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Induction of apoptosis by green tea catechins in human prostate cancer DU145 cells

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Abstract

Green tea catechins (GTCs) including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epicatechin (EC) were shown to suppress cell growth and induce apoptosis in various cell systems in addition to their chemo-preventive effect. In this study, except EC which was inactive, green tea extract (TE) and other 3 GTCs were found to suppress the growth and induce apoptosis in human prostate cancer DU145 cells largely through an increase in reactive oxygen species formation and mitochondrial depolarization. The conclusion was supported by the fact that the profiles for different GTCs in growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization are in a similar order, i.e. ECG > EGCG > EGC > EC. Although the molecular mechanisms are still not clear, apoptosis induced by GTCs is not related to the members of BCL-2 family as EGCG did not alter the expression of BCL-2, BCL-X_L and BAD in DU145 cells.

Keywords: Green tea catechins; Apoptosis; Free radicals; DU145 cells

Introduction

Tea consumption in the world is very high and ranks second to water consumption. Tea is prepared from the dried leaves of *Camellia sinensis.* Tea can be classified into black tea, green tea and oolong tea. The classification depends on the differences in the manufacturing process and fermentation time. Tea polyphenols are most abundant in green tea and they are mostly in the form of flavanols commonly known as catechins. Some major green tea catechins (GTCs) are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC),

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(−)-epicatechin-3-gallate (ECG), (−)-epicatechin (EC) and (+)-catechin. GTCs demonstrated profound biochemical and pharmacological activities including antioxidant activities, modulation of carcinogen metabolism and inhibition of cell proliferation. [1–3]

Epidemiological studies suggest that consumption of green tea is associated with a lower risk of several types of cancer, including stomach, esophagus, and lung. Administration of tea extract and GTCs reduced the growth of breast and prostate cancers in nude mice and *in vitro*. [1–4] The growth suppression effect of GTCs is at least partly due to the induction of apoptosis although the underlying mechanism is still not clear. [1, 5–7] The process of apoptosis is a multi-step process and is regulated by various factors including those in the BCL-2 family. Recently, mitochondrial depolarization is suggested to be one of early processes that leads to the final stage of apoptosis in addition to the cytochrome c release and activation of various caspases. [8–12] In an attempt to understand the process of GTCs induced apoptosis, human prostate cancer DU145 cells were used as a model. It is concluded from this study that GTCs induced apoptosis in DU145 cells through the reactive oxygen species (ROS) formation and mitochondrial depolarization.

Materials and methods

Green tea extract (TE) was prepared by the method as described by Agarwal et al [13] with modification. In brief, dried jasmine tea leaves were soaked three times with 80°C distilled water. The total combined extracts were cooled, filtered and then extracted with an equal volume of chloroform to remove caffeine and pigments. The aqueous layer was extracted twice with equal volume of ethyl acetate. The ethyl acetate phase was saved and later removed using a vacuum rotary evaporator. The crude extracts were dissolved in water and freeze-dried. The light brown residue obtained was called TE. GTCs are EGCG, ECG, EGC, EC and they were obtained from Sigma Chemical Co., USA. Human prostate carcinoma DU145 cells were obtained from American Type Culture Collection. Cells were grown in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum.

Growth suppression by MTT assay

Cells were seeded on a 96-well plate for 2 days and then incubated with different concentrations of TE or GTCs for 5 days; up to 50 μg/ml for TE or up to 100 μM for GTCs. Thereafter, medium was removed and the cells were incubated with 1mg/ml MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution for 4 hours to allow the formation of formazan precipitate which subsequently was dissolved in dimethyl sulfoxide. The absorbance in each well was then read by a microplate reader at 540nm.

DNA fragmentation assay

After 3 day treatment with, cells were trypsinized and centrifuged. The pellet was incubated with lysis buffer (10mM Tris-HCl pH7.5, 1 mM EDTA, 1% SDS and 80 μg/ml proteinase K) at 37°C overnight. After extraction with phenol/chloroform, the DNA was precipitated by 100% ethanol and then dissolved in Tris-EDTA buffer (pH8.0) with RNase A at 37°C overnight. The absorbance was then read at 260nm and 280nm. DNA was resolved in

1.5 % agarose gel containing 0.5 μg/ml ethidium bromide in 1× Tris-borate-EDTA buffer. The bands were visualized under UV transilluminator.

ROS and mitochondrial membrane potential by flow cytometry

The intracellular levels of peroxide (H_20_2) and superoxide anions (0_2-) were measured by flow cytometric analysis of cells stained respectively with DCF/DA (dichlorodihydrofluorescein diacetate) and HE (hydroethidine) (Molecular Probes, USA). The ROS convert the non-fluorescent DCF/DA and HE into the their respective fluorescent end-products and the reaction can be monitored by flow cytometry. [9] The mitochondrial membrane potential ($\Delta\Upsilon m$) of cells was measured by staining cells with JC-1 (Molecular Probes, USA) [14]. JC-1 selectively enters mitochondria as monomers and emits green fluorescence with an excitation at 488nm. With polarized mitochondria, JC-1 forms aggregates and emits red fluorescence under 488nm excitation. The red and the green fluorescence were measured simultaneously by flow cytometry. Cells after treatment with 50 μg/ml TE or 100 μM GTCs were incubated with DCF/DA, HE or JC-1 at room temperature. Thereafter, the cells were subjected to flow cytometric analysis with FACSort (Becton Dickinson, USA).

Western blot analysis

Cells were incubated with 100 μM EGCG for up to 48 h. At appropriate time, cells were lysed with lysis buffer (1% Triton X-100, 1 mM sodium ortho-vanadate, 4.9 mM $MgCl_2$, 1mM PMSF, 21 μg/ml aprotinin, and 0.5 μg/ml leupeptin). An equal amount of protein was resolved in SDS-polyacrylamide gel electrophoresis. The protein was transferred onto Immobilon P membrane (Millipore Co., USA), incubated with primary antibody and then with horseradish peroxidase conjugated secondary antibody. The primary antibodies are anti-BCL-2, BCL-X_L, BAD and CAS (Santa Cruz Biotechnology, Inc., USA). The signals were visualized using the ECL enhanced chemiluminescence kits (Amersham Pharmacia Biotech, UK).

Results

Growth suppression and apoptosis

The influences of TE and different GTCs on the growth of DU145 cells were shown in Fig. 1A and 1B. TE and GTCs demonstrated growth suppression effect on DU145. Among the GTCs, EC appeared to have little inhibitory effect on cells while others demonstrated significant growth suppression in the order as ECG, EGCG and EGC. The IC_{60} for ECG, EGCG and EGC are 60, 74 and 88 μM respectively.

One of the ways that leads to cell growth suppression is an increase in cell death that includes the process of apoptosis. Therefore, the influence of TE and GTCs on apoptosis in DU145 cells was examined by DNA fragmentation assay. As shown in Fig. 1C, 72h exposure to 50 μg/ml TE or 100 μM ECG, EGC and EGCG induced DNA fragmentation in DU145 cells while the fragmentation was less detectable in cells treated with EC. ECG was the most effective isomer in inducing apoptosis in DU145 cells followed by EGCG and then with EGC. The profile for GTCs induced apoptosis is comparable with the observed growth sup-

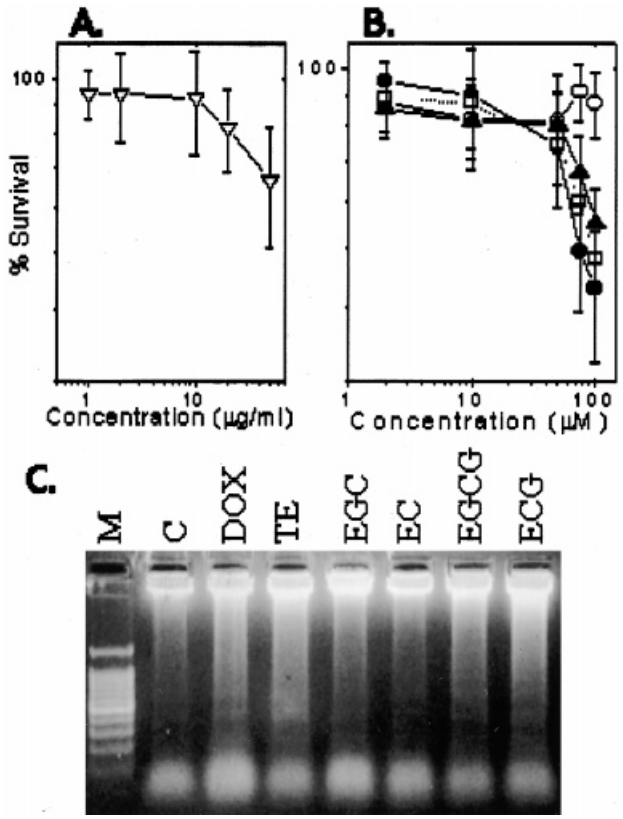

Fig. 1. The influence of (A) TE and (B) GTCs on the growth of DU145 cells as measured by MTT assay. ▽: TE; ▲: EGC; ○: EC; □: EGCG; ●: ECG. Both x- and y-axis were in log-scale. Error bar: standard deviation. Results were average from at least 5 separate experiments. (C) Induction of DNA fragmentation by TE and GTCs in DU145 cells. The cells were incubated for 72 h with 50 μg/ml of TE, 100 μM of EGC, EC, EGCG and ECG. M: DNA marker; C: cells with no treatment; DOX: cells incubated with 0.2 μg/ml of doxorubicin for 72 h, as a positive control. The experiments have been repeated for more than three times and the one shown is the representative one.

pression effect as shown in Fig. 1B. It is therefore suggested that the growth suppression by GTCs in DU145 cells may be related to the induction of apoptosis.

ROS and ΔΥm

One of the ways to induce apoptosis in cells is through ROS formation and mitochondrial depolarization. To confirm these, ROS levels that included peroxide and superoxide anions in cells treated with GTCs were measured by flow cytometry with DCF and HE and ΔΥm by JC-1. After 48h incubation, TE, EGCG and ECG increased significantly the peroxide levels in cells while there was almost no change for EGC and EC; the effect of ECG is more significant than that of the EGCG. In the case of superoxide anions, no detectable change was observed for TE and all 4 GTCs isomers. (Fig. 2 A)

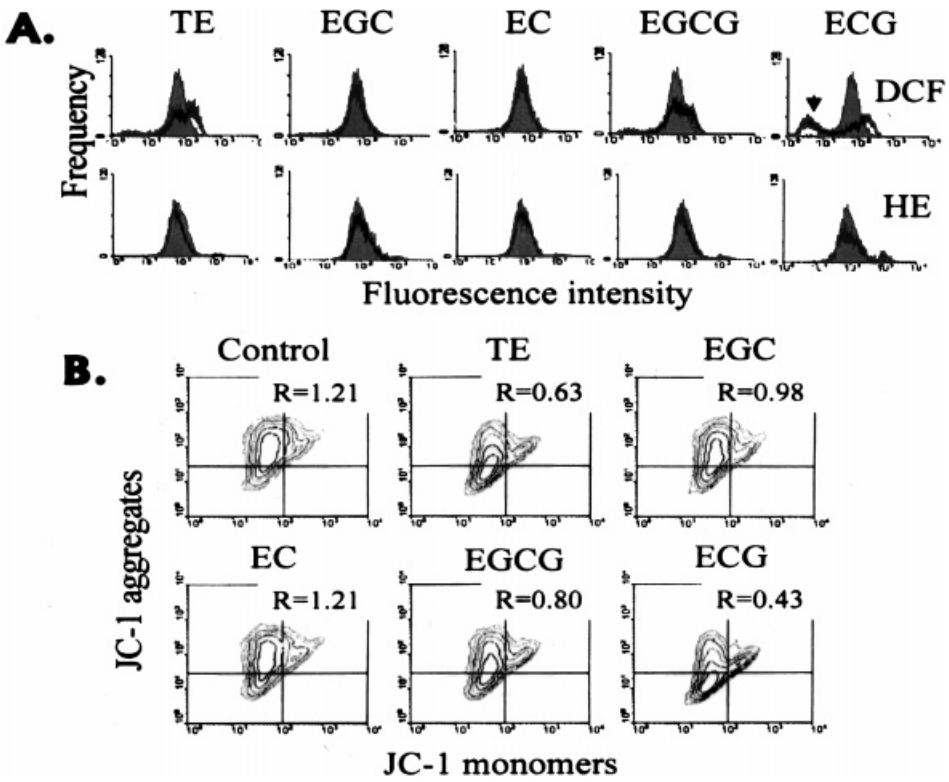

Fig. 2. The effects of TE and GTCs on (A) the levels of intracellular ROS and (B) ΔΥm in DU145 cells. The levels of peroxide and superoxides anions were measured by flow cytometry respectively with DCF/DA and HE. μm was ΔΥ by JC-1 staining. The cells were incubated with 50 μg/ml TE or 100 μM of different GTCs for 48 h. In Fig. 2A, shaded area: cells with no treatment; thick solid line: cells treated with TE or GTCs. In the DCF fluorescence profile for ECG treated cells, cells represented by the first peak (as indicated by an arrow) were permeable to propidium iodide and therefore were considered as dead cells. In Fig. 2B, control: cells with no treatment. R value is calculated as the G mean ratio between the fluorescence profiles of JC-1 aggregates and JC-1 monomers.

TE, EGCG, ECG and EGC induced mitochondrial depolarization in DU145 cells while it was not detected for those treated with EC. Mitochondrial depolarization is indicated by the increase in the fluorescence intensity of JC-1 monomers and simultaneously with the decrease in the intensity of JC-1 aggregates. Such changes can be estimated by an R value, a ratio of the fluorescence intensity between JC-1 aggregates and JC-1 monomers. A decrease in the R value of the treatment group as compared to the control represents mitochondrial depolarization. As shown in Fig. 2B, the R value for different GTCs was in the order of: control = EC > EGC > EGCG > ECG and therefore the extent of depolarization was ECG>EGCG>EGC>EC.

Apoptosis protein expression

To understand the molecular mechanisms for GTCs induced apoptosis, the influence of EGCG, which constitutes more than 50% of GTEs isomers in TE, on the expression of vari-

ous apoptosis-related proteins was examined. The process of apoptosis is regulated by systems that included members in BCL-2 family, e.g. BCL-2, BCL-X_L and BAX. However, treatment of DU145 cells with 100μM EGCG was found to have no significant effect on the levels of BCL-2, BCL-X_L and BAX. For other apoptosis related protein, CAS, EGCG was found to suppress its expression in DU145 cells.

Discussion

Induction of apoptosis by TE and GTCs may be related to their effect on ROS formation and mitochondrial depolarization. Induction of apoptosis by GTCs has been reported in other systems while the underlying mechanism is not clear. Although the ways to trigger apoptosis may be varied for different agents, the roles of ROS and mitochondria in many cases are thought to be important. [8–12] In the present study, a close correlation was observed for the levels of growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization in cells treated with 4 different GTCs isomers. Among these GTCs, ECG was the most effective isomer in growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization and *vice versa.* Such correlation supports the idea that GTCs may trigger apoptosis through ROS formation and mitochondrial depolarization and thus result in growth suppression. Although it is not clear why GTCs will induce peroxide in cells, the results agreed with those reported from human lung cancer H661 cells. Incubation of H661 cells with EGCG induced a dose-dependent formation of peroxide and also apoptosis in cells. Furthermore, the EGCG induced apoptosis in both H661 cells [6] and DU145 cells (results not shown] was inhibited by exogenously added catalase, a free radical scavenger. In Ehrlich ascites cells, the treatment of cells with EGCG suppressed the glutathione levels and at the same time inhibited the cell growth [15]. Under the catalysis of glutathione peroxidase, glutathione will convert hydrogen peroxide into water while the metabolism for superoxides is regulated by pathways indirectly with glutathione. Therefore, it is quite likely that the accumulation of peroxide in DU145 cells upon treatment with EGCG might be the end result of glutathione reduction by EGCG and the accumulation may then lead to mitochondrial depolarization and apoptosis. [12]

The proteins in BCL-2 family may either be pro-apoptotic or anti-apoptotic and they include BCL-2, BCL- X_L, BAD. [8, 11] Results from this study however suggested that these proteins might not be directly involved in GTCs induced apoptosis as EGCG did not alter their expression but induced apoptosis in DU145 cells (Fig. 1C and 3). Although there was no change in the expression of BCL-2 family members, EGCG however reduced the level of CAS in DU145 cells. CAS is the homologue of yeast chromosome segregation gene product CSE1. Antisense CAS suppressed the process of apoptosis induced by ADP-ribosylating toxins and tumour necrosis factor but not those by staurosporine or etoposide. In addition, the level of CAS was high in cells active in proliferation but low in non-proliferating cells. [16, 17] The decreased expression of CAS upon treatment with EGCG suggested that CAS would therefore likely be involved in growth suppression rather than apoptosis induction in DU145 cells.

The 3-gallate and 5′-hydroxyl groups at the trihydroxyphenyl B ring of GTCs isomers might be the important functional groups in GTCs for regulation of cell growth and apopto-

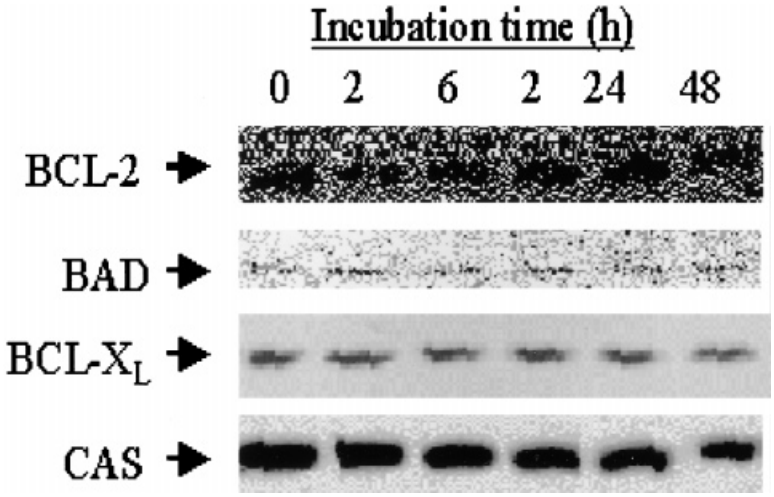

Fig. 3. The effect of 100 μM EGCG on the levels of BCL-2, BAD, BCL-X_L, and CAS in DU145 cells. The experiments have been repeated for more than three times and the one shown is the representative one.

sis. The hypothesis is supported as comparing the chemical structures of the isomers with their biological activities. Among the 4 GTCs, the effectiveness in growth suppression, apoptosis induction, ROS formation and mitochondrial depolarization is in the descending order as ECG, EGCG, EGC and EC. As shown in Fig. 4, the gallate moiety is present in ECG and EGCG but not in EGC and EC while the 5′-hydroxyl group is in EGC but not in EC. In the case where both groups were present, the effectiveness of the isomer may be reduced as a result of steric hindrance; EGCG vs ECG. To confirm such hypothesis, a detailed analysis of the relationship between chemical structure and biological activity among different isomers should be carried out. The less effectiveness of EC in inducing apoptosis and growth suppres-

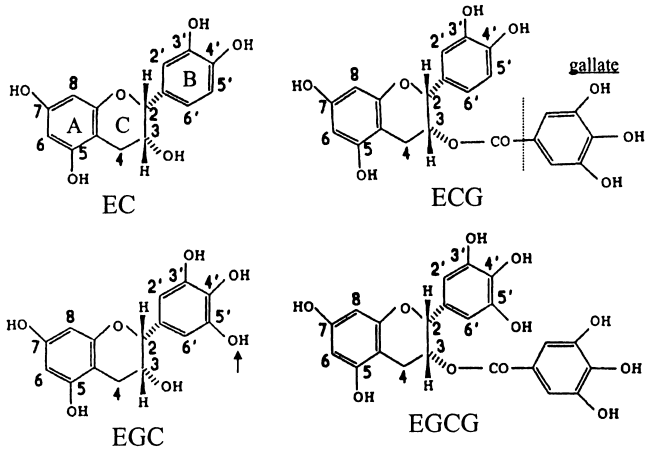

Fig. 4. Chemical structure of 4 different GTCs isomers. The gallate group and the 5′-hydroxyl group (pointed by an arrow) were indicated.

sion is at least in agreement with results from many other studies of which EC was always found to be less active isomer. [1, 5–7] The significance of EC in TE is to enhance the biological functions of other GTCs isomers by helping the uptake of other isomers into cells and into the body. [18]

GTCs have been shown to demonstrate chemoprevention activity in various types of cancer, including prostate cancer. The compounds are also effective in inducing growth suppression and cell death in different cells including DU145 cells. Although GTCs induced apoptosis and growth suppression in DU145 cells appeared to be correlated (Fig. 1), the importance of apoptosis in GTCs induced growth suppression is still not clear. Nevertheless, as suggested from the present study, the GTCs induced apoptosis might be related to their effect in ROS formation and mitochondrial depolarization. The underlying molecular mechanisms however will still need further investigation.

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