Effect of *Enterococcus faecalis* Lipoteichoic Acid on Apoptosis in Human Osteoblast-like Cells

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Abstract

Introduction: *Enterococcus faecalis* is commonly detected in persistent apical periodontitis characterized by unimproved periradicular bone resorption. The aim of the present study was to examine the effect of lipoteichoic acid (LTA), a major virulence factor of *E. faecalis*, on apoptosis of osteoblasts. **Methods:** Human osteoblast-like MG63 cells were treated with LTA from *E. faecalis* at a series of concentrations for 48 hours. The proliferation of the MG63 cells was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5- diphenyl-tetrazolium bromide assay. To examine the apoptosis, the LTA-treated cells were analyzed by flow cytometry by using annexin V–fluorescein isothiocyanate and propidium iodide double staining. Hoechst 33258 staining was performed to observe the morphologic changes of the cells. To investigate the apoptosis at the molecular level, the protein levels of Bcl-2 and Bax were determined by Western blot. Meanwhile, the caspase-3 activity was detected with caspase colorimetric protease assay. **Results:** The proliferation of MG63 cells was inhibited by *E. faecalis* LTA in a dose-dependent manner. Flow cytometry assay indicated that LTA had a stimulating effect on MG63 cell apoptosis. Typical morphologies of apoptotic cells were observed under fluorescence microscope. Furthermore, the cell apoptosis was confirmed by (1) the down-regulation of the antiapoptotic protein (Bcl-2), (2) the up-regulation of the proapoptotic protein (Bax), and (3) the elevated caspase-3 activity. **Conclusions:** LTA of *E. faecalis* could inhibit the proliferation and induce apoptosis of human osteoblast-like MG63 cells. *(J Endod 2013;39:632–637)*

Key Words

Apoptosis, *Enterococcus faecalis*, lipoteichoic acid, osteoblast

Microorganisms play a fundamental role in the pathogenesis of apical periodontitis, in large part resulting from their cell wall virulence factors (1). *Enterococcus faecalis* is a gram-positive bacterium, a facultative anaerobe commonly detected in root-treated teeth with persistent periapical lesions (2). *E. faecalis* can colonize dentin and survive even in harsh conditions (3). This organism contains several virulence factors such as lipoteichoic acid (LTA), peptidoglycan, aggregation substance, cytolysin, and lytic enzymes (4). Among virulence factors, LTA is closely involved in pathogenicity according to the following aspects:

1. LTA from *E. faecalis* (Ef LTA) could stimulate leukocytes to release several mediators that are known to play a role in various phases of the inflammatory response (5).
2. Ef LTA is also involved in biofilm formation and adhesion to teeth because of its adsorptive activity to hydroxyapatite (6).
3. Opsonic antibodies to *E. faecalis* are mostly generated against epitopes on the LTA (7).

Hence, Ef LTA is considered an important virulence factor in persistent apical periodontitis.

Apical periodontitis refers to a group of inflammations around the root apex that affect periodontal ligament, bone, cementum, and also dentin when there is root resorption. Apical periodontitis most commonly causes periapical bone destruction. The healing of resorbed periapical bone is mainly dependent on the number and function of osteoblasts. In physiological conditions, bone resorption is followed by osteoblast-mediated bone formation (8). During bone formation, osteoblasts undergo an orderly developmental progression that ultimately ends in apoptosis (9). The balance of osteoblast proliferation and apoptosis determines the osteoblast population (10). Thus, the increased apoptosis of osteoblastic cells may negatively affect the repair of periapical bone destruction.

Apoptosis is a programmed cell death that can be triggered by various signals (11). Studies have shown that the Bcl-2 protein family and the caspase family are involved in cell apoptosis and determine the cellular commitment to apoptosis (12). Members of the Bcl-2 protein family can be divided into 2 types: (1) proteins such as Bcl-2 that suppress apoptosis and (2) others such as Bax that promote apoptosis (13). Caspases are a family of cysteine proteases that are highly conserved in multicellular organisms. An increased caspase activity is a hallmark of apoptosis induction. Caspase-3 is considered the central executioner member of this family (14).

It has been found that there is a positive correlation between the amount of apoptotic osteoblast and the size of periapical lesion (15). Although *E. faecalis* is closely associated with persistent apical periodontitis (16, 17), there are no reports investigating the effects of LTA on bone-forming osteoblasts, particularly osteoblast

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apoptosis. The purpose of this study was to assess the effect of LTA of *E. faecalis* on the apoptosis in osteoblasts.

Materials and Methods

Chemicals and Reagents

LTA from *E. faecalis* was provided by Sigma-Aldrich (product number L4015; St Louis, MO). Dulbecco modified Eagle medium was obtained from Gibco (Grand Island, NY). Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining kit and caspase-3 activity assay kit were obtained from Beyotime Institute of Biotechnology (Beyotime, Haimen, China). Mouse/rabbit monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The chemicals used in the experiments were purchased from Sigma-Aldrich unless otherwise stated.

Cell Culture and Preparation of LTA

Human osteosarcoma MG63 cells have been shown to have an osteoblast-like phenotype (18) and have been widely used as a model for human osteoblast. The cells were purchased from the American Type Culture Collection and cultivated in Dulbecco modified Eagle medium (pH 7.4) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified (90%) incubator with 5% CO_2. The culture medium was renewed every 3 days, and cells from passages 4–6 were used for the experiments. Ef LTA was dissolved in phosphate-buffered saline at 5 mg/mL as stock solution and sterilized by passage through a 0.22-μm filter. Before application, stock LTA solution was diluted by culture medium to selected concentrations. All solutions were made under room temperature, and the pH was adjusted to 7.4.

Cell Proliferation Inhibition Assay

The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to evaluate the effect of Ef LTA on the proliferation of the MG63 cells. In brief, MG63 cells were seeded in 96-well plates (Corning Inc, Corning, NY) at 5×10^3 cells/well. After overnight incubation, the culture medium was replaced by fresh medium containing Ef LTA at the final concentrations 0.2, 2, 20, 100, and 200 μg/mL, respectively, for 48 hours. For each group, 4 wells were taken. Twenty microliters MTT (5 mg/mL) was added to each well, and the cells were incubated for extra 4 hours. After the medium was discarded, the purple formazan crystal was treated by 150 μL dimethyl sulfoxide for 15 minutes. The optical density (OD) of each well was determined at 490 nm by using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). Nontreated samples were set as control. Cell proliferation inhibition rate was defined as $(1 - OD_{\text{experimental group}}/OD_{\text{control group}}) \times 100\%$. All the observations and assays were repeated in triplicate.

Quantification of Apoptotic Cells by Flow Cytometry

Apoptotic cells were detected by annexin V-FITC and PI staining. Briefly, after the treatment with or without LTA at the indicated concentrations for 48 hours, the cells were centrifuged at 1000 rpm for 5 minutes, and the pellet was resuspended in binding buffer to a concentration of 1×10^6 cells/mL. An aliquot of 500 μL cell suspension was stained simultaneously with 5 μL FITC-conjugated annexin-V and 5 μL PI for 20 minutes in the darkness. The apoptotic cells were measured by fluorescence-activated cell sorter (Becton Dickinson Bioscience, Franklin Lakes, NJ). Early apoptotic cells were positive for annexin V and negative for PI, whereas late apoptotic cells were positive for both annexin V and PI.

Morphologic Examination of Cell Apoptosis by Hoechst 33258 Staining

MG63 cells were seeded in 6-well plates at 2.5×10^4 cells/well. After overnight incubation, the cells were treated or untreated with LTA at the indicated concentrations for 48 hours. Triple wells were used for each group. The cells were then stained with Hoechst 33258 at a final concentration of 5 mg/mL for 30 minutes. Nuclear morphology was examined under a fluorescent microscope (Olympus, Tokyo, Japan). Quantitative analysis was performed by counting the apoptotic cells (chromatin condensation, nuclear margination, and disintegration of the nuclear membrane) from 5 randomly selected fields at ×200 magnification. Values were expressed as the percentage of apoptotic cells relative to the total number of cells per field.

Western Blotting for Bcl-2 and Bax Expression

After the treatment with or without LTA at the indicated concentrations for 48 hours, the MG63 cells were harvested and lysed in lysis buffer. Lysates were centrifuged at 12,000 rpm for 15 minutes, and the supernatant was collected. The total protein concentration was measured by using a bicinchoninic acid assay kit (Pierce, Rockford, IL). The total protein of 30 μg per sample was separated by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After being blocked with 5% nonfat milk, the membrane was incubated with the primary antibody at 4°C overnight. Then the membrane was washed and incubated with the horseradish peroxidase–conjugated secondary antibody for 2 hours. The band intensity was measured by using Quantity One Software (Bio-Rad Laboratories). The β-actin antibody was used as loading control.

Analysis of Caspase-3 Activity by Using a Colorimetric Method

To investigate caspase-3 activation after treatment with LTA, a caspase-3 colorimetric assay kit was used. Briefly, 1×10^6 cells treated with different concentrations of LTA for 48 hours were collected, and

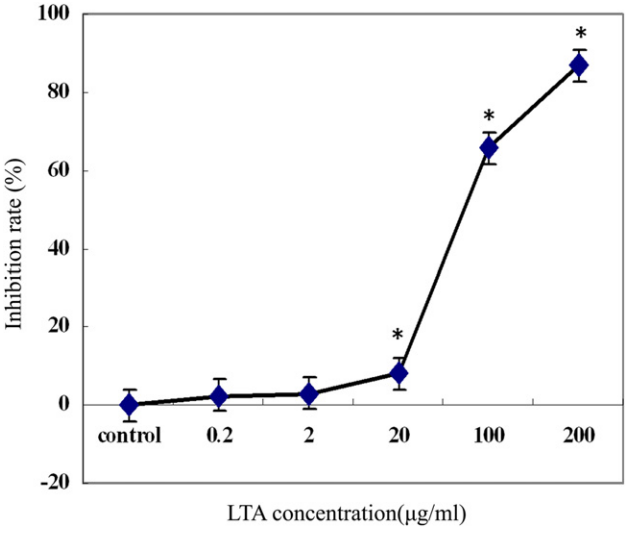

Figure 1. Inhibition of MG63 cell proliferation by LTA from *E. faecalis*. MG63 cells were treated with different concentrations of LTA (0, 0.2, 2, 20, 100, and 200 μg/mL, respectively) for 48 hours, and the proliferation of cells was detected by MTT assay. After LTA treatment, the cell proliferation inhibition rate was higher than that in control group in a dose-dependent manner. *$P < .05$ versus control group.