Expression of Interleukin-1β and Matrix Metalloproteinase-8 in Cytolytic and Noncytolytic Enterococcus faecalis–induced Persistent Apical Periodontitis: A Comparative Study in the Rat

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Abstract

Introduction: Cytolytic Enterococcus faecalis possesses a highly toxic and proinflammatory capacity. Cytokines and proteases play important roles in the host inflammatory response. The aim of this study was to compare the local expression of interleukin (IL)-1β and matrix metalloproteinase-8 (MMP-8) between persistent apical periodontitis (AP) infected by cytolytic and noncytolytic E. faecalis. Methods: Eighty-four left upper first rat molars were divided into 4 groups: chronic AP group (n = 6), disinfection group (n = 6), cytolytic E. faecalis–infected persistent AP group (n = 36), and noncytolytic E. faecalis–infected persistent AP group (n = 36). Periradicular lesions were established after pulp exposure. After 3 weeks, root canals were prepared, and disinfected. E. faecalis strains ATCC 29212 or ATCC 700802 suspensions were inoculated into root canals 2 weeks later. Six samples were collected at different time points (1, 2, 3, 4, 5, and 6 weeks). The expression levels of IL-1β and MMP-8 were detected by immunohistochemical staining. Results: IL-1β and MMP-8 expression trends in the cytolytic groups were similar to those of the noncytolytic group although at different time points the expression levels in the cytolytic group were significantly higher than those in the noncytolytic group (P < .01). IL-1β expression enhancement occurred during the early phase of infection, whereas increased MMP-8 expression lasted for a prolonged period. Conclusions: Both E. faecalis strains could induce local IL-1β and MMP-8 expression in persistent AP. Compared with noncytolytic E. faecalis, cytolytic E. faecalis may cause more severe local inflammation and tissue destruction in persistent AP. (J Endod 2015;41:1288–1293)

Key Words

Cytolytic Enterococcus faecalis, interleukin-1β, matrix metalloproteinase-8, persistent apical periodontitis, rat

Bacteria that survive root canal treatment procedures induce secondary intraradicular infections that lead to persistent apical periodontitis (AP). Enterococcus faecalis is one of the predominant bacteria in persistent endodontic infections and can survive under harsh environmental conditions such as alkalinity or malnutrition in the root canal environment (1, 2). Removing this bacterium by mechanical instrumentation or intracanal medication is difficult (3) because of its virulent ability to form biofilms (4), to penetrate dentinal tubules (5), and to resist several antibiotics (6).

As an important virulence factor of E. faecalis, cytolysin (Cyl) is exclusively expressed by cytolytic E. faecalis. Studies have reported high detection rates of cytolytic activator in clinical isolates of E. faecalis strains from persistent endodontic infection (7). The association between Cyl expression and enhanced virulence has been documented by a number of animal models (8, 9) and in clinical observations (10, 11). However, although much evidence exists regarding the correlation between the presence of cytolytic strains of E. faecalis and increased toxicity and inflammation in other parts of the body, little is known regarding their performance in persistent endodontic infection.

Inflammatory processes are thought to play key roles in the pathogenesis of AP (12). The interleukin (IL)-1 family is a group of cytokines that induces a complex network of proinflammatory cytokines and regulates and initiates inflammatory responses via the expression of integrins on leukocytes and endothelial cells (13). IL-1β is the predominant form of interleukin found in human periapical lesions and their exudates (14, 15). IL-1β is responsible for inducing the breakdown of alveolar bone in patients with periodontitis (16). Moreover, researchers have observed that gene polymorphisms with increased IL-1β production could contribute to increased susceptibility to persistent AP (17). Matrix metalloproteinase-8 (MMP-8) is expressed in both humans and rats and belongs to one of the subgroups of matrix metalloproteinases (MMPs), namely, collagenases, which are capable of degrading native type I, II, and III fibrillar collagens (18). Previous studies have shown that MMP-8 is closely related to the inflammation and development of periradicular lesions, implying the involvement of MMP-8 in tissue extracellular matrix degradation.

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including bone resorption (19, 20). Furthermore, IL-1β and MMP-8 are predominantly secreted by macrophages (21) and pleomorphic nuclear neutrophils (PMNs), respectively (22), which are both primary target cells of Cyl (23). Therefore, in this study, IL-1β and MMP-8 were chosen to monitor the periapical inflammatory process.

Considering the prevalence of cytolytic *E. faecalis* in persistent AP (7) and the association between Cyl expression and enhanced virulence, we hypothesized that cytolytic *E. faecalis* could cause more severe local inflammation and tissue degradation in persistent AP than noncytolytic *E. faecalis* could. To test this hypothesis, in this study, we selected 2 standard bacterial strains, namely, ATCC 29212 (cytolytic; American Type Culture Collection, Manassas, VA) and ATCC 700802 (noncytolytic; American Type Culture Collection). These 2 *E. faecalis* strains have similar genetic backgrounds for major virulence factors, except for the cyl gene (7). Accordingly, persistent AP models of rats infected with these 2 strains were established to compare the local levels of IL-1β and MMP-8 expression between these models.

### Materials and Methods

#### Animals

The care and handling of the animals were performed according to the guidelines of the Institutional Authority for Laboratory Animal Care, Fujian Medical University, Fujian, China. This study was reviewed and approved by Fujian Medical University.

In total, 84 male Sprague Dawley rats (8 weeks old, weighing 200–250 g) were obtained from the animal resource center (SLAC Laboratory Animal Co, Ltd, Shanghai, China). The rats were fed standard rodent chow and water ad libitum and housed at 6 rats per cage under a 12-hour light-dark cycle.

#### Establishment of Chronic AP Models with Primary Infection

After the rats were injected intraperitoneally with 10% ketamine HCl (62.5 mg/kg) (Fujian Gutian Pharmaceutical Co, Ltd, Fujian, China), the rats were mounted. The pulps of the left upper first molars were exposed using a 1/4 size round bur (Dentsply Maillefer, Ballaigues, Switzerland) and left open to the oral environment. Chronic AP developed after 3 weeks and was confirmed by periapical radiography.

#### Root Canal Preparation and Disinfection

At 3 weeks after pulp exposure, the rats were anesthetized and mounted. The mesial root canals of the teeth were shaped with reamers and files (Dentsply Maillefer) along with alternative irrigation using 15% EDTA (Xilong Chemical Co, Ltd, Guangdong, China) and 2.5% sodium hypochlorite (Zyi Reagent Factory, Shanghai, China). After a final irrigation with standard saline solution (Sinopharm Group, Rongsheng Pharmaceutical Co, Ltd, Henan, China), the root canals were dried with sterile paper points. Finally, a small cotton pellet soaked with formocresol (Second Medical Zhangjiang Biological Material Co, Ltd, Shanghai, China) was left in the pulp cavity followed by access cavity sealing with zinc oxide–eugenol (GC, Aichi, Japan).

#### E. faecalis Suspension Preparation

The cytolytic *E. faecalis* strain ATCC 29212 and the noncytolytic *E. faecalis* strain ATCC 700802 were cultivated in brain-heart infusion broth for 48 hours. Subsequently, the bacteria were seeded in agar plates and cultured under an aerobic environment at 37°C. After 6 hours, the bacteria grew logarithmically. Then, saline suspensions of both strains of *E. faecalis* at $1 \times 10^9$ colony-forming units/mL were prepared.

#### Establishment of Persistent AP Models of Rats Infected with Cytolytic and Noncytolytic *E. faecalis* Strains

At 2 weeks after the preparation and disinfection of root canals, the rats were anesthetized and mounted again. Subsequently, the temporary sealing material and formocresol cotton pellets were removed. After each mesial root canal was irrigated with normal saline, it was injected with 10 µL (10^7 colony-forming units) *E. faecalis* strain ATCC 29212 or ATCC 700802 suspension accordingly, and the access cavity was sealed using glass ionomer cement (ShoFu, Kyoto, Japan).

#### Animal Groups

**Disinfection Group.** At 3 weeks after pulp exposure, the root canals were prepared and disinfected. Then, 6 rats were sacrificed after being fed for another 2 weeks.

**Chronic AP Group.** In total, 6 rats were included in this group. After pulp exposure to induce chronic AP, the rats were fed for 11 weeks.

#### Groups with Persistent AP Infections Caused by Cytolytic and Noncytolytic *E. faecalis*. In total, 36 rats were used in each group. At 3 weeks after pulp exposure, the root canals were prepared and disinfected. *E. faecalis* strain ATCC 29212 or ATCC 700802 suspension was inoculated into the root canals 2 weeks later. In total, 6 rats were randomly selected and sacrificed at different time points (1, 2, 3, 4, 5, and 6 weeks) (Fig. 1).

#### Histopathological Slices

After the rats were sacrificed, the maxillae were collected and fixed in phosphate-buffered formalin (10%) for 24 hours, demineralized in 0.05 mol/L EDTA (pH = 7.2) for 20 days, dehydrated in a graded ethanol series, and clarified in xylol. After the samples were embedded in paraffin, serial sections (4-µm thickness) were prepared for histologic and immunohistochemical analyses.

#### Immunohistochemical Assays

Samples were analyzed using the indirect immunoperoxidase method. First, the sections were deparaffinized via immersion in xylene and ethanol followed by incubation in 3% hydrogen peroxide diluted in phosphate-buffered saline (PBS, pH = 7.2) for 40 minutes. Then, the sections were incubated with goat anti–IL-1β and goat anti–MMP-8 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), which were diluted in 1% PBS–bovine serum albumin (BSA) at 4°C overnight. Next, the sections were washed in PBS with Triton X-100 p.a. (Merck, Darmstadt, Germany) and incubated with biotinylated rabbit antigoat immunoglobulin G (Pierce, Rockford, IL) in 1% PBS-BSA for 60 minutes at room temperature. Subsequently, the sections were washed in PBS with Triton X-100 p.a. and incubated with avidin–biotin complex (K0492; Dako, Glostrup, Denmark) for 45 minutes at room temperature. After the sections were further incubated with 3,3-diaminobenzidine plus H2O2 (Dako) for 5 minutes at room temperature, they were washed with distilled water. Finally, the sections were counterstained with Mayer hematoxylin. Breast cancer sections served as positive controls for IL-1β and MMP-8 expression. Negative controls consisted of sections in which the primary antibodies were omitted and replaced with nonimmune murine serum (Dako) or with 1% PBS-BSA. Immunoreactivity was evaluated blindly by 2 observers who independently assessed the immunostaining using a light microscope (high-power field, 400× magnification).
Immunohistochemical Analysis

The immunohistochemical analysis referenced the method introduced by Xavier et al. (24). The intensity of the immunostaining for IL-1β and MMP-8 under 400× magnification was graded according to light staining (faint yellow), moderate staining (claybank), and intense staining (brownish black) using an image analysis system (Image-Pro Plus; Media Cybernetics Corp, Houston, TX). Five areas were observed around the apical foramen in each slice. The integral optical density (IOD) is the sum of each pixel point optical density value of positive images in the area (ie, IOD = \sum \text{optical density} \times \text{area}). The IOD was calculated to reflect the expression levels of cytokines. Each slice was measured 3 times and then averaged.

Statistical Analysis

The results of all measurements are presented as the mean value ± standard deviation (\(\bar{x} \pm \text{standard deviation}\)). The statistically significant differences were determined through analysis of variance wherein the significance was set at \(P < .05\).

Results

Immunohistochemical Assessment of IL-1β Expression

The IL-1β expression trends of cytolytic and noncytolytic E. faecalis–infected persistent AP groups were similar although the expression levels in the cytolytic group at different time points were significantly higher than those in the noncytolytic group (\(P < .05\)) (Fig. 24). Immediately after inoculation, the first week of infection witnessed a dramatic increase in IL-1β expression in the periadicular tissue followed by a gentle increase during the second week and then a peak during the third week. Afterward, a rapid decrease was observed for the next 2 weeks. During the sixth week, IL-1β expression decreased slowly. Moreover, the expression levels at different time points in both E. faecalis–infected persistent AP groups were statistically higher than those in the disinfection group and the chronic AP group (\(P < .05\)).

To validate our results, negative controls were performed during all experiments. In both E. faecalis–infected groups, most positive cells were densely aggregated and intensely stained during the third week. IL-1β–stained cells were observed in some bone resorption bays, particularly in the cytolytic group (Fig. 2C), whereas no positive cells could be investigated in the negative control of the same group (Fig. 2B). The number of IL-1β–positive cells decreased rapidly at 5 weeks and 6 weeks. For instance, at 6 weeks, only a small number of lightly stained IL-1β–positive cells still existed in the bone resorption bays in the cyto-

Immunohistochemical Assessment of MMP-8 Expression

A similar tendency of continuous increase was observed for the time-related expression levels of MMP-8 in both E. faecalis–infected persistent AP groups. However, at each time point, the expression levels of MMP-8 in the cytolytic group were significantly higher than were those in the noncytolytic group (\(P < .05\)) (Fig. 24). A gradual increase in MMP-8 expression was found during the first 2 weeks. During the following 4 weeks, a more obvious increase in MMP-8 expression was noticed in both groups. Furthermore, the expression levels at different time points, except for the first week, in both cytolytic and noncytolytic groups were statistically higher than those in the disinfection group and chronic AP group (\(P < .05\)).

The numbers of MMP-8–positive cells in both E. faecalis–infected groups were few during the first 2 weeks. However, beginning the third week, the numbers of MMP-8–positive cells increased markedly up to the sixth week. MMP-8–positive cells in the periapical tissue presented moderate to intense staining and were identified in the cytolytic group during the sixth week (Fig. 3C); whereas we could see no positive cells in the negative control of the same group (Fig. 3B). Simultaneously, a large number of MMP-8–positive cells could be observed in fibroblasts and bone resorption bays.

Discussion

Most studies have shown that E. faecalis is one of the primary pathogens in persistent AP, with a prevalence ranging from 24%–77% (25). Conversely, other investigators have reported extremely low detection rates or even the absence of E. faecalis in persistent endodontic infection, leading to controversy regarding the role of this bacterium in persistent endodontic infection (26). This controversy might be caused by the following factors: various sample sizes, sampling techniques, and diagnostic methods were used in different studies and this bacterial species might be harmful even at low levels because of its high virulence. Our study showed enhanced IL-1β and MMP-8 responses to cytolytic E. faecalis, indicating an additional pathogenic mechanism for E. faecalis in persistent AP.

The phenotype of cytolytic E. faecalis is modulated by the environment. Cyl gene expression is up-regulated upon the depletion of surrounding oxygen (27). In addition, recent data have indicated that Cyl can monitor bacterial population size and probe the environment for target cells (28). After root canal treatment, the oxygen concentration in the endodontic region decreases, the serum provided by the periapical tissue becomes the primary nutrient source, and bacterial and target cell densities change dynamically. Thus, Cyl expression in E. faecalis in the endodontic region differs from that in E. faecalis cultured in vitro. Therefore, investigating the performance of cytolytic E. faecalis in an environment similar to refractory AP is a prerequisite to access to evidence that is more reliable regarding the pathogenic mechanism in persistent endodontic infection. To mimic the clinical situation, an in vitro model was developed in the rat.

The experimental results indicated that the expression levels of both IL-1β and MMP-8 in the cytolytic group at most time points were significantly higher than those in the noncytolytic group, confirming the hypothesis that cytolytic E. faecalis causes more severe local inflammation in persistent AP than noncytolytic E. faecalis does. The differences in IL-1β and MMP-8 expression levels between the cytolytic and noncytolytic groups could most likely be caused by cyl gene expression, which is the major difference between E. faecalis strains ATCC 29212 and ATCC 700802. Therefore, Cyl may enhance the expression
of IL-1β and MMP-8 in AP; however, the underlying mechanism remains unclear. Cyl targets PMNs and macrophages (23), which are the principal cells involved in IL-1β (14) and MMP-8 (19) expression in AP. One possible explanation is that Cyl promotes IL-1β and MMP-8 expression via its effects on PMNs and macrophages. The results of this study suggest that Cyl may promote IL-1β and MMP-8 expression in persistent AP. However, further studies using gene silencing techniques are obligatory before a solid conclusion confirming the effects of cyl can be drawn.

Similar expression trends for IL-1β and MMP-8 were observed in both groups. Therefore, regardless of whether persistent AP was infected by cytolytic or noncytolytic E. faecalis strains, IL-1β and MMP-8 expression can be induced in the periapical region. Clearly, IL-1β and MMP-8 expression tendencies differed during the inflammation process (Figs. 2A and 3A), thereby suggesting that these proteins play different roles in E. faecalis–induced periradicular infection processes.

IL-1β is a major protein with osteoclast activating factor activity and reflects both a high level of expression and biological potency (29). IL-1β expression experienced a sharp increase during the first 3 weeks followed by a rapid decrease during the next 3 weeks. This finding was consistent with a previous study conducted by Martinez et al (30). In their study, experimental animal models of periapical lesions in rats were established. Both IL-1β gene up-regulation and strong IL-1β staining were observed in periapical areas during the inflammation phase. However, other researchers reported no significant differences in IL-1β messenger RNA expression between clinical samples taken from teeth presenting periapical lesions refractory to endodontic treatment and from healthy teeth with pulp vitality (31). One possible explanation for this controversy could be the dynamics of the development of AP. This disease process consists of 2 distinct phases, an active phase and a chronic phase with insignificant lesion expansion (32). IL-1β expression may be enhanced only during the active phase of AP. Because symptomatic lesions represent an immunologically active stage of this disease, this speculation may also explain the recent finding of significantly higher levels of IL-1β expression in symptomatic lesions compared with those in asymptomatic lesions (33).
MMPs have low expression levels in normal tissues but are up-regulated during inflammation (34), causing significant tissue destruction during the acute phase of the inflammatory process (35). The results of our study showed a continuous increase in MMP-8 expression after *E. faecalis* inoculation. This finding is consistent with the observation by Matsui et al (20). Their research revealed that MMP-8 gene expression increased gradually from 2 to 4 weeks followed by a slight decrease at 6 weeks in experimentally induced AP. However, in our study, until the end of the observation period (6 weeks), no decrease in MMP-8 expression was observed. Because MMP-8 is involved in the progression of tissue destruction including bone resorption (20), it is likely that *E. faecalis*–infected persistent AP may cause more severe periradicular tissue degradation compared with commensal bacteria-infected primary AP.

This study has some limitations. In our experiment, monospecies infection of *E. faecalis* in the endodontic region was established. Although some studies have shown that persistent AP infection is primarily colonized by multispecies bacteria (36), this design simplified the analysis of the effect of *E. faecalis* on the local inflammatory process. Another limitation is that to obtain the profiles of the time-related expression tendencies of IL-1β and MMP-8, we chose 6 different time points. Additionally, this study design had a low sample size at each time point, which is a drawback of this study.

To the best of our knowledge, this study is the first to compare the inflammatory responses of host periapical tissue to cytolytic and noncytolytic strains of *E. faecalis*. A novelty of the present study is that an *E. faecalis*–infected persistent AP animal model was established to mimic the clinical situation. This model could open new paths of investigation regarding the virulence of *E. faecalis* in persistent AP, facilitate the understanding of the pathogenesis of persistent AP, and eventually contribute to the development of effective therapies for persistent AP.

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The authors deny any conflicts of interest related to this study.
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