

Green tea extract and (–)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1 α protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells

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Abstract

Green tea extract and its major component (–)-epigallocatechin-3-gallate (EGCG) exhibit antiangiogenic activities in various experimental tumor models. A growing body of evidence has established that hypoxia-inducible factor-1 α (HIF-1 α) and its downstream target, vascular endothelial growth factor (VEGF), play a critical role in tumor angiogenesis. In this study, we investigated the effect of green tea extract and EGCG on HIF-1 α and VEGF expression in human cervical carcinoma (HeLa) and hepatoma (HepG2) cells. Our results showed that green tea extract and EGCG significantly inhibited hypoxia- and serum-induced HIF-1 α protein accumulation in these cancer cells but had no effects on HIF-1 α mRNA expression. Suppression of HIF-1 α protein by green tea extract and EGCG also resulted in a drastic decrease in VEGF expression at both mRNA and protein levels. The mechanisms of green tea extract and EGCG inhibition of hypoxia-induced HIF-1 α protein accumulation seem to involve the blocking of both phosphatidylinositol 3-kinase/Akt and extracellular signal-regulated kinase 1/2 signaling pathways and the enhancing of HIF-1 α protein degradation through the proteasome system. In addition, green tea extract and EGCG inhibited serum-induced HIF-1 α protein and VEGF expression by interfering with the phosphatidy-

linositol 3-kinase/Akt/mammalian target of rapamycin signaling pathways, which play a crucial role in the protein translational machinery cascade. Functionally, green tea extract and EGCG abolished both chemoattractant- and hypoxia-stimulated HeLa cell migration. Our data suggested that HIF-1 α /VEGF function as therapeutic target for green tea extract and EGCG in the context of cancer chemoprevention and anticancer therapy. [Mol Cancer Ther 2006;5(5):1227–38]

Introduction

Green tea is one of the most widely consumed beverages in the world. The water-extractable fraction of green tea contains several polyphenolic compounds known as catechins. Generally, a typical cup of green tea contains 100 to 150 mg catechins, including 50% of (–)-epigallocatechin-3-gallate (EGCG), 15% of (–)-epigallocatechin (EGC), 15% of (–)-epicatechin-3-gallate, and 8% of (–)-epicatechin (1). Numerous studies have shown that green tea extract derived from the dried fresh leaves of the plant *Camelia sinensis* and one of its major constituents, EGCG, possess obvious antiproliferative (2–4), antiangiogenic (5–11), antimetastatic (4, 12–15), proapoptotic (2, 16, 17), and cell cycle perturbation (18, 19) activities in various *in vitro* and *in vivo* tumor models. These experimental studies together with several epidemiologic studies have suggested that green tea extract and EGCG harbor strong anticancer and cancer preventive effects in numerous human cancers (2, 20, 21).

As the most potent component in green tea with respect to antitumor activity, EGCG has been shown to inhibit several critical signal transduction pathways (22). Previous studies have shown that treatment of cancer cells with EGCG leads to the inactivation of epidermal growth factor receptor (erbB1), HER-2/*neu* (erbB2), and HER-3 and subsequently the inhibition of multiple downstream signaling pathways, particularly phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase 1/2 (ERK1/2), in human head and neck squamous cell carcinoma, breast cancer, and colon cancer cell lines (23–29). These receptors belong to subclass I of the receptor tyrosine kinase superfamily (23–29). In addition to epidermal growth factor receptor, it has been shown that EGCG also inhibits tyrosine kinase activities of other receptor tyrosine kinases, such as platelet-derived growth factor receptor and fibroblast growth factor receptor in human A-431 epidermoid carcinoma cells, and insulin-like growth factor-I receptor in a mouse model of prostate cancer and human colon cancer cells (10, 30, 31). Collectively, these

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Note: Q. Zhang and X. Tang contributed equally to this work.

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studies suggest that these specific receptor tyrosine kinases and their downstream signaling pathways may serve as potential molecular targets of EGCG with respect to its antiproliferative and proapoptotic activities. It has been well established that neovascularization or angiogenesis plays a key role in the growth of malignant solid tumor and metastasis (32). One of the principal mediators of tumor angiogenesis is vascular endothelial growth factor (VEGF). Previous studies have indicated that treatment with green tea extract or EGCG not only decreased VEGF production (33–36) but also inhibited the expression (7), binding activity (5), and phosphorylation (37) of VEGF receptors, thus contributing to their potent antiangiogenic activities. However, up to date, the detailed biochemical and molecular mechanisms underlying the antiangiogenic effect of green tea extract and EGCG are still not fully understood.

Hypoxia-inducible factor-1 (HIF-1) belongs to the basic helix-loop-helix-periodic acid-Schiff domain transcription factor family and consists of two subunits, HIF-1 α and HIF-1 β . Only the expression and activation of HIF-1 α is tightly regulated by cellular oxygen concentration (38). In the presence of O₂, HIF-1 α is hydroxylated at the proline residues Pro⁴⁰² (39) and Pro⁵⁶⁴ (40, 41) in the oxygen-dependent domain, which is catalyzed by specific HIF-1 α prolyl hydroxylases (42). The prolyl-hydroxylated HIF-1 α interacts with the von Hippel-Lindau gene product, an important component of the E3 ubiquitin ligase complex, resulting in ubiquitination and subsequent degradation via the 26S proteasome system (43, 44). Under hypoxic conditions, due to the inhibition of prolyl hydroxylation, HIF-1 α escapes from degradation, accumulates rapidly, translocates into the nucleus, and forms a heterodimer with HIF-1 β . The HIF molecule then binds to the *cis*-acting element (hypoxia-responsive elements) within the promoter regions of hypoxia-responsive genes and activates their expression (45). In addition, accumulating evidence has shown that other pathways independent of hypoxia can also promote HIF-1 α protein synthesis and stabilization. These pathways include the inactivation of some tumor suppressor genes, such as von Hippel-Lindau gene product (44) and phosphatase and tensin homologue deleted on chromosome 10; ref. 46, the oncogenic activation of Src (47), and the stimulation by some inflammatory cytokines, hormones, or growth factors, such as interleukin-1, tumor necrosis factor- α (48–50), interleukin-6 (51), insulin, and insulin-like growth factor-I (52, 53) via translational or post-translational mechanisms. Recent studies have shown that HIF-1 α plays an important role in tumor growth, angiogenesis (54), metastasis (55), and apoptosis (54, 56, 57). Its overexpression is related to a more aggressive tumor phenotype (58–60), thus supporting the notion that hypoxia/HIF-1 system is a potential molecular target in cancer therapeutics (61).

In this study, we have shown for the first time that green tea extract and EGCG suppressed both hypoxia- and serum-induced HIF-1 α protein accumulation and VEGF expression in human cervical carcinoma (HeLa) and

hepatoma (HepG2) cells but had no apparent inhibitory effects on HIF-1 α mRNA levels. The mechanisms by which green tea extract and EGCG inhibited hypoxia- and serum-induced HIF-1 α protein expression seemed to involve an increase in HIF-1 α protein degradation via the ERK1/2 and/or PI3K/Akt signaling pathways. These unique findings provided further understanding of the molecular mechanisms underlying the antiangiogenic effects of green tea extract and EGCG and help to delineate further targets of therapeutic intervention and chemoprevention of human cancers.

Materials and Methods

Reagents

Green tea extract was obtained from Pharmanex, Inc. (Provo, UT) and dissolved in distilled water to make a stock solution of 10 mg/mL. The purity and components of green tea extract were described previously (15). EGCG was purchased from Sigma (St. Louis, MO), dissolved in distilled water at a concentration of 100 mmol/L, and stored at -80°C as a stock solution. LY294002, PD98059, U0126, wortmannin, and rapamycin were purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. The final DMSO concentration did not exceed 0.1% throughout the study. The selective proteasome inhibitor, MG132 (Z-Leu-Leu-Leu-CHO), and protein synthesis inhibitor, cycloheximide, were from Sigma. Primary antibody for HIF-1 α was from BD Transduction Laboratories (San Diego, CA). Antibodies for total or phosphorylated p42/p44 mitogen-activated protein kinases (Thr²⁰²/Tyr²⁰⁴) and total or phosphorylated Akt (Ser⁴⁷³) were from New England Biolabs (Beverly, MA). Antibodies for VEGF, phosphorylated M_r 70,000 ribosomal protein S6 kinase 1 (p70^{S6K1}; Thr⁴²¹/Ser⁴²⁴), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; Ser⁶⁵/Thr⁷⁰) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for β -actin was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Pierce (Rockford, IL).

Cell Culture

Human cervical carcinoma (HeLa) and hepatoma (HepG2) were obtained from the American Type Culture Collection (Rockville, MD). HeLa and HepG2 cells were maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$; Invitrogen) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Establishment of Hypoxic Culture Condition

Cells were cultured to $\sim 80\%$ confluence and transferred to a hypoxic chamber with an auto-purge airlock (Coy Laboratory Products, Inc., Grass Lake, MI). Environmental hypoxic conditions (1%) were achieved in an airtight humidified chamber continuously flushed with a gas mixture containing 5% CO₂ and 95% N₂. Maintenance of the desired O₂ concentration was constantly monitored during incubation using a microprocessor-based oxygen controller (Coy Laboratory Products).

Treatment of Cancer Cells with Green Tea Extract and EGCG

Exponentially growing cells (~80% confluence) in complete medium were pretreated for 1 hour with different concentrations of green tea extract and EGCG followed by incubation under normoxic or hypoxic conditions for indicated periods according to the purpose of experiment. To study the effects of green tea extract and EGCG on the half-life or degradation of hypoxia-induced HIF-1 α protein accumulation, HeLa cells were exposed to hypoxia for 16 hours followed by treatment with 10 μ g/mL cycloheximide in the presence or absence of green tea extract (80 μ g/mL) or EGCG (100 μ mol/L). Cells that were pretreated with 20 μ mol/L MG132 for 30 minutes were cultured in the presence or absence of green tea extract or EGCG under normoxic or hypoxic conditions. HIF-1 α protein levels were determined by Western blot analysis.

To study the effect of green tea extract and EGCG on serum-induced HIF-1 α protein expression, exponentially growing HepG2 cells were serum starved for 24 hours followed by pretreatment with green tea extract or EGCG for 1 hour in serum-free medium. Afterward, pretreated cells were cultured in complete medium containing 10% FBS in the presence or absence of green tea extract or EGCG for indicated periods. HIF-1 α protein levels were determined by Western blot analysis.

Transient Transfection and Luciferase Reporter Assays

The luciferase reporter plasmids (pGL2-Luc) harboring human VEGF promoter region (-1,175/+336) was kindly provided by Dr. David K. Ann (University of Southern California School of Pharmacy). HeLa cells were transiently transfected with 0.5 μ g VEGF reporter plasmids. One-tenth microgram of the *Renilla* luciferase pRL-TK plasmid was cotransfected as an indicator for normalization of transfection efficiency. Afterward, cells were pretreated with different concentrations of green tea extract or EGCG for 30 minutes followed by exposure to normoxia or hypoxia for 24 hours. Cell lysates were subsequently assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Western Blot Analysis

The protein samples from cultured cells were prepared as described previously (57). Western blot analyses were carried out using specific primary antibodies against HIF-1 α , VEGF, total or phosphorylated p42/p44 mitogen-activated protein kinases (Thr²⁰²/Tyr²⁰⁴) or Akt (Ser⁴⁷³), phosphorylated p70^{S6K1}, or phosphorylated 4E-BP1 followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:2,000). The signals were visualized by the enhanced chemiluminescence detection system. As a loading control, the blots were reprobed with a specific antibody against human β -actin (1:4,000).

Reverse Transcription-PCR Analysis for HIF-1 α and VEGF mRNA Levels

Total RNA was isolated from cancer cells using Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) analysis of HIF-1 α , VEGF, and β -actin mRNA levels was

done using the One-Step RT-PCR kit (Qiagen, Valencia, CA) with primers specific to HIF-1 α (forward primer 5'-TCAC-CACAGGACAGTACAGGATGC-3' and reverse primer 5'-CCAGCAAAGTTAAAGCATCAGGTTCC-3'), VEGF (forward primer 5'-AGGAGGGCAGAATCATCACG-3' and reverse primer 5'-CAAGGCCACAGGGATTTCT-3'), and β -actin (forward primer 5'-TCATGAAGTGTGACGTT-GACATCCGT-3' and reverse primer 5'-CCTAGAAG-CATTTGCGGTGCACGATG-3'). All the primers were synthesized by Microchemical Core Facility at Norris Comprehensive Cancer Center of University of Southern California.

ELISA for VEGF Production

VEGF production in the conditioned medium was assayed with Quantikine Human VEGF ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocols. Results were normalized to cell count (1×10^5).

Cell Migration Assay

The cell migration kit (Chemicon, Temecula, CA) was used according to the manufacturer's protocol. HeLa cells were added to the inner chamber of the insert in 300 μ L serum-free medium. Medium (500 μ L) with or without 10% FBS was added to the lower chamber. To determine the effect of green tea extract or EGCG on cell migration, 80 μ g/mL green tea extract or 100 μ mol/L of EGCG was added to the lower chamber followed by incubating under normoxia or hypoxia for 48 hours at 37°C. The non-migrating cells and the collagen gel from the interior side of the inserts were gently removed using a cotton-tipped swab. The cells that migrated through the gel insert to the lower surface of the membrane were stained and photographed using a computer imaging system.

Cell Viability Assay

HeLa or HepG2 cells were plated in 96-well plates (10^4 per well) with or without serum starvation for 24 hours and subsequently treated with different concentrations of green tea extract and EGCG under normoxia or hypoxia for 24 hours. Viable cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay kit (Chemicon) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean \pm SD for three separate experiments. One-way ANOVA and Bonferroni were employed for statistical analysis using SPSS 11.0 for Windows software. $P < 0.05$ was considered statistically significant.

Results

Green Tea Extract and EGCG Inhibited Hypoxia-Induced HIF-1 α Protein Accumulation and VEGF Expression in HeLa and HepG2 Cells

Most recently, two independent studies have reported that (-)-epicatechin-3-gallate and EGCG, two major components in green tea, induce HIF-1 activation and protein stabilization in T47D human breast carcinoma cells (62) and

PC-3 prostate cancer cells (63), respectively. These results have somewhat compromised the intended anticancer activity of green tea extracts and catechins and thus prompted us to test the effect of green tea extract and EGCG on HIF-1 α expression in other cancer cells. To this end, we examined the effects of green tea extract and EGCG on hypoxia-induced HIF-1 α expression in HeLa and HepG2 cells. Our results showed that under normoxic conditions neither green tea extract nor EGCG had any obvious effect on HIF-1 α protein accumulation in both types of cancer cells (Fig. 1A and C). On the contrary, pretreatment of HeLa and HepG2 cells with green tea extract or EGCG significantly inhibited hypoxia-induced HIF-1 α protein accumulation in a dose-dependent manner (Fig. 1A and B and Fig. 1C and D). It has been reported that prolonged treatment with green tea extract and EGCG can inhibit cell growth and induce apoptosis in a variety of cancer cells (16, 17). To rule out the possibility that the inhibitory effect of green tea extract and EGCG on HIF-1 α protein was due to cellular toxicity, cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. No obvious changes in cell viability were observed in HeLa cells after treatment with different concentrations of green tea extract and EGCG under both normoxic and hypoxic conditions for 16 hours (Fig. 1F). Similar results were obtained in HepG2 cells (data not shown).

To determine whether the reduction of hypoxia-induced HIF-1 α protein accumulation by green tea

extract and EGCG was the result of transcriptional inhibition, HIF-1 α levels were determined by RT-PCR. As shown in Fig. 1E, no apparent changes in HIF-1 α mRNA levels were observed in HeLa cells after exposure to hypoxia for 16 hours. Treatment of HeLa cells with different concentrations of green tea extract or EGCG did not alter HIF-1 α mRNA expression under both normoxic and hypoxic conditions (Fig. 1E). Similar results were obtained in HepG2 cells under the same experimental conditions (data not shown). These results suggest that green tea extract and EGCG decreased hypoxia-induced HIF-1 α protein accumulation via a post-transcriptional mechanism.

VEGF is an immediate downstream target gene of HIF-1 α and plays a critical role in tumor angiogenesis (64). To determine whether green tea extract and EGCG can inhibit hypoxia-induced VEGF expression in HeLa cells, VEGF protein secretion in the conditioned medium and mRNA levels were determined by ELISA and RT-PCR, respectively. Our results showed that green tea extract and EGCG suppressed hypoxia-induced VEGF protein production and mRNA expression in a dose-dependent manner in HeLa cells ($P < 0.01$; Fig. 2A and B and Fig. 2C). To further confirm the effects of green tea extract and EGCG on VEGF transcriptional activation, HeLa cells were transiently transfected with a luciferase reporter plasmid (pGL2-Luc) harboring human VEGF promoter region (-1,175/+336) followed by treatment with various doses of green tea extract or EGCG for 16 hours. Our results indicated that the

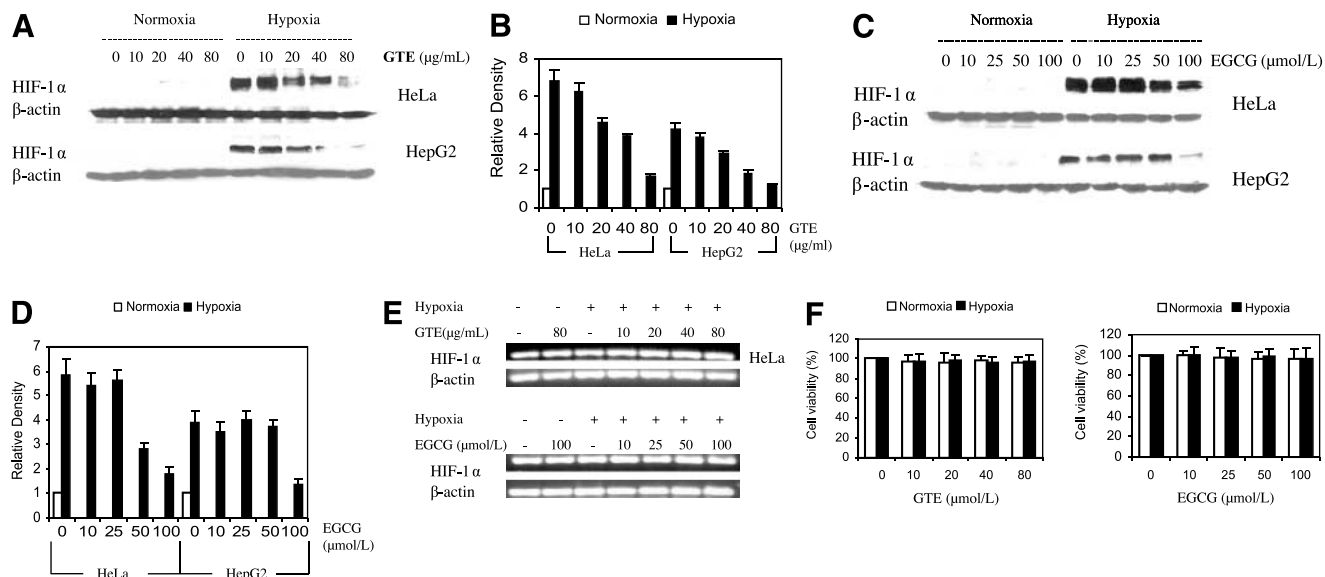
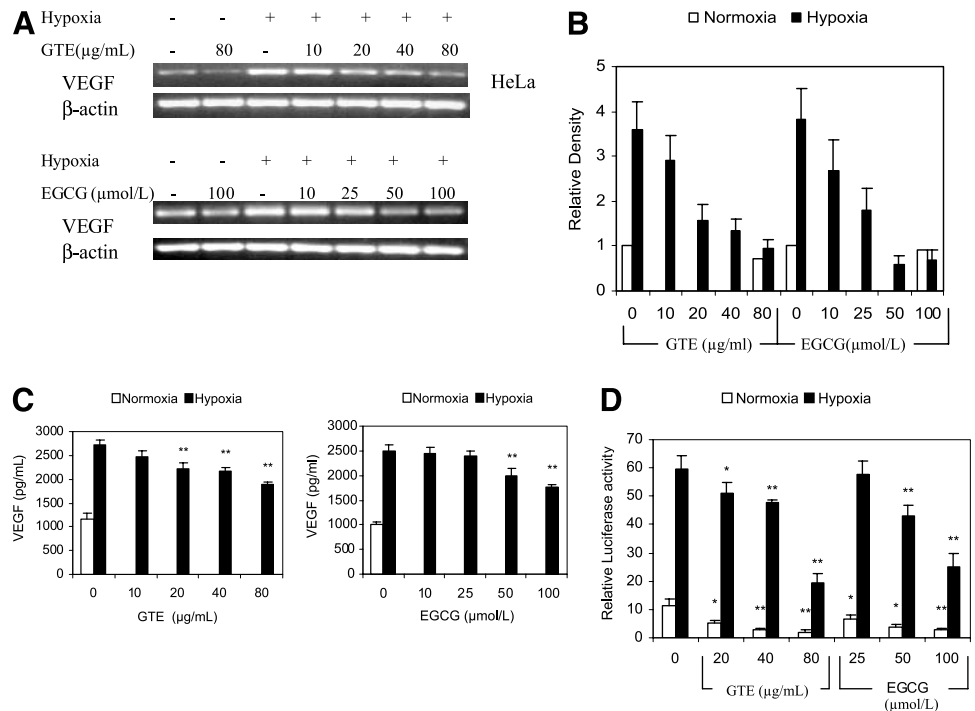


Figure 1. Green tea extract and EGCG inhibit hypoxia-induced HIF-1 α protein accumulation in HeLa and HepG2 cells. **A, C,** and **E,** HeLa and HepG2 cells were pretreated for 1 h with different concentrations of green tea extract (GTE) or EGCG in normal culturing conditions followed by exposure to normoxia or hypoxia (1% O₂) for 16 h. HIF-1 α protein and mRNA levels were determined by Western blot analysis (**A** and **C**) and RT-PCR (**E**), respectively. **B** and **D,** densitometry analyses of **A** and **C**. The relative density ratio of HIF-1 α protein band to β -actin with nontreatment under normoxia was arbitrarily set as 1.0. **F,** HeLa cells were treated with various concentrations of green tea extract or EGCG for 16 h under both normoxic and hypoxic conditions and cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Representative of three independent experiments. **Columns,** mean percentage of viable cells from three replicate experiments; **bars,** SD.

Figure 2. Green tea extract and EGCG inhibit VEGF expression and transcriptional activation in HeLa cells in response to hypoxia. **A–C**, HeLa cells were pretreated with different concentrations of green tea extract or EGCG for 1 h in normal conditions followed by exposure to normoxia or hypoxia (1% O₂) for 16 h. VEGF mRNA levels and VEGF protein production in the conditioned medium were determined by RT-PCR (**A**) and ELISA (**C**), respectively. **B**, densitometry analyses of **A**. The relative density ratio of VEGF mRNA band to β -actin with nontreatment under normoxia was arbitrarily set as 1.0. **D**, following transient transfection with the luciferase reporter plasmids (pGL2-Luc) harboring human VEGF promoter region (–1,175/+336), HeLa cells were pretreated with various concentrations of green tea extract or EGCG for 1 h and then exposed to normoxia or hypoxia for 16 h. Luciferase activities were assayed using the Dual-Luciferase Assay kit. *Columns*, mean of three replicate experiments; *bars*, SD.



strikingly increased VEGF promoter activities induced by hypoxia in HeLa cells were effectively suppressed by green tea extract or EGCG in a dose-dependent manner ($P < 0.01$; Fig. 2D).

Green Tea Extract and EGCG Inhibited Serum-Induced HIF-1 α Protein and VEGF Expression in HepG2 Cells

Accumulating evidence has shown that serum and some growth factors can increase HIF-1 α protein expression in several cancer cell lines (48, 65). To explore whether green tea extract and EGCG inhibit serum-induced HIF-1 α protein accumulation, HepG2 cells were cultured in serum-free medium for 24 hours and then exposed to 10% FBS in the presence or absence of various doses of green tea extract or EGCG for additional 16 hours. As shown in Fig. 3A and B, the serum-induced HIF-1 α protein accumulation in HepG2 cells was robustly inhibited by both green tea extract and EGCG in a dose-dependent manner. There were no obvious changes in cell viability in serum-starved HepG2 cells exposed to 10% FBS in the presence of various doses of green tea extract or EGCG for 16 hours (Fig. 3D). These findings indicate that the decrease in HIF-1 α protein expression by green tea extract or EGCG was not due to cellular toxicity. Similar to above findings, HIF-1 α mRNA levels were not affected in serum-starved HepG2 cells after exposure to 10% FBS in the presence or absence of different concentrations of green tea extract or EGCG for 16 hours (Fig. 3C). These results indicated that green tea extract and EGCG inhibited serum-induced HIF-1 α protein accumulation through a translational and/or post-transcriptional mechanism. Likewise, studies on VEGF expression showed that treatment of HepG2 cells

with green tea extract or EGCG suppressed serum-induced VEGF expression at both mRNA and protein levels in a dose-dependent manner ($P < 0.01$; Fig. 3E and F and Fig. 3G).

Green Tea Extract and EGCG Inhibited Hypoxia-Mediated Activation of PI3K/Akt and ERK1/2 Signaling Pathways

Previous studies have shown that several major signal transduction pathways, including PI3K/Akt and ERK1/2 pathways, are involved in hypoxia-induced HIF-1 α protein accumulation and its downstream target gene expression (66). We also showed that exposure to hypoxia led to transient activation of both ERK1/2 and Akt in HeLa cells (Fig. 4A). To explore whether green tea extract and EGCG can inhibit hypoxia-mediated activation of Akt and ERK1/2 in HeLa cells, cultured cells were pretreated with various concentrations of green tea extract or EGCG under hypoxic condition, whereas cells pretreated with specific inhibitors of ERK1/2 (50 $\mu\text{mol/L}$ PD98059 or 50 $\mu\text{mol/L}$ U0126) or specific inhibitors of PI3K (50 $\mu\text{mol/L}$ LY294002 or 100 nmol/L wortmannin) served as positive controls. Our results showed that green tea extract and EGCG decreased hypoxia-stimulated phosphorylated ERK1/2 and Akt levels in a dose-dependent manner (Fig. 4B and C), which were consistent with their inhibitory effects on hypoxia-induced HIF-1 α protein accumulation (Fig. 1A and C) and VEGF expression (Fig. 2A and C). To further confirm whether the activated ERK1/2 and PI3K/Akt are involved in hypoxia-induced HIF-1 α protein accumulation and transactivation, HeLa cells were transiently transfected with pGL2-Luc-VEGF promoter. The transfected and

nontransfected HeLa cells were pretreated with different doses of LY294002 or PD98059 for 1 hour followed by exposure to normoxia or hypoxia for 16 hours. Our results showed that pretreatment of HeLa cells with LY294002 and PD98059 dramatically suppressed both hypoxia-induced HIF-1 α protein accumulation in a dose-dependent manner (Fig. 4D and E) and VEGF promoter activity (Fig. 4F; $P < 0.01$). Taken together, these results suggest that green tea extract and EGCG inhibited hypoxia-induced HIF-1 α protein accumulation and VEGF expression, at least in part, via blocking PI3K/Akt and ERK1/2 signaling pathways.

Green Tea Extract and EGCG Inhibited Hypoxia-Induced HIF-1 α Protein Expression by Promoting Its Degradation in HeLa Cells

It has been well established that hypoxia induces HIF-1 α protein accumulation mainly by promoting its stability rather than increasing its synthesis (66, 38–43). To explore

whether green tea extract and EGCG have any effects on hypoxia-induced stabilization of HIF-1 α protein, we exposed HeLa cells to hypoxia for 16 hours followed by treatment with cycloheximide to block ongoing protein synthesis in the presence or absence of green tea extract or EGCG for different periods. Our results showed that green tea extract or EGCG dramatically promoted the degradation of hypoxia-induced HIF-1 α protein (Fig. 5A, *middle* and *bottom*, and B) compared with that treated with cycloheximide alone (Fig. 5A, *top*, and B). These findings suggested that green tea extract and EGCG decreased hypoxia-induced HIF-1 α protein levels mainly by promoting its degradation. To find out whether the increased degradation of hypoxia-induced HIF-1 α protein caused by green tea extract and EGCG was through the 26S proteasome system, HeLa cells were incubated with various concentrations of green tea extract or EGCG in the presence or absence of 20 $\mu\text{mol/L}$ MG132, a specific and

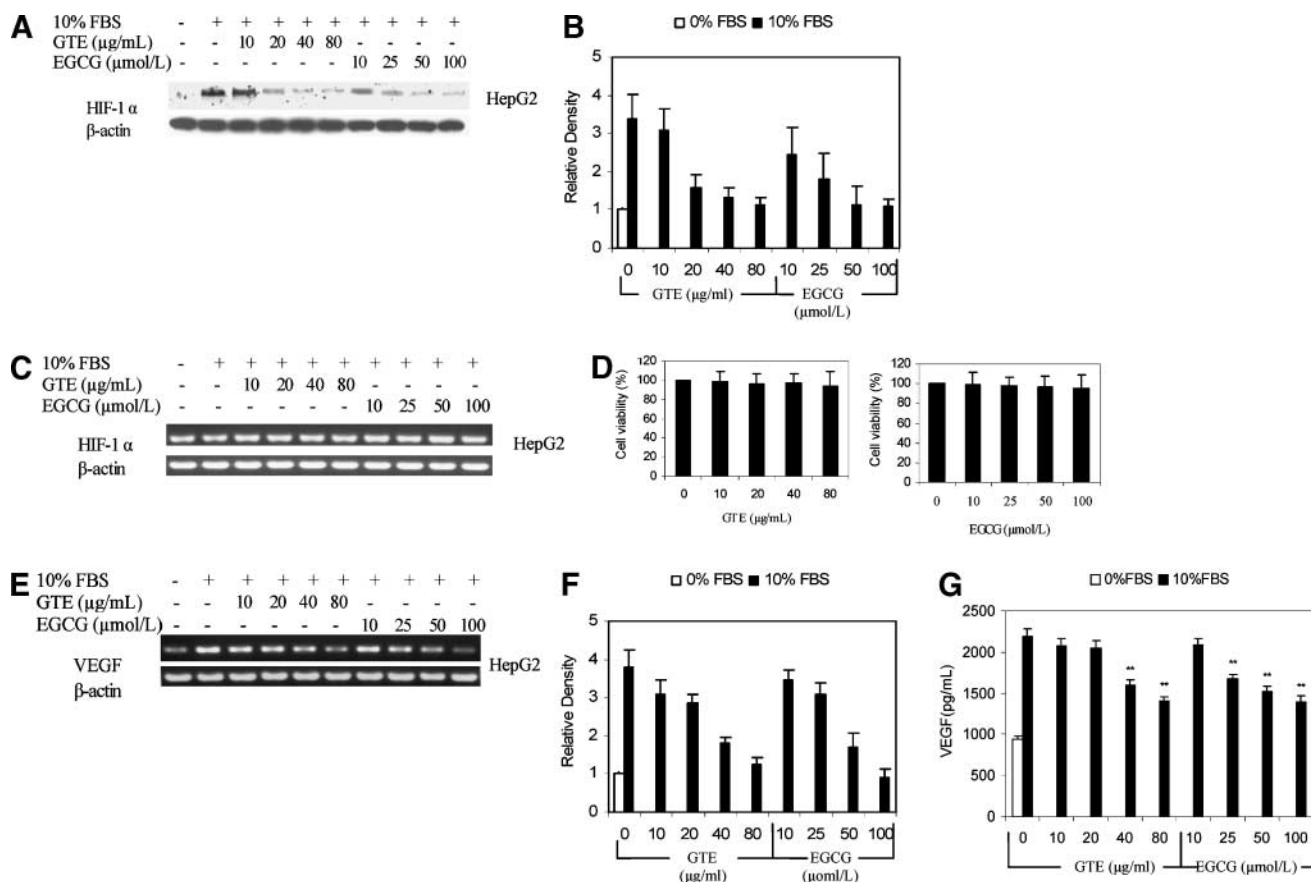


Figure 3. Effects of green tea extract and EGCG on serum-induced HIF-1 α protein and VEGF expression in HepG2 cells. **A–C**, HepG2 cells were serum starved for 24 h followed by pretreatment with various doses of green tea extract or EGCG for 1 h in serum-free medium. Afterward, the pretreated cells were cultured in complete medium containing 10% FBS in the presence of same doses of green tea extract or EGCG for 16 h under normoxia. HIF-1 α protein and mRNA levels were determined by Western blot analysis (**A**) and RT-PCR (**C**), respectively. **B**, densitometry analyses of **A**. The relative density ratio of HIF-1 α protein band to β -actin with nontreatment under normoxia was arbitrarily set as 1.0. **D**, HepG2 cells were serum starved for 24 h followed by incubation in complete medium containing 10% FBS in the presence of various doses of green tea extract or EGCG for 16 h under normoxia, and cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. **E–G**, following the same experimental procedures, VEGF mRNA levels and protein production in the conditioned medium were determined by RT-PCR (**E**) and ELISA (**G**), respectively. **F**, densitometry analyses of **E**. The relative density ratio of VEGF mRNA band to β -actin with nontreatment under normoxia was arbitrarily set as 1.0. *Columns*, mean of three replicate experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the control.

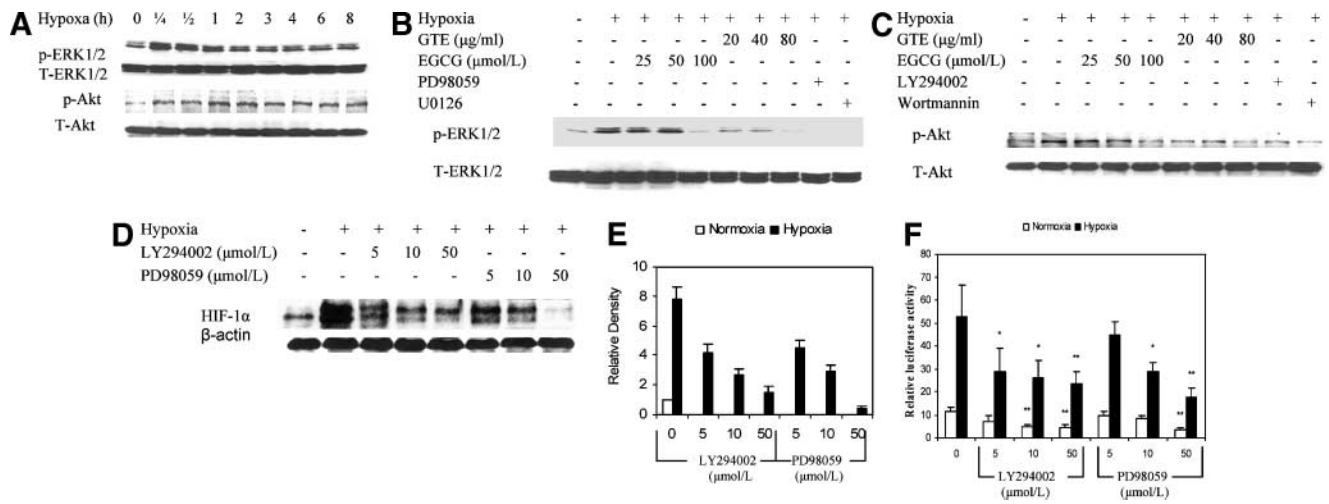


Figure 4. Green tea extract and EGCG inhibited hypoxia-induced activation of ERK1/2 and Akt in HeLa cells. **A**, time-course study on hypoxia-induced activation of ERK1/2 and Akt in HeLa cells. Following exposure to hypoxia for different periods, phosphorylated protein levels of ERK1/2 and Akt were analyzed by Western blot. **B** and **C**, HeLa cells were pretreated with different concentrations of green tea extract or EGCG for 1 h in normal conditions followed by exposure to hypoxia (1% O₂) for 1 h, and phosphorylated ERK1/2 (**B**) and Akt (**C**) levels were analyzed by Western blot. **D**, HeLa cells were pretreated with various concentrations of LY294002 or PD98059 for 1 h in normal culturing conditions followed by exposure to hypoxia (1% O₂) for 16 h. HIF-1α protein levels were analyzed by Western blot. **E**, densitometry analyses of **D**. The relative density ratio of HIF-1α protein band to β-actin with nontreatment under normoxia was arbitrarily set as 1.0. **F**, following transient transfection with the luciferase reporter plasmids (pGL2-Luc) harboring human VEGF promoter region (-1,175/+336), HeLa cells were pretreated with various concentrations of LY294002 or PD98059 for 1 h and then exposed to normoxia or hypoxia for 16 h. Luciferase activities were assayed using the Dual-Luciferase Assay kit. Representative of three independent experiments. Columns, mean of three replicate experiments; bars, SD.

potent 26S proteasome inhibitor, under both normoxic and hypoxic conditions. Our results showed that under normoxic conditions treatment with MG132 increased both the ubiquitinated fraction and the total HIF-1α protein levels. However, the increased HIF-1α protein level induced by MG132 could not be attenuated by green tea extract and EGCG (Fig. 5C, top). As expected, under hypoxic conditions, MG132 treatment further induced total

HIF-1α protein accumulation, whereas the inhibitory effects of green tea extract and EGCG on hypoxia-induced HIF-1α protein level were abolished in the presence of MG132 (Fig. 5C, bottom). Taken together, these results suggest that green tea extract and EGCG promoted the degradation of hypoxia-induced HIF-1α protein accumulation possibly via the proteasome degradation pathway in HeLa cells.

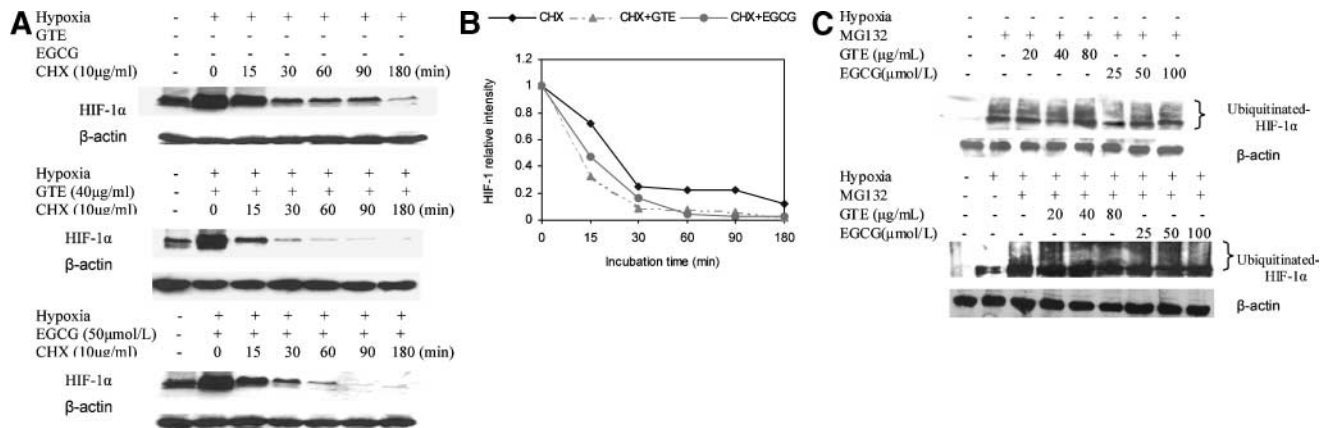


Figure 5. Effects of green tea extract or EGCG on the degradation of hypoxia-induced HIF-1α protein in HeLa cells. **A**, HeLa cells were exposed to hypoxia for 16 h followed by treatment with cycloheximide (CHX; 10 μg/mL) in the presence or absence of 40 μg/mL green tea extract or 50 μmol/L EGCG for different periods. HIF-1α protein levels were determined by Western blot analysis. **B**, quantitative densitometric analysis of results from **A**. **C**, HeLa cells were treated with 20 μmol/L MG132 for 30 min and cultured in the presence of different concentrations of green tea extract or EGCG for 6 h under normoxic (top) or hypoxic (bottom) conditions. Western blot was done to determine HIF-1α protein levels. Representative of three independent experiments.

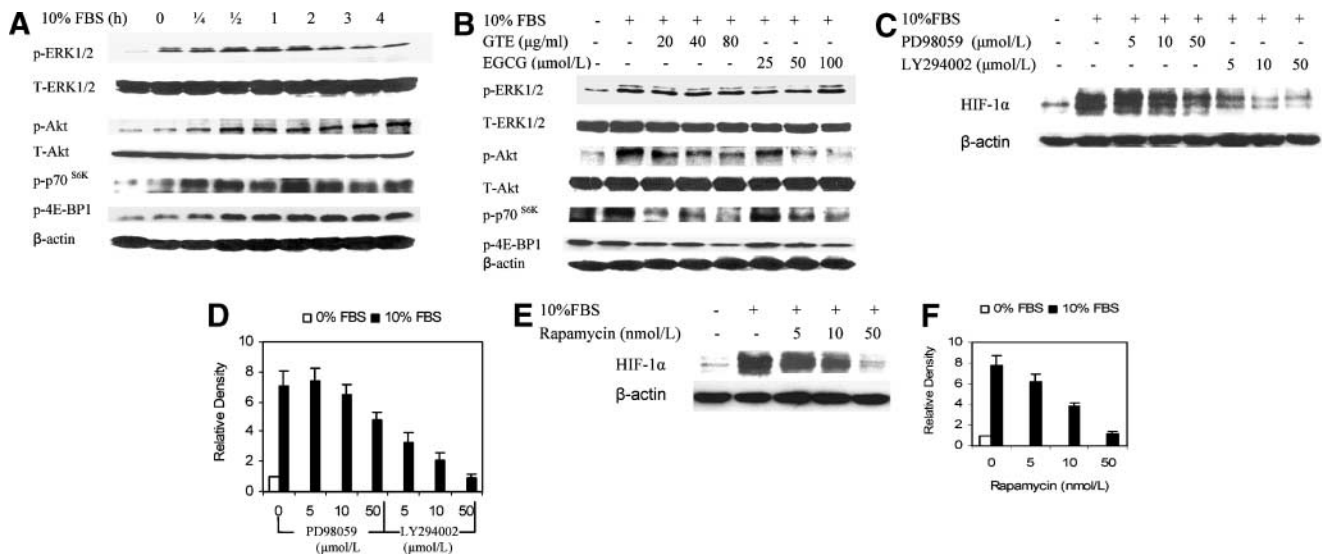


Figure 6. Effects of green tea extract and EGCG on serum-stimulated activation of ERK1/2 and Akt and phosphorylation of p70S6K and 4E-BP1 in HepG2 cells. **A**, after serum starvation for 24 h, HepG2 cells were cultured in complete medium for different periods, and phosphorylated protein levels of ERK1/2, Akt, p70S6K, and 4E-BP1 were analyzed by Western blot. **B**, after serum starvation for 24 h, HepG2 cells were pretreated with different concentrations of green tea extract or EGCG for 1 h in serum-free conditional medium followed by incubation in complete medium in the presence of the same doses of green tea extract or EGCG for 1 h. Phosphorylated ERK1/2, Akt, p70S6K, and 4E-BP1 levels were analyzed by Western blot. **C–F**, after serum starvation for 24 h, HepG2 cells were pretreated with various concentrations of PD98059, LY294002, or rapamycin for 1 h in serum-free conditional medium followed by incubation in complete medium for 16 h. HIF-1 α protein levels were analyzed by Western blot (**C** and **E**). **D** and **F**, densitometry analyses of **C** and **E**. The relative density ratio of HIF-1 α protein band to β -actin with nontreatment under normoxia was arbitrarily set as 1.0. Representative of three independent experiments.

Green Tea Extract and EGCG Inhibited Serum-Induced HIF-1 α Protein Expression by Interfering with PI3K/Akt/Mammalian Target of Rapamycin Signaling Pathway in HepG2 Cells

Previous studies have shown that growth factors and serum-stimulated activation of PI3K/Akt and ERK1/2 signaling pathways play an important role in their up-regulation of HIF-1 α protein accumulation (65, 67). In the present study, we also found serum greatly increased phosphorylated ERK1/2 and Akt levels in HepG2 cells (Fig. 6A). We next examined whether green tea extract and EGCG affected serum-induced activation of Akt and ERK1/2. The pretreatment of HepG2 cells with green tea extract or EGCG resulted in a dose-dependent decrease in the phosphorylated Akt levels but had no obvious inhibitory effect on ERK1/2 activation (Fig. 6B), which paralleled their similar inhibitory effects on serum-induced HIF-1 α protein expression in HepG2 cells (Fig. 3A). To further confirm our findings, we pretreated serum-starved HepG2 cells with various doses of LY294002 or PD98059 followed by exposure to 10% FBS for 16 hours. Our results showed that serum-induced HIF-1 α protein accumulation was greatly inhibited by LY294002 but only slightly inhibited by PD98059 (Fig. 6C and D). Collectively, these results suggested that the inhibitory effect of green tea extract and EGCG on serum- or growth factor-induced HIF-1 α protein expression was due to the inhibition of PI3K/Akt activation in HepG2 cells.

Previous studies have shown that serum- and growth factor-mediated activation of PI3K/Akt is essential to regulate HIF-1 α protein synthesis via phosphorylating protein translational regulators, including p70^{S6K1} and 4E-BP1 (52, 53, 67). Next, we investigated whether green tea extract and EGCG affected the protein translational machinery. Our results showed that serum obviously stimulated the phosphorylation of both p70^{S6K1} and 4E-BP1 in HepG2 cells (Fig. 6A). Treatment of cells with green tea extract or EGCG inhibited serum-induced phosphorylation of p70^{S6K1} and 4E-BP1 (Fig. 6B), paralleled to similar inhibitory effects on serum-induced HIF-1 α protein expression (Fig. 3A). Rapamycin is a specific inhibitor of mammalian target of rapamycin (mTOR), which has been reported to phosphorylate and activate p70^{S6K1} and 4E-BP1 (68, 69). To explore whether mTOR contributes to the pathways, we pretreated HepG2 cells with rapamycin and found it strikingly inhibited serum-induced HIF-1 α protein expression (Fig. 6E and F). Taken together, these results indicated that green tea extract and EGCG affected protein translational machinery, thus contributing to their inhibitory effects on serum-induced HIF-1 α protein expression.

Effects of Green Tea Extract and EGCG on the Migration of HeLa Cells

Recent studies have shown that hypoxia has stimulatory effects on cancer cell invasion and migration (55, 70). To investigate whether green tea extract and EGCG suppress hypoxia-induced cancer cell migration, an *in vitro* cell migration assay was done. As shown in Fig. 7, 10% FBS

obviously stimulated HeLa cell migration compared with the serum-free condition (Fig. 7B versus Fig. 7A). The chemoattractant migration of HeLa cells was significantly decreased in the presence of 80 $\mu\text{g}/\text{mL}$ green tea extract or 100 $\mu\text{mol}/\text{L}$ EGCG (Fig. 7C and D). Exposure to hypoxia for 48 hours potently promoted the 10% FBS-stimulated migration of HeLa cells (Fig. 7F versus Fig. 7B), and the hypoxia-induced migration of HeLa cells was drastically inhibited by treatment with 80 $\mu\text{g}/\text{mL}$ green tea extract or 100 $\mu\text{mol}/\text{L}$ of EGCG (Fig. 7G and H). These results indicated that both green tea extract and EGCG had inhibitory effects on chemoattractant- and hypoxia-stimulated migration of HeLa cells.

Discussion

Solid tumors cannot grow beyond 2 to 3 mm in diameter without the capability to recruit their own blood supply through new blood vessel formation (angiogenesis). Therefore, the growth and metastasis of tumors are strictly dependent on angiogenesis (32). As one of the principal mediators of tumor angiogenesis (64), VEGF can be potently stimulated by hypoxia via the major hypoxia-responsive transcription activator, HIF-1 α (45, 61, 66). Up to date, accumulating evidence indicates that HIF-1 α /VEGF can be a potential promising target for the antiangiogenic therapy of cancer.

Numerous experimental studies have shown that green tea extract and EGCG have antiangiogenic effects in several tumor angiogenic models (8–10), and the underlying mechanisms seemed to be associated with their inhibitory effects on VEGF production and VEGF receptor activity (5, 7, 33–37). Most recently, Basini et al. reported that treatment with EGCG decreased VEGF production

induced by mild (5% O₂) and severe (1% O₂) hypoxia in swine granulosa (36). However, the mechanism of green tea extract-mediated or EGCG-mediated reduction of VEGF expression has not been fully understood. In this study, we showed for the first time that green tea extract and EGCG significantly inhibited hypoxia- and serum-induced HIF-1 α protein accumulation (Figs. 1 and 3) and the corresponding VEGF expression at both mRNA and protein levels in HeLa and HepG2 cells (Figs. 2 and 3). Our results also indicated that green tea extract and EGCG function to suppress hypoxia-mediated increase in VEGF promoter activity (Fig. 2D), which harbors hypoxia-responsive elements and can be transactivated by HIF-1 α . These findings suggest that the suppression of hypoxia-induced VEGF expression by green tea extract and EGCG was due to, at least in part, their inhibitory effects on HIF-1 α transactivation of VEGF gene in HeLa cells. However, it is noteworthy that in a recent study Zhou et al. reported that (–)-epicatechin-3-gallate (1–100 $\mu\text{mol}/\text{L}$), another major green tea catechin, activated HIF-1 α and VEGF expression in T47D human breast carcinoma cells, whereas EGCG showed minor effect (62). Similarly, Thomas and Kim reported that treatment of prostate cancer cells with EGCG (20–40 $\mu\text{g}/\text{mL}$, 43.6–87.3 $\mu\text{mol}/\text{L}$) led to an increase in HIF-1 α protein level and HIF-1-mediated gene transcription even under normoxic conditions (63). These findings together with our present data suggest that the different effects of green tea extract and tea catechins on HIF-1 α expression and its downstream transcriptional activity are unique to the cancer cell type and probably the specific tumor targets. Of significant observation are the facts that both cancer cells used in this study, HeLa and HepG2, were initially derived from human cervical and hepatocellular cancers, respectively, with possibility of a viral-associated carcinogenesis. We are in the process to screen other infection-associated cancer cells to delineate the potential chemopreventive and antitumor effect of green tea extract and catechins and their underlying mechanisms.

In the present study, we also found that neither hypoxia nor serum stimulation had any obvious effects on HIF-1 α mRNA expression, and treatment with green tea extract or EGCG did not affect HIF-1 α mRNA levels in both HeLa and HepG2 cells (Fig. 1E and Fig. 3C), suggesting that green tea extract and EGCG inhibited HIF-1 α protein expression through a post-transcriptional mechanism (i.e., by affecting HIF-1 α protein synthesis and/or degradation). Under hypoxic conditions, HIF-1 α protein stabilizes and accumulates dramatically due to an inhibitory effect on its own degradation (41–43). Our results showed that green tea extract and EGCG significantly promoted the degradation of hypoxia-induced HIF-1 α protein accumulation in HeLa cells (Fig. 5A and B), and their inhibitory effects on hypoxia-induced HIF-1 α protein expression were abolished in the presence of MG132 (Fig. 5C). These results suggest that green tea extract and EGCG suppressed hypoxia-induced HIF-1 α protein accumulation in HeLa cells mainly by promoting its degradation via the 26S proteasome

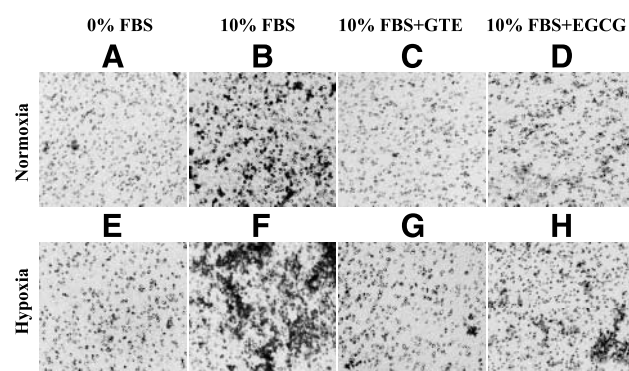


Figure 7. Effects of green tea extract and EGCG on the migration of HeLa cells. Cancer cells (5×10^4 per well) were seeded in 300 μL serum-free medium on the interior side of inner chambers containing a polycarbonate membrane of 8- μm pore size and a thin layer of rehydrated collagen. Medium (500 μL) with or without 10% FBS was added to the lower chamber. Then, the cells were cultured at 37°C for 48 h under normoxia (A–D) or hypoxia (E–H) in the absence or presence of 80 $\mu\text{g}/\text{mL}$ green tea extract (C and G) or 100 $\mu\text{mol}/\text{L}$ EGCG (D and H). The cells that migrated from the upper to the lower surface of the membrane were stained and photographed using a computer imaging system. Representative of three independent experiments.

system. On the contrary, Thomas and Kim recently reported that EGCG inhibits prolyl hydroxylation of HIF-1 α protein in prostate cancer cells by preventing its degradation (63). The apparent contradictory effect of EGCG on HIF-1 α protein stability remains to be elucidated. Further studies are in progress to clarify why green tea extract and EGCG exert different effects on HIF-1 α stabilization in different cancer types.

Studies to date have shown that multiple signaling pathways are involved in the regulation of hypoxia-induced HIF-1 α protein stabilization and transactivation (66). To further define the signaling mechanisms by which green tea extract and EGCG inhibited hypoxia-induced HIF-1 α protein accumulation, we next examined the effects of green tea extract and EGCG on the activation of PI3K/Akt and ERK1/2 pathways in HeLa cells in response to hypoxia. Our findings indicated that both green tea extract and EGCG significantly inhibited hypoxia-mediated activation of ERK1/2 and Akt in HeLa cells (Fig. 4B and C), which was consistent with their inhibitory effects on hypoxia-induced HIF-1 α protein accumulation and VEGF expression (Fig. 1A and C and Fig. 2A and C). Treatment of HeLa cells with LY294002 or PD98059 led to a dose-dependent inhibition of hypoxia-induced HIF-1 α protein accumulation and VEGF promoter activity (Fig. 4D and F). Collectively, these findings suggested that green tea extract and EGCG inhibited hypoxia-induced HIF-1 α protein accumulation via blocking the activation of PI3K/Akt and ERK1/2 signaling pathways.

An increasing body of evidence indicates that induction of HIF-1 α protein expression by the activation of certain oncogenes and growth factors is due to an increase in HIF-1 α protein synthesis, whereas the PI3K/Akt/mTOR signaling pathway plays a critical role (48–53). We next explored the effect of green tea extract and EGCG on HIF-1 α protein translational machinery. It is well known that eukaryotic initiation factors and p70^{S6K1} play a crucial role in the regulation of the protein translation. The full activation of p70^{S6K1}, a downstream target of PI3K, depends on mTOR activity. Eukaryotic initiation factors phosphorylate the S6 ribosomal protein of the 40S subunit of the ribosome and stimulate the translation of mRNAs with a 5'-oligopyrimidine tract (68, 69). On the other hand, eukaryotic initiation factor 4E is the key enzyme for cap-dependent initiation of protein translation, whose functions are mainly regulated by 4E-BP1 that can also be phosphorylated by mTOR and other unidentified kinases (68). Recent studies showed that expression of eukaryotic initiation factor 4E was sufficient to elevate HIF-1 α protein levels under normoxia (47). Herein, we found that treatment with green tea extract or EGCG led to a dose-dependent decrease in serum-induced phosphorylation of Akt, p70^{S6K1}, and 4E-BP1 in HepG2 cells but had no inhibitory effect on ERK1/2 activation (Fig. 6B). Consistently, treatment of HepG2 cells with LY294002 or rapamycin, a specific inhibitor of mTOR, drastically inhibited serum-induced HIF-1 α protein accumulation, but only a slight inhibitory effect was observed after treatment with

PD98059 (Fig. 6C and E). Taken together, our findings suggested that green tea extract and EGCG inhibited serum- or growth factor-induced HIF-1 α protein synthesis via interfering with PI3K/Akt/mTOR signaling pathway and translational machinery.

HIF-1 α overexpression, as a result of either intratumoral hypoxia or genetic alterations, has been found in several human cancer biopsies at the invading tumor margins (58–60). Previous studies have shown that green tea extract and EGCG have potential inhibitory effects on cancer cell migration and invasion in several tumor models (4, 12–15). In the present study, we have shown that green tea extract and EGCG significantly inhibited chemoattractant- and hypoxia-simulated migration of HeLa cells (Fig. 7), which could be attributed to its potent inhibitory effects on hypoxia- and serum-induced HIF-1 α protein accumulation in cancer cells. Additional studies are under way to identify the associated genes that are directly or indirectly involved in green tea extract- or EGCG-regulated cancer cell migration in response to hypoxia and/or HIF-1 α overexpression.

In summary, in this study, we have shown, to our knowledge for the first time, that green tea extract and EGCG inhibited hypoxia- and serum-induced HIF-1 α protein accumulation and VEGF expression in HeLa and HepG2 cells via promoting HIF-1 α protein degradation and/or interfering with protein translational machinery and thus provided a novel mechanism for the antiangiogenic action of green tea extract and EGCG.

References

- Horie N, Hirabayashi N, Takahashi Y, et al. Synergistic effect of green tea catechins on cell growth and apoptosis induction in gastric carcinoma cells. *Biol Pharm Bull* 2005;28:574–9.
- Lambert JD, Yang CS. Mechanisms of cancer prevention by tea constituents. *J Nutr* 2003;133:3262–7S.
- Nihal M, Ahmad N, Mukhtar H, et al. Anti-proliferative and proapoptotic effects of (–)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. *Int J Cancer* 2005;114:513–21.
- Baliga MS, Meleth S, Katiyar SK. Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems. *Clin Cancer Res* 2005;11:1918–27.
- Kondo T, Ohta T, Igura K, et al. Tea catechins inhibit angiogenesis *in vitro*, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Lett* 2002;180:139–44.
- Tang FY, Nguyen N, Meydani M. Green tea catechins inhibit VEGF-induced angiogenesis *in vitro* through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule. *Int J Cancer* 2003;106:871–8.
- Kojima-Yuasa A, Hua JJ, Kneddy DO, et al. Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. *Life Sci* 2003;73:1299–313.
- Fassina G, Vene R, Morini M, et al. Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. *Clin Cancer Res* 2004;10:4865–73.
- Liao J, Yang GY, Park ES, et al. Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea. *Nutr Cancer* 2004;48:44–53.
- Adhami VM, Siddiqui IA, Ahmad N, et al. Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. *Cancer Res* 2004;64:8715–22.

11. Yamakawa S, Asai T, Uchida T, et al. (-)-Epigallocatechin gallate inhibits membrane-type 1 matrix metalloproteinase, MT1-MMP, and tumor angiogenesis. *Cancer Lett* 2004;210:47–55.
12. Jung YD, Ellis LM. Inhibition of tumor invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. *Int J Exp Pathol* 2001;82:309–16.
13. Yang J, Wei D, Liu J. Repressions of MMP-9 expression and NF- κ B localization are involved in inhibition of lung carcinoma 95-D cell invasion by (-)-epigallocatechin-3-gallate. *Biomed Pharmacother* 2005;59:98–103.
14. Slivova V, Zaloga G, Demichele SJ, et al. Green tea polyphenols modulate secretion of urokinase plasminogen activator (uPA) and inhibit invasive behavior of breast cancer cells. *Nutr Cancer* 2005;52:66–73.
15. Lu QY, Jin YS, Pantuck A, et al. Green tea extract modulates actin remodeling via Rho activity in an *in vitro* multistep carcinogenic model. *Clin Cancer Res* 2005;11:1675–83.
16. Qanungo S, Das M, Haldar S, et al. Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Carcinogenesis* 2005;26:958–67.
17. Roy AM, Baliga MS, Katiyar SK. Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation. *Mol Cancer Ther* 2005;4:81–90.
18. Liang YC, Lin-Shiau SH, Chen CF, et al. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. *J Cell Biochem* 1999;75:1–12.
19. Ahmad N, Cheng P, Mukhtar H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem Biophys Res Commun* 2000;275:328–34.
20. Yang CS. Inhibition of carcinogenesis by tea. *Nature* 1997;389:134–5.
21. Sato D. Inhibition of urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine in rats by green tea. *Int J Urol* 1999;6:93–9.
22. Shimizu M, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. *Mutat Res*. In press 2005.
23. Sah JF, Balasubramanian S, Eckert RL, et al. Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases. *J Biol Chem* 2004;279:12755–62.
24. Shimizu M, Deguchi A, Lim JT, et al. (-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin Cancer Res* 2005;11:2735–46.
25. Shimizu M, Deguchi A, Joe A, et al. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. *J Exp Ther Oncol* 2005;5:69–78.
26. Masuda M, Suzui M, Weinstein IB. Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin Cancer Res* 2001;7:4220–9.
27. Masuda M, Suzui M, Lim JT, et al. Epigallocatechin-3-gallate inhibits activation of HER-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. *Clin Cancer Res* 2003;9:3486–91.
28. Pianetti S, Guo SQ, Kavanagh KT, et al. Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/Neu signaling, proliferation, and transformed phenotype of breast cancer cells. *Cancer Res* 2002;62:652–5.
29. Liang YC, Lin-shiau SY, Chen CF, et al. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J Cell Biochem* 1997;67:55–65.
30. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol* 2002;42:25–54.
31. Shimizu M, Deguchi A, Hara Y, et al. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. *Biochem Biophys Res Commun* 2005;334:947–53.
32. Folkman J. Fighting cancer by attacking its blood supply. *Sci Am* 1996;275:150–4.
33. Jung YD, Kim MS, Shin BA, et al. EGCG, a major component of green tea, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. *Br J Cancer* 2001;84:844–50.
34. Masuda M, Suzui M, Lim JT, et al. Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. *J Exp Ther Oncol* 2002;2:350–9.
35. Sartippour MR, Shao ZM, Heber D, et al. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002;132:2307–11.
36. Basini G, Bianco F, Grasselli F. EGCG, a major component of green tea, inhibits VEGF production by swine granulosa cells. *Biofactors* 2005;23:25–33.
37. Lamy S, Gingras D, Beliveau R. Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res* 2002;62:381–5.
38. Wang GL, Jiang BH, Rue EA, et al. Hypoxia inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* 1995;92:5510–4.
39. Masson N, Willam C, Maxwell PH, et al. Independent function of two destruction domain in hypoxia-inducible factor- α chains activated by prolyl hydroxylation. *EMBO J* 2001;20:5197–206.
40. Ivan M, Kondo K, Yang H, et al. HIF- α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 2001;292:464–8.
41. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 2001;292:468–72.
42. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulates HIF by prolyl hydroxylation. *Cell* 2001;107:43–54.
43. Huang LE, Gu J, Schau M, Buun HF. Regulation of hypoxia-inducible factor-1 α is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 1998;95:7987–92.
44. Kamura T, Sato S, Iwai K, et al. Activation of HIF-1 α ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci U S A* 2000;97:10430–5.
45. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor-1. *Mol Cell Biol* 1996;16:4604–13.
46. Zundel W, Schindler C, Haas-Kogan D, et al. Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev* 2000;14:391–6.
47. Karni R, Dor Y, Keshet E, et al. Activated pp60^{c-Src} leads to elevated hypoxia-inducible factor (HIF)-1 α expression under normoxia. *J Biol Chem* 2002;277:42919–25.
48. Stiehl DP, Jelkmann W, Wenger RH, et al. Normoxia induction of the hypoxia-inducible factor 1 α by insulin and interleukin-1 β involves the phosphatidylinositol 3-kinase pathway. *FEBS Lett* 2002;512:157–62.
49. Sandau KB, Zhou J, Kietzmann T, et al. Regulation of the hypoxia-inducible factor 1 α by the inflammatory mediators nitric oxide and tumor necrosis factor- α in contrast to desferrioxamine and phenylarsine oxide. *J Biol Chem* 2001;276:39805–11.
50. Hellwig-Burgel T, Rutkowski K, Metzen E, et al. Interleukin-1 β and tumor necrosis factor- α stimulate DNA binding of hypoxia-inducible factor-1. *Blood* 1999;94:1561–7.
51. Xu Q, Briggs J, Park S, et al. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene* 2005;24:5552–60.
52. Fukuda R, Hirota K, Fan F, et al. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J Biol Chem* 2002;277:38205–11.
53. Treins C, Giorgetti-Peraldi S, Murdaca J, et al. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem* 2002;277:27975–81.
54. Carmeliet P, Dor Y, Herbert JM, et al. Role of HIF-1 α or in hypoxia-mediated apoptosis, cell proliferation and tumor angiogenesis. *Nature* 1998;394:485–90.
55. Krishnamachary B, Berg-Dixon S, Kelly B, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003;63:1138–43.

56. Piret J, Lecocq C, Toffoli S, et al. Hypoxia and CoCl₂ protect HepG2 cells against serum starvation- and t-BHP-induced apoptosis: a possible anti-apoptotic role for HIF-1. *Exp Cell Res* 2004;295:340–9.
57. Zhang QZ, Zhang ZF, Rao JY, et al. Treatment with siRNA and antisense oligonucleotides targeted to HIF-1 α induced apoptosis in human tongue squamous cell carcinomas. *Int J Cancer* 2004;111:849–57.
58. Birner P, Schindi M, Obermair A, et al. Overexpression of hypoxia-inducible factor 1 α is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res* 2000;60:4693–6.
59. Zagzag D, Zhong H, Scazitti JM, et al. Expression of hypoxia-inducible factor 1 α in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* 2000;88:2606–18.
60. Jiang BH, Agani F, Passaniti A, et al. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res* 1997;57:5328–35.
61. Powis G, Kirkpatrick L. Hypoxia inducible factor-1 α as a cancer drug target. *Mol Cancer Ther* 2004;3:647–54.
62. Zhou YD, Kim YP, Li XC, et al. Hypoxia-inducible factor-1 activation by (–)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts. *J Nat Prod* 2004;67:2063–9.
63. Thomas R, Kim MH. Epigallocatechin gallate inhibits HIF-1 α degradation in prostate cancer cells. *Biochem Biophys Res Commun* 2005;334:543–8.
64. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005;23:1011–27.
65. Haddad JJ, Harb HL. Cytokines and the regulation of hypoxia-inducible factor (HIF)-1 α . *Int Immunopharmacol* 2005;5:461–83.
66. Semenza GL. Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol* 2002;64:993–8.
67. Cao Z, Fang J, Xia C, et al. *trans*-3,4,5'-Trihydroxystibene inhibits hypoxia-inducible factor 1 α and vascular endothelial growth factor expression in human ovarian cancer cells. *Clin Cancer Res* 2004;10:5253–63.
68. Gingras A, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev* 2001;15:807–26.
69. Berven LA, Crouch MF. Cellular function of p70S6K: a role in regulating cell motility. *Immunol Cell Biol* 2000;78:447–51.
70. Zhang QZ, Tang XD, Lu QY, et al. Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1 α and VEGF expression in human tongue squamous cell carcinoma and hepatoma cells. *Mol Cancer Ther* 2005;4:1465–74.