Catechins Inhibit CCL20 Production in IL-17A-Stimulated Human Gingival Fibroblasts

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Key Words

Catechins • IL-17A • CCL20 • Human gingival fibroblasts

Abstract

CC chemokine ligand 20 (CCL20) plays a pivotal role in the recruitment of Th17 cells and thus in the development of periodontal disease. Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), the major catechins in green tea, have multiple beneficial effects, but the effects of catechins on CCL20 production in human gingival fibroblasts (HGFs) are not known. In this study, we investigated the mechanisms by which EGCG and ECG inhibit interleukin (IL)-17A-induced CCL20 production in human gingival fibroblasts. IL-17A increased CCL20 production in HGFs in a concentration-dependent manner. EGCG and ECG prevented IL-17A-mediated CCL20 production in HGFs. Inhibitors of p38 mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) decreased IL-17A-induced CCL20 production. EGCG and ECG prevented IL-17A-induced phosphorylation of p38 MAPK and ERK in HGFs. In addition, EGCG and ECG attenuated IL-17 receptor expression on HGFs. These

data provide a novel mechanism through which the green tea flavonoids catechins could be used to provide direct benefits in periodontal disease.

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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. Immunohistochemical studies have revealed dense inflammatory cell infiltration, involving T cells, B cells and macrophages in periodontitis affected regions [1-3]. Recently, a new type of T cell, Th17, has been linked with several inflammatory diseases [4], and it was reported that Th17 cells are present in periodontally diseased tissues [5]. However, the effect of IL-17A, which is produced from Th17, on Th17 migration in periodontally diseased tissues is uncertain.

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 secrete various soluble mediators of inflammation such as IL-8 and chemokines [6-12]. These fibroblast-derived mediators are thought to play an important role in the periodontal inflammatory response.

Catechins are naturally occurring polyphenolic compounds, which have been shown to have anti-inflammatory, antioxidant, and free radical scavenging properties *in vitro*. [13, 14] 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 [15]. Catechins have also been shown to decrease the production of the proinflammatory cytokines IL-1 β and tumor necrosis factor (TNF)- α and to enhance the production of the anti-inflammatory cytokine IL-10 [16, 17]. However, reports concerning the effects of catechins on chemokine production are rare.

It has been reported that the CC chemokine CCL20 attracts activated Th17 cells through its interaction with CC chemokine receptor 6 (CCR6) [18]. We previously reported that IL-1 β , TNF- α and lipopolysaccharide induce CCL20 production in HGFs [8] and that CCR6 positive cells infiltrate into periodontally diseased tissues [19]. However, the effect of IL-17A on CCL20 production in HGFs is unknown.

The aim of this study was to examine the effect of IL-17A on CCL20 production in HGFs. Moreover, we examined the effects of the catechins EGCG and epicatechin gallate (ECG) on CCL20 production in IL-17A-stimulated HGFs. Furthermore, we investigated whether catechin treatment modified the phosphorylation of mitogen-activated protein kinases (MAPK) in IL-17A-stimulated HGFs.

Materials and Methods

Gingival tissue biopsies and cell culture

We used HGFs that were isolated from 3 clinically healthy gingiva during routine distal wedge surgical procedures. The gingival specimens were cut into small pieces and transferred to culture dishes. The HGFs that grew from the gingivae were primarily cultured on 100mm² uncoated plastic dishes in Dulbecco's modified Eagle medium (DMEM: Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS: Gibco, Grand Island, NY, USA) and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 μ g/ml) at 37°C in humidified air with 5% CO₂. 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.

CCL20 production in HGFs

The HGFs were stimulated with IL-17A (Peprotech, Rocky Hill, NJ, USA) for 24 hours. The supernatants from the HGFs were collected, and the CCL20 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 hour in the presence or absence of EGCG (0.5, 5, or 50 μ g/ml: Sigma), ECG (0.5, 5, or 50 μ g/ml: Sigma), SB203580 (20 μ M: Santa Cruz Biotechnology, Santa Cruz, CA, USA), or PD98059 (20 μ M: Santa Cruz Biotechnology) prior to their incubation with IL-17A.

Western blot analysis

To confirm the IL-17A-induced phosphorylation of signal transduction molecules, Western blot analysis was performed. HGFs stimulated with IL-17A (100 ng/ml) were washed once with cold PBS, before being incubated on ice for 30 min with lysis buffer (Cell signaling technology, Danvers, MA, USA) supplemented with protease inhibitors (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 μ g protein sample was loaded onto a 4-20% SDS-PAGE gel, before being electrotransferred to a PVDF membrane. The activation of p38 MAPK and ERK was assessed using phospho-p38 MAPK rabbit monoclonal antibody (Cell signaling technology), phospho-ERK rabbit monoclonal antibody (Cell signaling technology), p38 MAPK rabbit monoclonal antibody (Cell signaling technology), or ERK rabbit 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.

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-4mmol/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 minutes on ice) in PBS-1% bovine serum albumin (BSA; Sigma). The cells were incubated with mouse anti-human IL-17 receptor A (IL-17RA) antibody (R&D systems; 5 μ g/ml), IL-17 receptor C (IL-17RC) antibody (R&D systems; 5 μ g/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,

Fig. 1. Effects of IL-17A on CCL20 production by HGFs. HGFs were treated with IL-17A (0.1, 1, 10, or 100 ng/ml), and the supernatants were collected after 24 hours. The expression levels of CCL20 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. * = $P < 0.05$ significantly different from the non-stimulated HGFs.

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).

Statistical analysis

Statistical significance was analyzed using the Student's *t* test. P values < 0.05 were considered significant.

Results

The effect of IL-17A on CCL20 production by HGFs

We first investigated whether IL-17A was able to induce CCL20 production in HGFs. As shown in Fig. 1, IL-17A induced CCL20 production in HGFs in a concentration dependent manner.

Effects of catechins on CCL20 production in IL-17A-stimulated HGFs

To investigate the effects of EGCG and ECG on IL-17A-stimulated CCL20 production, HGFs were pretreated with EGCG or ECG at the indicated concentrations for 1 hour and stimulated with IL-17A for 24 hours. Treatment with 50 $\mu\text{g/ml}$ of EGCG or ECG significantly inhibited CCL20 production in IL-17A-stimulated HGFs (Fig. 2).

P38 MAPK and ERK pathways are related to CCL20 production in IL-17A-stimulated HGFs

We previously reported that MAPKs are related to CCL20 production in HGFs [8]. Therefore, we examined

Fig. 2. Effects of catechins on CCL20 production in IL-17A-stimulated HGFs. HGFs were pretreated with EGCG (0.5, 5, or 50 $\mu\text{g/ml}$) (A) or ECG (0.5, 5, or 50 $\mu\text{g/ml}$) (B) for 1 hour, and then the HGFs were stimulated with IL-17A (100 ng/ml), and the supernatants were collected after 24 hours. The expression levels of CCL20 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. * = $P < 0.05$ significantly different from the IL-17A-stimulated HGFs without catechins.

Fig. 3. Effects of signal transduction inhibitors on IL-17A-stimulated CCL20 release by HGFs. The cells were pre-incubated with SB203580 (20 μM) or PD98059 (20 μM) for 1 hour and then incubated with IL-17A (100 ng/ml). After 24 hours incubation, the supernatants were collected, and CCL20 production was measured by ELISA. Data are representative of HGFs 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. ** = $P < 0.01$ significantly different from the IL-17A-stimulated HGFs without inhibitors.

the effects of a p38 MAPK inhibitor (SB203580) and a MEK inhibitor (PD98059) on CCL20 production in IL-17A-stimulated HGFs. Fig. 3 shows that SB203580 and PD98059 significantly suppressed CCL20 production in IL-17A-stimulated HGFs.

Effects of EGCG and ECG on the p38 MAPK and ERK phosphorylation induced by IL-17A stimulation in HGFs

Next, we examined the effects of catechins on the signal pathways involved in CCL20 production in IL-17A-stimulated HGFs. EGCG and ECG treatment inhibited p38 MAPK and ERK phosphorylation in IL-17A-stimulated HGFs (Fig. 4). EGCG treatment strongly inhibited p38 MAPK and ERK phosphorylation compared to ECG treatment.

EGCG and ECG suppressed IL-17 receptor expression on HGFs

We hypothesized that catechins might modulate IL-17 receptor expression because they inhibit the CCL20 production induced by IL-17A stimulation. As shown in Fig. 5, EGCG and ECG treatment suppressed both IL-17RA and IL-17RC expression on HGFs.

Discussion

In this study, we demonstrated for the first time that IL-17A is able to induce CCL20 production in HGFs. It has been reported that CCL20 is involved in the migration of Th17 cells because Th17 cells preferentially express CCR6 [18]. Moreover, it was suggested that Th17 cells are involved in the exacerbation of periodontal disease [20]. Therefore, IL-17A might induce periodontal tissue destruction by enhancing Th17 cell migration.

In the present study, we revealed that EGCG and ECG significantly suppressed the IL-17A-stimulated induction of CCL20 in HGFs. Therefore, EGCG and/or ECG might be targets of treatment for Th17-related diseases, such as periodontal disease and arthritis because CCL20 is involved in Th17 cell migration into peripheral tissues. Further investigation is necessary to test the use of catechins for treating Th17-related disease.

We investigated the mechanisms underlying the inhibitory effects of EGCG and ECG on IL-17A-stimulated CCL20 production. It has been reported that IL-17A is able to preferentially activate the p38 MAPK and MEK/ERK pathways in fibroblasts [21]. Therefore, we used several inhibitors to elucidate which signaling

Fig. 4. Effects of EGCG and ECG on the IL-17A-induced phosphorylation of p38 MAPK or ERK. The cultured cells were pretreated with EGCG (50 μg/ml) or ECG (50 μg/ml) for 60 min and then stimulated with 100ng/ml IL-17A for 15, 30, or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific ERK, or ERK. (A) A representative Western blot displaying phospho-p38 MAPK and total-p38 MAPK levels in HGFs from three independent experiments (top). Bar graphs of phospho-p38 MAPK expression normalized to total-p38 MAPK (n=3). The data are shown as the mean and SD. * $P<0.01$ vs OSM for 15 min; stimulated HGFs without catechins. # $P<0.01$ vs OSM for 30 min; stimulated HGFs without catechins. ** $P<0.01$ vs OSM for 60 min; stimulated HGFs without catechins. (B) A representative Western blot displaying phospho-ERK and total-ERK levels in HGFs from three independent experiments. (top). Bar graphs of phospho-ERK expression normalized to total-ERK (n=3). The data are shown as the mean and SD. * $P<0.01$ vs OSM for 15 min; stimulated HGFs without catechins. # $P<0.01$ vs OSM for 30 min; stimulated HGFs without catechins. ** $P<0.01$ vs OSM for 60 min; stimulated HGFs without catechins.

Fig. 5. Effects of EGCG or ECG on IL-17 receptor expression on HGFs. HGFs were treated with EGCG (50 $\mu\text{g/ml}$) or ECG (50 $\mu\text{g/ml}$), and the cells were collected after 24 hours. The expression levels of IL-17RA and IL-17RC on HGFs were measured using flow cytometry as described in Materials and Methods. The light grey line represents the background level of fluorescence caused by the isotype matched antibody. The dark grey line represents IL-17RA or IL-17RC expression on non-stimulated HGFs. The black line represents EGCG (A) or ECG (B)-treated HGFs. One of three experiments with similar results is shown.

pathways are involved in CCL20 production in HGFs. Our study revealed that the p38 MAPK and ERK pathways are involved in IL-17A-induced CCL20 production in HGFs. We previously reported that SB203580 and PD98059 suppressed CCL20 production in lipopolysaccharide-stimulated HGFs [8]. Our previous

report agrees with this report. Furthermore, we revealed that EGCG and ECG treatment suppressed the p38 MAPK and ERK phosphorylation induced by IL-17A stimulation. We recently reported that EGCG and ECG treatment inhibited p38 MAPK activation in oncostatin M-stimulated HGFs [22]. Moreover, Chae et al. reported that EGCG suppressed ERK phosphorylation in angiotensin 2-treated human umbilical vein endothelial cells [23]. Bigelow et al. also reported that EGCG and ECG suppressed ERK activation in hepatocyte growth factor-treated tumorigenic breast epithelial cells [24]. Their reports support the results found in this study.

We demonstrated that EGCG and ECG treatment suppressed IL-17 receptor expression after 24 hours of catechin treatment. Recently, some researchers examined the effects of catechins on cytokine receptor expression. Ahn et al. reported that EGCG treatment suppressed TNF receptor 1 expression in vascular endothelial cells [25]. We recently discovered that EGCG and ECG treatment down-regulated oncostatin M receptor β expression on HGFs [22]. Previous reports and this report suggest that catechins are able to modulate the inflammatory response as a means of regulating cytokine receptor expression.

We used 50 $\mu\text{g/ml}$ catechins 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 use of the catechins for pocket irrigation.

In summary, the current study demonstrates that IL-17A causes CCL20 release by cultured HGFs. Catechins suppressed IL-17A-induced CCL20 production in HGFs. In addition, we revealed that catechins inhibited IL-17A-induced p38 MAPK and ERK phosphorylation and suppressed IL-17 receptor expression in HGFs. We think that the direct effects of catechins inhibit CCL20 production in IL-17A-stimulated HGFs in order to inhibit the phosphorylation of signal transduction molecules. After that, catechins gradually suppress IL-17 receptor expression so as to inhibit the effects of IL-17A on HGFs. These data provide a novel mechanism through which the green tea flavonoids EGCG and ECG could be used to provide periodontal benefits in periodontal disease.

Acknowledgements

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