Antiinflammatory effect of (−)-epigallocatechin-3-gallate on *Porphyromonas gingivalis* lipopolysaccharide-stimulated fibroblasts and stem cells derived from human periodontal ligament

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Directed by Professor : Seong-Ho Choi

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감사의 글

이 논문이 완성되기까지 기틀을 잡아주시고 귀중한 조언과 지도를 아끼지 않아주신 최성호 지도교수님께 마음속 깊이 감사드립니다. 그리고 많은 관심과 격려를 해주신 김종관 교수님, 채중규 교수님, 조규성 교수님, 김창성 교수님, 정의원 교수님, 윤정호교수님께도 감사 드립니다.

연구와 실험 과정 내내 많은 도움을 준 정임희 연구원 외 모든 치주과 의국원 여러분께도 감사의 인사를 전합니다.

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항상 든든한 지원군이 되어 주시고 사랑으로 감싸주시는 아버지, 어머니 사랑합니다. 또한 아기를 키우며 공부를 이어갈 수 있도록 여러모로 도와주신 우리 가족들, 시부모님, 동준이, 외삼촌, 이모 모두 감사합니다.

마지막으로 뜻을 펼칠 수 있게 격려와 지원을 아끼지 않는 평생의 친구이자 남편 김태희에게 감사의 마음을 전하며 부족한 엄마를 보며 늘 웃어주는 아들 시형아, 사랑한다.

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Abstract

Antiinflammatory effect of (–)-epigallocatechin-3-gallate on *Porphyromonas gingivalis* lipopolysaccharide-stimulated fibroblasts and stem cells derived from human periodontal ligament

Purpose:(–)-epigallocatechin-3-gallate (EGCG) has been reported to exert antiinflammatory and antibacterial effects in periodontitis. However, its exact mechanism of action has yet to be determined. The present *in vitro* study evaluated the antiinflammatory effects of EGCG on human periodontal ligament fibroblasts (hPDLFs) and human periodontal ligament stem cells (hPDLSCs) affected by bacterial lipopolysaccharide (LPS) extracted from *Porphyromonas gingivalis.*

Methods: hPDLFs and hPDLSCs were extracted from healthy young adults and were treated with EGCG and/or *P. gingivalis* LPS. After 1, 3, 5, and 7 days from treatment, cytotoxic and proliferative effects were evaluated using a MTT assay and BrdU assay, respectively. And then, the gene expressions of hPDLFs and hPDLSCs were observed for *IL-1β, IL-6, TNF-α, OPG, RANKL*, and *RANKL/OPG* using RT-PCR at 0, 6, 24, and 48 hours after treatment. The experiments were performed with the following groups for hPDLFs and hPDLSCs; (1) No treat, (2) EGCG alone, (3) *P. gingivalis* LPS alone, (4) EGCG + *P. gingivalis* LPS.

Results: The 20 μM of EGCG and 20 μg/mL of *P. gingivalis* LPS had the lowest cytotoxic effects, so those concentrations were used for further experiments. The proliferations of hPDLFs and hPDLSCs increased in all groups, though the 'EGCG alone' showed less increase. In RT-PCR, the hPDLFs and hPDLSCs of 'EGCG alone' showed similar gene expressions than those cells of 'no treat'. The gene expressions of '*P. gingivalis* LPS alone' in both hPDLFs and hPDLSCs were highly increased at 6 hours for *IL-1β, IL-6, TNF-α, RANKL,* and *RANKL/OPG,* except the *RANKL/OPG* in hPDLSCs. However, those increased gene expressions were down-regulated in 'EGCG + *P. gingivalis* LPS' by the additional treatment of EGCG,

Conclusions: Our results demonstrate that EGCG could exert an antiinflammatory effect in hPDLFs and hPDLSCs against a major pathogen of periodontitis, *P. gingivalis* LPS.

Keywords: Epigallocatechin gallate, Porphyromonas gingivalis, Lipopolysaccharide, Periodontal ligament, Osteoclastogenesis inhibitory factor.

Antiinflammatory effect of (–)-epigallocatechin-3-gallate on *Porphyromonas gingivalis* lipopolysaccharide-stimulated fibroblasts and stem cells derived from human periodontal ligament

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I. Introduction

Periodontitis is one of the most widespread infectious diseases, and the associated periodontal tissue destruction is caused directly from the presence of periodontal pathogenic bacteria. Periodontal pathogens are considered to chronically activate inflammatory cells and osteoclasts, leading to gingival attachment loss and alveolar bone destruction. Yamamoto et al. reported that human periodontal ligament (hPDL) cells may play an important role in the production of inflammatory cytokines in periodontal diseases(Yamamoto T et al., 2006). Furthermore, periodontal ligament (PDL) stem cells are regarded to be highly proliferative and capable of regenerating cementum- and PDL-like tissues, and to have therapeutic potential for the reconstruction of tissues destroyed by periodontal diseases (Seo BM et al., 2004).

Porphyromonas gingivalis is a gram-negative, black-pigmented anaerobic bacterium that is found in periodontal pockets, and is thought to play a principal role in periodontitis (Slots J et al., 1986). Lipopolysaccharide (LPS), a bacterial membrane protein, is known to be able to induce the secretion of high levels of several cytokines and proteinases from host cells, which leads to periodontal tissue destruction (Birkedal-Hansen H et al., 1993). LPS stimulates inflammatory cells, such as neutrophils, macrophages, and fibroblasts, to secrete interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha (TNF-α) (Nair SP et al., 1996; Aznar C et al., 1990), and these mediators have been reported to potently accelerate formation of osteoclast and bone resorption both in vivo and *in vitro* (Boyce BF et al., 1989; Gowen M et al., 1983). Moreover, the receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANKL) stimulates bone resorption, whereas osteoprotegerin (OPG) inhibits it, and this bimolecular system is involved in periodontal tissue destruction. *P. gingivalis* was reported to induce *RANKL* and reduce *OPG* mRNA expressions in hPDL cells and gingival fibroblasts, resulting in an increased *RANKL/OPG* expression ratio (Belibasakis GN et al., 2007).

Several studies demonstrated that (–)-epigallocatechin-3-gallate (EGCG) suppresses

LPS-induced bone resorption by inhibiting IL-1β production or by directly inhibiting osteoclastogenesis (Yun JH et al., 2004; Rogers J et al., 2005; Yun JH et al., 2007). Furthermore, EGCG was found to inhibit RANKL-induced osteoclast differentiation via the suppression of NF-κB transcriptional activity (Lee YL et al., 2009). Several studies have reported the biological effects of EGCG, one of the major constituents of green tea, including its cell-preserving cytostatic properties, antibacterial and anti-inflammatory effects, and antioxidant effects (Cabrera C et al., 2006; Crespy V et al., 2004; Higdon JV et al., 2003). In the field of dentistry, EGCG was demonstrated to improve the host conditions in periodontitis and periapical lesions, and those effects are due to the bactericidal effect of EGCG against periodontal pathogens (Lee YL et al., 2009; Sakanaka S et al., 1996), and due to the inhibitory effect on the production of related cytokines and their inflammatory pathways of gingival fibroblasts or osteoblasts (Higdon JV et al., 2003; Hosokawa Y et al., 2009).

The present *in vitro* study evaluated the antiinflammatory effects of EGCG on human periodontal ligament fibroblasts (hPDLFs) and human periodontal ligament stem cells (hPDLSCs) affected by a periodontal pathogen (*P. gingivalis*).

II. Materials & Methods

1. Isolation and culture of hPDLFs and hPDLSCs

We isolated hPDLFs and hPDLSCs from the nondecayed healthy teeth of three young adults (aged 11 to 19 years), that had been extracted for orthodontic purposes. The experimental protocols in this study were approved by the Ethics Committee of the College of Dentistry at Yonsei University, and before enrollment, written and informed consent was obtained from each subject.

After washing the extracted teeth with α-modified Eagle's medium (α-MEM; GIBCO, Grand Island, NY, USA) including 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO), the PDL tissues of the root from the middle-third to the apex were scraped and collected. For hPDLFs, the collected PDL tissues were minced and attached to the bottom of culture plates (T75 cell culture flask, Nunclon Delta Surface, NUNC, Roskilde, Denmark) and cultured with α-MEM supplemented with 10% fetal calf serum (GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2. On the other hand, hPDLSCs were prepared using the enzymatic method by Seo et al. (Seo BM et al. 2004). The minced small pieces of PDL tissues were digested with a solution of enzymes containing α-MEM (GIBCO), 2 mg/mL collagenase type I (Waco Pure Chemical Industries, Tokyo, Japan), and 1 mg/mL dispase (GIBCO) for 1 hour at 37°C. Then, single cell suspensions were collected using a 70-μm strainer (BD Falcon, BD Biosciences, Bedford, MA, USA). Cells (5×10^5) were cultured in a T75 flask at 37˚C in 5% CO_2 with α-MEM containing 15% fatal calf serum, 10 μmol/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich Co., St. Louis, MO, USA), 200 μmol/mL L-glutamine (GIBCO) and 100 U/mL penicillin, and 100 μg/mL streptomycin.

hPDLFs outgrown from PDL tissues and colonies of hPDLSCs were observed after approximately 14 days and 10 days, respectively. On days 21 and 14, respectively, the cells (hPDLFs and hPDLSCs) were detached by treating them with 0.25% trypsin-ethylenediaminetetraacetic acid solution (GIBCO) for 3 minutes at 37˚C. Culturing medium was exchanged every three to four days, and the third or fourth passage of hPDLFs and hPDLSCs were used in this experiment.

2. Preparation of *P. gingivalis* LPS

The extraction of *P. gingivalis* LPS was performed as follows. Any bioactive extracellular material was removed by suspending *P. gingivalis* in saline, stirring the suspension gently for 1 hour at 4˚C, and then harvesting them by centrifugation. After repeating this surface-washing process, primary extraction of the endotoxin was performed by using butanol as reported by Morrison and Leive (Morrison DC et al. 1975). In brief, the bacteria were suspended in 0.15 M NaCl (Sigma-Aldrich Co.) and an equal volume of butanol (Sigma-Aldrich Co.) was mixed in thoroughly at 4°C for 10 minutes. After centrifuging the mixture at 35,000×g for 20 minutes, the aqueous supernatant was removed and saved. Then, the same volume of butanol was put into the insoluble residue, and approximately half the volume of saline was added. This butanol extraction was performed in tricplicate. To eliminate any insoluble residues, the combined aqueous extracts were centrifuged and dialyzed with distilled water at 4°C for 8 hours, and lyophilization was performed. The products, however, also contained lipid-A-associated proteins as well as LPS, so a subsequent purification was prepared. The crude products were resuspended in water and ultracentrifuged at 105,000×g for 3 hours, and the procedures were repeated one more time before lyophilizing the endotoxin. Pure LPS was then prepared through the standard hot phenol-water method (Apicella MA et al. 2008). In short, endotoxin was resuspended into pyrogen-free distilled water, 90% phenol (Sigma-Aldrich Co.) was added, and the mixture was extracted twice at 68°C for 20 minutes. After cooling, the supernatants were centrifuged at 35,000×g for 15 minutes, dialyzed extensively with distilled water at 4°C, and centrifuged again at 105,000×g for 3 hours. After subsequent freeze-drying of the aqueous LPS solution, the mixture of DNase (25 μg/mL; Sigma-Aldrich Co.) and RNase (25 μg/mL; Sigma-Aldrich Co.) in 0.1 M Tris-HCl (pH 8.0; Sigma-Aldrich Co.) was treated overnight at 37°C to remove nucleic acids. Any contaminating protein was then hydrolyzed with proteinase K (50 μg/mL; Sigma-Aldrich Co.), followed by heating at 60°C for 1 hour and incubation overnight at 37°C.

3. Assay of the cytotoxicity of EGCG and *P. gingivalis* LPS

The cytotoxic activities of EGCG and *P. gingivalis* LPS on hPDLFs or hPDLSCs were confirmed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. hPDLFs or hPDLSCs were seeded onto 96-well plates and then treated with EGCG (at 0, 0.1, 1, 10, 20, 50, or 100 μM) and/or *P. gingivalis* LPS (at 0, 0.1, 0.5, 1, 5, 10, 20, or 50 μg/mL) for 1, 3, 5, and 7 days. MTT reagent (Amresco, Solon, OH, USA) was put into each well at a final concentration of 0.5 mg/mL, and after a 3-hour incubation at 37°C, the supernatant of each well was replaced with the same volume of dimethyl sulfoxide (Amresco) and further incubated for 30 minutes. The optical density was measured at a wavelength of 570 nm with a microplate reader (enzyme-linked immunosorbent assay [ELISA]; Bio-Rad Laboratories Inc., Hercules, CA, USA).

4. Cell proliferation assay

Using a bromodeoxyuridine (BrdU) cell proliferation kit (Roche Diagnostics, Mannheim, Germany), the proliferation of hPDLFs and hPDLSCs was measured. Cells were seeded onto 96-well plates, and after 6 hours they were treated with EGCG or/and *P. gingivalis* LPS at the concentrations that were selected on the basis of the aforementioned MTT cytotoxicity assays as follows: 1) No treatment, 2) EGCG alone (20 μM), 3) *P. gingivalis* LPS alone (20 μg/mL), and 4) EGCG (20 μM) + *P. gingivalis* LPS (20 μg/mL). After 24 horrs, the assay was performed according to the manufacturer's instructions. In brief, BrdU-labeling solution was added and cells were incubated for 2 hours at room temperature. Then, cells were fixed for 30 minutes and reincubated at room temperature with anti-BrdU-peroxidase antibody in the kit. After 90 minutes, cells were rinsed with phosphate-buffered saline and substrate solution was added until sufficient color had developed. The absorbance of the BrdU incorporation was measured by a microplate spectrophotometer (ELISA; Bio-Rad Laboratories Inc.) at 370 nm.

5. Quantitative real-time polymerase chain reaction (RT-PCR)

The mRNA of several cytokines was detected in order to analyze the changes in secretions of inflammatory factors under the influence of EGCG. The following representative inflammatory cytokines and osteoclastogenic factors involved in periodontitis were sought: IL-1β, IL-6, TNF-α, RANKL, and OPG (Belibasakis GN et al. 2007, Yamamoto T et al. 2006). RT-PCR analysis was performed, and mRNA expressions of *IL-1β, IL-6, TNF-α, RANKL*, and *OPG* were detected. hPDLSCs and hPDLFs were seeded onto 6-well plates, and were treated with EGCG (20 μM) and/or

P. gingivalis LPS (20 μg/mL) when they reached 80% confluence. At 6, 12, and 48 hours after treatment, the mRNA expressions of mediators were detected according to the manufacturer's instructions for each product. Using Trizol (Invitrogen, Carlsbad, CA, USA), cells were harvested and total RNA was prepared. For synthesis of cDNA, isolated RNA templates were mixed with Oligo dT (Maxime RT Premix, iNtRON Biotechnology Inc., Seongnam, Korea). The subsequent RT-PCR amplificatory reactions were performed using a SYBR real-time PCR kit (Premix Ex Taq, Takara Bio, Otsu, Japan), an ABI 7300 real-time PCR machine and software (Applied Biosystems, Foster City, CA, USA), and specific primers (Table 1). The quantification of each mRNA was calculated relative to the results of an internal standard (β-actin). Each data production was performed in triplicate with the same total RNA.

6. Statistical analysis

The statistical analysis was performed with IBM SPSS ver. 19 (IBM Co., Armonk, NY, USA). The data in the present study was analyzed using a Wilcoxon rank-sum test, Kruskal-Wallis test, and Tukey's post hoc multiple comparison test, and the level of significance was set as 0.1 or 0.05 ($P<0.05$).

III. Results

1. MTT cytotoxicity assays of EGCG and *P. gingivalis* LPS

The cell toxicities of EGCG and *P. gingivalis* LPS were tested on hPDLFs and hPDLSCs. The additional treatments of EGCG or *P. gingivalis* LPS produced significantly different cell viabilities of hPDLFs and hPDLSCs, especially at concentrations of 50 and 100 μM for EGCG, and 50 μg/mL for *P. gingivalis* LPS. For EGCG treatment, the cellular activities of hPDLFs and hPDLSCs were gradually increased until day 5 or 7, except the concentrations of 50 and 100 μM EGCG (Fig. 1A and B). The groups of 50 and 100 μM EGCG showed significantly low cell viabilities, while the cells of most concentrations (0, 0.1, 1, 10, and 20 μM EGCG) were comparable at each day. On day 7, the proliferations of hPDLSCs in 0.1, 1, 10, and 20 μM EGCG were less extensive than those of the untreated cells (0 μM EGCG), but there was no significant difference ($P<0.1$). On the other hand, the additional treatment of *P. gingivalis* LPS also produced a gradual increase in cell proliferation of hPDLFs and hPDLSCs, and they were similar to EGCG treatment. Comparing with other concentrations in hPDLFs, the only group of 50 μg/mL showed a significant decrease in cell viability at day 7 (Fig. 1C). However, the additional treatment of *P. gingivalis* LPS (0.1, 0.5, 1, 5, 10, 20, and 50 μg/mL) exerted effects on hPDLSCs, showing less activities at day 7 than untreated hPDLSCs (0 μg/mL) (Fig. 1D). Although there was a significant decrease in cell viabilities, the lower concentrations of *P. gingivalis* LPS (0.1, 0.5, 1, 5, 10, and 20 μg/mL) were relatively less influential than 50 μg/mL ($P<0.05$). Therefore, the less cytotoxic concentrations, 20 μM for EGCG and 20 μg/mL for *P. gingivalis* LPS, were used in the subsequent studies.

2. BrdU cell-proliferation assay

The effects of EGCG and/or *P. gingivalis* LPS on cell characteristics were determined by performing a cell-proliferation assay over 7 days with BrdU (Fig. 2). hPDLSCs and hPDLFs generally proliferated continuously during the experimental period, with an exponential increase occurring from days 5 to 7. More importantly, the population of hPDLSCs was significantly greater than that of hPDLFs. However, for both hPDLFs and hPDLSCs, the degree of conjugation with BrdU was significantly lower in the EGCG-treated groups ('EGCG alone' and 'EGCG + *P. gingivalis* LPS') than in the EGCG-untreated groups ('No treat' and '*P. gingivalis* LPS alone'); BrdU conjugation was unaffected by the presence of *P. gingivalis* LPS ($P<0.05$). This suggests that the reduction in hPDLSC and hPDLF proliferation observed in the EGCG-treated groups is attributable to the presence of EGCG.

3. Evaluation of inflammatory mRNA expression by RT-PCR

The inflamed condition of PDL cells was simulated by irritating hPDLFs and hPDLSCs with *P. gingivalis* LPS, and the antiinflammatory effect of EGCG was tested by comparing cells treated with EGCG and those left untreated. Analysis was conducted using RT-PCR, and the presented data is the representative of separate experiments in triplicate. For inflammatory genes (*IL-1β*, *IL-6*, and *TNF-α*) in general, hPDLFs and hPDLSCs in 'EGCG alone' showed similar or lower gene expressions than those cells of 'No treat', but the gene expressions of '*P. gingivalis* LPS alone' were highly increased at 6 hours (Fig. 3). The increased gene expressions were notably higher in *IL-1β* and *IL-6* for hPDLFs and in *IL-6* and *TNF-α* for hPDLSCs. Unlike '*P. gingivalis* LPS alone', the 'EGCG + *P. gingivalis* LPS' group of cells showed similar gene expressions with the cells of 'no treat'. The increased gene expressions of '*P. gingivalis* LPS alone' at 6 hours were down-regulated by the additional treatment with EGCG, though there was an increase in the *IL-1β* gene expression of hPDLSCs after 48 hours. Based on the *RANKL* and *OPG* gene expressions, the osteclastogenesis of the groups was evaluated with the ratio of *RANKL/OPG* (Fig. 4). The groups of 'no treat' and 'EGCG alone' showed similar and rarely increased gene expressions for both types of cells, hPDLFs and hPDLSCs. In the groups of '*P. gingivalis* LPS alone', however, the gene expression of hPDLFs was up-regulated at 6 hour in *RANKL*, so the ratio of *RANKL/OPG* was higher than the other groups. On the other hand, the ‘EGCG + *P. gingivalis* LPS’ group showed much lower gene expression at 6 hours in *RANKL* and *RANKL/OPG*. For hPDLSCs, the ‘*P. gingivalis* LPS alone’ group showed much higher gene expressions at 6 hours in both *RANKL* and *OPG*. Although the *RANKL* gene was strongly expressed, the relative gene expressions of *RANKL/OPG* were not increased since that of the *OPG* gene was also strongly observed. However, those up-regulated gene expressions of *RANKL* and *OPG* were not observed in the ‘EGCG + *P. gingivalis* LPS’ group, and there was an increase in *OPG* gene expression at 48 hours.

IV. Discussion

The PDL fibroblast, which is the major constitutive cell in the PDL, is known to participate with other immunogenic cells in immune responses to resist the harmful action of oral bacteria and toxins. However, since multipotent mesenchymal stem cells have recently been found in PDL tissues, so it has been suggested that the hPDLSCs may play an important role in biologic reactions in the periodontium. Few studies have investigated the immune reaction of hPDLSCs against periodontal pathogens.

It was reported recently that EGCG can improve the periodontal condition in periodontitis and periapical lesions because of its bactericidal effects against periodontal pathogens (Lee YL et al., 2009; Hirasawa M et al., 2002). With the aim of understanding the antiinflammatory mechanisms of EGCG, several studies have been performed on various cells, such as human chondrocytes, intestinal epithelial cells, and gingival fibroblasts (Hosokawa Y et al., 2009; Ahmed S et al., 2004; Yang F et al., 2001) . However, the effect of EGCG on PDL cells has yet to be established, and there are no reports on the effect of EGCG on the gene expressions of inflammatory cytokines in PDL cells. We therefore examined the effect of EGCG on the release of inflammatory cytokines from *P. gingivalis*-stimulated hPDLFs and hPDLSCs. We also determined the effect of EGCG on the proliferation of hPDLFs and hPDLSCs.

The cytotoxicities of *P. gingivalis* LPS and EGCG were tested using the MTT assay. Among the various concentrations of EGCG (0, 0.1, 1, 10, 20, 50, and 100 μM) and *P. gingivalis* LPS (0, 0.1, 0.5, 1, 5, 10, 20, and 50 μg/mL) tested, only 50 and 100 μM EGCG and 50 μg/mL *P. gingivalis* LPS exerted cytotoxic effects on the cell viability of hPDLFs and hPDLSCs. We tested the effects of both *P. gingivalis* LPS and EGCG on cell proliferation using the BrdU assay with the maximum concentrations that showed no cytotoxicity (i.e., 20 μg/mL and 20 μM, respectively).

The BrdU assay enables a more precise detection of changes in proliferation; the synthetic nucleoside BrdU can be incorporated into the newly synthesized DNA of replicating cells, and application of anti-BrdU can thus detect the newly proliferated cells. Although the number of both types of cells increased during the entire experimental period, the proliferation of hPDLSCs was greater than that of hPDLFs. This higher proliferation by hPDLSCs may be due to their potential for self-renewal, which is a typical property of stem cells.

On the other hand, the addition of EGCG to the culture medium reduced the cellular proliferation regardless of treatment with *P. gingivalis* LPS. Such an inhibitory effect of EGCG on cell proliferation might be attributable to its cytostatic nature. Previous studies have supported that EGCG pauses cell growth due to its ampiphathic property, which makes EGCG readily combine with cellular proteins, thus making possible the storage of intact cells (Liao S et al., 2001; Bae JY et al., 2009; Han DW et al., 2008) . Another study, however, has reported that EGCG suppresses tumorigenesis by interfering with signal transducer and transcription activator protein (Tang SN et al., 2012). In chronic peridontitis, PDL cells including PDLF are involved in the breakdown of periodontal tissues by secreating inflammatory cytokines (Birkedal-Hansen H et al., 1993). If the inflamed PDL cells that are releasing destructive inflammatory mediators could be decreased or at least limited in their rate increase during periodontitis, less destruction and better healing of periodontal tissues would be possible.

In the RT-PCR analysis, we observed the changes in gene expressions of hPDLFs and hPDLSCs, which *were IL-1β, IL-6, TNF-α, RANKL, OPG,* and the *RANKL/OPG* ratio. In previous reports, significantly large quantities of IL-1β, IL-6, and TNF-α were reportedly found at sites of periodontal disease, where they play a central role in the breakdown of periodontal tissues (Okada H et al., 1998; Pathirana RD et al., 2000) by stimulating monocytes and fibroblasts to release extracellular matrix-degradable proteins (Graves DT et al., 2003), helping the maturation of B cells into immunoglobulin-producing plasma cells (Ishimi Y et al., 1990). Therefore, those *P. gingivalis* LPS changes in gene expression induced by *P. gingivalis* LPS in this study may be the inflammatory reactions of hPDLFs and hPDLSCs. However, there was unexpected result, in that an exponential increase of *IL-1β* gene expression at 48 hours was observed in hPDLSCs of the 'EGCG + *P. gingivalis* LPS' group. We were not able to find related or similar results in other papers. Therefore, it further experiments are needed to understand the therapeutic mechanism by EGCG more thoroughly.

In addition, osteoclastogenesis, a differentiating and maturing procedure of osteoclasts, is the main mechanism underlying the break down of periodontal tissue(Hasegawa T et al. 2002) and IL-1β, IL-6, and TNF-α were demonstrated to be associated in tissue destruction by promoting the differentiation of osteoclast precursors and subsequently activating osteoclasts (Pfeilschifter J et al., 1989). For these reasons, changes in osteoclastogenic mediators, RANKL and OPG, should also be detected as periodontal inflammatory reactors. RANKL, a surface-bond molecule, is a key factor in the differentiation and activation of osteoclasts, while OPG, a decoy receptor for RANKL, blocks RANKL by binding RANKL-RANK ligands of osteoclast precursors and osteoclasts (Perez-Sayans M et al., 2010), so the ratio of RANKL/OPG is a value to consider as a possible marker of osteoclastogenesis**.**

We therefore investigated the gene expressions of *IL-1β, IL-6, TNF-α, RANKL,* and *OPG* in hPDL cells stimulated by *P. gingivalis* LPS. At 6 hours after *P. gingivalis* LPS stimulation, the challenged hPDLFs exhibited up-regulated gene expressions of *IL-1β, IL-6, TNF-α,* and *RANKL,* and an increased *RANKL/OPG* ratio, suggesting a bone-destruction response. These results are consistent with those of other studies: (Imatani T et al., 2001) found that *P. gingivalis* induced the secretion of IL-1β, IL-6, IL-8, and TNF-α by human gingival fibroblasts, and Yamamoto et al (Yamamoto T et al., 2006) found that stimulation with *P. gingivalis* induced mRNA expressions of *IL-1β, IL-6, IL-8, TNF-α,* and *RANKL* in hPDL cells. Similar research has revealed that *P.*

gingivalis-challenged PDL cells up-regulate *RANKL* and down-regulate *OPG*, resulting in a significantly higher *RANKL/OPG* ratio, and indicating a transient increase in their osteoclastogenic potential (Belibasakis GN et al., 2007). However, the gene expression levels of *TNF-α, RANKL*, and *OPG* in *P. gingivalis*-LPS-stimulated hPDLSCs were higher than those of *IL-1β* and *IL-6*. Moreover, although the *RANKL* gene was strongly expressed in *P. gingivalis*-LPS-stimulated hPDLSCs, the expression level of the *OPG* gene was also similarly high, indicating that the *RANKL/OPG* ratio was not changed markedly by *P. gingivalis* LPS treatment. These findings show that the main inflammatory reaction by hPDLSCs was due to increased *TNF-α* expression rather than *IL-1β* and *IL-6* expressions, which is different from the responses in hPDLFs. Moreover, the osteoclastogenesis induced by hPDLSCs would be down-regulated in comparison to hPDLFs, since the compensative *OPG* was expressed more strongly in *P. gingivalis*-stimulated hPDLSCs. This is thought to be due to cellular differences between fibroblastic hPDLFs and stem-cell-like hPDLSCs.

It is known that mesenchymal stem cells have immune-modulating effects, and several studies have found that PDLSCs also have immune-suppressing potential by inhibiting the proliferation of monocytes and T cells (Kim HS et al., 2010). Therefore, we assumed that the down-regulated osteoclastogenic *RANKL/OPG* ratio of hPDLSCs would be associated with the immune-modulating effect of stem cells, but further in-depth studies are required to investigate this relationship. The findings of such studies would be helpful for understanding the antiinflammatory mechanisms of

EGCG in periodontal inflammation.

Additional treatment with EGCG caused a down-regulation of the *P. gingivalis*-LPS-induced increase in the gene expressions of *IL-1β, IL-6, TNF-α, RANKL*, and *OPG* in both hPDLFs and hPDLSCs. The anti-inflammatory effects of EGCG have been reported mainly in fields other than dentistry, although a few studies have recently investigated this in the dental field. Nakanishi et al. (Nakanishi T et al., 2010) confirmed that treatment with EGCG causes down-regulation of the secretion of IL-6 by human dental pulp cells simulated by *Escherichia coli* LPS, and Hosokawa et al. showed that EGCG can reduce IL-6 production by human gingival fibroblasts stimulated by TNF-14. In a clinical study, Hirasawa et al (Hirasawa M et al. 2002) found significant reductions in markers of gingivitis after the slow-release application of EGCG over an 8-week period.

V. Conclusions

We treated hPDLFs and hPDLSCs with a major green tea extract, EGCG, and *P. gingivalis* LPS. The findings in the present study show that EGCG treatment induces the down-regulation of cell proliferative activity. The hPDLFs and hPDLSCs showed up-regulated gene expression against *P. gingivalis* LPS stimulation, while EGCG decreased those up-regulated gene expressions of *IL-1 β, IL-6, TNF-α*, as well as the osteoclastogenic gene expression of *RANKL*. Our results demonstrated that EGCG may exert an anti-inflammatory and antiosteoclastogenic effect in hPDLFs and hPDLSCs against a major pathogen of periodontits, *P. gingivalis* LPS.

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Legends

Figure 1 Effects of (–)-epigallocatechin-3-gallate (EGCG) and *Porphyromonas gingivalis* lipopolysaccharide (LPS) on the viability of human periodontal ligament fibroblasts (hPDLFs) and human periodontal ligament stem cells (hPDLSCs). Most of the hPDLFs (A) and hPDLSCs (B) exhibited comparable cell viability in the EGCG-containing media (0, 0.1, 1, 10, and 20 μM) at days 3 to 7, except in 50 and 100 μM ($P<0.1$). The proliferation of hPDLFs (C) and hPDLSCs (D) treated with 50 μg/mL *P. gingivalis* LPS exhibited the most significant down-regulation at day 7, while remained unaffected for hPDLFs and less affected for hPDLSCs in other concentrations (i.e., 0, 0.1, 0.5, 1, 5, 10, and 20 μg/mL) ($P<0.05$). MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OD: optical density. [a)], [b)], or [c)], there is a statistically significant difference; [a)] vs. [b)], [a)] vs. [c)], and [b)] vs. [c)] ($P<0.1$). [d)], [e)], or [f)], there is a statistically significant difference; [d)] vs. [e)], [d)] vs. [f)], and [e)] vs. [f)] ($P<0.1$).

Figure 2. . Proliferative changes of human periodontal ligament fibroblasts (hPDLFs) and hPDLSCs treated with *Porphyromonas gingivalis* lipopolysaccharide (LPS) and (–)-epigallocatechin-3-gallate

(EGCG). hPDLFs (A) and human periodontal ligament stem cells (hPDLSCs) (B) generally proliferated continuously during the experimental period, with an exponential increase in cell growth being observed from days 5 to 7. The cellular proliferation was generally higher in hPDLFs than hPDLSCs. However, for both hPDLFs and hPDLSCs, the degree of conjugation with bromodeoxyuridine (BrdU) was significantly lower in EGCG-treated groups than in EGCG-untreated groups. Conjugation with BrdU was not affected by treatment with *P. gingivalis* LPS (P<0.05). OD: optical density. [a)], [b)], or [c)], there is a statistically significant difference; [a)] vs. [b)], [a)] vs. [c)], and [b)] vs. [c)] (P<0.05).

Figure 3. Effects of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and/or (–)-epigallocatechin-3-gallate (EGCG) on inflammatory mRNA expressions. EGCG-treated cells (human periodontal ligament fibroblasts [hPDLFs] and human periodontal ligament stem cells [hPDLSCs]) showed similar or lower gene expressions than those cells of 'No treat'. The gene expressions of *P. gingivalis* LPS alone were highly increased at 6 hours, but those increased gene expressions were down-regulated in 'EGCG + *P. ginvialis* LPS' group by the additional treatment of EGCG. *IL-1β:*

interleukin 1β, IL-6: interleukin 6, TNF-α: tumor necrosis factor-α.

Figure 4. Effects of *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS) and (–)-epigallocatechin-3-gallate (EGCG) on osteoclastogenesis-related mRNA expression. The gene expressions of only cells and EGCG-alone were similar and few increased. (A, B and C) The gene expression of *P. gingivalis* LPS-stimulated human periodontal ligament fibroblasts (hPDLFs) was increased at 6 hours in receptor activator of nuclear factor kappa-B ligand (*RANKL*) and *RANKL*/osteoprotegerin (*OPG*), but it was down-regulated in 'EGCG + *P. gingivalis* LPS' group. (D, E and F) The '*P. gingivali* LPS alone' of human periodontal ligament stem cells (hPDLSCs) showed much higher gene expressions at 6 hours in both of *RANKL* and *OPG*, but those up-regulated gene expressions of *RANKL* and *OPG* were not increased in 'EGCG + *P. gingivalis* LPS'group

Tables

Table 1 Primer Sequences for the quantitative real-time polymerase chain reaction.

Gene (GenBank no.)	Primer sequence (forward and reverse)	Size (bp)
IL-1β (NM_000088)	GAT CTG CGT CTG CGA CAA C (forward) GGC AGT TCT TGG TCT CGT CA (reverse)	68
IL-6 (NM_000090)	GCC AAA TAT GTG TCT GTG ACT CA (forward) GGG CGA GTA GGA GCA GTT G (reverse)	145
TNF-α (NM_002317)	CGG CGG AGG AAA ACT GTC T (forward) TCG GCT GGG TAA GAA ATC TGA (reverse)	128
OPG (NM_005576)	CCA CTA CGA CCT ACT GGA TGC (forward) GTT GCC GAA GTC ACA GGT G (reverse)	97
RANKL (NM_002318)	GGG TGG AGG TGT ACT ATG ATG G (forward) CTT GCC GTA GGA GGA GCT G (reverse)	137
β-actin (NM_001101)	CAT GTA CGT TGC TAT CCA GGC (forward) CTC CTT AAT GTC ACG CAC GAT (reverse)	250

IL-1β: interleukin 1β, IL-6: interleukin 6, TNF-α: tumor necrosis factor-α, OPG: osteoprotegerin, RANKL: receptor activator of nuclear factor kappa-B ligand.

Figures

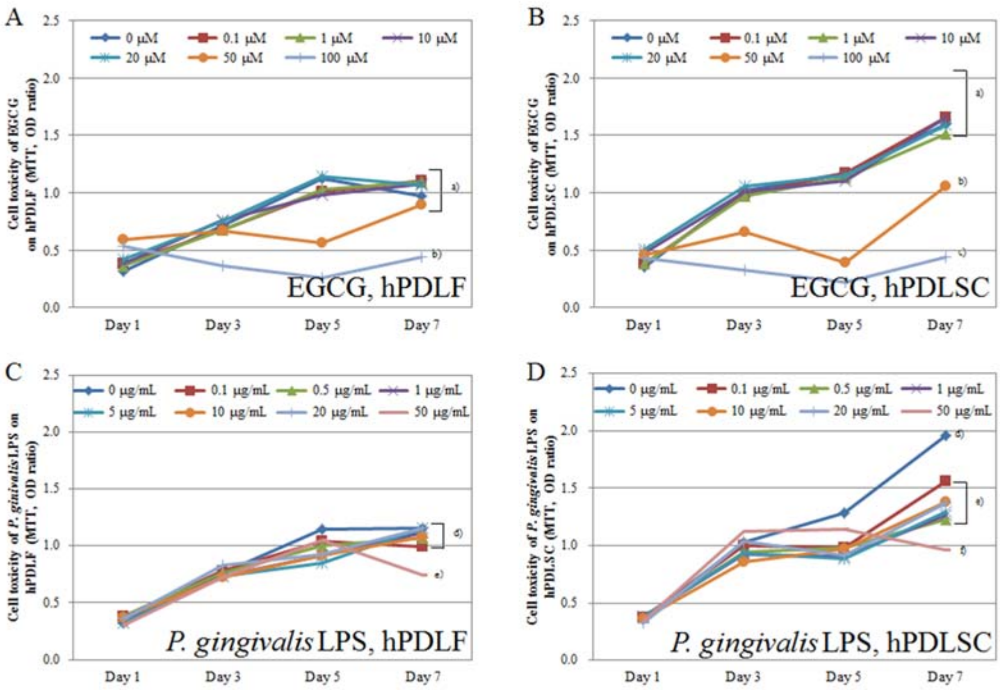

Figure 1

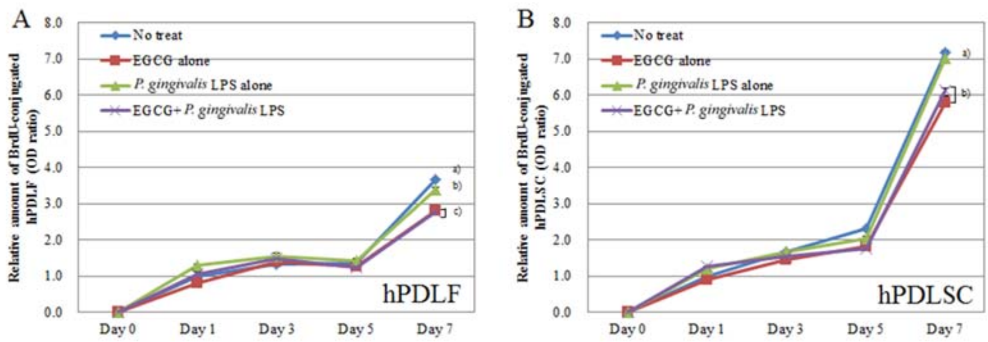

Figure 2

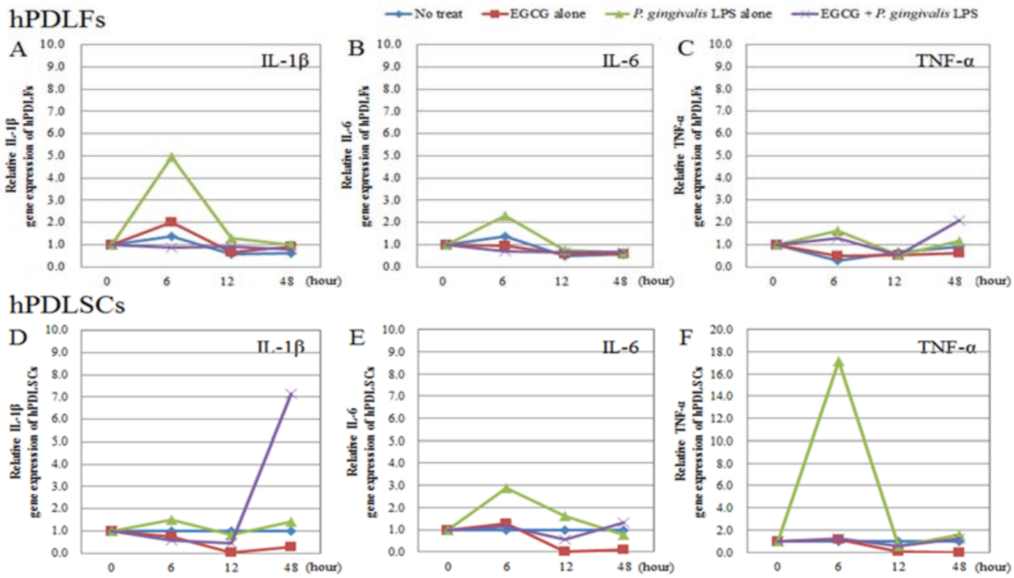

Figure 3

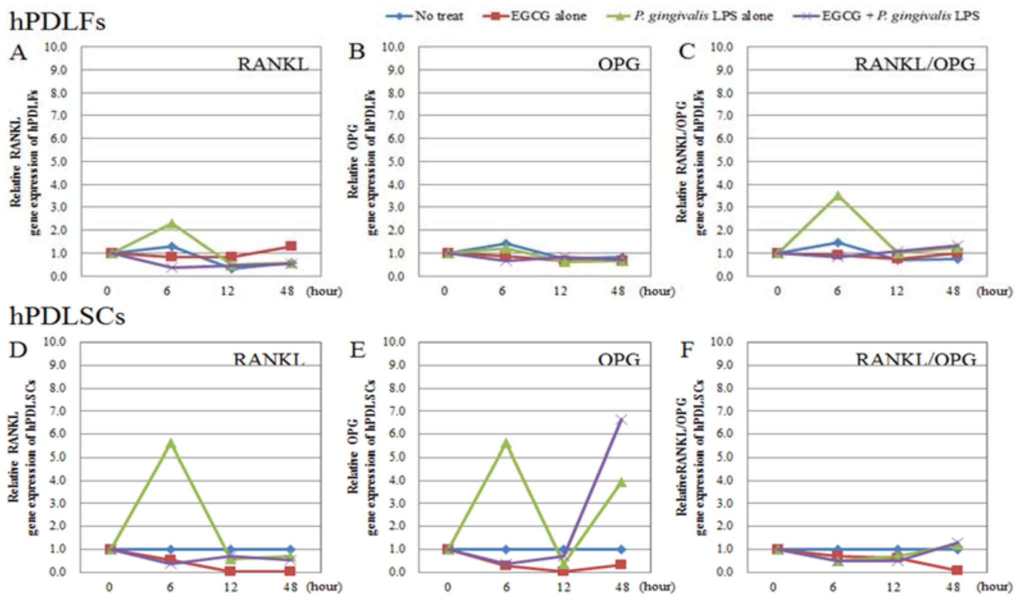

Figure 4

국문요약

인간 치주인대 섬유모세포와 줄기세포에서 ***Porphyromonas gingivalis*** 지질 다당류 자극에 의한 **(–)-epigallocatechin-3-gallate**의 항염효과

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이 동 은

녹차 추출물의 주성분인 (-)-epigallocatechin-3-gallate는 치주염에서 항염효과와 항균효과를 보인다고 보고되어 왔다. 그러나 그 정확한 기전은 알려져 있지 않다. 이에 본 연구의 목적은 *Porphyromonas gingivalis* 지질 다당류에 의해 자극받은 인간 치주인대 섬유모세포와 치주인대 줄기세포에서 (-)-epigallocatechin-3-gallate의 항염효과를 평가하는 것이다.

성인의 건강한 치아에서 추출한 hPDLFs와 hPDLSCs를 EGCG 및/또는 *P. gingivalis* LPS로 처리하였다. 1, 3, 5, 7일간 처리한 후에 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 와 bromodeoxyuridine assay (Brdu) assay를 이용하여 각각의 세포독성과 증식능을 측정하였다. 0, 6, 24, 48 시간 후의 치주인대

섬유모세포와 줄기세포의 *IL-1*β, *IL-6*, *TNF*-α, *RANKL*, *OPG* 유전자 표현형의 변화는 Quantitative real-time polymerase chain reaction (PCR)을 통해 관찰하였다. hPDLFs실험군과 hPDLSCs실험군은 다음과 같이 처리하였다. 1)아무처치 하지 않음, 2)EGCG만 처치, 3) *P. gingivalis* LPS만 처치, 4)EGCG+ *P. gingivalis* LPS 처치

가장 낮은 세포독성을 보인 20μM의 EGCG와 20 μg/mL의 *P. gingivalis* LPS를 이후의 실험에서 이용하였다. 모든 실험군에서 hPDLFs와 hPDLSCs실험군의 증식능은 증가하였으나 'EGCG만' 처치한 실험군에서 가장 낮은 증가를 보였다.

Real-time PCR실험에서는 hPDLFs실험군과 hPDLSCs실험군에서 모두 'EGCG'만을 처치한 경우와 '아무처리 하지않은' 경우 유사한 유전자 표현형의 변화를 보였다. 실험 6 시간후, hPDLSCs실험군의 *RANKL/OPG* 수치를 제외한 '*P. gingivalis* LPS만' 처치한 hPDLFs실험군과 hPDLSCs 실험군에서 *IL-1*β, *IL-6*, *TNF*-α, *RANKL*, *RANKL/OPG*수치는 모두 높은 증가를 보였다. 반면에 'EGCG+ *P. gingivalis* LPS'처치한 실험군에서 부가적인 EGCG의 처치는 증가된 염증 세포 유전자를 감소시켰음을 관찰할 수 있었다.

EGCG는 인간 치주 인대 섬유모세포와 줄기세포에서 치주염의 주요 병원체인 *P. gingivalis* LPS에 항염효과를 가지는 것으로 사료된다.

핵심되는 말 : 항염효과; 치주인대; 재생; 지질다당류; *P. gingivalis*