

Epigallocatechin-3-gallate suppresses cell proliferation and promotes apoptosis in Ec9706 and Eca109 esophageal carcinoma cells

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Abstract. Epigallocatechin-3-gallate (EGCG) is a component of green tea with anticancer effects that have been demonstrated in multiple types of cancer, but few reports exist concerning its effect in esophageal squamous cell carcinoma cells. The present study investigated apoptosis induced by EGCG treatment and the underlying molecular mechanisms in Eca109 and Ec9706 human esophageal squamous cell carcinoma cells. The apoptosis rate following treatment with various concentration of EGCG for 24 h was detected by flow cytometry. The effect of EGCG on esophageal cancer cell viability was detected via MTT assay. Mitochondrial membrane potential and caspase-3 protein expression was detected in Eca109 and Ec9706 cells following treatment with EGCG by flow cytometry. The telomerase activity of Eca109 and Ec9706 cells following treatment with EGCG was assayed using the polymerase chain reaction-telomeric repeat amplification protocol (PCR-TRAP) argmentation method. EGCG was demonstrated to inhibit the viability of Eca109 and Ec9706 cells in a dose-and time-dependent manner. The flow cytometry results revealed that EGCG treatment induced apoptosis, decreased the mitochondrial membrane potential and increased caspase-3 protein expression levels. PCR-TRAP argmentation analysis revealed that EGCG inhibited telomerase activity. The results of the present study suggested that EGCG functions as an antitumor agent in esophageal cancer cells. The induction of apoptosis may be a viable method for treating esophageal cancer. It is possible to induce apoptosis by modulating the expression level of telomerase activity,

mitochondrial membrane potential and caspase-3 protein expression levels.

Introduction

Esophageal squamous cell carcinoma is a common malignant tumor (1-3). The initiation and progression of esophageal cancer is a complicated process that results from the loss of the normal regulatory pathways controlling cell proliferation, differentiation and apoptosis. However, present therapeutic strategies, including chemotherapy, are characterized by low efficacies (4). Drug resistance and side effects of chemotherapy drugs are major barriers to the success of chemotherapy (5). Therefore, the identification of novel drugs for use in chemotherapy against esophageal squamous cell carcinoma is required (6).

Certain natural products have been suggested to be effective agents for cancer prevention, including epigallocatechin-3-gallate (EGCG). EGCG is the ester form of epigallocatechin/gallic acid; it is the main catechin in green tea and contributes to its beneficial therapeutic effects, which include antioxidant and immunomodulatory effects (7-10). Due to its reported anti-oxidant and immunomodulatory effects, EGCG has been extensively investigated against various types of cancer (11-13). EGCG has not been observed to cause adverse effects against normal cells and tissues, whereas it has anti-proliferative, anti-invasive and chemo-preventive effects against cancer cells (14). However, the number of investigations concerning the effect of EGCG on esophageal cancer is limited, and the potential function of EGCG in esophageal cancer therapy remains poorly understood. Cell growth and apoptosis are regulated through complex signaling systems in the human body; their disorder or imbalance may induce the development of tumors (15). The efficacy of chemotherapy drugs can be evaluated by their ability to induce apoptosis. The upregulation of caspase-3 and the reduction of mitochondrial membrane potential may result in apoptosis, so an ideal chemotherapy drug would cause these alterations (16,17).

The present study investigated the anticancer effects of EGCG in esophageal squamous cell carcinoma and the underlying molecular mechanisms in human esophageal squamous cell carcinoma cells.

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Materials and methods

Cancer cell lines and culture. Human esophageal Eca109 cancer cells were obtained from the Cancer Institution, The Fourth Hospital of Hebei Medical University (Shijiazhuang, China). Human esophageal Ec9706 cancer cells were obtained from the Molecular Oncology State Key Laboratory Cancer Institute and Hospital of the Chinese Academy of Medical Sciences (Beijing, China).

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Chemicals and reagents. EGCG was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was purchased from Beckman Coulter, Inc. (Brea, CA, USA). Mouse anti-human caspase-3 monoclonal antibodies (cat. no. sc-7272) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). FITC-conjugated goat anti-mouse secondary IgG antibody (cat. no., 115-095-003) was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA).

Cytotoxicity assay. The sensitivity of Eca109 and Ec9706 cells to EGCG was determined using an MTT assay, in which the capacity of viable cells to metabolize MTT reagent salt to purple formazan crystals via mitochondrial succinate dehydrogenase was assessed. Cells were seeded into 96-well culture plates at a density of 5x10⁴ cells/ml. Serial concentrations of EGCG (0, 25, 50, 100, 200 and 400 mg/l) were added in a final volume of 200 µl per well. Following treatment for 24 or 48 h at 37°C, the medium was replaced with an equal volume of fresh medium containing 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA) and incubated for 4 h. Then, the medium was removed and 180 µl DMSO was added and incubated for 10 min at room temperature. The cytotoxic effects of drugs were determined according to the OD values using a microplate reader at an absorption wavelength of 490 nm. Cell viability was expressed as the relative formazan formation in treated samples when compared with control cells: Growth inhibitory rate=[(1-A490 treated cells/A490 control cells)x100%].

Apoptosis analysis. Cultured Eca109 and Ec9706 tumor cells treated with 0, 100, 200 or 300 mg/l EGCG for 24 h at 37°C were harvested. The cells (1x10⁶) were stained with PI and Annexin V-FITC, according to the manufacturer's protocol and analyzed using an Epics-XL flow cytometer (Beckman Coulter, Inc.). Expo32 v1.2 software (Beckman Coulter, Inc.) was used to analyze the flow cytometric data. Early apoptotic cells were positive for Annexin V and negative for PI staining, whereas late apoptotic cells undergoing secondary necrosis were positive for Annexin V and PI staining.

Analysis of mitochondrial membrane potential expression level in Eca109 and Ec9706 cells. Cultured Eca109 and Ec9706 tumor cells (1x10⁶) were harvested following treatment with 0, 100, 200 or 300 mg/l EGCG for 24 h at 37°C.

Following two washes with ice-cold PBS, the cells were dyed in 1 ml of 10 µg/ml Rhodamine 123 dissolved in distilled water. Following incubation for 30 min in the dark at 37°C and two washes with ice-cold PBS, the stained cells were resuspended in 1 ml PBS. The stained cells were analyzed using an Epics-XL flow cytometer. Expo32 v1.2 software was used to analyze the flow cytometric data.

Analysis of caspase-3 protein. Cultured Eca109 and Ec9706 tumor cells (1x10⁶) were harvested following treatment with 0, 100, 200 or 300 mg/l EGCG for 24 h at 37°C. Cells were fixed overnight with 70% ice-cold ethanol. Following two washes with ice-cold PBS, the fixed cells were resuspended in 1 ml PBS containing caspase-3 antibody (dilution, 1:100) and incubated for 30 min in the dark at room temperature. Following two washes with PBS, cells were resuspended in 1 ml PBS containing secondary FITC-conjugated immunoglobulin G antibodies and incubated for 30 min in the dark at room temperature. An isotype control group with no primary antibody was used to exclude nonspecific binding. Following two washes with PBS, cells were resuspended in 1 ml PBS. The stained cells were analyzed using an Epics-XL type flow cytometer. Expo32 v1.2 software was used to analyze the flow cytometric data.

Analysis of telomerase activity. Telomerase activity was determined using a telomeric repeat amplification protocol silver staining kit (cat. no. GMS 20106.1C.1; Genmed Scientifics, Inc., Shanghai, China) according to the manufacturer's protocol. Subsequent to silver staining, a positive outcome was bands with a 6 bp interval. Cultured Eca109 and Ec9706 tumor cells (1x10⁶) were harvested following treatment with 0, 100, 200 or 300 mg/l EGCG for 24 h at 37°C, then telomerase activity was detected.

Statistical analysis. All data were presented as the mean ± standard deviation and were statistically analyzed using a one-way analysis of variance followed by the Newman-Keuls method for post hoc comparisons, using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory effect of EGCG on Eca109 and Ec9706 cells. The effect of EGCG on the viability of Eca109 and Ec9706 cells was analyzed by MTT assay. The viability of Eca109 and Ec9706 cells was significantly inhibited by EGCG in a dose and time dependent manner (Fig. 1).

EGCG induced esophageal cancer cell apoptosis. Experiments were performed using Eca109 and Ec9706 human esophageal cancer cells. Exposure to EGCG for 24 h was demonstrated to induce apoptosis in a dose-dependent manner in Eca109 and Ec9706 cells. Annexin V/PI staining revealed that EGCG treatment induced apoptosis in the range of 100 to 300 mg/l (Fig. 2).

EGCG modulates Eca109 and Ec9706 cell mitochondrial membrane potential. Eca109 and Ec9706 cells were treated with 0, 100, 200 or 300 mg/l EGCG for 24 h, washed with

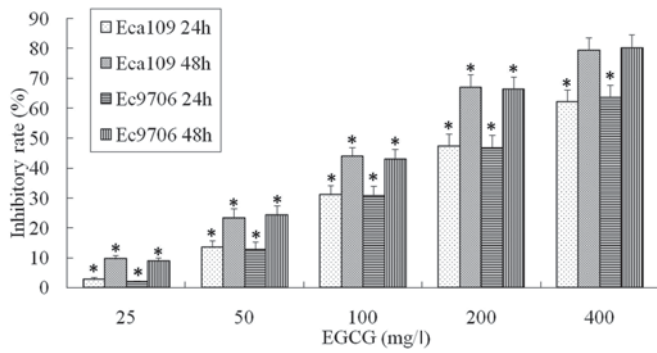


Figure 1. Inhibitory effect of EGCG on Eca109 and Ec9706 cells, as assessed by MTT assay. The viability of Eca109 and Ec9706 cells was significantly inhibited by EGCG in a dose and time dependent manner. *P<0.05 vs. 400 mg/l EGCG 48 h group. EGCG, epigallocatechin-3-gallate.

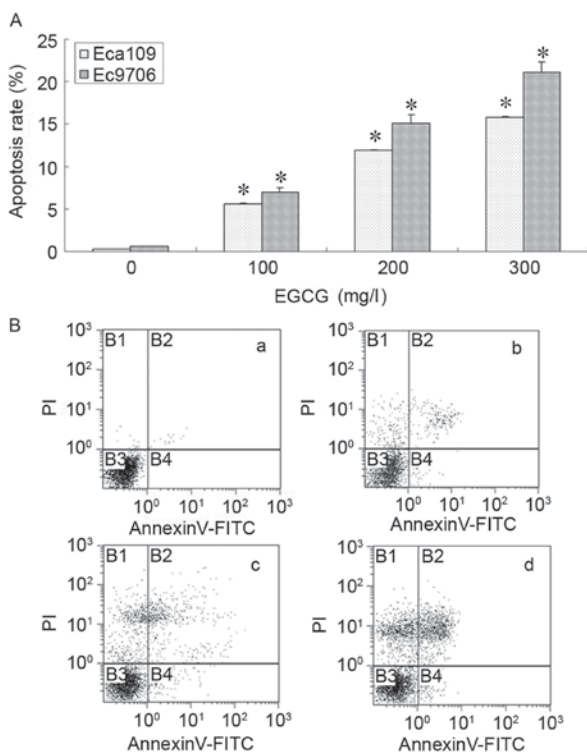


Figure 2. Eca109 and Ec9706 cell apoptosis following treatment with 100, 200 or 300 mg/l EGCG. (A) Apoptosis rate of Eca109 and Ec9706 cells following EGCG treatment. (B) Apoptosis rate of Ec9706 cells following treatment with EGCG for 24 h, detected by flow cytometry. a, control group; b, 100 mg/l EGCG group; c, 200 mg/l EGCG group; and d, 300 mg/l EGCG group. *P<0.05 vs. 0 mg/l EGCG group of that cell type. EGCG, epigallocatechin-3-gallate; FITC, fluorescein isothiocyanate; PI, propidium iodide.

cold PBS, and flow cytometry was used to analyze the mitochondrial membrane potential. The mitochondrial membrane potential in the EGCG-treated groups was significantly lower compared with the control group (P<0.01; Fig. 3). The 300 mg/l EGCG group had significantly lower mitochondrial membrane potential compared with the 100 and 200 mg/l EGCG groups (P<0.01; Fig. 3).

Caspase-3 expression levels were increased following EGCG treatment in Eca109 and Ec9706 cells. Eca109 and Ec9706 cells were treated with 0, 100, 200 or 300 mg/l EGCG for 24 h,

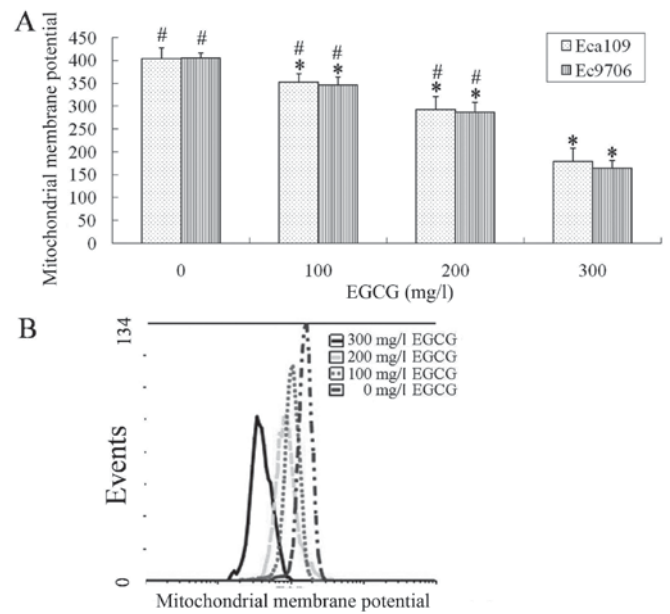


Figure 3. Mitochondrial membrane potential of Eca109 and Ec9706 cells following treatment with EGCG. (A) Mitochondrial membrane potential of the EGCG group was significantly lower compared with the control group. (B) Mitochondrial membrane potential of Ec9706 cells following treatment with 100, 200 or 300 mg/l EGCG, detected by flow cytometry. *P<0.01 vs. 0 mg/l EGCG group; #P<0.01 vs. 300 mg/l EGCG group of that cell type. EGCG, epigallocatechin-3-gallate.

washed with cold PBS, and caspase-3 protein expression was analyzed using flow cytometry. Caspase-3 protein expression level was significantly upregulated in the treatment groups compared with the controls (P<0.05; Fig. 4). Additionally, caspase-3 protein expression level was significantly higher in the 300 mg/l EGCG group compared with the 100 and 200 mg/l EGCG groups (P<0.05).

EGCG inhibited telomerase activity in Eca109 and Ec9706 cells. Following treatment with 100, 200 or 300 mg/l EGCG for 24 h, the telomerase activity in Eca109 and Ec9706 cells was significantly inhibited compared with control cells (P<0.05; Fig. 5). Additionally, telomerase activity was significantly lower in the 300 mg/l EGCG group compared with the 100 and 200 mg/l EGCG groups (P<0.05).

Discussion

EGCG is the most abundant catechin in green tea, and it possesses anti-inflammatory, antioxidant, immunomodulatory and anticancer functions (18-20). Previous studies have suggested that EGCG is associated with the potential health benefits attributed to green tea consumption (21). The anti-cancer effect of EGCG has been explored in various tumor cells (22-24), but there are few articles concerning the anti-cancer effect of EGCG on esophageal cancer. In the present study, EGCG was demonstrated to suppress the viability of esophageal cancer Eca109 and Ec9706 cells via inducing apoptosis in an EGCG dose-dependent manner. EGCG has been reported to induce cancer cell apoptosis through different pathways involving the pro-oxidant, epigenetic modulation of apoptosis-related genes, including human telomerase reverse

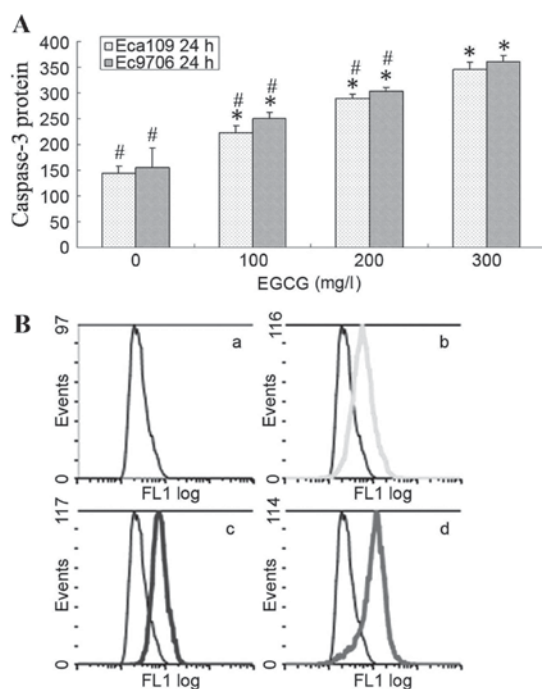


Figure 4. Caspase-3 protein expression of Eca109 and Ec9706 cells following treatment with EGCG. (A) Caspase-3 protein expression levels of the EGCG group were significantly increased compared with the control group. (B) Caspase-3 protein expression following treatment with 100, 200 or 300 mg/l EGCG was detected by flow cytometry. a, control group; b, 100 mg/l EGCG group; c, 200 mg/l EGCG group; and d, 300 mg/l EGCG group. * $P < 0.05$ vs. 0 mg/l EGCG group; # $P < 0.05$ vs. 300 mg/l EGCG group of that cell type. EGCG, epigallocatechin-3-gallate; FL1, fluorescence channel 1.

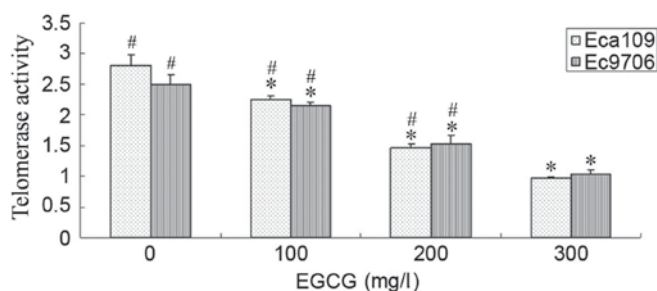


Figure 5. EGCG inhibited telomerase activity in Eca109 and Ec9706 cells. * $P < 0.05$ vs. 0 mg/l EGCG group; # $P < 0.05$ vs. 300 mg/l EGCG group of that cell type. EGCG, epigallocatechin-3-gallate.

transcriptase, and to reduce cell proliferation through the modulation of cell cycle progression (25-27).

In the present study, two esophageal cancer cell lines, Eca109 and Ec9706, were selected to test the potential anticancer effects of EGCG on esophageal squamous cell carcinoma cells. The tumor-suppressive effects of EGCG against esophageal cancer cells were thus investigated *in vitro*. The results of an MTT assay demonstrated that EGCG inhibited the viability of Eca109 and Ec9706 cells in a dose- and time-dependent manner. Flow cytometric results indicated that the EGCG also induced apoptosis in Eca109 and Ec9706 cells in a dose-dependent manner. However, the molecular mechanisms underlying EGCG-induced apoptosis in esophageal cancer cells remain poorly understood. In the present study, EGCG was reported to exert cytotoxic effects on human

esophageal cancer cell lines *in vitro*. This cytotoxicity was revealed to be mediated by apoptosis, a conclusion supported by apoptosis detection and expression of the apoptosis-associated protein caspase-3. Apoptosis is involved in the maintenance of cell homeostasis, and dysfunction of apoptotic signaling may result in serious conditions, including cancer.

At present, apoptosis is the most well-studied mechanism associated with anticancer therapy. Apoptosis is cell death under genetic control, involving complicated regulatory mechanisms. Mitochondrial transmembrane potential loss is able to induce apoptosis. In the present study, the pro-apoptotic effect of EGCG on Eca109 and Ec9706 cells was assessed *in vitro*, with focus on the mitochondrial pathway. The apoptosis rate was detected using Annexin V/PI staining and a flow cytometer. Treatment with various concentrations of EGCG was revealed to promote apoptosis of Eca109 and Ec9706 cells in a dose-dependent manner. The reduction of cell proliferation is associated with apoptosis. Mitochondrial transmembrane potential, an essential effector of the intrinsic pathway of apoptosis, was downregulated following EGCG treatment. Mitochondrial transmembrane potential downregulation is associated with the induction of apoptosis. Caspase-3 protein was upregulated in a dose-dependent manner in Eca109 and Ec9706 cells following treatment with EGCG. In the present study, EGCG induced Eca109 and Ec9706 cell apoptosis by downregulating mitochondrial membrane potential and upregulating caspase-3 expression levels.

Telomerase activity allows eukaryotic cells to have unlimited division potential. While functioning, telomerase synthesizes short DNA repeats at the 3'-end of DNA within chromosomes, ensuring genome stability during cell division. Telomerase is active in the majority of cancer cell types. Meanwhile, telomerase activity is essential for survival of malignant cells. The present study revealed that telomerase activity in Eca109 and Ec9706 cells was downregulated following EGCG treatment.

The specific mechanism inhibiting the growth of esophageal cancer cells by EGCG was explored in the present study. EGCG inhibited cell viability and induced esophageal cancer cell apoptosis, reducing the mitochondrial membrane potential and telomerase activity while increasing caspase-3 expression levels. As EGCG has the characteristics of low toxicity and few side effects, if it can be developed as an anti-tumor drug, it will have broad application prospects.

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