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Catechins inhibit CXCL10 production from oncostatin M-stimulated human gingival fibroblasts

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Abstract

CXC chemokine ligand 10 (CXCL10) plays a pivotal role in the recruitment of Th1 cells and, thus, in the development of periodontal disease. Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), the major catechins derived from green tea, have multiple beneficial effects, but the effects of catechins on CXCL10 production from human gingival fibroblasts (HGFs) is not known. In this study, we investigated the mechanisms by which EGCG and ECG inhibit oncostatin M (OSM)-induced CXCL10 production in HGFs. HGFs constitutively expressed glycoprotein 130 and OSM receptor beta (OSMRβ), which are OSM receptors. OSM increased CXCL10 production in a concentration-dependent manner. EGCG and ECG prevented OSM-mediated CXCL10 production by HGFs. Inhibitors of p38 mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-OH kinase and signal transducer and activator of transcription (STAT)3 decreased OSM-induced CXCL10 production. EGCG significantly prevented OSM-induced phosphorylation of JNK, Akt (Ser473) and STAT3 (Tyr705 and Ser727). ECG prevented phosphorylation of JNK and Akt (Ser473). In addition, EGCG and ECG attenuated OSMRβ expression on HGFs. These data provide a novel mechanism through which the green tea flavonoids, catechins, can provide direct benefits in periodontal disease.

Keywords: Catechins; Oncostatin M; CXCL10; Human gingival fibroblasts

1. Introduction

Oncostatin M (OSM) belongs to the interleukin (IL)-6 family of cytokines, which includes IL-6, leukemia-inhibitory factor, IL-11, cardiotrophin-1 and ciliary neurotrophic factor [1]. The IL-6 family of cytokine receptors requires dimerization with glycoprotein 130 (gp130), a glycoprotein cell surface receptor, for intracellular signaling. OSM and several members of the IL-6 cytokine family are known to activate fibroblasts and to regulate the synthesis of matrix metalloproteinases and their inhibitors in these cells [2–4]. However, the results of studies in humans, rats and mice support the notion that OSM is uniquely involved in the regulation of inflammation [5–7]. OSM is primarily produced in and released by activated monocytes, T lymphocytes and neutrophils [8–10], and it is found in a variety of inflammatory sites. In the human lung during acute lung injury, infiltrating neutrophils secrete OSM [5]. OSM levels also are elevated in the sera of patients with rheumatoid arthritis [11] as well as in patients with inflamed skin [12] and periodontitis [13]. In addition, in vitro studies have demonstrated that OSM not only regulates the remodeling function of fibroblasts but also elicits inflammatory responses in these cells. OSM induces the CC chemokines eotaxin [7] (an eosinophil chemoattractant) and monocyte chemoattractant protein 1 [3] in the mouse lung and synovial fibroblasts, respectively. Moreover, overexpression of OSM in the mouse lung results in increased recruitment of eosinophils [7]. Taken together, these studies suggest that OSM regulates inflammatory function in fibroblasts and that fibroblasts may be implicated in the recruitment of leukocytes upon activation by OSM.

Periodontitis is a chronic bacterial infection of tooth-supporting structures. It causes destruction of periodontal connective tissues and bone. The disease initiation and progression result from the host response to plaque bacteria. Immunohistochemical studies have revealed dense inflammatory cell infiltration, including T and B cells and macrophages in periodontitis-affected regions [14–19]. Recently, several studies demonstrated that Th1 cells are involved in bone resorption in oral cavities. Kawai et al. [20] reported that the activation of Th1-type T cells appeared to trigger inflammatory periodontal bone resorption. Stashenko et al. [21] reported that intrapulpal challenge with viable *Porphyromonas gingivalis* results in massive periapical bone destruction during systemic Th1 response. However, the effects of OSM on the Th1 response in periodontal disease are uncertain.

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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 as well as with bacterial pathogens, human gingival fibroblasts (HGFs) secrete various soluble mediators of inflammation such as IL-1β, IL-6 and IL-8 [22–25] and up-regulate expression of HLA-DR, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 [26]. These fibroblast-derived mediators and surface antigens 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 [27,28]. 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 [29]. Catechins have also been shown to decrease the production of the proinflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α and to enhance the production of the anti-inflammatory cytokine IL-10 [30,31]. However, reports concerning the effects of catechins on chemokine production are rare.

CXC chemokine ligand 10 (CXCL10) was discovered as an IFN-γ-inducible protein of 10 kDa in the monocytic U937 cells [32]. CXCL10 attracts activated Th1 cells through interaction with CXC chemokine receptor 3 (CXCR3) [33,34]. CXCL10 shares this receptor and, hence, biological activity with two more recently identified CXC chemokines, CXCL9 and CXCL11 [35–37]. In vivo, enhanced levels of CXCL10 have been reported in several inflammatory diseases that are predominantly associated with a Th1 phenotype. It is reported that CXCL10 and CXCR3 are detected in inflamed gingival tissues [38,39]. However, it is unknown whether HGFs are related to CXCL10 production in inflamed gingival tissues.

The aim of this study was to examine the effect of OSM on CXCL10 production by HGFs. Moreover, we examined the effects of the catechins EGCG and epicatechin gallate (ECG) on CXCL10 production from OSM-stimulated HGFs. Furthermore, we investigated whether catechin treatment modified phosphorylation of mitogen-activated protein kinases (MAPK), Akt or signal transducer and activator of transcription (STAT)3 in OSM-stimulated HGFs.

2. Materials and methods

2.1. Gingival tissue biopsies and cell culture

We used HGFs isolated from three clinically healthy gingiva during routine distal wedge surgical procedures. 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 Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (penicillin G, 100 U/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 by 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.

2.2. RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from gingival biopsies or HGF 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 the oligo$(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA, USA) and superscript3 reverse transcriptase (Invitrogen) under the conditions indicated by the manufacturer. Specific primers were designed from cDNA sequences for gp130, OSM receptor beta (OSMRβ) and glyceraldehyde-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: gp130-F, 5′-CATGCTTTGGGTGGAATGGAC-3′; gp130-R, 5′-CATCAACAGGAAGTTGGTCCC-3′; OSMRβ-F, 5′-GTGTGGGTGCTTCTCCTGCTTCTGTA-3′; OSMRβ-R, 5′-TCTGTGCTAATGACTGTGCTTGTGGT-3′; GAPDH-F, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; GAPDH-R, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The conditions for PCR were as follows: 1× (95°C for 15 min), 35× (94°C for 1 min, 59°C for 1 min, 72°C for 1 min) and 1× (72°C for 10 min). The products were analyzed on a 1.5% agarose gel containing ethidium bromide. We did not detect any bands when we performed PCR without adding the cDNA template in this study.

2.3. Flow cytometric analyses

Following the required culture time, the cells were washed twice with ice-cold PBS. 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 (20 min on ice) in PBS–1% bovine serum albumin (BSA). The cells were incubated with mouse anti-human gp130 antibody (R&D Systems, Minneapolis, MN, USA; 5 μg/ml), mouse anti-human OSMRβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 5 μg/ml) or an isotype control antibody on ice for 30 min. After being washed three times with PBS–1% BSA (Sigma), the cells were incubated with an FITC-conjugated rabbit anti-mouse F $(ab')_2$ fragment (DAKO, Kyoto, Japan) or FITC goat anti-rat IgG(H+L) Conjugate (ZYMED Laboratories, South San Francisco, CA, USA) 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).

2.4. CXCL10 production by HGFs

HGFs were stimulated with OSM (PeproTech, Rocky Hill, NJ, USA) for 24 h. The supernatants from HGFs were collected, and the CXCL10 concentrations of the culture supernatants were measured in triplicate with ELISA. Duoset (R&D Systems) was used for the determinations. 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, HGFs were cultured for 1 h in the presence or

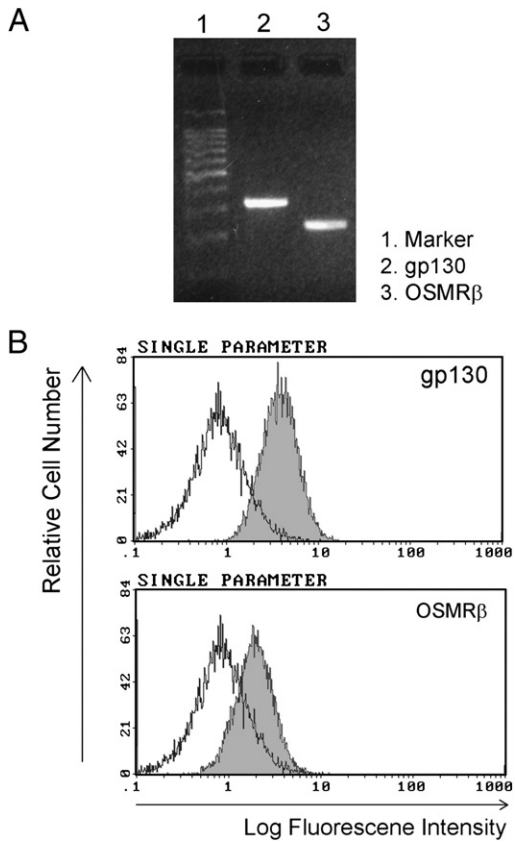

Fig. 1. gp130 and OSMRβ expression by HGFs. (A) Total RNA was prepared from nonstimulated HGFs. The expression of gp130 and OSMRβ mRNA in nonstimulated HGFs was analyzed by RT-PCR, as described in Materials and methods. (B) Flow cytometric analysis of gp130 and OSMRβ expression by nonstimulated HGF. The filled area represents gp130 or OSMRβ-specific fluorescence, and the empty area represents the background level of fluorescence caused by the secondary antibody.

absence of EGCG (5 or 50 μg/ml; Sigma), ECG (5 or 50 μg/ml; Sigma), SB203580 (20 μM; Santa Cruz Biotechnology), PD98059 (20 μM; Santa Cruz Biotechnology), SP600125 (20 μM; Sigma), LY294002 (20 μM; Calbiochem, La Jolla, CA, USA) or AG490 (20 μM; Calbiochem) prior to their incubation with OSM.

2.5. Western blot analysis

To confirm OSM-induced phosphorylation of signal transduction molecules, Western blot analysis was performed. HGFs stimulated with OSM (10 ng/ml) were washed once with cold PBS, followed by incubation 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. Protein (20 μg) was loaded onto a 4–20% SDS-PAGE gel, followed by electrotransfer to a PVDF membrane. Activations of p38 MAPK, c-Jun N-terminal kinase (JNK), Akt and STAT3 were assessed using phospho-p38 MAPK (Thr180/Tyr182) rabbit monoclonal antibody (Cell Signaling Technology), phospho-SAPK/JNK (Thr183/Tyr185)(81E11) rabbit monoclonal antibody (Cell Signaling Technology), Phospho-Akt (Ser473)(193H12) rabbit monoclonal antibody (Cell Signaling Technology), phospho-STAT3 (Tyr705) rabbit monoclonal antibody, phospho-STAT3 (Ser727) rabbit monoclonal antibody, p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), SAPK/JNK rabbit monoclonal antibody (Cell Signaling Technology), pan-Akt rabbit antibody (Cell Signaling Technology) or STAT3 rabbit monoclonal antibody (Cell Signaling Technology) 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).

2.6. Statistical analysis

Statistical significance was analyzed using the Student's *t* test. *P* values <.05 were considered significant.

3. Results

3.1. OSM receptor expression by HGFs

It has been reported that the OSM receptor is a heterodimer of gp130 and OSMRβ [1]. Therefore, we first examined the expression of gp130 and OSMRβ on HGFs. RT-PCR analysis showed that nonstimulated HGFs express gp130 and OSMRβ mRNA (Fig. 1A). Flow cytometric analysis showed a higher fluorescence from the cells incubated with anti-gp130 or OSMRβ mAb than those incubated with control antibody, indicating that gp130 and OSMRβ were significantly expressed on the cell surface (Fig. 1B).

3.2. The effects of OSM on CXCL10 production by HGFs

Since cultured HGFs express the OSM receptor, we then investigated whether OSM could stimulate HGFs. We examined Th1 chemokine production, because previous studies have shown that Th1 cells are involved in the pathogenesis of periodontal disease [20]. As shown in Fig. 2A, OSM induced CXCL10 production by HGFs in a concentration-dependent manner.

3.3. The effects of catechins on CXCL10 production from OSM-stimulated HGFs

To investigate the effects of EGCG and ECG on the OSM-stimulated CXCL10 production, HGFs were pretreated with EGCG or ECG at the indicated concentrations for 1 h and stimulated with OSM for 24 h. Treatment with 5 μg/ml EGCG slightly inhibited CXCL10 production. Treatment of HGFs with 5 mg/ml ECG had no effect on CXCL10 production. Treatment of HGFs with 50 mg/ml EGCG and ECG significantly abolished the effect of OSM on CXCL10 production from HGFs.

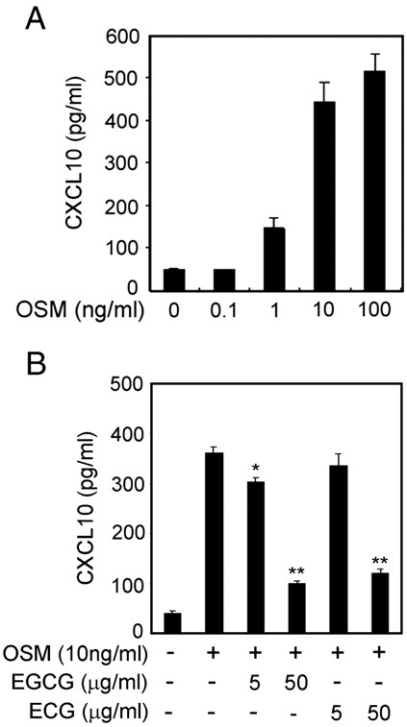

Fig. 2. Effects of OSM and catechins on CXCL10 production by HGFs. (A) HGFs were treated with OSM (0.1, 1, 10 or 100 ng/ml), and the supernatants were collected after 24 h. The expression levels of CXCL10 in the supernatants were measured using ELISA. (B) HGFs were pretreated with EGCG (5 or 50 μg/ml) or ECG (5 or 50 μg/ml) for 1 h and then were stimulated with OSM (10 ng/ml), and the supernatants were collected after 24 h. The expression levels of CXCL10 in the supernatants were measured using ELISA. Data are representative of three different HGFs samples from three different donors. The results were calculated as the mean and standard deviation of one representative experiment performed in triplicate. The error bars show the standard deviation of the values. $^{*}P<.05$ and $^{**}P<.01$, significantly different from the OSM-stimulated HGFs without catechins.

3.4. p38 MAPK, JNK, Akt and STAT3 pathways are related to CXCL10 production from OSM-stimulated HGFs

It has been reported that OSM can preferentially activate the p38 MAPK, extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK, JNK, phosphatidylinositol-3-OH kinase (PI3K)/Akt and STAT3 pathways [1]. To determine whether the activations of p38 MAPK, MEK, JNK, PI3K and STAT3 are required for the production of CXCL10 in response to OSM, the effects of several inhibitors on the production of CXCL10 by HGFs were examined (Fig. 3). At a concentration of 20 μM, SB203580 (a selective p38 MAPK inhibitor), SP600125 (a selective JNK inhibitor), LY294002 (a selective PI3K inhibitor) and AG490 (a STAT3 inhibitor) prevented CXCL10 production from HGFs stimulated with OSM. PD98059, a selective MEK inhibitor, did not modulate CXCL10 production in this experiment.

3.5. The effects of EGCG and ECG on the p38 MAPK, JNK, Akt and STAT3 phosphorylation induced by OSM in HGFs

Next, we examined the effects of catechins on the signal pathways involved in CXCL10 production from OSM-stimulated HGFs. EGCG completely inhibited JNK, Akt and serine STAT3 phosphorylation in OSM-stimulated HGFs. EGCG treatment inhibited tyrosine STAT3 phosphorylation in HGFs at 60 min. ECG treatment inhibited JNK and Akt phosphorylation in OSM-stimulated HGFs at 60 min. Serine STAT3 phosphorylation was slightly suppressed by ECG treatment. EGCG and ECG treatment did not modulate p38 MAPK phosphorylation in OSM-stimulated HGFs (Fig. 4).

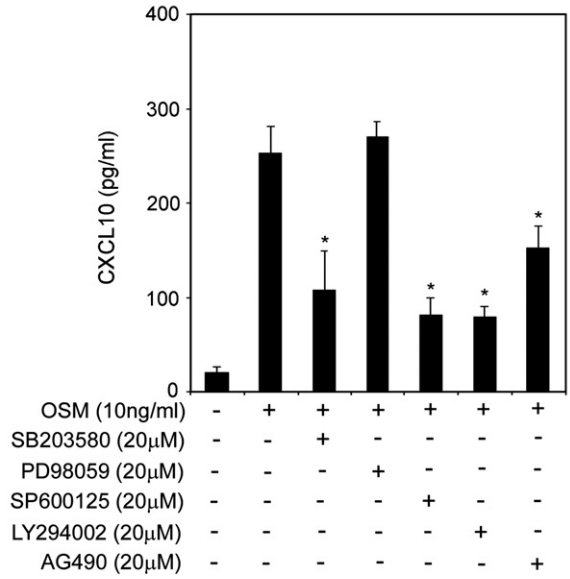

Fig. 3. Effects of signal transduction inhibitors on the OSM-stimulated CXCL10 release by HGFs. The cells were preincubated with SB203580 (20 μM), PD98059 (20 μM), SP600125 (20 μM), LY294002 (20 μM) or AG490 (20 μM) for 1 h and then incubated with OSM (10 ng/ml). After 24 h incubation, the supernatants were collected, and CXCL10 expression was measured by ELISA. Data are representative of HGFs from three different donors. The results were calculated as the mean and standard deviation of one representative experiment performed in triplicate. The error bars show the standard deviation of the values. *P<.05, significantly different from the OSM-stimulated HGFs without inhibitors.

3.6. EGCG and ECG suppressed OSMRβ expression on HGFs

We hypothesized that catechins might modulate OSM receptor expression because they inhibit the CXCL10 production induced by OSM treatment. As shown in Fig. 5, high concentrations of EGCG (50 μg/ml) and ECG (50 μg/ml) suppressed OSMRβ expression on

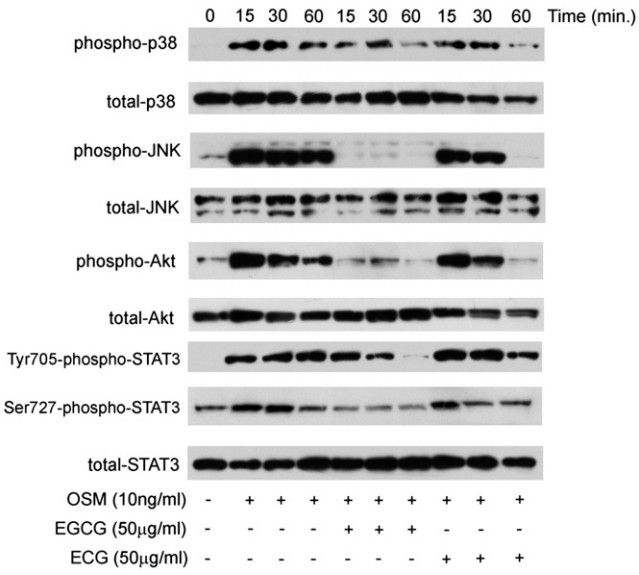

Fig. 4. The effects of EGCG and ECG on the OSM-induced phosphorylation of p38 MAPK, JNK, Akt or STAT3 in HGFs. The cultured cells were pretreated with EGCG (50 μg/ml) or ECG (50 μg/ml) for 60 min and then stimulated with 10 ng/ml OSM for 15, 30 or 60 min. The cell extracts were subjected to SDS-PAGE followed by Western blotting analysis with antibodies against phospho-specific p38 MAPK, p38 MAPK, phospho-specific JNK, JNK, phospho-specific Akt, Akt, tyrosine phospho-specific STAT3, serine phospho-specific STAT3 or STAT3.

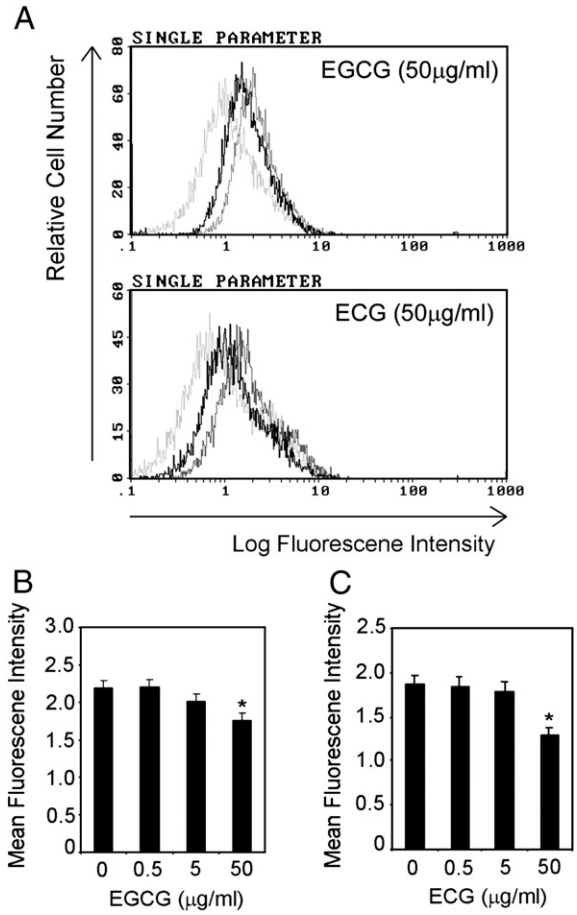

Fig. 5. The effects of EGCG or ECG on OSMRβ expression on HGFs. (A) HGFs were treated with EGCG (50 μg/ml) or ECG (50 μg/ml), and the cells were collected after 24 h. The expression levels of OSMRβ on HGFs were measured using flow cytometry as described in Materials and methods. The light gray line represents the background level of fluorescence caused by the isotype-matched antibody. The dark gray line represents OSMRβ expression on nonstimulated HGFs. The black line represents EGCG- or ECG-treated HGFs. HGFs were treated with EGCG (0.5, 5 or 50 μg/ml) (B) or ECG (0.5, 5 or 50 μg/ml) (C) and were collected after 24 h. The expression level of OSMRβ on the surface of the HGFs was determined by flow cytometry. The results are expressed as mean fluorescent intensity. The data are representative of three different HGFs samples from three different donors. The results were calculated as the mean and standard deviation of one representative experiment performed in triplicate. The error bars show the standard deviation of the values. *P<.05, significantly different from OSMRβ expression on HGFs without EGCG or ECG treatment.

HGFs. On the other hand, EGCG and ECG treatment did not modulate gp130 expression on HGFs (data not shown). These results show that inhibition of OSMRβ expression by catechin treatment might be related to the suppression of CXCL10 production from OSM-stimulated HGFs because catechins did not change the gp130 expression on HGFs.

4. Discussion

In this study, we demonstrated for the first time that OSM is able to induce CXCL10 production in HGFs. It has been reported that CXCR3 ligands are involved in the migration of Th1 cells because Th1 cells preferentially express CXCR3 [40]. Moreover, Kawai et al. [20] reported that Th1 cells are involved in bone resorption in periodontally diseased tissues. Therefore, OSM might induce periodontal tissue destruction by enhancing Th1 cell migration.

In the present study, we revealed that EGCG and ECG significantly suppressed the OSM-stimulated induction of CXCL10 in HGFs.

Therefore, EGCG or ECG might be a target of treatment for Th1-related diseases, such as periodontal disease and arthritis, because CXCL10 is involved in Th1 cells' migration into peripheral tissues. Further investigation is necessary to test the use catechins for treating Th1-related disease.

We investigated the mechanisms of EGCG and ECG underlying the inhibitory effects on OSM-stimulated CXCL10 production. It has been reported that OSM can preferentially activate the p38 MAPK, MEK/ERK, JNK, PI3K/Akt and STAT3 pathways [1]. Therefore, we used several inhibitors to elucidate which signaling pathways are related to CXCL10 production in HGFs. Our study revealed that the p38 MAPK, JNK, PI3K/Akt and STAT3 pathways are involved in OSM-induced CXCL10 production in HGFs. Shen et al. [41] reported that p38 MAPK and JNK inhibitors suppressed interferon-gamma-induced CXCL10 production in microglial cells. Kanda et al. [42] reported that p38 MAPK and PI3K inhibitors inhibited interferon-gamma and IL-18-induced CXCL10 production in keratinocytes. Their reports agree with our report. Furthermore, we revealed that EGCG treatment suppressed the JNK, Akt and STAT3 phosphorylation induced by OSM stimulation. Yun et al. [43] reported that EGCG treatment suppressed the JNK phosphorylation in TNF-α-stimulated synovial cells. Sah et al. [44] reported that EGCG treatment suppressed the phosphorylation of Akt in cervical tumor cells. Park et al. [45] reported that EGCG treatment inhibited STAT3 phosphorylation in keloid fibroblasts. Their reports support our results in this study.

We also revealed that ECG suppressed JNK, Akt and STAT3 phosphorylation in OSM-stimulated HGFs. Huang et al. [46] reported that ECG suppressed JNK phosphorylation in UV-treated keratinocytes. Bigelow et al. [47] reported that ECG inhibited Akt phosphorylation in tumorigenic breast epithelial cells. Their reports agree with ours. This report is the first to show that ECG can suppress STAT3 phosphorylation.

We demonstrated that EGCG and ECG treatment suppressed OSMRβ expression after 24 h of catechin treatment. However, we also found that 1 h catechin treatment did not change OSMRβ expression on HGFs (data not shown). Therefore, the suppression of OSMRβ expression is not related to the inhibition of signal transduction. However, we think that the suppression of OSMRβ expression might be involved in the reduction of CXCL10 production from OSM-stimulated HGFs. Recently, Ahmed et al. reported that EGCG treatment enhanced the expression of soluble gp130 and suppressed that of IL-1β-induced membrane-bound gp130 on synovial fibroblasts. They also reported that EGCG treatment did not modulate the expression of membrane-bound gp130 on nonstimulated synovial fibroblasts [48]. We found that EGCG or ECG did not change the expression of membrane-bound gp130 on nonstimulated HGFs (data not shown). Further investigation should be necessary to clarify the effects of catechins on gp130 in detail, especially those of soluble gp130.

In summary, the current study demonstrates that OSM causes CXCL10 release by cultured HGFs. Catechins suppressed OSM-induced CXCL10 production from HGFs. In addition, we revealed that catechins inhibited OSM-induced JNK, Akt and STAT3 phosphorylation and suppressed OSMRβ expression in HGFs. We think that the direct effects of catechins inhibit CXCL10 production from OSM-stimulated HGFs in order to inhibit the phosphorylation of signal transduction molecules. After that, catechins gradually suppress OSMRβ expression to inhibit the effects of OSM on HGFs. These data provide a novel mechanism through which the green tea flavonoids, EGCG and ECG, can provide periodontal benefits in periodontal disease.

Acknowledgments

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