

Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2

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Abstract. Platelet-derived growth factor (PDGF) has been known to induce vascular endothelial growth factor (VEGF) expression in human vascular smooth muscle cells (hVSMCs). We previously reported that Erk-1/2 and AP-1 pathways are crucial in the PDGF-induced VEGF expression in hVSMCs. In this study, we investigated the effect of epigallocatechin-3-gallate (EGCG), the major green tea catechin, on the PDGF-induced VEGF expression in hVSMCs and the underlying mechanisms. EGCG were found to inhibit dose-dependently the VEGF expression and activation of PDGF receptor, Erk-1/2 and AP-1 induced by PDGF. In addition, cell free studies demonstrated that EGCG could directly inhibit the Erk-1/2 activity. Conditioned media from the hVSMCs treated with PDGF could remarkably stimulate the *in vitro* growth of human umbilical vein endothelial cells (HUVECs) but the media from the EGCG-pretreated hVSMCs lost its stimulatory activity for HUVEC proliferation. These results suggest that EGCG may exert the anti-angiogenic effect by inhibiting the PDGF-induced VEGF expression at multiple signaling levels.

Introduction

Epidemiological studies have suggested that ingestion of green tea may decrease cancer risk (1,2). In addition, many pre-clinical studies have provided convincing evidence that some substances of green tea may afford protection against cancer (3,4). The anti-carcinogenic effects of green tea have been attributed to the biological activity of its polyphenol components. Green tea extract contains catechins such as (-)-epigallocatechin gallate (EGCG), (-)-epigallo-catechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin

(EC) (5). EGCG has been reported to prevent tumor invasion and angiogenesis that are essential processes for tumor growth and metastasis (6). Our previous results showed that treatment of mice with EGCG resulted in marked inhibition of vascularity and proliferation of human colon cancer xenografts in nude mice (3).

Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are the major cellular components of small tumor vessels. Interactions between these cells play significant roles in the homeostasis of the structure and function of the blood vessel (7). Angiogenesis, the growth of novel capillaries from pre-existing vessels, is an essential step in tumor growth and metastasis (8). The formation of new blood vessels is mediated by angiogenic growth factors (9). The growth factors not only bind their receptors on ECs and stimulate the cell proliferation initiating new blood vessel formation but also bind receptors on accessory cells such as VSMCs that maintain vessel integrity (10). One of the most potent angiogenic factors is vascular endothelial growth factor (VEGF), which stimulates capillary formation and has specific mitogenic and chemotactic activity for vascular ECs (11). VEGF is produced by various cell types, including ECs, VSMCs, fibroblasts, epithelial cells, mesenchymal cells, and macrophage (11-13). A variety of cytokines and growth factors, including IL-1, IL-6, PDGF-BB, TGF, basic fibroblast growth factor, and hepatocyte growth factor have been shown to induce VEGF expression in malignant and non-malignant cell lines (11). In previous reports, we suggested that ERK-1/2 and AP-1 signaling pathways are involved in the PDGF-induced VEGF expression in human VSMCs (hVSMCs) and that these paracrine signaling pathways induce endothelial cell proliferation (14).

In the present study, we have found that EGCG inhibits the PDGF-induced VEGF expression in hVSMCs and investigated the effects of EGCG on the signaling pathways which are suggested to be involved in the VEGF expression.

Materials and methods

Cell culture and reagents. hVSMCs were obtained from American Type Culture Collection (Rockville, MD) and cultured in Hanks modified Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 U/ml penicillin-streptomycin at 37°C in

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5% CO₂. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection and cultured in DMEM supplemented with 15% FBS and 5 ng/ml basic fibroblast growth factor (FGF). PDGF (PDGF-BB) was purchased from R&D Systems, Inc. (McKinley Place, NE). EGCG, EC, ECG, and EGC were obtained from Sigma Chemical Co. (St. Louis, MO). The rabbit polyclonal anti-phosphospecific p44/42 MAPK (Erk-1/2), anti-phosphospecific JNK, and anti-phosphospecific P38 MAPK antibodies were purchased from New England Biolabs, Inc. (Beverly, MA), and anti-PDGF- β R and anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Northern blot analysis. Total RNA extraction and Northern blot hybridization were performed as previously described (14). The cDNA probe for VEGF was generated by PCR using sense (5'-TCCAGGAGTACCCTGATGAG-3') and anti-sense (5'-ATTCACATTTGTTGTGCTGT-3') primers (15). The glyceraldehyde-phosphate dehydrogenase (GAPDH) probe was purchased from American Type Culture Collection. Each cDNA probe was radiolabeled with [α -³²P]deoxyribonucleoside triphosphate by the random-priming technique using the Rediprime labeling system (Amersham Corp., Arlington, IL). The probed nylon membranes were exposed to radiographic films (Life Technologies, Inc., Grand Island, NY). To examine the effects of catechins on VEGF induction by PDGF, hVSMCs were pretreated with 0-50 μ M catechin for 1 h prior to exposure to PDGF, and the level of VEGF mRNA was measured by Northern blot analysis.

ELISA for VEGF concentration. The concentration of VEGF in the culture supernatants was measured by ELISA using a commercially available kit (Immunoassay Kit Human VEGF; BioSource International, Camarillo, CA). Briefly, samples were incubated in microtiter plates precoated with a monoclonal antibody specific for VEGF. After incubation at room temperature for 2 h and washing, the substrate solution was added. Color development was stopped after 30 min at room temperature, and the color intensity was read at 450 nm within 30 min.

Measurement of VEGF promoter activity. A 2951-bp fragment containing the 5' flanking region and transcriptional start site of the VEGF gene (-2362 - +589 bp) was prepared by PCR with genomic DNA of VEGF as a template. The specific primers contained KpnI and SmaI linker sites (16). This PCR product was cloned into the pGL3-Basic vector (Promega, Madison, WI) to make a VEGF promoter-luciferase reporter construct (pGL3-VEGF). hVSMCs (5 \times 10⁵) were seeded and grown to 60-70% confluence, and then pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-VEGF were cotransfected into cells using FuGENE (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. pRLTK and pGL3 were cotransfected as a negative control. Cells were incubated in the transfection medium for 20 h and treated with PDGF for 8 h. The effect of EGCG on VEGF promoter activity was determined by pretreating cells with EGCG for 1 h

prior to addition of PDGF. Cells were harvested with passive lysis buffer (Dual-Luciferase Reporter Assay System; Promega), and the luciferase activity was determined using a single sample luminometer according to the manufacturer's protocol.

Immunoprecipitation. hVSMCs, after treatment with EGCG at the indicated concentration, were lysed in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Nonidet P-40, 0.6% sodium deoxycholate, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 0.2 units of aprotinin). The proteins (100 μ g) in each sample were incubated with 1/10 volume of Protein G-Sepharose beads (Amersham Corp.) for 1 h to eliminate non-specific binding. The protein samples were incubated with the 1:100 dilutions of anti-PDGF- β R, anti-Erk-1/2, anti-JNK and anti-P38 MAPK antibodies at 4°C overnight. Protein G-Sepharose (1/10 volume) was then added and incubated for 1 h at 4°C. The beads were washed four times with lysis buffer followed by two washing with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM dithiothreitol, 5 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, and 1 mM NaF). Samples were boiled for 3 min before Western blot analyses. For *in vitro* kinase assays, the immunoprecipitated kinase bound to the beads was incubated in kinase buffer supplemented with 20 pM ATP, and 1 μ g protein substrate. Reactions were stopped by the addition of equal volume of 2X Laemmli buffer followed by SDS-PAGE. The specific kinase activities were measured by the non-radioactive kinase assay using the kits purchased from Cell Signaling Technologies (Danvers, MA).

Western blot analysis. Western blot analysis was performed as previously described (14). The primary antibody preparations used in this study were 1:1000 dilutions of rabbit polyclonal anti-phosphotyrosine and anti-phosphospecific ERK-1/2 antibodies. The secondary antibody was horseradish peroxidase-labeled anti-rabbit immunoglobulins from donkey (Amersham Corp.) used at a 1:3000 dilution. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham Corp.). Total protein levels were assayed by washing the blotted membrane with stripping solution [(100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl (pH 6.7))] for 30 min at 50°C and then reprobing the membrane with rabbit polyclonal anti-PDGF- β R and anti-p44/42 antibodies diluted at 1:1000.

Extraction of nuclear proteins. hVSMCs (80-90% confluent) were incubated overnight in medium containing 1% FBS and treated with 0-10 ng/ml PDGF for 8 h. The cells were then resuspended in 500 μ l cold buffer A [50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM MgCl₂]. After the cells were allowed to swell for 5 min on ice, they were lysed with 500 μ l buffer B [identical to buffer A except containing 0.05% Nonidet P-40 (Sigma)]. The homogenate was gently layered onto an equal volume cushion of buffer C [10 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM MgCl₂] and centrifuged for 5 min at 200 \times g. The white nuclear pellet was resuspended in 75 μ l cold high-salt lysis buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM

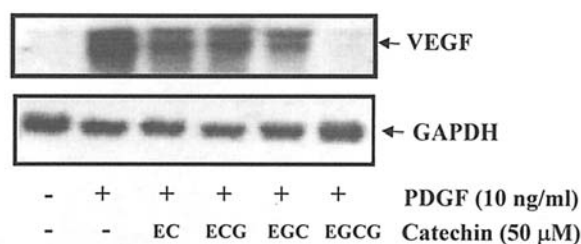


Figure 1. Effects of tea catechins on the PDGF-induced VEGF expression in hVSMCs. hVSMCs pretreated with 50 μ M EC, ECG, EGC and EGCG for 1 h were incubated with 10 ng/ml PDGF for 4 h and Northern blot analysis was performed for the VEGF and GAPDH mRNA expression.

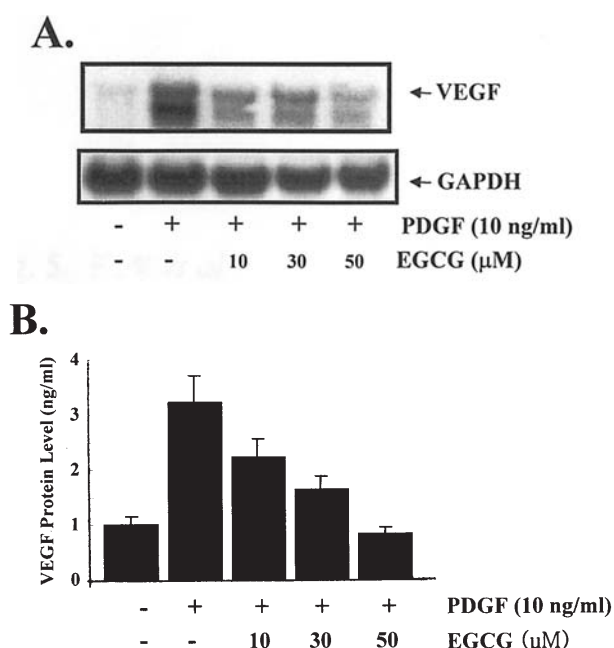


Figure 2. Inhibitory effect EGCG on the PDGF-induced VEGF mRNA and protein expression in hVSMCs. hVSMCs pretreated with 0-50 μ M EGCG for 1 h were incubated with 10 ng/ml PDGF for 4 h and Northern blot analysis was performed for the VEGF mRNA expression (A). The cells pretreated with 0-50 μ M EGCG for 1 h were incubated with 10 ng/ml PDGF for 48 h and culture medium was collected. ELISA was performed to determine the VEGF protein level in the culture medium. Data represent the means \pm SD from triplicate measurements (B).

EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride]. This suspension was agitated for 30 min at 4°C and then microcentrifuged for 15 min at 4°C. The resulting supernatant was stored in aliquots at -80°C. Protein was quantitated spectrophotometrically using the BCA assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA). EMSA was performed with the Gel Shift Assay System (Promega). Briefly, oligonucleotides containing the consensus sequence for AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') were end-labeled with [α - 32 P]adenosine triphosphate (3000 mCi/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase, purified in a Microspin G-25 column (Sigma) and used as a probe for EMSA. Nuclear extract proteins (6 μ g) were pre-incubated with the binding

buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly(deoxyinosine-deoxycytosine)] for 5 min and then incubated with the labeled probe for 15 min at 37°C. Each sample was electrophoresed in a 5% non-denaturing polyacrylamide gel in 0.5X Tris borate-EDTA buffer at 150 V for 4 h. The gel was dried and subjected to autoradiography.

Effect of EGCG pretreatment on proliferation of HUVECs stimulated by conditioned medium (CM) derived from PDGF-treated hVSMCs. CM derived from hVSMCs was prepared as follows. Cells were grown to 95-100% confluence and incubated for 48 h in DMEM with 1% FBS and 10 ng/ml PDGF. After incubation, the supernatant (conditioned medium, CM) was centrifuged, filtered, and stored at -20°C. To determine the effect of CM on endothelial cell proliferation, HUVECs (5x10³) were seeded on 96-well plates (Falcon Laboratories, McLean, VA) and incubated for 24 h in DMEM containing 15% FBS and 10 ng/ml bFGF. The medium was replaced with CM, and the cells were incubated for 24 h. To examine the effect of EGCG pretreatment on CM (derived from PDGF-treated hVSMCs)-induced HUVEC proliferation, hVSMCs were pretreated with 0-50 μ M EGCG for 1 h prior to exposure to PDGF. The cell proliferation was determined by the MTT assay.

Results

Effect of EGCG on the PDGF-induced VEGF expression in hVSMCs. To examine whether tea catechins could inhibit the PDGF-induced VEGF expression in hVSMCs, cells were pretreated with 0-50 μ M EGCG and other catechins for 1 h prior to 8-h incubation with 10 ng/ml PDGF, and VEGF mRNA and protein were measured by Northern blotting and ELISA, respectively. EGCG at 50 μ M inhibited remarkably the PDGF-induced VEGF mRNA expression. However, other tea catechins such as EC, ECG and EGC inhibited only slightly the PDGF-induced VEGF mRNA expression at the same concentration (Fig. 1). EGCG inhibited the PDGF-induced VEGF mRNA and protein expression in a dose-dependent manner (Fig. 2). EGCG at the concentrations used did not affect the cell viability (data not shown).

Effect of EGCG on the PDGF-induced VEGF promoter activity. Next, we sought to examine the effect of EGCG on the transcriptional regulation of the VEGF gene induced by PDGF. To this end, hVSMCs were transiently transfected with the promoter-reporter construct (pGL3-VEGF) of the human VEGF gene fused to the luciferase gene. hVSMCs transfected with pGL3-VEGF showed an 8-fold increase in promoter activity after PDGF treatment (Fig. 3). When the transfected cells were pretreated with 0-50 μ M EGCG before PDGF treatment, the induction of VEGF promoter activity by PDGF was inhibited dose-dependently (Fig. 3).

Effects of EGCG on signaling pathways involved in PDGF-induced VEGF expression. Our previous studies have suggested that Erk-1/2 and AP-1 signaling pathways are involved in the PDGF-induced VEGF expression in hVSMCs (14). Therefore, to study the underlying mechanisms responsible for the

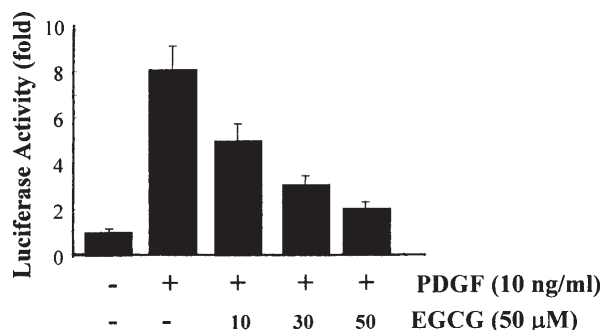


Figure 3. EGCG blocks the PDGF-induced VEGF promoter activity in hVSMCs. hVSMCs were transiently transfected with 1 μ g pGL3-VEGF. The transfected cells, after being pretreated with 0-50 μ M EGCG for 1 h, were incubated with 10 ng/ml PDGF for 8 h. The cells were lysed and the luciferase activity was measured using a luminometer. Data represent the means \pm SD from triplicate measurements.

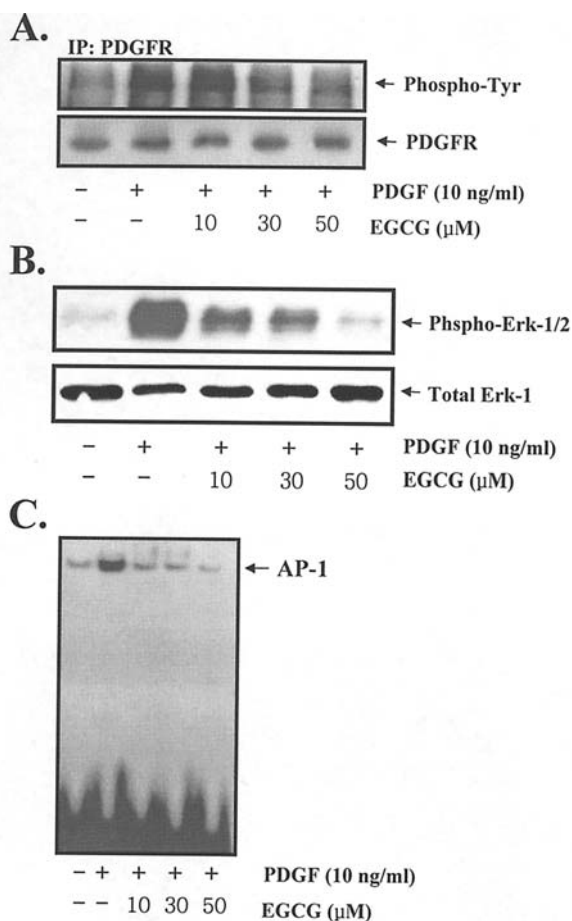


Figure 4. EGCG blocks the PDGF-induced activation of PDGF receptor, Erk-1/2 and AP-1. hVSMCs pretreated with 0-50 μ M EGCG for 1 h were incubated with 10 ng/ml PDGF for 15 min. PDGF receptor was immunoprecipitated (IP) from the cell lysates and then analyzed for phosphorylated and total PDGF receptor by Western blot analysis (A). The cell lysates were determined for the phosphorylated and total ERK-1/2 by Western blot analysis (B). The cells pretreated with 0-50 μ M EGCG for 1 h were incubated with 10 ng/ml PDGF for 8 h and nuclear proteins were extracted. EMSA was performed to determine the activated AP-1 using a radiolabeled oligonucleotide probe (C).

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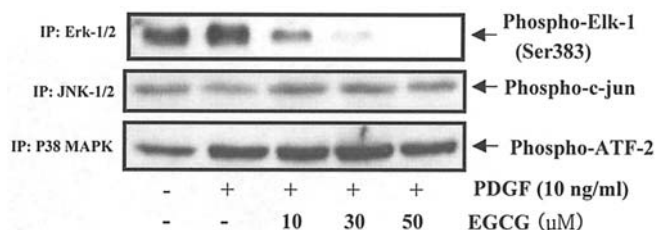


Figure 5. Inhibition by EGCG of the Erk-1/2 activity. The cell lysates from the hVSMCs treated with 10 ng/ml PDGF for 30 min were prepared. The indicated kinases were immunoprecipitated (IP) from the lysates and incubated with the appropriate protein substrates and ATP in the presence of 0-50 μ M EGCG for 30 min. After incubation, the reaction mixtures were electrophoresed and the incorporation of phosphate into the protein substrates was detected using the appropriate phosphate-specific antibodies. The immunoblots depicted correspond to Erk-1/2 (phospho-Elk-1), JNK-1/2 (phospho-c-jun) and P38 MAPK (phospho-ATF-2).

the PDGF receptor, Erk-1/2 and AP-1 were examined. The results showed that EGCG inhibited the PDGF-induced phosphorylation of the PDGF receptor in a dose-dependent manner without affecting the protein level of the PDGF receptor (Fig. 4A). EGCG inhibited the PDGF-induced phosphorylation of Erk-1/2 in a dose-dependent manner, too. The level of total Erk-1/2 was not significantly altered by EGCG treatment (Fig. 4B). In addition, EGCG inhibited remarkably the PDGF-induced activation of AP-1, as revealed by EMSA (Fig. 4C). Thus, EGCG was shown to inhibit not only the PDGF-induced tyrosine phosphorylation of the PDGF receptor but also the downstream activation of Erk-1/2 and AP-1.

Effect of EGCG on Erk-1/2 activity. Since EGCG has been reported to be a potent inhibitor of some enzymes (17), we next examined whether activated Erk-1/2 could be directly affected by EGCG. Extracts were prepared from PDGF-stimulated and -non-stimulated cells, and Erk-1/2 and other MAP kinases were collected by immunoprecipitation. The precipitated kinases were then incubated in *in vitro* kinase reactions in the presence of 0-50 μ M EGCG. As shown in Fig. 5, EGCG inhibited potently both the PDGF-induced and constitutive Erk-1/2 activity. In contrast, however, JNK-1/2 and P38 MAPK activity was not significantly affected by EGCG. The above results suggest that EGCG could inhibit not only the PDGF-induced activation of Erk-1/2 but also the activity of activated Erk-1/2.

Effect of EGCG pretreatment on proliferation of HUVECs stimulated by conditioned medium (CM) derived from PDGF-treated hVSMCs. Our results showed that EGCG could remarkably inhibit the PDGF-induced VEGF expression in hVSMCs. Also, our previous studies showed that conditioned medium (CM) obtained from PDGF-treated hVSMCs could stimulate the growth of HUVECs and this stimulatory effect was significantly abolished by anti-VEGF antibody, suggesting that VEGF derived from PDGF-treated hVSMCs may stimulate the EC proliferation. Therefore, the effect of EGCG pretreatment on the proliferation of HUVECs stimulated by CM derived from PDGF-treated hVSMCs was examined. As shown in Fig. 6, the CM derived from PDGF-treated hVSMCs remarkably stimulated the *in vitro* growth of HUVECs, and

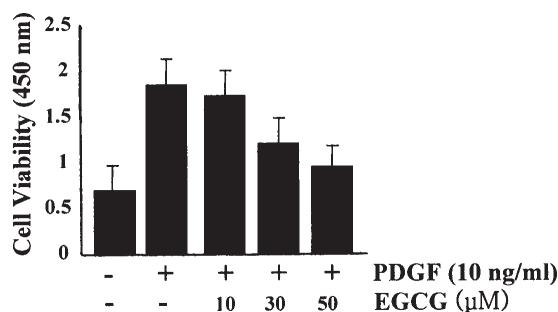


Figure 6. EGCG pretreatment inhibits the proliferation of HUVECs stimulated by conditioned medium (CM) derived from PDGF-treated hVSMCs. hVSMCs, after being pretreated with 0-50 μ M EGCG for 1 h, were incubated with 10 ng/ml PDGF for 48 h and the medium (CM) was collected. HUVECs were incubated with CM for 24 h and the number of cells was counted using MTT. Data represent the means \pm SD from triplicate measurements.

when the CM was collected from the cells pretreated with EGCG, its proliferation-stimulatory effect was significantly abolished.

Discussion

The interest in green tea as a cancer chemopreventive agent in humans has increased for several reasons: a) in epidemiological studies, green tea lowers the risk of various cancers among people who consume a large amount of green tea (18); b) green tea protects against the development and progression of skin, lung, mammary gland, and gastrointestinal tract cancers in animal models (4); c) green tea extracts stimulate apoptosis of various cancer cell lines, such as prostate, lymphoma, colon, and lung *in vitro* (19); and d) green tea consumption is associated with few adverse events and can be obtained easily at a low cost (20).

The anti-carcinogenic effects of green tea have been attributed to the biological activity of its polyphenol components. EGCG is the most abundant of the green tea polyphenols, accounting for >40% (5). Several molecular mechanisms have been suggested for EGCG's observed anticancer effect, including suppression of ligand binding to the EGF receptor (21), inhibition of protein kinase C (22), lipoxygenase and cyclooxygenase activity (5), and induction of apoptotic cell death and arrest of the cell cycle in tumor cells (20).

In this study, we demonstrated that EGCG inhibits VEGF induction by PDGF in hVSMCs at a transcriptional level. Recently, it was proposed that EGCG may exert at least part of its anti-cancer effect by inhibiting angiogenesis through blocking the induction of vascular endothelial growth factor (VEGF). Our results also showed that EGCG inhibited HUVEC proliferation stimulated by CM derived from PDGF-treated hVSMCs (14), supporting our earlier contention that VEGF is an *in vivo* survival factor for tumor endothelium (23). In another tumor system, Bertolini *et al* (24) demonstrated that treatment with green tea led to an inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis in an animal model of human high-grade non-Hodgkin's lymphoma. These findings suggest that down-regulation of VEGF by EGCG may not only inhibit the new blood vessel

formation and cell proliferation within tumors, but also lead to tumor cell apoptosis.

PDGF is a potent mitogen and survival factor for VSMC and tyrosine phosphorylation in the PDGF receptor serves as a critical line between extracellular PDGF stimulation and intracellular signaling. In this study, we demonstrated that EGCG inhibited the phosphorylation of tyrosines in the PDGF receptor elicited by PDGF without affecting the protein expression of PDGF receptors in hVSMCs. Several possible mechanisms by which EGCG inhibits the phosphorylation of the PDGF receptor are suggested. Liang *et al* (21) reported that EGCG inhibited receptor type protein (epidermal growth factor receptor, fibroblast growth factor receptor and PDGF receptor) tyrosine kinase whereas it scarcely inhibited serine- and threonine-specific protein kinases such as protein kinase A and C. EGCG, like tyrphostin AG1296, may induce conformational changes at the ATP-binding site of the PDGF receptor, thereby inhibiting its tyrosine phosphorylation (25,26). On the other hand, Sakata *et al* (27) suggested that the inhibition of PDGF-induced receptor phosphorylation and its downstream activation by EGCG may be attributed to the inhibition of PDGF and its receptor binding. EGCG also can form complexes with biologic macromolecules such as lipids, carbohydrates, protein, and nucleic acids (19). Weber *et al* (28) showed that EGCG was incorporated into different cellular components including cell surface membranes, which lead to a trapping of PDGF to non-receptor binding sites and reduced PDGF binding to its receptors.

Previously, we reported that ERK-1/2 and AP-1 signaling pathways were involved in PDGF-induced VEGF expression in hVSMCs and suggested that these paracrine signaling pathways induce endothelial cell proliferation (14). In this study, EGCG was shown to inhibit the activation of Erk-1/2 and transcription factor AP-1 in PDGF-treated hVSMCs. Since EGCG inhibited the phosphorylation of PDGF receptor, it would be logical to assume that the reduction in activity of kinases downstream of the PDGF receptor was due to the reduction in PDGF receptor activity. In addition, it was shown that EGCG could directly inhibit the Erk-1/2 activity in a cell free system, although the mechanism by which EGCG inhibited the Erk-1/2 activity remained unknown. Thus, activation of Erk-1/2 might be inhibited by a dual effect of EGCG in the cells: the suppression of upstream PDGF receptor phosphorylation and the direct inhibition of Erk-1/2 activity.

Our results suggest that EGCG may exert anti-angiogenic and anti-proliferative effects by inhibiting PDGF-induced VEGF expression at multiple signal levels, supporting the role of green tea as a cancer chemopreventive agent. Further studies are needed to elucidate the detailed mechanisms by which EGCG inhibits the VEGF expression and to examine whether EGCG exerts the same effects *in vivo*.

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