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Original Paper

Epigallocatechin-3-Gallate Inhibits IGF-I-Stimulated Lung Cancer Angiogenesis through Downregulation of HIF-1α and VEGF Expression

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Key Words
Angiogenesis · Epigallocatechin-3-gallate · HIF-1α · VEGF · Lung cancer

Abstract
Background/Aims: Numerous studies have shown that epigallocatechin-3-gallate (EGCG), a polyphenol component extracted from green tea, can inhibit the growth and induce apoptosis of various types of human tumor cells. In this study, we evaluated the inhibitory effects of EGCG on the proangiogenic capabilities of A549 cells. ***Methods:*** A549 cells starved in serum-free culture medium for 24 h were pretreated with EGCG at various concentrations (0, 10, 25, 50, and 100 μmol/l) for 1 h, followed by the addition of insulin-like growth factor-I (IGF-I) at the final concentration of 40 ng/ml and continued culturing for an additional 16 h. The in vitro angiogenesis analyzing test kit with ECMatrix™ gel was used to detect the formation of capillary tube-like structures. The mRNA expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) was determined by real-time PCR. The protein expression of HIF-1α and VEGF was detected by Western blotting and ELISA, respectively. ***Results:*** EGCG significantly inhibited the formation of capillary tube-like structures on the surface of ECMatrix induced by IGF-I both in vitro and in vivo and reduced the level of hemoglobin in Matrigel plugs. In addition, EGCG was shown to significantly inhibit the IGF-I-induced upregulation of HIF-1α protein expression. Meanwhile, EGCG at the concentration of 25 and 100 μmol/l exhibited obvious inhibitory effects on IGF-I-induced VEGF expression ($p < 0.01$). ***Conclusion:*** Our results suggest that EGCG has potent inhibitory effects on tumor angiogenesis induced by IGF-I in human non-small cell lung cancer cells, which may possibly contribute to the downregulation of HIF-1α and VEGF expression.

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Introduction

In the process of tumor formation and progression, angiogenesis plays a critical role in the growth and invasion of most solid tumors and serves as an important factor to determine the fate and treatment outcome of a tumor-like regression, metastasis or recurrence. Therefore, one of the hot topics in the research of cancer therapies is how to effectively curb or inhibit angiogenesis or neovascularization in tumors. The hypoxia-inducible factor-1α (HIF-1α) and one of its immediate downstream target genes, the vascular endothelial growth factor (VEGF), are essential for promoting the complex process of angiogenesis in tumor tissues. Green tea is one of the most commonly consumed beverages, and its water-soluble extracts contain multiple polyphenolic compounds, with epigallocatechin-3-gallate (EGCG) as the most abundant and active monomer [1–3]. It has been observed that EGCG has multiple biological and pharmacological effects, such as free radical scavenging and antagonistic activities against oxidation, inflammation, virus infection, gene mutation, and tumor angiogenesis. Additionally, EGCG also exhibits potent inhibitory effects on growth [4–9] and angiogenesis in a variety of tumors [10–12], but the underlying mechanisms of the anti-angiogenic activity of EGCG are still not completely understood. The present study was designated to observe both the in vitro and in vivo effects of EGCG on the angiogenic capabilities of human non-small cell lung cancer (NSCLC) cells (A549) in response to insulin-like growth factor-I (IGF-I) stimulation and the possible molecular mechanisms.

Materials and Methods

Materials

EGCG (a monomer with purity at 98%), diethyl pyrocarbonate, acrylamide, and N,N'-methylenebisacrylamide were from Sigma Co. Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials Co. (Hangzhou, China). Pepsin and TRIzol were purchased from Invitrogen Co. (USA). The real-time fluorescent quantitative PCR kit was obtained from TaKaRa Biological Reagent Co. The monoclonal anti-human HIF-1α antibody raised in mouse, the multiclonal anti-human β-actin antibodies raised in goat, and the ECL chemiluminescent kit were all purchased from Santa Cruz Biological Technology Co. The VEGF ELISA kit was obtained from Boster Bio-Engineering Co. The primers were synthesized by TaKaRa Biological Reagent Co.

Cell Line and Cell Culture

The human NSCLC (A549) cell line was purchased from the American Type Culture Collection. A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of EGCG and Treatment of the Cells

EGCG was dissolved in distilled water at the concentration of 100 mmol/l as the stock solution, which was aliquoted and stored at –70°C for further use. The normally maintained A549 cell cultures were changed with FBS-free medium for 24 h, followed by pretreatment with EGCG at the final concentration of 10, 25, 50, and 100 μmol/l for 1 h, and then stimulated by IGF-I at the concentration of 40 ng/ml and continued culturing for another 16 h.

In vitro Angiogenesis Assay

The 96-well cell culture plates were coated with ECMatrix™ and then inoculated with human umbilical venous endothelial cells (HUVEC) at the density of 5×10^3 cells/well. Subsequently, the conditioned media, derived from A549 cells under various treatments, were added into each HUVEC-containing well. The formation of capillary tube-like structures in each group was observed by the phase contrast microscopy and photographed. Six fields were randomly chosen from each well. The Scion Image software was used for the analysis of the total length of the capillary tube-like structures. The experiments were repeated 3 times.

In vivo Angiogenesis Assay

The A549 cells pretreated with IGF-I (40 μg/ml) and EGCG (100 μmol/l) were diluted with FBS-free medium at the density of 3×10^6 cells/ml. Then, each 0.2-ml cell dilution was mixed with 0.4 ml of BD Matrigel Matrix, which was finally injected subcutaneously into both flanks of a 6- to 8-week-old nude mouse. On day 11, the nude mice were sacrificed and the subcutaneous Matrigel plugs were isolated. After the removal of the residual blood and tissues from the Matrigel plugs, the angiogenic conditions were observed and photographed. Additionally, part of the Matrigel plugs were weighed and used to determine the hemoglobin content as described previously [13]. Briefly, Matrigel plugs were digested in 0.1% Triton X-100 and processed for hemoglobin content at 405-nm absorbance using a standard curve of purified hemoglobin (Sigma).

Immunohistochemistry

The subcutaneous Matrigel plugs were isolated from the nude mice, and part of the Matrigel plugs was fixed with 10% formalin and embedded in paraffin. Then, serial 5-μm sections were cut. Afterwards, they were processed for immunohistochemical studies on the expression of human HIF-1α and VEGF using a mouse anti-human HIF-1α monoclonal antibody (BD Transduction Laboratories) or a rabbit anti-human VEGF polyclonal antibody (Bioss Inc.) according to the standard immunohistochemical procedures.

Detection of HIF-1α and VEGF mRNA Expressions by Real-Time PCR

The total RNA of the cells in each group was extracted using Trizol. The expression of HIF-1α and VEGF mRNA in the cells in each treatment was determined by real-time PCR. The primers for HIF-1α, VEGF, and β-actin were designed using primer synthesis software Premier 5 and Oligo 6. The primer sequences for real-time PCR were as follows: for HIF-1α, forward 5′-TCT GGG TTG AAA CTC AAG CAA CTG-3′ and reverse 5′-CAA CCG GTT TAA GGA CAC ATT CTG-3′; for VEGF, forward 5′-TGC TTC TGA GTT GCC CAG GA-3′ and reverse 5′-TGG TTT CAA TGG TGT GAG GAC ATA G-3′; for β-actin, forward 5′-TGG CAC CCA GCA CAA TGA A-3′ and reverse 5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′. The thermocycling conditions for real-time PCR were as follows: stage 1 for the reverse transcription (42°C for 5 s), stage 2 for predenaturing (95°C for 10 s), and stage 3 for the PCR process (95°C for 5 s and 60°C for 31 s; running for 40 cycles).

Detection of HIF-1α Protein Expression by Western Blot Analysis

A cell lysing solution was added to the cells in each treatment. The proteins were then extracted and separated by 10% SDS-PAGE and afterwards electroblotted onto nitrocellulose (PVDF) membranes. After blocking with 5% fat-free milk, the membrane was incubated at 4°C overnight with a primary monoclonal antibody against HIF-1α (with 1:1,000 as the working concentration), followed by incubation with horseradish peroxidase-labeled secondary antibodies (diluted at 1:1,000) at room temperature for 1 h. The fluorescent signals were detected using ECL chemiluminescence reagent and exposed to an X-ray film.

Detection of VEGF Protein Concentrations by ELISA

The culture media were collected from cells in the presence or absence of EGCG, followed by the detection of the VEGF protein content by ELISA according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as means ± SD. SPSS11.0 statistical software was used to analyze the data, and one-way ANOVA was performed for comparison between data of multiple groups. The experiments were repeated 3–5 times. $p < 0.05$ was defined as the statistical significance.

Results

Effects of EGCG on the in vitro Angiogenic Activity of NSCLC Cells Stimulated by IGF-I

The in vitro angiogenesis experiments indicated that the conditioned medium derived from IGF-I-treated A549 cells significantly promoted the formation of capillary tube-like structures by HUVECs on the surface of ECMatrix (fig. 1a). However, the formation of capillary tube-like structures stimulated by IGF-I was significantly abrogated by 25–100 μmol/l of

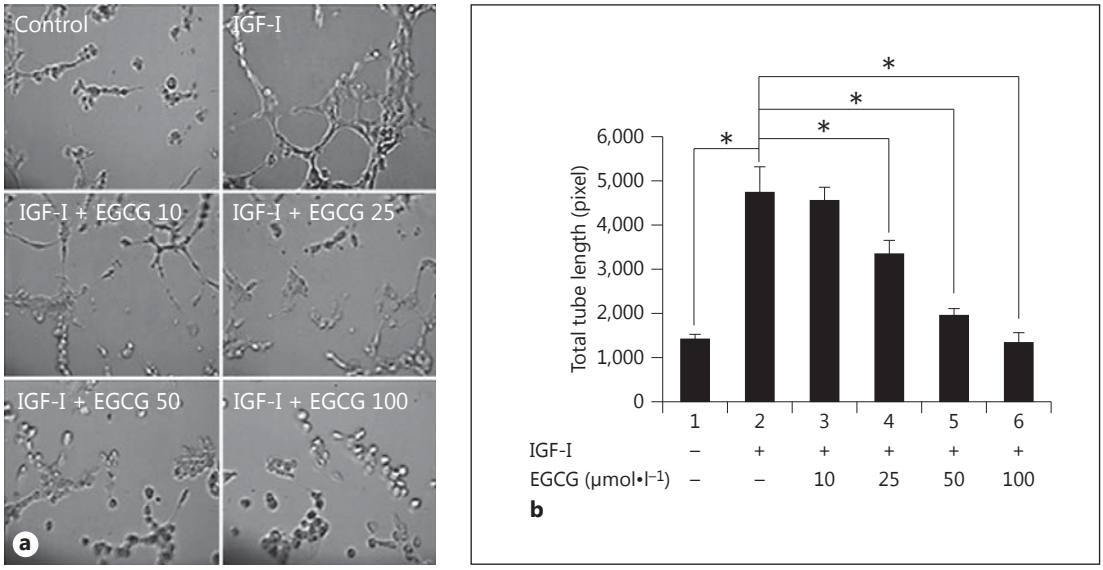


Fig. 1. Effects of EGCG on the in vitro formation of capillary tube-like structures by HUVECs induced by A549 cells in response to IGF-I. HUVECs (5×10^3 cells/well) were seeded onto the surface of 96-well culture plates precoated with polymerized ECMatrix and then incubated at 37°C for 6–8 h in the conditioned media derived from IGF-I-treated A549 cells in the presence or absence of EGCG. **a** Tube formation was observed under a phase-contrast microscope (10×). **b** Quantification of capillary tube formation. The averaged values of the formed branch points were calculated by analyzing the total tube length in 6 random view fields per well. All data presented are representative of 3 separate experiments. * $p < 0.01$.

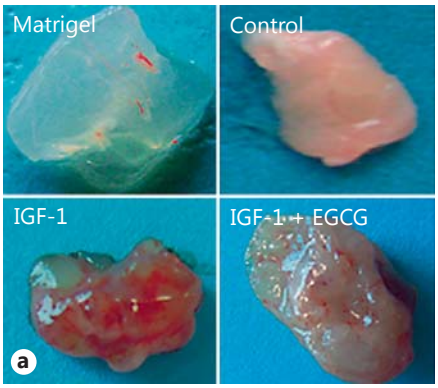
EGCG (fig. 1a). Further analysis of the total length of the formed capillary tubes by Scion Image software indicated that the pretreatment of EGCG resulted in a concentration-dependent inhibition of the angiogenic activity of A549 cells stimulated by IGF-I ($p < 0.01$; fig. 1b). These results suggest that IGF-I can promote the angiogenic capabilities of NSCLC cells in vitro, which could be inhibited by EGCG.

Effects of EGCG on the in vivo Lung Cancer Angiogenesis Stimulated by IGF-I

The in vivo angiogenesis assay indicated that Matrigel plugs mixed with the control-conditioned medium derived from untreated A549 cells did not induce angiogenesis (fig. 2a), which was indicated by a low or undetectable hemoglobin content (fig. 2b). However, stimulation by IGF-I greatly promoted the formation of apparent capillary tube structures in the transplanted tumor Matrigel plugs in nude mice (fig. 2a), which was reflected by a significant increase in hemoglobin content in the Matrigel plugs as compared with controls ($p < 0.01$; fig. 2b). In addition, our results also indicated that the treatment with EGCG (100 μmol/l) obviously reduced the number of capillary tube structures on the Matrigel plugs (fig. 2a) and dramatically decreased the hemoglobin levels ($p < 0.01$; fig. 2b). In consistency with our in vitro findings, these results suggest that IGF-I can promote A549 cell-induced tumor angiogenesis in vivo, which can also be significantly inhibited by EGCG.

Effects of EGCG on the IGF-I-Stimulated HIF-1α Protein Expression in A549 Cells

To further explore the potential mechanisms whereby EGCG inhibits IGF-I-induced tumor angiogenesis in lung cancer cells, the expression of HIF-1α induced by IGF-I in A549 cells in the presence or absence of EGCG was examined. As shown in figure 3, the stimulation of A549



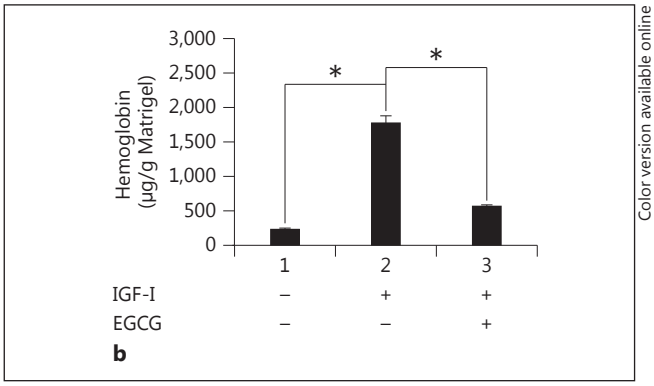
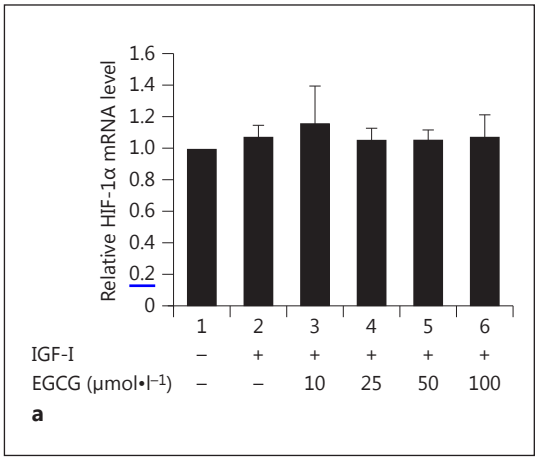
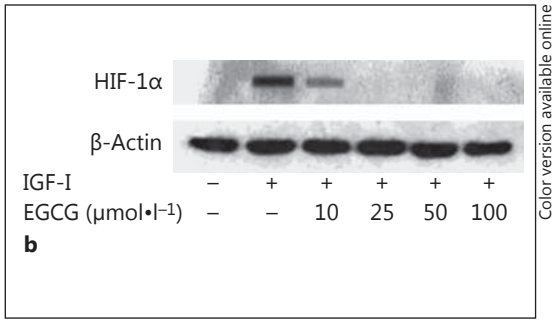


Fig. 2. EGCG inhibited in vivo lung cancer angiogenesis stimulated by IGF-I. A549 cells (0.2 ml, 6×10^5 cells/well) were mixed with 0.4 ml of BD Matrigel Matrix in the presence or absence of EGCG (100 μmol/l) and/or IGF-I (40 ng/ml) and injected subcutaneously into both flanks of nude mice (n = 5). Matrigel mixed with the serum-free medium alone was used as negative control. On day 11, mice were sacrificed and the Matrigel plugs were removed and photographed. **a** Representative Matrigel plugs. **b** Hemoglobin levels in Matrigel plugs: the hemoglobin content of Matrigel plugs is expressed. The results are representative of 5 independent experiments. * $p < 0.01$.





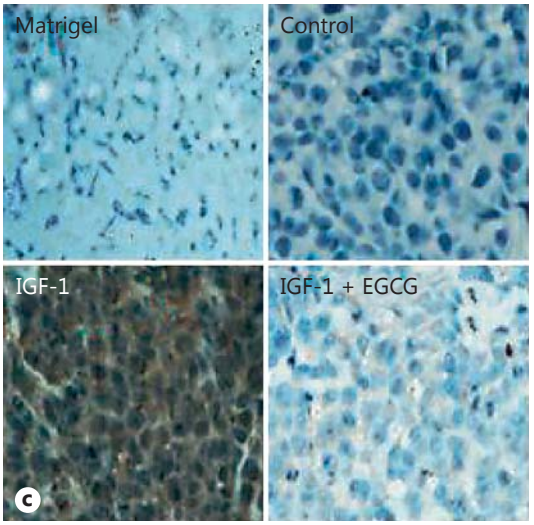
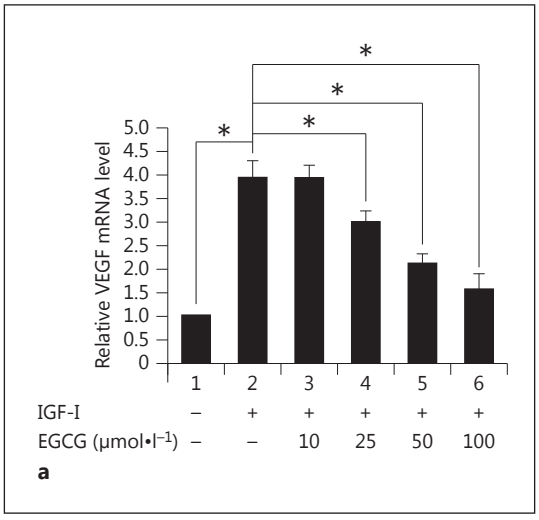
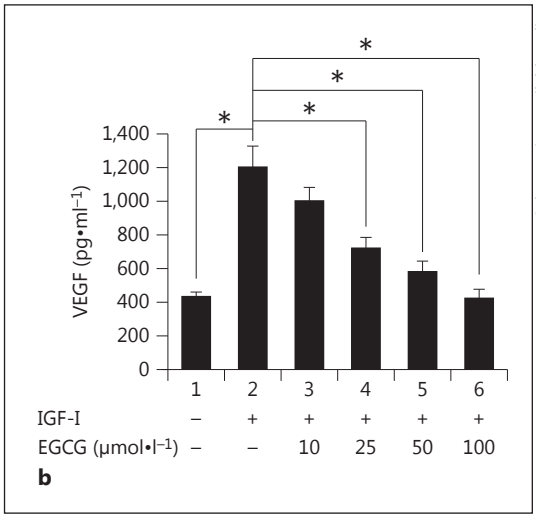


Fig. 3. EGCG inhibited IGF-I-induced HIF-1α expression in A549 cells. Serum-starved A549 cells were pretreated for 1 h with different concentrations of EGCG, followed by incubation with IGF-I (40 ng/ml) for 16 h. **a** HIF-1α mRNA levels were determined by real-time PCR. **b** HIF-1α protein levels were determined by Western blot analysis. **c** Immunohistochemical staining for HIF-1α protein expression in Matrigel plugs. Tumor sections were cut and processed for immunohistochemical staining using antibody against human HIF-1α as described in the Materials and Methods section.





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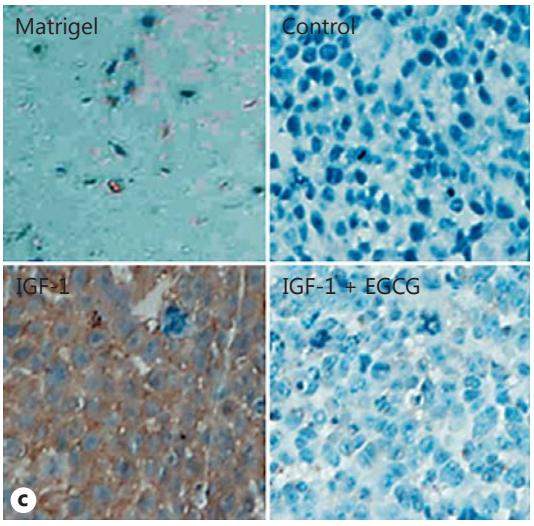

Fig. 4. EGCG inhibited IGF-I-induced VEGF expression in A549 cells. Serum-starved A549 cells were pretreated for 1 h with different concentrations of EGCG, followed by incubation with IGF-I (40 ng/ml) for 16 h. **a** VEGF mRNA levels were determined by real-time PCR. **b** VEGF protein levels were determined by ELISA analysis. **c** Immunohistochemical staining for VEGF protein expression in Matrigel plugs. Tumor sections were cut and processed for immunohistochemical staining using antibody against human VEGF as described in the Materials and Methods section. * $p < 0.01$.

cells by IGF-I significantly upregulated the expression of HIF-1α protein (fig. 3b), but had no obvious stimulatory effects on HIF-1α mRNA expression ($p > 0.05$) (fig. 3a). In addition, pretreatment of A549 cells with various concentrations of EGCG apparently inhibited, in a concentration-dependent manner, the IGF-I-induced upregulation of the HIF-1α protein expression, but not the HIF-1α mRNA expression (fig. 3a, b). Consistently, we also found that IGF-I significantly increased the protein expression of HIF-1α in A549 xenografts, which was drastically attenuated in the presence of EGCG (fig. 3c). Taken together, these results have demonstrated that EGCG can potently inhibit the IGF-I-induced expression of HIF-1α protein in A549 cells.

Effects of EGCG on the IGF-I-Stimulated VEGF Expression in A549 Cells

Next, the effects of EGCG on the IGF-I-induced VEGF expression were examined. As expected, stimulation of the A549 cells by IGF-I concomitantly upregulated the VEGF mRNA expression as well as the protein secretion in the supernatants ($p < 0.01$; fig. 4a, b). Invariably, the IGF-I-induced upregulation of VEGF mRNA expression and protein secretion in A549 cells was dose-dependently abrogated by pretreatment with different concentrations of EGCG

($p < 0.01$; fig. 4a, b). Consistently, we also found that IGF-I significantly increased the VEGF protein expression in A549 xenografts, which was drastically attenuated in the presence of EGCG (fig. 4c). These results suggest that EGCG can simultaneously inhibit the IGF-I-induced expression of HIF-1α protein and its downstream target gene VEGF at both mRNA and protein levels in A549 cells.

Discussion

EGCG is a polyphenol extracted from green tea. In recent years, accumulating experimental evidence has shown that EGCG can substantially inhibit growth and induce apoptosis in various types of human tumor cells [14–18]. Due to its anti-cancer potentials, EGCG has become a promising drug used for health care and cancer prevention. Further studies have suggested that EGCG may play a critical role in the inhibition of tumor angiogenesis [19–22], and the results of the present study have provided further evidence that EGCG is capable of inhibiting IGF-I-stimulated proangiogenic activity conferred by human NSCLC cells both in vitro and in vivo.

HIF-1 is a transcription factor composed of α and β subunits that is universally found in mammalian cells. Its fundamental function is to perceive changes in tissue oxygen level. Under hypoxic conditions, the HIF-1α protein is stabilized and accumulated and forms stable dimers with the HIF-1β subunit. With the assistance of a coactivator, the HIF-1 protein dimers bind to the hypoxia response elements of their target genes, thus leading to the transcriptional activation of these target genes. According to the related research findings, the target genes regulated by HIF-1α involve those engaged in tumor cell growth, angiogenesis, invasion and metastasis, and metabolism of ions and catechols [23–30]. Zhong et al. [31] have demonstrated that the HIF-1α protein does not exist in most (88%) normal tissues, while it is expressed in 53% of malignant tumors, suggesting a correlation of the HIF-1α protein overexpression with tumorigenesis. Lately, a growing body of evidence has implicated the critical role of HIF-1α and some of its downstream target genes, such as VEGF, p53, and COX-2, in the regulation of tumor angiogenesis [32–34]. Therefore, HIF-1α is supposed to play an important role in the growth of tumors through multiple mechanisms. Thus, it is emerging as a potential molecular target for the development of novel therapeutic modalities for cancer.

Recently, it has been shown that the expression of HIF-1α can be upregulated not only by hypoxia, but also by other nonhypoxic factors, such as the inactivation of tumor-suppressor genes (p53, pVHL, and PTEN [35–37]), activation of some viral oncoproteins (EBV and HPV-16 [38, 39]), and challenge by IGF-I [40–42], etc. In the present study, the human NSCLC cell line A549 was used to investigate the effect of EGCG on the IGF-I-induced HIF-1α and VEGF expression and the proangiogenic activity of human lung cancer cells.

The experimental results indicate that EGCG at concentrations of 10, 25, 50, and 100 μmol/l can obviously inhibit IGF-I which stimulates the in vitro and in vivo angiogenic activities conferred by A549 lung cancer cells. At the molecular level, our results showed that EGCG at various concentrations can significantly inhibit the IGF-I-induced expression of HIF-1α protein, and EGCG at relatively high concentrations (25–100 μmol/l) can also substantially inhibit the IGF-I-induced expression of VEGF protein and mRNA. The finding that EGCG has no inhibitory effect on HIF-1 α mRNA expression suggests that EGCG inhibits the expression of HIF-1α protein possibly through a posttranscriptional mechanism. Taken together, our results indicate that EGCG exerted its anti-angiogenic effects in lung cancer possibly via disrupting the HIF-1α/VEGF pathway that plays an important role in tumor angiogenesis [43–45]. Of note, in the present study, we also found that EGCG at relatively low concentrations (0–10 μmol/l) did not decrease the IGF-I-induced HIF-1α protein and mRNA expression

to a statistically significant level, suggesting the possibility that some other mechanisms might also be involved in the EGCG-mediated inhibition of tumor angiogenesis in lung cancer, which remains to be explored in future studies.

In conclusion, in the present study we have demonstrated that EGCG inhibited the IGF-I-stimulated in vitro and in vivo proangiogenic activity of NSCLC cells possibly through the inhibition of the HIF-1α protein accumulation and VEGF expression induced by IGF-I. Therefore, our study has provided evidence that the pharmacological action of EGCG may contribute to its potential application as a potent anti-angiogenic agent for the effective therapy of human NSCLC.

Acknowledgements

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