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Original Article

Effects of EGCG on LPS-induced elevation of inflammatory factors in human gingival fibroblasts and functional mechanisms

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Abstract: Periodontal disease is one bacterial infectious disorder featured with elevated inflammatory factor level. Epigallocatechin-3-gallate (EGCG) is one factor derived from green tea with anti-inflammation effects. The role of EGCG in bacterial periodontal disease is unclear, this study aimed to illustrate the role of green teat extract EGCG in LPS-induced inflammatory factors of human gingival cells along with related mechanism. *In vitro* cultured human gingival fibroblasts were stimulated by LPS. Inflammatory cytokine IL-8 and IL-6 secretion level was measured after treatment using serial concentrations of EGCG (20-100 μM). Western blotting and p65-DNA analyzed the effect of EGCG on NF-κB pathway. Phosphorylation of Akt, p38, ERK and JNK was measured by Western blotting. EGCG treatment significantly suppressed LPS-induced expression of inflammatory cytokines IL-8 ($P<0.01$ at 100 μM, $P<0.05$ at 20 μM) and IL-6 ($P<0.01$ at 100 μM, $P<0.05$ at 20 μM) in human gingival fibroblast in a dose-dependent manner. By mechanism analysis, we found that EGCG treatment remarkably suppressed LPS induced p65 and IκB phosphorylation in human gingival cells, and affected p65 nuclear translocation. Meanwhile, EGCG effectively suppressed Akt phosphorylation, and p38, ERK or JNK phosphorylation in MAPK pathway. EGCG can alleviate LPS-induced inflammatory response in human gingival fibroblast by modulating NF-κB, PI3K/Akt signal pathway and MAPK pathway.

Keywords: EGCG, human gingival fibroblast, inflammation, signal pathway

Introduction

Periodontal disease is one bacterial infectious disease, and is featured with gingival inflammation and destruction of supporting tissues. Body immunity and inflammation response can facilitate progression of periodontal disease [1]. Porphyromonas gingivalis is the major bacterial strain in periodontal disease [2], with LPS as the major toxicity factor [3]. As one LPS response cell surface receptor, Toll like receptor and related signal pathway participate in recognition of microbes and production of pro-inflammatory factors [4]. Human gingival fibroblast is the major subtype of periodontal cells. Previous studies showed the surface expression of Toll like receptor 4 in gingival fibroblast and its participation in LPS induced inflammatory factor production [5-7]. Gingival fibroblast induced inflammatory response is believed to participate in reconstruction of gingival tissues [7]. Therefore, the decrease of inflammatory factor level could reduce cell oxidative stress response and inflammatory response, thus effectively managing progression of periodontal disease.

Epigallocatechin-3-gallate (EGCG) is one polyphenol compound extracted from green tea, and exerts protective effects against multiple human diseases [8]. Previous studies found that EGCG could inhibit NF-κB activity in multiple malignant tumors including colorectal carcinoma, breast cancer, lung cancer and chronic inflammation [9-12]. Moreover, EGCG is believed to mediate cell growth and apoptosis pro-

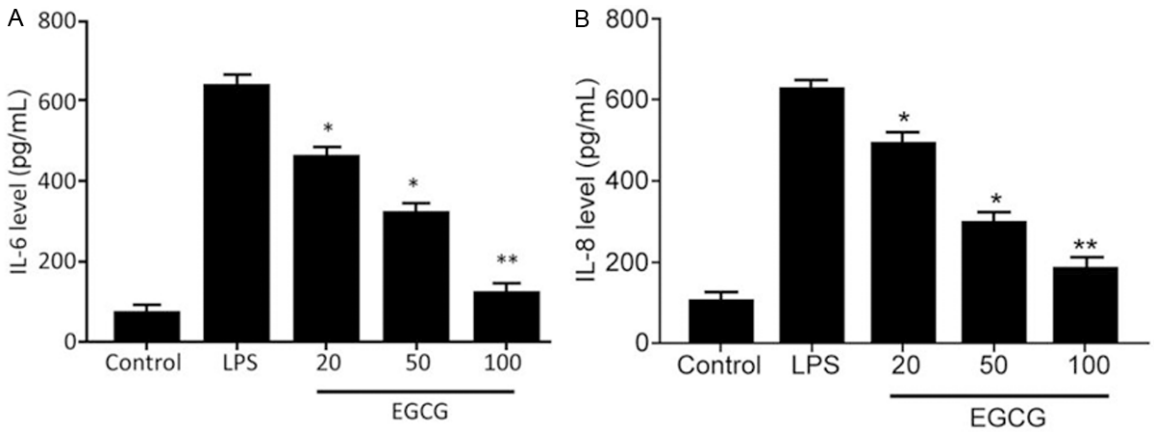

Figure 1. EGCG treatment decreased LPS induced inflammatory cytokine in human gingival fibroblast. Human gingival fibroblast was incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Cell culture supernatant was quantified for IL-6 (A) and IL-8 (B) by ELISA. (A) IL-6 concentrations in control, LPS and EGCG groups were 66.67±12.82, 638.46±53.85, 458.97±48.72, 320.51±48.72 and 120.51±38.46 pg/mL. (B) IL-8concentrations in control, LPS and EGCG groups were 100±31.11, 622.22±75.56, 488.89±57.78, 293.33±35.56 and 180.00±46.67 pg/mL. *, P<0.05 compare to LPS treatment group; **, P<0.01 compared to LPS treatment group.

cess via mediating PI3K-Akt signal pathway [13]. Recent study revealed that EGCG could inhibit inflammatory cytokine and chemokine production in human cells [14]. The effect of EGCG on LPS-induced elevation of inflammatory factors of human gingival fibroblast has not been reported.

In this study, we generated an *in vitro* system of LPS-stimulated human gingival fibroblast to confirm the protective effect of green tea extract EGCG on human gingival cells, along with related mechanism. Our results revealed that EGCG could reduce LPS-induced human gingival fibroblast inflammation by mediating NF-κB, PI3K/Akt and MAPK pathways.

Materials and methods

Materials

Green tea active extracts EGCG were purchased from Sigma-Aldrich (US). DMEM cell culture medium, fetal bovine serum (FBS) and reagent/disposable materials for cell culture were purchased from Gibco (US). P-p65, p-IκB, p-Akt, p-Erk, p-JNK, p-p38 and GAPDH were purchased from Cell signaling Technology (US). LPS derived from Porphyromonas gingivalis was purchased from InvivoGen (US). NF-κB p65 transcriptional factor analysis kit was purchased from Abcam (US). ELISA kits for IL-6 and IL-8 were purchased from R&D (US).

Cell culture

Human gingival fibroblast separation and culture were performed as previously described in literatures [15]. Transplant was obtained from patient impacted tooth. After PBS rinsing, tissues were cut into small pieces and were cultured in DMEM medium containing 10% FBS and 1% streptomycin-penicillin at 37°C with 5% CO_2. After 3 days culture, cells grew from tissues. After reaching 70% confluence, cells were digested for passage culture.

ELISA

Human gingival fibroblast was inoculated into 24-well plate at 2×10^5 cells per well density for 24 h to reach attached growth. Cells were then incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Supernatant was collected from measuring IL-6 and IL-8 levels by ELISA method, suing test kit purchased from R&D (US), following manual instruction.

Western blotting

Human gingival fibroblasts were inoculated into 6-well plate at 1×10^6 cells per well density for 24 h to make attached growth. Cells were then incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Cells were collected for twice rinsing in PBS. Cells

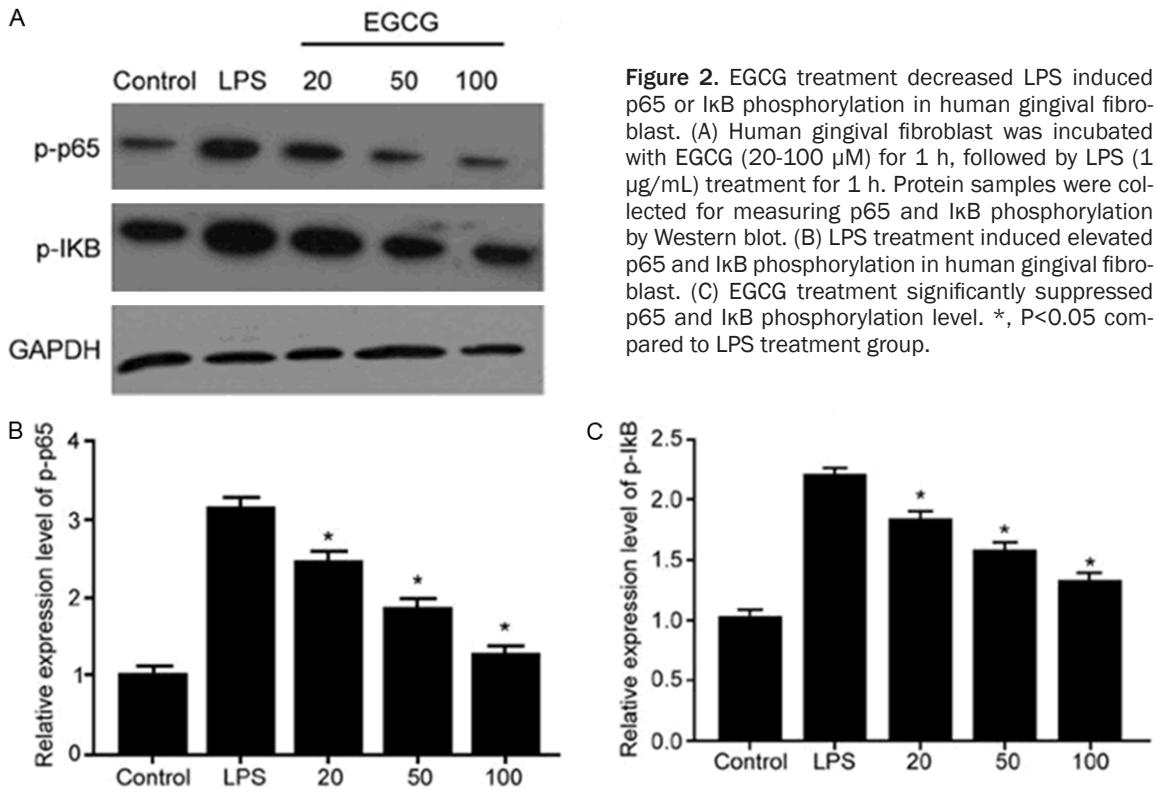

Figure 2. EGCG treatment decreased LPS induced p65 or IκB phosphorylation in human gingival fibroblast. (A) Human gingival fibroblast was incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Protein samples were collected for measuring p65 and IκB phosphorylation by Western blot. (B) LPS treatment induced elevated p65 and IκB phosphorylation in human gingival fibroblast. (C) EGCG treatment significantly suppressed p65 and IκB phosphorylation level. *, P<0.05 compared to LPS treatment group.

were then lysed in RIPA buffer containing phosphatase inhibitor. BCA method was used to quantify proteins. Equal volume of protein was separated by 12% SDS-PAGE, followed by transferring to PVDF membrane, which was blocked by TBST buffer containing 5% defatted milk powder. Rabbit anti-human monoclonal antibody (Cell Signaling Technology, 1:1000 dilution) was used for 4°C overnight incubation. Goat anti-rabbit secondary antibody (Vector Laboratory, 1:5000) was added for incubation. After washing, ECL was used for development. Image J software was used to measure optical density.

Statistical analysis

All statistical analysis was performed by SPSS 19.0 software. Measurement data were presented by mean ± standard deviation (SD). Comparison among multiple groups was performed by analysis of variance (ANOVA). A statistical significance was identified when P<0.05.

Results

EGCG treatment decreased LPS induced inflammatory cytokines in human gingival fibroblast

Human gingival fibroblast was incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Cell culture supernatant was quantified for IL-6 and IL-8 by ELISA. Our results (**Figure 1**) showed that EGCG treatment significantly depressed LPS induced human gingival fibroblast inflammatory factor IL-6 and IL-8 elevation in a dose dependent manner.

EGCG treatment suppressed LPS induced p65 or IκB phosphorylation in human gingival fibroblast

Protein samples were collected and quantified for p65 and IκB phosphorylation in human gingival fibroblast by Western blot. Our results showed that LPS treatment induced elevated p65 and IκB phosphorylation level in human gingival fibroblast, whilst EGCG treatment significantly suppressed p65 and IκB phosphorylation (**Figure 2**).

EGCG treatment decreased p65 nuclear translocation in LPS stimulated human gingival fibroblast

ELISA was used to measure binding affinity between NF-κB transcriptional and DNA. Our results showed that LPS treatment induced elevated binding affinity between p65 and DNA

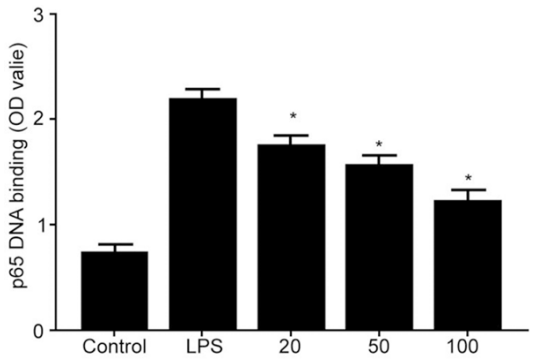

Figure 3. EGCG treatment suppressed LPS induced p65 nuclear translocation in human gingival fibroblast. Human gingival fibroblast was incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. ELISA was performed to test the binding affinity between NF-κB transcriptional and DNA. LPS treatment induced elevated binding affinity between p65 and DNA in human gingival fibroblast, indicating enhanced nuclear translocation, whilst EGCG treatment significantly depressed binding affinity between p65 and DNA. *, P<0.05 compared to LPS treatment group.

in human gingival fibroblast, indicating enhanced nuclear translocation, whilst EGCG treatment significantly depressed binding affinity between p65 and DNA (**Figure 3**).

EGCG treatment depressed PI3K-Akt or MAPK signal pathway activity in LPS stimulated human gingival fibroblast

Protein samples were collected and measured for AKt, p38, Erk1/2 and JNK phosphorylation level in human gingival fibroblast by Western blot. Our results showed that LPS treatment elevated phosphorylated level of Akt, p38, Erk1/2 and JNK in human gingival fibroblast, whilst EGCG treatment significantly depressed phosphorylation level of Akt, p38, Erk1/2 and JNK (**Figure 4**).

Discussion

Periodontal disease derives from inflammatory response of gingival tissues [16]. Gingival fibroblast is the major cell component of gingival mesenchymal tissues, and plays important roles in periodontal disease. LPS component of Porphyromonas gingivalis can stimulate expression of inflammatory cytokines in human gingival fibroblast [3]. Therefore, LPS is one important pathogenic factor of Porphyromonas gingivalis. In this study, we generated an *in vitro* system of human gingival fibroblast to illustrate the protective effect of green tea extracts EGCG on human gingival fibroblast along with the investigation of related mechanism. Our results showed that EGCG treatment significantly suppressed LPS induced expression of inflammatory factors IL-8 and IL-6 in human gingival fibroblast in a dosage dependent manner. The analysis of mechanism revealed that EGCG treatment remarkably suppressed LPS induced p65 or IκB phosphorylation in LPS induced human gingival fibroblast, and affected p65 nuclear translocation. Meanwhile, EGCG effectively down-regulated Akt phosphorylation and phosphorylation of p38, Erk and JNK in MAPK pathway. It is the first time that the role of EGCG in human gingival fibroblast inflammatory response was reported.

Previous study showed that various cells could express IL-6 and IL-8, both of which are believed to participate in tissue injury related with inflammation or neutrophil [17]. These ILs have strong anti-inflammatory effects, and are believed to be involved in pathological process of periodontal disease. Our results showed that EGCG significantly decreased levels of these two inflammatory cytokines.

Moreover, some study showed that LPS induced IL-6 and IL-8 production in human gingival fibroblast mainly via affecting NF-κB signal pathway activation [18]. As one transcriptional factor, NF-κB can enhance expression of multiple genes related with inflammatory response. P65 subunit and p50 subunit form heterodimer as one major activated form to interact with DNA binding site. Under normal circumstance, such heterodimer can bind with IκB protein in cytoplasm. Under LPS stimulation, IκB is undergone phosphorylation to induce the release of p65 and p50. These subunits then translocate into nucleus. After binding with DNA binding sites, transcription of target gene is initiated. Previous study showed that EGCG could inhibit NF-κB activation in human head-neck cancer H891 cell line and breast cancer MDA-MB-231 cells [19]. Other study showed that EGCG treatment could affect intracellular IκB level in dosage- and time-dependent manner, and inhibit NF-κB nuclear translocation [20]. Under UV irradiation induced NF-κB activation process of normal human epithelial keratocytes, EGCG could inhibit NF-κB activation and nuclear translocation [20]. We observed EGCG could affect p65

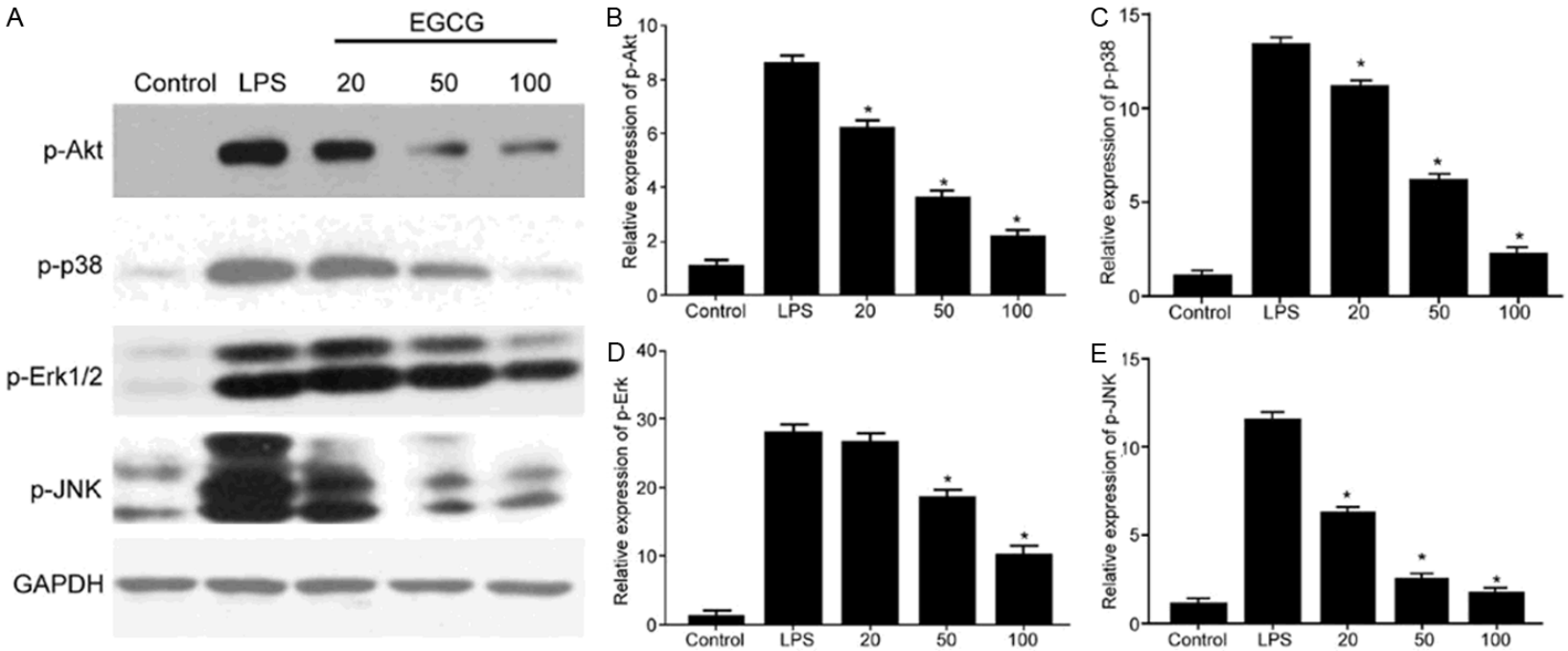

Figure 4. EGCG treatment depressed PI3K-Akt or MAPK signal pathway activity in LPS stimulated human gingival fibroblast. Human gingival fibroblast was incubated with EGCG (20-100 μM) for 1 h, followed by LPS (1 μg/mL) treatment for 1 h. Protein samples were collected and measured for AKt, p38, Erk1/2 and JNK phosphorylation level in human gingival fibroblast by Western blot. LPS treatment elevated phosphorylated level of Akt, p38, Erk1/2 and JNK in human gingival fibroblast, whilst EGCG treatment significantly depressed phosphorylation level of Akt, p38, Erk1/2 and JNK. *, P<0.05 compared to LPS treatment group.

nuclear translocation via modulating phosphorylation level of p65 and IκB.

PI3K/AKT signal pathway is one important regulatory pathway for NF-κB activation, and is involved in cell migration, cell growth facilitation and blockade of cell apoptosis [21]. Previous study showed the implication of PI3K/AKT pathway related molecules in treating periodontal diseases [22]. Therefore, this study observed the effect of EGCG on PI3K/AKT signal pathway. Results showed that in LPS-induced inflammatory model of human gingival fibroblast, EGCG significantly depressed PI3K/AKT signal pathway activity.

Besides PI3K/AKT signal pathway, MAPK signal pathway can also enhance expression of inflammatory cytokines in various immune cells. It is also involved in LPS induced inflammatory response of human gingival fibroblast [23]. Our study showed that EGCG treatment also affected phosphorylation of MAPK signal pathway related molecules p38, Erk1/2 and JNK, as consistent with previous study regarding the participation of EGCG in the suppression of MAPK signal pathway in mouse epithelium [24] and NHEK cells [25].

In summary, we confirmed that EGCG could exert anti-inflammatory effects via mediating phosphorylation level of p65 and IκB, as well as p65 nuclear translocation. Moreover, PI3K-Akt signal pathway and MAPK signal pathway was also found to be involved in anti-inflammatory effects of EGCG.

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Disclosure of conflict of interest

None.

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