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Cell Growth Inhibition and Gene Expression Regulation by (-)-Epigallocatechin-3-Gallate in Human Cervical Cancer Cells

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EGCG [(-)-epigallocatechin-3-gallate] has shown its antitumor ability and perhaps a potential regimen for cancer patients. The goal of this study was to investigate the effect of EGCG on human papilloma virus (HPV) positive cervical cancer cell lines. EGCG inhibited the growth of CaSki (HPV16 positive) and HeLa (HPV18 positive) cells in a time- and concentration-dependent manner. Cell cycle arrest and apoptosis were observed in two cell lines after EGCG exposure. More importantly, we focused on EGCG regulation ability on pivotal genes involved in cervical cancer: viral oncogenes E6/E7, estrogen receptor (ER) and aromatase. Our results suggested that EGCG may be suitable for prevention and treatment of cervical cancer.

Key words: EGCG, Cervical cancer, HPV, ER, Aromatase

INTRODUCTION

(-)-Epigallocatechin-3-gallate (EGCG), the most abundant and active tea catechin, is best known for its various biological and pharmacological properties including antioxidative, antibacterial, antiviral and antitumor activity. The chemopreventive and chemotherapeutic effect of EGCG has been verified against cancers of breast, ovarian, skin, lung, colon, liver, stomach, prostate and other sites both *in vitro* and *in vivo* (Yang et al., 2002; Mukhtar and Ahmad, 2000; Kim et al., 2005). In our opinion, given that EGCG blood concentration is extremely low through oral administration (Kim et al., 2000), it seems that EGCG is most likely to be used for skin cancer and cervical cancer due to the topical administration to reach a very high concentration.

Molecular epidemiologic study has been firmly established the etiological relationship between human papilloma virus (HPV) infection and cervical cancer (Walboomers et al., 1999). Approximately 96.6% of cervical cancer patients are infected with HPV. Type 16 and 18 are two major high-risk types (Munoz et al., 2003). Viral oncogenes E6 and E7 are vital for carcinogenesis, progression, invasion and metastasis of cervical cancer (Munger and Howley, 2002). Moreover, it has been reported that estrogen may play a synergistic role with HPV in cervical carcinogenesis by increasing expression of E6/E7 (Moodley et al., 2003). This means that inhibition of E6/E7 and estrogen may be a feasible way for prevention and treatment of cervical cancer.

The purpose of this study was to examine the effect of EGCG on HPV positive cervical cancer cell lines. We found that EGCG inhibited cell growth and regulated gene expression of E6/E7, estrogen receptor (ER) and aromatase which have high correlation with cervical cancer.

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MATERIALS AND METHODS

Cells and cell culture conditions

Human cervical cancer cell lines, CaSki (HPV16 positive) and HeLa (HPV18 positive), obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in RPMI 1640 and DMEM medium respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in humidified atmosphere of 5% CO_2.

EGCG (Sigma, St. Louis, MO) was dissolved in sterile 50% DMSO (dimethylsulfoxide) at 100 mM and stored at -20°C before use (Huh et al., 2004). The final concentration of DMSO in culture medium was 0.005-0.05% (v/v). In all experiments control cultures were made up of medium, DMSO and cells. DMSO in such low concentration had no effect on the parameters measured (Opare Kennedy et al., 2001).

MTT cell viability assay

The effect of EGCG on cell proliferation was examined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. CaSki and HeLa cells (10^4 per well) were seeded in 96-well plates and allowed to grow 24 h. Then EGCG or DMSO (vehicle control) was added at specified concentrations. Each treatment was quadruplicate. After 24 and 48 h, 20 μL of MTT solution (5 mg/mL; Sigma, St. Louis, MO) was added to each well and incubated for 4 h at 37°C. The MTT formazan crystal was then dissolved in DMSO, and the absorbance was measured by a microplate reader (Bio-Rad 680, USA) at a wavelength of 490 nm.

Flow cytometry

CaSki and HeLa cells were treated with 25 μM and 50 μM of EGCG for 24 h respectively. After treatment, cells were collected and resuspended in propidium iodide (PI) solution (0.1% Triton X-100, 0.2 mg/mL RNase A, 20 μg/mL PI) at room temperature for 30 min. Cell cycle distribution was analyzed on a EPICS ELITE ESP flow cytometer (Beckman Coulter) using Multicycle software. Each treatment was triplicate.

DAPI staining

To observe the nuclear morphology of apoptotic cells, CaSki and HeLa cells treated with EGCG for 24 h were stained with DAPI (4', 6-diamidino-2-phenylindole; Keygentec Biotechnology, Beijing), and examined immediately under a fluorescence microscope. The nuclei were visualized with blue staining under fluorescence and apoptotic cells were defined on the basis of nuclear morphology changes including nuclei shrinkage, chromatin condensation and nuclei fragmentation.

Quantitative polymerase chain reaction (QPCR)

CaSki and HeLa cells were treated with 25 μM and 50 μM EGCG for 6, 12 and 24 h respectively. DMSO vehicle controls were set at the same time. Then total RNA was isolated with Trizol reagent (Invitrogen Carlsbad, CA). First-strand cDNA was reverse transcribed (RT) according to the manufacturer's protocols. The QPCR primer sets of human ERα, ERβ, aromatase, E6 and E7 for HPV16 and HPV18, and β-actin were designed by Primer Express 2.0 software and the sequence and amplicon size were shown in Table I. Relative levels of mRNA were determined by QPCR using ABI Prism 7900HT real time PCR system (Applied Biosystems, Foster City, CA, USA). Amplifications

Table I. Oligonucleotide primers used in QPCR

Gene Name	Primer 5' → 3'	Amplicon Size
HPV16E6	5'-AGGAGCGACCCAGAAAGTTACC-3'	101 bp
	3'-TCGCAGTAACTGTTGCTTGCA-5'	
HPV16E7	5'-CCGGACAGAGCCCATTACAAT-3'	118 bp
	3'-TGCCCATTAACAGGTCTTCCA-5'	
HPV18E6	5'-GGTGCCAGAAACCGTTGAATC-3'	101 bp
	3'-CGAATGGCACTGGCCTCTATAG-5'	
HPV18E7	5'-CGAACCACAACGTCACACAAT-3'	102 bp
	3'-TGCTGGAATGCTCGAAGGT-5'	
ERα	5'-GAATGTGCCTGGCTAGAGATCCT-3'	101 bp
	3'-TTCCTGTCCAAGAGCAAGTTAGG-5'	
ERβ	5'-CAAGTTGGCCGACAAGGAGTT-3'	113 bp
	3'-CCATCCAACAGCTCTCCAAGAG-5'	
Aromatase	5'-TGATGAAAGCCATCCTCGTTAC-3'	105 bp
	3'-GGTGCAAGGACAAGTCGTGTAT-5'	
β-actin	5'-CCGAGGACTTTGATTGCACA-3'	101 bp
	3'-AGTGGGGTGGCTTTTAGGAT-5'	

were carried out using 2 μL of cDNA, 12 μL of 2 × SYBR Green PCR Master Mix (ABI), 0.3 μL of primers (F/R) (15 pmol/μL), and 10.4 μL of H_2O in a final reaction volume of 25 μL. The cycling parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1min, and the melting curve was analyzed at the end to ensure the product specificity. Each sample was performed in triplicate. The data were analyzed with the comparative C_T ($\Delta\Delta C_T$) method and the amount of target genes ($2^{\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference (β-actin) and compared to the respective control group (Livak and Schmittgen, 2001).

Western blotting

CaSki and HeLa cells were treated with 25 μM and 50 μM EGCG for 6, 12 and 24 h respectively. DMSO vehicle controls were set at the same time. Then cells were washed with PBS and lysed in a buffer containing: 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1% NP-40, and 1 mM PMSF. Protein concentration was determined with BCA protein assay kit. The equal amount of protein was subjected to electrophoresis in a 12% polyacrylamide-SDS gel and then transferred onto a PVDF membrane using semi-dry transfer system (Bio-Rad). The membrane was blocked in 5% non-fat milk for 1 h at room temperature then incubated overnight at 4°C with indicated primary antibodies. The primary antibodies were mouse monoclonal HPV16/18 E6 (1:500) and β-actin (1:4000), rabbit polyclonal ERα (1:300) and ERβ (1:300) (Santa Cruz, CA, USA), mouse monoclonal aromatase (1:200) (Serotec, Oxford, UK) and p53 (1:200) (Fisher Scientific, CA, USA). After washed, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. SuperSignal West Pico Chemiluminiscence Substrate (Pierce Biotechnology, USA) was used for detection antibodies. The membranes were wrapped by Sarah membrane and exposed to film for 1-3 min. After development, film was scanned for the quantification using Bio-Rad Quantity One software. All target signals were normalized with the β-actin in the same sample.

RESULTS

Inhibition of cell growth by EGCG

The chemical structure of EGCG is shown in Fig. 1. MTT assay showed that EGCG treatment resulted in a time- and concentration- dependent inhibition of cell proliferation, and 50% inhibition concentration (IC_{50}) at 24 h appeared to be 27.3 μM for CaSki, and 47.9 μM for HeLa cells. CaSki cells (Fig. 2A) appeared more sensitive to EGCG than HeLa cells (Fig. 2B).

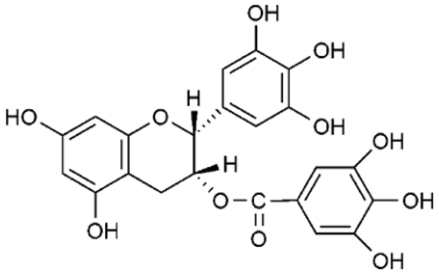

Fig. 1. Chemical structure of (-)-epigallocatechin-3-gallate (EGCG)

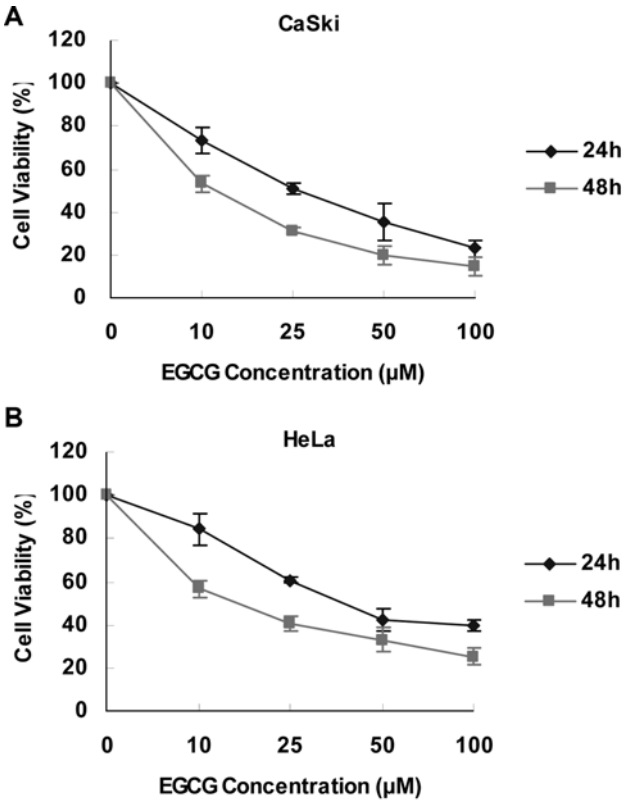

Fig. 2. Cell proliferation inhibition by EGCG was determined by MTT assay. CaSki (**A**) and HeLa (**B**) cells were treated with indicated EGCG concentration (μM) for 24 and 48 h. Control cultures were exposed to the vehicle control DMSO. Cell proliferation was expressed as a percentage of viable cells of control. Data are expressed as mean ± S.D., n = 3.

Induction of cell cycle arrest and apoptosis by EGCG

Flow cytometry showed EGCG modulated cell cycle progression in both cell lines. In CaSki cells, EGCG induced significant increase in S phase from 38.3% to 46.4%, G_2/M phase from 7.4% to 16.9%, while decrease in G_0/G_1 phase from 54.2% to 36.7% (Fig. 3A). In HeLa

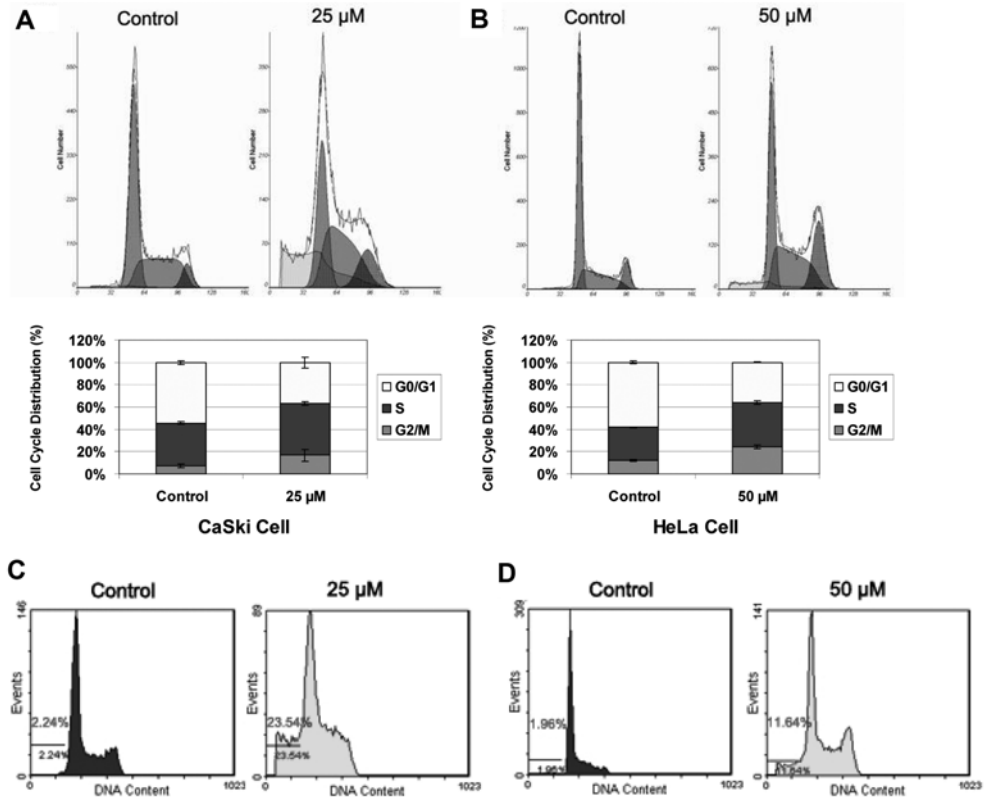

Fig. 3. Effect of EGCG on cell cycle distribution analyzed by flow cytometry. Treatment with 25 μM or 50 μM EGCG for 24 h led to significant G_2/M arrest and sub-G_0/G_1 augmentation in CaSki (**A**, **C**) and HeLa (**B**, **D**) cells.

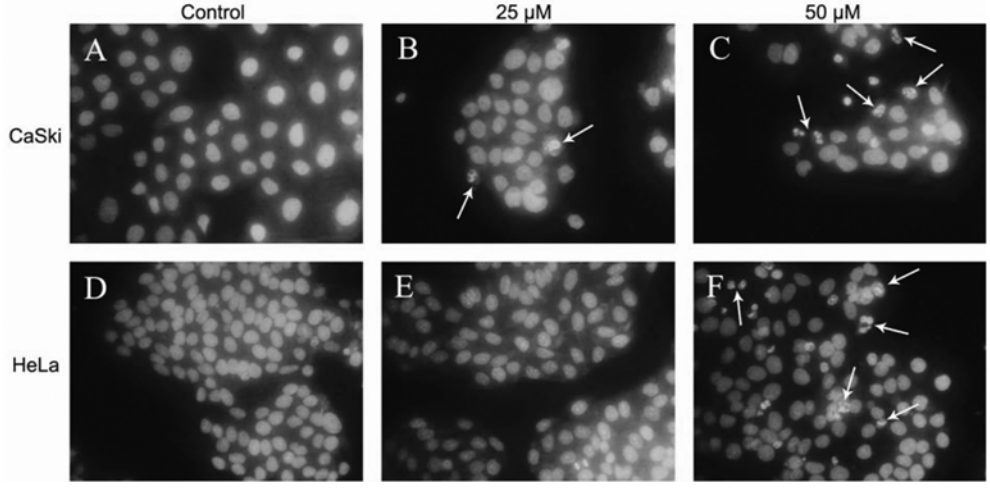

Fig. 4. Apoptosis induced by EGCG in CaSki (**A**, **B** and **C**) and HeLa (**D**, **E** and **F**) cells. Analysis was done by fluorescence microscope after nuclear staining with DAPI. A, D was control for CaSki and HeLa cells respectively. Cells were treated with 25 μM (B and E) or 50 μM (C and F) EGCG for 24 h. Cells undergone apoptosis showing nuclei shrinkage, chromatin condensation and nuclei fragmentation, indicated by arrows. Magnification is 400×.

cells, EGCG induced significant increase in S phase from 29.8% to 39.8%, G_2/M phase from 12.1% to 24.4%, while decrease in G_0/G_1 phase from 58.1% to 35.8% (Fig. 3B). Furthermore, EGCG increased the portion before G_0/G_1 phase (sub-G_0/G_1) from 2.24% to 23.54% in CaSki cells (Fig. 3C), from 1.96% to 11.64% in HeLa cells (Fig. 3D).

Moreover, DAPI staining showed that EGCG significantly increased the content of chromatin condensation and nuclear fragmentation, an indicator of apoptosis, compared with control in both CaSki (Fig. 4A) and HeLa cells (Fig. 4D). CaSki cells showed more

condensed and fragmented DNA staining at 50 μM (Fig. 4C) than at 25 μM (Fig. 4B). In contrast, less change was observed in HeLa cells at 25 μM (Fig. 4E), but 50 μM EGCG can effectively induced apoptosis in HeLa cells (Fig. 4F).

Inhibition of E6 and E7 expression by EGCG

QPCR showed that EGCG caused approximately 30% to 60% reduction of E6 and E7 mRNA expression in CaSki (Fig. 5A) and HeLa (Fig. 5B) cells compared to controls. The inhibition occurred as early as 6 h and persisted up to 24 h. Western blotting showed that E6 protein was decreased, while p53 protein was significantly increased by EGCG at 24 h in both cell lines (Fig. 7).

Regulation of ERα, ERβ and aromatase by EGCG

As QPCR shown, ERα mRNA was down-regulated by EGCG in CaSki cells after 6 h treatment and kept at a low level even after 12 and 24 h (Fig. 6A). However, HeLa cells needed higher concentration and longer time to response to EGCG treatment (Fig. 6B). On the other hand, ERβ mRNA was slightly increased in CaSki (Fig. 6C) and HeLa cells (Fig. 6D). Consistent with QPCR results, western blotting provided further evidence to reveal the effect of EGCG on ERs. As shown in Fig. 7, ERα protein significantly decreased while ERβ protein indeed increased at varying degree in both cell lines after EGCG treatment.

EGCG decreased mRNA expression level of aromatase in both CaSki (Fig. 6A) and HeLa (Fig. 6B) cells as early as 6 h after treatment. Especially there was a 76% reduction in CaSki cells after 12 h and a 90% reduction in HeLa cells after 24 h treatment. The

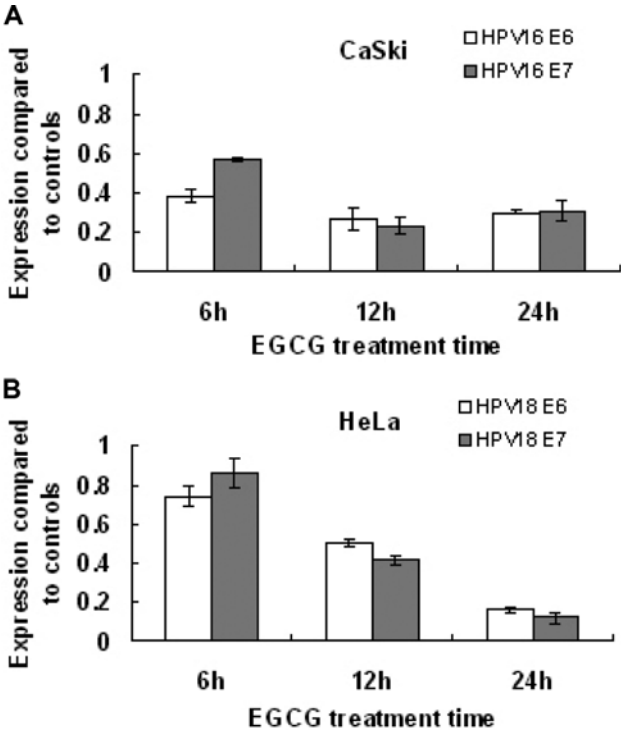

Fig. 5. Gene expression of E6 and E7 in CaSki cells (HPV16 positive) and HeLa cells (HPV18 positive) after treated with 25 μM and 50 μM EGCG respectively for 6, 12 and 24 h. Data are expressed as mean ± S.D., n=3.

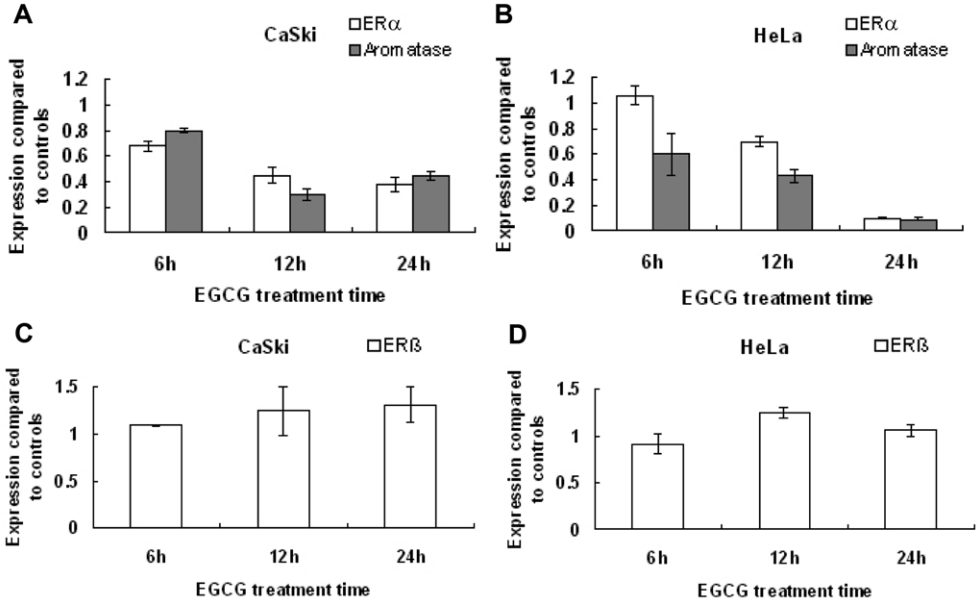

Fig. 6. Gene expression of ERα, ERβ, aromatase in CaSki and HeLa cells after treated with 25 μM and 50 μM EGCG respectively for 6, 12 and 24 h. Data are expressed as mean ± S.D., n=3.

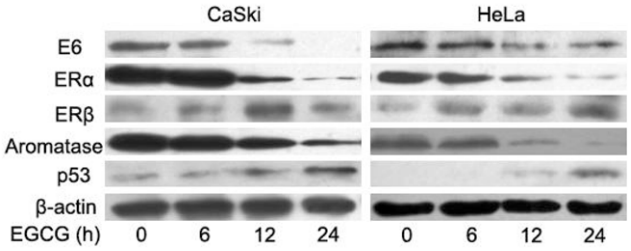

Fig. 7. Protein expression of E6, ERα, ERβ, aromatase and p53 in CaSki and HeLa cells after treated with 25 μM and 50 μM EGCG respectively for 6, 12 and 24 h

protein level of aromatase was significantly decreased in both cell lines with EGCG exposure time (Fig. 7).

DISCUSSION

The antiviral and antitumor properties make EGCG more feasible to be used against HPV positive cervical cancer. Recently, a clinical study showed that EGCG delivered in a form of ointment or capsule was effective in patients with HPV infected cervical lesions (Ahn et al., 2003). But the underlying mechanism was not focused on in that study.

In the present study we dwelt on the cell biology and molecular biology changes of HPV positive cervical cancer cell lines after EGCG exposure. We showed that EGCG inhibited growth of both CaSki and HeLa cells in a time- and concentration- dependent manner. We also observed CaSki cells were more sensitive to EGCG than HeLa cells, which was probably because the inhibitory effect of EGCG on squamous cancer was stronger than it on adenocarcinoma (Yokoyama et al., 2004). EGCG concentrations of 25 and 50 μM used here can be easily obtained around cervix if administrated through vagina. This low concentration has no toxicity for normal keratinocytes and is within the physiological range in humans, given the fact that daily topical application of 30 mg/ml EGCG (1309 times higher than 50 μM) for 30 days failed to induce dermal toxicity in BALB/c and SKH1 mice (Stratton et al., 2000; Hsu et al., 2003).

In this study, we observed EGCG induced cell cycle arrest at G_2/M phase in both cell lines. The portion before G_0/G_1 phase (sub-G_0/G_1) was significantly increased by EGCG, which indicates apoptosis happened. Furthermore, DAPI staining revealed typical morphology changes of cell apoptosis by EGCG in both cell lines.

HPV oncogenes E6 and E7 can disturb cell cycle regulation and impede apoptosis through binding to p53 and pRb (Mantovani and Banks, 2001; Scheffner and Whitaker, 2003). Our results demonstrated that EGCG strongly inhibited the expression of E6/E7 in both CaSki and HeLa cells at mRNA level. Western blot analysis showed that E6 protein decreased while p53 protein increased in a time-dependent manner after EGCG treatment. This perhaps meant that more p53 would be freed from E6 bound and restored to be function. So finally inhibited cervical cancer cell growth and induced apoptosis.

Estrogen has been reported to play an important role in HPV positive cervical cancer (Deligeoroglou et al., 2003; Elson et al., 2000; Moodley et al., 2003). Aromatase is the key enzyme for estrogen synthesis. Overexpression of aromatase in HPV positive cervical cancer cells resulted in increased expression and activity of ER, higher expression of E6/E7 and increased cell proliferation (Nair et al., 2005). Our experiment is the first time to demonstrate that EGCG can suppress mRNA and protein expression of ERα and aromatase in cervical cancer cell lines. It suggested that inhibition of ERα and aromatase by EGCG would limit expression of E6/E7, and thus indirectly inhibited cervical cancer cell growth and induced apoptosis. Interestingly, we observed that ERβ expression was slightly increased by EGCG both in CaSki and HeLa cells. Considering that ERα promotes while ERβ suppresses the proliferation and invasion of ovarian carcinoma (Bardin et al., 2004; O'Donnell et al., 2005), this is a good sign for EGCG against cervical cancer.

In all, our results showed that EGCG could inhibit the growth of HPV positive cervical cancer cells and induce apoptosis. Moreover, EGCG could inhibit expression of HPV E6/E7, ERα and aromatase which are pivotal genes involved in cervical cancer. Given that the high prevalence of HPV infection and the easy administration through vagina, our results strongly demonstrated that EGCG is a potential drug for prevention and treatment of cervical cancer.

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