EFFECTS OF SONICATED *PREVOTELLA INTERMEDIA*, *FUSOBACTERIUM NUCLEATUM* AND *LACTOBACILLUS CASEI* EXTRACTS ON INTERLEUKIN-8 PRODUCTION BY HUMAN DENTAL PULP CELLS

Boonyanit Thaweboon[1], Sroisiri Thaweboon[1], Suwan Choonharuangdej[1] and Prapan Suppakpatana[2]

[1]Department of Microbiology, [2]Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Abstract. The objective of this study was to determine the effects of *Prevotella intermedia*, *Fusobacterium nucleatum* and *Lactobacillus casei* on the production of IL-8 by human dental pulp cells. Human dental pulp cells from teeth of young patients (aged 18-25 years) were cultured and tested with sonicated *P. intermedia* ATCC 25611, *F. nucleatum* ATCC 25586 and *L. casei* ATCC 4646 extracts. IL-8 secreted into the culture supernatants were measured at 6, 12 and 24 hours using a quantitative sandwich enzyme immunoassay technique. Cell viability was evaluated using trypan blue exclusion technique. IL-8 production by human dental pulp cells increased significantly at 12 and 24 hours after exposure to *P. intermedia* and *F. nucleatum*, whereas *L. casei* extract exhibited low IL-8 production. The sonicated bacterial extracts did not significantly affect viability or total number of dental pulp cells.

INTRODUCTION

Bacterial infection of dental pulp and the persistence of this infection in the root canal system are the major causes of pulpal and periapical lesions. *Prevotella intermedia* and *Fusobacterium nucleatum*, gram-negative bacteria, have been documented in human dental pulp and periapical disease (Sundqvist *et al*, 1989; Gomes *et al*, 2004). These bacteria have been demonstrated to express a variety of factors that play many roles in pathogenicity, including fimbriae, proteases and lipopolysaccharide endotoxins. Lactobacilli, gram-positive bacterium, have been isolated in root canals of teeth with apical lesions persisting after treatment (Molander *et al*, 1998, Sundqvist *et al*, 1998). The invasion of host tissue by these microorganisms and their by-products could induce a variety of immunopathological reactions.

Correspondence: Boonyanit Thaweboon, Department of Microbiology, Faculty of Dentistry, Mahidol University, 6 Yothi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2644-8644-6 ext 4811-2; Fax: 66 (0) 2644-8634
E-mail: boonitdt@yahoo.com

Polymorphonuclear cells and mononuclear phagocytes are key components of the host defense against invading microbes. Polymorphonuclear cells tend to predominate in acute infections, whereas a mononuclear infiltrate is more often seen in chronic infections. The nature of the leukocyte infiltrate affects the progress of the disease. Therefore, factors that regulate the recruitment of specific leukocytes represent an important component of the host response.

Chemokines are a recently described family of chemotactic cytokines (Graves and Jiang, 1995). Unlike the classic leukocyte chemoattractants, they exhibit a relatively high degree of specificity. The best-studied member of this group is interleukin-8 (IL-8), which is secreted by several cell types, including monocytes and macrophages, fibroblasts, endothelial cells, and keratinocytes (Graves and Jiang, 1995). In addition, dental pulp fibroblasts, which are considered as cells primarily concerned with providing physical barriers and structural components in pulpal tissue, are thought to be one of the cellular sources of IL-8. The most important function of IL-8 is the attraction of polymorphonuclear leukocytes. It is also important in the

activation of leukocyte adhesion molecules and as chemoattractant in directed leukocyte migration. High levels of IL-8 have been demonstrated in various inflammatory diseases, including pulpal inflammation (Huang *et al*, 1999) and periapical lesions (Shimauchi *et al*, 2001).

To investigate the mechanism that may contribute to the pathologic reaction of pulpal tissue due to bacterial infection, we have examined the different expression of IL-8 by human dental pulp cells stimulated *in vitro* by sonicated extracts of *P. intermedia*, *F. nucleatum* and *L. casei*.

MATERIALS AND METHODS

Cell culture

Human dental pulp cells were obtained from teeth extracted from young patients (age 18-25 years) in the course of orthodontic treatment. After the dental pulps had been extracted aseptically, they were washed twice with phosphate-buffered saline solution (PBS) pH 7.4. The pulpal tissues were minced and then placed in 35 mm tissue culture dishes.

The culture medium used was Dulbecco's Modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (Gibco) and 10,000 units of penicillin G and 25 µg/ml of amphotericin B as fungisone. Cultures were grown at 37ºC in a humidified atmosphere of 5% CO_2. After confluency, cells were harvested with 0.05% trypsin in PBS and subcultured weekly. Cells from the third to sixth passage were used in this study.

Sonicated bacterial extracts

P. intermedia (ATCC 25611) *F. nucleatum* (ATCC 25586) and *L. casei* (ATCC 4646) were maintained in brain-heart infusion broth. The density of each inoculum was adjusted to a turbidity of 2 McFarland standard (6 x 10^8 CFU/ml). After centrifugation at 16,000*g* for 15 minutes at 4ºC, cells were washed three times in PBS, lyophilized and sonicated on ice with a Vibracell (Model VCX-750, Sonics, Conneticut, USA). Disruption of bacterial cells was confirmed microscopically, and these sonicates were collected and centrifuged at 85,000*g* for 60 minutes. The supernatants were dialyzed against PBS, and were designated as sonicated *P. intermedia*, *F. nucleatum* and *L. casei* extracts.

Treatments

Confluent cells were trypsinized, counted and plated at a concentration of 1 x 10^4 cells in a 24-well plate and allowed to achieve confluence. Cells in G_0 by serum deprivation (0.5% FBS for 48 hours) were immediately exposed to the sonicated *P. intermedia*, *F. nucleatum* and *L. casei* extracts for 6, 12 and 24 hours. Cultures in 0.5% FBS were used as control.

IL-8 immunoassay

After 6, 12 and 24 hours of incubation, the amounts of IL-8 present in the culture supernatants were assessed by quantitative sandwich enzyme immunoassay technique (IL-8 ELISA, Biosource, Belgium) according to the manufacturer's protocol. Three cultures were used for each experiment, and four independent experiments were performed.

Cell viability assay

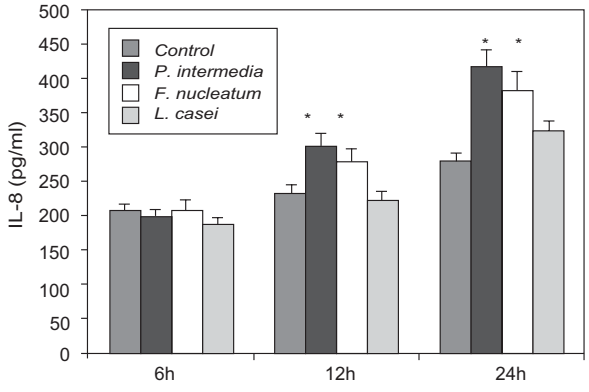
Trypan blue exclusion technique was used to evaluate cell viability. The dental pulp cell monolayers exposed to microorganisms and the non-exposed controls were detached using trypsin, centrifuged and resuspended in fresh media containing trypan blue, and inspected under a hemocytometer. Viable cells were expressed as a percentage of the values obtained in the initial incubation.

Statistical analysis

Three cultures were used for each experiment, and four independent experiments were performed. Statistical analysis was carried out by one-way ANOVA. Tests of differences of the treatments were analyzed by Duncan's test and a value of $p < 0.05$ was considered statistically significant.

RESULTS

The time-course of IL-8 production by human dental pulp cells in response to sonicated bacterial extracts were examined (Fig 1). Compared with the untreated control group, extracts from *P. intermedia* and *F. nucleatum* significantly



* statistically significant differences from control group ($p < 0.05$)

Fig 1–IL-8 production by human dental pulp cells in response to bacterial extracts measured by quantitative sandwich enzyme immunoassay technique. Data are mean ± SD from 4 independent experiments in triplicate assays.

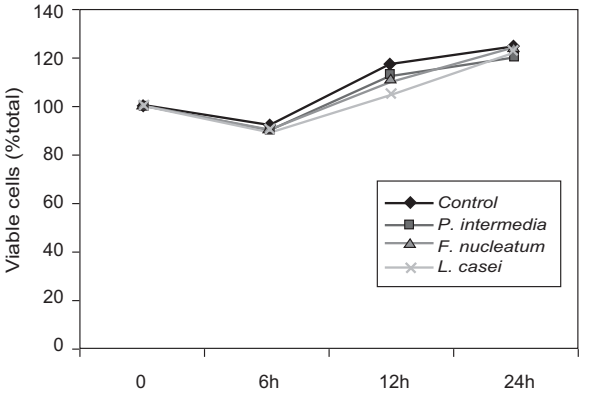


Fig 2–Percentage of viable dental pulp cells in response to bacterial extracts monitored by trypan blue exclusion technique at 6, 12 and 24 hours after exposure. Data are mean ± SD from 4 independent experiments in triplicate assays.

up-regulated the production of IL-8 after exposure for 12 and 24 hours. In contrast, *L. casei* slightly enhanced the production of IL-8.

Fig 2 shows the results of the trypan blue exclusion assays for the indicated time intervals. Up to 24 hours, there are no significant differences between treated cells and the untreated controls.

DISCUSSION

The hard tissues of the tooth normally protect the pulp by acting as physical barriers. These tissues can also function as structures, which, while protecting, can physically restrict pulpal inflammation during tissue injuries. Any pulpal injury can result in inflammation and its consequences, such as pain, hard tissue resorption and pulpal necrosis. Although the irritants can be physical, thermal, or chemical in nature, microorganisms are considered to be the main cause of pulpal and periapical pathosis. Based on the results of recent studies, it appears that infections are multibacterial and that anaerobic organisms such as *Prevotella* and *Fusobacterium* play a significant role in clinical signs and symptoms of the disease (Sundqvist *et al*, 1989; Gomes *et al*, 2004). These bacteria access and colonize the pulpal tissue and impair its function. The most common pathways to the pulp are direct extension from carious lesions and exposure of dentine following erosion or cracking of the enamel during restorative procedures.

Our study demonstrated that *P. intermedia* and *F. nucleatum* that are gram-negative bacteria had the ability to induce human dental pulp cells to produce IL-8. The results are in agreement with previous studies that endodontic pathogens can stimulate IL-8 production in human pulpal fibroblasts (Nagoaka *et al*, 1996). The induction pattern was dependent on the type of bacteria, its lipid structures as well as polysaccharide chains of the cell wall (Novotomy, 1984). These lipopolysaccharides have been demonstrated to be powerful immuno-stimulatory agents to initiate the local synthesis and release of proinflammatory cytokines from the cells of immune system (Dijkmans *et al*, 1990). IL-8, one of the mediators in chemokine family, is considered to be the primary regulatory molecule in the acute inflammation response (Callard, 1977). It is best known as a potent chemotactic factor for neutrophils, but it has been shown to demonstrate a wide range of proinflammatory activities such as lymphocyte migration, angiogenesis, neutrophil degranulation, increased expression of the cell adhesion molecule MAC-1 and the complement receptor CR-1 (Larsen *et al*, 1990). Although IL-8 expression has been documented

in committed immune effector cells, such as mononuclear phagocytes, large granular lymphocytes, and neutrophils (Graves and Jiang, 1995), its expression has also been confirmed in cells of the mesenchyme lineage, such as fibroblasts and osteoblasts, where it acts as inflammatory mediator of wound healing and connective tissue remodeling (Ohmori and Hamilton, 1994). Despite the fact that IL-8 has a documented role in the host defense, it has still been associated with certain pathological states (Harada *et al*, 1996). High level of IL-8 could induce the perpetuation and exacerbation of an acute inflammatory response within the tissue. Therefore, production of IL-8 in this study may lead to an excessive local amplification of the immune response, which may be partly responsible for pulpal and periapical tissue destruction.

Endodontic therapy or root canal treatment aims to eliminate microorganisms in the infected pulp tissue. However, in many cases, despite thorough mechanical instrumentation and disinfection of the root canal system, microorganisms have been recovered both at the end of the treatment procedure and at subsequent treatment sessions (Gomes *et al*, 1996; Molander *et al*, 1998). Such residual organisms are likely to play a role in treatment failures (Molander *et al*, 1998, Sundqvist *et al*, 1998). Lactobacilli were the most frequently isolated group of bacteria (Sundqvist and Carlsson 1974). In our study, *L. casei*, in contrast to *P. intermedia* and *F. nucleatum*, weakly enhanced IL-8 production. Recent data suggest that they are able to survive in restricted nutritional environments (Chavez *et al*, 2003) and have been recovered in root canal samples from teeth with apical lesions persisting after endodontic treatment. According to Molander *et al* (1998), the bacteria can survive in a quiescent phase with low metabolic activity for a period of time, and are also likely to be more resistant to antimicrobial and mechanical treatments. However, they have not been regarded as endodontic pathogen since their pathogenic potential has not well established. It may be of particular interest to further explore the mechanism by how these bacteria are implicated in pulpal and periapical inflammation after treatment.

In terms of cell viability, three microorganisms neither reduced the total number nor the viability of the exposed cells. These data suggest that the microorganisms act specifically on defense mechanism and perhaps on cell viability at a later phase (after 24 hours).

In conclusion, human dental pulp cells were found to produce IL-8 when stimulated by sonicated extracts from *P. intermedia* and *F. nucleatum*. These findings are significant because they demonstrate that endodontic pathogens can directly stimulate dental pulp cells to produce neutrophil-associated chemokine, mediator thought to be important in regulating recruitment of inflammatory cells.

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