Effect of Epigallocatechin Gallate on Apoptosis and Telomerase Activity of Hepatoma Cells

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Abstract **Objective** To explore the effects of EGCG on apoptosis and telomerase activity of hepatoma cells and its relativity. **Methods** The cytotocity of EGCG on Bel-7402 cells was tested by MTT assay. Apoptosis was determined by fluorescence microscope and flow cytometry analysis, and PCR-ELISA assay was used to detect telomerase activity. **Results** After Bel-7402 cells were treated with EGCG, apoptosis was induced and characterized by condensation of the nuclear, margination against the nuclear envelope and apoptosis body formation with fluorescence microscope and electronic microscope. Flow cytometry revealed that EGCG-induced apoptosis had a increased trendy of concentration and time dependency. Whereas, telomerase activity was decreased in a concentration time-dependency during EGCG induced apoptosis of Bel-7402 cells. Apoptosis of Bel-7402 cells correlated well with down-regulating telomerase during a certain concentration and time range (r=-0.948, p=0.014; r=0.967, p=0.007). **Conclusion** EGCG induced apoptosis in Bel-7402 cells by down-regulation telomerase activity. It may be a major mechanism for anticancer of tea.

Key Words Epigallocatechin-3-gallate(EGCG); Hepatoma cell; Apoptosis; Telomerase activity

Epigallocachin-3-gallate(EGCG) is a major component of green tea extracts. Several lines of evidence of the anticancer activities of tea catechins have emerged from animals and human epidemiological studies. Nevertheless, the exact molecular mechanisms are still indistinct and mostly speculative[1-2]. On the other hand, telomerase is the unique reverse transcriptase and plays an important role in the control of cellular proliferation capacity and senescence by maintaining the telomeres, the ends of chromosomes. Telomerase activity has been proved to associate with immortali zation of cells and cancer. More than 85% of all cancers express telomerase activity, whereas most somatic cells appear to lack detectable levels of telomerase activity [3]. Telomerase is expected as a target for cancer treatment. some reports have revealed that several drugs could inhibit telomerase activity. The aim of this experiment is to explore the effect of EGCG on apoptosis and telomerase activity in Bel-7402 cells.

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MATERIALS AND METHODS

Drugs and reagents

EGCG was purchased from Sigma Company and its purity was eighty percentage. EGCG was dissolved in redistilled water and filtered by 0.45μm filter membrane. RPMI-1640 was obtained from Gibco Laboratories. RNase A propidium iodide. Hochest 33258 were from Sigma Company (st Louis MO), Telomerase PCR ELISA kit was obtained from Roche Company (USA). The other reagents were of analytical purity.

Cell lines and culture

Bel- 7402 cells lines maintained in RPMI-1640 medium and incubated at 37℃ is 5% CO_2-95% air with a high humidity and subcultured every 2d or 3d.

MTT assay of cytotoxic activity

MTT assay was performed as follows: Bel-7402 cells were seeded with 96-well plates at 5×10^6 viable cell in a final volume of 200μL per well. After 24h incubation, the medium was removed, then add up the different concentration of EGCG (0μg/mL, 12.5μg/mL, 25μg/mL, 50μg/mL, 100μg/mL, 200μg/mL, 400μg/mL, respectively) to incubated 48h. MTT 0.5g/l [3-(4,5-dimethylthia-

zol-2-yl]-2, 5-diphenyltetrazolium bromide] was added to each well in a volume of 20μL and incubated for 4h. Afterwards, the medium was removed and 150μmol/l of Me2SO (DMSO) was added and incubated for 20 min at room temperature. The absorbance was measured on DJ-3022 ELISA Micro-Plats Reader at 570nm. Mean volume of each concentration (n=6 wells) was obtained. Absorbance of U (untreated controls) was taken as 100% survival and the percentage of inhibition was calculated as follows: cell inhibition rate (%) IR=(1-T/U)× 100%. Whereas T (treated) is the absorbance of treated cells.

Fluorescence microscope and electron microscope examination

Collecting Bel-7402 cells were treated with different concentration of EGCG for 48h and identified under an BH-2 fluorescence microscope after stained with hochest 33258 and Each concentration counted cells and calculated apoptotic rates.

Bel-7402 cells treated with EGCG of 200μg/L for 24h, then normal fixed with 3% glutaraldehyde, and then postfixed with 2% Osmium tetroxide. After dehydration, the samples were embedded in Epon 812 and ultramicrotomed. The sections were routinely stained and examined by electron microscope. and viewed with a Hitachi H-300 electron microscope.

Flow cytometry

In brief, The inducation of apoptosis of Bel-7402 hepatoma cells were treated with EGCG in different concentration and duration, then Bel-7402 cells were harvested and washed three times with PBS solution and fixed in sodium citrate buffer at 4℃ for 30 min, staining solution 500μL (PI 50μg/mL, RNase50μg/mL) was added to 1×10^6 cells and incubated at 4℃ for 15 min in dark, afterwards. Quantitation detection of apoptotic cells and analysis of cell cycle distribution in cultures were performed directly by flow cytometry.

Telomerase activity detection

Telomerose PCR ELISA kit is an extension of original method described by Kim et al. It allows hightly specific amplification of telomerase-mediated elongation products combined with non-radioactive detection following an ELISA protocol. 2×10^5 Bel-7402 cells of each group were harvested and washed with 0.01mol/L phosphate buffered saline (PBS) twice. Then centrifuged twice at 3000×g for 10 min in PBS. The PBS water and tips should be pretreated with diethyl pyrocarbonate (DEPC) in order to prevent RNase from destroying telomerase activity. The cell extracts were prepared by resuspending cells in 200μL of lysis reagent and incubated on ice for 30 min. Then centrifuged at 16000×g for 20 min at 4℃. For PCR amplification, 2μL of extract (equal to 20000 cells) and 20μL of reaction mixture were transferred into a suitable tube, then add nuclease-free water to a final volume of 50μL. An elongation/amplification was performed by the following protocol: mixture was kept at 25℃ for 10 min, 94℃-30s, 50℃-30s, 72℃-90s(30 cycles totally), and at 72℃ for 10 min. Amplification product (equal to 2000 cells) 5μL was mixed with 20μL of denaturation reagent and incubated at room temperature for 15 min. Hybridization buffer 225μL was added and mixed thoroughly, then 100μL of mixture per well was transferred into precoated MTT modules and incubated at 37℃ on a shaker for 2h. Anti-DIG-POD working solution 100μL was added and incubated at room temperature (15-25℃) for 30 min with shaking. The solution was removed completely and the precipitate was rinsed 5 times with 250μL of washing buffer per well for a minimum of 30s. After removing the washing buffer. 100μL of TMB substrate solution was added and incubated for color development at room temperature for 20 min with a slight shaking. Finally 100μL of stop reagent per well was added to stop color development. The absorbance of the sample at 450nm and 690nm (a reference wave length) within 30 min was measured. The result was reported as △A=A490−A690nm(blank).Negative control should be less than 0.25△A units. Positive control should be higher than 1.5△A units. Sample was regarded as telomerase-positive if the difference is absorbance (△A) is higher than 0.2△A units.

Statistics

Statistic analysis of data was performed using the Student´s t-test and biavariate process correlate by professional statistical computer software SPSS. The result are expressed as $\bar{\chi}\pm s$, when $P<0.05$ was considered statistically significant.

RESULTS

Growth inhibition

Bel-7402 cells exposed to EGCG 0-400mg/mL

revealed evident antiproliferative action after 48h in a concentration-dependent manner. As shown in table 1. they had a increased trendy of concentration dependency

Morphology of apoptosis After staining with hochest 33258, three type of cell morphology were visualized by fluorescence microscope i.e. live cells with normal chromatin. The early and later apoptotic cells with condensed and fragmented chromatin. Furthermore ,the ultra structural features of a reduction, cytoplasm shrinkage condensation, fragmentation of chromatin, and presence of apoptotic bodies under electron microscope.

Tab 1. Effects of EGCG on the growth of Bel−7402 cells by MTT assay. (n=6, $\bar{X}$±S)

EGCG/mg.l^{-1}	A630($\bar{x}$±s)	IR/%
Control	0.70±0.08	
25	0.61±0.05^{b}	12.81
50	0.48±0.06^{c}	31.43
100	0.38±0.05^{c}	45.71
200	0.17±0.04^{c}	75.71
400	0.09±0.03^{c}	87.54

^{a}P>0.05, ^{b}P<0.05, ^{c}P<0.01 VS control group

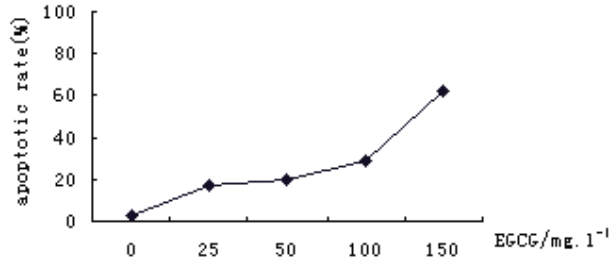

Fig.1 Apoptonic rate of hepatoma Bel−7402 cells induced by different dose EGCG in 48h (n=6)

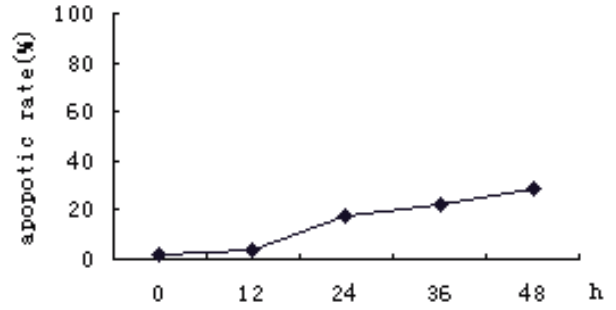

Fig.2 Apoptotic rates of hepatoma Bel−7402 cells induced by EGCG 150mg.l in different time (n=6)

Apoptosis Rates induced by different EGCG concentration

The apoptosis induced by EGCG was determined by fluorescence in microscopy and flow cytometer, flow cytometer showed a concentration-dependent manner from 1.52% to 62.38% during a certain concentration range (0-150 mg.l^{-1}) after incubation for 48 (Fig.1, Tab.2); and also showed a time-dependent manner from 0.95% to 30.25% during a certain time range (0-48h) by 100 mg.l^{-1} EGCG (Fig.2, Tab. 3).

Down-regulation of telomerase activity during EGCG-induced apoptosis

We examined whether level of telomerase activity changed in Bel-7402 cells undergoing EGCG-induced apoptosis. Telomerase activities were quantitated by PCR-ELISA assay. Following exposure to EGCG, there was a progressive decrease in telomerase activity of Bel-7402 cells during a certain concentration (0-150 mg.l^{-1}) (Tab. 2) and time range (0-48h) (Tab. 3).

Tab 2. The telomerase activity´s varieties and apoptotic rate of hepatoma Bel-7402 cells induced by different dose EGCG in 48h (n=6, $\bar{x}$±S)

EGCG/mg.l^{-1}	Apoptotic Rate/%	Telomerase/ΔA
Control	1.52	1.80±0.09
25	15.30	1.32±0.10^{c}
50	21.56	0.96±0.09^{c}
100	30.25	0.87±0.07^{c}
150	62.38	0.28±0.03^{c}

(r=-0.967, p=0.007), ^{a}P>0.05, ^{b}P<0.05, ^{c}P<0.01 vs control group

Tab.3 The telomerase activity´s varieties and apoptotic rate of hepatoma Bel-7402 cells induced by EGCG 150 mg.l in different time (n=6, $\bar{x}$±S)

Time/h	Apoptotic Rate/%	Telomerase/ΔA
0	0.95	1.81±0.11
12	5.25	1.79±0.03
24	16.36	1.63±0.10^{b}
36	22.64	1.13±0.09^{c}
48	30.25	0.87±0.07^{c}

(r=−0.948, p=0.014), ^{a}P>0.05, ^{b}P<0.05, ^{c}P<0.01 vs control group