Effects of *Enterococcus faecalis* sonicated extracts on IL-2, IL-4 and TGF-β1 production from human lymphocytes

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ABSTRACT

In order to examine the immunoresponse of host cells to *Enterococcus faecalis*, this in vitro study monitored the production of Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Transforming growth factor-β1 (TGF-β1) in human lymphocytes. Lymphocytes were activated with PHA in the presence or abscence of sonicated extracts of *E. Faecalis* (SEF) and further incubated for 72 hours. The level of each cytokine was measured by ELISA. Data were analyzed with Kruskal-Wallis test and Mann-Whitney U test ($P < 0.05$). PHA-activated group did exhibit higher level of IL-2 and IL-4 than untreated control group. The levels of expression of both cytokines were significantly decreased following the treatment of high (25 μg/ml) and medium concentration (12.5 μg/ml) of SEF ($P < .05$) than those of PHA activated group. But low concentration (5 μg/ml) of SEF showed the similar level of IL-2 and IL-4 production as those of PHA activated group. TGF-β1 was unaffected by SEF treatment. These results suggested that *E. faecalis* may suppress IL-2 and IL-4 production by lymphocytes and this could be one of possible factors why *E. faecalis* are found frequently in the teeth with failed endodontic treatment. [J Kor Acad Cons Dent 30(1):1-6, 2005]

Key words : Enterococus faecalis, Lymphocyte, IL-2, Il-4, TGF-β1, Sonicated extract

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I. Introduction

Bacteria and their by-products are considered to be the primary etiologic agents of pulpal necrosis and apical lesions[1]. Therefore, the aim of endodontic treatment is the elimination of micrroorganisms from the root canal system and the prevention of reinfection. But some cases fail even when apparently well treated. A number of factors have been identified as agents associated with failure of endodontic therapy. Most treatment failures are caused by microorganisms persisting in the apical parts of root canals of incomplete obturated teeth. Especially, single species of gram-positive organisms.

Enterococcus faecalis had been found to be one of the predominant bacteria in teeth in which root canal therapy failed[2]. There are many studies to identify a possible mechanism that would explain how *E. faecalis* could survive and grow within root canal system and reinfect an obturated root canal. *E. faecalis* has low sensitivity to antimicrobial agents and contribute to endodontic treatment failures[3]. *E. faecalis* persisted for at least 10 day after withdrawal of nutrient support,

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whereas the other organisms died within 4 to 48 hr[4]. *E. faecalis* maintained the capability to invade dentinal tubules and adhered to collagen in the presence of human serum[5]. Survival of *E. faecalis* in calcium hydroxide at high pH was related to a functional proton pump[6].

However, there have been few investigations about the immunologic effect of *E. faecalis*. In previous studies, sonic extracts from bacteria were studied for their effects on the immune response. For example, sonicated material of *Fusobacterium nucleatum* can evoke a concentration-dependent stimulatory or suppressive effect on the proliferation rate of accessory cell[7]. Sonicated extract of *Actinobacillus actinomycetemcomitans* suppressed interleukin-2 production of T-cells[8].

T helper cells have important roles in human immune system and are divided according to the production of cytokine. Th1 cells are primarily involved in macrophage-dependent immune responses, synthesize and secrete Interleukin-2 (IL-2), Interferon-γ (IFN-γ) and tumor-necrosis factor-β (TNF-β) and regulate cell-mediated immune response or delayed-type hypersensitivity. Th2 cells facilitate the synthesis of subclasses of antibody, synthesize and secrete IL-4, IL-5, IL-6, IL-13 and regulate humoral immune response[9]. Th3 cells suppress immune response to ingested antigen and their main lymphokine is TGF-β.

In a study by Hahn et al.[10] more lymphocytes were observed in inflamed pulps than in normal pulps, and the ratios of T4/T8 and B/T were changed with pulpal inflammation. T cells are responsible for the regulation of pulpal immunopathic changes under carious lesions.

IL-2 by Th1 cells is present in normal vital pulp and is significantly elevated in cases of symptomatic irreversible pulpitis[11]. IL-4 by Th2 cells was detected in human periapical granulation tissue and intraosseous inflammation[12,13]. In our previous study, the level of IL-2 and IL-4 was increased in experimentally induced pulpitis by *S. mutans* and *P. endodontalis* LPS[14]. Transforming growth factor-β (TGF-β) is a key mediator of immunological homeostasis in pulp and periapical regions. TGF-β1 is of particular importance in regulating inflammation with effects that are predominantly immunosuppressive. TGF-β1 exerts potent suppressive effects on the proliferation and differentiation of both T- and B-lymphocytes[15,16].

The purpose of this study was to investigate the capacity of peripheral lymphocytes to secrete IL-2, IL-4 and TGF-β1 after stimulation with sonicated extract of *E. faecalis* under proper mitogenic activation.

Ⅱ. Materials and methods

Preparation of bacterial sonicated extracts

Enterococcus faecalis (ATCC 29212) strains were used in this study. Strains were grown in 1-liter cultures in 85% N_2-10% H_2-5% CO_2 chamber for 3 days at 37℃. The medium was 3.7% brain heart infusion broth supplemented with 0.5% yeast extract, 0.5 ㎎ L-cysteine hydrochloride, and 0.5% Sodium bicarbonate.

Bacterial fractions were prepared as previously described[7]. Briefly, bacterial cells harvested from 1-liter cultures were washed, suspended in 20 ㎖ of phosphate-buffered saline (PBS), to minimize the loss of protein, 1 mM of Phenylmethylsul-fonylfluoride (PMSF) were added. PMSF acts as protease inhibitor at protein extraction. And Suspensions of bacterial cells were disrupted by sonication (100W output, Fisher sonicator) on ice for 5 min with 30-sec pulses-on and 10-sec pulse-off in the presence of glass beads. Disruption of the cells was confirmed microscopically. The sonicated material was centrifuged at 12000 rpm for 30min and at 30,000 rpm for 60 min. The protein that remained in suspension after high-speed centrifugation was designated the sonic extract of *E. faecalis* (SEF) and contained both cytoplasmic and periplasmic proteins. The supernatant was filtered with 0.22 ㎛ syringe. The extractes was resuspended with 10-15 ㎖ of Tris Buffer, Protein concentration was determined by the Bicinchoninic acid (BCA) protein assay (Pierce Chemical Corp., Rockford, IL, USA), and stocked in deep-freezer at -20℃.

Preparation of Human peripheral blood lymphocytes (HPBL)

HPBL were prepared from 20*ml* of EDTA-anticoagulated venous blood of healthy donors. The twice volume with Hanks balanced salt solution (HBSS) was added to the blood. The HPBL were isolated by buoyant density centrifugation on Ficoll-Hypaque (Phamacia LKB Biotechnology, Piscataway, N. J.; Lymphocyte separating medium). The blood was first centrifuged at 1500 rpm for 30min at room temperature. Lymphocytes were obtained by centrifugation layered over *Ficoll-Hypaque using a clean Pasteur pipette. The* HPBL were washed twice with HBSS, centrifuged at 1800 rpm for 10 min at 4℃, and diluted to 2×10^6 viable cells per ml with Hematocytometer for cytokine assays. HPBL were suspended in RPMI 1640 supplemented with 100 U of penicillin/*ml*, 100 *μg* of streptomycin and 1% fetal bovine serum.

Bacterial stimulation and Cytokine production

HPBL suspension (500 *μl*) containing 1×10^6 cells were placed into each well of flat-bottom 24-well plate. Each culture received medium or varying concentrations of SBE diluted in medium (400 *μl*). The cells were then incubated for 30 min at 37℃, at which time the cultures received an optimal mitogenic dose of Phytohemagglutinin (PHA) (4 *μg/ml*; Sigma, 100 *μl*). The cells were incubated for 72 hr at 37℃ in humidified air containing 5% CO_2.

Group 1: Medium only (negative control)
Group 2: 0 *μg* of SEF + PHA (positive control)
Group 3: 5 *μg* of SEF (low concentration) + PHA
Group 4: 12.5 *μg* of SEF (medium concentration) + PHA
Group 5: 25 *μg* of SEF (high concentration) + PHA

After incubation, the amount of IL-2, IL-4, TGF-β1 present in the culture supernatants were assayed by Enzyme-linked immunosorbent assay (ELISA, R & D Systems, Minneapolis, USA) *according to the manufacturer's protocols.*

Statistical analysis

Data were statistically analyzed using the Man-Whitney rank sum test and Kruskal-Wallis test. A value of $p < 0.05$ was considered statistically significant

Ⅲ. Results

The results of this study are summarized in Table 1 and Figure 1.

Bacterial extracts were evaluated for their ability to suppress the production of cytokine of lymphocyte to mitogens of PHA. Data of Table 1 and Figure 1 are the representative results of experiments. Results are the mean values of six samples of duplicate cultures. PHA-activated group

Table 1. Average concentration (± SD) of cytokines in cultures.

Group	Mean concentration ± SD (pg/*ml* protein)		
	IL-2	IL-4	TGF-β1
Medium only	17.66 ± 25.77	20.30 ± 24.57	37.10 ± 8.46
SEF 0 *μg/ml* + PHA	261.74 ± 75.43††	74.70 ± 25.04§*	29.41 ± 10.87
SEF 5 *μg/ml* + PHA	192.74 ± 59.81	72.01 ± 50.27	31.62 ± 27.14
SEF 12.5 *μg/ml* + PHA	95.05 ± 43.10†	41.28 ± 32.40§	10.90 ± 4.70
SEF 25 *μg/ml* + PHA	51.07 ± 47.71†	23.34 ± 23.89*	23.18 ± 11.52

SEF = sonicated extract of *Enterococcus faecalis*

"†, †, § and * are statistically significant different ($p < 0.05$) between groups

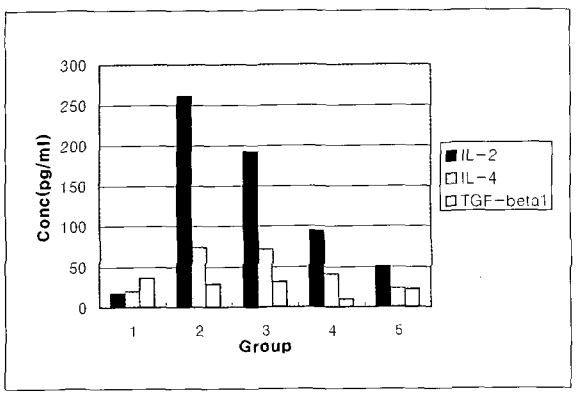

Figure 1. Response to SEF (Sonicated extract of E. faecalis) of IL-2, IL-4 and TGF-β1 production in the supernatants of lymphocytes. The cell was treated with PHA and various concentrations of each SEF for 72 hr. The data represent mean values (n = 6 duplicate culture for each group).

(Group 2) exhibited higher level of IL-2 and IL-4 than medium only control group (Group 1) ($p < 0.05$). As compared with PHA-activated (Group 2), high and medium concentration of SEF (Group 4, 5) decreased the production of IL-2 and IL-4 from lymphocyte ($p < 0.05$), but there was no statistically significant difference between low concentration of SEF (Group 3) and PHA-activated group (Group 2). So we can suggest that SEF cause a dose-dependent reduction in PHA-induced cytokine production of IL-2, and IL-4. But the production of TGF-β1 was independent of concentration of SEF ($p > 0.05$).

Ⅳ. Discussion

Microbial products represent an important source of immunoregulatory agents. In particular, several microorganisms are capable of suppressing the immune response through various products, including toxins, enzymes, cell wall components, and metabolites. These immunosuppressive products may alter the immune system via different mechanisms. In some instances these agents indirectly modify lymphocyte response by directly affecting monocytes or macrophage activities directly. For example, Spirochetes inhibited lymphocytes function and *Leishmania donovani* impaired the synthesis and release of IL-1 from macrophages[17,18]. Sonicated extract from *Prevotella intermedia, Porphylomonas asaccharolytica, Porphyromonas endodontalis* and *Prevotella melaninogenica* were capable of suppressing human T- and B-cell response in dose-dependent manner. The immunosuppressive activity is nondialyzable and heat labile[19].

This study showed that the level of IL-2 and IL-4 in lymphocytes stimulated with PHA were significantly higher than those of the negative control group. So, PHA can have sufficient mitogenic effect to lymphocytes. But sonicated extracts of *E. faecalis* suppressed *in vitro* IL-2, IL-4 production of human lymphocyte in a dose-dependent manner. Ability of *E. faecalis* to impair the production of IL-2 and IL-4 may be related to the suppression of T-cell activation. In our previous studies, in contrast, lipopolysaccharide of *Porphyromonas endodontalis in vitro* stimulated the production of macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta from human polymorphonuclear leukocyte[20]. In rat pulpitis experimentally induced by specific bacteria, *Streptococcus mutans, Porphylomonas endodontalis* stimulated the production of Interferon-γ, IL-2 and IL-4[14]. .These studies showed that *P. endodontalis* have inflammatory effects by stimulation, but in other reports, sonic extract of *P. endodontalis* in lymphocyte *in vitro* have immunosuppressive effects by inhibition[19], and in this study *E. faecalis* inhibited the cytokine production of lymphocytes. Much further investigation is required to clarify the stimulation and inhibition of cytokines. In contrast, there was no significant difference in the level of TGF-β1 in regardless of SEF, this results show that TGF-β1 is released by other mechanism.

When comparing with the level of each cytokine, Results showed that the level of IL-2 predominated over those of other cytokine IL-4, and TGF-β1. Considering the concentration of IL-2, Th1 lymphocytes may be dominant in human peripheral blood lymphocytes.

It has been proposed that impaired host defense may play a pivotal role in the pathogenesis of many infections. The data presented in this study

suggest that microbial mediated immunosuppression may contribute to the pathogenesis of endodontic infection by altering the nature and consequences of host-parasite interactions[21]. Microbial virulence may be the consequence of several properties, including the ability of certain species to resist, escape, or pervert host defense mechanism. Some causative virus infects and destroys a subpopulation of T lymphocytes, such as human immunodeficiency virus. Such inhibitory factors could lead to a state of immunological hyporesponsiveness that favors colonization by the initiating organism or by other opportunistic organisms. Our current finding that *E. faecalis* inhibits the immune response suggests that impaired host defense mechanism may contribute to the disease process.

This results suggested that bacterial protein sonicated extract of *E. faecalis* can inhibit the immune response by lymphocyte and this could be one of possible factors why *E. faecalis* are found frequently in the teeth with failed endodontic treatment.

Ⅴ. Conclusion

According to this study we could summarize as follows: PHA activated expression of IL-2 and IL-4 from lymphocytes compared with negative control group ($p < 0.05$). As compared with PHA-activated group, high and medium concentration of SEF decreased the production of IL-2 and IL-4 from lymphocytes ($p < 0.05$). There was no statistically significant difference between low concentration of SEF and PHA-activated group. The production of TGF-β1 was independent of concentration of SEF ($p > 0.05$). So we can suggest that SEF cause a dose-dependent reduction in PHA-induced cytokine production of IL-2, and IL-4.

References

1. Kakehashi S et al. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rat. *Oral Surg* 20:340-344, 1965.
2. Sundqvist G et al. Microbiologic analysis of the teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endo* 85:86-93, 1998.
3. Dahlen G et al. Identification and antimicrobial susceptibility of enterococci isolated from the root canal. *Oral Microbiol Immunol* 15: 309-312, 2000.
4. Orstavik D and Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endo Dent Traumatol* 6:142-149, 1990.
5. Love RM. *Enterococcus faecalis* - a mechanism for its role in endodontic failure. *Int Endo J* 34:399-405, 2001.
6. Evans et al. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int Endo J* 35:221-228, 2002.
7. Yoshida H et al. Effect of sonicated material from *Fusobacterium nucleatum* on the functional capacity of accessory cells derived from dental pulp. *Oral Microbiol Immunol* 10:208-212, 1995.
8. Kurita-Ochiai T et al. Immunosuppressive effect induced by Actinobacillus actinomycetemcomitans: effect on immunoglobulin production and lymphokine synthesis. *Oral Microbiol Immunol* 7:338-343, 1992.
9. Trowbridge H. Inflammation: A review of the process, 5th edition, *Quintessence Publishing Co.* p79-90, 1997.
10. Hahn CL et al. A study of T and B cells in pulpal pathosis. *J Endodon* 15:20-6, 1989.
11. Rauschenberger CR et al. Detection of human IL-2 in normal and inflamed dental pulps. *J Endodon* 23:366-70, 1997.
12. Kawashima N and Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. *Arch Oral Biol* 44:55-66, 1999.
13. Walker KF et al. Cytokine expression in periapical granulation tissue as assessed by immunohistochemistry. *Eur J Oral Sci* 108:195-201, 2000.
14. Kim SA and Lim SS. T lymphocyte subpopulations and interleukin-2, interferon-gamma, and interleukin-4 in rat pulpitis experimentally induced by specific bacteria. *J Endodon* 28:202-5, 2002.
15. Wahl SM. Transforming growth factor beta in inflammation : a cause and a cure. *J Clin Immunol* 12:1-14, 1992.
16. Wahl SM. Transforming growth factor β: the good, the bad, and the ugly. *J Exp Med* 180:1587-1590, 1994.
17. Shenker BJ et al. Suppression of human lymphocyte responses by oral spirochetes: a monocyte-dependent phenomenon. *J immunol* 132: 2039-2045, 1984.
18. Reiner. Parasite accessory cell interactions in murine heishmaniasis. I. Evasion and stimulus-dependent suppression of the macrophage interleukin-1 response by Leishmania donovani. *J Immunol* 138:1919-1925, 1987.
19. Shenker BJ and Slots J. Immunomodulatory effects of Bacteroides products on in vitro human lymphocyte functions. *Oral Microbiol Immunol* 4:24-29, 1989.
20. Ko HJ and Lim SS. Production of macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta by human polymorphonuclear neutrophils stimulated with *Porphyromonas endodontalis* lipopolysaccharide. *J Endod* 28:754-7, 2002.
21. Shenker BJ et al. Immunosuppressive effects of *Prevotella intermedia* on In vitro Human lymphocyte activation, *Infect Immun* 59: 4583-4589, 1991.

국문초록

Enterococcus faecalis 추출물이 임파구의 IL-2, IL-4, TGF-β1 분비에 미치는 영향에 관한 연구

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근관치료의 실패원인 중 중요한 세균으로 알려진 *Enterococcus faecalis*는 최근에 중요성이 더해지며 많은 연구들이 진행중이다. 여러가지 기전들이 보고되고 있으나 면역반응에 관한 연구는 거의 알려져 있지 않은 상태이다.

본 연구에서는 *Enterococcus faecalis*의 초음파 분쇄 추출물을 성인의 말초혈액으로부터 얻은 임파구에 적용시켜서 여기서 분비되는 interleukin-2, interleukin-4, transforming growth factor-β1의 농도를 Enzyme linked immunosorbent assay (ELISA)로 측정하여 비교, 평가하는 것을 목적으로 한다.

*E. faecalis*를 적절한 조건에서 배양한 뒤 초음파 분쇄를 하여 추출물을 얻어냈다. 임파구는 건강한 성인의 말초혈액에서 추출하여 분리하였다. 임파구를 적절한 농도의 mitogen (Phytohemagglutinin: PHA)으로 자극시킨 뒤에 다양한 농도의 *E. faecalis* 초음파 추출물을 적용시키고 72시간 동안 배양하였다. ELISA를 이용하여 IL-2, IL-4, TGF-β1의 농도를 측정하였다. 실험결과는 Kruskal-Wallis test, Man-Whitney rank sum test ($p < 0.05$)를 사용하여 통계처리 하였다.

실험결과 PHA로 처리한 군은 아무것도 처리하지 않은 군에 비해서 IL-2, IL-4의 수치가 유의성 있게 높았다 ($p < 0.05$). PHA로 처리한 군중에서 고농도와 중농도의 sonic extract of *E. faecalis* (SEF)로 처리한 군은 그렇지 않은 군에 비해서 IL-2, IL-4의 농도가 유의성 있게 낮았다 ($p < 0.05$). PHA로 처리한 군중에서 저농도의 SEF로 처리한 군은 그렇지 않은 군과 비교하여 유의할 만한 차이를 보이지 않았다. TGF-β1의 농도는 모든 군에서 유의할 만한 차이를 보이지 않았다 ($p > 0.05$). 따라서, *E. faecalis*의 추출물은 임파구의 IL-2, IL-4의 분비능력을 저하시킨다고 할 수 있다.

주요어 : *Enterococcus faecalis*, 임파구, 초음파 추출물, IL-2, IL-4, TGF-β1, ELISA