Epigallocatechin-3-gallate inhibits the growth of HPV positive cervical cancer HeLa cell line

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ABSTRACT

Aim: To elucidate the antiproliferative effects of Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, in the HPV 18 positive human cervical cancer HeLa cell line.

Material and Methods: The viability of the cells was assessed by MTT and Trypan blue dye exclusion method. Cell cycle analysis and apoptosis by FITC-Annexin V were measured by flow cytometry. Protein expression was detected by Western blotting. Morphological changes were observed by phase contrast microscope.

Results: EGCG strongly decreased HeLa cell line proliferation in a dose- and time-dependent manner. Microscopic examination and flow cytometry assay showed that EGCG cause a reduction in cell number by its antiproliferative property. Furthermore, western blot assay of EGCG treated HeLa cell lines showed activation of caspase-3, caspase-9, cleavage of PARP and decreased Bcl-2 expression.

Conclusion: EGCG strongly inhibited the growth of HPV-positive cervical cancer cells and induce apoptosis.

INTRODUCTION

Cancer of the uterine cervix accounts for 15% of all cancers diagnosed in women (Boyle and Feraly, 2005). In developing countries, it is often the most common cancer in women and may constitute up to 25% of all female cancers (Burd et al, 2003, Waggoner, 2003). It is the single largest female malignancy in India (Gajalakshmi et al., 2001). Approximately 96.6% of cervical cancer patients are infected with HPV. HPV 16 and 18 are two major high-risk types associated with cervical cancer (Munoz et al., 2003). HPV oncogenes E6 and E7 have been reported to be vital for carcinogenesis, progression, invasion and metastasis of cervical cancer (Munger and Howley, 2002).

EGCG is the most abundant of the green tea polyphenols, accounting for more than 40% of the total polyphenolic mixture (Stoner and Mukhtar, 1995). Many recent studies have verified the chemopreventive effect of EGCG against cancers of the breast, ovarian, skin, lung, colon, liver, stomach and prostate (Yang et al., 2008; Pellecchia et al., 2004; Kim et al., 2005). Several molecular mechanisms have been suggested for EGCG's observed anticancer effect, including suppression of ligand binding to the EGF receptor (Adachi et al., 2007); inhibition of urokinase (Siddiqui et al., 2008; Lim et al., 2008), protein kinase C (Kitano et al, 1997), lipoxygenase, and cyclooxygenase activities (Hwang et al., 2007) and induction of apoptotic cell death and arrest of the cell cycle (Hastak et al., 2003; Nihal et al., 2005) in tumour cells. The purpose of this study is to examine the effect of EGCG on HPV 18 positive HeLa cervical cancer cell line.

MATERIALS AND METHODS

All chemicals and EGCG were purchased from Sigma (St Louis, MO, USA). RPMI-1640 and fetal calf serum (FCS) were purchased from GIBCO (Grand Island, NY, USA). The primary antibodies against caspase-3, caspase-9, Bcl-2, and PARP as well as peroxidase conjugated anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Delaware Avenue Santa Cruz, CA, USA). Primary antibody against cleaved PARP (89 kDa) was purchased from Cell Signaling Technology.

Cell culture

The human cervical cancer HeLa cells, obtained from National Centre for Cell Science (NCCS, Pune, India), were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate at 37 °C in an atmosphere of 95% air and 5% CO2 under humidified conditions. Aminoguanidine (1 mmol/l) was added as an inhibitor of amine oxidase derived from FCS and had no effect on the various parameters of the cells measured in this study. We treated the Hela cell with 10 -80 µg of EGCG for 24 and 48 hr.

MTT cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide) assay was used to assess the effects of EGCG on cell viability. The assay relies on the production of a colored formazan by the action of mitochondrial enzymes on MTT. Exponentially growing HeLa cells were seeded (5×10^3 /well) onto 96-well flat-bottomed plates and allowed to attach overnight. The cells were treated with the 10, 20, 40 and 80 µg/ml of EGCG for 24 and 48 hr. 10µl MTT (5 mg/ml) was added to each well and then plates were incubated for another 4 h at 37 °C. DMSO was added to each well and thoroughly mixed to dissolve the dark blue crystals. The plates were read on a Qualisystems PR 601 Elisa reader, using a wavelength of 570 nm and a reference wavelength of 450 nm.

Trypan blue exclusion assay

Growth inhibition of cells was determined by trypan blue dye exclusion method. The exponentially growing HeLa cells were seeded onto 24-well, flat-bottomed plates at a density of 5×10^4/ml and allowed to attach overnight. The cells were treated with 10, 20, 40 and 80 µg/ml of EGCG for 24 and 48 hr. The cells were collected by trypsinization and counted by a Thoma hemocytometer (Shanghai, China) using 0.2% trypan blue dye for cell viability.

Caspase activity assay

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, DEVD-pNA (Asp-Glu-Val-Asp-p nitroanilide for caspase-3) and LEHD-pNA (Leu-Glu-His-Asp-p-nitroanilide for caspase-9). HeLa cells (1×10^6/well) in 12-well plate were treated with 20-80 µg/ml EGCG for 24 and 48 hr. Cells were collected and washed with PBS and suspended in 50 µl of cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 µg/ml aprotinin and 5 µg/ml leupeptin), incubated on ice for 10 min, and centrifuged for 1 min in at 10,000 g. Protein concentration (as determined by bicinchoninic acid assay) was adjusted with the cell lysis buffer. Equivalent amounts of cell lysates were incubated with peptide substrate (DEVD-pNA or LEHD-pNA) in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS), pH 7.4) for 2 h at 37°C. The release of chromophore p-nitroaniline (p-NA), after its cleavage by caspases from the labeled caspase-specific substrates, was measured spectrophotometrically at 405 nm. (Shen et al., 2007).

Western blot analysis

HeLa cells (8×10^6/dish) were seeded in a 10-cm dish. After 24 h of incubation, cells were treated with 80 µg/ml of EGCG for 24 and 48 h. Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 µg/ml aprotinin and 5 µg/ml leupeptin). Equivalent amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–12%) and transferred to polyvinylidene difluoride membranes. After the membrane was blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% non-fat powdered milk, the membranes were incubated with primary antibodies (the antibodies specific against caspase-3, -9, Bcl-2 and PARP) at 4 ºC for 16 h. After washing three times with Tris-buffered saline for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection performed using the enhanced chemiluminescence blotting detection system (Amersham, Arlington Heights, IL, USA) (Hsu et al., 2006).

Cell cycle analysis

Flow cytometric measurement of cellular DNA content was performed with ethanol fixed cells using the intercalating DNA fluorochrome, propidium iodide (PI) as described earlier (Zolzer et al., 1995). HeLa cells (5×10^6 cells/well) were cultured in 6-well plates. After 24 hr of incubation, cells were treated with 40-80 µg/ml of EGCG for 24 and 48 hr. Cells were harvested, washed with PBS by centrifugation at 600g for 5 min, fixed in 80% chilled ethanol and store at 4 C till measurement. Cells were washed with PBS after removing ethanol and treated

with RNase A (200 µg/ml) for 30 min at 37ºC. Subsequently, cells were stained with PI (25 µg/ml) for 15 min at room temperature. The measurement was made with an argon laser-based Flow cytometer (FACS-Calibur Becton Dickinson San Jose, CA, USA) using the Argon laser (488 nm) for excitation. Distribution of cells in different phases of cell cycle was calculated from the frequency.

FITC-annexin V assay

Apoptotic cells were detected by the labeling of externalized phosphatidylserine using FITC-Annexin V in unfixed cells (Verma and Mazumder, 1995). FITC-labeled Annexin V apoptosis detection kit was obtained from Becton Dickenson (San Jose, CA, USA). Briefly, cells (1x10^5) were cultured in 10 cm Petri dishes, treated with 20-80 µg/ml of EGCG for 48 h, harvested and washed with PBS. Subsequently, cells were resuspended in 100 l binding buffer (10 mM HEPES/NaOH, PH 7.4, 10 mM NaCl, 2.5 mM CaCl$_2$) and stained with 5 µl FITC-Annexin V and 10 µl PI (50 g/ml). After 15 min at room temperature, 400 µl of binding buffer were added to each sample and analyzed by flow cytometry. The percentage of Annexin V-positive and negative cells were estimated by applying appropriate gates and using regional statistics analysis facility provided in the CellQuest Software (Becton Dickenson, San Jose, CA, USA).

Morphological analysis

For assessing morphological changes, 50-60% confluent cells were treated with PBS alone or with 40 and 80 µg/ml concentration of EGCG. After 48 h of treatment, photographs were taken using a phase contrast microscope at 400X magnification.

RESULTS

EGCG inhibits cell growth

We observed that at the dose of 80 g/ml, EGCG inhibited cell growth after 24 hr of treatment, while significant inhibition of cell proliferation was seen at the dose of 40 and 80 g/ml of EGCG after 48 h of treatment (Figure 1). More or less similar observations were obtained with Trypan blue exclusion assay (Figure 2). These results indicated that EGCG inhibits cell growth in HeLa cells as a function of both dose and time. Inhibition of cell proliferation could be the result of the induction of cell cycle growth arrest or apoptosis. We hypothesized that EGCG induced inhibition of cell proliferation was due to alterations in cell cycle control and programmed cell death.
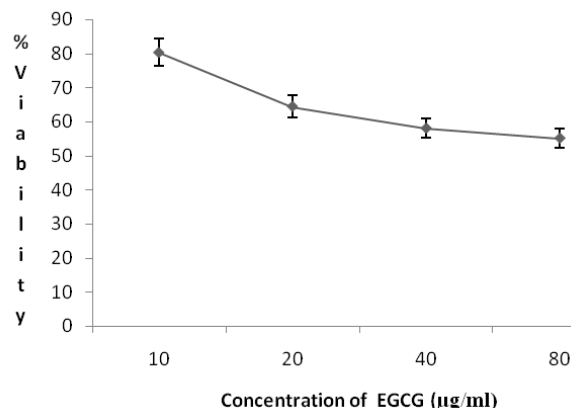

Figure 1. MTT cell viability assay.

For dose response effect of EGCG (0-80 µg/ml) after 48 hr on HeLa cell (plz note: in HeLa cell line at 24 hr negligible effect of EGCG was observed). Data represents mean ± SEM of 3 experiments.
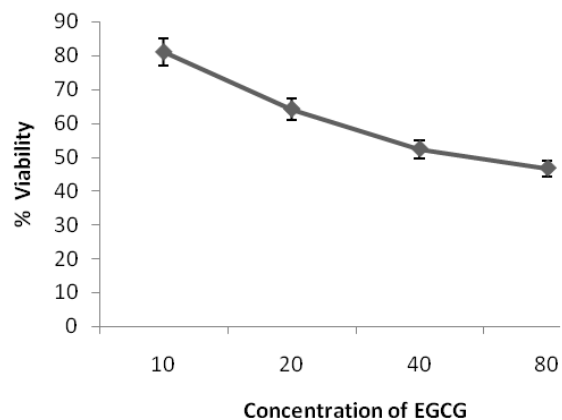

Figure 2. Trypan blue dye exclusion assay.

Dose dependent inhibition of growth of HeLa cervical cancer cells after treatment with EGCG (10-80 µg/ml). Data represent mean ± S.E.M. of three independent experiments.
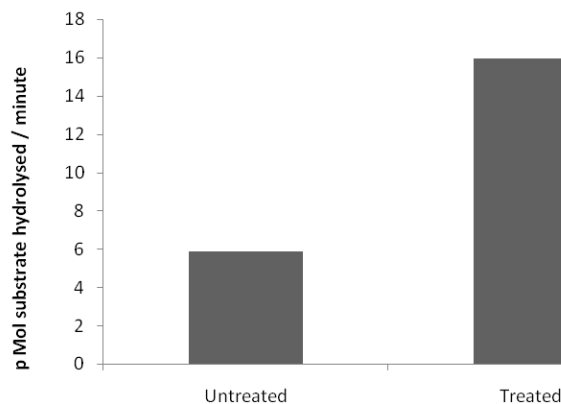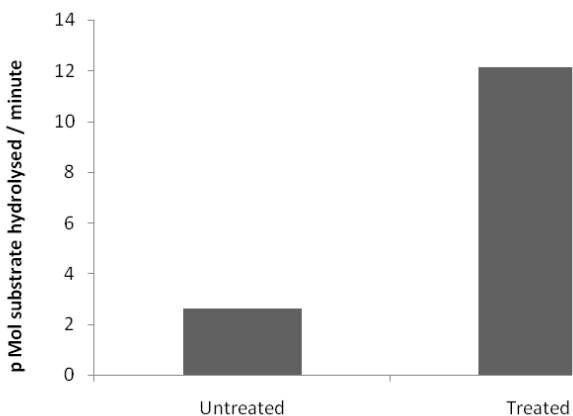

Figure 3.1. Caspase 3 activity.

Figure 3.2. Caspase 9 activity.

Dose-dependent activation of caspase 3& 9:

HeLa cells were treated with different concentrations of EGCG (20-80 µg/ml) for 24 and 48 hr. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase-3 & 9 was determined by incubation of 50 µg of total protein with substrate DEVD-pNA and LEHD-pNA respectively for 2 h at 37 °C. The release of *p*-NA was monitored spectrophotometrically at 405 nm. Data represent mean ± S.E.M. for three independent experiments.

EGCG induces activation of Caspase-3, -9 and cleavage of poly (ADP-Ribose) polymerase (PARP), bcl-2 during apoptosis

The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro to active forms, mediate the cleavage of Poly (ADP-Ribose) Polymerase (PARP). So, we then investigated whether caspases play some role in the apoptosis of HeLa cells induced by EGCG. As described previously, the cell growth inhibition by EGCG was strongly dose and time dependent; we examined the dose and time dependence of caspase activation as well. Cytosolic proteins were extracted and assayed for caspase activity by incubation with a chromogenic substrate, DEVD-pNA (for caspase-3) or LEHD-pNA (for caspase-9). Our results showed a significant induction of caspase-3 and -9 activities in HeLa cells after 48 hr of treatment at all the indicated doses of EGCG (Figure 3). Insignificant caspase-3 and -9 activities were observed after 24 h. The caspase activities were observed to be dose and time dependent and maximal at the dose of 80 µg/ml after 48 hr of treatment with EGCG.

Furthermore, we confirmed our above results by Western blot analysis of capase-3, -9 and PARP in total cell lysate of HeLa cells prepared after 24 and 48 hr of treatment with 80 µg/ml of EGCG. Our results showed that activation and cleavage of initiator caspase-9 which was

more pronounced after 48 h of treatment (Figure 4). The activation of initiator caspases resulted in activation and cleavage of downstream effector caspase-3. Our results also showed that 80 µg/ml of EGCG led to activation and cleavage of caspase-3 which was more evident after 48 h of treatment (Figure 4). The activation of the effector caspase-3 leads to cleavage of its substrate PARP. We also revealed PARP cleavage in EGCG (80 µg/ml) treated cells and its cleaved product of 89 kDa was more obvious after 48 h of treatment (Figure 4). Thus, the induction of caspase-3 and -9 activities and cleavage of PARP is a specific biochemical event, brought about by EGCG, inducing apoptosis.
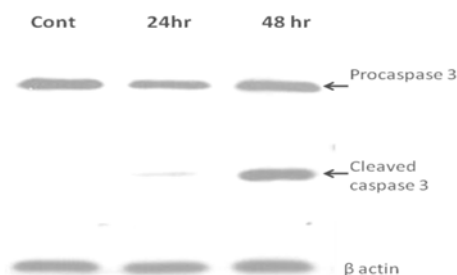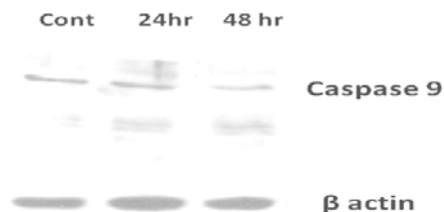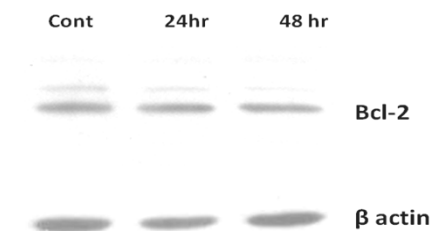

Figure 4a.

Figure 4b.

Figure 4c.

Figure 4d.

Western blotting of caspase-3, -9, Bcl-2 & PARP: HeLa cells were treated with 80 µg/ml of EGCG for 24 and 48 hr. Protein (50 µg) from total cell lysates was subjected to SDS–PAGE and Western blotting using caspase-3, -9, Bcl-2 & PARP antibody.
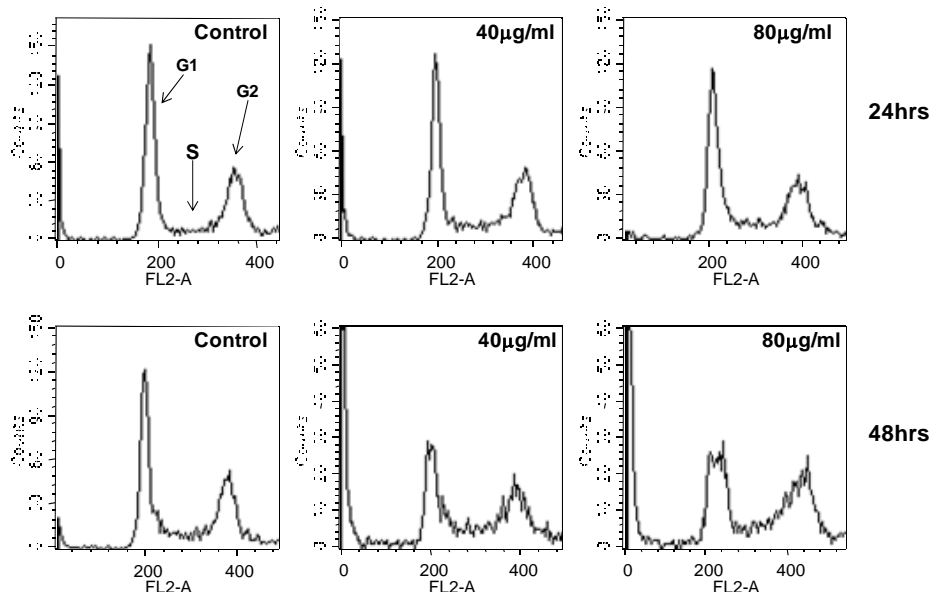
Figure 5. Cell cycle analysis:

HeLa cell were treated with the 40 & 80 µg/ml doses of EGCG for 24 & 48 hr and analyzed by a flow cytometer. The data shown here are from a representative experiment repeated three times with almost similar results. At 10 and 20 µg/ml effect are negligible (Data not shown).

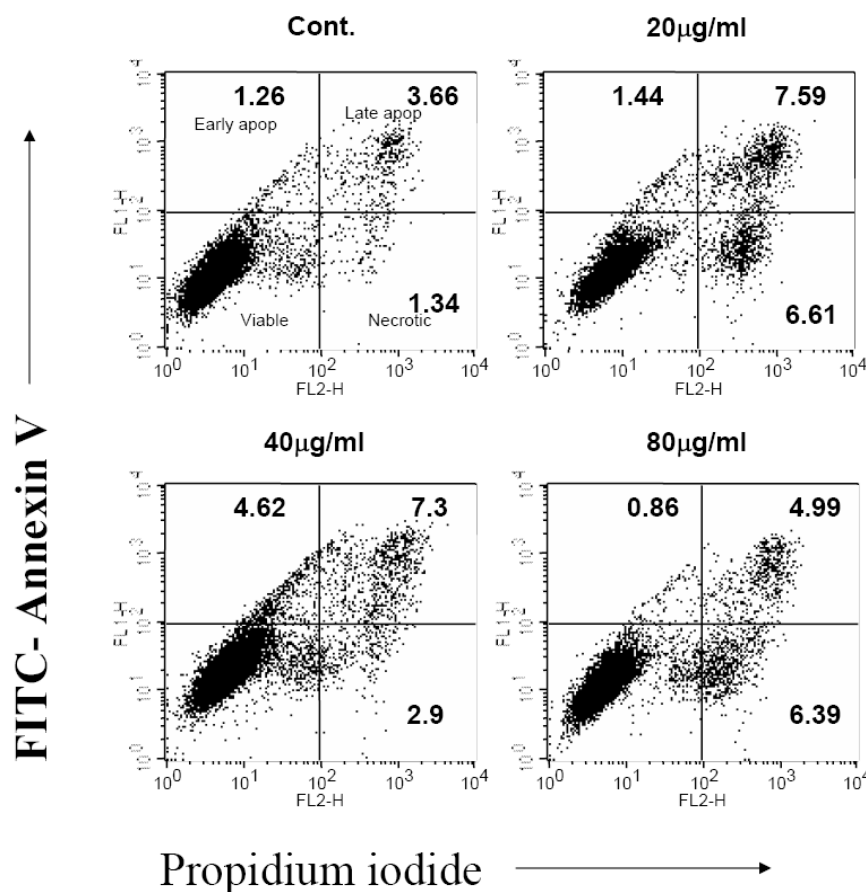**Figure 6.** Measurement of apoptosis by FITC-annexin V.

HeLa cells were treated with the 20-80 µg/ml indicated doses of EGCG for 48 hr and stained with FITC-Annexin V and Propidium iodide. Apoptosis was detected by using a Flow cytometer. The data shown here are from a representative experiment repeated three times with almost similar results. At 10 µg/ml, effects are negligible (Data not shown).

EGCG induced cell cycle arrest at G2/M phase

Previous studies have shown that EGCG induces G0/G1 phase arrest of cell cycle in breast cancer cells (Liang et al., 1999; Liberto and Cobrinik, 2000) and prostate cancer cells (Gupta et al., 2000; Ahmad et al., 2000), hence we investigated the effect of EGCG on the cell cycle distribution of HeLa cervical cancer cells. For this, HeLa cells were treated for 24 and 48 h with the dose of 20, 40 and 80 µg/ml of EGCG. Our results showed that there was a significant arrest at the G2/M phase of cell cycle in HeLa cells treated with the dose of 40 and 80 µg/ml of EGCG and the number of cells at this phase was significantly increased after 48 h of treatment (Figure 5). The insignificant change was observed in the cell cycle distribution of HeLa cells after 24 h of treatment with all the indicated doses of EGCG (Figure 5). Furthermore, we observed a little increase in the sub-G0/G1 population of HeLa cells after 48 h of treatment with the dose of 40 and 80 µg/ml of EGCG, which is an indicator of apoptosis (Figure 5). Since, in addition to cell cycle arrest, the growth inhibition induced by EGCG treatment could also be due to programmed cell death, hence, we investigated whether EGCG induces apoptosis in HeLa cells or not.

Annexin V assay

To determine whether the loss of viability induced by EGCG was due to apoptosis, we measured apoptosis in HeLa cells by FITC-Annexin V assay. Apoptotic cells were detected after treating HeLa cells with the dose of 20, 40 and 80 µg/ml of EGCG for 48 hr. We observed a significant amount of apoptosis in HeLa cells after 48 h of treatment with the dose of 40 and 80 µg/ml of EGCG (Figure 6). Moreover, the percentage of apoptotic cells did not coincide with the percent of viable cells which could be due to a cell cycle arrest at G2/M phase. In order to explore the mechanism(s) by which EGCG induces apoptosis, we investigated the alterations in the expression of selected genes that are involved in the complex processes of apoptosis.
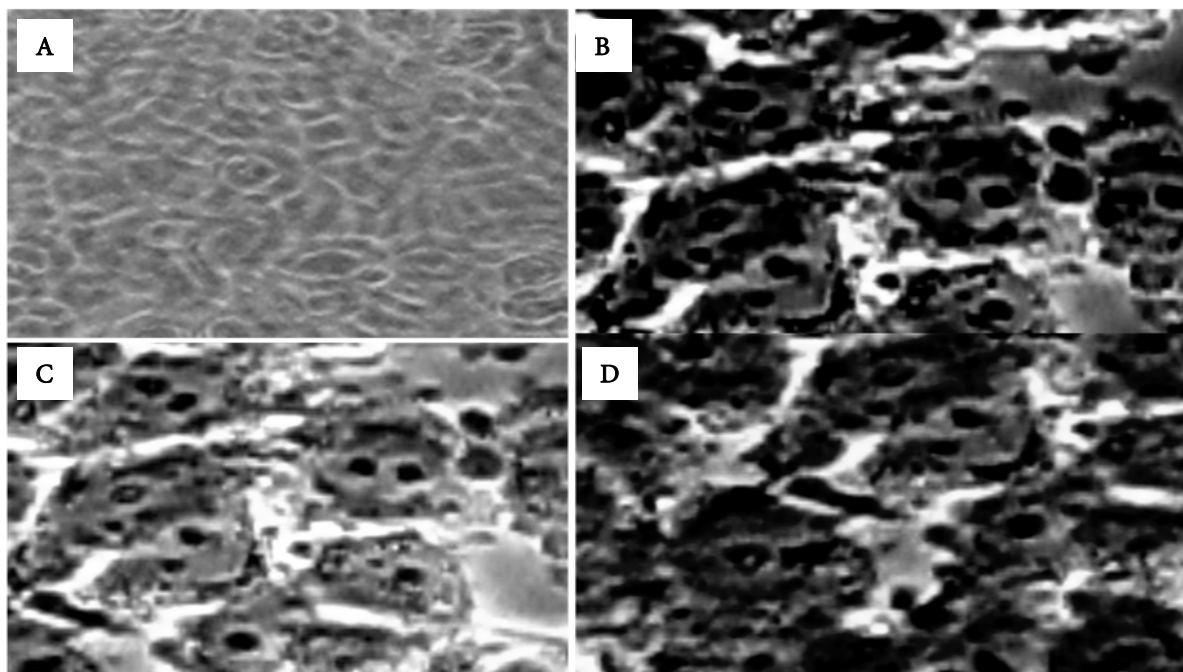

Figure 7. Morphological analysis of HeLa cell lines treated with EGCG, (A) Monolayer of HeLa Cell line, (B) Control at 48 hr, (C&D)- Treated with 40 & 80 (µg/ml) EGCG at 48 hr, picture were taken by using a phase contrast microscope at 400X magnification. The data shown here are from a representative experiment repeated three times with similar results.

Morphological Examination

Microscopic examination by phase contrast microscope (400X) revealed typical morphological changes of cell apoptosis in HeLa cells treated with EGCG. The morphological hallmark of apoptosis such as nuclear fragmentation was also observed (Figure 7).

DISSCUSSION

Many recent studies indicate that EGCG exert inhibitory effect on the activity of several enzymatic and metabolic pathways of relevance to the development and progression of cancer (Yang et al., 2008; Khan et al., 2006; Beltz et al., 2006). EGCG, by virtue of its ability to selectively induce apoptosis in cancer cells and not in normal cells, is potentially an important cancer chemopreventive agent.

We have investigated the effect of EGCG on HPV 18 positive cervical cancer HeLa cell line. Our results illustrated that EGCG induced cell death in HeLa cells in a dose- and time-dependent manner. Previous studies have shown a dose-dependent inhibition of proliferation by EGCG in cells of prostate cancer (Gupta et al., 2000; Gupta et al., 2003), colon cancer (Lambert et al., 2006) and breast cancer (Thangapazham et al., 2007; Stuart et al., 2006). Furthermore, we observed EGCG induced cell cycle arrest at G2/M phase in HeLa cells and the portion before G0/G1 phase (sub-G0/G1) was significantly increased by EGCG, which is an indicator of apoptosis. EGCG induced G_2/M phase arrest of cell cycle has been recently reported in HPV 18 positive HeLa and HPV 16 positive CaSki cervical cancer cell lines (Qiao et al., 2009). EGCG induced G_0/G_1 phase arrest of cell cycle has also been reported previously in MCF-7 and MCF10A breast cancer cells (Liberto and Cobrinik, 2000) and DU145 and LNCaP prostate cancer cells (Gupta et al., 2000; Ahmad et al., 2000).

Apoptosis plays a crucial role in the regulation of the development and homeostasis of multicellular organisms (Yan and Shi, 2005). Balance between cancer cell proliferation and spontaneous cell death via apoptosis has an important role in the regulation of cancer cell growth (Brown and Attardi, 2005). Anticancer drugs also function by inducing cancer cell death via induction of apoptosis in sensitive cells (Roninson, 2003; Johnstone et al., 2002). The downstream signals during apoptosis are transmitted via caspases. Caspases are synthesized as inactive proenzymes and their activation during apoptosis results in cleavage at specific aspartate cleavage sites (Thornberry et al., 1997). While initiator caspases-8 and -9 undergo autocatalytic activation, executioner pro-caspase-3 is processed by initiator caspases. Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP, followed by DNA fragmentation (Enari et al., 1998; Nagata, 2000). Thus, PARP is known to be cleaved in the execution phase of apoptosis.

In the present study, we have shown that treatment with EGCG caused an increase in caspase-9 and -3 activities. Furthermore, our western blot results have confirmed the finding of enhanced caspase-9 and -3 activities and cleavage of PARP, which precedes the onset of apoptosis. Many previous studies on different cancer cells have verified our observation. Increased caspase-3 activity has been reported in chondrosarcoma and breast cancer cells treated with EGCG (Islam et al., 2000; Roy et al., 2005). Caspase-3-induced PARP cleavage has been reported in

pancreatic cancer cells treated with EGCG (Qanungo et al., 2005).

The ratio of Bax to Bcl-2 is critical to cell survival which determines whether a cell undergo apoptosis (Oltavi et al., 1993). Bax, when overabundant, inserts into the outer mitochondrial membrane, leading to its oligomerization and release of cytochrome c (Wolter et al., 1997).Cytochrome c complexes with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 to form 'apoptosome'. The apoptosome recruits procaspase-3, which is cleaved and activated by caspase-9 to induce apoptosis. In this study, our western blot results have shown that there was an upregulation of Bax and downregulation of Bcl-2 in HeLa cells after EGCG treatment. EGCG has been shown to bound to the BH3 pocket of antiapoptotic Bcl-2 family proteins (Leone et al., 2003) suggesting a mechanism for EGCG to inhibit the antiapoptotic function of Bcl-2 proteins. In many previous studies, EGCG has been shown to modulate the Bax/Bcl-2 ratio in different cancer cells, including HNSCC, ovarian carcinoma, melanoma, pancreatic carcinoma, colorectal and prostate cancer cells (Nihal et al., 2005; Qanungo et al., 2005; Shimizu et al., 2005). Apoptosis is again confirmed by Microscopic examination of cancer cell, which show a clear damage of nucleus with EGCG.

CONCLUSION

Thus, our results demonstrated that EGCG treatment caused a significant decrease in the percentage of viable cell along with a concurrent induction of apoptosis in HeLa cervical cancer cells; however, the percentage of apoptotic cells did not coincide with the percent of viable cells. This difference in cell viability and apoptosis could be due to cell cycle arrest at G_2/M phase induced by EGCG.

Thus, our results strongly demonstrated that EGCG could be a potential drug for prevention and treatment of cervical cancer.

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