RESEARCH ARTICLE

(-)-Epigallocatechin-3-Gallate Induces Apoptosis and Inhibits Invasion and Migration of Human Cervical Cancer Cells

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Abstract

Invasion and metastasis are the major causes of cancer-related death. Pharmacological or therapeutic interventions such as chemoprevention of the progression stages of neoplastic development could result in substantial reduction in the incidence of cancer mortality. (-)-Epigallocatechin-3-gallate (EGCG), a promising chemopreventive agent, has attracted extensive interest for cancer therapy utilizing its antioxidant, anti-proliferative and inhibitory effects on angiogenesis and tumor cell invasion. In this study, we assessed the influence of EGCG on the proliferative potential of HeLa cells by cell viability assay and authenticated the results by nuclear morphological examination, DNA laddering assay and cell cycle analysis. Further we analyzed the anti-invasive properties of EGCG by wound migration assay and gene expression of MMP-9 and TIMP-1 in HeLa cells. Our results indicated that EGCG induced growth inhibition of HeLa cells in a dose- and time-dependent manner. It was observed that cell death mediated by EGCG was through apoptosis. Interestingly, EGCG effectively inhibited invasion and migration of HeLa cells and modulated the expression of related genes (MMP-9 and TIMP-1). These results indicate that EGCG may effectively suppress promotion and progression stages of cervical cancer development.

Keywords: Epigallocatechin-3-gallate - invasion - matrix metalloproteinases-9 - cervical cancer

Introduction

Cancer is one of the leading causes of mortality worldwide. The main reason of cancer mortality is tumor invasion and metastasis and is the greatest barrier to cancer cure. A rational and appealing treatment approach in cancer management is chemoprevention, the administration of natural or synthetic agents to inhibit, delay, block or reverse the initiation, promotion and progression stages of carcinogenesis, which could be the most prospective way to reduce morbidity and mortality (Clément et al., 1998; Agarwal et al., 2000). Accumulating scientific evidences on chemopreventive agents substantiate that a diet rich in fruits and vegetables could reduce 7–31% of all cancers worldwide (Bal et al., 2001).

One such bioactive compound, EGCG, an ester of epigallocatechin and gallic acid, is a major polyphenolic constituent of green tea and possesses remarkable cancer chemopreventive and therapeutic potential against various cancers (Khan et al., 2006; Seeram et al., 2006; Zaveri, 2006). It has also been found that EGCG assist the inhibition of tumorigenesis in a variety of organs including skin, lung, oral cavity, lymphoid leukemia cells, oesophagus, stomach, small intestine, colon, liver, pancreas, ovary and mammary gland, prostate cancer (Hibasami et al., 1996; Ahmad et al., 1997; Jung et al., 2001; Kazi et al., 2002; Yang et al., 2002; Zhang et al., 2002; Lambart and Yang, 2003). Gamut reports have shown that EGCG has the ability to inhibit tumor development by inducing apoptosis, inhibiting tumor cell proliferation and invasion and angiogenesis through various molecular targets such as Bcl-xl, p21/WAF1 VEGF, MMP family and may enhance gap junctional communication between cells (Kazi et al., 2002; Takahashi et al., 2004; Seeram et al., 2006; Zaveri, 2006; Shankar et al., 2008; Zhu et al., 2011). Recent findings also show that green tea may activate detoxification enzymes, such as glutathione S-transferase and quinone reductase and inhibit telomerase activity (Kim and Moon, 2005; Artali et al., 2009). Interestingly, clinical trials have indicated its relatively low toxicity and effective oral bioavailability (Stingl et al., 2011). Thus, EGCG seems to be a potentially ideal antitumor agent.

In this study, we investigated the effects of EGCG on human cervical cancer cells, with the aim of enhancing apoptosis of cancer cells and decreasing their invasion and metastasis potential. To confirm this hypothesis, we tested the effects of EGCG in vitro on human cervical cancer cell line (HeLa). Also, we tried to correlate the in vitro modulating effects of EGCG on various molecular
targets in signal transduction pathways, resulting in the suppression of cell proliferation as well as the inhibition of cell invasion and metastasis.

Materials and Methods

Cell culture

The human cervical carcinoma cell line, HeLa was maintained in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and 100X Pen-strep (Sigma, USA) in a humidified atmosphere of 5% CO₂ in air at 37 °C.

Preparation of Drug Solutions

EGCG was obtained from Sigma (USA). A stock solution of EGCG (10 mM) was prepared in water, sterile filtered with 0.2-µm filters, and stored at −20°C in aliquots. Fresh EGCG solution was used in each experiment and further dilutions were made in complete medium to required concentrations between 1 µM – 100 µM for the treatment of HeLa cells.

Cell Viability Assay

The anti-proliferative activity of EGCG on HeLa cells was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described (Sharma et al., 2011). Briefly, HeLa cells (~7000 cells/well) were plated in 96 well plates and incubated overnight in complete medium at 37 °C in order to obtain semi-confluent cultures. This was followed by treatment with varying concentrations of EGCG (1 µM – 100 µM) in complete medium for 24 h and 48 h respectively (in triplicates). After incubation, MTT (Sigma, USA) (final concentration 0.5 mg/ml) was added to each well at appropriate time and incubated for 2-4 h at 37 °C. The formazan crystals thus formed were dissolved in 100 µl DMSO (Sigma, USA). The absorbance was read at 570 nm using an Absorbance Microplate Reader (BioTek, USA). Cell viability was calculated as percent of control (untreated cells), and averaged from three independent experiments.

Morphological analysis of HeLa cells before and after treatment with EGCG

Morphological changes in HeLa cells were observed on treatment with EGCG at different concentrations (1-100 µM) for 24 h and 48 h using normal inverted microscope (Labomed, USA). The untreated cells were used as negative control.

Observation of Nuclear Morphological Changes by Propidium Iodide staining

The nuclear morphological changes associated with cells undergoing apoptosis were studied using Propidium iodide staining after the treatment with EGCG at varying time points (0, 6, 24 and 48 h). Briefly, cells (~10⁵ Cells/ml) were seeded on glass coverslips and left overnight to attach in complete medium at 37 °C, followed by treatment with EGCG at its IC₅₀ for varying time points (0, 6, 24, 48 h). After treatment with EGCG, cells were fixed in a mixture of acetone, methanol (1.1) at −20°C for 10 min, washed with 1X PBS (pH 7.4) twice and stained with Propidium Iodide (10 µg/ml in PBS) for 30 s in dark at RT. The coverslips were thoroughly washed with PBS and placed upturned onto a glass slide with mounting media (DPX). Slides were viewed at 515 nm under the Progress Fluorescent Microscope (Olympus, USA). The images were captured at 40X magnification.

DNA fragmentation assay

Internucleosomal cleavage of DNA was analysed as described previously with slight modifications (Moongkamdi et al., 2004). Briefly, cells were treated with EGCG at IC₅₀ for varying time intervals (0, 24 & 48 h) and were gently scraped and harvested by centrifugation. Cell pellets were resuspended in lysis buffer containing 10 mM Tris – HCl (pH 8.0), 20 mM EDTA and 0.5% Triton X-100 and incubated for 30 min on ice. Following centrifugation, DNA was extracted with phenol–chloroform–isoamyl alcohol and subjected to 2.0% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light transilluminator.

Cell cycle analysis

To determine the effect of EGCG on the cell cycle, HeLa cells (~1×10⁶ cells each) were treated with EGCG at a dose of 50 µM at various time points (0, 24 and 48 h) as described earlier (Sharma et al., 2006). After treatment, both adherent and floating cells were harvested, washed with phosphate buffered saline (PBS, pH 7.2) and fixed with ice-cold absolute ethanol at -20°C overnight. Cells were then washed with PBS prior resuspending in a buffer containing PI (50 µg/ml), 0.1% sodium citrate, 0.1% Triton- X-100 and 100 µg/ml of RNase A. The cells were analyzed using Flow cytometry (FACS Calibur; BectonDickinson, Franklin Lakes, NJ, USA). The data was analyzed using the Win-MDI software. The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at the sub-G₀ phase of the cell cycle.

Migration Assay

The effect of EGCG on tumor cell invasion was investigated in vitro by cell migration assay as described previously (Matsuura et al., 2006). Cells were seeded in 96-well plates at a density of ~5×10⁴ cells per well and cultured in complete medium until completely confluent. The medium was then replaced with serum-free DMEM. One linear scar was drawn in the monolayer using a yellow tip. A set of digital photographs were taken at the time of scarring. The plates were washed, and fresh serum-free medium containing 0.1% BSA in the absence or presence of EGCG (25 µM) was added at various time intervals (0, 8, 24 and 48 h). After each time point, a set of photos were taken and were superimposed on the first photo set to measure the migration of the cells. Each condition was tested in duplicate in three independent experiments.

Expression analysis of MMP-9 and TIMP-1 by RT-PCR

Reverse transcription-PCR was used to detect transcriptional regulation of MMP-9 and TIMP-1. Total RNA extraction from untreated and 25 µM EGCG-treated
HeLa cells at various time intervals (0, 6, 24 & 48 h) was carried out as per the manufacturer’s protocol using GenElute Mammalian Genomic Total RNA Kit (Sigma, USA). ProtoScript M-MuLV Taq RT-PCR Kit (New England Biolabs, USA) was used to synthesize cDNA strand by reverse transcription from 5 µg of total RNA (at 42 °C for 60 min) followed by RT-PCR using gene-specific primers. Amplification of 50 µl of these cDNA by PCR was performed using the following β-actin, MMP-9 and TIMP-1 gene-specific primers. forward 5'-CACGCCATGACGGTGATCCACAGGAC-3'; reverse 5'-GTTTCGAGGATCCACAGGAC-3'; forward 5'-CCACGCTGGGCCAACCCAGATGGC-3' and reverse 5'-GGCGTGCGGCAAACTGTG GGCGTGGCCAC-3'; forward 5'-GCAATTCCG ACCTCGTCATC-3' and reverse 5'-AGTGTAGTGGTTGGAAGGC-3' respectively. The PCR cycle was as follows. initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s), with final extension at 72 °C for 7 min. Amplified products were visualized on a 2% agarose gel containing ethidium bromide. β-actin was taken as an internal control.

Statistical analysis

All data are expressed as means±SD of at least 3 experiments. Fisher’s exact test was adopted for statistical evaluation of the results. Significant differences were established at p <0.05. All statistical analyses were performed in GraphPad InStat Software.

Results

**EGCG inhibits the cell viability of HeLa Cells**

The effect of EGCG on the viability of HeLa cells was determined by treating the cells with varying concentrations of EGCG (1 µM-100 µM) for 24 and 48 h respectively and evaluating the cell viability using MTT assay. Increasing concentrations of EGCG (1 µM-100 µM) for 24 h and 48 h showed significant growth inhibition in a dose and time dependent manner (Figure 1). The IC_{50} value of EGCG on HeLa cells was calculated at 100 µM for 24 h and 50 µM for 48 h. The experiment was reproduced at least three times.

**Morphological changes induced by EGCG treatment on HeLa cells**

The microscopic examination of cells treated with 25, 50 and 100 µM EGCG for 24 h and 48 h (Figure 2A and B) compared to untreated control showed characteristic rounding off of dying cells indicating the cell death induced by EGCG. An increase in cell death was observed with increasing concentrations and duration of treatment with EGCG.

**EGCG treatment induced nuclear morphological changes in HeLa Cells**

To validate EGCG induced apoptosis, Propidium Iodide staining was used to detect nuclear morphological changes. Nuclear morphology of HeLa Cells treated with 50 µM EGCG was analyzed at various time intervals (0, 6, 24, 48 h). Untreated HeLa cells showed uniform chromatin density. However, EGCG treatment for 6 h showed considerable increase nuclear condensation indicative of early apoptotic cells. With an increase in the duration of exposure to EGCG (24, 48 h), the cells showed accumulation of late apoptotic changes such as nuclear debris which could be identified as apoptotic cells in comparison to untreated cells (Figure 3). The data shown are means±SD from three independent experiments. A significant differences (p <0.05) was observed between the groups.

**Appearance of DNA ladders in EGCG treated HeLa cells**

The cytotoxicity of EGCG is possibly the result of apoptosis (Figure 6). To determine the degree of EGCG-induced apoptosis, DNA fragmentation, a hallmark of apoptosis, was detected on 2.0% agarose gel electrophoresis by treating HeLa cells with EGCG at 50 µM for 24 h and 48 h. Exposure of HeLa cells to 50 µM EGCG for 24 and 48 h resulted in fragmentation of the DNA in a ladder-like pattern (Figure 4). Thereby, these results suggest time dependent increase in cell death induced by the EGCG treatment was mediated through apoptotic pathway.

**Cell Cycle distribution after the treatment with EGCG on HeLa cells**

Figure 2. Morphological Changes in HeLa Cells at Varying Concentrations of EGCG Along with Their Respective Controls. Microscopic features of HeLa cells with (drug treated) & without treatment (controls) with EGCG at 25, 50 and 100 µM for 24 h (A) and 48 h (B) (Magnification 100×). Arrows represent rounding off of cells (indicative of cell death) with increasing concentrations and time of treatments.
Figure 3. The Nuclear Morphological Changes induced by 50 μM EGCG at Various Time Intervals on HeLa Cells were Visualized using Fluorescence Microscope with Propidium Iodide Staining. Untreated HeLa cells (0 h) showing no signs of apoptosis as nuclei are large and prominent. N. HeLa cells treated with 50 μM EGCG for 6, 24 and 48 h showing time-dependent increase in apoptotic morphological changes; shape distortion, plasma shrinkage, nuclear condensation (C), blebbing (B) & formation of apoptotic bodies (circled) (Magnification 40X).

Figure 4. Inter-Nucleosomal DNA Fragmentation in HeLa Cells Treated with 50 μM EGCG at Various Time Intervals. Lane 1 depicts untreated control cells (0 h), Lane 2 & 3 shows HeLa cells treated with 50 μM EGCG for 24 and 48 h respectively.

Figure 5. Effect of EGCG on Cell Cycle Progression Determined by Flow Cytometry Analysis. After treatment with 50 μM EGCG for 24 h (B) and 48 h (C), the apoptotic cells in the sub-G1 region were significantly increased as compared to the untreated control cells (A). The data presented here are the mean value of three independent experiments.

Cell cycle analysis was performed to determine the mode of cell death induced by EGCG treatment at various time points. Figure 5B depicts that the percentage of cells in the apoptosis phase (sub-G1-phase) was found to increase on treatment with EGCG for 24 h compared to the untreated controls wherein there was a proper distribution of cells throughout the cell cycle (Figure 5A). Furthermore, there was a marked increase in the number of apoptotic cells on treatment with EGCG for 48 h (Figure 5C).

EGCG inhibits HeLa cell migration in a time-dependent manner

Tumor cell migration is considered as a major event in the metastatic cascade. Here we examined the effect of EGCG on migration capacity and molecular mechanism using cervical cancer cells as a model. Using the cell migration assay, we observed that treatment of HeLa cells with 50 μM EGCG resulted in time-dependent inhibition of migration of these cells as compared to the control (untreated HeLa cells) wherein a gradual movement into the wound was observed with complete closure occurring by 48 h of incubation (Figure 6).

EGCG inhibit the migratory activity of HeLa cells by modulating the expression of MMP-9 and TIMP1

To decipher the probable anti-invasive mechanism of EGCG, we analyzed the expression of Matrix metalloproteinases-9 (MMP-9) and Tissue inhibitors of Matrix metalloproteinases-1 (TIMP-1) before and after treatment with 25 μM EGCG at various time points. Untreated HeLa cells showed a marked expression of MMP-9 (Figure 7). However, a significant time-dependent downregulation of MMP-9 expression was observed in EGCG treated HeLa cells (Figure 7). Thus, the invasion inhibitory effect of EGCG might be due to the suppression of MMP-9 expression, one of the mediators of tumor cell migration.

It is well established that MMP activity is controlled by endogenous inhibitors, such as TIMPs. Changes in TIMP-1 levels directly affect MMP-9 activity. Therefore, we evaluated TIMP-1 expression in untreated and EGCG-treated HeLa cells (Figure 7). A significant time-dependent upregulation of the expression of TIMP-1 was observed, indicating the migration inhibitory activity of EGCG is due to the modulation of MMP-9 and TIMP-1 expression.

Discussion

Failure of conventional treatment strategies in
conjunction with invasion and metastasis are the main causes of cancer morbidity and mortality necessitating the development of alternative treatment modalities such as the chemoprevention which reverses, suppresses, or prevents carcinogenic progression to invasive cancer (Sporn and Suh, 2000). Many dietary agents such as curcumin, resveratrol, gingerol, retinoic acid, gallic acid, genistein etc are being widely studied for their anti-cancer properties, which may act by suppression of proliferation, induction of apoptosis or inhibiting angiogenesis and invasion (Aziz et al., 2003; Matsuura et al., 2006; Sharma et al., 2006; Lee et al., 2008; Gupta et al., 2011; Qi et al., 2011). Various reports suggest that EGCG, a green tea catechin, possesses cancer–preventive, antioxidant, anti-mutagenic, apoptotic, and anti-inflammatory properties (Roy et al., 2001; Takahashi et al., 2004; Seeram et al., 2006; Shankar et al., 2008; Hsu and Liou, 2011; Zhu et al., 2011). Hence, this study focused on elucidating the chemopreventive action of EGCG in the cervical cancer. We observed that EGCG treatment decreased the cell viability in a dose dependent and time dependent manner in HeLa cells and the IC_{50} was found to be 100 µM for duration of 24 h and 50 µM for 48 h (Figure 1). Our study is consistent with other previous studies which also showed that EGCG inhibits cancer cell viability in a dose-dependent manner in various cancer cells such as human cervical cancer cells, human pancreatic cancer, human hepatic carcinoma cells, human ovarian cancer cells, human colon cancer cells and human T-cell leukemia cells (Takada et al., 2002; Harakeh et al., 2008; Qiao et al., 2009; Shirakami et al., 2009; Zhu et al., 2011). These results were further confirmed by microscopic examination of EGCG treated cells which showed characteristic rounding off of dying cells and detachment from the substratum compared to untreated cells indicating that the cell death induced by EGCG may be through the apoptotic pathway (Figure 2). The percentage of non-viable cells increased in a dose and time-dependent manner on treatment with 25, 50 and 100 µM EGCG for 24 and 48 h.

It is well established that chemopreventive agents exert their effects on cancer cells via apoptosis (D’Agostini et al., 2005; Sharma et al., 2006; Lee et al., 2008; Gupta et al., 2011). In order to confirm that the mode of cell death induced by EGCG is via apoptosis, we analyzed changes in nuclear morphology of HeLa cells on treatment with EGCG (50 µM) for varying time points (0, 6, 24 and 48 h) by PI staining. We observed that the untreated cells showed no signs of apoptosis indicated by large and prominent nuclei. However, EGCG treatment resulted in accumulation of apoptotic changes such as nuclear condensation, chromatin fragmentation and formation of nuclear debris identified as apoptotic bodies in these cells which increased in a time-dependent manner (Figure 3).

One of the biochemical hallmarks of apoptosis is genomic DNA fragmentation, an irreversible event that commits the cell to die (Matsuura et al., 2006; Rao and Pagidas, 2010). Thus, we performed the DNA fragmentation assay after treatment with 50 µM EGCG for 24 and 48 h. In untreated cells, no significant fragmentation of DNA was observed whereas EGCG treatment for 24 and 48 h induced DNA fragmentation in HeLa cells (Figure 4). Our results were consistent with other studies in bladder tumor cell line and ovarian cancer cells where EGCG induced apoptosis by causing DNA damage (Li et al., 2000; Chen et al., 2004; Rao and Pagidas, 2010).

We further examined whether DNA fragmentation induced upon EGCG administration was due to accumulation of cells possessing a sub-G_{1} DNA content, as a measure of apoptosis, using flow cytometry. We found that the percentage of EGCG-treated cells in the sub-G_{1} phase which may comprise of apoptotic cells was significantly increased as compared to the untreated cells after 24 and 48 h treatment with EGCG (Figure 5), suggesting a up-regulation of cell death machinery. This is in agreement with previous studies which established that apoptosis is the likely mechanism of action of green tea polyphenol, EGCG for inhibiting cell growth in various carcinomas (Yang et al., 1998; Gupta et al., 2003; Huh et al., 2004; Qanungo et al., 2005; Khan et al., 2006; Gu et al., 2009; Tang et al., 2010). It has also been shown that treatment of MCF7 breast cancer cells with EGCG resulted in G_{1}/G_{2} phase cell cycle arrest (Liang et al., 1999). Thus, the activation of apoptosis is believed to play a critical role in both the chemo prevention and treatment of human carcinomas.

During the development of most types of human cancer, primary tumor acquires the ability to invade adjacent tissues, and thus become capable to colonize distant sites thus resulting in metastases (Sporn, 1996; Wels et al., 2008). At this juncture, we determined EGCG mediated inhibition of HeLa cell migration, with the objective to assess its anti-invasive property. As determined by the in vitro scratch wound closure assay, EGCG inhibited HeLa cell migration observed by the overgrowth of an acellular line in the wounded areas of the confluent monolayer as compared to the untreated controls wherein complete wound closure was observed by 48 h of drug incubation (Figure 6). Our results are consistent with other studies showing the anti-invasive activity of EGCG in various carcinomas (Punathil et al., 2008; Chan et al., 2010; Park et al., 2010).

Two gene families, MMPs and TIMPs are involved in a wide range of proteolytic events, including tumor growth, migration, metastasis and angiogenesis (Khuri et al., 2001; Giannelli et al., 2005). To further elucidate the invasion inhibitory effect of EGCG, we studied the expression of MMP-9 and TIMP-1 on treatment of HeLa cells with EGCG. We found that untreated HeLa cells have significantly high expression of MMP-9, accounting for its invasive behaviour. Also, the results showed that the expression level of MMP-9 in the HeLa cells was downregulated after EGCG treatment in a time-dependent manner with no detectable expression by 48 h of incubation (Figure 7). These results were parallel with EGCG-induced suppression of MMP-9 in various carcinomas (Maeda-Yamamoto et al., 2003; Kim et al., 2004; Harakeh et al., 2008; Park et al., 2010; Roomi et al., 2010; Sen et al., 2010; 2011).

TIMP-1 has been shown to directly affect MMP activity (Gomis-Ruth et al., 1997; Holten-Andersen et
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These studies establish a promising area of investigation in understanding the EGCG-induced cellular signal transduction events that seems to have implications in the inhibition of cell proliferation, induction of apoptosis as well as inhibition of tumor invasion and metastasis. However, further studies on bioavailability of EGCG are needed to more effectively determine its potential usefulness as a cancer preventive agent.

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