JBUON 2014; 19(2): 435-439
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

EGCG decreases the expression of HIF-1α and VEGF and cell growth in MCF-7 breast cancer cells

Hai-qing Luo[1*], Meng Xu[5*], Wang-tao Zhong[2], Zhong-yi Cui[3], Fu-mei Liu[3], Ke-yan Zhou[3,4], Xiang-yong Li[3,4*]

[1]Center of Oncology, [2]Department of Neurology, The Affiliated Hospital of Guangdong Medical College, Zhanjiang; [3]Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Zhanjiang; [4]Key Laboratory for Biologically Active Molecules of Guangdong Provincial Department of Education, Guangdong Medical College, Zhanjiang, Guangdong; [5]Department of Oncology, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong, China

**These authors contributed equally to this work*

Summary

Purpose: *To investigate the effects of epigallocatechin-3-gallate (EGCG) on the expression of HIF-1a and vascular endothelial growth factor (VEGF) and cell growth in MCF-7 breast cancer cells.*

Methods: *MCF-7 human breast cancer cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L) for 48 h. The growth and proliferation of cells were analyzed by trypan blue staining in the pretreated MCF-7 cells. Furthermore, mRNA expression of HIF-1a and VEGF was detected by reverse transcriptase polymerase chain reaction (RT-PCR) analysis in the pretreated MCF-7 cells. Protein expression of HIF-1a was detected by Western blot, and the secreted protein level of VEGF in the supernatant of the culture medium was analyzed by enzyme linked immuno-sorbent assay (ELISA) in the MCF-7 cells pretreated with different concentrations of EGCG.*

Results: *Cell growth decreased dramatically in MCF-7 cells treated with different concentrations of EGCG, compared with untreated (control) cells. Moreover, protein expression of HIF-1a and VEGF declined in a dose-dependent manner in MCF-7 cells pretreated with increasing concentrations of EGCG.*

Conclusions: *EGCG inhibits cell growth and proliferation of MCF-7 breast cancer cells, possibly by inhibiting the protein expression of HIF-1a and VEGF.*

Key words: *breast cancer, epigallocatechin-3-gallate (EGCG), HIF-1a, vascular endothelial growth factor (VEGF)*

Introduction

Green tea is widely consumed in China and contains many water-soluble polyphenol compounds, including EGCG. EGCG is the most abundant monomer in green tea and has the highest bioactivities [1,2]. Research demonstrates that EGCG has many biologic activities and pharmacodynamic effects such as antioxidation, scavenging of reactive oxygen species (ROS), antiinflammatory, antiviral, antimutational, and antitumor properties. Moreover, EGCG has been shown to inhibit growth of several types of cancer cells [3]. However, the effect of EGCG on the growth of breast cancer cells has not been reported yet. The goal of the present study was to investigate the effects of EGCG on the growth of breast cancer cells and the expression of HIF-1α and VEGF in MCF-7 breast cancer cells, which may help elucidate the anticancer mechanism of EGCG.

Methods

The breast cancer cell line MCF-7 was from our institute. Monomer EGCG with a purity of >98% was purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Gibco (USA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biotech Company (Hangzhou, China). Trizol was purchased from Invitro-

Correspondence to: Xiang-yong Li, MD, PhD. Institute of Biochemistry and Molecular Biology, Guangdong Medical College, Zhanjiang 524023, China. Tel: +86 759 238 8581, E-mail: xyli75@126.com
Received: 15/09/2013; Accepted: 25/11/2013

gen (Carlsbad USA). Chemical reagents such as diethyl pyrocarbonate (DEPC), acrylamide and N,N' methylenebisacrylamide were purchased from Sigma (St. Louis, MO, USA). RT-PCR kits were purchased from QIAGEN (Hambourg, Germany). Mouse antihuman HIF-1α monoclonal antibody, goat antihuman β-actin polyclonal antibody, and ECL luminescent kits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). VEGF ELISA kits were purchased from Boster Co. Ltd (Wuhan, China). All primers were synthesized by Sangon Biotech Co Ltd (Shanghai, China).

Pharmaceutical preparation

EGCG was dissolved in DMSO at a final storage concentration of 100 mg/L, and solutions were stored at -70 °C. EGCG solutions were diluted to the required concentration before use. To prevent general toxicity, the concentration of DMSO in each sample did not exceed 0.1% throughout the experiment.

Cell culture

MCF-7 cells were cultured in RPMI 1640 culture medium containing 10% FBS, 100 U/ml penicillin, 100 mg/L streptomycin, and 0.5 U/ml insulin. Cells were cultured in a 5% CO_2 incubator at 37 °C. Cells in logarithmic growth phase were harvested and used in experiments.

Analyzing cell growth by trypan blue staining

MCF-7 breast cancer cells in logarithmic growth phase were plated and cultured. When cells were completely adhered to culture flasks, they were treated with EGCG at concentrations of 25, 50, and 100 mg/mL. Control samples were treated with water as vehicle. Triplicate samples treated with EGCG or vehicles were harvested each day over a 7-day period. Cell morphology was observed under an inverted microscope, detached by trypsin, stained by trypan blue, and analyzed for viable counts under a light microscope. Cell growth curves were constructed with culture time on the horizontal axis and cell count on the vertical axis.

RT-PCR

MCF-7 cells treated with different concentrations of EGCG were harvested and total RNA was extracted with Trizol kits. The mRNA expression of HIF-1α and VEGF were detected by one-step RT-PCR, with human GAPDH as internal control. The amplification primers were as follows: HIF-1α (150bp) / sense primer: 5'- TCT GGG TTG AAA CTC AAG CAA CTG -3', antisense primer: 5'- CAA CCG GTT TAA GGA CAC ATT CTG -3'; VEGF (176bp) /sense primer: 5'- TGC TTC TGA GTT GCC CAG GA -3', antisense primer: 5'- TGG TTT CAA TGG TGT GAG GAC ATA G -3' ; GAPDH (226bp) /sense primer: 5'- CCG CCG AGC CAC ATC GCT C -3', antisense primer: 5'- ATG AGC CCC AGC CTT CTC CAT -3'. The system of RT-PCR was as follows: stage 1 was a process of reverse transcription at 42 °C for 5 sec. Stage 2 was a process of preheat denaturing at 95 °C for 10 sec. Stage 3 was a process of PCR with 30 cycles of 95 °C for 5 sec and then 60 °C for 30 sec.

SDS-PAGE and Western blot

MCF-7 cells treated with increasing concentrations of EGCG were harvested and protein was extracted following cell lysis. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a gel concentration of 10%. Proteins were then transferred to nitrocellulose (NC) membrane. NC membranes were first blocked with 5% dried skim milk, incubated with the primary antibody solution (mouse antihuman HIF-1α monoclonal antibody, at a working concentration of 1:600) at 4 °C overnight, and incubated with the horseradish peroxidase-labeled secondary antibody (1:4000). Finally, protein bands were imaged by the ECL luminescence system and analyzed by image J software.

Analyzing protein expression of VEGF by ELISA

The supernatants of cultured cells treated with different concentrations of EGCG were harvested and analyzed for VEGF protein expression by ELISA according to the kit manufacturer's instructions. The A492 values of samples were determined by an ELISA reader at an absorbance of 492 nm.

Statistics

Measurements of data were expressed as mean ± SD. Statistical significance between means was determined and analyzed by one-way analysis of variance (ANOVA) with SAS 8.0 software. A p-value <0.05 was considered significant, and <0.01 was considered extremely significant.

Results

Effects of EGCG on cell growth and proliferation of breast cancer cells

Results from trypan blue analysis (Figure 1) demonstrated that the untreated breast cancer cells grew quickly in the control group. In comparison, although the number of viable cells increased, cells grew slowly when pretreated with different concentrations of EGCG in both time- and concentration-dependent manner, indicating that EGCG inhibits cell growth and proliferation of MCF-7 breast cancer cells ($p<0.001$).

Effects of EGCG on mRNA expression of HIF-1a and VEGF in breast cancer cells

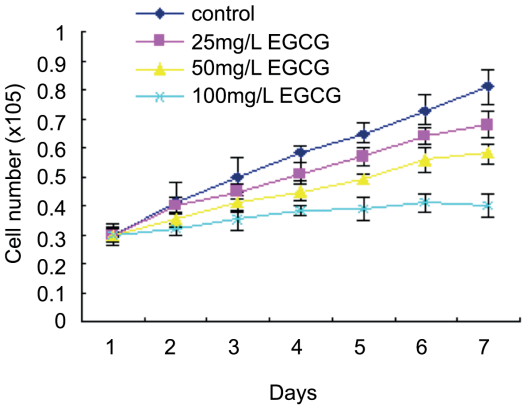

Figure 1. Effects of EGCG on cell growth of MCF-7 cells. MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L) for 7 days, and then subjected to trypan blue analysis. Quantitative data from 3 independent experiments (mean ± SD).

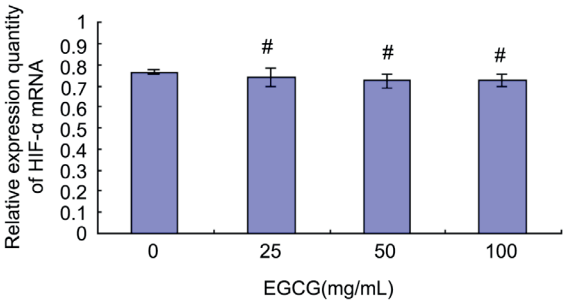

Figure 4. Quantitative analysis of mRNA expression of HIF-1α in MCF-7 cells pretreated with different concentrations of EGCG (25, 50, 100 mg/L). Cells were subjected to RT-PCR analysis. This Figure shows the relative expression of HIF-1α mRNA. Quantitative data from 3 independent experiments (mean± SD). #p>0.05 compared with the vehicle control group.

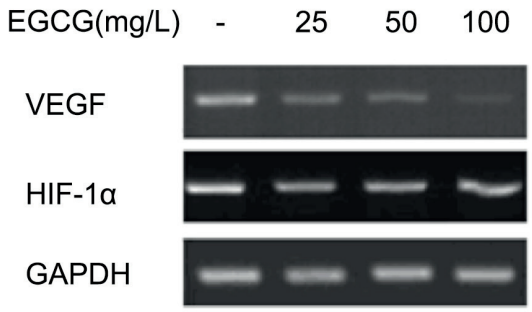

Figure 2. Analysis of mRNA expression of VEGF and HIF-1α by RT-PCR. MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L), and then they were subjected to RT-PCR analysis. GAPDH served as internal control. Results are representative of 3 independent experiments.

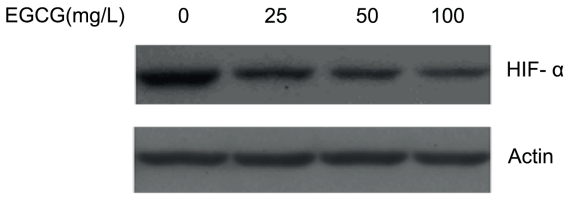

Figure 5. Analysis of the protein expression of HIF-1α in MCF-7 cells by Western blot. MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L), and then cells were subjected to Western blot analysis. Actin levels demonstrate similar loading. Results are representative of 3 different experiments.

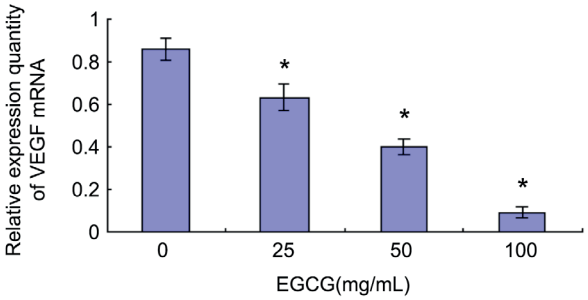

Figure 3. Quantitative analysis of VEGF mRNA expression. MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L), and then they were subjected to RT-PCR analysis. This Figure shows the relative expression of VEGF mRNA. Quantitative data from 3 independent experiments (mean± SD). * p<0.05 compared with the vehicle control group.

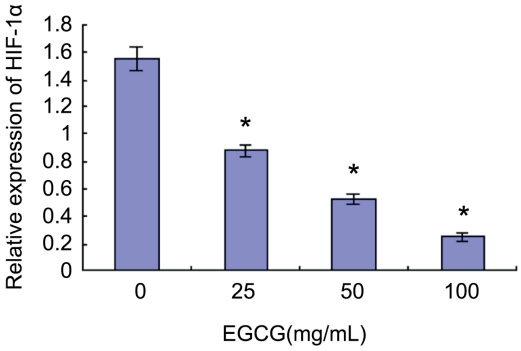

Figure 6. Quantitative analysis of VEGF protein expression.MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L), and then cells were collected and subjected to Western blot analysis. Quantitative data from 3 independent experiments (mean±SD) are shown. The relative expression of HIF-1α equals arbitrary quantitation of HIF-1α/ arbitrary quantitation of actin. *p<0.05 compared with the vehicle control group.

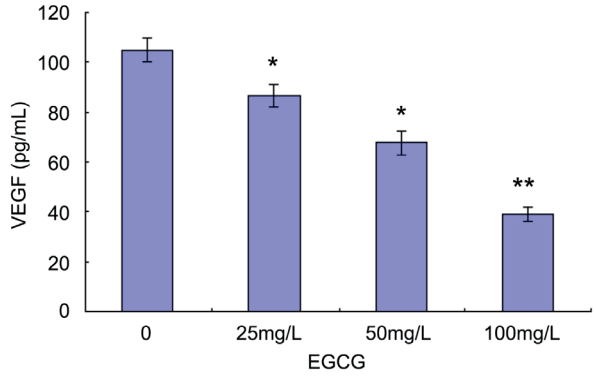

Figure 7. Analysis of the protein expression of VEGF in MCF-7 by ELISA. MCF-7 cells were pretreated with different concentrations of EGCG (25, 50, 100 mg/L), and then the supernatants of the cultured cells were collected and subjected to ELISA analysis. Shown are quantitative data from 3 independent experiments (mean± SD). *p<0.05 compared with the vehicle control group. **p<0.01 compared with the vehicle control group.

As shown in Figure 2, the mRNA expression of HIF-1α showed no obvious changes following treatment with increasing concentrations of EGCG (Figures 2,4). However, the mRNA expression of VEGF decreased dramatically in MCF-7 cells treated with EGCG in a dose-dependent manner (Figures 2,3).

Collectively, these data indicated that EGCG inhibited the mRNA expression of VEGF in a dose-dependent manner, but showed no obvious effect on the mRNA expression of HIF-1α in MCF-7 breast cancer cells. This may represent one mechanism by which EGCG inhibits MCF-7 cell growth.

Effects of EGCG on the protein expression of HIF-1a and VEGF in breast cancer cells

According to the results of protein expression (Figures 5,6), HIF-1α protein expression decreased dramatically in breast cancer cells treated with different concentrations of EGCG in a dose-dependent manner, indicating that EGCG inhibits the growth of breast cancer cells possibly through suppressing the protein expression of HIF-1α.

Analyzing VEGF protein levels in the supernatant of cultured medium by ELISA

As shown in Figure 7, the level of VEGF protein decreased significantly in the supernatants of the cultured MCF-7 cells pretreated with different concentrations of EGCG in a concentration-dependent manner, indicating that EGCG dose-dependently inhibited the protein expression of VEGF in MCF-7 cells.

Discussion

Green tea is widely consumed in China and around the world and contains a high quantity of polyphenol compounds such as EGCG, EGC, and ECG. It has been demonstrated that EGCG has anticancer activity and inhibits cell growth and proliferation of various tumor cells, including nasopharyngeal carcinoma cells, lung cancer cells, and pancreatic cancer cells both *in vitro* and *in vivo* [4-6]. Although the antitumor mechanisms of EGCG remain unknown, they are possibly associated with apoptosis of tumor cells and inhibition of cell cycle progression [7]. HIF-1α is a transcription factor that is selectively expressed in mammalian cells. It consists of α and β subunits. Research reveals that most normal tissues do not express HIF-1α protein. In contrast, 53% of malignant tumor tissues express HIF-1α protein, indicating that expression of HIF-1α protein is involved in the occurrence and development of tumors [8]. Other studies have shown that the target genes regulated by HIF-1α are probably associated with tumor cell growth, angiogenesis, tumor invasion and metastasis, ion transport, and catecholamines, metabolism [9,10]. One of the common characteristics of tumors is high activity of HIF [11]. The HIF-1 transcription complex upregulates the expression of VEGF and induces the corresponding biological effects by interacting with the hypoxia reaction element of the VEGF gene, indicating that VEGF is one of the target genes of HIF-1 [12]. Li et al. [13] also confirmed in a recent study that VEGF expression was induced by the HPV tumor protein in lung cancer cells, which was dependent on HIF-1. HIF-1 and VEGF are potentially important targets in the prevention and treatment of tumors, so it is meaningful to investigate the relationship between the expression of these two factors and the occurrence and development of tumors.

In the present study, the effects of EGCG on cell growth and survival of MCF-7 breast cancer cells was investigated by trypan blue staining. The results showed that EGCG significantly suppressed cell growth and proliferation of MCF-7 breast cancer cells in both a time and dose-dependent manner, which is consistent with the findings of Zhang et al. [14] and Shirakami et al. [15]. Next, the inhibitory mechanisms governing EGCG's effect on MCF-7 cells were further explored. EGCG

inhibited signal transduction pathways that were related to angiogenesis. However, it is unknown if EGCG inhibits the activation of the angiogenesis initiator HIF-1α and expression of VEGF. The results of the current study reveal that EGCG not only inhibited the mRNA expression of VEGF, but also suppressed the protein expression of VEGF in MCF-7 cells in a dose-dependent manner, suggesting that inhibition of the expression of VEGF by EGCG may be one of the mechanisms behind the antitumor effect of EGCG in breast cancer cells. Although EGCG had no obvious effect on the mRNA expression of HIF-1α, it dose-dependently inhibited the protein expression of HIF-1α in MCF-7 cells, indicating that EGCG inhibits the protein expression of HIF-1α possibly through posttranscriptional regulation. However, this must be investigated further.

In conclusion, the data of the present study show that EGCG inhibits the cell growth and proliferation of breast cancer cells by inhibiting the expression of HIF-1α protein and secreted VEGF, which might provide new thoughts for uncovering the antitumor mechanism of EGCG and indicate new targets for the prevention and treatment of breast cancer.

Acknowledgements

This work was supported by grants from National Science Foundation of China (81272434, 81273814), Science and Technological Program for Higher Education, Science and Research, and Health Care Institutions (2011108102044), Guangdong Medical Research Funding (B2012280), Scientific and Technological Project of Zhanjiang city (2009C3101012, 2011C3108012), Doctor Initial Funding of Guangdong Medical College (B2011022) and Science & Technology Innovation Fund of Guangdong Medical College (STIF201105).

References

1. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3 gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. Natl Cancer Inst 1997;89:1881-1886.
2. Zhang Y, Yang ND, Zhou F et al. (-)-Epigallocatechin-3-Gallate Induces Non-Apoptotic Cell Death in Human Cancer Cells via ROS-Mediated Lysosomal Membrane Permeabilization. PLoS One 2012;7:e46749. doi:10.1371/journal.pone.0046749.
3. Wang H, Bian S, Yang CS. Green tea polyphenol EGCG suppresses lung cancer cell growth through upregulating miR-210 expression caused by stabilizing HIF-1α. Carcinogenesis 2011; 32:1881-1889.
4. Lin CH, Shen YA, Hung PH, Yu YB, Chen YJ. Epigallocathechin gallate, polyphenol present in green tea, inhibits stem-like characteristics and epithelial-mesenchymal transition innasopharyngeal cancer cell lines. BMC Complement Altern Med 2012;12:201-213.
5. Zhang Q, Fu H, Pan J et al. Effect of dietary Polyphenol E and EGCG on lung tumorigenesis in A/J Mice. Pharm Res 2010;27:1066-1071.
6. Shankar S, Suthakar G, Srivastava RK. Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer. Front Biosci 2007;1:5039-5051.
7. Kang SU, Lee BS, Lee SH, Baek SJ, Shin YS, Kim CH. Expression of NSAID-activated gene-1 by EGCG in head and neck cancer: involvement of ATM-dependent p53 expression.J Nutr Biochem 2012 Sep 24. pii: S0955-2863(12)00196-9. doi: 10.1016/j.jnutbio.2012.07.003.
8. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clin Cancer Res 2010;16:5928-5935.
9. Hung JJ, Yang MH, Hsu HS, Hsu WH, Liu JS, Wu KJ. Prognostic significance of hypoxia-inducible factor-1 alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. Thorax 2009;12:1082-1089.
10. Dai CX, Gao Q, Qiu SJ et al. Hypoxia-inducible factor-1 alpha, in association with inflammation, angiogenesis and MYC, is a critical prognostic factor in patients with HCC after surgery. BMC Cancer 2009;9:418-429.
11. Ellis L, Hammers H, Pili R. Targeting tumor angiogenesis with histone deacetylase inhibitors. Cancer Lett 2009;280:145-153.
12. Semenza GL. Expression of hypoxia-inducible factor 1: mechanisms and consequences. Biochem Pharmacol 2000;59:47-53.
13. Li G, He L, Zhang E, Shi J et al. Overexpression of human papillomavirus (HPV) type 16 oncoproteins promotes angiogenesis via enhancing HIF-1α and VEGF expression in non-small cell lung cancer cells. Cancer Lett 2011;311:160-170.
14. Zhang Q, Tang X, Lu Q, Zhang Z, Rao J, Le AD. Green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1 alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. Mol Cancer Ther 2006;5:1227-1238.
15. Shirakami Y, Shimizu M, Adachi S et al. (-)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor-vascular endothelial growth factor receptor axis. Cancer Sci 2009;100:1957-1962.