

Green Tea Catechins Inhibit Vascular Endothelial Growth Factor Receptor Phosphorylation¹

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Abstract

Vascular endothelial growth factor (VEGF) receptors (VEGFR) play a major role in tumor angiogenesis and, thus, represent attractive targets for the development of novel anticancer therapeutics. In this work, we report that green tea catechins are novel inhibitors of VEGFR-2 activity. Physiological concentrations (0.01–1 μM) of epigallocatechin-3 gallate, catechin-3 gallate, and, to a lesser extent, epicatechin-3 gallate induce a rapid and potent inhibition of VEGF-dependent tyrosine phosphorylation of VEGFR-2. The inhibition of VEGFR-2 by epigallocatechin-3 gallate was similar to that induced by Semaxanib (SU5416), a specific VEGFR-2 inhibitor. The inhibition of VEGFR-2 activity by the catechins displayed positive correlation with the suppression of *in vitro* angiogenesis. These observations suggest that the anticancer properties of green tea extracts may be related to their inhibition of VEGF-dependent angiogenesis.

Introduction

There is increasing evidence supporting the central role of angiogenesis in tumor growth and metastasis (1). As a consequence, tremendous efforts have been made to identify antiangiogenic molecules with antitumor properties (2), leading to the development of a variety of molecules targeting critical aspects of angiogenesis such as cell adhesion, degradation of the extracellular matrix, and stimulation of endothelial cells by angiogenic cytokines (3). VEGF³ is an endothelial cell-specific mitogen that is often associated with tumor-induced angiogenesis. At the endothelial cell surface, VEGF binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR; Flk-1 in mice), the latter being responsible for most of the mitogenic and chemotactic effects of VEGF (4). For this reason, intense research has focused on the development of anti-VEGF and anti-VEGFR-2 compounds to inhibit angiogenesis (5).

Green tea, the beverage made from the unfermented leaves of *Camellia sinensis*, is one of the most ancient and widely consumed beverages in the world. Green tea polyphenols have demonstrated significant antioxidant, anticarcinogenic, antimutagenic, anti-inflammatory, and antimicrobial properties (6). On the basis of a large body of evidence, it has become clear that green tea is an effective chemopreventive agent for many types of cancer in animal tumor models,

including those involving tumors of the skin, breast, lung, liver, esophagus, forestomach, small intestine, pancreas, and colon (7).

The chemical components found in green tea consist mainly of polyphenols (flavanols), commonly known as catechins. The major catechins in green tea are EC, ECG, EGC, and EGCG (8). Most of the biological effects of green tea appear to be mediated by its major polyphenolic constituent, EGCG. The anticarcinogenic properties of green tea polyphenols are likely to be the result of many biological responses (7), including the inhibition of urokinase activity, an enzyme crucial for cancer growth (9). However, the concentrations of EGCG used in most of these studies seem too high to account for the anticancer activity associated with green tea, based on the levels measured in human blood and serum after oral consumption (10). Green tea and one of its components, EGCG, were shown recently to prevent the growth of new blood vessels in a chick CAM assay (11), suggesting the presence of antiangiogenic molecules within green tea. In this work, we present evidence that these antiangiogenic effects of green tea are correlated with potent inhibitory effects of a number of green tea catechins on VEGFR-2 activity.

Materials and Methods

Materials. DMEM low glucose, penicillin/streptomycin, and endothelial basal medium MCDB 131 were obtained from Life Technologies, Inc. (Burlington, Ontario, Canada). Epidermal growth factor and Matrigel basement membrane matrix were obtained from Becton Dickinson Labware (Bedford, MA). Bovine calf serum and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT) and Medicorp (Montreal, Quebec, Canada), respectively. Hydrocortisone and (+)C, (–)EC, (–)CG, (–)ECG, and (–)EGC catechins were purchased from Sigma Chemical Co. (St. Louis, MO). (–)EGCG catechin was obtained from ICN Biomedicals, Inc. (Aurora, OH). Human recombinant VEGF was obtained from R&D Systems, Inc. (Minneapolis, MN). SU5416 was from Calbiochem (La Jolla, CA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). Protein A-Sepharose and protein G-Sepharose were obtained from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). The anti-VEGFR-1 and -VEGFR-2 polyclonal antibodies, and the anti-Tyr(P) PY99 monoclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antimouse IgG horseradish peroxidase-linked whole antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA) and Western Blot Cheluminescence Reagents Plus were from NEN Life Science Products, Inc. (Boston, MA).

Cell Culture. BAECs were purchased from Clonetics (San Diego, CA) and maintained in DMEM low glucose containing 10% heat-inactivated bovine calf serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cells were grown, serum-starved, and stimulated with 1 nM of VEGF as described previously (12). HMEC-1 was obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and maintained in MCDB 131 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10 ng/ml epidermal growth factor, and 1 mg/ml hydrocortisone. All of the cells were cultured at 37°C under a humidified atmosphere containing 5% CO₂.

Immunoprecipitation and Immunoblotting Procedures. For immunoblotting analysis of VEGFRs after immunoprecipitation, cells were lysed with ice-cold lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% NP40, and 1% Triton X-100] containing 1 mM Na₃VO₄. After each

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³The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; C, catechin; CG, catechin-3 gallate; EC, epicatechin; ECG, epicatechin-3 gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3 gallate; Tyr(P), phosphotyrosine; CAM, chorioallantoic membrane; BAEC, bovine aortic endothelial cell; HMEC, human microvascular endothelial cell; RTK, receptor tyrosine kinase; PDGFR, platelet-derived growth factor receptor; Flt-1, fms-like tyrosine kinase receptor (or VEGFR-1); KDR, kinase insert domain-containing receptor (or VEGFR-2).

treatment, equal amounts of protein (100 μg) were precleared by incubation with a mixture of protein A/protein G-Sepharose beads for 1 h at 4°C. After removal of the Sepharose beads by low-speed centrifugation, the supernatants were transferred to fresh tubes. Lysate supernatants were incubated in lysis buffer overnight at 4°C in the presence of 2 $\mu\text{g}/\text{ml}$ of specific antibodies. Immunocomplexes were collected by incubating lysates with 25 μl of protein A-Sepharose beads (50% suspension) for another 2 h at 4°C. Immunoprecipitates were then washed three times with lysis buffer and once with PBS (pH 7.4) containing 1 mM Na_3VO_4 . The proteins were extracted with 2-fold concentrated Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β -mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, separated on 7.5% SDS-PAGE, and analyzed by Western blotting as described previously (12).

In Vitro Kinase Assay. Confluent BAEC were serum starved for 24 h, and the cells were then stimulated with VEGF and lysed as described above. Precleared cell lysates (450 μg proteins) were incubated overnight with anti-VEGFR-2 antibodies, and the resulting immune complexes were collected with protein A-Sepharose beads and washed as described above. The washed beads were incubated for 1 h on ice in 15 μl of kinase buffer [100 mM Tris-HCl (pH 7.0), 0.2% β -mercaptoethanol, 20 mM MgCl_2 , and 0.2 mM Na_3VO_4] containing the specific catechin. The kinase reaction was initiated by the addition of 5 μCi of [γ - ^{32}P]ATP (ICN Biochemicals) into a final volume of 20 μl and incubated at 30°C for 15 min. The reaction was stopped by adding Laemmli sampler buffer, and proteins were separated by electrophoresis on 7.5% SDS gels. The resulting gels were stained for 30 min in 0.1% (w/v) Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie Blue dye. The gels were then washed extensively in this solution and exposed to Fuji X-ray film.

Matrigel Endothelial Cell Tube Formation Assays. Matrigel (12.5 mg/ml) was thawed at 4°C, and 50 μl were quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37°C. Once solid, the wells were incubated for 30 min with HMEC-1 (25,000 cells/well). After adhesion of the cells, the medium was removed and replaced by fresh medium supplemented (or not) with 50 μM of C, ECG, EGCG, or SU5416, and incubated at 37°C for 18 h. The tubes of growth were visualized with an inverted Nikon/TMS microscope at a magnification of $\times 4$, and the length of the capillary network was quantified with a map scale calculator.

Results

Effect of Green Tea Polyphenols on VEGF-induced Tyrosine Phosphorylation of VEGFRs. To better understand the inhibitory action of green tea polyphenols on the induction of *in vitro* angiogenesis, we examined their effect on the function of the most potent angiogenic factor, VEGF. Strong evidence shows that blocking VEGFR-2 activity limits the ability of most tumors to stimulate the formation of blood vessels (13). Thus, we examined whether the different polyphenols from green tea could have an effect on VEGF-induced tyrosine phosphorylation of VEGFR-2. Quiescent BAEC were incubated in serum-free medium in the presence or absence of 25 μM of each catechin (C, EC, ECG, CG, EGC, and EGCG) for 24 h. The medium was then replaced with fresh serum-free medium without catechins, and the cells were stimulated with 1 nM of recombinant VEGF for 1 min. The phosphorylation state of the VEGFR-2 was assessed by immunoprecipitation of the receptor followed by Tyr(P) immunodetection. Under these conditions, VEGF caused a marked increase in the tyrosine phosphorylation state of VEGFR-2 in untreated BAEC (Fig. 1A, top panel). The increase in tyrosine-phosphorylated VEGFR-2 was completely inhibited in BAEC by three of the catechins, CG, ECG, and EGCG. The total amount of VEGFR-2 in each sample of cells was unaffected by the catechins (Fig. 1A, bottom panel). Furthermore, the treatment of the cells with catechins at 25 μM did not induce significant cell death nor apoptosis (data not shown). These results suggest that CG, ECG, and EGCG are potent inhibitors of VEGF-induced tyrosine phosphorylation of VEGFR-2. We have shown previously that the phosphorylation of another VEGFR, VEGFR-1, in BAEC was stimulated to a much lesser extent by VEGF

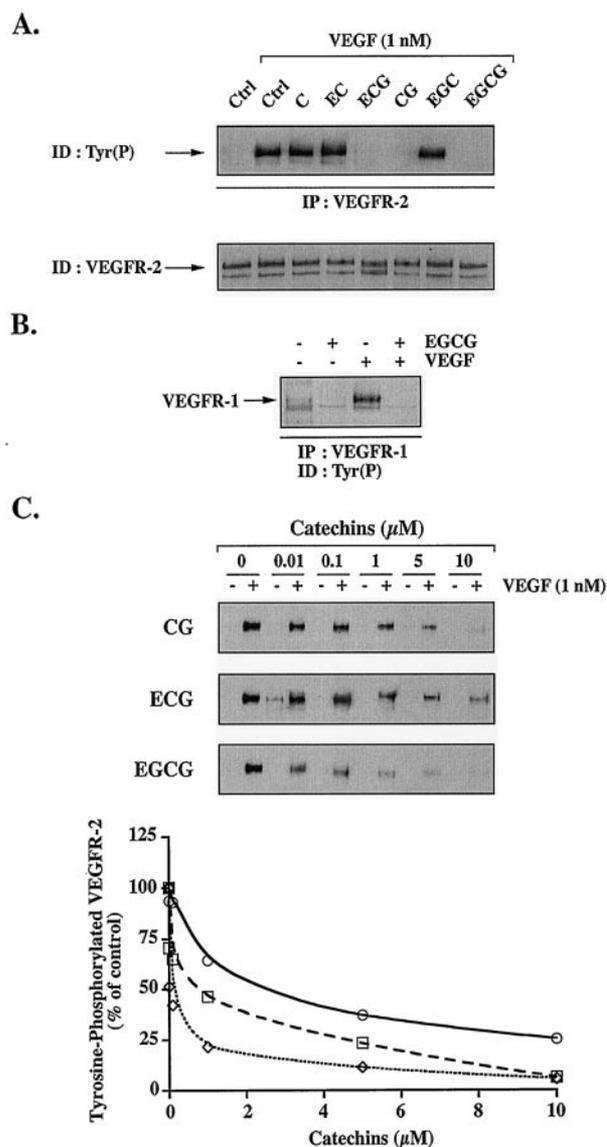
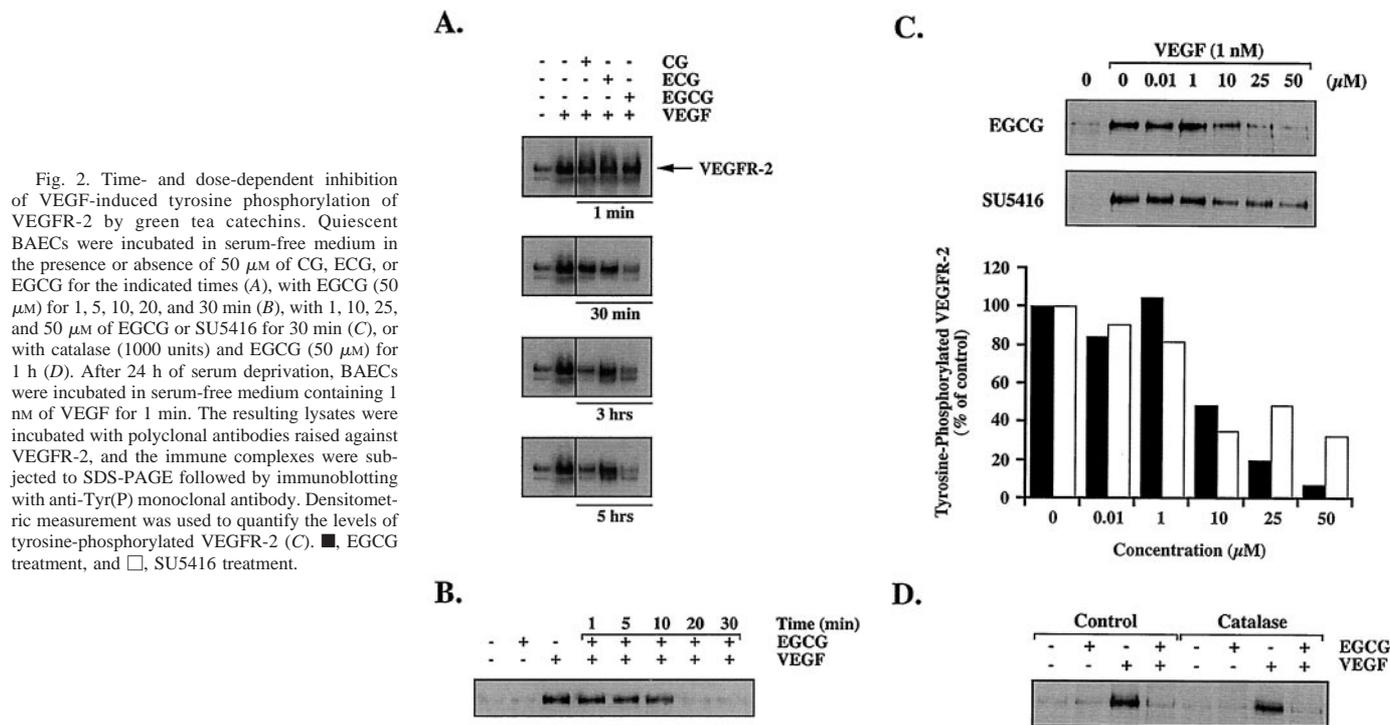


Fig. 1. Effect of VEGF on the tyrosine phosphorylation of VEGFRs in BAEC treated with green tea catechins. A, quiescent cells were incubated in serum-free medium in the presence or absence of 25 μM of each indicated catechin for 24 h. The medium was replaced with serum-free medium without catechins, and BAEC were stimulated with 1 nM of recombinant VEGF for 1 min. The extent of tyrosine phosphorylation was determined by immunoprecipitation as described in "Materials and Methods" (top panel). The effects of the treatments on the amount of VEGFR-2 were assessed by Western blotting using anti-VEGFR-2 antibodies (bottom panel). B, BAEC were pretreated with 25 μM of EGCG for 24 h and then stimulated with VEGF. Lysates were subjected to immunoprecipitation with anti-VEGFR-1 antibody, and the Tyr(P) content of the receptor was visualized by immunoblotting with the anti-Tyr(P) antibody. C, BAECs were pretreated with different concentrations of CG, ECG, or EGCG for 24 h and then stimulated with VEGF. The levels of tyrosine-phosphorylated VEGFR-2 were monitored by immunoprecipitation with anti-VEGFR-2 and immunoblotting with anti-Tyr(P) monoclonal antibody. Laser densitometric analysis of the bands was performed to quantify the effect of VEGF on tyrosine phosphorylation of VEGFR-2 in BAEC after treatment with various concentrations of ECG (\circ), CG (\square), and EGCG (\diamond).

compared with that of VEGFR-2 (12). Thus, we examined the inhibitory effect of the most potent inhibitor catechin, EGCG, on the tyrosine phosphorylation levels of VEGFR-1. As shown in Fig. 1B, addition of EGCG completely inhibits VEGF-dependent phosphorylation of VEGFR-1.

Tyrosine Phosphorylation of VEGFR-2 Is Inhibited by Physiological Concentrations of CG, ECG, and EGCG. Each potent inhibitory catechin, CG, ECG, and EGCG (0.01–10 μM), caused a dose-dependent inhibition of VEGF-induced tyrosine phosphorylation



of VEGFR-2 (Fig. 1C). For CG and EGCG, the inhibition became apparent at a relatively low concentration of 0.01 μ M compared with 1 μ M for ECG. Laser densitometric analysis of the bands of tyrosine-phosphorylated VEGFR-2 shows that EGCG is the most potent inhibitor followed by CG and ECG, respectively. The inhibition observed for the catechins EGCG and CG was almost complete at 10 μ M (94.4% and 93.7%, respectively), whereas ECG was less potent (74.8% inhibition).

Time Course of the Inhibitory Effect of Green Tea Polyphenols on the Tyrosine Phosphorylation of VEGFR-2. We conducted time course studies to characterize the inhibition by the specific catechin compounds. Serum-starved quiescent BAECs were incubated with a catechin (50 μ M) for varying lengths of time and then stimulated with VEGF. As shown in Fig. 2A, the tyrosine phosphorylation of VEGFR-2 by VEGF was rapidly inhibited by CG and EGCG (<3 h), unlike ECG, which had produced no effect during the 5-h experiment. Moreover, the inhibitory effect of EGCG was extremely rapid, being observed after as little as 30 min. The rapid kinetics of EGCG inhibitory activity prompted us to more precisely examine the time course between 1 and 30 min. The results show that the inhibition by EGCG was time-dependent and was already complete at 20 min where a return to a basal level of phosphorylation was observed (Fig. 2B). To additionally explain the mechanisms involved in the inhibitory action of EGCG, we compared its effect with that of a potent and selective synthetic inhibitor of VEGFR-2, SU5416 (14). For both of these two molecules, significant inhibition occurred at 10 μ M for 30 min of incubation with the cells (Fig. 2C). Interestingly, VEGFR-2 was more sensitive to the inhibitory effect of EGCG than to that of SU5416.

Recent studies have provided evidence that the addition of green tea polyphenols and other phenolic compounds to cell culture medium leads to the generation of substantial amounts of hydrogen peroxide (15, 16). This generation of H₂O₂ has been suggested to account for some of the effects reported for the phenolic compounds on cells in culture. To investigate whether the inhibition of VEGF-induced tyrosine phosphorylation of VEGFR-2 by EGCG was mediated by H₂O₂

production, the effect of catalase was examined in this system. The addition of catalase (1000 units) to the culture medium with or without EGCG (50 μ M) for 1 h did not prevent the inhibition of tyrosine phosphorylation by catechins (Fig. 2D). Therefore, these results argue against a nonspecific effect of EGCG on VEGFR-2 activity, which would have been mediated through H₂O₂ production.

Effect of Green Tea Polyphenols on the Tyrosine Kinase Activity of VEGFR-2. To determine whether the inhibition of tyrosine phosphorylation of VEGFR-2 by the catechins resulted in a diminution of its kinase activity, anti-VEGFR-2 immunoprecipitates from treated cells were subjected to *in vitro* kinase assays. A concentration of 10 μ M of EGCG was used in this study, because at this concentration EGCG inhibited the VEGF-dependent kinase activity of VEGFR-2 but did not affect its basal kinase activity (data not shown). Moreover, we used C catechin as a control, because it had no effect on the tyrosine phosphorylation of VEGFR-2. As shown in Fig. 3, immunoprecipitates from CG- and EGCG-treated cells showed a diminution in the radiolabeling of the receptor, indicating that both catechins reduced the kinase activity of the receptor at 10 μ M com-

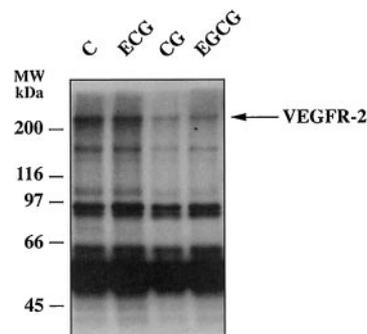


Fig. 3. Effect of CG, ECG, and EGCG on the tyrosine kinase activity of VEGFR-2. Anti-VEGFR-2 immunoprecipitates from treated cells (+)-C, (-)-CG, (-)-ECG, and (-)-EGCG were subjected to an *in vitro* kinase assay as described in "Materials and Methods." Samples were separated by SDS-PAGE followed by autoradiography.

pared with ECG, which shows no significant effect. At 50 μM , the tyrosine kinase activity of VEGFR-2 was also reduced by ECG (data not shown). These data confirm that CG and EGCG are the most potent catechin inhibitors and that ECG inhibits VEGFR-2 less effectively. Overall, these results suggest a potential direct interaction of the catechins with the catalytic activity associated with VEGFR-2.

Green Tea Catechins Inhibit the Formation of Endothelial Cell Capillary-like Structures. Green tea polyphenols, and especially EGCG, have been shown to possess antiangiogenic activity based on their inhibitory effects on neovascularization in the CAM *ex ovo* assay (11). To determine whether other catechins could also inhibit angiogenesis, we examined their effect on Matrigel-induced tube formation, another widely used angiogenesis assay (17). As shown in Fig. 4, HMEC-1 cells cultured on Matrigel migrated and organized into tubular networks, which resemble capillaries. Treatment of these cells with 50 μM of ECG, CG, or EGCG for 18 h affected tube formation; this process was almost completely abolished by CG and EGCG (82.5% and 91.6% inhibition, respectively). The C catechin had no effect. Interestingly, SU5416 also apprehended the formation of capillary-like structures (38.2% inhibition) but to a lesser extent than did EGCG.

Discussion

Angiogenesis, the process of blood-vessel growth, is a critical event during development, and during tumor invasion and metastasis. The growth of almost all types of tumor is dependent on angiogenesis, and the expansion and metastasis of tumors is disrupted when it is sup-

pressed (1). VEGF, the ligand for the VEGFRs, is the most potent angiogenic factor. It has been demonstrated recently that EGCG inhibited VEGF-induced angiogenesis, because it suppressed both bovine capillary endothelial cells growth *in vitro* and the formation of new blood vessels in the chick CAM model (11), as well as the induction of VEGF by human colon carcinoma cells (18). In this study, we have shown for the first time that VEGF stimulation of the tyrosine phosphorylation of VEGFR-2 in endothelial cells is inhibited in a dose- and time-dependent manner by green tea. This inhibitory effect seems to be specific to ECG, CG, and EGCG, because the other catechins tested had no effect. Whereas the structural determinants involved in the inhibitory effect of these catechins remain to be established, it is noteworthy that the ester bond present in these structures was reported recently to be crucial for their inhibitory action against the chymotrypsin-like activity of the proteasome (19).

In contrast to most studies on green tea catechins, which have used rather high concentrations of these compounds, we observed that low physiological concentrations of EGCG were sufficient to significantly inhibit VEGFR-2 activity. Indeed, a 50% inhibition of VEGF-dependent tyrosine phosphorylation of VEGFR-2 could be observed at a concentration of 0.01 μM EGCG, \sim 10-fold less than its plasma concentration in tea drinkers (11, 20, 21). We also observed that, under our experimental conditions, EGCG was as potent as SU5416 in inhibiting VEGFR-2 tyrosine phosphorylation. In *in vitro* Matrigel assays, EGCG was even more inhibitory than was SU5416, possibly reflecting its activity against other components of the angiogenesis process such as matrix metalloproteinases (22). Because SU5416 is known to inhibit angiogenesis both *in vitro* and *in vivo*, these results strongly support the concept that EGCG represents a potent angiogenesis inhibitor.

RTKs (also known as growth factor receptors) play an important role in many cellular processes such as proliferation, differentiation, morphogenesis, and angiogenesis (23). Disturbances in the expression of growth factors, their cognate RTKs, or constituents of their downstream signaling pathways are commonly associated with many types of cancer and other nonmalignant proliferative diseases (23–25). Therefore, the targeting of RTKs to inhibit tumor growth has received considerable attention in recent years (24). EGCG has been demonstrated previously to inhibit the protein tyrosine kinase activities of the PDGFR (21) and fibroblast growth factor receptor (26). We extend these observations by showing that EGCG also interferes with the activity of VEGFR-2 at the endothelial cell level. It seems that EGCG is more active against VEGFR than PDGFR, because the reported inhibitory effect on tyrosine phosphorylation of PDGFR- β only appeared at 50 μM (21) as compared with that of VEGFR-2 (0.01 μM) observed in our study. Moreover, it is possible that, like SU5416, EGCG could be a potent, competitive (in regard to ATP) inhibitor of the tyrosine kinase activity of VEGFR-2, thereby inhibiting its tyrosine phosphorylation. These results suggest that EGCG may act as a multifunctional anticancer agent through its inhibitory effect on several aspects of both tumor growth and tumor angiogenesis. Our findings may be helpful in designing future strategies for the development of green tea as a practical chemopreventive agent and a potential clinical therapy in combination with current anticancer drugs.

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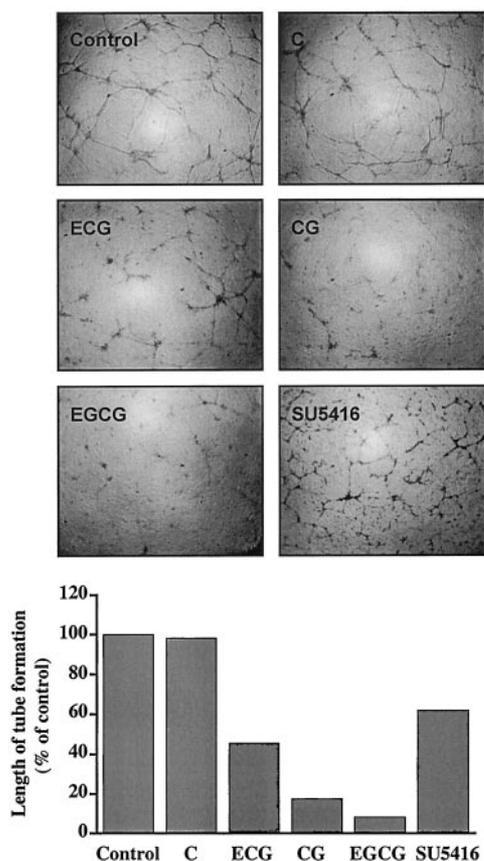


Fig. 4. Effect of green tea catechins and SU5416 compound on the Matrigel-induced tube formation of HMEC-1. Representative microscopy photographs of HMEC-1 cultured for 18 h on Matrigel in the presence or absence of 50 μM of the indicated compounds are illustrated. The length of the capillary network was quantified as described in "Materials and Methods."

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