Epigallocatechin-3-O-gallate Decreases Tumor Necrosis Factor- α -induced Fractalkine Expression in Endothelial Cells by Suppressing NF- κ B

Ae Sin Lee¹, Yu Jin Jung¹, Duk Hoon Kim¹, Tae Hwan Lee¹, Kyung Pyo Kang¹, Sik Lee¹, Nae Ho Lee², Mi Jeong Sung³, Dae Young Kwon³, Sung Kwang Park¹ and Won Kim¹

¹Renal Regeneration Laboratory and Department of Internal Medicine, ²Department of Plastic and Reconstructive Surgery, Institute for Medical Sciences, Chonbuk National University Medical School, Jeonju, ³Food Function Research Division, Korea Food Research Institute, Seongnam, South Korea

Key Words

Fractalkine • Endothelial cells • Epigallocatechin-3-O-gallate

Abstract

Epigallocatechin-3-O-gallate (EGCG), the main catechin in green tea, has anti-oxidant, anti-atherosclerotic and anti-inflammatory properties. Fractalkine, a chemokine involved in inflammation and early atherosclerotic processes, acts as a chemoattractant as well as an adhesion molecule in endothelial cells activated by proinflammatory cytokines. In the present study, we investigated the effect of EGCG on fractalkine expression in TNF- α -induced human umbilical vein endothelial cells (HUVECs). EGCG decreased TNF- α -induced fractalkine mRNA and protein expression in HUVECs in a time-dependent manner. EGCG suppressed the TNF- α -induced phosphorylation and degradation of I κ B- α , thereby decreasing the phosphorylation and nuclear translocation of the NF- κ B p65 subunit in HUVECs. The DNA binding activity of the NF- κ B p65 subunit was lower in EGCG-pretreated HUVECs than in those treated with TNF- α alone. Furthermore, EGCG inhibited monocyte adhesion to HUVECs

stimulated by TNF- α . The silencing of fractalkine with an siRNA or treatment with a blocking antibody against fractalkine suppressed the TNF- α -induced increase in monocyte adhesion. These results demonstrate that EGCG prevents TNF- α -induced vascular endothelial fractalkine expression.

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Introduction

Green tea is a popular beverage with beneficial health effects [1]. Epidemiologic and animal studies suggest that green tea has a preventive effect against cardiovascular disease and can also reduce cancer risk [2]. Epigallocatechin-3-O-gallate (EGCG), a polyphenolic compound which is the major constituent of green tea, has been reported to have a beneficial effect on atherogenesis by modulating lipid peroxidation in atherosclerosis models [3, 4]. Ludwig et al. [5] reported that EGCG reduces the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and reduces monocyte adhesion to endothelial cells. EGCG also decreases the expression of monocyte chemoattractant protein-1 (MCP)-1 in endothelial cells and

contributes to a reduction in monocyte migration [6].

Fractalkine (CX3CL1) is the only CX3C-chemokine, composed of an extracellular domain that is connected to an extended mucin-like stalk across the cell membrane [7]. Fractalkine is expressed by activated vascular endothelial cells and involved in the recruitment of cells which express the fractalkine receptor, CX3CR1 [8, 9]. As the expression of CX3CR1-positive cells were reported in macrophages [8], T cells [10], natural killer cells [8] and dendritic cells [11], the fractalkine expression in inflamed endothelial cells may play an important role in the adhesion and migration of these inflammatory cells [12]. The role of fractalkine in the development of atherosclerosis has been proved using fractalkine-deficient mice [13]. Furthermore, it has been demonstrated that fractalkine has a role in sepsis [14], allograft rejection [15, 16], human immunodeficiency virus infection [17] and rheumatoid arthritis [18]. Therefore, regulation of fractalkine expression in vascular endothelial cells may be a useful therapeutic target for treating inflammation and atherosclerosis [14, 19]. However, the effect of EGCG on fractalkine expression in endothelial cells has not been investigated.

In this study, we examined whether EGCG decreases the expression of TNF- α -induced fractalkine in human umbilical vein endothelial cells (HUVECs) and the adhesion of monocytes to the vascular endothelium. We also investigated the signaling mechanism that mediates the suppressive effect of EGCG on fractalkine expression in endothelial cells.

Materials and Methods

Materials and endothelial cell culture

Anti-fractalkine antibody (full-length fractalkine: Torrey Pines BioLabs, Houston, TX, USA), anti-phospho-p65 antibody (Ser536) (Cell Signaling Technology, Beverly, MA, USA), anti-p65 antibody (Upstate Biotechnology, Lake Placid, NY, USA), recombinant human TNF- α (R&D Systems, Minneapolis, MN, USA) were used in this study. Fractalkine-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF- κ B p65 siRNA (human specific) (Cell Signaling Technology), negative control siRNA (Dharmacon Research, Lafayette, CO, USA) were used. EGCG, lipopolysaccharide (LPS), media, and most other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

The human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville (Walkersville, MD, USA). HUVECs were cultured in media 199 supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum at 37°C in 5% CO₂.

Reverse transcriptase-polymerase chain reaction

HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Total RNA was extracted from the HUVECs homogenates using Trizol (Invitrogen Corp., Carlsbad, CA, USA). The Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) was used according to the manufacturer's protocol to synthesize cDNA from total RNA. The resulting first-strand DNA was amplified in a final volume of 20 μ L containing 10 pmol of each primer and 1U of *Taq* polymerase (TaKaRa, Shuzo, Japan). The primers used for fractalkine were: forward 5'-TGT GTC TCT TGG TCC TGC TG-3' and reverse 5'-GAT CAG AAC CCA GCC TTC AG-3'. To confirm the use of equal amounts of RNA in each reaction, all samples were checked in parallel for GAPDH mRNA expression (primers for GAPDH were: forward 5'-AGACTTCCCACAACC CAC AG-3' and reverse 5'-AAC AGC CGT TAG GTG AAT GC-3'). The amplification conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 30 s, and extension at 62°C for 30 s. Amplification products (2 μ L each) were subjected to electrophoresis on 1.5% agarose gels, followed by ethidium bromide staining.

Western blot analysis

HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 6 h. HUVECs were transfected with NF- κ B p65 siRNA or control siRNA and treated with TNF- α (10 ng/mL) for 6 h. Western blot analysis was performed as described previously [19]. Primary antibodies against NF- κ B p65 (Upstate Biotechnology, Lake Placid, NY, USA), phospho-NF- κ B p65, phospho-I κ B- α and I κ B- α (Cell Signaling Technology) were used. All signals were visualized using chemiluminescent reagents (Amersham, Buckinghamshire, UK) and analyzed by densitometric scanning (LAS-3000; Fuji Film, Tokyo, Japan). The membranes were reblotted with anti-actin antibody to verify equal loading of protein in each lane.

Cytosolic and nuclear protein extraction

HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Cytosolic and nuclear protein extractions from HUVECs were performed with a Nuclear Extraction Kit (Panomics, Inc., Fremont, CA, USA). The resulting nuclear pellets were agitated by vortex mixing and then centrifuged at 14,000g for 5 min at 4°C. The supernatant consisted of soluble nuclear proteins. The nuclear lysates (20 μ g) were western blotted with anti-NF- κ B p65 antibody.

Electrophoretic mobility shift assay

HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Nuclear extracts were prepared and analyzed for nuclear translocation of NF- κ B. Control cells (CB) received vehicle alone. Five micrograms of nuclear protein was used in each experiment. Electrophoretic mobility shift assay (EMSA) was performed as described previously [19].

Fig. 1. EGCG suppresses TNF- α -induced fractalkine mRNA in HUVECs. (A) RT-PCR analysis of fractalkine mRNA expression in TNF- α -stimulated HUVECs co-treated with EGCG. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and exposed to TNF- α (10 ng/mL) and the indicated amount of EGCG for 4 h. Control cells (CB) received vehicle alone. RNA samples were analyzed by RT-PCR with the indicated primers (upper panel). GAPDH was used as a reference for gel loading (middle panel). Densitometric analyses (lower panel) indicate the relative ratio of fractalkine mRNA to GAPDH mRNA. The relative ratio measured to control is arbitrarily defined as 1. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs CB; #, $p < 0.05$ vs. TNF- α . (B) RT-PCR analysis of fractalkine mRNA expression in TNF- α -stimulated HUVECs co-treated with EGCG. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and exposed to TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for the indicated times. Control cells (CB) received vehicle alone. RNA samples were analyzed by RT-PCR with the indicated primers (upper panel). GAPDH was used as a reference for gel loading (middle panel). Note that EGCG suppressed TNF- α -induced fractalkine mRNA expression in a time-dependent manner. Densitometric analyses are presented as the relative ratio of fractalkine mRNA to GAPDH mRNA. The relative ratio measured to control is arbitrarily defined as 1. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α .

Monocyte adhesion assay

THP-1 cells (American Type Culture Collection, Manassas, VA, USA) were incubated with Celltracker (Invitrogen) for 15 min at 37°C. Monocyte-endothelial adhesion was determined by fluorescence labeling of monocytes as described previously [19]. HUVECs were pre-treated with EGCG (10, 20, or 50 μ mol/L) for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (10, 20 and 50 μ mol/L) for 4 h. To evaluate the knockdown effect of fractalkine in the monocyte adhesion assay, some HUVECs were transfected with fractalkine-specific siRNA or control siRNA with HUVEC Nucleofector Kit (Amaxa, Cologne, Germany) and stimulated with TNF- α (10 ng/mL) for 4 h. We also used anti-fractalkine antibody (20 μ g/mL) in some HUVECs to evaluate the role of fractalkine in the adhesion assay. Fluorescence-labelled monocytes were added to the HUVEC monolayer and allowed to adhere for 2 h. The number of monocytes adhering to HUVECs was expressed as a percent calculated by the formula: % adherence = (adherent signal/total signal) $\times$ 100.

Fig. 2. EGCG suppresses TNF- α -induced fractalkine protein expression in HUVECs. (A) Immunoblot analysis of fractalkine protein expression in TNF- α -stimulated HUVECs co-treated with EGCG. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG at the indicated concentrations. Each lane contains 20 μ g of cellular protein. The blot was probed with anti-fractalkine antibody (upper panel) and reprobed with anti-actin antibody (middle panel) to demonstrate equal loading of protein in each lane. Densitometric analyses (lower panel) are presented as the relative ratio of fractalkine to actin. The relative ratio compared to the control is arbitrarily defined as 1. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. control (CB); #, $p < 0.05$ vs. TNF- α . (B) Immunoblot analysis of fractalkine protein in TNF- α -stimulated HUVECs co-treated with EGCG. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and incubated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for the indicated times. Each lane contains 20 μ g of cellular protein. Note that EGCG suppressed TNF- α -induced fractalkine protein expression in a time-dependent manner. Densitometric analyses are presented as the relative ratio of fractalkine to actin. The relative ratio compared to the control is arbitrarily defined as 1. Bars represent the mean $\pm$ SD from 5 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α .

Statistical analysis

Data are expressed as mean $\pm$ standard deviation. Student's t test was used to compare the means of normally distributed continuous variables. Statistical significance was set at $P < 0.05$.

Results

EGCG suppresses TNF- α -induced fractalkine mRNA in HUVECs

We previously reported the maximal expression of TNF- α (10 ng/mL)-induced fractalkine mRNA at 4 h in HUVECs [14], so we used this dose and time course for TNF- α -induced fractalkine mRNA expression measured by RT-PCR in this experiment. Pre-treatment with 50 μ mol/L of EGCG and TNF- α decreased fractalkine

mRNA by approximately 44% compared to treatment with TNF- α alone (Figure 1A). EGCG also suppressed TNF- α -induced fractalkine mRNA in a time-dependent manner, with maximal suppression at 4 h after EGCG treatment (Figure 1B). EGCG alone did not induce fractalkine mRNA expression. These data suggest that EGCG suppresses TNF- α -induced fractalkine mRNA expression in HUVECs.

EGCG suppresses TNF- α -induced fractalkine protein expression in HUVECs

We previously reported that TNF- α induces the maximal fractalkine protein expression at 6 h in HUVECs [14]. Therefore, in this experiment, we used this dose and time course for TNF- α -induced fractalkine protein expression. Co-treatment with 50 μ mol/L of EGCG and TNF- α decreased fractalkine protein expression by approximately 49% compared to treatment with TNF- α alone (Figure 2A). EGCG also suppressed TNF- α -induced fractalkine protein expression in a time-dependent manner, with maximal suppression at 6 h after EGCG treatment (Figure 2B). EGCG alone did not induce fractalkine protein expression. These data suggest that EGCG suppresses TNF- α -induced fractalkine protein expression in HUVECs.

Silencing NF- κ B p65 decreases TNF- α -induced fractalkine protein expression

NF- κ B p65 siRNA treatment decreased the TNF- α -induced fractalkine expression in HUVECs. Control siRNA alone did not show any effect on the expression of fractalkine. EGCG did not change fractalkine protein expression after treatment with NF- κ B p65 siRNA (Figure 3).

EGCG suppresses TNF- α -induced I κ B- α phosphorylation, degradation of I κ B- α and p65 phosphorylation

Immunoblot analyses using phospho-I κ B- α -specific and I κ B- α antibodies showed that stimulation of the cells with TNF- α increased rapid phosphorylation and subsequent degradation of I κ B- α , as compared to control buffer-treated cells. Pretreatment with EGCG (50 μ mol/L) suppressed TNF- α -induced phosphorylation and subsequent degradation of I κ B- α in HUVECs (Figures 4A and 4B). We next evaluated the effect of EGCG on NF- κ B activities using an anti-phospho-NF- κ B p65 antibody that detects the p65 subunit of NF- κ B. TNF- α -induced phosphorylation of the p65 subunit of NF- κ B was increased (about ~3.4 fold) compared to

Fig. 3. Immunoblot analysis of fractalkine protein expression in HUVECs after silencing NF- κ B p65. HUVECs were transfected with NF- κ B p65 siRNA or control siRNA (Cont siRNA) and treated with TNF- α with or without EGCG (50 μ mol/L) for 6 h. Actin was used as the loading control. Densitometric analyses are presented as the relative ratio of fractalkine to actin. The relative ratio compared to the control is arbitrarily defined as 1. Note that silencing NF- κ B p65 reduced TNF- α -induced fractalkine protein expression. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. Cont siRNA only; #, $p < 0.05$ vs. TNF- α plus Cont siRNA.

control or EGCG only. However, co-treatment with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) resulted in decreased phosphorylation of the p65 subunit of NF- κ B after 1 h of EGCG treatment and almost completely inhibited the phosphorylation of the p65 subunit of NF- κ B at 4 h after EGCG treatment. EGCG alone had no significant effect on the phosphorylation of the p65 subunit of NF- κ B (Figure 4C). These data demonstrate that EGCG suppresses TNF- α -induced phosphorylation of NF- κ B in HUVECs.

EGCG suppresses TNF- α -induced translocation of NF- κ B p65

To assess the nuclear translocation of the NF- κ B p65 subunit, nuclear extracts of HUVECs were examined by western blot analysis. TNF- α -induced nuclear translocation of the p65 subunit of NF- κ B was increased by about ~3.9 fold compared to control. After a 4 h co-treatment with TNF- α (10 ng/mL) and EGCG (50 μ mol/L), the nuclear translocation of the p65 subunit of NF- κ B was decreased by about 66% compared to after treatment with TNF- α alone. Treatment with EGCG alone had no effect on the nuclear translocation of the NF- κ B p65 subunit (Figure 4D). These data demonstrate

Fig. 4. EGCG suppresses TNF- α -induced I κ B- α phosphorylation, degradation of I κ B- α and p65 phosphorylation (A) Immunoblot analysis of phosphorylated I κ B- α in total cell extract. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 1 h. Control cells (CB) received vehicle alone. Total cell extracts were prepared and analyzed for phosphorylation of I κ B- α . Twenty micrograms of total protein was used in each experiment. Densitometric analyses are presented as the relative ratio of phospho-I κ B- α to I κ B- α . Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α . (B) Immunoblot analysis of I κ B- α in total cell extract. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Control cells (CB) received vehicle alone. Total cell extracts were prepared and analyzed for I κ B- α . Twenty micrograms of total protein was used in each experiment. Densitometric analyses are presented as the relative ratio of I κ B- α to actin. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α . (C) Immunoblot analysis of phosphorylated p65 subunit of NF- κ B in total cell extracts. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for the indicated times. Control cells (CB) received vehicle alone. Total cell extracts were prepared and analyzed for phosphorylation of NF- κ B p65. Twenty micrograms of nuclear protein was used in each experiment. Densitometric analyses are presented as the relative ratio of phospho-p65 to p65 subunit of NF- κ B. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α . (D) Immunoblot analysis of the p65 subunit of NF- κ B in nuclear and cytoplasmic protein extracts. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Nuclear and cytoplasmic protein extracts were prepared as described in the Material and Methods sections. Twenty micrograms of nuclear protein was used in each experiment. Note that TNF- α -induced nuclear translocation of NF- κ B p65 was increased by about ~4.0 fold compared to control. However, co-treatment with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) suppressed nuclear translocation of NF- κ B p65 compared to treatment with TNF- α alone. EGCG alone had no effect on the nuclear translocation of NF- κ B p65. Densitometric analyses are presented as the relative ratio of nuclear p65 to cytosolic p65 of NF- κ B. Bars represent the mean $\pm$ SD from 3 different experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α .

that EGCG suppresses TNF- α -induced translocation of NF- κ B in HUVECs.

EGCG suppresses TNF- α -induced NF- κ B-DNA binding activity

To examine the effect of EGCG on the NF- κ B-DNA binding induced by TNF- α , nuclear extracts of TNF- α -

Fig. 5. EGCG suppresses TNF- α -induced NF- κ B-DNA binding in nuclear extracts from HUVECs. HUVECs were pre-treated with 50 μ mol/L EGCG for 30 min and stimulated with TNF- α (10 ng/mL) and EGCG (50 μ mol/L) for 4 h. Nuclear extracts were prepared and analyzed for nuclear translocation of NF- κ B. Control cells (CB) received vehicle alone. Five micrograms of nuclear protein was used in each experiment. Note that the increased NF- κ B-DNA binding in nuclear extracts from HUVECs was decreased significantly by treatment with EGCG (50 μ mol/L). Bars represent the mean $\pm$ SD from 3 experiments. *, $p < 0.05$ vs. CB; #, $p < 0.05$ vs. TNF- α .

stimulated HUVECs were examined by EMSA. TNF- α (10 ng/mL) treatment increased DNA binding of the NF- κ B p65 by about 2.3-fold compared with control buffer in the nuclear extracts of HUVECs. The increased NF- κ B-DNA binding induced by treatment with TNF- α (10 ng/mL) in nuclear extracts from HUVECs was decreased significantly (about ~90%) by treatment with

Fig. 6. EGCG reduces adhesion of monocytes to HUVEC monolayers. (A) Representative images of the adhesion of monocytes to HUVEC monolayers. HUVECs were incubated with the indicated doses of EGCG, fractalkine-specific siRNA, control siRNA, antibody against fractalkine, control antibody (Cont antibody) and TNF- α (10 ng/mL) for 4 h. Fluorescence-labelled monocytes were added to the HUVEC monolayer and allowed to adhere for 2 h. The adhered cells were measured as described in Materials and Methods. (B) Quantification of monocyte adhesion to HUVECs. Data are from three experiments and are expressed as a percentage of adhesion to TNF- α -induced HUVECs. Bars represent the mean $\pm$ SD from three experiments. *, $p < 0.05$ vs. control (CB); #, $p < 0.05$ vs. TNF- α .

EGCG (50 μ mol/L). Treatment with EGCG alone had no effect on NF- κ B-DNA binding (Figure 5). These data suggest that EGCG suppresses TNF- α -induced fractalkine expression through regulation of NF- κ B activities in HUVECs.

EGCG suppresses adhesion of monocytes to TNF- α -stimulated HUVECs

We examined whether EGCG decreases monocyte adhesion to TNF- α -stimulated HUVECs through fractalkine expression. Monocytes were fluorescently labeled with Celltracker and were then added to confluent monolayers of HUVECs, which were treated with control buffer, EGCG (50 μ mol/L), TNF- α (10 ng/mL), or co-treated with TNF- α and EGCG. Stimulation of HUVECs with TNF- α (10 ng/mL) induced a significant increase in monocyte adhesion compared to treatment with control buffer. However, treatment with EGCG (50 μ mol/L) decreased monocyte adhesion to TNF- α -stimulated HUVECs by 52%. EGCG alone had no effect on monocyte adhesion in HUVECs (Figure 6). The antibody against fractalkine decreased TNF- α -stimulated monocyte adhesion by about 43%. The control antibody alone had no effect on adhesiveness of monocytes to HUVECs. Silencing fractalkine effectively suppressed the TNF- α -induced increase of monocyte adhesion by 42%, while control siRNA had no effect on the monocyte adhesiveness to HUVECs. These findings suggest that EGCG decreases monocyte adhesion to TNF- α -stimulated HUVECs through fractalkine expression.

Discussion

In the present study, we demonstrate that EGCG suppresses TNF- α -induced fractalkine expression through regulation of the NF- κ B signaling pathway in primary cultured HUVECs. These data suggest that EGCG has an anti-inflammatory effect mediated by the down-regulation of fractalkine expression.

Proinflammatory mediators such as cytokines, adhesion molecules and leukocytes are involved in the pathophysiologic process of inflammation [20]. Among inflammatory mediators, fractalkine is expressed mainly in inflamed endothelial cells with its major role being leukocyte adhesion and migration from the vascular compartment to inflamed tissues [7, 9]. Because TNF- α is a major cytokine associated with inflammation, we previously reported that fractalkine is up-regulated in TNF- α -treated primary cultured HUVECs [14, 19]. In the present study, EGCG suppressed the TNF- α -induced fractalkine mRNA and protein expression in primary cultured HUVECs in a dose- and time-dependent manner. It has been reported that EGCG decreases VCAM-1 and MCP-1 expression in endothelial cells [5, 6]. Although fractalkine acts like an adhesion molecule such as VCAM-1 and MCP-1 in endothelial cells, fractalkine is not regulated by all of the molecules that regulate VCAM-1. For example, adrenomedullin suppresses TNF- α -induced VCAM-1 expression in HUVECs [21], however, adrenomedullin did not affect TNF- α -induced fractalkine expression in HUVECs in our preliminary data.

(data not shown). Therefore, fractalkine-regulating molecules may differ from VCAM-1-regulating molecules.

Activation of NF- κ B is a key step after TNF- α stimulation of endothelial cells [14, 22]. We reported previously that fractalkine expression was increased through an NF- κ B-dependent pathway in primary cultured HUVECs [19]. Recently, Scaldaferrì F et al. [23] demonstrated that fractalkine was associated with TNF- α -induced phosphorylation of MAPK, and p38 or p42/44 inhibition significantly suppressed the increase in fractalkine expression stimulated by TNF- α in human intestinal microvascular endothelial cells. Therefore, the MAPK pathway may be a signaling pathway associated with fractalkine expression in endothelial cells.

Because nuclear translocation of the NF- κ B p65 subunit is an important process in NF- κ B signal pathway activation, EGCG-induced down regulation of nuclear translocation of NF- κ B p65 subunit may be critical in regulating the nuclear binding activity of NF- κ B in adhesion molecule expression. Ludwig et al. [5] demonstrated that EGCG prevented VCAM-1 expression after stimulation with TNF- α in endothelial cells. They demonstrated that EGCG did not decrease TNF- α -induced nuclear translocation of p65 and p50 in endothelial cells. However, we showed that EGCG does decrease TNF- α -induced phosphorylation and nuclear translocation of the NF- κ B p65 subunit in primary cultured endothelial cells. Our EMSA data also demonstrate that EGCG decreases the nuclear binding of NF- κ B in TNF- α -treated endothelial cells. These data suggest that EGCG may act in different signaling pathways to suppress TNF- α -induced fractalkine expression and TNF- α -induced VCAM-1 expression in endothelial cells.

Leukocyte adhesion to activated endothelial cells is a key step in inflammatory responses and the development of atherosclerosis. Recently, Jeong et al. [24] demonstrated that the flavanols of EGCG not inhibited the THP-1 cell adhesion to oxidized LDL-activated human endothelial cells. Contrary to those results, the present study showed that EGCG treatment decrease TNF- α -induced monocyte adhesion to primary cultured HUVECs. The differences in the endothelial cell stimulating agents (LDL and TNF- α) used in the two experiments, may be the possible reason for the difference in the results observed.

The decrease of atherosclerosis has been documented in CX3CR1 knockout mice [25]. Scaldaferrì et al. [23] demonstrated that fractalkine is independently involved in macrophage accumulation and atherosclerotic

lesion formation in mice. Fractalkine and its receptor are also present in patients with abdominal aortic aneurysm [26]. Thus, fractalkine may be a key therapeutic target for the treatment of atherosclerosis. Our data demonstrate that EGCG down regulates fractalkine expression in activated endothelial cells. As green tea has been reported for its protective role in cardiovascular disease [2], the regulation of fractalkine expression by EGCG may be a putative mechanism for the anti-atherosclerotic effects of green tea catechins.

It has been reported that fractalkine and the fractalkine receptor, CX3CR1 are involved in metastasis of neuroblastoma to bone-marrow [27, 28]. The antitumor response was decreased in CX3CR1-deficient mice [28] and fractalkine-mediated antitumor immunity through activation of NK cell cytotoxicity [29]. The antitumor property of the green tea polyphenol, EGCG, is well documented. Thus, EGCG-induced regulation of fractalkine may be a potential mechanism for the antitumor effects of green tea.

One of the limitations for *in vivo* application of EGCG is the dosage in humans. In this *in vitro* experiment, we used a high concentration of EGCG (50 μ mol/L) to confirm the effects of EGCG in HUVECs. However, it has been reported that the peak plasma concentration of EGCG is greater than 1 μ mol/L after an oral dose of more than one gram EGCG in humans [30]. Therefore, a high plasma concentration (50 μ mol/L) is typically not systemically observed after oral administration in humans. Future studies should focus on the effects of intravenous administration of EGCG or the combination of EGCG plus compounds that decrease fractalkine expression.

In conclusion, our data suggest that EGCG, a major polyphenol of green tea, suppresses TNF- α -induced fractalkine mRNA and protein expression in primary cultured HUVECs through regulation of the NF- κ B signaling pathway. EGCG also decreases TNF- α -induced monocyte adhesiveness to endothelial cells.

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