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EGCG Enhances TRAIL-mediated Apoptosis in Human Melanoma A375 Cell Line

Qin SHEN (沈 琴)†, Fen TIAN (田 芬)†, Ping JIANG (蒋 萍)†, Yanqiu LI (李艳秋), Li ZHANG (张 丽), Jingjing LU (卢静静), Jiawen LI (李家文)#
Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

Summary: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent. Epigallocatechin-3-gallate (EGCG) is a polyphenolic constituent of green tea. In this study, inhibitory effect of combined use of EGCG and TRAIL on human melanoma A375 cells was examined and the possible mechanism investigated. The cells were divided into 4 groups: control group, EGCG group (EGCG: 10, 20 μg/mL), TRAIL group (TRAIL: 25 ng/mL) and EGCG＋TRAIL group (combined group). The growth inhibition was measured in the A375 cells treated with different concentrations of TRAIL ((25, 50, 75, 100, 125, 150 ng/mL) by MTT assay. The apoptosis was assessed by flow cytometry. The expressions of DR4 and DR5 were detected by flow cytometry and western blotting. The activities of caspase-8 and caspase-3 were determined by colorimetric assay. The results showed that TRAIL could dose-dependently inhibit the growth of A375 cells and the IC_{50} of TRAIL was 150 ng/mL. The apoptosis rate was 11.8% in the TRAIL group, 5%–7% in the EGCG group and 48.9%–59.1% in the combined group. Significant difference was found in the apoptosis rate between the combined group and the EGCG or TRAIL group (P<0.05 for each). The expression of DR4 instead of DR5 was significantly increased in the EGCG group. The activity of caspase-3 rather than caspase-8 was substantially enhanced in the EGCG group. These results suggest that EGCG is useful for the TRAIL-based treatment for melanoma.
Key words: epigallocatechin-3-gallate; tumor necrosis factor-related apoptosis-inducing ligand; death receptor 4; death receptor 5; apoptosis; melanoma

Melanoma, the most deadly form of skin cancer, is very aggressive. When detected early, melanoma can be removed successfully by surgery, with a 5-year survival rate of 95%[1]. However, melanoma at more advanced stages would metastasize to a variety of organs. Available therapies, such as chemotherapy, radiation therapy, are of limited efficacy[2]. So, it has been desirable to develop novel treatments for malignant melanoma.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent for its capacity to selectively kill cancer cells without toxic effects on normal cells[3–5]. TRAIL induces apoptosis by binding to TRAIL receptors DR4 and DR5. It was found that the activation of these receptors leads to formation of homo- or hetero-complexes that can recruit and activate Fas-associated death domain (FADD) and then activate initiator caspases, including caspase-8, -9 and -10, and effector caspases such as caspase-3, a factor ultimately responsible for apoptosis. However, some studies showed that fresh melanoma isolates are resistant to TRAIL-induced apoptosis due to low level of TRAIL-death receptors on the surface of melanoma cells [6]. Moreover, the level of TRAIL-R2/DR5 was found to be substantially reduced on the membrane of the melanoma cells which acquired TRAIL resistance by prolonged exposure to TRAIL[7, 8]. Recent studies showed that the resistance of melanoma cells against TRAIL is related to down-regulation of initiator caspases and TRAIL-R1/DR4[9, 10]. Taken together, these studies indicate that clinical potential of TRAIL in the treatment of melanoma may be limited unless agents that are able to increase the cell-surface expression of TRAIL death receptors are used jointly.

Epigallocatechin-3-gallate (EGCG) is a naturally polyphenolic constituent obtained from green tea. EGCG has been shown to have significant chemopreventive and chemotherapeutic effects on tumor cells[11–13]. The biological responses of EGCG include antioxidant function, carcinogen modulation, inhibition of tumor growth, invasion, metastasis and angiogenesis, cell cycle arrest and induction of apoptosis[14,15]. In this study, the combined effect of EGCG and Apo2L/TRAIL on human melanoma cells A375 was examined and the underlying mechanism explored.

1 MATERIALS AND METHODS

1.1 Materials

EGCG (purity > 98％) was bought from Alexis Biochemicals, USA. RPMI 1640 medium were obtained

Qin SHEN, E-mail:shenqin1220@tom.com
†These authors contributed equally to this work.
#Corresponding author

from Hyclone Co, USA. Fetal bovine serum was procured by Hangzhou Sijiqing Co, China. PE-conjugated mouse anti-human DR4 antibody (TRAIL-R1, CD261) and PE-conjugated mouse anti-human DR5 antibody (TRAIL-R2, CD262) were products of San Diego Co, China. Recombinant human soluble TRAIL/Apo2L was bought from Pepro Tech Inc, USA. Human malignant melanoma cell line A375 was purchased from Institute of Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Science, China. Annexin Ⅴ-FITC apoptosis detection kit was from Keygen Co, China.

1.2 Cell Culture and Grouping

A375 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 300-mg *L*-glutamine, 100-U/mL penicillin and 25-U/mL streptomycin in 5%CO_2 at 37°C. EGCG was dissolved in PBS (pH 7.4) to give a 10,000 μmol/L stock solution. The cells were divided into 4 groups: control group, EGCG group, TRAIL group, and EGCG and TRAIL-combined group. In these treatment groups, cells were incubated with corresponding agents respectively. The cells incubated with the equal amount of PBS only served as control.

1.3 Cell Growth and Viability

A375 cells at a density of 2×10^5/mL were seeded into a 96-well plate and treated with different concentrations of TRAIL (25, 50, 75, 100, 125, 150 ng/mL) for 12 h for MTT assay as described previously[1]. The control group was treated with PBS only. The absorbency (*A* value) was measured at a wavelength of 570 nm on a microplate reader. The experiment was conducted in triplicate. The growth inhibition rate (IR) of cells was calculated according to the following formulate: IR= $A_{control} - A_{treatment}/ A_{control}\times100\%$.

1.4 Analysis of Cell Apoptosis by Flow Cytometry

A375 cells (1×10^6/mL) were seeded into culture plates and harvested after treatment with EGCG (10, 20 μg/mL) or/and TRAIL (25 ng/mL) for 24 h. The cell pellets were suspended in 500-μL 1×binding buffer at a density of approximately 1×10^6/mL. Samples were incubated with 1-μL annexin Ⅴ-FITC and 5-μL PI for exactly 5 min at room temperature in the dark and then measured on a FACSCalibur cytometer (Becton Dickinson, USA). Annexin Ⅴ-FITC and PI fluorescence were detected in the FL-1 (green) and FL-2 (red) channels respectively after correction to the spectral overlap between the two channels. Data were analyzed by using CellQuest software (Becton Dickinson, Country).

1.5 Detection of DR4 and DR5 Expression by Flow Cytometry

The expressions of DR4 and DR5 were evaluated by a direct immunofluorescence staining procedure. Cells were treated with EGCG (10, 20 μg/mL) for 24 h and harvested after trypsinization. Cell pelles were washed with PBS and incubated with PE-conjugated mouse anti-human antibodies against DR4 and DR5 or PE-conjugated mouse IgG1 monoclonal isotype antibody (as control) for 30 min at 4°C in the dark. After washes with PBS, cells were re-suspended in PBS and the fluorescence (wavelength: 488 nm) of 10,000 events per sample was analyzed by FACSCalibur cytometry (Becton Dickinson, USA).

1.6 SYBR Green Real-time RT-PCR

A375 cells were seeded into 6-well plates at a density of 1×10^6/mL and treated with plumbagin (10 μm). They were harvested at different time points (0, 12, 24, 36 h), and total RNA was extracted with a QIAamp RNA kit (Qiagen, the Netherlands). RNA (1 μg) was subjected to reverse transcription by using RevertAidTM first strand synthesis kit (Fermentas, USA). Equal amounts of cDNA were subjected to PCR, in the presence of SYBR green dye by using the QuantiTect SYBR Green RT-PCR kit (Qiagen, the Netherlands) and the ABI PRISM 6700 Real Time PCR system (Fengling Biotechnology Inc., China). The primers used were as follows: DR5, 5'-GCAGACTTGGTGCCCTTTG-3' and 5'-TGTTGACCCACTTTATCAGCAT-3'; DR4, 5'-TAC GCCCTGGAGTGACATCG-3' and 5'-CACCAACAG-CAACGAACAA-3'; β-actin, 5'-GTCCACCGCAAATG CTTCTA-3' and 5'-TGCTGTCACCTTCACCGTTC-3'. PCR was performed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 45 s at 72°C. Analysis of cDNA for β-actin served as control. The threshold cycle value (Ct) was normalized against β-actin cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

1.7 Measurement of Activities of Caspase-3 and Caspase-8

Cells (1×10^6/well) were seeded into 6-well plates, and treated with different concentrations of EGCG (10, 20 μg/mL) or/and TRAIL (25 ng/mL) for 12, 24 and 36 h. Caspase-3 and caspase-8 activities were measured by using colorimetric assay kits (Keygen Co., China). Caspase-8 and caspase-3 can hydrolyze peptide substrate Ac-IETD-PNA and Ac-DEVD-PNA respectively, thereby leading to the release of a pNA moiety. The concentration of pNA was calculated according to the absorbency measured at a wavelength of 405 nm and calibration curve based on standard pNA solutions. Values were expressed as nmol pNA/mg of total protein.

1.8 Statistical Analysis

Statistical comparisons of mean values were performed by using one-way ANOVA. Correlation of the variables was assessed by using Bivariate correlation analysis. All *P*-values were determined by two-sided tests. A significant criterion of $P<0.05$ was employed. The statistical analysis was performed by using SPSS 12.0 software.

2 RESULTS

2.1 Effect of EGCG on TRAIL-mediated Apoptosis in A375 Cells

MTT assay showed that TRAIL dose-dependently inhibited the proliferation of A375 cells and the IC_{50} of TRAIL was 150 ng/mL. Flow cytometry revealed apoptosis rate was 5%–7% in the cells treated with EGCG alone and it was significantly increased to 48.9%–59.1% after treatment with EGCG plus TRAIL (fig. 1A and 1B).

2.2 Effect of EGCG on Protein Expression of DR5 and DR4

Flow cytometry showed that DR4 expression was increased in the EGCG-treated cells as compared with that in the non-treated cells. However, there was no sig-

nificant change in the protein levels of DR5 in the cells after EGCG treatment (fig. 2).

2.3 Effect of EGCG on DR5 and DR4 mRNA Level in A375 Cells

Real-time PCR showed that EGCG ((20 μg/mL) up-regulated the level of DR4 mRNA in A375 cells in a time-dependent manner. In contrast, the level of DR5 mRNA in EGCG-treated A375 cells was not significantly increased with time (fig. 3).

2.4 Effect of EGCG on Caspase-3 and Caspase-8 Expression

EGCG significantly increased the expression of activated caspase-3 in a time-dependent manner in A375 cells (fig. 4). However, EGCG did not cause any change in the expression of caspase-8 in A375 cells (data not shown). In contrast to treatment with EGCG alone, the combined application of EGCG and TRAIL was more effective in activating caspase-8 (fig. 4).

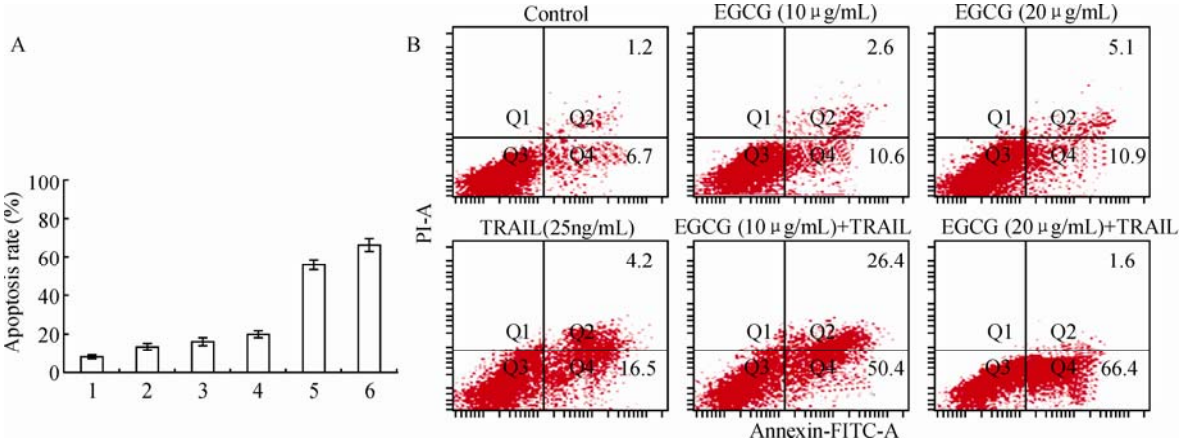

Fig. 1 EGCG enhances TRAIL-induced apoptosis in human melanoma A375 cells
A and B: Apoptosis of A375 cells treated with EGCG (10, 20 μg/mL) and/or TRAIL (25 ng/mL) for 24 h by flow cytometry. 1: Control; 2: EGCG (10 μg/mL); 3:EGCG (20 μg/mL); 4:TRAIL (25 ng/mL); 5: EGCG (10 μg/mL)＋TRAIL (25 ng/mL); 6:EGCG (20 μg/mL)＋TRAIL (25 ng/mL)

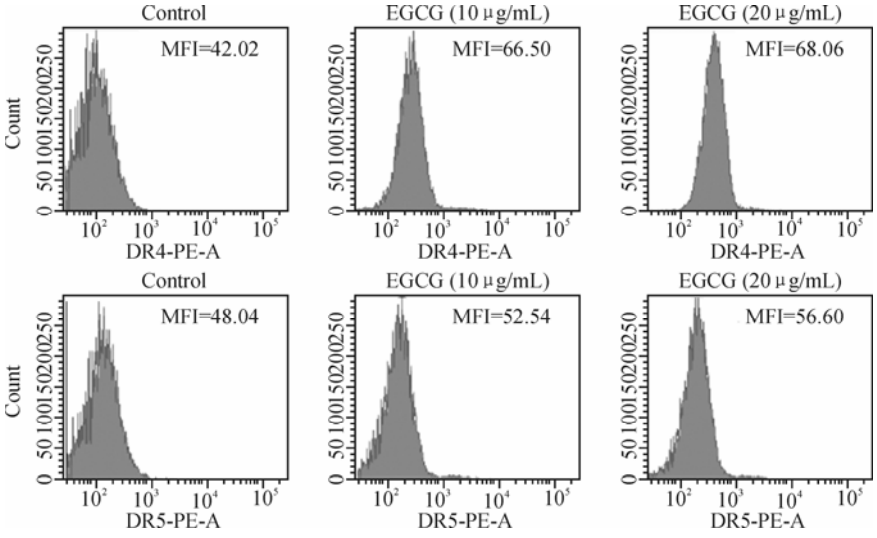

Fig. 2 Expression of DR5 and DR4 protein in the EGCG-treated A375cells

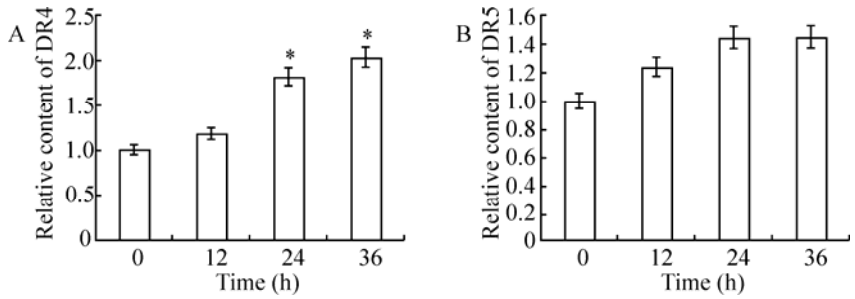

Fig.3 Expression of DR4 (A) and DR5 (B) mRNA in the EGCG-treated A375cells
*P <0.05 as compared with 0 h

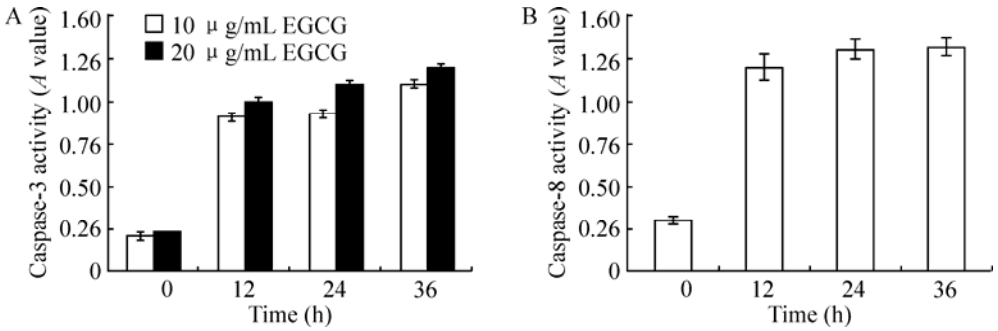

Fig. 4 Effect of EGCG or/and TRAIL on caspase-3 and caspase-8 activities
A: Effect of EGCG on the expression of caspase-3
B: Effect of EGCG (20 μg/mL) and TRAIL (25 ng/mL) on the expression of caspase-8

3 DISCUSSION

TRAIL/Apo2L is a promising candidate for cancer therapy for it can induce apoptosis in many kinds of cancer cells while has no marked toxic effects on normal cells[3–5]. However, *in vitro* studies showed that many cancer cells were resistant to TRAIL in various degree[1–3], which limits the potential use of TRAIL. Some studies also indicated fresh melanoma isolates were resistant to TRAIL-induced apoptosis due to low levels of TRAIL-death receptor expression on melanoma cells[6,16]. Recently, research found some agents and treatment approaches, such as chemotherapeutics, histone deacetylase inhibitors and γ-irradiation, can promote the TRAIL-induced apoptosis by up-regulating death receptors DR4 and/or DR5, and thus enhance the effectiveness of TRAIL[17–20].

In this study, MTT assay showed that TRAIL could inhibit the proliferation of A375 cells, yet the concentration of TRAIL high up to 150 ng/mL was required for 50% inhibition of cell growth. Flow cytometry indicated that A375 cells were not sensitive to TRAIL. EGCG is a special polyphenol found in green tea that possesses anti-tumor effects[4, 5]. Our results found that combined application of EGCG and TRAIL was more effective on the cell growth of A375 than treatment with EGCG, or TRAIL alone and suggested combined treatment may decrease the concentration of TRAIL and therefore reduce the drug-resistance.

TRAIL-induced apoptosis is mediated by its receptors. Four types of membrane receptors of TRAIL, namely death receptors DR4 and DR5, decoy receptors DcR1 and DcR2, have so far been identified. DR4 and DR5 extensively express on the membrane of various cells, including tumor cells and normal cells, while DcR1 and DcR2 selectively express on the surface of normal cells instead of most tumor cells. In this study, EGCG was found to up-regulate the expression of DR4 in A375 cells, suggesting that EGCG contributes to the apoptosis of A375 cells by enhancing the expression of TRAIL receptors.

Recent studies demonstrated that caspases represent the common final path of apoptosis and many apoptosis regulators produce effects through regulation of caspases[21]. The caspase cascade is activated in a manner like the blood coagulation cascade, eventually triggering the apoptotic process. In the protease cascade, caspase-8 acts as an initiator of apoptosis while caspase-3, also called executor of apoptosis, as an effector, responsible for the cleavage of some key proteins at the final executive stage of apoptosis[22]. Our study showed that EGCG could induce cell apoptosis through caspase-3-dependent signal pathway, thereby killing tumor cells. In the EGCG-treated cells, caspase-3 activity was increased, significantly higher than that in the non-treated cells. Moreover, EGCG alone did not increase the activity of caspase-8 as was further found to be enhanced by EGCG plus TRAIL, suggesting that caspase-3 and caspase-8 are both involved in the apoptosis regulation. Based on other literatures, the underlying mechanisms of synergistic effects of TRAIL and EGCG are speculated as follows. EGCG reduces the generation of c-flice like inhibitory protein (c-FLIP), thereby amplifying the caspase cascade, continually transmitting and enlarging the apoptosis signals, eventually arousing the apoptosis of A375 cells. EGCG still can strength the TRAIL-induced apoptosis.

In conclusion, this study demonstrates that EGCG can enhance TRAIL-mediated apoptosis in human melanoma A375 cells by increasing the expression of TRAIL receptor TRAIL-R1/DR4 and activating caspase-8 and caspase-3. The combined use of TRAIL and EGCG is promising to be an effective adjuvant treatment for melanoma.

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