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 and Hoechst 33258 staining. 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, cytolsins, 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...
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% CO2. 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 × 10^4 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<sub>experimental group</sub>/OD<sub>control group</sub>) × 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 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.

**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.
lysed in a lysis buffer. The cell lysates were tested for protease activity by using Asp-Glu-Val-Asp-pNA (DEVD-pNA), a tetrapeptide p-nitroanilide substrate. After incubation with this substrate for 2 hours, the absorbance was measured at 405 nm by using a microplate reader. The caspase enzymatic activities in cell lysates were directly proportional to the color reaction.

**Statistical Analysis**

These data were from experiments performed in triplicate. Values were expressed as the mean ± standard deviation. Statistical analyses were performed by using one-way analysis of variance, followed by Student-Newman-Keuls multiple-comparison test. Statistical significance was set at \( P < .05 \).

Figure 2. Analysis of apoptosis induced by LTA from *E. faecalis* in MG63 cells by flow cytometry by using annexin V–FITC and PI staining. Quadrant analysis of fluorescence intensity of gated cells in FL1-H (annexin V–FITC) and FL2-H (PI) channels was assessed from 10,000 events. Cells in the lower right quadrant represent early apoptotic cells, and cells in the upper right quadrant represent late apoptotic cells. (A) Control group, (B) 25 µg/mL LTA-treated group, (C) 50 µg/mL LTA-treated group, (D) 100 µg/mL LTA-treated group, and (E) the early and late apoptotic rates of MG63 cells induced by different concentrations of LTA. *\( P < .05 \) versus control group.
Results

**E. faecalis LTA Inhibited the Proliferation of MG63 Cells**

As shown in Figure 1, the cell proliferation inhibition rate was significantly increased in a LTA–dose-dependent manner. No significant difference was found in the 0.2 μg/mL and 2 μg/mL LTA-treated samples, when compared with the control. The proliferation inhibition rate was remarkably increased from 8.05% to 65.56% at the concentration of LTA increasing from 20 μg/mL to 100 μg/mL. MTT assay showed that Ef LTA inhibited the proliferation of MG63 cells in vitro in a dose-dependent manner.

**Figure 3.** Observation of MG63 cell apoptosis by Hoechst 33258 staining under a fluorescence microscope (original magnification, × 200). After MG63 cells were treated with different concentration of LTA from *E. faecalis* for 48 hours, Hoechst 33258 staining was used to assess apoptotic cells (arrows). The number of apoptotic cells was increased in a LTA–dose-dependent manner with marked morphologic changes found in cell apoptosis: condenser chromatin and disintegration of the nuclear membrane. (A) Control group, (B) 25 μg/mL LTA-treated group, (C) 50 μg/mL LTA-treated group, (D) 100 μg/mL LTA-treated group, and (E) the percentages of apoptotic cells in control group and LTA-treated groups were compared. *P* < .05 versus control group.
Effect of LTA from *E. faecalis* on activation of caspase-3 in MG63 cells. After MG63 cells were treated with different concentrations of LTA from *E. faecalis* for 48 hours, caspase-3 activity was examined by colorimetric assay. Caspase-3 activity was significantly increased in response to LTA treatment. *P < .05 versus control group.

**Figure 5.**

Effects of LTA on apoptosis of MG63 cells. (A) LTA from *E. faecalis* induced apoptosis of MG63 cells. 

**Discussion**

LTA was found on the cell surfaces of many gram-positive bacteria and was shed during bacterial replication (19). Therefore, LTA might accumulate in and diffuse from the tissues where *E. faecalis* resides and proliferates. When confronted with the host immune response, *E. faecalis* that invaded in the periapex can be broken down (20). The products of *E. faecalis* breakdown may also be released into the neighboring tissues. Hence, LTA in periapical lesions seems to be a combined result of the diffusion of LTA from *E. faecalis* in root canal system and the products of *E. faecalis* breakdown in the periapex. Indeed, there is an epithelial lining in many periapical lesions, which may act as a barrier and prevent the neighboring host cells from being affected. However, researches have proved that *E. faecalis* has the ability to adhere to and invade through epithelial cells (21, 22). In addition, LTA from gram-positive bacteria has been shown to disrupt epithelial barriers and to promote bacterial invasion (23). Therefore, although the protective epithelial lining in periapical lesions can slow down the invasion of bacteria and their products to the host tissues, LTA may still be capable of affecting osteoblasts in the vicinity of the periapex. Despite the frequent presence of *E. faecalis* in persistent apical periodontitis, its impact on periapical lesions is rarely investigated. The present study was designed to explore the effect of LTA on osteoblast proliferation and apoptosis.

The effect of LTA on proliferation inhibition rate of MG63 cells was assessed by use of MTT assay. The concentrations of LTA used in the present study, ranging from 0.2 to 200 μg/ml, were based on our pilot experiments. Our results showed that LTA exhibited a potent inhibition on MG63 cell proliferation, particularly when the concentration was increased from 20 to 100 μg/ml. This indicated that LTA at high concentration could significantly decrease the proliferation of osteoblasts. On the basis of this finding, the LTA concentrations of 25, 50, and 100 μg/ml were selected for the rest of the experiment. The

**E. faecalis* LTA Down-regulated the Protein Expression Ratio of Bcl-2/Bax and Elevated the Caspase-3 Activity**

Bcl-2 family proteins, which have either antiapoptotic or proapoptotic activities, have been suggested to play a critical role in the regulation of apoptosis. The ratio of antiapoptotic to proapoptotic proteins determines the fate of cells. We examined the levels of expression of Bcl-
rationale for this selection is to focus on the range of LTA concentrations at which active apoptosis may most likely be induced. Therefore, we have reasons to believe that the findings of the present in vitro study can be clinically relevant. Opposite to our results, a previous study by Kargianni et al (24) found that heat-inactivated endodontic E. faecalis stimulated the proliferation of ovine osteoblast-like cells. This discrepancy might be due to the species specificity, which may result in a different biological response and sensitivity of cells when exposed to LTA. Another reason for the discrepancy might be related to the fact that the compositions of heat-inactivated E. faecalis were different from LTA.

Next, the causes to inhibit the proliferation of MG63 cells were investigated. The flow cytometry analysis showed that the differences in the apoptosis rates between control cells and LTA-treated cells were significant. The apoptosis in MG63 cells was induced by LTA treatment in a dose-dependent manner. Meanwhile, the characterized apoptotic morphologies, including chromatin condensation, nuclear margination, and disintegration of the nuclear membrane, were observed in LTA-treated cells. Parallel to the present results, the sonicated extract of E. faecalis, which may contain secreted virulence factors, induced apoptosis in human lymphocytes as well (25). However, a limitation of the present study should be noted. We cannot completely exclude the possibility that the osmotic pressure of different concentration dilutions might have an effect on apoptosis.

To further provide insight into Ef LTA–induced cell apoptosis, molecular parameters of apoptosis were also evaluated. The results of the Western blot showed that the protein level of Bcl-2 in MG63 cells was down-regulated by Ef LTA, whereas the protein level of Bax was up-regulated. The ratio of Bcl-2 to Bax in response to LTA revealed a significant promoting effect of LTA on apoptosis in MG63 cells. Moreover, caspase colorimetric assay was used to assess the caspase-3 activity in the present Ef LTA. Our data showed that the treatment of Ef LTA significantly increased the caspase-3 activity in MG63 cells, leading to the final irreversible execution step of the MG63 cells apoptosis.

Alveolar bone destruction commonly occurs under conditions such as periodontitis and apical periodontitis. Current literature has proved that periodontitis is partly due to the elimination of osteoblasts (26, 27). Meanwhile, the progression of periapical lesions is related to the significance of osteoblast apoptosis (15). These findings of our study provided evidence that Ef LTA may actively induce osteoblast apoptosis. Considering the frequent association of E. faecalis (among other diverse bacterial populations) with the chronic periapical lesions (28), the increased osteoblast apoptosis in response to Ef LTA may partly explain the delayed reconstruction of the periapical lesion.

Recently, Lee and Baek (29) studied the antibacterial effect of human β-defensins (HBDs) on E. faecalis and E. faecalis LTA. The HBDs exhibited neutralizing effects on the activity of Ef LTA, resulting in a decreased binding of Ef LTA to the host cells. When the findings of the present study were taken into account, the HBDs may contribute not only to prevent Ef and Ef LTA invasion but also to alleviate Ef or Ef LTA–induced osteoblast apoptosis. Other research revealed that calcium hydroxide could inactive Ef LTA through deacylation of the lipid moiety (30). In view of these findings, further studies should be performed to better clarify the protective effect of HBDs and calcium hydroxide on Ef LTA–induced osteoblast apoptosis.

In conclusion, our study indicated that Ef LTA significantly inhibited proliferation of human osteoblast-like MG63 cells and induced apoptosis of MG63 cells. The apoptotic effect of Ef LTA on osteoblast was in a dose-dependent manner.

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References