Epigallocatechin gallate, the main ingredient of green tea induces apoptosis in breast cancer cells

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1. ABSTRACT

Green tea has been suggested for prevention of cancers. In this study, the effect of the main constituent of green tea, epigallocatechin gallate (EGCG), on apoptosis of breast cancer cells was examined. EGCG induced apoptosis in T-47D cells through caspase cascade and the cells were detained at the G1 phase. The rate of apoptosis and activity of caspase-3 induced by EGCG was time and dose dependent. These findings suggest that EGCG might be useful in treatment and/or prevention of breast cancer by inducing apoptosis.

2. INTRODUCTION

Green tea has been an essential part of Chinese daily life for almost 2000 years. Chinese people regard green tea as a beneficial beverage. A number of monographs of traditional Chinese medicine mentioned the pharmacological functions of green tea. Modern scientific research has also reported that green tea is salubrious. In 1986, Hirota Fujiki *et al* reported that epigallocatechin gallate (EGCG), the main constituent of green tea, has a possible effect on prevention of cancers (1). As a non-toxic agent beneficial to multiple organs of the body, green tea attracted worldwide interest. With the development of chemotherapy, chemoprevention has played an increasingly important role in the treatment of tumors (2, 3). The chemopreventive effects of EGCG have been investigated by a variety of rodent tumor models (4, 5). One of the most plausible mechanisms of its chemopreventive activity might be its ability to suppress the promotion of carcinogenesis, demonstrated in cultured cells as well as in animals. The molecular mechanism of the antiproliferative action of EGCG in cancer cells has, in part, attributed to the induction of a highly regulated cellular process called

apoptosis (6). In recent years, the incidence of breast cancer in China has been very high, and is on the rise year by year. Some previous studies demonstrated the ability of EGCG to suppress growth and invasion of breast cancer cells in a dose- dependent manner (7). In our study, we observed the change of Caspase-3, a key signal molecule to induce cell apoptosis, to determine whether the antiproliferative effect of EGCG in breast cancer cell lines is mediated through apoptosis.

3. MATERIALS AND METHODS

3. 1. Main reagents and cell culture

Human breast cancer cell lines T-47D were purchased from ATCC. EGCG was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). Caspase-3 colorimetric activity assay kit was purchased from Chemicon International and stored at -20°C. This kit included 5× Cell Lysis Buffer, 5×Assay Buffer, Caspase-3 Substrate (Ac-DEVD- PNA), Caspase-3 Inhibitor (Ac-DEVD-CHO) and pNA Standard. T-47D was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml gentamicin at 37 ^{o}C in a 5% CO_2 humidified atmosphere.

3. 2. The 3-(4, 5-dimethylthiazo-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay

For MTT assay, $2×10^6$ cells of T-47D were cultured in a 96-well-plate with or without 0.1, 0.2 and 0.3 mM EGCG for 24, 48, and 72h. MTT (1mg/ml, Sigma) solution was then put in and the cells were cultured for another 4 h. Then DMSO was applied and quaked for 10 min. Viable cells could cleave MTT and produce dark brown formazan in the cells. The amount of MTT formazan product was determined by measuring absorbance with a microplate reader at a test wavelength of 490 nm. The absorbance values were used to express the level of cell survival.

3. 3. Comet assay

The Comet assay is an indirect measurement of endonuclease activity and an initial confirmation of chemical-induced apoptosis. The assay was conducted under alkaline conditions essentially according to the procedure of Janusz *et al.* (8) with modification. A newly prepared suspension of cells, which had been cultured under the optimal concentration and time selected by MTT assay in 0.75% low melting point agarose (Sigma Chemicals) dissolved in phosphate buffered saline (PBS; Sigma Chemicals), was cast onto microscope slides precoated with 0.5% normal melting agarose. Then, cells treated for 48h with 0.2 mM EGCG were lysed for 1 h at 4 ^{o}C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10 mM Tris, with pH=10. After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution containing 300 mM NaOH, and 1 mM EDTA, with pH > 13. Electrophoresis was conducted at 4 ^{o}C for 30 min at electric field strength 0.73 V/cm (30 mA). The slides were then neutralized with 0.4M Tris, pH 7.5, stained with 20 mg/ml EB (ethidium bromide) and covered with cover slips. The slides were examined at 200×magnification in an Eclipse fluorescence microscope

(Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu) equipped with a UV filter block containing an excitation filter (359 nm) and barrier filter (461 nm) and connected to a PC-based image analysis system CASP (Dutch). Fifty images were randomly selected from each sample and the comet tail DNA was measured. Each experiment was conducted twice. Percentage of DNA in the tail (% tail DNA) was analyzed. It was positively correlated with the level of DNA breakage or/and alkali-labile sites in the cells and was negatively correlated with the level of DNA cross links. The mean value of the % tail DNA in a particular sample was taken as an index of DNA damage in this sample.

3. 4. Cell cycle analysis by flow cytometry

After the designated treatment period with 0.2 mM EGCG, T-47D cell lines were collected into centrifuge tube by trypsinization. After centrifugation (1000rpm, 5min), the supernatant was removed, and cells were plated at $1×10^5$ cells into PBS. Then, washed with Hank's, the pellet of cells was fixed by resuspending in cold 70% methanol and placed at -20 ^{o}C. At the time of flow cytometry, the fixed cells were washed in PBS and resuspended in 1 ml of propidium iodide staining solution (0.1% Triton X-100, 40 μg propidium iodide and 100 μg RNAse A in PBS). The samples were incubated at room temperature for 30min in the dark and analyzed on a Becton-Dickerson FACScan (BD Company, USA).

3. 5. The Caspase-3 colorimetric activity assay

Cells were collected as described before and analyzed by Caspase-3 colorimetric activity assay kit. In brief, cells were incubated in the lysis buffer for 10min on ice and then centrifuged at 10,000 rpm for 1min. Enzymatic reactions were carried out in a 96-well flat bottom microplate. To each reaction sample 50 μl of cell lysate (100–200 μg total protein) was applied. Additional controls, one free from cell lysate and the other lacking substrate, had been included. After being stored at room temperature for 2h, the OD value of Caspase-3 was measured in ELISA reader. The results were expressed as activity (IU/mL) and specific activity (IU/mg protein) of caspase-3.

3. 6. Statistical analysis

All data are subjected to the statistical processing, including X^2 test, 4-chess table exact probability method, with significant level of two-side alpha = 0.05.

4. RESULTS

4. 1. MTT assay

Green tea contains catechins, which are believed to be efficacious in the prevention of cancers, and EGCG is the most significant catechin in this beverage. Therefore, we performed MTT analysis to evaluate the cell survival following exposure to EGCG. As shown in table 1, the presence of EGCG significantly reduced the number of apoptotic T-47D cells, in a time (24, 48 and 72h) and dose (0, 0.1, 0.2 and 0.3 mM) dependent manner (P<0.05). The significant inhibition of cell proliferation,

Table 1. Changes in the *A* values of T-47D cells induced by EGCG

Time (Hour)	CK (EGCG N/A)	EGCG (0.1 mM)	EGCG (0.2 mM)	EGCG (0.3 mM)
24 h	0.875±0.077	0.702±0.061*	0.532±0.044*	0.797±0.055
48 h	0.846±0.048	0.648±0.049*	0.357±0.041**	0.841±0.057
72 h	0.859±0.054	0.863±0.052	0.793±0.067	0.852±0.069

* *P* <0.05, ** *P* <0.01, CK *vs* EGCG group

Table 2. Change in the rate of apoptosis in T-47D cells induced by EGCG

Time (Hour)	CK(EGCG N/A)	EGCG (0.1 mM)	EGCG (0.2 mM)	EGCG (0.3 mM)
24	0.12±0.118	2.40±0.451*	9.53±0.644*	0.08±0.100
48	0.21±0.239	4.64±0.549*	31. 57±1.341**	0.11±0.107
72	0.09±0.110	0.13±0.122	0.23±0.317	0.12±0.139

* *P* <0.05, ** *P* <0.01, CK *vs* EGCG group

Table 3. Changes in the OD values of T-47D cells induced by EGCG

Time (Hour)	CK (EGCG N/A)	EGCG (0.1 mM)	EGCG (0.2 mM)	EGCG (0.3 mM)
24	0.465±0.034	0.302±0.037*	0.232±0.044*	0.457±0.035
48	0.446±0.039	0.248±0.029*	0.157±0.041**	0.441±0.047
72	0.459±0.044	0.463±0.032	0.447±0.038	0.452±0.042

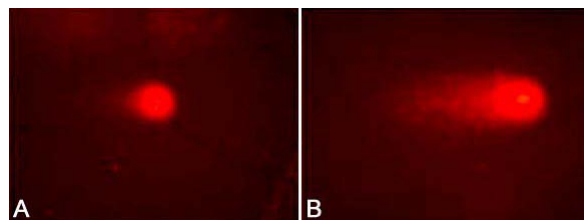*P<0.05,**P<0.01,CK*vs* EGCG group, OD: optical density

Figure 1. Evaluation of DNA damage using the COMET assay. Cells treated with EGCG (0 or 0. 2mM) for 48 h were submitted to the COMET assay (see materials and method). DNA was stained with ethidium bromide (20 mg/ml) and the comet tail DNA was visualized under an Eclipse fluorescence microscope (Nikon, Tokyo, Japan). The length of the comet tail is positively correlated with the level of DNA damage in the sample. Representative control and treated samples are shown on **A** and B, respectively.

corresponding to the lowest absorbance value (0.357±0.041), was observed in the group of cells treated with 0.2 mM EGCG for 48h (*P*=0.004).

4.2. Comet assay

From the first experiment, EGCG at 0.2 mM for a treatment period of 48h appeared as the optimal combination for the following experiments. In order to determine whether the suppression of cell proliferation by EGCG was due to apoptosis (DNA damage), the COMET assay was applied on cells (10^6 cells) treated for 48h with 0.2 mM EGCG. Figure 1 displays the representative comet tails of the alkaline version for controls and breast cancer patients.

While comet tails originating from controls (A) had a nearly symmetrical shape with practically no tails, cells treated for 48h with 0.2 mM EGCG were characterized by a fairly long tail (B). It's also notably observed that these comet profiles had very long tails and small heads, and their density (i.e., the number of comets

per slide) was much higher than that of the cells without EGCG. This might indicate that EGCG resulted in complete fragmentation of DNA in considerable number of cells. In our study, the mean endogenous DNA damage measured as the comet tail moment of the T-47D cells was 4.92±0.64 and 18.76±1.37 for controls and treated groups, respectively. The values were significantly different (*P* = 0.003).

4.3. Flow cytometric analysis of cell cycle and apoptosis

Apoptotic cells could be recognized and distinguished from necrotic cells with FCM of cellular DNA. Apoptotic cells showed a diminished staining below the G_0/G_1 population of normal diploid cells and the DNA specific fluorochrome PI identified a distinct hypo-diploid cell population. As shown in figure 2 and table 2, exposure of T-47D cells strains to EGCG significantly increased the incidence of apoptosis in a dose and time dependent manner (P<0.05). The highest induction was found with an exposure of 48h to 0.2 mM EGCG (*P*=0.003). In EGCG-treated cells, a notable accumulation of cells was observed at the G_1 stage associated with a remarkable decrease at the S stage. None of these parameters were affected by 0.3 mM EGCG or 72h treatment.

4. 4. Expression of Caspase-3

The Caspase-3 colorimetric assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by Caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. P-Nitroaniline had a high absorbance at 405 nm. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm. As shown in Table 3 and Figure 3, the presence of EGCG, and the activity of Caspase-3 appeared to be time and dosage dependent. The combination of 0.2 mM EGCG and 48h treatment yielded the lowest activity peak value (*P*<0.05). Compared to the cells with the Caspase-3
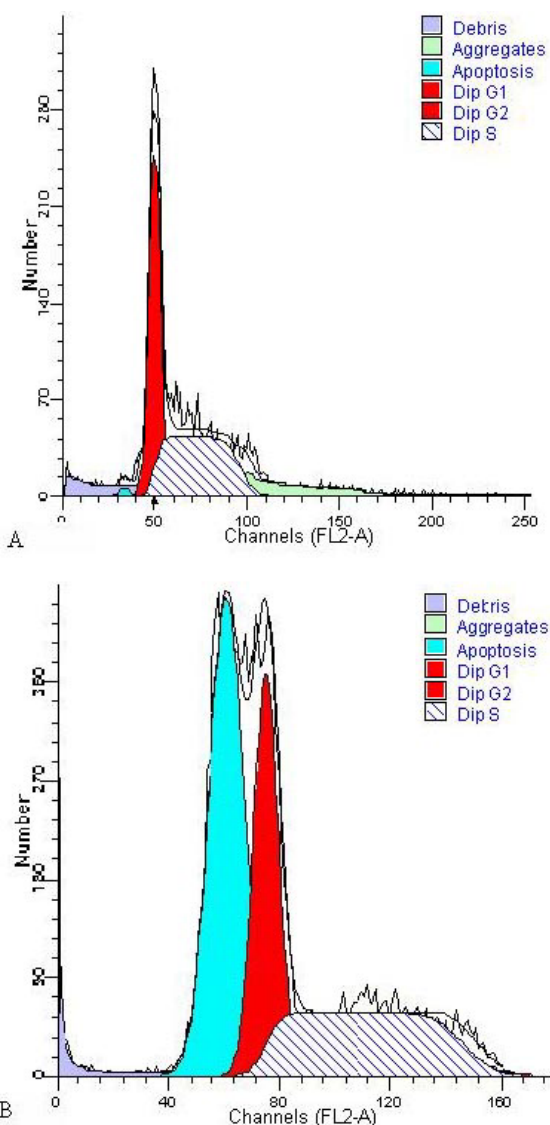
Figure 2. Effect of EGCG on the incidence of apoptosis. The CK cells were treated with EGCG (0 or 0.2mM) for 48 h and submitted to evaluation of the apoptosis levels using the flow cytometry. A representative curves obtained with the control and treated cells are shown on **A** and B, respectively.

inhibitor applied, the Caspase-3 activity of those without the Caspase-3 inhibitor rose remarkably (P<0.05). The cells with the inhibitor applied were not time and dosage dependent on EGCG. Thus, kinetically, the activation of Caspases preceded the induction of apoptosis. Further confirmation of the involvement of Caspase-3 in the EGCG-induced apoptosis in the T-47D cell line was provided by the results obtained from cells treated with the Caspase-3 inhibitor.

5. DISCUSSION

Cancer is described as a disease that involves excessive proliferation of cells and abandonment of their ability to die. Normally, cells can kill themselves in a balanced process known as 'apoptosis' (9-10). It is now clear that too little cell suicide by apoptotic process can lead to a variety of cancers, including breast cancer (11, 12). Our study suggests that EGCG has an antiproliferative effect on breast cancer cells and the mechanism is to mediate through programmed cell death or apoptosis.

EGCG, which is water-soluble with limited permeability through the plasma membrane of cells, may bind to certain receptors on the cell surface, thereby provoking receptor-like intracellular signals. In addition, EGCG may utilize the kinase cascade (Caspase cascade) in a similar manner to toxic substances or cellular stress (13, 14). Caspases are a group of cysteinyl aspartate-specific proteinase, and the cascade activation of these families of proteinases is a very important way of apoptosis. It seems that Caspase-3 is necessary to apoptosis, and hydrolysis of the substrate by Caspase-3 is the strongest. Overall, our understanding of the molecular mechanisms by which EGCG exerts the antitumorigenic effect is not clear. But induction of apoptosis by EGCG is one observed fact to explain the antitumorigenic activity. Although the effect of green tea on human breast cancer is still not clear from epidemiologic studies, experimental evidence has indicated clearly that green tea components can induce antitumorigenic activity by apoptosis in breast cancer cells.

Although some studies indicate the antiproliferative effect of EGCG on breast cancer (15-17), the mechanism of action has not been investigated clearly. In this study, we provide Caspase-3 as a novel mechanism of catechin-induced apoptosis, and show that EGCG has a variable effect on the expression of Caspase-3 protein, apoptosis induction and cell growth rate in human breast cancer cells. Interestingly, our observation indicates that the cell apoptosis rate and the activity of Caspase-3 are time and dosage dependent on EGCG. The requirement of optimal concentration of EGCG to suppress cell proliferation or to induce apoptosis in breast cancer cells may be due to the differential cell permeability of EGCG or sequestering of EGCG by other proteins yet to be identified and optimal time of EGCG to induce apoptosis may be due to the activity of enzyme. Takada et al. (18) demonstrated the suppression of growth inhibition, as well as invasion of pancreatic cancer cells, at 0.1- 0.2 mM concentration of EGCG. It's presumed that 0.1 mM concentration of EGCG is often required to trigger stress signal in cancer cells. At present, two major pathways that link apoptosis have been identified: (1) intrinsic or mitochondrial and (2) extrinsic or death receptor-related (19-21). The intrinsic pathway involves the cell sensing stress that triggers the mitochondria-dependent processes, resulting in activation of caspase-9. The extrinsic pathway is triggered by binding of ligands such as TNF or FasL to their receptors, leading to the recruitment of adaptor proteins such as FADD and the subsequent activation of procaspase-8. Finally, both pathways pass through the Caspase-3 to induce cell apoptosis. As observed in our study, EGCG-induced apoptosis in breast cancer cells may be orchestrated by the cooperative effects of both 'extrinsic' and 'intrinsic' pathways.
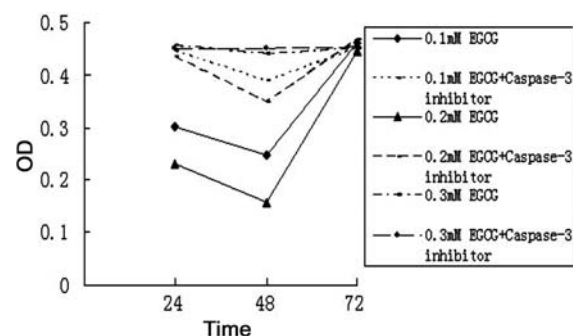
Figure 3. The optical density (*OD*) values of T-47D cell line affected by different density EGCG with Caspase-3 inhibitor added at different times. X refer to time/hour, Y refers to OD values

However, apoptosis induced by green tea in breast cancer needs further investigation. Our study has confirmed that green tea can be a better cancer preventive agent which is non-toxic and beneficial to other organs. Applying green tea in chemoprevention may be a good choice in the future.

6. ACKNOWLEDGMENTS

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