(–)-Epigallocatechin Gallate Induced Apoptosis in Human Adrenal Cancer NCI-H295 Cells through Caspase-dependent and Caspase-independent Pathway

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Abstract. *(–)-Epigallocatechin-3-gallate (EGCG) is a major constituent of green tea and has been identified as an excellent anticancer agent. Nevertheless, there are no reports to date about the molecular mechanisms and signal pathways of EGCG on the induction of apoptosis in human adrenal NCI-H295 cancer cells. The purpose of this study was to investigate the anticancer effect and molecular mechanisms of EGCG on human adrenal NCI-H295 cancer cells. The results showed that EGCG induced growth inhibition in a dose- and time-dependent manner. Moreover, it exerted low cytotoxicity on Detroit 551 normal human embryonic skin cell. When NCI-H295 cells were treated with 20 μM EGCG, the mitochondrial membrane potential decreased and intracellular free Ca^{2+} increased in a time-dependent manner as analysed by flow cytometry. EGCG decreased the protein levels of Bcl-2, Bcl-xl, xIAP, cIAP, Hsp70 and Hsp90, but increased the protein expression of Bad, Bax, Fas/CD95, cytochrome c, Apaf-1, AIF, GADD153, GRP78, and caspase-3, -7,-8 and -9 as observed by Western blotting examination. EGCG promoted caspase-8, -9 and -3 activities in a time-dependent manner. However, pretreatment of cells with inhibitors of caspase-8, -9 and -3 led to a decrease in caspase-8, -9 and -3 activities and an increase in the percentage of viable cells. Based on the above findings, it was confirmed that EGCG may be a drug candidate for the treatment of human adrenal cancer in the future.*

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Green tea is an infusion of the leaves from the *Camellia sinensis* plant rich in polyphenolic compounds (1). Many studies have shown that green tea can prevent a number of chronic diseases and tumors in humans (1-2). Phase I and II clinical trials have been conducted to explore the anticancer effects of green tea in humans (3-5). The major polyphenols in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) (6). EGCG was repeatedly shown to have chemo-preventive and anti-carcinogenic actions (7). *In vitro* studies show that EGCG has potently anti-oxidative effects (8), protection of DNA damage (9) and methylation (10), inhibition of proteasome activity (11), regulates cell cycle progression (12) and induces apoptosis (13) in human cancer cells (8-13) but not in their normal cell counterparts (14). Various animal *in vivo* studies have revealed that treatment with EGCG inhibits tumor growth in different organs such as skin, lung, liver, stomach and colon (15).

Apoptosis plays an important role in the embryogenesis and homeostasis of multi-cellular organisms, and impairment of apoptotic function has been associated with several human diseases (16-17), including neurodegenerative disorders and cancer (18-19). The death receptor pathway and the mitochondrial pathway are the two major apoptosis process pathways (20-21). In the death receptor pathway, Fas ligand (FasL)/tumor necrosis factor-α (TNF-α) binds to cell surface receptor Fas/TNF, resulting in activation of caspase-8 followed by the activation of caspase-3 and -7 (22, 23). In the mitochondrial pathway, cytochrome *c*, Apaf-1, pro-caspase-9,

AIF and Endo G are released from the mitochondrial membrane into the cytosol when reactive oxygen species (ROS) are increased or mitochondrial membrane potential (MMP, $\Delta\psi m$) is decreased (24-26). Cytochrome *c* and Apaf-1 bind to pro-caspase-9 inducing activation of caspase-9 followed by the activation of caspase-3 and -7. Caspase-8 activation induces cleavage of Bid to truncated Bid (tBid) triggering Bax activation, and resulting in a change of the mitochondrial permeability and the induction of the mitochondrial pathway (24-26). Recently, it has been demonstrated that a novel endoplasmic reticulum (ER)-specific apoptotic pathway operates with GADD153, GRP78 protein change and caspase-12 activation. Active caspase-12 is capable of directly activating caspase-3 or -7 (27).

The mechanism by which EGCG exerts cytotoxicity remains largely unknown in human adrenal NCI-H295 cancer cells. In the present study, the cytotoxic effects and molecular mechanisms of EGCG on human adrenal NCI-H295 cancer cells were investigated and evaluated. The results suggest that EGCG induces apoptosis *via* the death-receptor, mitochondrial and ER stress pathways in human adrenal tumor NCI-H295 cells.

Materials and Methods

Chemicals and reagents. (–)-Epigallocatechin gallate (EGCG), dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase A, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Tris-HCl and Triton® X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Caspase-8 inhibitor, z-Ile-Glu-Thr-Asp-fluoromethyl ketone (Z-IETD-FMK), caspase-9 inhibitor, z-Leu-Glu-His-Asp-fluoromethyl ketone (Z-LEHD-FMK), Caspase-3 inhibitor z-Asp-Met-Gln-Asp-fluoromethyl ketone (Z-DEVD-FMK) (R&D Systems, USA) were dissolved in DMSO and diluted in cell culture medium before use. 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was obtained from Calbiochem (La Jolla, CA, USA). Indo 1/AM was obtained from Molecular Probes (Eugene, OR, USA). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Cell cultures. Human adrenal NCI-H295 cancer cells and Detroit 551 normal human embryonic skin cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks and cultured in RPMI-1640 (NCI-H295) or minimum essential medium (Detroit 551) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine at 37˚C in a humidified atmosphere with 5% CO_2 and 95% air at 1 Atm.

Cell viability and morphological changes. The NCI-H295 cancer cells were seeded in 96-well cell culture plates at an initial density of 1×10^5 cells/mL and incubated with 10, 20, 30 or 40 μM of EGCG for 24, 48 and 72 h. After incubation for the indicated time, cells were treated by the addition of MTT dye to each well. After an additional 4 h incubation, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with DMSO in isopropanol. The absorbance was measured at 490 nm and the cell survival ratio was expressed as a percentage of the control. For morphological changes, cells were examined and photographed under phase-contrast light microscopy (28-29).

Measurement of mitochondrial membrane potential ($\Delta\psi m$). The NCI-H295 cancer cells were seeded in 24-well cell culture plates at an initial density of 2.5×10^5 cells/mL and incubated with 20 μM of EGCG for 6, 12 or 24 h. The cells were harvested and washed twice with PBS, re-suspended in 500 μL of $DiOC_6$ (4 mol/L) and incubated at 37˚C for 30 min before being analyzed to detect the changes of $\Delta\psi m$ using flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA) with excitation and emission settings of 484 nm, respectively (30).

Determination of intracellular free Ca^{2+} using Indo-1/AM assay. The NCI-H295 cancer cells were seeded in 24-well cell culture plates at an initial density of 2.5×10^5 cells/mL and incubated with 20 μM of EGCG for 6, 12 or 24 h. The cells were harvested and washed twice with PBS, re-suspended in Indo-1/AM (3 μg/mL), incubated at 37˚C for 30 min, then analyzed to detect the changes of intracellular free Ca^{2+} levels using flow cytometry with excitation and emission settings of 484 nm, respectively (31).

Western blotting analysis of specific proteins. The NCI-H295 cancer cells were seeded in 10 cm-dish at an initial density of 1.0×10^7 cells and incubated with 20 μM of EGCG for 12, 24, 36 or 48 h. Total proteins were separated by 10% gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were blotted onto nitrocellulose membranes at 1.5 mA/cm^2 for 1.5 h (Invitrogen, Carlsbad, Germany). The membrane was then blocked in 5% powdered non-fat milk in PBST solution (0.1% Tween 20 in PBS) for 1 h. The Bcl-2, Bcl-xL, Bad, Bax, cIAP, IAP, Hsp70, Hsp90, cytochrome c, AIF, GADD153, GRP 78, Fas/CD95 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3, -7, -8 and -9 (R&D Systems) primary antibodies were diluted in blocking solution and then incubated with the membrane overnight. The membrane was then covered with an alkaline HRP-conjugated secondary IgG antibody (goat anti-rabbit and goat anti-mouse) for 1 h after which blots were incubated with ECL reagents (Amersham Pharmacia, Buckinghamshire, UK) and exposed to X-OMAT AR films (Eastman Kodak, Rochester, NY, USA). The auto-radiograms were scanned on a UMAX PowerLook Scanner (UMAX Technologies, Fremont, CA, USA) using Photoshop software (Adobe Systems, Seattle, WA, USA) (32).

Caspase activity determination. The NCI-H295 cancer cells were seeded in 12 well cell culture plates at an initial density of 5.0×10^6 cells and incubated with 20 μM of EGCG or caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK) for 1 h prior to treatment with EGCG for 12, 24, 36 or 48 h. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT). About 50 μg of cytosol proteins were incubated with caspase-3-, -8- and -9-specific substrates [Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA (R&D Systems)] for 1 h at 37˚C. The caspase activity was determined by measuring OD_{405} (33).

Apoptosis by flow cytometry analysis. The NCI-H295 cancer cells were seeded in 24-well cell culture plates at an initial density of 2.5×10^5/mL cells and incubated with 20 μM of EGCG or caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK) for 1 h prior to treatment with EGCG for 48 h. Cells were harvested by centrifugation and washed twice with PBS. The cells were fixed gently (drop by drop) with 70% ethanol (in PBS) in ice overnight and were then washed twice with PBS and re-suspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase (Sigma Chemical, Co.) and 0.1% Triton® X-100 in a dark room. After 30 min at 37˚C, the cells were immediately analyzed with a flow-cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm wave-length. The cell cycle was then determined and analyzed, and data were acquired with CellQuest software (33).

Statistical analysis. Student's *t*-test was used to analyze the differences between the EGCG-treated and control groups.

Results

Effects of EGCG on cell viability and morphology of NCI-H295 cancer cells and Detroit 551 normal human embryonic skin cells. NCI-H295 cells were treated with EGCG at different concentrations (10, 20, 30 and 40 μM). The number of viable cells was determined by MTT method 24, 48 and 72 h later. As shown in Figure 1A, EGCG exerted time- and dose-dependent anti-proliferation action in NCI-H295 cells. The concentration required to inhibit growth by 50 % (IC_{50}) was approximately 20.34 μM at 48 h. Morphological examinations in Figure 1B show that the cells were significantly different between EGCG-treated and control groups. The EGCG-treated group had cells detached from the surface and contained some debris whereas the control cells were well spread with a flattened morphology. NCI-H295 and Detroit 551 normal human embryonic skin cells were treated with EGCG at different concentrations (50, 100 and 200 μM). The number of viable cells was determined by MTT method 24 h later. As shown in Figure 1C, EGCG exerted low cytotoxicity on Detroit 551 normal human embryonic skin cells (IC_{50}>100 μM). This therefore indicated that EGCG reduced the proportion of viable NCI-H295 cells in a dose- and time-dependent manner and induced cell apoptosis on NCI-H295 cells, but with low toxicity to normal cells.

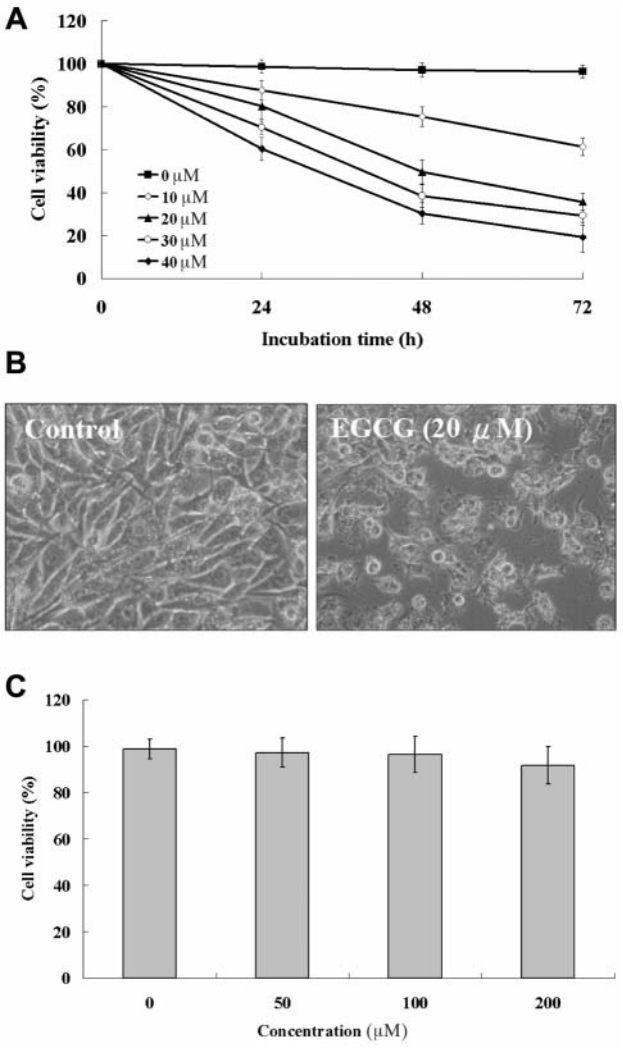

Figure 1. *Effects of EGCG on cell viability (A) and morphology (B) of NCI-H295 cells, and cell viability of Detroit 551 normal human cells (C). NCI-H295 cells were cultured with 10, 20, 30 and 40 μM of EGCG for 24, 48 and 72 h, Detroit 551 normal human cells were cultured with 50, 100 and 200 μM of EGCG for 24h. Percentages of viable cells were determined by MTT assay as described in Materials and Methods. Each point is mean±S.D. of three experiments. The cells were examined and photographed under phase-contrast microscopy (×400).*

Effects of EGCG on mitochondrial membrane potential (Δψm) and intracellular free Ca^{2+} in NCI-H295 cancer cells. The NCI-H295 cells were treated with 20 μM EGCG for 6, 12 and 24 h. The changes of Δψm were determined by staining with $DiOC_6$ and then analyzed by flow cytometry. The representative data is shown in Figure 2A. Results showed that Δψm decreased on 12, 24, 36 and 48 h treatment with EGCG in a time-dependent manner. The cells were harvested for determination of intracellular free Ca^{2+} levels. The changes of Ca^{2+} levels in response to the effect of EGCG was studied by staining with Indo-1/AM and then analyzed by flow cytometry. The data are shown in Figure 2B. Results showed that intracellular free Ca^{2+} increased after 6, 12, 24, 36 and 48 h treatment with EGCG in a time-dependent manner. This suggests EGCG-induced apoptosis is mediated by induction of the endoplasmic reticulum stress and mitochondrial pathways.

Effects of EGCG on the levels of apoptosis associated proteins in NCI-H295 cancer cells. The Bcl-2 family of proteins

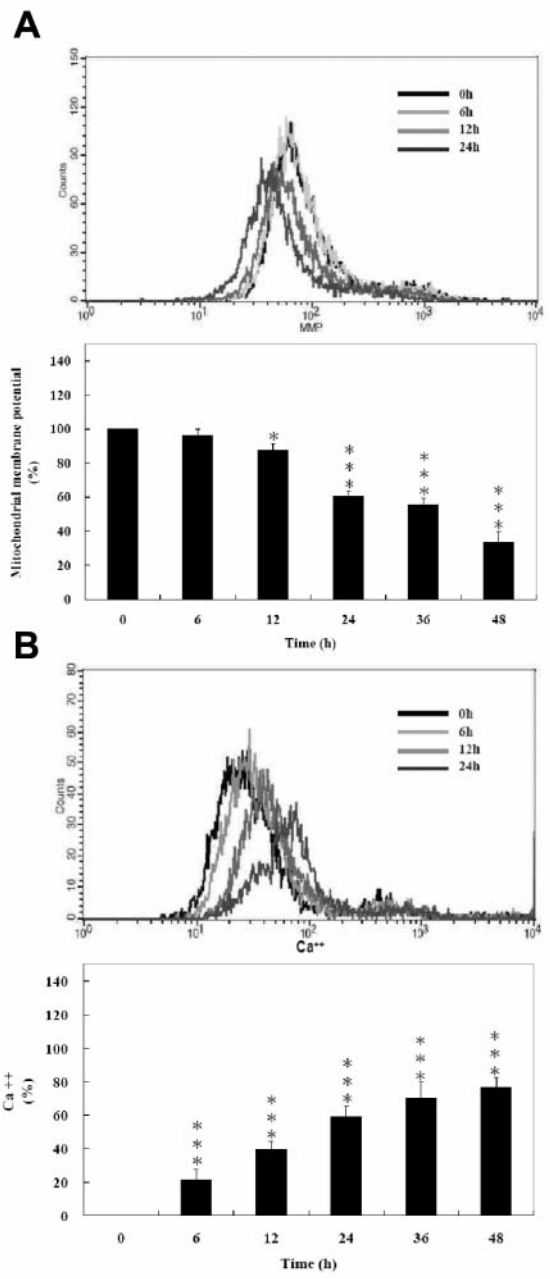

Figure 2. *EGCG decreased the levels of mitochondria membrane potential (Δψm) (A) and increased intracellular free Ca^{2+} (B) in NCI-H295 cells. NCI-H295 cells were treated with 20 μM EGCG for various time periods then cells were collected, stained with $DiOC_6$ for Δψm and with Indo-1/AM for intracellular free Ca^{2+} level determination as described in Materials and Methods. Data represents mean±S.D. of three experiments. *p<0.05, ***p<0.001.*

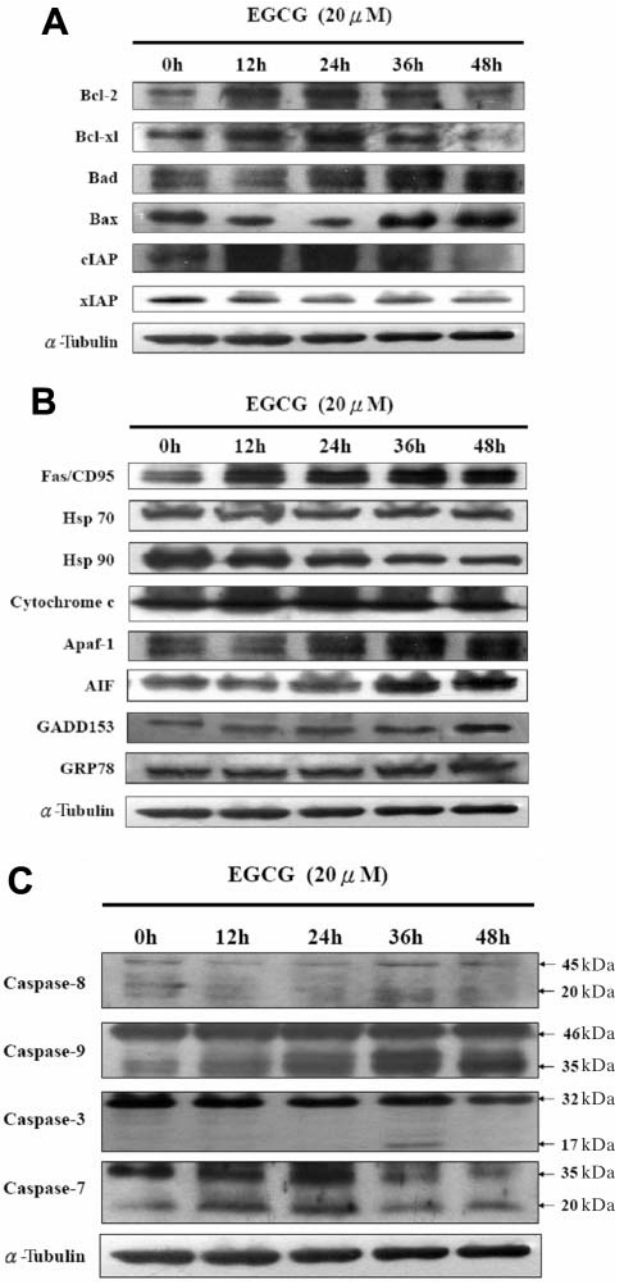

Figure 3. *EGCG affected the levels of associated proteins in apoptosis of NCI-H295 cells. Cells were treated with EGCG at 20 μM for various time periods and were then harvested for total protein determination, as described in Materials and Methods. The associated protein expressions (A: Bcl-2, Bcl-xl, Bad, Bax, cIAP and xIAP; B: Fas, Hsp70, Hsp90, cytochrome c, Apaf-1, AIF, GADD153 and GRP78; C: caspase-8, caspase-9, caspase-3 and caspase-7) were estimated by Western blotting, as described in Materials and Methods.*

located on the mitochondrial membrane is important for suppression of mitochondrial manifestations of apoptosis (34). Whether or not EGCG-induced apoptosis is associated with Bcl-2 family proteins, and xIAP and cIAP proteins, which are caspase cascade inhibitors, was examined by Western blotting. As shown in Figure 3A, 20 μM of EGCG decreased the protein expression of Bcl-2, Bcl-xl, xIAP and cIAP for 48 h. EGCG increased the protein expression of Bad and Bax, the

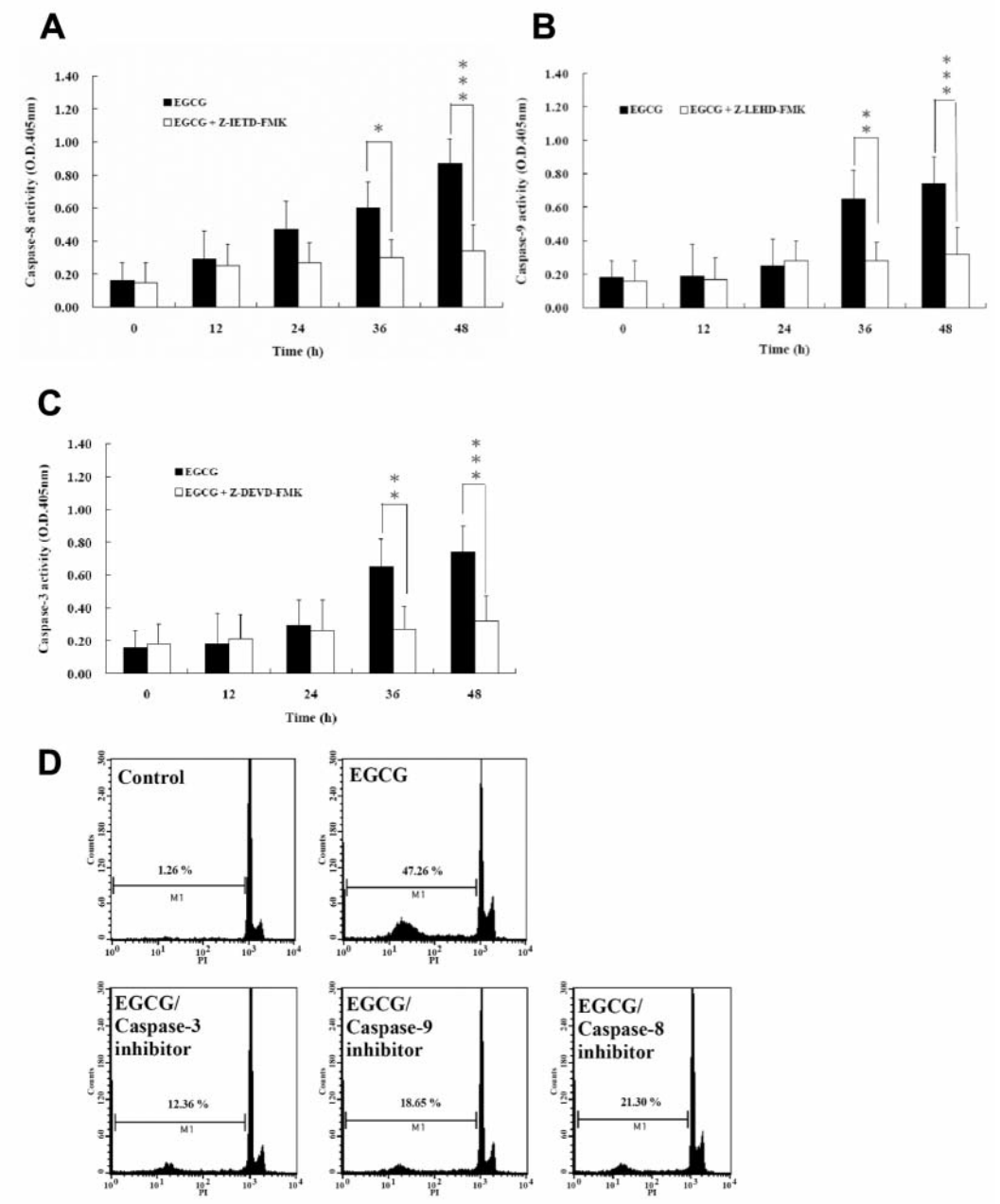

Figure 4. *EGCG and caspase-8, -9 and -3 inhibitors induced the activity of caspase-8 (A), caspase-9 (B) and caspase-3 (C) in NCI-H295 cells. (D) Caspase-8, -9 and -3 inhibitors inhibited EGCG-induced apoptosis. NCI-H295 cells were treated with 20 μM EGCG in the presence or absence of 50 μM of caspase-8, -9 or -3 inhibitor. Data represents mean±S.D. of three experiments. *p<0.05, **p<0.01, ***p<0.001.*

pro-apoptotic members in Bcl-2 family, over Bcl-2. Whether EGCG-induced apoptosis is related with apoptosis associated proteins, including the death receptor pathway (Fas/CD95 and caspase-8), mitochondrial pathway (cytochrome *c*, Apaf-1, AIF and caspase-9), stress pathway (Hsp70 and Hsp90) and ER stress pathway (GADD153 and GRP78) proteins, was also assessed. The data shown in Figure 3B and 3C indicate that EGCG promoted the levels of Fas/CD95, cytochrome *c*, Apaf-1, AIF, GADD153, GRP78, caspase-3, -7, -8 and -9, but decreased the expression of Hsp70 and Hsp90, suggesting that EGCG-induced apoptosis is mediated by induction of the death receptor, mitochondrial and ER stress pathways.

Effects of EGCG on the caspase-8, -9 and -3 activities and the percentages of viable NCI-H295 cancer cells. In order to evaluate the effects of EGCG on the activities of caspase-8,

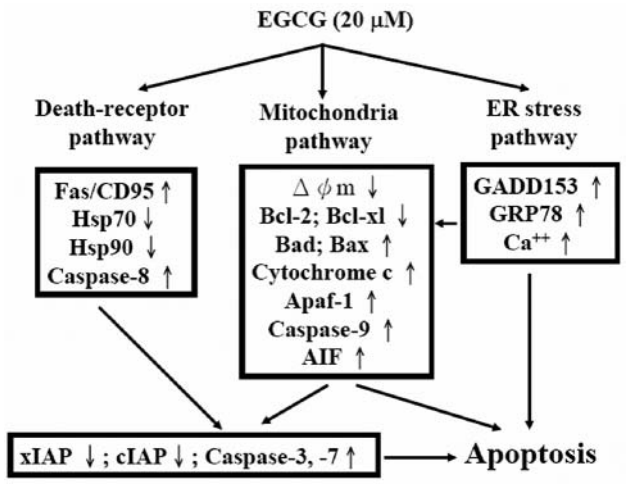

Figure 5. *The proposed model of EGCG-mediated apoptosis in human adrenal cancer NCI-H295 cells.*

-9 and -3, and the percentage of viability in NCI-H295 cells, caspase activity assay was used and the cell cycle and sub-G1 population were analysed by flow cytometry. The results in Figure 4 show that 20 μM of EGCG promoted caspase-8, -9 and -3 activities in a time-dependent manner. However, pretreatment of cells with inhibitors of caspase-8, -9 and -3, respectively, led to a decrease in caspase-8, -9 and -3 activities and an increase in the percentage of viable cells (control: 1.26±0.12; EGCG treatment: 47.26±4.98; caspase-3 inhibitor pretreatment: 12.36±3.44; caspase-9 inhibitor pretreatment: 18.65±4.31; caspase-8 inhibitor pretreatment: 21.30±5.21). This confirms that EGCG-induced apoptosis is mediated by induction of caspase-8, -9 and -3 activities.

Discussion

Polyphenols are a group of chemical substances widely present in the human diet (35). Major polyphenol dietary sources are fruits, vegetables and beverages (fruit juice, wine, tea, coffee) and chocolate (36). The total intake of polyphenols is approximately 1,000 mg/day, depending on lifestyle and dietary preferences (37). There are four sub-groups of polyphenols: flavonoids, anthocyanins, proanthocyanidins and xanthones (38). The major polyphenol in green tea is EGCG which accounts for 50-80% of catechin and representing 200-300 mg in a brewed cup of green tea (39-40). Many studies have been reported that EGCG has growth inhibitory effects in human cancer cell lines (8-13), but few have reported on its inhibitory effect in human adrenal cancer. The presented data showed that 20 μM of EGCG significantly inhibited the proliferation of human adrenal cancer cell line NCI-H295 cells. In contrast, it exerted low cytotoxicity on Detroit 551 normal human cells and IC_{50}>100 μM (Figure 1C). These data indicated that EGCG represents a promising candidate as an anti-adrenal cancer agent with low toxicity to normal cells.

EGCG is considered to suppress cancer cell growth effect by anti-metastasis and anti-angiogenic action, activation of immune function and directly through cell-cycle arrest and induction of apoptosis (41-44). Previous studies have reported that EGCG induces G_0/G_1 phase arrest and apoptosis in human HCC cell lines, including leukemia, pancreatic cancer, human breast cancer, human YT and Jurkat T-cells, oral carcinoma, human melanoma, human ovarian carcinoma, human prostate carcinoma and human colon carcinoma cell lines (44-51). In the present studies, it is shown that EGCG induced morphological changes and reduced the percentage of viable cells in the human adrenal cancer cell line NCI-H295 in a dose- and time-dependent manner. In Figure 4D, it an be seen that EGCG induced the accumulation of the sub-G_1 population (apoptosis) but did not induce the accumulation of the G_0/G_1 population. Cerquetti *et al*. used sequence analysis of *p53* and revealed a large deletion of exons 8 and 9 in NCI-H295 cells (52). It is hereby proposed that p53 plays pivotal roles in the regulation of the cell cycle progression by EGCG.

EGCG induced cell-cycle arrest and apoptosis through Fas-Fas ligand pathways in HepG2 cells and its binding to Fas triggers the Fas-mediated apoptosis in U937 cells (53-54). In the present study, it has been demonstrated that EGCG promoted the levels of Fas/CD95 and caspase-8 activity, and pretreatment with caspase-8 inhibitors led to decreased caspase-8 activity and an increase in the percentage of viable cells in NCI-H295 cells. This indicates that EGCG-induced apoptosis is primarily mediated by induction of the death receptor pathway and Fas/CD95 may be a major target in NCI-H295 cells.

Alteration in the ratio of Bax/Bcl-2 stimulates the release of cytochrome *c* from mitochondria into the cytosol (55-56). Cytosolic cytochrome *c* then binds to Apaf-1 and leads to the activation of caspase-3 and caspase-7. Activated caspase-3 and -7 are key mediators of cell apoptosis (57). In this study, it was demonstrated that EGCG increased Bax and Bad, and inhibited the levels of Bcl-2 and Bcl-xL which led to the disruption of $\Delta\psi m$ and the release of cytochrome *c*, AIF and Apaf-1. It was also demonstrated that EGCG promoted the levels of ER stress associated proteins, such as GADD153 and GRP78 and induced an increase in intracellular free Ca^{2+} levels in NCI-H295 cells. EGCG increased caspase-9 and -3 activities, and pretreatment with caspase-9 and -3 inhibitors led to a decrease in caspase-9 and -3 activities, and an increase in the percentage of viable cells. It is therefore suggested that EGCG-induced apoptosis is also mediated by induction of the mitochondrial and ER stress pathways in conjunction with the death receptor pathway discussed above.

In conclusion, the results demonstrate that EGCG induced cytotoxicity on NCI-H295 cells operates through the induction of apoptosis *via* death receptor, mitochondrial and ER dependent pathways (Figure 5). These findings provide important possible molecular mechanisms of the anti-human adrenal cancer activity of EGCG and confirm that EGCG may be an anti-adrenal cancer drug candidate.

Acknowledgements

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