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Effects of EGCG on proliferation and apoptosis of gastric cancer SGC7901 cells via down-regulation of HIF-1α and VEGF under a hypoxic state

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Abstract. – OBJECTIVE: To investigate the effects of epigallocatechin-3-gallate (EGCG) on proliferation and apoptosis of human gastric cancer SGC7901 cells under a hypoxic state.

MATERIALS AND METHODS: Human gastric cancer SGC7901 cells were sub-cultured, and the cobalt chloride (CoCl2) hypoxia model was established. The blank control group (normoxia group), hypoxia control group (hypoxia group) and hypoxia + different concentrations of EGCG subgroups (20, 40, 60, 80, 100 μg/mL EGCG) were set up. Cell viability was detected via methyl thiazolyl tetrazolium (MTT) assay, apoptosis was detected via flow cytometry, and expressions of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) were detected via reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

RESULTS: Relatively low concentrations of EGCG (20-80 μg/mL) presented no significant inhibiting effect on SGC7901 cell growth within a short time (24 h) ($p>0.05$). The increasing concentration of EGCG inhibited cell proliferation under a hypoxia state ($p<0.05$). EGCG induced apoptosis in a dose-dependent manner under hypoxia ($p<0.05$). EGCG could significantly impede expressions of HIF-1α and VEGF proteins ($p<0.05$), and down-regulate the level of VEGF mRNA ($p<0.05$), but it showed no significant effect on the HIF-1α mRNA expression ($p>0.05$).

CONCLUSIONS: EGCG inhibited cell proliferation under hypoxia via the downregulation of HIF-1α and its downstream target gene VEGF levels, providing a theoretical basis for the early diagnosis and treatment of gastric cancer in clinic.

Key Words:
EGCG, Hypoxia, Gastric cancer cells, Hypoxia-inducible factor-1α, Vascular endothelial growth factor.

Introduction

Studies have shown that tea polyphenol extracted from green tea is the most important beneficial ingredient for the health of human body, among which epigallocatechin-3-gallate (EGCG) is the critical component for anti-cancer effect. Accumulative evidence indicated that EGCG can inhibit the occurrence and development of a variety of tumors, such as gastric cancer and colon cancer without toxic and side effects on normal cells[1-5]. Therefore, EGCG is hailed as one of the most promising anti-oxidants and anti-cancer drugs nowadays[6]. It has been found that hypoxia is one of the growth characteristics of many tumors, including gastric cancer, which leads to resistance to radiotherapy and chemotherapy[7]. Hypoxia-inducible factor-1α (HIF-1α) plays a major regulatory role in this process. Activators induce the increasing expression of the downstream target gene vascular endothelial growth factor (VEGF), and VEGF is the most potent tumor angiogenic factor that can trigger angiogenesis, tumor cell growth and metastasis[8-10]. In this study, the effects of EGCG on proliferation and apoptosis of gastric cancer cells under a hypoxic state were detected, and its influences on expressions of HIF-1α and its downstream target gene VEGF were also determined.

Materials and Methods

Main Reagents and Equipment

Dimethylsulfoxide (DMSO), fetal bovine serum (FBS) and cobalt chloride ($CoCl_2$) were from Sigma-Aldrich (St. Louis, MO, USA).

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Roswell Park Memorial Institute-1640 (RPMI-1640) medium were purchased from HyClone (South-Logan, UT, USA). TRIzol reagent was collected from Invitrogen (Carlsbad, CA, USA). Mouse anti-human HIF-1 monoclonal antibody, mouse anti-human VEGF-A monoclonal antibody and mouse anti-human GAPDH monoclonal antibody were bought from Abcam (Cambridge, MA, USA). Complementary deoxyribonucleic acid (cDNA) kits and SYBR Green polymerase chain reaction (PCR) kits were provided from Toyobo (Osaka, Japan). Propidium iodide (PI) and Annexin V-FITC apoptosis detection kits were offered by BD Biosciences (Franklin Lakes, NJ, USA). Heracell CO_2 cell incubator and optical microscope were from Olympus (Shinjuku, Tokyo, Japan). Vertical electrophoresis apparatus and membrane transfer device were obtained from Bio-Rad (Hercules, CA, USA). PCR primers were designed and synthesized by Sangon (Shanghai, China).

Cell Culture

Human gastric cancer cell line SGC7901 was subcultured by our laboratory. Well-grown cells in the logarithmic growth phase were selected for experiment. SGC7901 cells were placed into RPMI-1640 medium (10% fetal bovines serum (FBS) + 1% double antibodies) and incubated at 37°C 5% CO_2 for 2-3 d. 95% EGCG was added into serum-free RPMI-1640 medium and prepared into 2 mg/mL solution for standby application.

Determination of Cell Survival Rate

SGC7901 cells in the logarithmic growth phase were digested, prepared into single-cell suspension and inoculated into a 96-well plate (10^3-10^4/well, 200 μL/well). Cells were divided into following groups in the experiment: 1) normoxia group: Cells were cultured routinely using RPMI-1640 medium containing 10% FBS; 2) hypoxia group: 150 μmol/L $CoCl_2$ group; and 3) hypoxia induction group. EGCG in different concentrations was added into RPMI-1640 medium containing 10% FBS to establish the hypoxia induction model, and the model was divided into 5 subgroups according to the concentrations of EGCG: 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL, respectively. Cells in each group were cultured in the incubator with 5% CO_2 and saturated humidity at 37°C under corresponding conditions. After cells were cultured for a certain period of time, methyl thiazolyl tetrazolium (MTT) solution was added into each well to culture cells for another 4 h. After the culture was terminated, the supernatant in each well was discarded. An appropriate amount of DMSO was added, and shaken carefully for 10 min to fully dissolve crystals in wells. The optical density (OD) value of each well was measured. The cell growth inhibition rate = (1-average OD value $_{\text{experimental group}}$/ average OD value $_{\text{normoxia group}}$) × 100%, and results were recorded.

Determination of Apoptosis Rate

Cells were grouped as mentioned above, and the operation was performed according to instructions of Annexin-V/PI bidirectional labeling kit. SGC7901 cells in the logarithmic growth phase were inoculated to a 6-well plate (2 mL/well). RPMI1640 cell culture solution containing different concentrations of drugs was added, followed by routine culture in the incubator with 5% CO_2 at 37°C for 48 and 72 h. Apoptosis rate was detected using a flow cytometer, 10,000 cell fluorescence signals were collected in each sample, and detection results were recorded and analyzed.

Detection of HIF-1Ð and VEGF Messenger Ribonucleic Acid (mRNA) Expressions via RT-PCR

Tissue RNA extraction: the tissue was grinded in liquid nitrogen and treated with TRIzol (100 mg tissue: 1 ml TRIzol). Next, the solution was moved to an EP (Eppendorf) tube and added with 200 μl chloroform (Solarbio, Beijing, China). After vibrated for 15 s, the upper aqueous phase was added with 500 μl isopropanol (SolarBio, Beijing, China) for 10 min. After centrifuged at 12000 g for 10 min, the precipitation was added with 1 ml ethanol (75%). After centrifuged at 4°C and 7500 g for 5 min, the supernatant was removed and the tube was dried for 10 min. Next, the RNA was solved in DEPC water. RNA content and purity were determined by ultraviolet spectrophotometer.

Reverse transcription: reaction solution was prepared according to the instruction, including 2 μg total RNA, 1 μl oligo primer (50 μM), 1 μl dNTP mix (10 μM), and ddH_2O. The solution was predegenerated at 65°C for 5 min. Next, cDNA first chain synthesis reaction system was prepared, including 2 μl 10×RT buffer, 4 μl $MgCl_2$ (25 μM), 2 μl DTT (0.1 M), 1 μl RNAase OUT (40 U/μl), 1 μl SuperScrip III RT (200 U/μl) (Invitrogen, Carlsbad, CA, USA), and ddH_2O. The reaction condition was composed by 50°C for 50 min and 85°C for 5 min.

Real-time PCR was then performed by using SYBR Premix Ex Taq GC kit (TaKaRa, Otsu, Shiga, Japan) (7.5 μl 2×premix, 10 mM forword and reverse primers, dH_2O to a final volume of 15 μl) in the following condition: 95°C denature for 2 min, followed by 45 cycles each containing 94°C denature for 10 s, and 60°C annealing for 45 s with LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Primer sequence and amplification length were shown in Table I. GAPDH was selected as internal reference. Relative gene expression was semi-quantitative analyzed by $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta Ct}$ = gene copy number in test group/gene copy number in control. Experiments were carried out in triplicates.

Detection of HIF-1α and VEGF Protein Expressions via Western blotting

Cells were homogenized to extract total protein. Proteins were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred into polyvinylidene difluoride (PVDF) membranes, blocked with 5% nonfat milk for 1 h, and incubated with primary antibody (Mouse anti-human HIF-1 monoclonal antibody 1:2000 and mouse anti-human VEGF-A monoclonal antibody 1:1000, mouse anti-human GAPDH monoclonal antibody 1:2000) overnight at 4°C. The membrane was washed with Tris-buffered saline and Tween-20 (TBST) 30 min for 3 times, followed with incubation with horseradish-peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies (1:5000) for 60 min. After washed three times with PBST, enhanced chemiluminescence (ECL) detection reagent was used to develop and fix. GIS-2020D gel image system was used to analyze the band density of HIF-1α, VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression level of HIF-1α protein = $\text{HIF-1}\alpha_{\text{gray value}}/\text{GAPDH}_{\text{gray value}}$, and the relative expression level of VEGF-A protein = $\text{VEGF-A}_{\text{gray value}}/\text{GAPDH}_{\text{gray value}}$.

Flow Cytometry

PI staining was used to test apoptotic rate of cells. In brief, cells were digested and collected to adjust to concentration of 10^5 cells/ml. 0.4 ml buffer was added to mix cells well, followed by adding 5 μl of annexin V-FITC and 5 μl PI dye for 10 min dark staining at room temperature. Flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA) was then performed in dual parameter analysis.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) software was used. Measurement data were presented as mean ± standard deviation (Mean±SD), and *t*-test or analysis of variance was used for intergroup comparisons. $p<0.05$ suggested that the difference was statistically significant.

Results

Inhibiting Effect of EGCG on SGC7901 Cell Growth Under Hypoxia Conditions

After culture under hypoxia conditions for 24 h, no statistical difference of SGC7901 cell growth was shown between groups with relatively low concentrations of EGCG (20-80 μg/mL) and hypoxia group ($p>0.05$), but high-concentration of EGCG (100 μg/mL) significantly inhibited the cell growth compared with that in hypoxia group ($p<0.05$). After culture under hypoxia conditions for 48 and 72 h, the inhibiting effect of EGCG on SGC7901 cell proliferation in a time- and dose-dependent manner ($p<0.05$), with the highest inhibition rate of 76.2±2.91% (Table I).

Table I. Inhibition rates of EGCG in different concentrations on SGC7901 cell proliferation ($\bar{x}$±s, n=6).

Group	24 h	48 h	72 h
Hypoxia group	25.0±1.57	26.3±2.58	33.2±1.36
Hypoxia + 20 μg/mL EGCG group	11.7±2.38	34.3±0.72§*	42.7±2.91§*
Hypoxia + 40 μg/mL EGCG group	24.5±5.09	42.2±0.65§*	53.9±0.83§*
Hypoxia + 60 μg/mL EGCG group	25.3±3.47	51.5±2.07§*	66.4±2.86§*
Hypoxia + 80 μg/mL EGCG group	21.5±1.88	56.9±0.77§*	71.0±1.00§*
Hypoxia + 100 μg/mL EGCG group°	39.06±0.79	62.3±2.25§*	76.2±2.91§*

Note: °$p<0.05$, §$p<0.01$ *vs.* hypoxia group, *$p<0.01$ vs. 24 h group.

Effect of EGCG on SGC7901 Cell Apoptosis Rate Under Hypoxia Conditions

After culture for 48 h, cell apoptosis rates of SGC7901 cells in normoxia group and hypoxia group were 1.62±0.18% and 4.15±0.54%, respectively. Only a few apoptotic cells were found in normoxia group were, suggesting that hypoxia can induce apoptosis. After culture for 48 h under hypoxia conditions, the apoptosis rate was increased by 4.57±0.71%. The apoptosis rate of SGC7901 cells was remarkably increased with the treatment of EGCG (Figure 1). Apoptosis rates of SGC7901 cells after being treated with 80 μg/mL EGCG for 48 h and 72 h were 17.17±2.15% and 24.88±1.58%, respectively, indicating that the apoptotic role of EGCG was enhanced as the time of treatment extended (p<0.05) (Figure 1).

Effects of EGCG on HIF-1α and VEGF Expressions in SGC7901 Cells Under Hypoxia Conditions

There were no significant differences in the expression of HIF-1α mRNA among normoxia group, hypoxia group and EGCG in different concentrations of EGCG + hypoxia groups (p>0.05). However, the expression of VEGF mRNA in hypoxia group was significantly increased, and the inhibiting effect on VEGF mRNA transcription was also significantly increased in a EGCG concentration dependent manner (p<0.05) (Figure 2). Interestingly, the levels of HIF-1α and VEGF protein in hypoxia group were significantly higher than those in normoxic group (p<0.05). Compared with those in hypoxia group, the HIF-1α protein expression was decreased in 20 μg/mL EGCG group (p<0.05), but the VEGF protein expression was not significantly affected (p>0.05). However, with the growing concentration of EGCG, the expressions of HIF-1α and VEGF proteins were suppressed (p<0.05) (Figure 3).

Discussion

A large number of epidemiological studies have suggested that green tea can exert an anti-cancer effect on a variety of tumor cells, and long-term consumption of green tea can reduce the risk of various cancers. Since Fujiki et al[11] reported that EGCG could inhibit human cancer cells for the first time in 1987, many scholars have conducted a multitude of studies on EGCG and confirmed that EGCG can inhibit the occurrence and development of a variety of cancers, such as gastric cancer, colon cancer, lung cancer, liver cancer and pancreatic cancer. It has been indicated that EGCG exerts an anti-tumor activity mainly through the following ways: (1) it induces tumor cell apoptosis and cell cycle arrest. Lee et al[12] found that EGCG down-regulated the expression of B-cell lymphoma-2 (Bcl-2) in human fibrosarcoma cells in a time- and dose-dependent manner, resulting in an increase in the Bcl-2 associated X protein (Bax)/Bcl-2 ratio, and played an apoptotic role. In addition, EGCG can arrest tumor cells in the G1 phase through down-regulating the expression of cyclin-dependent kinases[13]. (2) It affects cell signal transduction pathway. EGCG can inhibit the binding of Raf-1 and mitogen-activated protein kinase 1 (MAPK1) in cells of mouse transfected with mutant H-ras gene, and reduce the phosphorylation

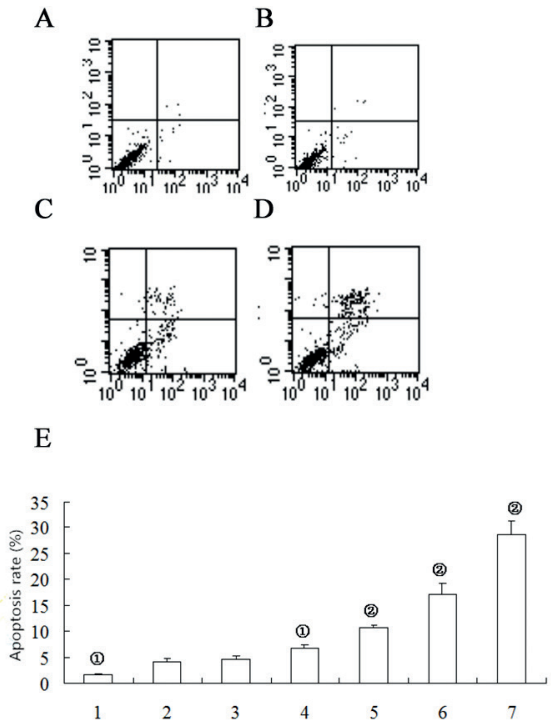

Figure 1. Detection of apoptosis rate of gastric cancer SGC7901 cells in each group after 48 h via flow cytometry (n=3). A, normoxia group. B, hypoxia group. C, intervention with 80 μg/mL EGCG for 48 h. D, intervention with 100 μg/mL EGCG for 48 h. E, the effect of different concentrations of EGCG on apoptosis rates of gastric cancer SGC7901. 1: Normoxia group, 2: hypoxia control group, 3: hypoxia + 20 μg/mL EGCG group, 4: hypoxia + 40 μg/mL EGCG group, 5: hypoxia + 60 μg/mL EGCG group, 6: hypoxia + 80 μg/mL EGCG group, 7: hypoxia +100 μg/mL EGCG group. ^1p<0.05, ^2p<0.01 *vs.* hypoxia control group. Note: first and third quadrants: dead cells and normal cells, second and fourth quadrants: late apoptotic cells and early apoptotic cells.

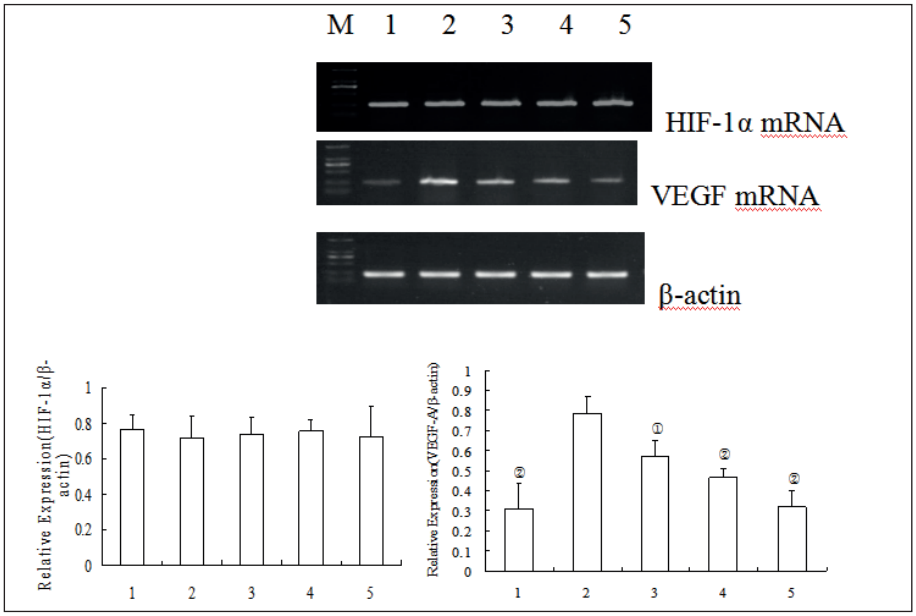

Figure 2. Detection of HIF-1α and VEGF mRNA expressions in gastric cancer SGC7901 cells in each treatment group via RT-PCR. M: DL2000 DNA maker, 1: normoxia group, 2: hypoxia control group, 3: hypoxia + 20 μg/mL EGCG group, 4: hypoxia + 60 μg/mL EGCG group, 5: hypoxia + 100 μg/mL EGCG group. $^{1}p<0.05$, $^{2}p<0.01$ *vs.* hypoxia control group.

levels of extracellular signal regulated kinase 1 (ERK1)/2 and MAPK1/2 proteins, thereby contributing to a suppressing function to the transduction of Ras-MAPK signaling pathway[14]. Moreover, evidence also revealed that EGCG down-regulated the expressions of nuclear factor-κB (NF-κB), epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2)[15]. (3) It inhibits tumor cell invasion and metastasis. Neovascularization is a necessary condition for tumor invasion and metastasis. Aggarwal et al[16] reported that EGCG deterred angiogenesis via the reduction of expression of VEGF, through blocking signal transduction and activating transcription factor 3. The research found that the suppression of EGCG can induce the apoptosis of proliferative vascular smooth muscle cells by enhancing the stability of P53 and formation pathway of NF-κB complex in vascular smooth muscle. EGCG can also elevate the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) through the thiol oxidation or the activation of protein kinases, such as phosphatidylinositol 3-hydroxy kinase (PI3K) and MAPK, and conduct to restrict the growth and metastasis of tumor cells[17]. (4) Others. EGCG contains active hydroxyl hydrogen that can exert a potent antioxidant activity, and make up for the defects of anti-oxidant system in tumor[18]. It was shown that EGCG limited the telomerase activation by inhibiting the expression of human telomerase reverse transcriptase (hTERT) in human breast cancer cells[19].

Hypoxia is a type of the growth characteristics of solid tumors, such as gastric cancer. In this study, SGC7901 cells were treated with low-concentration $CoCl_2$ for the establishment of the hypoxia model. Results of MTT cell proliferation inhibition assay showed that EGCG induced gastric cancer cell apoptosis in a dose-dependent manner ($p<0.05$). Of note, previous study found that the inhibiting effect of EGCG on oral cancer cells HSG was constrained by $CoCl_2$[20]. Therefore, EGCG at a low concentration has no inhibiting effect on gastric cancer cells under hypoxia within a short time, and it is speculated that such a phenomenon may be related to the inhibiting effect of $CoCl_2$ on EGCG. HIF-1α is the most important and central regulator in a series of regulatory responses of tumor cells to adapt to the hypoxic environment. HIF-1 triggers various biological effects through transcriptional activation of a large number of downstream target genes, so that tumor cells can adapt to the hypoxic environment and continue to survive, followed by malignant transformation, proliferation, metastasis and resistance to radiotherapy and chemotherapy. VEGF is one of the important target genes of HIF-1 pathway. VEGF is the most potent angiogenic factor known currently, and its expression maintains at an extremely low level under normal circumstances, except in tissues with vigorous metabolism and rich blood supply, such as myocardial cells and glomerular podocytes. However, its expressions are increased in lung cancer, gastrointestinal cancer, etc. Our

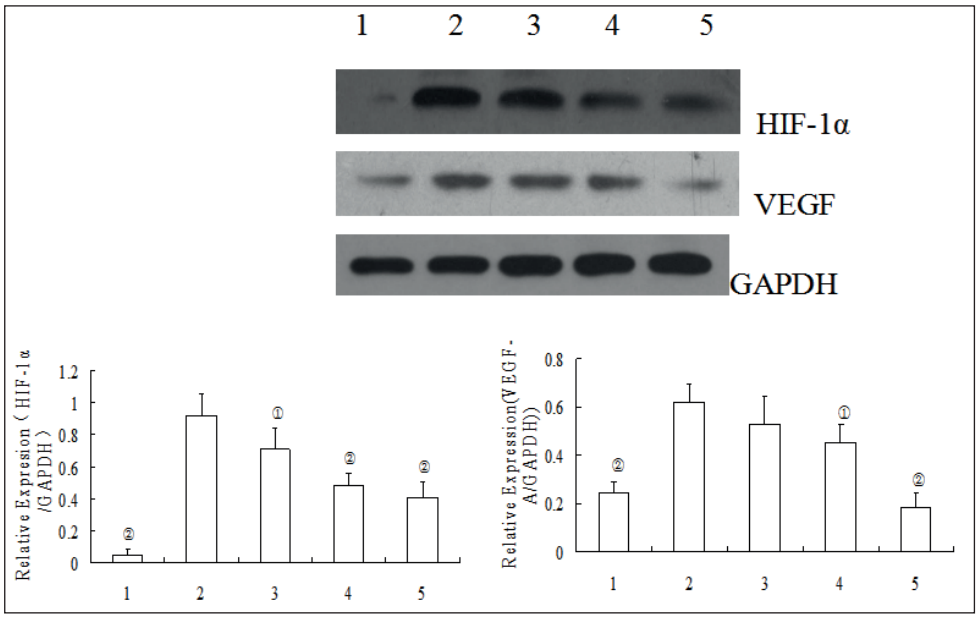

Figure 3. Detection of HIF-1α and VEGF protein expressions in gastric cancer SGC7901 cells in each treatment group via Western blotting. 1: Normoxia group, 2: hypoxia control group, 3: hypoxia + 20 μg/mL EGCG group, 4: hypoxia + 60 μg/mL EGCG group, 5: hypoxia + 100 μg/mL EGCG group. [1]$p<0.05$, [2]$p<0.01$ *vs.* hypoxia control group.

study found that the expression of VEGF in gastric cancer SGC7901 cells was exceptionally low under a normoxic state. However, the expression of VEGF in SGC7901 cells was significantly increased after the HIF-1α expression in SGC7901 cells was up-regulated in a hypoxic status induced by $CoCl_2$. The expression of VEGF is regulated by many factors, such as transforming growth factor, hypoxia factor and platelet-derived growth factor[21], among which hypoxia factor, especially HIF-1, plays a major regulatory role[22,23]. HIF-1α regulates VEGF expression at multiple levels[24,25]. The 5' end of VEGF contains the hypoxia response element (5'-TACGT-GGC-3'), and the HIF-1 transcription complex binds to this element after formation, thereby resulting in the elevation of VEGF. Semenza et al[26] found that after HIF-1α antisense oligonucleotides were transfected into cells, the expression of VEGF expression in cells was inhibited. At present, it is generally believed that HIF-1 in tumor cells is activated and VEGF is highly expressed under hypoxia, which causes vascular endothelial cell proliferation, migration and neovascularization. Therefore, HIF-1α and VEGF are proposed as potential targets in antitumor therapy. In our data, EGCG had significant inhibiting effects on expressions of VEGF mRNA, VEGF and HIF-1α proteins in a dose-dependent manner, but presented no obvious effect on the transcription of HIF-1α gene, which are consistent with previous findings[27-30]. However, the specific mechanisms as well as the *in vivo* evaluation need further study.

Conclusions

We observed that EGCG induces apoptosis of gastric cancer SGC7901 cells via down-regulating HIF-1α and VEGF under a hypoxic state, which provides insight for the future therapy of gastric cancer.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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