Green Tea Polyphenol Epigallocatechin-3-Gallate Inhibits TNF-α-Induced Production of Monocyte Chemoattractant Protein-1 in Human Umbilical Vein Endothelial Cells

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Key Words
Green tea • Epigallocatechin-3-gallate • Monocyte chemoattractant protein-1 • Human umbilical vein endothelial cells

Abstract

\textbf{Aims:} Epigallocatechin-3-gallate (EGCG), a major catechin found in green tea, displays a variety of pharmacological properties and recently received attention as a prospective dietary intervention in cardiovascular diseases (CVD). This study was conducted to test the hypothesis that EGCG was able to inhibit tumor necrosis factor-α (TNF-α)-induced production of monocyte chemoattractant protein-1 (MCP-1) in human umbilical vein endothelial cells (HUVECs) and investigated the underlying molecular mechanisms.\textbf{Methods:} The inhibitory effect of EGCG on TNF-α-induced expression of MCP-1 was measured using ELISA and RT-qPCR. The effect of EGCG on TNF-α-induced nuclear factor-kappa B (NF-κB) activation was investigated by western blot and luciferase assays. Monocyte adhesion assay was detected by microscope.\textbf{Results:} EGCG significantly suppressed the TNF-α-induced protein and mRNA expression of MCP-1. Investigation of the mechanism suggested that EGCG suppressed the TNF-α-mediated NF-κB activation. In addition, the 67-kD laminin receptor (67LR) was involved in EGCG-mediated suppression of MCP-1 generation. Furthermore, EGCG potently inhibited monocyte adhesion to activated HUVECs.\textbf{Conclusion:} EGCG suppresses TNF-α-induced MCP-1 expression in HUVECs. This effect was mediated by 67LR and was via the inhibition of NF-κB activation. Our results demonstrated that EGCG might be a possible medicine for CVD prevention and treatment.

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Wang et al.: EGCG inhibits TNF-α-Induced Production of MCP-1 in HUVECs

Introduction

Green tea, produced from the leaves of the plant Camellia sinensis, is widely consumed beverage throughout the world. Epidemiological studies and associated meta-analyses have reported that daily consumption of green tea is associated with many health benefits, such as a reduced risk of coronary artery disease (CAD) [1, 2]. Green tea contains many biologically active polyphenolic flavonoid, commonly known as catechins, which is a family that includes (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG) [3, 4]. As the principal constituent, EGCG has a variety of biological and pharmacological properties and has received attention as a prospective dietary intervention in cardiovascular diseases (CVD) [4].

Dysregulated expression of molecules involved in inflammation which results from endothelial dysfunction is associated with the initiation and the progression of CVD [5]. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, can induce the activation of the vascular endothelium and plays a pivotal role in vascular disease development by directly promoting inflammation, oxidative stress, vascular remodeling and atherogenesis [6, 7]. It has been suggested that many cardiovascular risk factors contribute to the pathogenesis of endothelial dysfunction through the modulation of TNF-α signaling [6]. Monocyte chemotactic protein-1 (MCP-1) is a chemoattractant and can be produced by many cell types, including endothelial, epithelial, fibroblasts, smooth muscle cells and macrophages [8-10]. In patients at risk for CAD, elevated MCP-1 serum levels have been suggested as a direct marker of the inflammatory activity [10, 11].

The 67-kDa laminin receptor (67LR) is a non-integrin cell-surface receptor with high affinity for laminin; it plays a key role in tumor invasion and metastasis [12]. Recently, 67LR has been identified as a cell surface EGCG receptor that mediates the anti-inflammatory activity of EGCG. In macrophages, 67LR was shown to be involved in the inhibitory effect of EGCG on the LPS-induced TLR4 signaling and peptidoglycan-induced TLR2 signaling pathway [13, 14]. It was also shown to be involved in the inhibitory effect of EGCG on the TLR4 signaling pathway in dendritic cells [15]. Recently, it has been demonstrated that 67LR as a cell-surface EGCG receptor mediates the anti-inflammatory action of EGCG in endotoxin-stimulated human cerebral microvascular endothelial cells (hCMECs) [16]. Nonetheless, the relationship between 67LR and anti-inflammatory activity of EGCG in TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) has not yet been established.

In the present study, we tried to investigate the effects of EGCG on HUVECs exposed to TNF-α by evaluating the expression of MCP-1. Furthermore, the mechanisms through which EGCG exerts its action were investigated.

Materials and Methods

Cell Culture and Treatment

The HUVECs-derived EA.hy 926 cells were gifted by Atherosclerosis Research Lab of Nanjing Medical University. They obtained this cell line from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CBTCCAS, Shanghai, China). Cells were maintained in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) without antibiotic in a humidified atmosphere at 37°C in 5% CO2 as previously described [17]. HUVECs at passage 5-7 were used for the experiments. The culture medium was changed to RPMI 1640 supplemented with 0.1% FBS for an additional 12 h before the experiments. HUVECs were treated with different concentrations of EGCG (Sigma-Aldrich St. Louis, MO, USA) (10, 25, and 50 μM) for 1 h prior to TNF-α (10 ng/ml) treatment and further incubated together for different time periods. For the blockage of 67LR, cells were incubated with anti-67LR Ab (clone MluC5; 20 μg/ml; NeoMarkers, Fermont, CA, USA) or isotype-matched control mouse IgM (20 μg/ml) for 1 h before the treatment of EGCG.
Enzyme-linked immunosorbent assay (ELISA)

HUVECs were seeded in 6-well microplates and grown to confluence. They were pretreated with different concentrations of EGCG (10, 25, and 50 μM) for 1 h and stimulated by TNF-α (10 ng/ml) for 24 h. After treatment, supernatants of cell cultures were collected and concentrated using centrifugal filter units to remove cellular debris; the MCP-1 levels were quantified using ELISA (Boster Biotech, Wuhan, China). Assays were performed according to the manufacturer’s instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR) and reverse transcriptional PCR (RT-PCR) assay

Total cellular RNA was extracted using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions and reverse transcribed into cDNA using PrimeScript RT reagent kit Perfect Real (Takara, Dalian, China). The conditions for quantitative real-time PCR were: 95°C for 30 s, then 40 cycles at 95°C for 5 s, and 60°C for 34 s, and a final extension of 95°C for 1 min and 95°C for 15 s. The mRNA level for MCP-1 of each sample was normalized to Ct values of the GAPDH amplified from the same sample, \( \Delta C_t = C_{MCP-1} - C_{GAPDH} \) and the 2\(^{-\Delta\Delta C_t}\) method was used to calculate gene expression change. Samples were measured in triplicates to ensure the reproducibility of the results. 67LR mRNA expression was evaluated by RT-PCR. cDNA was subsequently amplified in 50μl reaction volumes containing 0.4 μmol/l of each 67LR primer and 1.25 U Taq DNA polymerase (TaKaRa, Dalian, China). The amplification conditions were pre-denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 40 s, and a final extension of 72°C for 7 min. PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide and then visualized under UV light. The primer sequences are shown in Table 1.

Cell counting kit-8 assay

The viability of HUVECs was determined using the Cell Counting Kit-8 Assay Kit (Beyotime Institute of Biotechnology, China), as described previously [18]. Briefly, 5 × 10\(^3\) cells/well were seeded in a 96-well plate and cultured to confluence. Subsequently, cells were treated with EGCG at increasing concentrations (0, 10, 25, and 50 μM) for 24 h. After 10 μl CCK-8 solution was added to each well, the absorbance at 450 nm was measured 4 h after incubation in the dark.

Western blot analysis

Cells were harvested and lysed, and then protein concentration was measured using Pierce BCA protein assay reagent (Thermo-Fisher Scientific, Rockford, III., USA). Cell lysates were electrophoresed through 12% polyacrylamide gels and transblotted onto polyvinylidene difluoride (PVDF) membranes in a semidy transfer system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After blocking with 5% BSA in TBST buffer (10 m M Tris, pH 7.5, 150 m M NaCl, and 0.05% Tween-20) for 2 h at room temperature, the membrane was probed with rabbit polyclonal antibody to P-p65 (1: 5,000; Santa Cruz, Calif., USA), mouse monoclonal antibody to IκBα (1: 1000; Cell Signaling Technology, MA., USA), and mouse monoclonal antibody to β-actin (1: 1,000; Santa Cruz, Calif., USA) overnight at 4 °C. The antibodies were detected using 1: 5,000 goat anti-rabbit (Biosynthesis, Beijing, China) and 1: 2,000 goat anti-mouse (Santa Cruz, Calif., USA) horseradish peroxidase-conjugated antibodies for 1 h at room temperature. Membranes were developed with enhanced chemiluminescence detection (ECL, Amersham, Bucks, UK). The immunoreactivity was quantitated using Image J software.

**Table 1. Primers of MCP-1, GAPDH and 67LR for RT-qPCR and RT-PCR**

| Target | Primers |
|--------|---------|
| MCP-1  | Sense: 5’-CATAGCACCCGACCTTTATTCC-3’<br>Antisense: 5’-TCTGCACTGATCTCTCTATGGG-3’ |
| GAPDH  | Sense: 5’-GGTGTTCTCCTGACCTCAACA-3’<br>Antisense: 5’-GTTTCTGATGCAAAATCTCTGTG-3’ |
| 67LR   | Sense: 5’-AGGGGCTGCTGACTTTTGG-3’<br>Antisense: 5’-CCAGTCAGTGGTGGTCTCTAC-3’ |
Luciferase assay
HUVECs were seeded in six-well plates (1.5×10^5 cells per well) overnight and cotransfected with NF-κB reporter plasmid (Promega) and pRL-TK (Promega) as an internal control. Transfection complexes were prepared by mixing FuGENE HD Transfection Reagent (Roche, Basel, Switzerland) and DNA in a 3:1 ratio in serum-free medium (3 μl FuGENE HD to 1μg DNA per plasmid per well). The complexes were incubated for 15 min before added to cells. Six hours after the transient transfection, the cells were serum-starved for 12 h and incubated with or without EGCG (10, 25, and 50μM) for 1 h, then subjected to TNF-α-stimulation for an additional 6 h. Firefly and Renilla luciferase activities in cell extracts were assayed using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity represents the ratio of the activity of firefly to control Renilla luciferase activity [19].

Monocyte adhesion assay
We used monocyte adhesion assay to evaluate the physiological consequences of the inhibitory effect of EGCG on induction of MCP-1 [19, 20]. Briefly, HUVECs were seeded on 2% gelatin-coated six-well plates and cultured to confluence. The cells were pre-treated with EGCG (10, 25, 50 μM) for 1 h and stimulated with TNF-α (10 ng/ml) for 6 h. U937 cells (CBTCCCAS, Shanghai, China) were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100μg /ml streptomycin. After washing three times with serum-free RPMI 1640 medium, approximately 1 mL of U937 cells (20,000 cells/mL) was added to the wells and incubated for 20 min at 37°C to allow the adhesion. The plates were then carefully washed out three times with serum-free RPMI 1640 medium to remove non-adherent cells. The adherent monocytes were counted in five randomly selected optical fields in each well under a microscope (Olympus).

Statistical analysis
Values are expressed as mean ± standard error. Statistical significance between groups was assessed by using one-way ANOVA followed by Fisher’s exact test. It was considered statistically significant when the P-value was less than 0.05.

Results

Effect of EGCG on TNF-α-induced MCP-1 generation in HUVECs
The results in Fig. 1 showed that MCP-1 in the culture supernatant was markedly elevated after the exposure of HUVECs to 10ng/ml TNF-α for 24 h. However, pretreatment of the cells with different concentrations of EGCG for 1 h prior to TNF-α stimulation significantly inhibited TNF-α-induced MCP-1 production in a concentration-dependent manner.

Effect of EGCG on TNF-α-induced mRNA expression of MCP-1 in HUVECs
MCP-1 mRNA expression in HUVECs was assayed by RT-qPCR. As shown in Fig. 2, the level of MCP-1 mRNA in HUVECs was also markedly increased following exposure of the cells to 10 ng/ml TNF-α for 4 h. After the cells were preincubated with different concentrations of EGCG for 1 h, TNF-α-induced mRNA expression of MCP-1 was significantly down-regulated in a concentration-dependent manner. The study also demonstrated that EGCG alone did not affect the mRNA expression of MCP-1.

Effect of EGCG on the cell viability of HUVECs
We performed a Cell counting kit-8 assay to assess the cell viability. The HUVECs were treated with 0, 10, 25 or 50μM EGCG for 24 h. As shown in Fig. 3, EGCG up to 50μM did not display any cellular toxicity against HUVECs. This finding suggested that the effects of EGCG were not due to cytotoxicity.

EGCG inhibits TNF-α-induced NF-κB activation
It is well known that NF-κB activation is required for the TNF-α-induced inflammatory cytokine production in HUVECs [7]. In order to determine whether the effect of EGCG on MCP-1 generation was due to suppression of NF-κB activation, we investigated the effects
Wang et al.: EGCG inhibits TNF-α-Induced Production of MCP-1 in HUVECs

Cellular Physiology and Biochemistry

Effect of EGCG on TNF-α-induced MCP-1 generation in HUVECs. HUVECs were pretreated with different concentrations of EGCG for 1 h before exposure to 10 ng/ml TNF-α for 24 h. The MCP-1 concentration in the supernatant was measured by ELISA. Data are presented as mean ± S.D. of three independent experiments. *P<0.01, **P<0.001 vs. control; *P<0.05, **P<0.01 vs. TNF-α alone.

Fig. 2. Effect of EGCG on TNF-α-induced mRNA expression of MCP-1 in HUVECs. HUVECs were pretreated with different concentrations of EGCG for 1 h before exposure to 10 ng/ml TNF-α for 4 h. Quantitative data of relative MCP-1 mRNA were achieved by the comparative Ct method (2−ΔΔCt). Data are presented as mean ± S.D. of three independent experiments. *P<0.01 vs. control; *P<0.05, **P<0.01 vs. TNF-α alone.

Fig. 3. Effect of EGCG on the cell viability of HUVECs. HUVECs were treated with EGCG at increasing concentrations (0, 10, 25, and 50 μM) for 24 h. Cell viability was measured using the CCK-8 assay. Data are presented as mean ± S.D. of three independent experiments. *P> 0.05 compared with the control group (0μM EGCG).

Effect of EGCG on NF-κB activation in HUVECs. As shown in Fig. 4, HUVECs treated with TNF-α led to NF-κB activation, including IκB-α degradation and p65 phosphorylation. TNF-α-induced NF-κB activation was potently inhibited by EGCG treatment. We next evaluated the effects of EGCG on NF-κB-dependent transcriptional activity by transient transfection of HUVECs with a NF-κB-luciferase reporter plasmid. As shown in Fig. 5, TNF-α-induced NF-κB-dependent transcriptional activity was potently inhibited by EGCG in a dose-dependent manner.

67LR is involved in EGCG-mediated suppression of TNF-α-induced MCP-1 generation

As shown in Fig. 6A and B, the protein and mRNA expression of 67LR in HUVECs were determined by western blot and RT-PCR analysis. It was next investigated whether the 67LR could mediate the inhibitory effects of EGCG on the TNF-α induction of MCP-1 expression. For this purpose, we treated HUVECs with neutralization antibody against 67LR (20 μg/ml) or control IgM for 1 h prior to TNF-α and/or EGCG treatment. As shown in Fig. 6 C, the blockage of 67LR attenuated the inhibitory effect of EGCG on the TNF-α induction of MCP-1 expression. Moreover, the inhibitory effect of EGCG on TNF-α-mediated NF-κB activation was counteracted by antibody against 67LR pre-treatment (Fig. 6 D). Our results suggest that the 67LR is involved in EGCG-mediated suppression of TNF-α-mediated MCP-1 generation.
**Fig. 4.** Effect of EGCG on TNF-α-induced NF-κB activation in HUVECs. (A) HUVECs were stimulated with TNF-α (10 ng/ml) for various periods as indicated. Representative immunoblots showing expression of phosphorylated p65, total IκBα and β-actin after various periods of TNF-α stimulation. Representative blots of 3 separate experiments are shown. (B) HUVECs were pretreated with EGCG (50 μM) for 1 h, then stimulated with TNF-α (10 ng/mL) for 30 min. Western blotting analysis was carried out to evaluate the levels of phosphorylated p65, total IκBα, and β-actin. Data represent the mean ± S.D. of at least three independent experiments. #P<0.05 vs. control; *P<0.05 vs. TNF-α alone.

**Fig. 5.** Effect of EGCG on TNF-α-induced NF-κB-dependent promoter activity in HUVECs. HUVECs transfected with NF-κB-luciferase reporter plasmid were pretreated in the absence or presence of various doses of EGCG (10, 25, or 50 μM) for 1 h before treatment with or without TNF-α (10 ng/mL) for 6 h. Cells were lysed and the lysate was analyzed using a luciferase assay system. Data represent the mean ± S.D. of three independent experiments. #P<0.01 vs. control; *P<0.05, **P<0.01 vs. TNF-α alone.

**Effect of EGCG on the adhesion of monocytes to TNF-α-stimulated HUVECs**

We used monocyte adhesion assay to evaluate the physiological consequences of the inhibitory effect of EGCG on induction of MCP-1. As shown in Fig. 7, monocytes bound readily to TNF-α-activated HUVECs, whereas EGCG pretreatment for 1 h reduced adhesion of monocytes in a concentration dependent manner.
Discussion

In the present study, we demonstrated that EGCG, a major green tea catechin, inhibited the expression MCP-1 induced by TNF-α in HUVECs. We found that EGCG reduced the MCP-1 expression. The mechanism involves the inhibition of TNF-α-induced NF-κB activation and the involvement of 67LR in the EGCG-mediated suppression of TNF-α-induced MCP-1 generation. EGCG also inhibits monocyte adhesion to HUVECs, further supporting its anti-inflammatory effects.

Fig. 6. 67LR is involved in EGCG-mediated suppression of TNF-α-induced MCP-1 generation. (A) Protein expression of 67LR in HUVECs was determined by western blot analysis using 67LR antibody. Cells were treated with or without EGCG (50 μM) for 24 h. (B) RT-PCR analysis of 67LR mRNA expression in HUVECs at 24 h after treated with or without EGCG (50 μM). (C) Effect of 67LR antibody on EGCG-mediated suppression of TNF-α-induced MCP-1 generation. For the blockage of 67LR, HUVECs were pretreated with anti-67LR Ab (20 μg/ml) or control IgM for 1 h, then treated with EGCG (50 μM) for 1 h before exposure to TNF-α (10 ng/ml) for 24 h. MCP-1 concentration in the supernatant was measured by ELISA. (D) Effect of 67LR antibody on EGCG-mediated inhibition of TNF-α-induced NF-κB activation. Cells transfected with NF-κB-luciferase reporter plasmid were pretreated with anti-67LR Ab (20 μg/ml) or control IgM for 1 h, then treated with EGCG (50 μM) for 1 h before exposure to TNF-α (10 ng/ml) for 6 h. Cells were lysed and the lysate was analyzed using a luciferase assay system. Data represent the mean ± S.D. of three independent experiments. *P<0.05.

Fig. 7. EGCG inhibits monocyte adhesion of HUVECs. HUVECs were pretreated with or without EGCG (10, 25, 50 μM) for 1 h prior to TNF-α (10 ng/mL) treatment for an additional 6 h. U937 cells were then added and incubated for 20 min at 37°C to allow the adhesion. Quantitative U937 cells adhering to HUVECs were taken under a microscope. Data are presented as mean ± S.D. of three independent experiments. #P<0.01 vs. control; *P<0.05, **P<0.01 vs. TNF-α alone.
1 mRNA and protein levels induced by TNF-α. EGCG treatment also inhibited monocytes adhesion to TNF-α-activated HUVECs in a concentration dependent manner. Our results provide the evidence for the potential development of EGCG in the prevention of atherosclerosis.

NF-κB acts as a central mediator for the regulation of genes important in immune and inflammatory responses [21]. To understand the potential mechanisms for EGCG attenuation of MCP-1 production by TNF-α, we confirmed in the HUVECs that a predominant signal transduction pathway by which TNF-α induces MCP-1 secretion is through inhibition of NF-κB activation. Our results demonstrated that EGCG significantly suppressed TNF-α-induced IκB-α degradation. On the other hand, we also observed that EGCG inhibited TNF-α-induced phosphorylation of NF-κB p65. Previous study has shown that EGCG significantly inhibits NF-κB transcriptional activity and nuclear translocation in RAW 264.7 macrophages [22]. Also it has been reported that EGCG can regulate the production of inflammatory cytokines via inhibiting the activation of NF-κB pathway in human mast cell line [23]. In addition, EGCG was suggested to have the ability to block NF-κB activation in intestinal epithelial cell line IEC-6 [24], respiratory epithelial cells [25], human endothelial ECV304 cells [26], and in hCMECs [16]. These are compatible with our data suggesting one mechanism of action for EGCG on endothelial cell function is the inhibition of the NF-κB pathway following TNF-α stimulation.

In our study, the inhibitory actions of EGCG were mediated through 67LR, suggesting that 67LR has a pivotal role as a cell-surface receptor that mediates the inhibitory action of EGCG in TNF-α-mediated MCP-1 generation in HUVECs. However, the expression of other inflammatory cytokines still warrants further investigation.

Green tea consumption is now considered to be an inexpensive, readily applicable, and accessible approach to CVD control and management [4]. In our previous study, we estimated that the risk of CAD decreased by 10% with each increase in green tea consumption of 1 cup/d [2]. Green tea catechins have been associated with a wide spectrum of beneficial effects on CAD; they have been found to inhibit vascular inflammation, oxidation, thrombogenesis, and atherogenesis and to favorably modulate the vascular reactivity and plasma lipid profile [2, 4]. However, the mechanism of the chemopreventive action of most catechins, including EGCG, is still unknown. Our data suggested that EGCG down regulates MCP-1 expression in activated HUVECs; this may be a putative mechanism for the anti-atherosclerotic effects of green tea consumption.

In our study, the concentrations of EGCG (10–50μM) are generally within the range used in most published in vitro studies [27-29]. Nevertheless, the relatively high concentrations of EGCG obtained in cell culture studies have been considered to far exceed the levels found in human plasma after green tea consumption. The pharmacokinetic studies in humans demonstrated that the peak plasma concentration after single oral dose administration of EGCG is less than 1.0 μM [30, 31]. Therefore, the rather poor bioavailability of EGCG needs to be considered when we extrapolate the activities observed with high EGCG doses in cell culture conditions to situations in vivo. However, efforts in improving the bioavailability of EGCG with promising approaches are being undertaken; recently reported attempts such as encapsulation of EGCG, the semisynthesis O-acyl derivatives of EGCG, the transdermal delivery of EGCG are worthy of further exploration to enhance the EGCG concentrations in the plasma [32].

Taken together, our results suggest that EGCG, a major polyphenol of green tea, could suppress TNF-α-induced MCP-1 mRNA and protein expression via inhibition of NF-κB in HUVECs. In addition, the present data demonstrate that the 67LR is involved in EGCG-mediated suppression of TNF-α-mediated MCP-1 generation. Our result shows that EGCG plays a protective role against endothelial dysfunction induced by TNF-α and may help us to better understand the beneficial effects of the green tea polyphenol proved in previous epidemiologic studies.
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