The Effect of *Treponema denticola* immunoinhibitory protein on cytokine expression in T cells

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ABSTRACT

Immunoinhibitory protein extracted from sonicated *Treponema denticola* have been shown to suppress cell cycle progression of human lymphocytes. To study in detail about the effect of this microorganism on the function of lymphocytes, we investigated the levels of Interleukin-2 (IL-2) and Interleukin-4 (IL-4) production by T lymphocytes before and after the addition of 12.5 ㎍/㎖ *T. denticola* sonicated extracts. In this study, levels of IL-2 and IL-4 produced from T cells pretreated with sonicated extracts were evaluated by using the quantitative sandwich enzyme immunoassay technique. In response to phytohemagglutinin (PHA) stimulation, T cell produced increased levels of IL-2 and IL-4. However, the expressions of both cytokines were significantly inhibited when PHA activated-T cells were pre-exposed to sonicated *T. denticola* extracts ($p < 0.05$). These findings suggest that the *T. denticola* sonicated extracts induced-immunosuppression in Th1 and Th2 cell functions could be a part of the pathogenic mechanism of the endodontic failure associated with this microorganism. (J Kor Acad Cons Dent 29(5):479-484, 2004)

Key words : Immunoinhibitory protein, *T. denticola,* Sonicated extracts, Interleukin-2, Interleukin-4

Ⅰ. INTRODUCTION

Treponema denticola has long been considered as a periodontal pathogen[1]. Listgarten also reported that spirochetes are conspicuous inhabitants of subgingival plaque in patients with gingivitis and periodontitis[2]. Although this bacteria is well known to be important periodontal pathogens, several studies suggest that spirochetes may be involved in pulpal and periapical infections as well. For instance, Thilo et al.[3] reported that the root canal flora of decayed teeth contained as much as 6% of spirochetes. Likewise, Nair[4] reported that the spirochetes form a significant component of the flora of periapical specimen. Recent studies[5,6] using 16S rDNA-directed polymerase chain reaction (PCR) technique confirmed that the occurrence of *Treponema denticola* accounted for 52.4% of the flora in infected root canals and 50% in acute alveolar abscesses of endodontic origin. Thus, spirochetes or their byproducts may contribute to the inflammatory process and pathogenesis of the periradicular infection. However, it is not clear how spirochetes are involved in the etiology of the disease. In this regard, Shenker et al.[7] demonstrated that soluble sonic extracts of several strains of *T. denticola* inhibit human peripheral blood lymphocyte (HPBL) proliferative responses to both mitogen and antigens *in vitro* with no effect on cell viability. They have also observed that these effects are due to a protein compound

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of two polypeptides of 50 and 56 kDa.

Among the lymphocyte populations in the host defense system, T cell has been well known to play an important role in periapical lesion development and maintenance. 60% of peripheral T cells are CD4 positive T helper cells and these cells are subdivided into Th1 and Th2 cells. Due to the lack of definitive phenotypic markers for these subsets, cytokine production can be a good indicator for their activities in response to bacterial stimulations[8]. In fact, Interleukin-2 (IL-2) and Interleukin-4 (IL-4) are the major effector cytokines released from Th1 and Th2, respectively. IL-2 stimulates T cells proliferation and involved in cell mediated immunity while IL-4 promotes the B cell to differentiate into plasma cell that produces antibodies and thus mediating humoral immunity[9].

Recent studies[10-13] have investigated that the suppression of lymphocyte proliferation by pathogenic bacteria related to endodontic treatment failures. Furthermore, Lee et al.[14] clearly showed that immunoinhibitory protein extracted from *T. denticola* suppressed proliferative ability of lymphocytes by arresting cell cycle. However, studies on the effects of *T. denticola* on cytokine-producing function of lymphocytes are lacking. Therefore, the objective of this study was to compare the IL-2 and IL-4 expression by human T cells before and after the addition of sonicated *T. denticola* extract.

Ⅱ. MATERIALS AND METHODS

1. T lymphocyte preparation

T lymphocytes were prepared from 20㎖ of EDTA-anticoagulated venous blood of healthy donors. Cells were isolated by buoyant density centrifugation on Ficoll-Hypaque (Pharmacia LKB Biot- echnology, Piscataway, NJ, USA). T Cells were then washed twice with Hanks' balanced salt solution, centrifuged at 16,000 × g for 10 min at 4℃, and diluted to 2×10^6 viable cells per ㎖ culture medium consisting of RPMI 1640, 2% penicillin/streptomycin, and 2% fetal bovine serum.

2. Sonicated bacterial extracts preparation

Treponema denticola strain LL2513 was grown in 500ml of TYGVS (Trypticase, yeast extract, glucose, volatile fatty acid, serum) spirochete medium containing veal infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with yeast extract (BBL), trypticase peptone, ammonium sulfate, *L*-cycteine hydrochloride, glucose, and volatile fatty acid solution (acetic acid, propionic acid, *n*-butyric acid, isovaleric acid and D,L-methylbutyric acid in dH_2O). Anaerobic cultures of *T. denticola* were grown at 37℃ in 20-㎖ glass tubes. After 72 h, bacterial cell suspensions were harvested by centrifugation at 16,000 × g for 15 minutes at 4℃ and washed 3 times in Phosphate buffered saline (PBS) containing PMSF. The washed bacterial cells were lyophilized and sonicated on ice with a Sonic Dismembrator (Model 550, Fisher Scientific, Pittsburg, PA, USA) for a total of 7 min with 30-s pulses. Disruption of the bacterial cell was confirmed microscopically and these sonicates were collected and centrifuged at 85,000 × g for 60 minutes. The supernatant was dialyzed against PBS, and the protein that remained in suspension was designated as sonicated *T. denticola* extracts.

3. Sonicated extracts response experiment

For the experiment, T cell suspension containing 1×10^6 cells was placed into each well of flat-bottomed 24-well plate. Each culture received medium (RPMI supplemented with 100U of penicillin/ml, 100㎎ of streptomycin and 2% fetal bovine serum) or optimal concentrations (12.5 ㎍/㎖) of sonicated extracts diluted in medium. Additionally, all experimental cultures received 100 ㎕ of phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO, USA) as an antigenic stimulant. Experimental groups were arranged in 3 groups: Group 1 was designated untreated control cells; group 2 consisted of cells with PHA activation alone;; group 3 contained cells pretreated with 12.5 ㎍/㎖ of sonicated extracts and PHA activation.

4. Cytokine assay

After 72h incubation, the amount of IL-2 and IL-4 present in the culture supernatants were assessed by Enzyme-linked immunosorbent assay (Quantikine colorimetric sandwich ELISA kits; R&D System, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, monoclonal antibodies specific for IL-2 and IL-4 were precoated onto enzyme-linked immunosorbent assay plate. Dilutions of standards and culture supernatants were then added to individual wells in duplicate. After any unbound substances were washed away, an enzyme-linked polyclonal antibody specific for IL-2 and IL-4 was added to the wells. After a further wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and the color was developed. The optical density was read at an absorbance of 450 ㎚, and the cytokine concentration was quantified from the standard curve. Three samples in each group were analyzed in duplicate.

5. Statistical analysis

Data were statistically analyzed between the experimental groups using Mann-Whitney rank sum test with P value set at 0.05.

Ⅲ. RESULTS

The means and standard deviations of IL-2 and IL-4 expressions are summarized in Table 1.

Compared to the control (Group 1), T lymphocytes were well responded to the PHA stimulation. As shown in Table 1, PHA activated cells (Group 2) exhibited high levels of both IL-2 and IL-4. These cytokine productions were significantly suppressed when the cells were pre-exposed to 12.5 ㎍/㎖ (Group 3) of sonicated *T. denticola* extracts ($p < 0.05$).

Table 1. Means and standard deviations, concentration calculated from the equation and value after multiplied by dilution factor of IL-2 and IL-4 expressions. † and * represent statistical differences between groups.

	IL-2 (pg)	conc. By equation	multiply by dilution factor	IL-4 (pg)	conc. By equation	multiply by dilution factor
Group 1	0.1095 ± 0.019	0.075	7.5	0.170 ± 0.014	3.825	382.5
Group 2	0.1345 ± 0.0021	1.6375	163.75†	0.338 ± 0.0071	14.325	1432.5*
Group 3	0.1145 ± 0.0049	0.3875	38.75†	0.2545 ± 0.016	9.106	910.63*

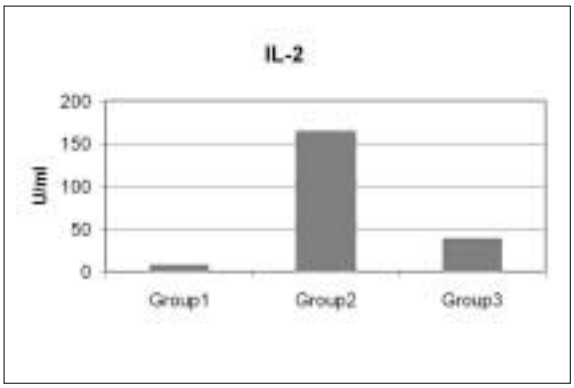

Figure 1. Expression of IL-2 in each group. The data represent mean values of three experiments done in duplicate.

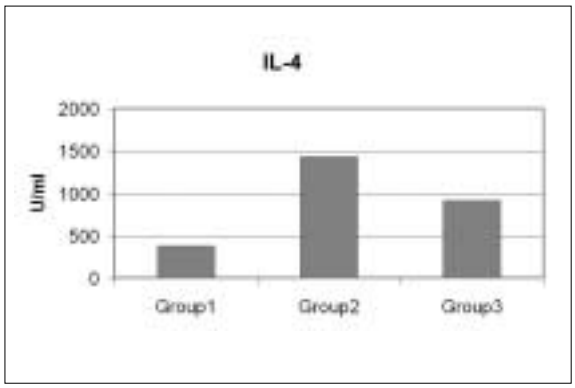

Figure 2. Expression of IL-4 in each group. The data represent mean values of three experiments done in duplicate.

Ⅳ. DISCUSSION

Treponema denticola have been rarely found associated with endodontic diseases, however, unidentified spirochete species have now been found in endodontic infections by employing polymerase chain reaction (PCR) method[6,15-17]. In this regard, Smallwood et al.[18] recently reported the occurrence of spirochetes in all samples collected from infected canals using PCR. Likewise, Siqueira et al.[5] demonstrated that Treponema denticola was detected 11 of 21 infected root canal cases.

T. denticola is a gram-negative, anaerobic, helically shaped, highly motile bacteria. It has an assembly of virulence factors that can contribute to its pathogenicity. *T. denticola* can adhere to diverse host cells and other tissue components and it has also been demonstrated to invade cells and tissues. In addition, it may have immunomodulatory effect, including a number of specific effects on polymorphonuclear leukocytes and lymphocytes. Some *T. denticola* surface-expressed proteins have adhesion, cytotoxic and tissue destructive activities, including the major surface protein and the chymotrypsin-like protease complex. Other extracellular membrane-associated proteolytic and hydrolytic enzymes, short chain fatty acids and volatile sulfur compounds are other potentially virulence factors of oral spirochetes[19-20].

There are accumulating evidences that pathogenic bacteria associated with endodontic infection and have been shown to alter the host defense system. For example, Yoshida et al.[13] reported that sonicated material from *Fusobacterium nucleatum* can either suppress or stimulate T cell proliferation in the presence of dental pulp accessory cells. Likewise, Gentry-Weeks et al.[21] reported that *Enterococcus faecalis* can survive in mouse peritoneal macrophages. Recent study of Lee et al.[12] demonstrated that sonicated *Enterococcus faecalis* extracts inhibit lymphocyte proliferation by arresting cell cycle progression. Thus, this immunosuppressive process is relevant to the pathogenesis of the periapical disease. This kind of immunosuppression by bacterial byproducts may act differently by interfering with either the induction or the expression of immune reaction. Sometimes these immunomodulatory agents can activate T cells, sometimes can suppress T cells, or have direct effect on both precursor and mature effector cells.

In this regard, Shenker et al.[7] have previously shown that extracts of the oral spirochete, *T. denticola,* contain an immunosuppressive protein which impairs human lymphocyte proliferation. Recently, Lee et al.[14] confirmed this immunoinhibitory effect by inducing irreversible G_1 arrest in activated human lymphocytes. Spirochetes may contribute to the pathogenesis of a member of disorders including periodontal and periradicular diseases; however, the mechanism by which these organisms act to cause the disease is unknown.

In the present study, we have investigated whether the sonicated *T. denticola* extracts have the immunosuppressive effect on cytokine-producing function of T cells. As shown in data, PHA stimulation has sufficient antigenic effect on the T cells (Group 2 in Table 1). The levels of both IL-2 and IL-4 expressions were significantly increased when cells were activated with PHA (Group 2), compared to those of the unstimulated control (Group 1). These cytokine productions were down-regulated when T cells were exposed to the sonicated *T. denticola* extracts prior to PHA activation (Group 3). This suppression of cytokine expression indicated that the immunoinhibitory protein extracted from *T. denticola* has the ability of inhibiting lymphocyte function. This impaired lymphocyte function, in turn, would adversely affect the development of normal immunologic defense mechanism and as a result, it contributes the pathogenesis of periapical lesion.

T helper cells actively participate in immune defense via producing cytokines in response to the antigenic stimulation. Mediators produced by subset Th1 cells increase inflammation and bone resorption while Th2 cell-derived cytokines are inhibitory[22]. Th1 cells initiate cell-mediated immune reactions whereas Th2 cells orchestrate humoral immune responses. The balance between

Th1 and Th2 cell is critical in periapical lesion development and thus, protects hard tissue from direct bacterial invasion[9]. Therefore, Bacteria-induced reduction or hypo-production of selected cytokines derived from the Th1 and Th2 cell is responsible for the host tissue destruction.

In conclusion, the cytokine-producing functions of both Th1 and Th2 cells were significantly perturbed by sonicated *T. denticola* extracts. This observation suggests that the *T. denticola* induced-immunosuppression in T cell function could be a part of the pathogenic mechanism of the periapical lesion.

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국문초록

Treponema denticola 면역억제 단백질이 T 세포의 cytokine 발현에 미치는 영향

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감염근관내 spirochetes의 존재 유무에 대한 논란이 많았으나 최근 PCR을 사용한 세균검출 실험에서 *Treponema denticola* 균주가 감염근관의 50%이상의 경우에서 발견됨에 따라 이 세균이 치수 및 치근단 질환의 병인과정에 관여하는 지에 대한 관심이 높아지고 있다. 이와 관련하여 Shenker 등이 *T. denticola*의 sonicated extract에서 순수분리된 면역억제 단백질 (immunoinhibitory protein)이 T 임파구의 proliferation을 방해함을 보고한 바 있다. 하지만 이 세균성 단백질이 T 임파구의 기능에 어떤 영향을 미치는 지에 대한 연구는 부족한 실정이다. 따라서 *T. denticola*의 면역억제 단백질이 처리되기 전과 후의 T 세포에서 분비되는 cytokine Interleukine-2와 Interleukine-4의 발현 정도를 비교하여 그 작용기전을 밝히는 것이 본 연구의 목적이다.

Treponema denticola LL2513를 혐기성 상태에서 TYGVS 배지에 배양한 다음 PBS 세척과 lyophilize 과정을 거친 후 sonication을 시행한다. 이 과정을 거쳐 추출된 상층액이 *T. denticola*의 면역억제 단백질인 sonicated extract이다. 실험을 위해 건강한 혈액 공여자로부터 T 세포를 Buoyant density 방법으로 추출해 낸 다음 24-well plate에 100만개의 세포를 주입한 다음 Group 1에는 2% FBS의 medium만으로 배양하고, Group 2에는 100 ㎕ 의 PHA로만 증식 자극을 하였고, Group 3에는 PHA 처리 전 sonicated extract로 T 세포를 자극하였다. 72시간 동안 배양한 다음 상층액을 추출하여 ELISA assay를 사용하여 IL-2와 IL-4의 발현정도를 측정하였다. PHA로 자극받은 Group 2에서는 IL-2와 IL-4가 대조군인 Group 1에서보다 높은 수준으로 발현되었다. 하지만 12.5 ㎍/㎖의 *T. denticola* sonicated 추출물로 전처리한 Group 3에서는 IL-2와 IL-4의 수준이 유의성 있게 억제되어 발현되었다 ($p < 0.05$). 이러한 결과를 통하여 *T. denticola*에서 추출된 면역억제 단백질이 Th1과 Th2의 cytokine 분비 기능을 억제하는 것으로 확인 되었으며 이 기전이 감염 근관에서 발견되는 *T. denticola*의 치수 및 치근단 질환에 대한 병인기전과 관련이 있는 것으로 사료된다.

주요어 : *Treponema denticola*, Sonicated 추출물, 면역억제 단백질, IL-2, IL-4