Green Tea Compound in Chemoprevention of Cervical Cancer

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Objectives: Human papillomavirus (HPV) infection is closely associated with the development of more than 95% of cervical cancer. Clinical trials using several chemopreventive agents are underway, but results are inconclusive. Most agents used in trials inhibited the growth of cancer cells in vitro, and about half of patients had some degree of clinical responses; however, the therapeutic effect was confounded by high rates of spontaneous regression and relapse. The selection of nontoxic agents especially food, beverage, and natural products that suppress oncogenic HPV, inhibit malignant transformation, and can additionally be used long term may be important for cervical cancer prevention.

Methods: We evaluated green tea compound (epigallocatechin gallate and polyphenols E) effects on immortalized cervical epithelial and cervical cancer cells. HPV–immortalized cervical epithelial cells, TCL1, and HPV-positive cervical cancer cells, Me180 and HeLa, were used in the study. The effects of green tea compounds on cell growth, apoptosis, cell cycle, and gene expression were examined and characterized.

Results: Both epigallocatechin gallate and polyphenols E inhibited immortalized cervical epithelial and cancer cell growth. Apoptosis induction and cell cycle changes were observed in a dose-dependent manner. Western blot analysis of apoptosis-related proteins, p53 and p21, showed dose-dependent increase, whereas p27 was not affected. HPV–E7 protein expression was decreased by green tea compounds.

Conclusions: This study provides information on the potential mechanisms of action of green tea compounds in suppression of HPV-related cervical cells, and it will enable us to assess the feasibility of using these agents.

Key Words: Cervical cancer, HPV-E7, p53, p21

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Cervical cancer is the second leading cause of cancer deaths in women worldwide with 500,000 new diagnoses annually, most occurring in the developing world.[1] In the United States, there were approximately 9710 new diagnoses and nearly 3700 deaths in 2006.[2] Although the 5-year survival rate of cervical cancer in the US and Western Europe ranges from 70% to 65%, for the rest of the world, the rates are significantly lower (40%), reflecting the disproportionate burden of the disease in developing countries.[3] Despite a relatively low incidence and improved 5-year survival rates of cervical cancer for the US in general, important disparities contribute to substantial gaps for black, Hispanic, and economically disadvantaged patients.[2] Mortality rates have declined over the past decade because of the success of cytology-based screening for cancer precursors in developed world settings;

however, even in these locations, women with poor access to health care services continue to present clinically with high-grade cervical cancer precursors.

A persistent human papillomavirus (HPV) infection is a prerequisite for the development of precursor lesions and invasive cervical cancer. Invasive cervical cancer has a long premalignant phase termed cervical intraepithelial neoplasia (CIN). The protracted course from HPV infection to CIN and invasive disease make CIN an ideal candidate for chemoprevention.[4,5] During the past decade, the relationship between tea consumption and cancer has been a subject of research interest for many investigators. Recent reports have thoroughly reviewed and summarized epidemiological and experimental studies on tea and cancer prevention,[6–10] especially tea compound in inhibition of cervical carcinogenesis.[11–15] Experimental studies demonstrating the chemopreventive effects of tea have been conducted primarily with green tea, given the presence of highly polymerized components in black tea, which are not well characterized.

The evidence obtained from both in vitro and in vivo studies concerning potentially protective effects of green tea or green tea components is compelling. Green tea, green tea extract, green tea polyphenols, and epigallocatechin gallate (EGCG) have been shown to inhibit carcinogenesis induced by a wide variety of carcinogens in rodent cancer models.[16,17] Moreover, the cancer chemopreventive activity of these compounds has been demonstrated in variety of tissues and organs such as the colon, duodenum, esophagus, fore stomach, large intestine, liver, lung, mammary glands, and skin.[6–10]

A clinical trial using green tea compounds in subjects with persistent oncogenic HPV infection and low-grade cervical disease (CIN1) is being carried out in our group. In preparation for this effort, we conducted a series of experiments using immortalized human cervical epithelial cell lines or precancerous cell lines that mimic the CIN in vivo and carcinoma cell lines as in vitro models to explore potential mechanisms for the activity of green tea compounds and its precursors in cervical cancer. We investigated the effects of 2 green tea compounds, EGCG and polyphenol E (poly E), on growth inhibition, cell cycle, and apoptosis induction in cervical cancer and precancerous cell lines. The results will improve our understanding of the potential role of green tea compounds in cervical cancer chemoprevention.

MATERIALS AND METHODS

Cell Culture

The human cervical epithelial cell line, TCL1, and cervical carcinoma cell lines, HeLa and Me180, were provided by Reuben Lotan, PhD (MD Anderson Cancer Center, Houston, TX). The human cervical epithelial cell lines, TCL1, were primary cells electroporated with cloned viral DNAs from HPV types 16 and 18.[18] Cervical cells were grown in monolayer culture in a 1:1 (v:v) mixture of Dulbecco modified Eagle minimal essential medium and Ham F12 medium containing 5% fetal bovine serum at 37°C in the humidified atmosphere of 5% CO_2:95% air.

Green Tea Compounds

Epigallocatechin gallate was provided by Dr Shun-Jun Cheng's laboratory (Department of Chemical Etiology and Carcinogenesis, Cancer Institute, Peking Union Medical College, Beijing, China), and poly E was obtained from the National Cancer Institute Consortium Program. Each compound was dissolved in dH_2O for a final concentration of 10 mg/mL and stored in an atmosphere of N_2 at −80°C (for EGCG) and 4°C (for poly E).

Growth Inhibition Assay in Monolayer Culture

Exponentially growing cells (TCl1, Me180, and HeLa) were seeded at densities ranging from 1000 to 3000 cells per well in 96-well culture plates and treated the second day with concentrations of 0, 1, 5, 10, 25, and 50 μg/mL of EGCG or poly E. Cell growth inhibition was determined after 5 days of treatment using the crystal violet method as described previously.[18] Briefly, cells were fixed by 5% glutaraldehyde in phosphate buffered saline solution (PBS), rinsed with distilled water, and dried completely. Cells were incubated in a mixture (v/v) of 200 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid (pH 9.5) and 0.2% crystal violet at 25°C for 30 minutes and then washed and dried. Fixed and stained cells were rendered soluble with 10% glacial acetic acid, and the absorbance at 590 nm was determined using a plate reader. The percentage of growth inhibition was calculated according to the equation: inhibition = $(1 - N_t/N_c) \times 100$, where N_t and N_c are the numbers of cells in treated and control cultures, respectively. All experiments were performed in triplicate, and the mean ± SD were calculated and tested for statistical significance ($P < 0.05$).

Analysis of Apoptosis Induced by EGCG and Poly E

TCL1, Me180, and HeLa cells were cultured in the presence of 0, 5, 25, and 50 μg/mL of EGCG or poly E. After 2 days of incubation, cells were collected and fixed in 1% formaldehyde in PBS (pH 7.4). The terminal deoxynucleotidyl transferase–mediated fluorescein-deoxyuridine triphosphate nick-end labeling assay from Phoenix Flow Systems (San Diego, CA) was used to detect the apoptotic cells. Fixed cells were washed twice with 1 mL of wash buffer, and the pellet ($\sim 10^6$ cells) was resuspended in 50 μL of staining buffer. Cells were incubated at 37°C for 60 minutes and stained with 500 μL of propidium iodide/RNase A solution in the dark for 30 minutes at room temperature.

Cells were then analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA) with a 15-mW argon laser used for excitation at 488 nm. Fluorescence was measured at 542 to 585 nm. The Phoenix flow cytometry kit included suspensions of cells that served as negative and positive controls. The Cellquest Pro computer (BD Biosciences) with ModFit LT by Verity Software (Topsham, ME) was used to determine the percentage of apoptotic cells.

Cell cycle analysis by propidium iodide staining. TCl1, Me180, and HeLa cells were cultured in the presence of 0 and 50 μg/mL of poly E. After 2 days of incubation, cells were harvested by trypsinization, fixed in 1% paraformaldehyde for 30 minutes on ice then washed in PBS and resuspended in 70% ethanol containing 1% HCl for 10 minutes at −20°C. After washing, cells were stained with 500 μL of propidium iodide/RNase A solution in the dark for 30 minutes at room temperature.

Cells were analyzed using a FACScan flow cytometer (BD Biosciences) with a 15-mW argon laser used for excitation at 488 nm. Fluorescence was measured at 542 to 585 nm. The Cellquest Pro computer (BD Biosciences) with ModFit LT by Verity Software was used to determine the proportion of cells in G_1, S, and G_2 phases of the cell cycle. All experiments were performed in triplicate and the mean ± SD were calculated and tested for statistical significance ($P < 0.05$).

Western Blot Analysis

The cervical carcinoma Me180 cells were treated with different concentrations of EGCG (0, 5, 25, and 50 μg/mL) for 2 days. Total protein level was extracted, and 30 μg were loaded in each well. Protein was separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk followed by incubation with mouse IgG monoclonal antibodies against p53, p21, and p27 proteins (Santa Cruz Biotech, Santa Cruz, CA). Membranes were washed before incubating with a peroxidase-conjugated anti-mouse antibody (GE Healthcare, Piscataway, NJ), and immunoreactive bands were detected using an Enhanced Chemiluminescence reagent (GE Healthcare). After the x-ray detection of immunoreactive bands, the membranes were stripped and reused for detection of β-actin.

Immunohistochemistry

Cervical cancer cells (TCl1 and HeLa) were treated with 50 μg/mL of EGCG and poly E. After 2 days of incubation, cells were collected and fixed on pathologic slides. Cell membranes were made soluble, followed by a 30-minute inactivation of the endogenous peroxidase in 0.015% peroxide in methanol and a 10-minute rehydration in PBS. Cells were blocked for 30 minutes with 3% horse serum (normal horse serum) diluted in PBS, followed by 60-minute incubation with a mouse anti-HPV-E7 antibody. After washing, cells were incubated for another 60 minutes with a biotinylated secondary antibody against the mouse anti-HPV-E7antibody. Then, slides were washed in PBS and

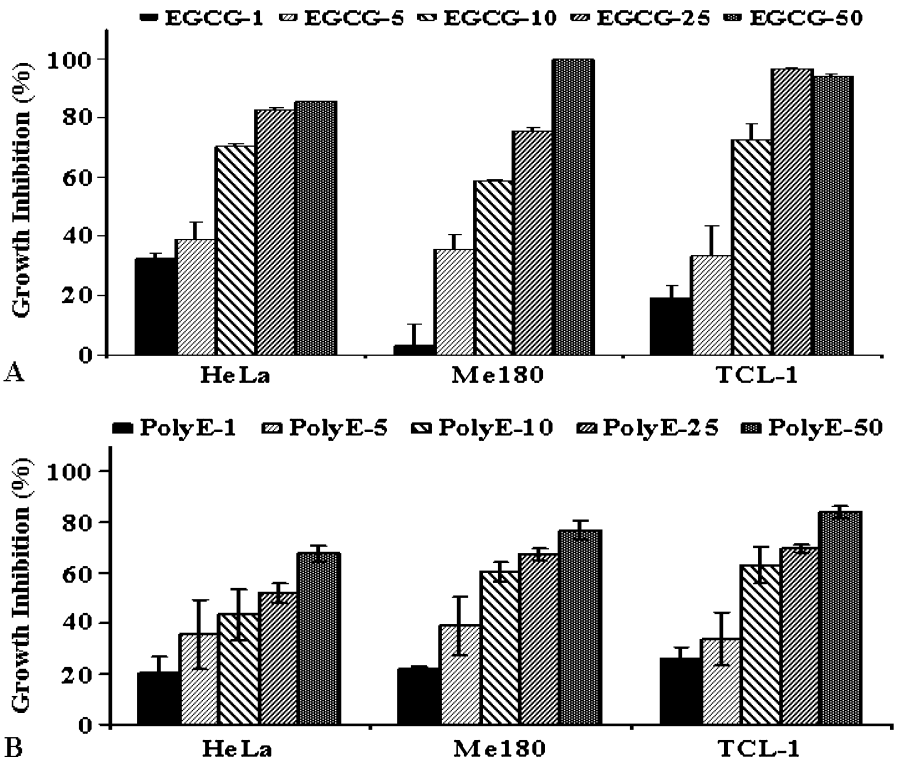

FIGURE 1. Effect of green tea compound on the growth of cervical epithelial cell line TCL1 and cervical cancer cell lines HeLa and Me180. Cells were treated with either EGCG (upper panel) or poly E (lower panel) at concentrations of 0, 1, 5, 10, 25, and 50 μg/mL for 5 days. Cells without any treatment were used as a control. The percentage of growth inhibition was calculated, as described in the ''Materials and Methods'' section. Cells were fixed and stained with crystal violet to measure the cell viability. The data was presented as the mean ± SD of triplicate determinations.

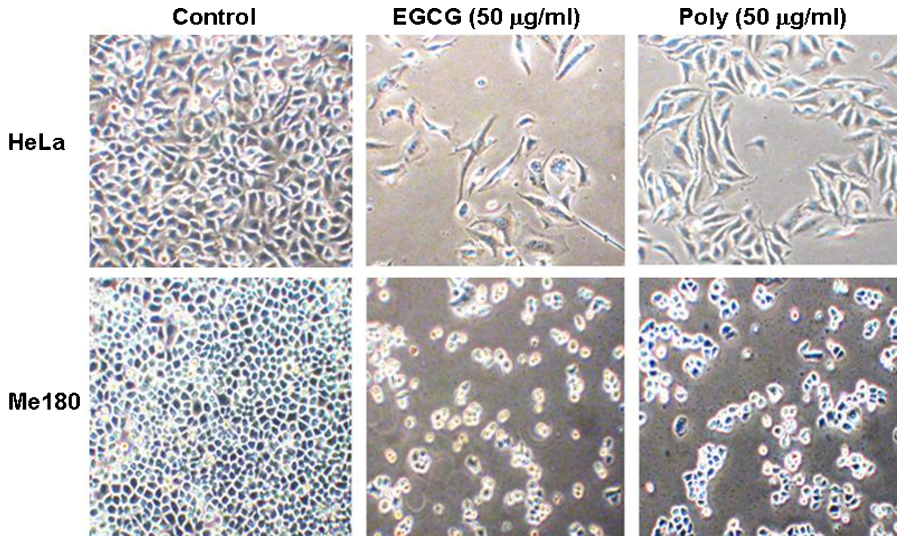

FIGURE 2. Representative morphologic changes induced by EGCG or poly E at 50 μg/mL in cervical cancer cell lines HeLa and Me180.

incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 minutes. The peroxidase reaction was developed with diaminobenzidine/peroxide, and the slides were washed, counterstained with hematoxylin, (Thermo Fisher Scientific, Inc., Waltham, MA) and observed under a light microscope. Positive and negative controls were included for each experiment, and all procedures were carried out at room temperature.

RESULTS

Growth Inhibitory Effect of EGCG and Poly E on Human Cervical Cell Lines

EGCG and poly E were tested at different concentrations (0, 1, 5, 10, 25, and 50 μg/mL) to compare the effect on the growth of immortalized human cervical epithelium (TCL1) cells and 2 cervical cancer cell lines (HeLa and Me180) grown in monolayer culture. The results summarized in Figure 1 demonstrate that at lower concentrations (1 and 5 μg/mL), EGCG and poly E induced a similar level of cell growth inhibition. The average inhibition rates were about 17.7% and 37.3% in cancer cells and 19.2% and 33.4% in TCL1 cells after EGCG treatment compared with 21% and 37% in cancer cells and 25.9% and 33.8% with ploy E treatment. At high concentrations (10, 25, and 50 μg/mL), EGCG caused a stronger growth inhibitory effect than poly E, irrespective of the cell types: 64.6%, 79.26%, and 92.8% in cancer cells and 72.7%, 96.6%, and 94.2% in TCL1 cells for EGCG; and 52%, 59.6%, and 72.2% in cancer cells and 62.9%, 69.6%, and 83.9% in TCL1 for poly E. Mean differences between low- and high-dose comparisons were significantly different. Figure 2 illustrates a representative image of the morphological changes induced by green tea compounds after 3 days of incubation and how these changes correlate with the agents' effects on cell growth.

Effects of Green Tea Compounds on Apoptosis and Cell Cycle

To assess the possible mechanisms of growth inhibitory effect of EGCG and poly E, apoptosis induction and cell cycle analysis were performed. Figure 3 shows that apoptosis is induced by EGCG in a dose-dependent statistically significant manner in all cell lines (TCl1, HeLa, and Me180), whereas a slight increase was observed only at 50 μg/mL with poly E (data not shown).

The cell cycle analysis performed with 50 μg/mL poly E shows an increase of cells in G_1 phase and a decrease of cells in S phase (data not shown, available upon request).

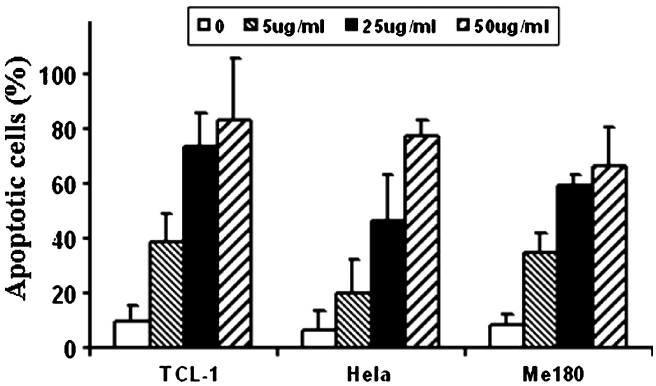

FIGURE 3. Effect of EGCG on apoptosis induction in TCL1, HeLa, and Me180 cells. Cells were treated with 50 μg/mL of EGCG for 5 days. The cells were stained with fluorescein-labeled fluorescein-deoxyuridine triphosphate to label DNA fragments by the terminal deoxynucleotidyl transferase–mediated fluorescein-deoxyuridine triphosphate nick-end labeling method. The percentage of apoptosis cell population is shown in histogram.

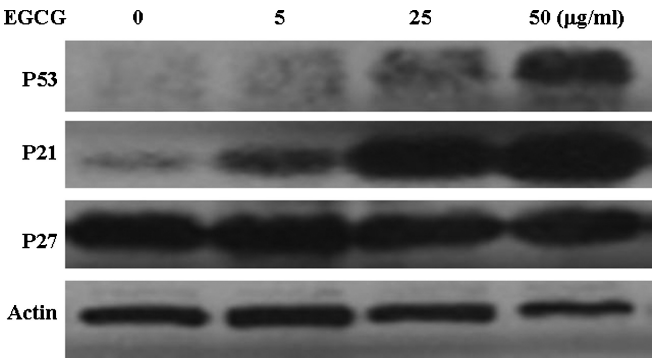

FIGURE 4. Representative effect of EGCG on p53, p21, and p27 protein levels in cervical cancer Me180 cells. Proteins were extracted from cells treated with 5, 25, and 50 μg/mL for 3 days. Thirty micrograms of proteins per lane was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The p53, p21, and p27 proteins were identified by blotting with monoclonal antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence method (Materials and Methods section). The blots were stripped and then reblotted to mouse anti-β-actin antibody for assessment of loading in each lane.

The same profile was observed with both immortalized (TCL1) and cancer (HeLa and Me180) cells.

Modulation of Gene Expression Green Tea Compounds

The effects of different concentrations of EGCG (0, 5, 25, and 50 μg/mL) on the expression of the apoptosis-associated genes p53, p21, and p27 were examined in Me180 cells (Fig. 4). The results show a dose-dependent increase in p53 and p21 protein expression. The level of p27 protein expression was unchanged.

Immunohistochemistry Staining of HPV-E7 Expression in Cervical Cells

The expression of HPV-E7 was examined by immunohistochemistry in TCL1 and HeLa cells treated with 0 or 50 μg/mL EGCG or poly E. A strong positive staining of HPV-E7 expression was observed in control group, whereas both EGCG- and poly E-treated groups showed a decrease in HPV-E7 expression (Fig. 5).

DISCUSSION

Cancer of the cervix is the third most common malignancy worldwide in women and the most common gynecologic cancer in the developing world. In developed countries, prevention of cervical cancer achieved by the widespread and systematic use of cervical cytologic screening has contributed to the successful decrease in the incidence of invasive cervical carcinomas. In the developing world, cervical cancer remains a common malignancy impacting the lives of women during their period of highest productivity. Especially in low-resource settings, an inexpensive dietary chemopreventive intervention would be an attractive adjunct to existing cervical cancer prevention programs.

It is well known that the regular consumption of fruits and vegetables is highly associated with the reduced epidemiologic risk of different types of cancer,[18–21] and green tea consumption is associated with lowering certain cancer incidences. This has not been demonstrated for cervical cancer specifically.

Poly E and (−)-EGCG are one of the various polyphenols found in green tea. In this study, we investigated the

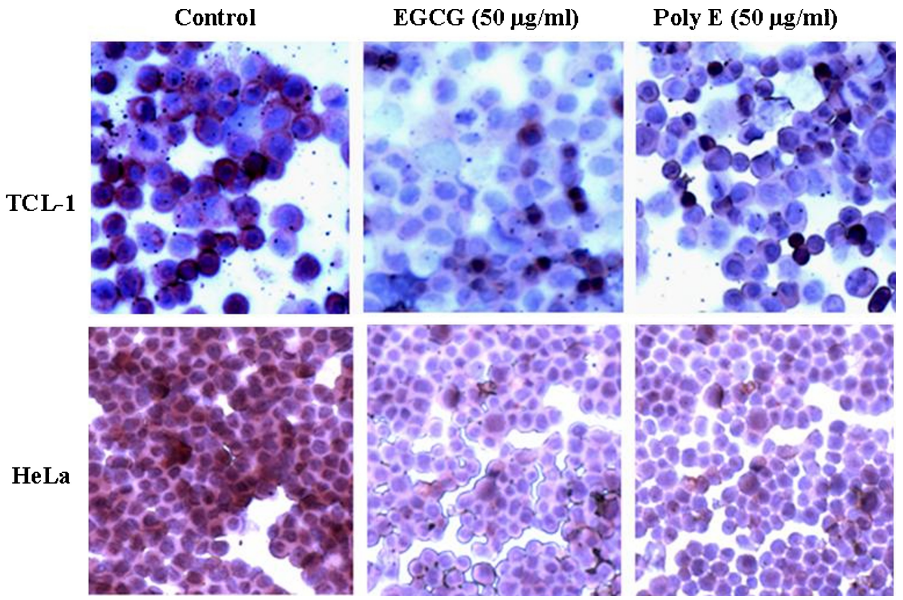

FIGURE 5. Representative immunohistochemistry staining of HPV-E7 in cervical cells treated with green tea compound. The expression of HPV-E7 in TCL1 and HeLa cells treated with 0 or 50 μg/mL of EGCG or poly E were stained by immunohistochemistry.

chemopreventive effect of EGCG, the major component of green tea polyphenols, and poly E on the growth of HPV-positive (HeLa, adenocarcinoma and Me180, and squamous carcinoma) and HPV-immortalized cervical epithelial (TCL1) cells. Results showed that EGCG and poly E inhibit the growth of both immortalized TCL1 cells and cancer cell lines HeLa and Me180. In cancer cell lines, cell growth rate was inhibited by the high concentration of poly E and EGCG (50 μg/mL: 76.9% and 100%) in squamous cell carcinoma cell line, Me180, whereas growth inhibition was less in adenocarcinoma cell lines, HeLa (67.5% and 85.6%), suggesting that growth inhibition by treatment with green tea polyphenol may be modulated by cellular type.

The EGCG-induced growth inhibition was stronger than that of poly E. Nevertheless, the effect of poly E on cell growth was observed at higher concentrations (25 and 50 μg/mL) than that of EGCG (from 10 μg/mL). Most studies focused only on EGCG, which is by far the most pharmacologically active green tea polyphenol, and our findings are consistent with previous studies reporting the beneficial effects of EGCG on different types of human cancer and cancer cell lines (lung, colon, breast, cervix, etc.).[8,11,12,14,15,22–24] Our result suggests that green tea poly E at higher concentrations may be a potential chemopreventive agent.

In this study, the concentrations of EGCG and poly E, 0, 1, 5, 10, 25, and 50 μg/mL, were consistent with the previous studies showing an inhibition of growth of HPV-18 immortalized cervical cells and cervical cancer cell lines.[14,15] The dose-dependent effects of EGCG and poly E on growth inhibition were also consistent with previous reports.[14,15] At present, the physiological extra- and intra-cellular levels of green tea polyphenols are unknown. Even the amount of tea ingestion necessary to obtain noticeable benefits is still under debate. It has been reported that a cup of tea contains approximately 120 to 150 mg of EGCG, which is equivalent to 300 μmol.[9,25] For a single cup of tea, Lin et al[26] estimated a maximum of 60 μmol/L of EGCG in the human blood, whereas Lee et al[7] measured a range of 0.2 to 0.65 μmol/L in human blood plasma after consumption of 2 to 3 cups of green tea. The difference between these data may be attributed to the biodegradability of the ingested tea and polyphenols and/or the delay between ingestion and EGCG measurement. Other studies have suggested amounts of about 10 cups.[27,28] Our data suggest that a regular and high consumption of green tea may contribute to the prevention of cervical cancer.

Several mechanisms of cancer inhibition by green tea polyphenols have been proposed. We investigated the regulation of apoptosis and cell cycle progression, which could be important targets for cancer chemoprevention.[9,29–33] For this purpose, experiments were run 1 day before the onset of the significant inhibition of cell growth, allowing a better evaluation of events occurring downstream.

The dose-dependent increase of apoptosis in immortalized and cancer cells in the presence of EGCG is in agreement with other reports on human carcinoma cells.[11,12,15,34] The oxidative properties of EGCG in cancer cells have been suggested to explain the increase of apoptosis.[34,35] Indeed, higher concentrations (100–200 μmol/L) of EGCG appear to be associated with an intracellular production of free radicals, whereas low concentrations (10 μmol/L) have antioxidative properties.[14,34] In the present study, 5 μg/mL (equivalent of 10 μmol/L) of EGCG induced a significant increase of apoptosis in both immortalized TCL1 and squamous carcinoma Me180 cell lines, whereas only 25 and 50 μg/mL (50 or 100 μmol/L) of EGCG induced the same significant effect with adenocarcinoma HeLa cells. These results also suggest a differential sensitivity of cervical cancer cell types to EGCG exposure. Some studies have reported an association between the induction of apoptosis and disruption or loss of telomere structure.[36–38] A loss of telomerase activity has been observed in cervical adenocarcinoma cancer cells (HeLa: 13 and OMC-4/TMCC1)[39] exposed to the same range of EGCG concentrations (50 and/or 100 μmol/L). A similar pattern in apoptosis induction was not observed with poly E treatment, where only a slight increase was found at 50 μg/mL. However in a parallel experiment, we found that the concentration of 100 μg/mL of poly E induced a high proportion of apoptotic cells (data not shown). These data suggest that poly E is less effective than EGCG to induce apoptosis in these cells.

The ability of EGCG to modify cell cycle progression has been reported by many groups;[40–42] however, the effect of poly E on the cell cycle is still not clear. In the present study, only the effect of poly E (50 μg/mL) on the cell cycle was evaluated. All cell lines showed a modified cell cycle profile, which was characterized by an accumulation of cells in G_1 phase and a decrease of those in S phase. The same pattern was observed both in immortalized (TCL1) and cancer (Me180 and HeLa) cells. These results are comparable with the EGCG effect on cell cycle, as reported in the literature. Epigallocatechin gallate is known to induce cell cycle arrest at G_0/G_1 phase, with a perturbation of expression of cell cycle regulatory genes.

Several cell cycle checkpoint genes are involved in the regulation of cell growth and apoptosis.[42–44] Here, we evaluated the expression of p53, $p27^{KIP1}$, and $p21^{WAF1/Cip1}$ proteins on the squamous cervical carcinoma Me180 cells, which were the most sensitive to the lowest concentrations of EGCG. The p53 and $p21^{WAF1/Cip1}$ protein expression increased in a dose-dependent fashion in the presence of EGCG (0, 5, 25, and 50 μg/mL). Similar dose- and time-dependent increases have been observed by Gupta et al.[42] The up-regulation of p53 by EGCG at low concentrations (1 to 5 μg/mL) has been suggested to induce an arrest of mitosis and DNA damage repair, whereas at higher concentrations (25–50 μg/mL), it may act for induction of apoptosis. Many studies have shown that certain exogenous stimuli may result in a p53-dependent or p53-independent induction of $p21^{WAF1/Cip1}$, an inhibitor of cyclin-dependent kinases.[45–47] This in turn may trigger a series of events such as cell cycle arrest and apoptosis.[44,45] Therefore, our data showing an increase of $p21^{WAF1/Cip1}$ by EGCG appear p53-dependent in Me180 cancer cell line with wild type of p53.

The effect of the 2 green tea compounds on the high-risk HPV-E7 oncoprotein expression in cervical immortalized and adenocarcinoma cell lines was also evaluated. The E7

protein is one of the early proteins expressed by the viral genome to ensure its reproduction in a differentiated cell. The binding of E7 to proteins involved in the cell cycle (ie, phosphorylated retinoblastoma [pRb] and p107RB) leads to the degradation of pRb. The release of its transcription factor E2F allows the cell to enter into the S phase, resulting in uncontrolled cell proliferation.[48–51] Our results show a down-regulation of E7 expression by both EGCG and poly E, irrespective of the cancer cell lines. However, the mechanism by which these 2 green tea polyphenol compounds exert their effects is still unknown. The hypothesis of a down-regulation of E7 by p27 protein as observed by Lee et al[52] in HeLa cells is unlikely because the p27 protein level was not affected by the EGCG treatment. Therefore, the repression of E7 protein level by p27 might not be a general mechanism.

CONCLUSIONS

We demonstrate the ability of 2 green tea polyphenols (EGCG and poly E) to inhibit the proliferation of HPV-immortalized and HPV-positive cancer cells through cell cycle arrest at the G_0/G_1 phase. Their effects seem dependent on the type of cervical cancer cells (adeno or squamous carcinoma), and poly E was less effective than EGCG on growth inhibition and apoptosis. The cell growth inhibition appears more likely to be mediated via an up-regulation of apoptotic-related genes or a down-regulation of HPV-E7 oncoprotein, which triggers the pRb and allows the deregulation of cell cycle control. Further studies are required to investigate protein downstream of p53 activation by green tea polyphenols. We will be able to examine the in vivo effect of green tea compounds on persistent oncogenic HPV infection and low-grade cervical disease (CIN1) in the context of the currently ongoing chemoprevention trial.

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