Original Article

Effects of tea polyphenols on proliferation and apoptosis of cadmium-transformed cells

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Abstract: To investigate the roles and mechanism(s) of epigallocatechin gallate (EGCG) in carcinogenesis in malignant transformed cell line, cadmium-induced malignant transformed cells were treated with different doses of EGCG. Then cell proliferation, cell apoptosis, hTERT mRNA and protein level, and c-Myc protein levels were measured at different time points. EGCG was found to inhibit cell proliferation in a dose-dependent manner. Cell cycle was changed in the transformed cells after EGCG treatment with significantly increased cell numbers in G0/G1 phase and decreased cell numbers in S phase compared to control group, \( P < 0.001 \). EGCG was also found to promote cell apoptosis with a time-dependent manner. Both mRNA and protein levels of hTERT gene were significantly decreased in cells after treated with EGCG, \( P < 0.001 \). c-Myc protein level was significantly decreased after EGCG treatment, especially in the highest dose group (i.e. 200 µg/ml). The decrease in c-Myc protein level was accompanied by the reduction of hTERT protein levels. EGCG can inhibit cell proliferation and promote apoptosis in malignant cadmium-transformed cell line. The mechanism may be its ability to reduce c-Myc gene expression and consequently inhibits hTERT gene expression, which in turn decrease the telomerase activity.

Keywords: Tea polyphenols, cadmium, cell proliferation, apoptosis, telomerase

Introduction

Cadmium is a heavy metal existing widely in nature. Human can expose to cadmium via polluted air, water, and soil [1, 2]. Cadmium can cause damages in multiple organs and systems, including kidneys, liver, respiratory system, nerve system and skeleton system [1, 3-5], it can also induce various cancers [6]. The International Agency for Research on Cancer (IARC) classified cadmium as group 1 carcinogen in 1993, and the United Nations Environment Program and the International Commission on Occupational Health considered cadmium as the main environmental contaminants [17]. There are studies about the mechanisms of cadmium carcinogenesis. However, the mechanism is still not clear.

Human telomerase is a ribonucleoprotein enzyme, which comprises a reverse transcriptase protein (human telomerase reverse transcriptase, hTERT), the telomerase RNA (TERC/TR) and telomerase-associated protein (hTEPI). The hTERT is the catalytic subunit of telomerase. It is also a determinant factor of activation of telomerase. The hTERT is associated with tumorigenesis and the degree of malignancy, and its expression is regulated by a variety of factors [7]. hTERT can promote cell proliferation and inhibit apoptosis through extending the shortened telomere during DNA replication [8]. Most of immortalized cells and tumor cells have an ability to replicate and express telomerase activity infinitely [9]. Telomerase activation can stabilize telomeres, thereby making cell infinite replication. Recent studies have found activation of telomerase in more than 90% of tumors, indicating that activation of telomerase plays an important role in tumorigenesis [10]. Study showed that cadmium chloride increased the hepatocellular telomerase activity in a dose-dependent manner and significantly induced the apoptosis of hepatocytes [11]. Therefore, cadmium may activate telomerase and consequently increase the risk of carcinogenesis.

Tea polyphenols is the main ingredient in tea, mostly in green tea, accounting for 25-35% of tea dry weight. It is related to many beneficial
effects found in tea. Tea polyphenols consist of more than 30 kinds of polyphenols, including catechins, flavonoids, anthocyanins, phenolic acids. Among these polyphenols, catechins is the major one and accounts for 20-30% of tea dry weight. Epigallocatechin gallate (EGCG) is the most abundance catechins [12, 13]. It was found that EGCG had antioxidant ability without known side effects. Animal and human studies showed that EGCG can inhibit the occurrence and progress of a variety of tumors [12-15], mainly through inhibiting tumor cell proliferation [16-18]. However, it is not clear whether EGCG can inhibit cadmium-induced carcinogenesis.

The present study investigated the roles and mechanisms of EGCG in cellular proliferation of cadmium-induced malignant transformed cell line. The results showed the potential use of EGCG in preventing/inhibiting cancer cells growth.

Materials and methods

Cell line

Malignant transformed human bronchial epithelial cell line (16HBE) induced by cadmium chloride was used in the present study. The cell line was established previously [19]. The cell line had the ability to form tumor in naked mouse after inoculation. The histological morphology showed squamous cell carcinoma with low level of differentiation [19].

Cell culture

The cell line was cultured in MEM media containing 10% fetal bovine serum (FBS) in a humid incubator at 37°C with 5% CO₂. This condition was used in all the experiments. For cell passaging, a solution with 0.25% of trypsin and 0.02% EDTA was used (1:1 in v/v). Cells were cultured in 96-well plates with approximately 3000 cell/well.

EGCG preparation

EGCG was purchased from Chengdu Ke Rui Fa Research & Development Company (Sichuan, P.R. China). The purity of the product was 95%. EGCG was dissolved in MEM medium.

CCK-8 assay

After 24-48 h of culture, cells were washed twice with PBS. Then cells were treated with different doses of EGCG, i.e. 0, 50, 100, 150, and 200 µg/ml of EGCG. Each dose group contained 4 wells of cells. The cells were then treated for 0, 12, 24, 48, and 72 h. At the end of each time point, cells were washed twice with PBS and then 100 µl of fresh MEM media without EGCG was added to each well. Immediately 10 µl of CCK-8 reagent was added and well mixed. The reaction took place in a 37°C incubator for 30 min. Then the plates were read in a plate reader with wavelength of 560 nm. Inhibition rate was calculated by the formula as follows, inhibition rate = (mean A value in control group-mean A value in treatment group)/mean A value in control group.

Cell cycle measurement

Log-phase cells were determined, counted, and passaged. The passaged cells were made sure to grow well at 24 h after the passage. Then fresh MEM media was added with different doses of EGCG, i.e. 0, 50, 100, 150, and 200 µg/ml of EGCG and the cells were cultured for 48 h. Another experiment was carried out with 100 µg/ml of EGCG, but the cells were cultured for 0, 12, 24, 48, and 72 h.

At the end of the experiment, cells were washed twice with cold PBS and then collected by centrifugation. The cell pellets were washed another 2 times with cold PBS, and then were fixed with 70% ethanol overnight at 4°C or -20°C for long time preservation. After fixation, cells were washed once with PBS. The cells were then suspended in 500 µl of PBS containing 0.2% Triton X-100, and 1 µl of RNase A (100 µg/ml).

Table 1. Inhibition rates (%) of cell growth by EGCG

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>EGCG concentrations (µg/ml)</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>22±3.9</td>
<td>30±4.3</td>
<td>47±3.1</td>
<td>55±5.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>38±4.5</td>
<td>51±3.4</td>
<td>63±3.7</td>
<td>70±4.6</td>
<td></td>
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<tr>
<td>48</td>
<td>48±3.2</td>
<td>60±4.8</td>
<td>72±5.1</td>
<td>79±5.1</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>62±3.6</td>
<td>74±2.1</td>
<td>87±4.5</td>
<td>93±5.4</td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean ± standard error. Each concentration group included 4 replicates. A significant dose-response relationships was observed as each time point, P < 0.05. With time, cell growth was significantly inhibited at different dose group, P < 0.05.
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Table 2. Effects of EGCG (100 µg/ml) on cell cycle of Cd-transformed cells

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time (h)</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td></td>
<td>39.24±1.12</td>
<td>47.12±1.28*</td>
<td>56.45±3.25*</td>
<td>64.38±2.06*</td>
<td>73.18±2.22*</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>41.09±2.60</td>
<td>33.03±0.88*</td>
<td>27.52±4.20*</td>
<td>25.95±0.93*</td>
<td>18.16±1.67*</td>
</tr>
<tr>
<td>G2/M</td>
<td></td>
<td>19.67±2.81</td>
<td>19.85±1.83</td>
<td>16.03±1.54</td>
<td>9.66±1.60</td>
<td>8.66±0.64</td>
</tr>
</tbody>
</table>

Data represent mean ± standard error. Each concentration group included 3 replicates.

*Comparisons between 0 h and other time points showed significantly different, same phase, P < 0.002.

The cells were incubated at 37°C for 30 min. Then 5 ml of propidium iodide (PI) (50 µg/ml) was added into each tube, and incubated in dark at room temperature for 30 min. Cells were then analyzed through flow cytometry. The cell number was between 10000 to 20000. The cell cycle was further analyzed with ModFit software.

Apoptosis assay

Cells in log-phase were collected and passaged. The passaged cells were sure to grow well at 24 h after the passage. Then cells were treated with 0 or 200 µg/ml of EGCG for various times, i.e. 12, 24, 48, and 72 h. At the end of each time points, cells were washed twice with cold PBS and then collected by centrifugation. The cell pellets were washed another 2 times with cold PBS, and then were fixed with 70% ethanol overnight at 4°C or -20°C for long time preservation. After fixation, cells were washed once with PBS. The cells were then suspended in 400 µl of medium. Cell number was adjusted to 10000 to 20000 per tube before filtered through a 50 µm membrane. Then 5 µl of propidium iodide (PI) (50 µg/ml) and 3 µl of Annexin-V FITC were added into each tube, and incubated in dark at room temperature for at least 10 min. Cells were then run through a flow cytometry for detection of apoptotic cells.

Expression of hTERT mRNA by RT-PCR

Passaged cells as described above were allowed to grow till 60-70% confluence in dishes. After changed with fresh media, the cells were then treated with EGCG as follows. 1) Cells were treated with 100 µg/ml of EGCG for 0, 12, 24, 48, and 72 h; or 2) cells were treated with various doses of EGCG (i.e. 0, 50, 100, 150, and 200 µg/ml) for 48 h.

At each end point, cells were washed 3 times with cold D-Hanks. Then RNA was extracted according to the instructions provided with the Trizol kit (GIBCO BRC). RNA concentration and purity were measured to make sure the RNA integrity.

For RT-PCR, the first step was carried out in 15 µl of reaction buffer containing 2 µl of primer (0.5 µM) and 1 µg of total RNA. The reaction took place at 65°C for 5 min and then put on ice for at least 2 min. The next step was performed in 20 µl of reaction mixture with 15 µl of mixture from the first step, 4 µl of 5 X iScript reaction mix, 1 µl of reverse transcriptase (Bio-Rad, US) at the following condition, i.e. 25°C for 5 min followed by 42°C for 30 min. The reaction was stopped by heating to 85°C for 5 min. The final product was then diluted 5 X with ddH₂O and stored at -20°C for future use.

hTERT mRNA was then measured by SYBR assay (Ambion, US). The total reaction volume was 10 µl, consisting of 5 µl of 2 X SYBR Premix DimerEraser, 0.5 µl of 20 µM reverse primer, 0.5 µl of 20 µM forward primer, 0.2 µl of ROX Reference Dye, 1 µl of cDNA synthesized above, and 2.8 µl of ddH₂O. The reverse primer of hTERT gene was 5'-CTGATGAAATGGGAGCTGACG-3', and the reverse primer was 5'-TGTGCACCAACATCTACAAGATC-3'. The reaction was first carried out at 95°C for 30 s followed by 45 cycles with 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s.

Western blot

Cells were passaged as described above and then treated with various doses of EGCG, i.e. 0, 100, 150, and 200 µg/ml for 48 h. Cells were collected by centrifugation at 4°C, and then lysed with cold lysis buffer for protein extraction. The lysates were centrifuged 12000 rpm at 4°C for 15 min. The supernatants were collected and measured for protein concentration with BCA method. Totally 50 µg of protein at different time points was used for western blot. Primary antibodies against hTERT and c-Myc were diluted 1000 times. The secondary antibody was diluted 2000 times.
Figure 1. Effects of single dose of EGCG on cell cycle at different times. Cell were treated with 100 µg/ml of EGCG and percentages of cells in different phases of cell cycle were then determined at 12 h, 24 h, 48 h and 72 h.
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Statistical analysis

Data were presented as mean ± standard deviations of three to four independent experiments. Student t-test and one way ANOVA were used to analyze the data. SPSS 15.0 software was used. A \( P < 0.05 \) was considered statistically significant.

Results

EGCG inhibited growth of malignant transformed cells

Cell growth was significantly inhibited at different times after exposure to EGCG (Table 1). For example, the inhibit rate was greater than 60% in the lowest dose group (50 µg/ml) and greater than 93% in the highest dose group (200 µg/ml) at 72 h after treatment with EGCG (Table 1). There were strong dose-response relationships between EGCG treatments and cell growth inhibition, \( P < 0.05 \). With time after same dose of EGCG treatment, cell inhibition rates were significantly increased, \( P < 0.05 \).

EGCG interrupted cell cycle

The proportion of cells at G0/G1 phase was gradually increased with time after treatment with 100 µg/ml of EGCG. Correspondingly the proportion of cells at S phase was gradually decreased with time, \( P < 0.002 \) (Table 2; Figure 1). For example, cells at G0/G1 phase accounted for 73.18% at 72 h after treatment with EGCG, which was contrasting to 39.2% in the control group, whereas cells at S phase decreased from 33.3% to 18.6% at 72 h after treatment of EGCG (Table 2).

There was no significant dose-dependent effect between EGCG treatment and cell cycle after 48 h of treatment with EGCG (Table 3). However, each of the dose group caused significantly change in the proportion of cells at G0/G1 and S phases compared to the control group (\( P = 0.000 \)) (Figure 2).

EGCG induced apoptosis at different times after exposure to 200 µg/ml of EGCG, \( P = 0.000 \) (Table 4). The number of cells that went through apoptotic death significantly increased with time, e.g. 26.37% and 45.61% of cells died at 24 h and 72 h, respectively, after treatment with EGCG, \( P < 0.01 \).

hTERT mRNA levels were decreased after treatment with EGCG

At 48 h after EGCG treatment, cells showed significant decrease of hTERT mRNA levels in all treatment groups compared to the control group, \( P < 0.01 \) (Table 5). A dose-response relationship was observed between EGCG treatment and hTERT mRNA levels. When cells were treated with a single dose of EGCG (100 µg/ml), significantly decreased hTERT mRNA levels were found at different times after treatment compared to the control group (Table 6), whereas no significant differences between hTERT mRNA levels among different times after treatment was observed.

hTERT and c-Myc protein levels were reduced by EGCG treatment

Cells treated with EGCG showed decrease levels of hTERT and c-Myc protein levels, especially at the highest dose group (200 µg/ml) (Figure 3).

Discussion

The present study demonstrated that EGCG inhibited proliferation and promoted apoptosis in cadmium transformed malignant cell line. EGCG is a Chinese medicine with efficacy of antitumor and regulation of immune system. However, the mechanism is not clear. Studies indicated that the antitumor function of EGCG was related to its inhibition of the enzymes involved into cell proliferation, e.g. urokinase

Table 3. Effects of EGCG on cell cycle of Cd-transformed cells at 48 h

<table>
<thead>
<tr>
<th>Phase</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>43.08±1.02</td>
<td>50.84±1.59*</td>
<td>61.83±1.05*</td>
<td>52.91±2.61*</td>
<td>57.54±2.12*</td>
</tr>
<tr>
<td>S</td>
<td>47.73±1.32</td>
<td>30.45±1.22*</td>
<td>29.90±0.78*</td>
<td>24.66±0.68*</td>
<td>20.51±2.35*</td>
</tr>
<tr>
<td>G2/M</td>
<td>9.28±2.05</td>
<td>18.71±0.47</td>
<td>8.26±0.94</td>
<td>22.43±1.97</td>
<td>12.95±0.25</td>
</tr>
</tbody>
</table>

Data represent mean ± standard error. Each concentration group included 3 replicates.
*Comparisons between 0 ug/ml and other dose groups showed significantly different, same phase, \( P < 0.001 \).
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Figure 2. Effects of EGCG on cells cycle at 48 h after treated with different dose of EGCG. The percentages of cells in different phases of cell cycle were determined at 48 h after treated with 50, 100, 150, and 200 µg/ml of EGCG.
Table 4. Effects of EGCG on cell apoptosis (%) of Cd-transformed cells

<table>
<thead>
<tr>
<th>EGCG (µg/ml)</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.55±0.68</td>
<td>4.73±0.58</td>
<td>6.07±0.25</td>
</tr>
<tr>
<td>200</td>
<td>26.37±2.20*</td>
<td>29.67±3.80*</td>
<td>45.61±3.96*</td>
</tr>
</tbody>
</table>

Data represent mean ± standard error. Each concentration group included 3 replicates. *Comparisons between 0 µg/ml and 200 µg/ml at different time points showed significantly different, P < 0.000.

Table 5. hTERT mRNA expression at 48 h after EGCG treatment

<table>
<thead>
<tr>
<th>EGCG concentration (µg/ml)</th>
<th>GAPDH</th>
<th>hTERT</th>
<th>2-ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.10±0.03</td>
<td>29.67±0.15</td>
<td>100%</td>
</tr>
<tr>
<td>50</td>
<td>15.61±0.19</td>
<td>28.95±0.05</td>
<td>58.78%</td>
</tr>
<tr>
<td>100</td>
<td>17.34±0.05</td>
<td>31.53±0.23</td>
<td>32.95%</td>
</tr>
<tr>
<td>150</td>
<td>16.76±0.11</td>
<td>31.42±0.07</td>
<td>23.6%</td>
</tr>
<tr>
<td>200</td>
<td>19.02±0.08</td>
<td>35.72±0.18</td>
<td>5.75%</td>
</tr>
</tbody>
</table>

Table 6. Time-dependent hTERT mRNA expressions in cadmium-transformed cells after treated with 100 µg/mL of EGCG

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>GAPDH</th>
<th>hTERT</th>
<th>2-ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.40±0.04</td>
<td>25.86±0.17</td>
<td>100%</td>
</tr>
<tr>
<td>12</td>
<td>17.62±0.21</td>
<td>26.57±0.05</td>
<td>37.28%</td>
</tr>
<tr>
<td>24</td>
<td>15.94±0.17</td>
<td>28.21±0.13</td>
<td>48.21%</td>
</tr>
<tr>
<td>48</td>
<td>17.59±0.11</td>
<td>28.66±0.18</td>
<td>34.57%</td>
</tr>
<tr>
<td>72</td>
<td>17.61±0.07</td>
<td>28.63±0.12</td>
<td>37.89%</td>
</tr>
</tbody>
</table>

In the present study, mRNA and protein levels of hTERT gene were found significantly decreased in cadmium transformed cells after treated with EGCG. It is known that hTERT can promote cell proliferation and inhibit apoptosis, therefore facilitating occurrence of tumor [21, 22]. Furthermore, hTERT gene is also found associated with the degree of tumor malignancy [7]. By targeting hTERT activity can inhibit tumor occurrence and progress. For example, nucleoside inhibitors such as AZT (3-azido-3' deoxythymidine) inhibit the activity of hTERT by forming a complex with hTERT and incorporating into telomere DNA to interrupt the bindings between the telomerase and DNA. This consequently resulted in tumor cell death in colorectal cancer, breast, liver, and lung cancers [23]. Inhibition of hTERT gene by shRNA markedly reduced the activity of telomerase in nasopharyngeal cancer cell lines, and consequently resulted in inhibiting cancer cell growth and promoting apoptosis [24].

Acknowledgements

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Disclosure of conflict of interest
None.

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References


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