts. The sections were deparaffinized in xylol, ethanol, and ethanol/acetone and left to dry at room temperature. Next, a solution was prepared with 10 mL acetic acid buffer, 0.1 mL N-N-dimethylformamide, 5 mg Fast Red Violet LB Salt (Sigma-Aldrich, St Louis, MO), and 1 mg Naphthol AS-BI phosphoric acid (Sigma-Aldrich) and then pipetted onto the sections that were maintained in a dark chamber at 37°C for 30 minutes. After incubation, the sections were counterstained with hematoxylin for 1 minute and examined with the microscope in the bright field. Quantitative analysis of the number of osteoclasts was determined by counting the number of multinucleate TRAP-positive cells in the resorption lacunae in direct contact with the alveolar bone around the periapical lesion. The results were expressed as the number of cells per lesion.

qRT-PCR Array

Global evaluation of genes involved in the inflammatory response was performed using a commercially available qRT-PCR array (Mouse inflammatory cytokines and receptors RT^2 Profiler PCR Array, PAMM-011A; Qiagen Inc). Total RNA was amplified starting with 300 ng using a commercially available kit (PreAMP cDNA synthesis, Qiagen Inc). *Gusb*, *Hprt*, *Hsp90ab1*, *Actb*, and *Gapdh* were used as reference genes. qRT-PCR reactions were performed in duplicate in an Eppendorf Mastercycler ep Realplex (Eppendorf AG) using SYBR Green (Qiagen Inc). Amplification was done under the following conditions: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The dissociation curve was performed to determine the specificity of the primers considering the melting temperature of the amplicon under the following conditions: increasing the temperature to 95°C for 15 seconds followed by decreasing the temperature to 60°C for 15 seconds, gradually increasing the temperature to 95°C for 20 minutes, and maintaining the temperature at 95°C for 15 minutes. Relative quantification was performed using the ΔΔCt method.

Statistical Analysis

Data were analyzed using 1-way analysis of variance followed by the Dunnett test for comparison of gene expression over time in teeth with and without AP or using 2-way analysis of variance followed by the Bonferroni test for comparison of the effects of 5-lipoxygenase inhibition on AP development over time (α = 0.05).

Results

Root Canal Contamination Induces 5-LO, BLT1, and BLT2 Gene Expression and Administration of 5-LO Inhibitor Reduced Early RANK and RANKL Synthesis but Augmented Late RANKL and OPG Expression in AP

Root canal contamination induced 5-lipoxygenase (*Alox5*), LTB_4 receptor 1 (*Ltb4r1*), and LTB_4 receptor 2 (*Ltb4r2*) gene expression compared with healthy teeth at 14 days after AP induction. At 7, 21, and 28 days, *Alox5* and *Ltb4r2* were not modulated by root canal bacterial contamination, whereas *Ltb4r1* expression was inhibited compared with healthy teeth (Fig. 1*A–C*).

Because 5-lipoxygenase and LTB_4 receptors were induced by root canal contamination, we sought to investigate the functional role of 5-