RESEARCH ARTICLE

Tea polyphenols inhibit IL-6 production in tumor necrosis factor superfamily 14-stimulated human gingival fibroblasts

Yoshitaka Hosokawa[1], Ikuko Hosokawa[1], Kazumi Ozaki[2], Tadashi Nakanishi[1], Hideaki Nakae[1] and Takashi Matsuo[1]

[1] Department of Conservative Dentistry and Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Tokushima, Japan
[2] Department of Oral Health Care Promotion, School of Oral Health and Welfare, Faculty of Dentistry, The University of Tokushima, Tokushima, Tokushima, Japan

IL-6 is well recognized to be a potent bone resorptive agent and thus in the development of periodontal disease. Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), the major catechins in green tea, and theaflavin-3,3′-digallate (TFDG), polyphenol in black tea, have multiple beneficial effects, but the effects of catechins and theaflavins on IL-6 production in human gingival fibroblasts (HGFs) are not known. In this study, we investigated the mechanisms by which EGCG, ECG, and TFDG inhibit tumor necrosis factor superfamily 14 (TNFSF14)-induced IL-6 production in HGFs. We detected TNFSF14 mRNA expression in human diseased periodontal tissues. TNFSF14 increased IL-6 production in HGFs in a concentration-dependent manner. EGCG, ECG, and TFDG prevented TNFSF14-mediated IL-6 production in HGFs. EGCG, ECG, and TFDG prevented TNFSF14-induced extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and nuclear factor-κB activation in HGFs. Inhibitors of ERK, JNK, and nuclear factor-κB decreased TNFSF14-induced IL-6 production. In addition, EGCG, ECG, and TFDG attenuated TNFSF14 receptor expression on HGFs. These data provide a novel mechanism through which the green tea and black tea polyphenols could be used to provide direct benefits in periodontal disease.

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1 Introduction

Periodontitis is a chronic bacterial infection of tooth-supporting structures. It causes destruction of periodontal connective tissues and bone. The initiation and progression of the disease result from the host response to plaque bacteria [1]. IL-6 is a proinflammtory cytokine, which has been considered to be related to the pathogenesis of periodontal disease. It was reported that IL-6 levels in inflamed gingival tissues were higher that those in healthy tissues [2]. Recently, it has been reported that IL-6 is involved in bone

Correspondence: Dr. Yoshitaka Hosokawa, Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, Tokushima 770-8504, Japan
E-mail: hosokawa@dent.tokushima-u.ac.jp
Fax: +81-886-33-7340

Abbreviations: ECG, epicatechin gallate; **EGCG,** epigallocatechin gallate; **ERK,** extracellular signal-regulated kinase; **GAPDH,** glyceraldehydes-3-phosphate dehydrogenase; **HGFs,** human gingival fibroblasts; **HVEM,** herpes simplex virus entry mediator; **JNK,** c-Jun N-terminal kinase; **LTβR,** lymphotoxin β receptor; **MAPK,** mitogen-activated protein kinase; **NF,** nuclear factor; **TFDG,** theaflavin-3,3′-digallate; **TNFSF14,** tumor necrosis factor superfamily 14

resorption to induce receptor activator of nuclear factor κB ligand expression in some types of cells [3].

Gingival fibroblasts, the major cell type in periodontal connective tissues, provide a tissue framework for tooth anchorage. Until recently, they were presumed to be immunologically inert. Currently, however, researchers recognize their active role in host defense. Upon stimulation with cytokines or bacterial pathogens, human gingival fibroblasts (HGFs) secrete various soluble mediators of inflammation such as IL-6, IL-8, and chemokines [4–10]. These fibroblast-derived mediators are thought to play an important role in the periodontal inflammatory response.

Tumor necrosis factor superfamily 14 (TNFSF14; lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes simplex virus entry mediator (HVEM), a receptor expressed by T lymphocytes (LIGHT)) is a cytokine in the TNF superfamily that is involved in innate and adaptive immune responses. TNFSF14 is signaling through two distinct members of the TNF receptor superfamily, HVEM and lymphotoxin β receptor (LTβR), and can also bind to the soluble decoy receptor 3. It has been reported that TNFSF14 is important for the initiation of various autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis through effects on T cells and T-cell homing into inflamed tissues [11]. However, the effect of TNFSF14 on the pathogenesis of periodontal disease is uncertain.

Catechins are naturally occurring polyphenolic compounds, which have been shown to have anti-inflammatory, antioxidant, and free radical scavenging properties *in vitro* [12, 13]. For example, epigallocatechin gallate (EGCG), one of the major isoforms of the catechins, has been shown to inhibit the infiltration of leukocytes and myeloperoxidase activity and to decrease UV-B induced erythema [14]. Catechins have also been shown to decrease the production of the proinflamatory cytokines IL-1β and TNF-α and to enhance the production of the anti-inflammatory cytokine IL-10 [15, 16]. However, reports concerning the effects of catechins on IL-6 production are few.

Theaflavins are the major polyphenols in black tea. Theaflavins are categorized into following forms: theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (TFDG). It was reported that TFDG has strong biological effects, including antioxidant effect, compared with other theaflavins [17]. However, the effect of TFDG on cytokine production is still unknown.

The aim of this study was to examine the effect of TNFSF14 on IL-6 production in HGFs. Moreover, we examined the effects of EGCG, epicatechin gallate (ECG), and TFDG on IL-6 production in TNFSF14-stimulated HGFs. Furthermore, we investigated whether catechins and theaflavin treatment modified mitogen-activated protein kinases (MAPK) and nuclear factor κB (NF-κB) activation in TNFSF14-stimulated HGFs.

2 Materials and methods

2.1 Gingival tissue biopsies and cell culture

Tissue samples were obtained at surgery from the inflamed gingiva of patients diagnosed with chronic periodontitis or from the gingivae of clinically healthy subjects. All gingival biopsy sites in the chronic periodontitis group exhibited radiographic evidence of bone destruction, as well as having clinical probing depths greater than 4 mm, with sulcular bleeding on probing; otherwise the patients were systemically healthy. We collected samples after basic periodontal therapy such as scaling. Samples of gingival tissues were obtained from nine chronic periodontitis patients (four males and five females, average age: 61.0 ± 9.8, average probing depth: 6.33 ± 2.06, average attachment loss: 7.02 ± 2.26) and five healthy control subjects (five females, average age: 31.2 ± 9.8, average probing depth: 2.4 ± 0.54, average attachment loss: 2.7 ± 0.57). We used two clinically healthy gingival samples and eight chronic periodontitis samples for RT-PCR. We used HGFs that were isolated from three clinically healthy gingvae. The gingival specimens were cut into small pieces and transferred to culture dishes. The HGFs that grew from the gingivae were primarily cultured on 100 mm^2 uncoated plastic dishes in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (penicillin G: 100 units/mL, streptomycin: 100 μg/mL) at 37°C in humidified air with 5% CO_2. Confluent cells were transferred and cultured for use in the present study. After three to four subcultures with trypsinization, the cultures contained homogeneous, slim, and spindle-shaped cells growing in characteristic swirls. The cells were used for experiments after five passages. Informed consent was obtained from all subjects participating in this study. The study was performed with the approval and compliance of the University of Tokushima Ethical Committee (329).

2.2 RNA extraction and RT-PCR analysis

Total RNA was prepared from gingival biopsies using the Rneasy total RNA isolation Kit (Qiagen, Hilden, Germany). Single-strand cDNA for a PCR template was synthesized from 48 ng of total RNA using a primer, oligo$(dT)_{12–18}$ (Invitrogen, Carlsbad, CA, USA) and superscript 3 reverse transcriptase (Invitrogen) under the conditions indicated by the manufacturer. Specific primers were designed from cDNA sequence for TNFSF14 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Each cDNA was amplified by PCR using Hot star *Taq* DNA polymerase (Qiagen). The sequences of the primers were as follows: TNFSF14-F (5′-CAAGAGCGAAGGTCTCACGAGGTC-3′), TNFSF14-R (5′-TCACACCATGAAAGCCCCGAAGTAAG-3′), GAPDH-F (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′), and

GAPDH-R (5′-CATGTGGGCCATGAGGTCCACCAC-3′). The conditions for PCR were 1 × (95°C, 15 min), 35 × (94°C, 1 min, 59°C, 1 min, 72°C, 1 min) and 1 × (72°C, 10 min). The products were analyzed on a 1.5% agarose gel containing ethidium bromide. We could not detect any bands when we performed PCR without adding the cDNA template in this study.

2.3 IL-6 production in HGFs

The HGFs were stimulated with TNFSF14 (Peprotech, Rocky Hill, NJ, USA) for 24 h. The supernatants from the HGFs were collected, and the IL-6 concentrations of the culture supernatants were measured in triplicate with ELISA. Duoset (R&D systems, Minneapolis, MN, USA) was used for the determination. All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using the standard curve prepared for each assay. In selected experiments, the HGFs were cultured for 1 h in the presence or absence of EGCG (5 or 50 μg/mL: Sigma), ECG (5 or 50 μg/mL: Sigma), TFDG (5 or 50 μg/mL: Nagara Science, Gifu, Japan), PD98059 (20 μM: Santa Cruz Biotechnology, Santa Cruz, CA, USA), SP600125 (20 μM; Sigma), or Bay11-7085 (20 μM: BIOMOL, Plymouth Meeting, PA, USA) prior to their incubation with TNFSF14.

2.4 Western blot analysis

To confirm the TNFSF14-induced phosphorylation of signal transduction molecules, Western blot analysis was performed. HGFs stimulated with TNFSF14 (100 ng/mL) were washed once with cold PBS, before being incubated on ice for 30 min with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin; Cell signaling technology, Danvers, MA, USA) supplemented with Protease Inhibitor Cocktail (104 mM AEBSF, 0.085 mM aprotinin, 4 mM, bestatin, 1.4 mM, E-64, 2 mM leupeptin, and 1.5 mM pepstatin A; Sigma). After removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20 μg protein sample was loaded onto a 4–20% SDS-PAGE gel, before being electrotransfered to a PVDF membrane. The activation of p38 MAPK and extracellular signal-regulated kinase (ERK) was assessed using phospho-p38 MAPK rabbit monoclonal antibody (Cell signaling technology), phospho-ERK rabbit monoclonal antibody (Cell signaling technology), phospho-c-Jun N-terminal kinase (JNK) rabbit monoclonal antibody (Cell signaling technology), phospho-IκB-α mouse monoclonal antibody (Cell signaling technology), p38 MAPK rabbit monoclonal antibody (Cell signaling technology), ERK rabbit monoclonal antibody (Cell signaling technology), JNK rabbit monoclonal antibody (Cell signaling technology), or IκB-α mouse monoclonal antibody according to the manufacturer's instructions. Protein bands were visualized by incubation with the HRP-conjugated secondary antibody (Sigma), followed by detection using the ECL system (GE Healthcare, Uppsala, Sweden). The quantitation of the chemiluminescent signal was analyzed using NIH image.

2.5 Flow cytometric analyses

Following the required culture time, the cells were washed twice with ice-cold PBS. The HGFs were harvested by incubation with PBS-4 mmol/L EDTA. Most of the cells were rounded up following this treatment and removed by gentle agitation. Any cells that failed to detach were removed with gentle scraping. The cells were washed twice with ice-cold PBS and incubated (for 20 min on ice) in PBS-1% BSA (Sigma). The cells were incubated with mouse anti-human HVEM antibody (Thermo Scientific, Cheshire, UK; 5 μg/mL), mouse anti-human LTβR antibody (Biolegend, San Diego, CA, USA; 5 μg/mL), or an isotype control antibody on ice for 30 min. After being washed three times with PBS-1% BSA, the cells were incubated with an FITC-conjugated rabbit anti-mouse F $(ab')_2$ fragment (DAKO, Kyoto, Japan) for 30 min on ice. After being washed three times with PBS-1% BSA, the cells were immediately analyzed with flow cytometry (Epics XL-MCL; Coulter, Hialeah, FL).

2.6 Statistical analysis

Experimental data were expressed as standard errors of the means and were analyzed using one-way ANOVA to compare the difference between tea polyphenols or signal inhibitors treatment groups and the control groups. Any p values smaller than 0.05 were considered to be significant.

3 Results

3.1 TNFSF14 mRNA expression in human gingival tissues

We first examined TNFSF14 expression in human gingival tissues. We could not detect TNFSF14 mRNA expression in healthy gingival tissues (Fig. 1). On the other hand, we detected TNFSF14 mRNA in five to nine human diseased periodontal tissues (Fig. 1).

3.2 Effects of EGCG, ECG, or TFDG on IL-6 production in TNFSF14-stimulated HGFs

We next investigated whether TNFSF14 was able to induce IL-6 production in HGFs. As shown in Fig. 2A, TNFSF14

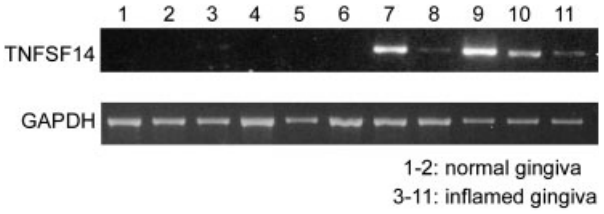

Figure 1. TNFSF14 mRNA expression in human gingival tissues. Total RNA was prepared from two clinically healthy gingival samples (pocket depth, 2 mm) and nine diseased gingival samples (pocket depth, 4–10 mm). The expressions of TNFSF14 and GAPDH mRNA in periodontal tissues were analyzed by RT-PCR as described in Section 2.

induced IL-6 production in HGFs in a concentration-dependent manner. To investigate the effects of EGCG, ECG, or TFDG on IL-6 production from TNFSF14-stimulated HGFs, HGFs were pretreated with EGCG, ECG, or TFDG at the indicated concentrations for 1 h and stimulated with TNFSF14 for 24 h. At first, we examined the effect of EGCG, ECG, or TFDG on HGFs proliferation. Method of transcriptional and translational (MTT) assays revealed treatment with 50 μg/mL of EGCG, ECG, or TFDG for 24 h did not modulate HGFs proliferation (data not shown). Treatment with 50 μg/mL of EGCG, ECG, or TFDG significantly inhibited IL-6 production in TNFSF14-stimulated HGFs (Fig. 2B).

3.3 Effects of EGCG, ECG, or TFDG on MAPKs and NF-κB pathways in TNFSF14-stimulated HGFs

It was reported that MAPKs and NF-κB pathways were involved in IL-6 production [18, 19]. Therefore, we examined the effects of EGCG, ECG, or TFDG on MAPKs and NF-κB pathways in TNFSF14-stimulated HGFs. EGCG or ECG treatment inhibited ERK and IκB-α phosphorylation in TNFSF14-stimulated HGFs (Fig. 3). TFDG treatment apparently suppressed ERK and JNK phosphorylation. However, IκB-α phosphoryeation did not change in TFDG-treated HGFs. (Fig. 4). We demonstrated that EGCG, ECG, and TFDG suppressed ERK, JNK, or NF-κB signal transduction pathways in Figs. 3 and 4. We next examined whether ERK, JNK, and NF-κB inhibitors diminished IL-6 production from TNFSF14-stimulated HGFs. Figure 5 shows that PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and Bay11-7085 (NF-κB inhibitor) significantly inhibited IL-6 production. Therefore, EGCG, ECG, and TFDG suppressed IL-6 production from TNFSF14-stimulated HGFs through the inhibition of ERK, JNK, or NF-κB activation.

3.4 Effects of EGCG, ECG, and TFDG on TNFSF14 receptor expression on HGFs

We hypothesized that EGCG, ECG, and TFDG might modulate TNFSF14 receptor expression because they inhibit

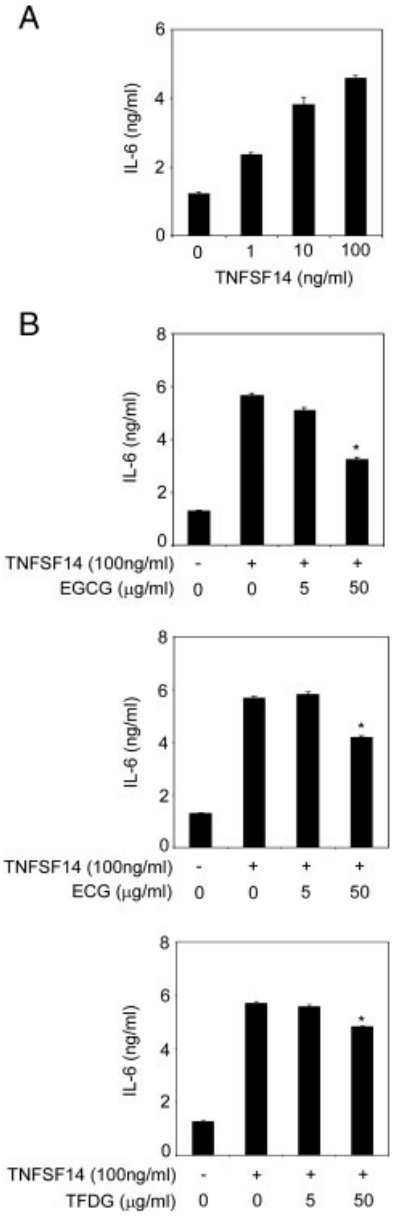

Figure 2. Effects of EGCG, ECG, or TFDG on IL-6 production in TNFSF14-stimulated HGFs. (A) HGFs were treated with TNFSF14 (1, 10, or 100 ng/mL), and the supernatants were collected after 24 h. The expression levels of IL-6 in the supernatants were measured using ELISA. Data are representative of three different HGFs samples from three different donors. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. (B) HGFs were pretreated with EGCG (5 or 50 μg/mL), ECG (5 or 50 μg/mL), or TFDG (5 or 50 μg/mL) for 1 h, and then the HGFs were stimulated with TNFSF14 (100 ng/mL), and the supernatants were collected after 24 h. The expression levels of IL-6 in the supernatants were measured using ELISA. Data are representative of three different HGFs samples from three different donors. Data indicate the mean ± SD of three cultures. The error bars show the SD of the values. $^{*}p<0.05$ significantly different from the TNFSF14-stimulated HGFs without EGCG, ECG, or TFDG.

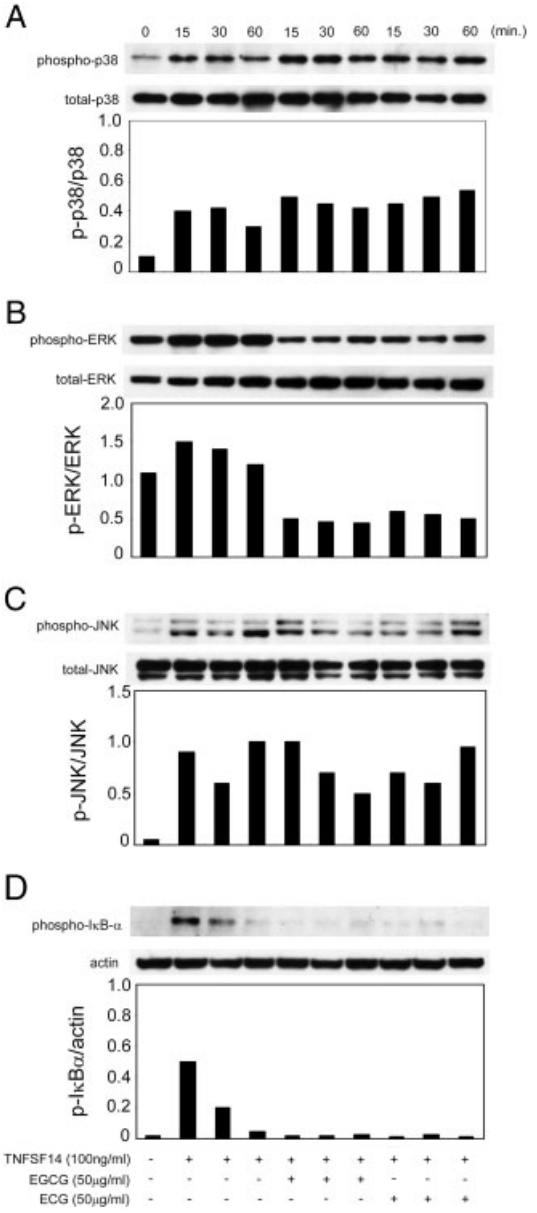

Figure 3. Effects of EGCG and ECG on the TNFSF14-induced phosphorylation of MAPKs or IκB-α. The cultured cells were pretreated with EGCG (50 μg/mL) or ECG (50 μg/mL) for 60 min and then stimulated with 100 ng/mL TNFSF14 for 15, 30, or 60 min. (A) Cellular lysates were collected and the levels of phospho-specific p38 MAPK or p38 MAPK were measured using a Western blot analysis. Bar graphs of phospho-p38 MAPK expressions were normalized to total-p38 MAPK. (B) Cellular lysates were collected and the levels of phospho-specific ERK or ERK were measured using a Western blot analysis. Bar graphs of phospho-ERK expressions were normalized to total-ERK. (C) Cellular lysates were collected and the levels of phospho-specific JNK or JNK were measured using a Western blot analysis. Bar graphs of phospho-JNK expression were normalized to total-JNK. (D) Cellular lysates were collected and the levels of phospho-specific IκB-α or actin were measured using a Western blot analysis. Bar graphs of phospho-IκB-α expression were normalized to actin.

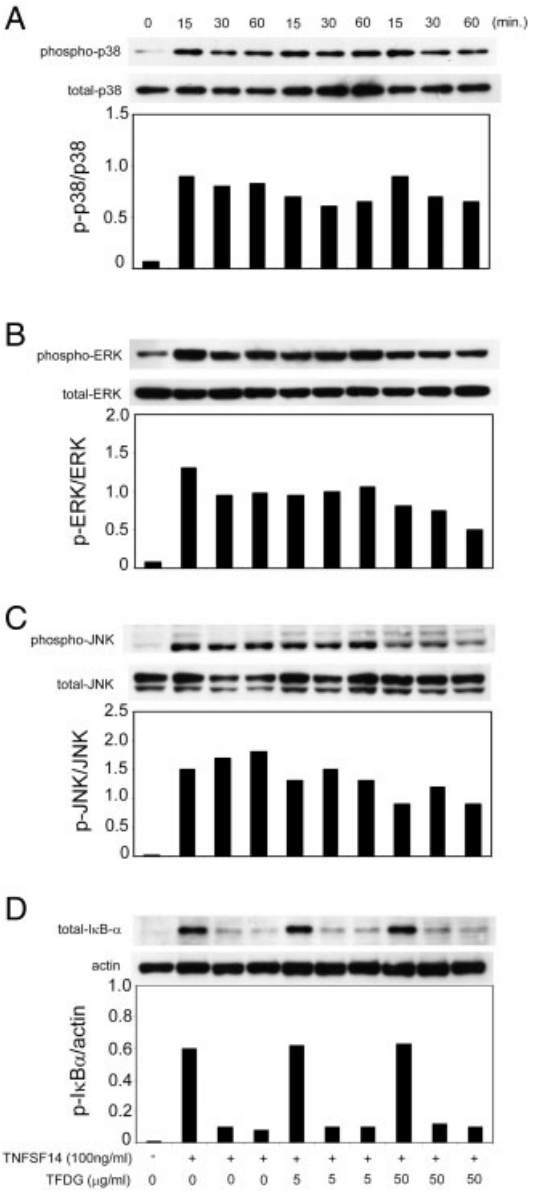

Figure 4. Effects of TFDG on the TNFSF14-induced phosphorylation of MAPKs or IκB-α. The cultured cells were pretreated with TFDG (5 or 50 μg/mL) for 60 min and then stimulated with 100 ng/mL TNFSF14 for 15, 30, or 60 min. (A) Cellular lysates were collected and the levels of phospho-specific p38 MAPK or p38 MAPK were measured using a Western blot analysis. Bar graphs of phospho-p38 MAPK expressions were normalized to total-p38 MAPK. (B) Cellular lysates were collected and the levels of phospho-specific ERK or ERK were measured using a Western blot analysis. Bar graphs of phospho-ERK expressions were normalized to total-ERK. (C) Cellular lysates were collected and the levels of phospho-specific JNK or JNK were measured using a Western blot analysis. Bar graphs of phospho-JNK expression were normalized to total-JNK. (D) Cellular lysates were collected and the levels of phospho-specific IκB-α or actin were measured using a Western blot analysis. Bar graphs of phospho-IκB-α expression were normalized to actin.

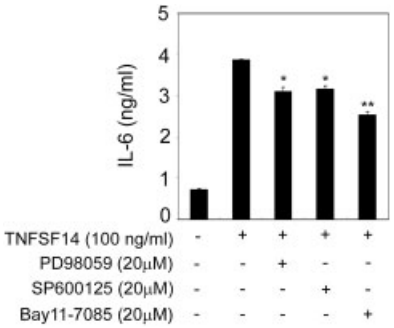

Figure 5. Effects of signal transduction inhibitors on TNFSF14-stimulated IL-6 release by HGFs. The cells were pre-incubated with PD98059 (20 μM), SP600125 (20 μM), or Bay11-7085 (20 μM) for 1 h and then incubated with TNFSF14 (100 ng/mL). After 24 h incubation, the supernatants were collected, and IL-6 production was measured by ELISA. Data are representative of HGFs from three different donors. Data indicate the mean ± SD of three cultures. The error bars show the SD of the values. $^{*}p<0.05$, $^{**}p<0.01$ significantly different from the TNFSF14–stimulated HGFs without inhibitors.

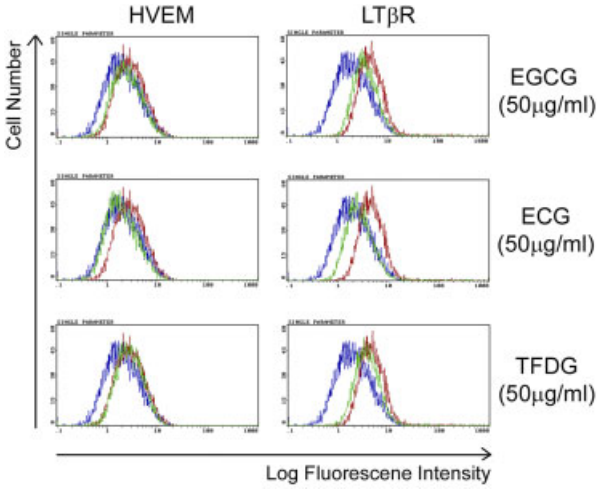

Figure 6. Effects of EGCG, ECG, or TFDG on TNFSF14 receptor expression on HGFs. HGFs were treated with EGCG (50 μg/mL), ECG (50 μg/mL), or TFDG (50 μg/mL), and the cells were collected after 24 h. The expression levels of HVEM and LTβR on HGFs were measured using flow cytometry as described in Section 2. The blue line represents the background level of fluorescence caused by the isotype matched antibody. The red line represents HVEM or LTβR expression on non-stimulated HGFs. The green line represents EGCG, ECG, or TFDG-treated HGFs. One of three experiments with similar results is shown.

the IL-6 production induced by TNFSF14 stimulation. As shown in Fig. 6, EGCG, ECG, and TFDG treatment significantly suppressed LTβR expression on HGFs. On the other hand, only ECG apparently down-regulated HVEM expression on HGFs, though EGCG slightly suppressed HVEM expression on HGFs (Fig. 6).

4 Discussion

In this study, we demonstrated that TNFSF14 is able to induce IL-6 production in HGFs. It has been reported that IL-6 is involved in the pathology of periodontal disease [20]. Moreover, IL-6 is well recognized to be a potent bone resorptive agent and induce osteoclast formation [21]. Therefore, TNFSF14 might induce periodontal tissue destruction, including bone resorption, by enhancing IL-6 production from HGFs.

In the present study, we revealed that EGCG significantly suppressed the TNFSF14-stimulated induction of IL-6 in HGFs to inhibit ERK and NF-κB pathway. Tokuda *et al.* reported that EGCG treatment suppressed endothelin-1-induced IL-6 production from osteoblast-like MC3T3-E1 cells to inhibit ERK activation [22]. Shin *et al.* also reported that EGCG inhibited IL-6 production from phorbol 12-myristate 13-acetate-stimulated human mast cell line to suppress ERK and NF-κB activation [23]. Our report agrees with their reports.

We reported ECG also suppressed IL-6 production from TNFSF14-stimulated HGFs. The reports about the effect of ECG on cytokine production are few. Kim *et al.* reported that ECG suppressed IL-8 production from IL-1β-stimulated human nasal fibroblasts and bronchial epithelial cells [24]. Ichikawa *et al.* reported ECG inhibited IL-12 p40 production from LPS-stimulated macrophage [25]. They also reported that ECG suppressed NF-κB activation in macrophages induced by LPS stimulation, though they reported ECG did not modify ERK activation. This discrepancy might reflect differences of cell type and stimulator.

The reports about effects of theaflavin treatment on immune reaction are few compared with catechins. Ukil *et al.* reported that oral administration of TFDG significantly improved colitis in mouse model associated with decreased mRNA and protein levels of TNF-α, IL-12, and IFN-γ in colonic mucosa [26]. They also reported that oral administration of TFDG inhibited NF-κB activation in colon tissues. In this report, we demonstrate that TFDG suppresses IL-6 production from HGFs through the inhibition of ERK and JNK activation. This report is the first one to mention the role of theaflavins on immune response in cell levels. Further investigations are necessary to clarify the role of theaflavins on the immune system.

We reported that EGCG, ECG, and TFDG suppressed TNFSF14 receptors expression on HGFs. Ahn *et al.* reported that EGCG suppressed TNF-α receptor 1 expression on vascular endothelial cells [27]. We recently reported that EGCG and ECG down-regulated oncostatin M receptor β expression on HGFs [28]. We show catechins and theaflavins could modulate cytokine receptor expression in this report. The role of catechins and theaflavins on cytokine receptor expression on various types of cells should be examined.

We used 50 μg/mL catechins and TFDG to treat the HGFs in this experiment. It is known that the epithelial cell layer is broken at the bottom of periodontal pocket. This means that catechins might come into contact with HGFs when they are used for periodontal pocket irrigation. Further investigation is necessary to test the use of EGCG, ECG, or TFDG for pocket irrigation.

In summary, the current study demonstrates that TNFSF14 causes IL-6 release by cultured HGFs. EGCG, ECG, and TFDG suppressed TNFSF14-induced IL-6 production in HGFs. In addition, we revealed that EGCG, ECG and TFDG inhibited TNFSF14-induced ERK, JNK, and NF-κB activation and suppressed TNFSF14 receptor expression in HGFs. These data show that we could use EGCG, ECG, or TFDG for periodontal disease treatment.

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The authors have declared no conflict of interest.

5 References

[1] Seymour, G. J., Importance of the host response in the periodontium. *J. Clin. Periodontol.* 1991, *18*, 421–426.

[2] Wang, P. L., Ohura, K., Fujii, T., Oido-Mori, M. *et al.*, DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem. Biophys. Res. Commun.* 2003, *305*, 970–973.

[3] Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A. *et al.*, Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem. Biophys. Res. Commun.* 2000, *275*, 768–775.

[4] Takashiba, S., Takigawa, M., Takahashi, K., Myokai, F. *et al.*, Interleukin-8 is a major neutrophil chemotactic factor derived from cultured human gingival fibroblasts stimulated with interleukin-1 beta or tumor necrosis factor alpha. *Infect. Immun.* 1992, *60*, 5253–5258.

[5] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H. *et al.*, CXCL12 and CXCR4 expression by human gingival fibroblasts in periodontal disease. *Clin. Exp. Immunol.* 2005, *141*, 467–474.

[6] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., Increase of CCL20 expression by human gingival fibroblasts upon stimulation with cytokines and bacterial endotoxin. *Clin. Exp. Immunol.* 2005, *142*, 285–291.

[7] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., CXC chemokine ligand 16 in periodontal diseases: expression in diseased tissues and production by cytokine-stimulated human gingival fibroblasts. *Clin. Exp. Immunol.* 2007, *149*, 146–154.

[8] Hosokawa, I., Hosokawa, Y., Ozaki, K., Nakae, H., Matsuo, T., Adrenomedullin suppresses tumour necrosis factor alpha-induced CXC chemokine ligand 10 production by human gingival fibroblasts. *Clin. Exp. Immunol.* 2008, *152*, 568–575.

[9] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., CC chemokine ligand 17 in periodontal diseases: expression in diseased tissues and production by human gingival fibroblasts. *J. Periodontal Res.* 2008, *43*, 471–477.

[10] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., Cytokines differentially regulate CXCL10 production by interferon-gamma-stimulated or tumor necrosis factor-alpha-stimulated human gingival fibroblasts. *J. Periodontal Res.* 2009, *44*, 225–231.

[11] Schneider, K., Potter, K. G., Ware, C. F., Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol. Rev.* 2004, *202*, 49–66.

[12] Chan, M. M., Fong, D., Ho, C. T., Huang, H. I., Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem. Pharmacol.* 1997, *54*, 1281–1286.

[13] Yen, G. C., Chen, H. Y., Scavenging effect of various tea extracts on superoxide derived from the metabolism of mutagens. *Biosci. Biotechnol. Biochem.* 1998, *62*, 1768–1770.

[14] Katiyar, S. K., Matsui, M. S., Elmets, C. A., Mukhtar, H., Polyphenolic antioxidant (–)-epigallocatechin-3-gallate from green tea reduces UVB-induced inflammatory responses and infiltration of leukocytes in human skin. *Photochem. Photobiol.* 1999, *69*, 148–153.

[15] Yang, F., de Villiers, W. J., McClain, C. J., Varilek, G. W., Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. *J. Nutr.* 1998, *128*, 2334–2340.

[16] Crouvezier, S., Powell, B., Keir, D., Yaqoob, P., The effects of phenolic components of tea on the production of pro- and anti-inflammatory cytokines by human leukocytes *in vitro*. *Cytokine* 2001, *13*, 280–286.

[17] Leung, L. K., Su, Y., Chen, R., Zhang, Z. *et al.*, Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr.* 2001, *131*, 2248–2251.

[18] Patil, C., Rossa, C., Jr.,, Kirkwood, K. L., *Actinobacillus actinomycetemcomitans* lipopolysaccharide induces interleukin-6 expression through multiple mitogen-activated protein kinase pathways in periodontal ligament fibroblasts. *Oral Microbiol. Immunol.* 2006, *21*, 392–398.

[19] Haddad, J. J., Fahlman, C. S., Nuclear factor-kappa B-independent regulation of lipopolysaccharide-mediated interleukin-6 biosynthesis. *Biochem. Biophys. Res. Commun.* 2002, *291*, 1045–1051.

[20] Irwin, C. R., Myrillas, T. T., The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis.* 1998, *4*, 43–47.

[21] Heymann, D., Rousselle, A. V., gp130 Cytokine family and bone cells. *Cytokine* 2000, *12*, 1455–1468.

[22] Tokuda, H., Takai, S., Hanai, Y., Matsushima-Nishiwaki, R. *et al.*, (–)-Epigallocatechin gallate suppresses endothelin-1-induced interleukin-6 synthesis in osteoblasts: inhibition of p44/p42 MAP kinase activation. *FEBS Lett.* 2007, *581*, 1311–1316.

[23] Shin, H. Y., Kim, S. H., Jeong, H. J., Kim, S. Y. *et al.*, Epigallocatechin-3-gallate inhibits secretion of TNF-alpha, IL-6 and IL-8 through the attenuation of ERK and NF-kappaB in HMC-1 cells. *Int. Arch. Allergy Immunol.* 2007, *142*, 335–344.

[24] Kim, I. B., Kim, D. Y., Lee, S. J., Sun, M. J. *et al.*, Inhibition of IL-8 production by green tea polyphenols in human nasal fibroblasts and A549 epithelial cells. *Biol. Pharm. Bull.* 2006, *29*, 1120–1125.

[25] Ichikawa, D., Matsui, A., Imai, M., Sonoda, Y., Kasahara, T., Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line, J774.1. *Biol. Pharm. Bull.* 2004, *27*, 1353–1358.

[26] Ukil, A., Maity, S., Das, P. K., Protection from experimental colitis by theaflavin-3,3′-digallate correlates with inhibition of IKK and NF-kappaB activation. *Br. J. Pharmacol.* 2006, *149*, 121–131.

[27] Ahn, H. Y., Xu, Y., Davidge, S. T., Epigallocatechin-3-O-gallate inhibits TNFalpha-induced monocyte chemotactic protein-1 production from vascular endothelial cells. *Life Sci.* 2008, *82*, 964–968.

[28] Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakanishi, T. *et al.*, Catechins inhibit CXCL10 production from oncostatin M-stimulated human gingival fibroblasts. *J. Nutr. Biochem.* 2009, DOI: 10.1016/j.jnutbio.2009.04.005.

