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(−)-Epigallocatechin-3-gallate Induces Apoptosis of Human Hepatoma Cells by Mitochondrial Pathways Related to Reactive Oxygen Species

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The aim of this study was to investigate the effects of (−)-epigallocatechin-3-gallate (EGCG) on the induction of apoptosis in hepatocarcinoma cell lines in vitro and further examine the molecular mechanisms of EGCG-induced apoptosis. In the present study, it was observed that EGCG rapidly induced apoptosis in hepatocarcinoma SMMC7721 cells. EGCG-induced apoptosis was in association with the attenuation of mitochondrial transmembrane potentials ($\Delta\psi_m$), the alteration of Bcl-2 family proteins, the release of cytochrome *c* from mitochondria into the cytosol, and the activation of caspase-3 and caspase-9. Elevation of intracellular reactive oxygen species (ROS) production was also shown during EGCG-induced apoptosis of hepatocarcinoma SMMC7721 cells. The antioxidant *N*-acetyl-L-cysteine (NAC) significantly reduced ROS production and EGCG-induced apoptosis, suggesting that ROS plays a key role in EGCG-induced apoptosis in hepatocarcinoma SMMC7721 cells. In summary, EGCG-induced apoptosis through mitochondrial pathways, and ROS affected EGCG-induced apoptosis in hepatocarcinoma SMMC7721 cells.

KEYWORDS: Green tea; (−)-epigallocatechin-3-gallate; apoptosis; reactive oxygen species; hepatocarcinoma

INTRODUCTION

Hepatocellular carcinoma belongs to the most common tumor diseases worldwide (*1*, *2*). Despite the continuing efforts for the early detection of hepatocellular carcinoma, the mortality rate and prognosis have not been improved yet. Surgical resection has been considered the optimal treatment approach, but only a small number of patients are suitable candidates for surgery, and the relapse rate is high. Severe adverse effects and complications such as serious infection due to anticancer drugs are also major problems in the clinical setting (*3*). In particular, side effects of drugs might be fatal in older or immunocompromised patients. In addition, repeated episodes of relapse of the disease may lead to refractory or chemotherapy resistance (*4*). Therefore, novel strategies and agents, which specifically target hepatocellular carcinoma but have lower toxicity for normal liver cells, are seen as a method of detection of enormous potential.

Tea is the most widely consumed beverage in the world, besides water. Green tea has gained attention of both consumers and researchers in the past few years for its potential health benefits. Because of its widespread and long use, it is considered to be safe (*5*). Some studies have shown that green tea consumption is associated with a reduced risk of cardiovascular diseases, degenerative diseases, and cancer (*6*, *7*). The potential health benefits associated with tea consumption have been partially attributed to the antioxidative properties of polyphenols, particularly catechins, among which (−)-epigallocatechin-3-gallate (EGCG) is the most effective (*8*, *9*). Moreover, EGCG has been shown to induce apoptosis of human hepatocelluar carcinoma in vitro (*10*).

Apoptosis, or programmed cell death, is a very important phenomenon in cytotoxicity induced by anticancer treatment (*11*). It is a tightly controlled multistep mechanism of cell death, and mitochondria are considered to play a central role in this process. Mitochondria initiate two distinct apoptosis pathways: one caspase-dependent and the other caspase-independent. Most chemotherapeutic agents induce apoptosis through at least one of these pathways (*12*). In addition, mitochondrial production of reactive oxygen species (ROS) seems to play a role in apoptosis. ROS, the byproduct of normal cellular oxidative processes, have been suggested to regulate the process involved in the initiation of apoptotic signaling. The cytotoxicity of anticancer chemotherapeutic drugs may largely depend on the intracellular level of ROS (*13*). EGCG has a potent antioxidant property because of hydroxyl functional groups in its chemical structure. Several studies have demonstrated that EGCG can protect the heart, brain, and kidney from oxidative injury. EGCG provides both short- and long-term protection against oxidative stress through a variety of mechanisms (*14*). The antioxidant activity of EGCG has been reported in various models; however, it can also behave as a pro-oxidant under certain conditions. It has also been reported that EGCG may induce the production of H_2O_2 in the culture media (*15*). Therefore, we hypothesized that EGCG induced apoptosis of human hepatoma cells via mitochondrial pathways related to ROS.

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MATERIALS AND METHODS

Materials and Reagents. EGCG was purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai China) and dissolved in phosphate-buffered saline. It was diluted to appropriate concentrations in culture media before being used in each experiment. Cell culture products were obtained from Life Technologies (Paisley, Scotland). 5(6)-Carboxy-2′,7′-dichlorofluorescein diacetate (cDCFH-DA) and Rhodamine-123 were purchased from Molecular Probes Inc. (Eugene, OR). Annexin V-FITC apoptosis detection kits were obtained from BD Biosciences (San Jose, CA). *N*-Acetyl-L-cysteine (NAC) was obtained from Sigma Chemical Co. (St. Louis, MO). Anti-β-actin, anti-cytochrome *c*, anti-Bcl-2, and anti-Bax primary antibodies, as well as the horseradish peroxidase (HRP)-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Cell Culture. The hepatocarcinoma SMMC7721 cell line was generously provided by Prof. Ming He (Department of Pharmacology, Nanchang University). Hepatocarcinoma cell line (BEL-7402) and liver cell line (L-02) were purchased from Chinese Academy of Sciences Cell Bank. These hepatocarcinoma cell lines were cultured in RPMI-1640 medium with 2.0 g/L sodium bicarbonate plus 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. L-02 was cultured in RPMI-1640 medium with 2.0 g/L sodium bicarbonate plus 20% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C.

Cytotoxicity Assay. The cytotoxicity of EGCG was assessed by trypan blue dye exclusion. Bel-7402 cells, SMMC7721 cells, and L-02 cells were seeded in 12-well plates at 2.5×10^3 cells/mL, respectively. Cells were allowed to grow for 1 day before being exposed to EGCG. For the control group, an equal volume of PBS was added into the medium. Cell culture medium was changed every alternative day. After incubation for the indicated time, trypan blue dye exclusion was used to determine cytotoxicity.

Flow Cytometric Analysis Using Annexin V and PI. EGCG-induced apoptosis was determined by Annexin V and PI double labeling. Cells were centrifuged to remove the medium, washed with PBS, and stained with Annexin V and PI in binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM $CaCl_2$). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson).

Measurement of Caspase Activities. Caspase activitives were evaluated by use of caspase-3 and caspase-9 Colorimetric Assay Kit (Biovision, CA). The assay is based on spectrophotometric detection of the chromophore ρ-nitroanilide (ρNA) after cleavage from the labeled substrate DEVD-ρNA. The absorbance at 405 nm of the released ρNA was monitored with a spectrophotometer.

Preparation of Mitochondria and Cytosolic Fractions. Mitochondrial and cytosolic fractions of cells were prepared using a mitochondrial/cytosol fractionation kit (Biovision, Mountain View CA). Cells at 1×10^7/mL with or without different treatments were harvested by centrifugation at 700*g* for 5 min and washed twice with cold phosphate-buffered saline. Afterward, the cells were resuspended in 250 μL of extraction buffer containing protease inhibitor mixture and dithiothreitol. After incubation on ice for 30 min, the cells were homogenized using a Kontes Dounce tissue grinder on ice. Homogenates were centrifuged at 700*g* for 10 min at 4 °C, and the supernatant was collected. Then the collected supernatant was centrifuged again at 10000*g* for 30 min at 4 °C. The resulting supernatants were harvested and designated cytosolic fractions, and the pellets were resuspended in an appropriate buffer and designated mitochondrial fractions.

Western Blotting. Samples were mixed with sample buffer (Laemmli sample buffer, Bio-Rad) and then boiled for 5 min and loaded onto 12 or 15% SDS−polyacrylamide gels. Electroblotted onto polyvinylidene fluoride (PVDF) membranes, the membranes were blocked for 2 h with 5% nonfat milk powder in TBST solution (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, and 0.1% v/v Tween 20) at room temperature. Membranes were incubated with the antibodies overnight at 4 °C and probed with different antibodies in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST). To ensure equivalent protein loading, the membranes were also incubated with anti-β-actin mouse monoclonal antibody and, subsequently, with a corresponding HRP-conjugated secondary antibody IgG and developed using Chemiluminescence Reagent Plus. The scan densitometric analysis was carried out using a GDS-8000 UVP photoscanner and LAB WOEK45 Image software (Bio-Rad).

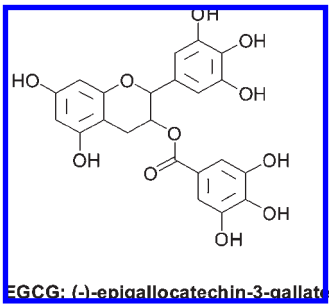

Figure 1. Chemical structure of (−)-epigallocatechin-3-gallate (EGCG) used in this study.

Determination of Mitochondrial Membrane Potential ($\Delta\psi_m$). To determine the change of $\Delta\psi_m$, flow cytometry was applied using Rhodamine-123 staining (*15*). The cells were harvested and washed with ice-cold PBS twice by centrifugation at 1000*g* for 5 min, and then 1 mL of PBS containing 10 μg/mL Rhodamine-123 was added to the cells. The tubes were vortexed gently and incubated at 37 °C for 20 min. Rhodamine-123 fluorescence was measured using a FACStar Plus cell sorter (Becton Dickinson) with excitation and emission wavelengths of 488 and 530 nm, respectively.

Measurement of ROS Generation. The generation of ROS was assessed using the cell-permeable probe 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (cDCFH-DA). This dye is cleaved by cellular esterases to nonfluorescent 2′,7′-dichlorofluorescin (DCFH) and oxidized by ROS to a fluorescent product dichlorofluorescein (DCF). After incubation with or without EGCG, cells were harvested and washed with cold phosphate-buffered saline. Washed cells were further incubated with 10 μM DCFH-DA at 37 °C for 20 min. Dye oxidation (increase in fluorescence) was measured using a FACStar Plus flow cytometer (Becton Dickinson) with excitation and emission settings of 488 and 530 nm, respectively.

Statistical Analysis. Values are expressed as means ± SEM. One-way analysis of variance followed by the Student−Newman−Keuls test was applied to calculate the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of EGCG on Cellular Proliferation of BEL-7402, SMMC7721, and L02 Cell Lines. The chemical structure of EGCG is shown in **Figure 1**. In the present study, we examined whether EGCG inhibited the cellular growth of hepatocarcinoma cell lines (BEL-7402 and SMMC7721) and normal liver cell line (L-02). EGCG did not affect the viability of normal liver L-02 cells (data not shown), but significantly ($P < 0.01$) inhibited the cellular growth of hepatocarcinoma cells (BEL-7402 and SMMC7721) in a dose- and time-dependent manner (**Figure 2**). Consumption of tea has been suggested to prevent cancer and other diseases. In humans, tea polyphenols undergo glucuronidation, sulfation, methylation, and ring fission. The peak plasma concentration of EGCG is approximately 1 μM. However, numerous studies reported effects of EGCG at submicromolar levels, and most experiments required concentrations of >10 or 20 μM to demonstrate its effect (*16*). In our study, we analyzed the half-maximal inhibitory concentration (IC_{50}) from the dose−response curves. The hepatocarcinoma SMMC7721 cells were more sensitive to EGCG with an IC_{50} of 22 μM. Thus, we used hepatocarcinoma SMMC7721 cells for the series of experiments, and the cells were exposed to 22 μM EGCG.

Effects of EGCG on Apoptosis in Hepatocarcinoma SMMC7721 Cells. To examine whether EGCG induced apoptosis, the hepatocarcinoma SMMC7721 cells were incubated with EGCG. Flow

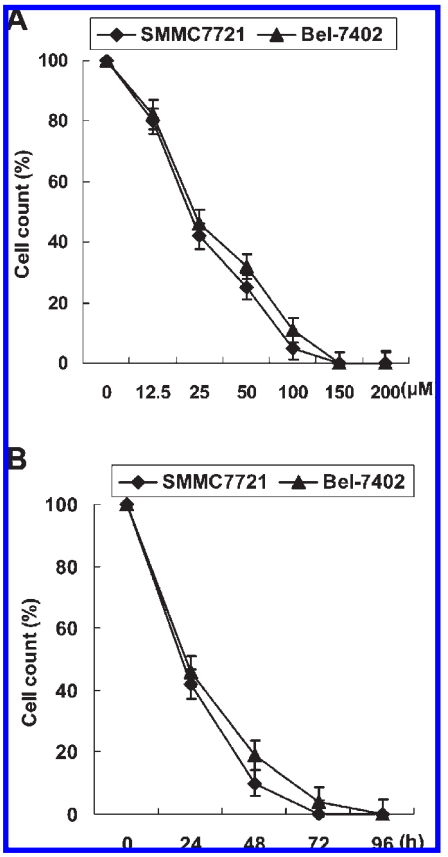

Figure 2. Effects of (−)-epigallocatechin-3-gallate (EGCG) on cellular proliferation of hepatocarcinoma cell lines: (**A**) hepatocarcinoma cell lines (BEL-7402 and SMMC7721) were treated with various concentrations (0−100 μmol/L) of EGCG for 24 h; (**B**) hepatocarcinoma cell lines (BEL-7402 and SMMC7721) were treated with 25 μmol/L EGCG for indicated times (0−96 h). Cell viability was assessed by trypan blue dye exclusion. Eight independent experiments were done, and all gave similar results.

cytometry analysis was used to quantify the rate of cell apoptosis using double staining of Annexin V and PI. Interestingly, a significant increase of apoptosis was observed at a time point as early as 4 h in hepatocarcinoma SMMC7721 cells treated with EGCG compared with the control group (**Figure 3**).

Effects of EGCG on Caspase Activity in Hepatocarcinoma SMMC7721 Cells. Caspases are believed to play a central role in mediating various apoptotic responses. To analyze the apoptotic pathway in EGCG-treated hepatocarcinoma SMMC7721 cells, we next examined the caspase-9 and -3 activities using the synthetic caspase substrates DEVD-ρNA and LEHD-ρNA. Both caspase-3 and caspase-9 in hepatocarcinoma SMMC7721 cells increased after treatment with EGCG for 4 h (**Figure 4**). These results suggested that EGCG-induced apoptosis is associated with the activation of caspase-3 and caspase-9.

Expression of Apoptosis-Associated Proteins. To investigate the molecular mechanism of EGCG-induced apoptosis in hepatocarcinoma SMMC7721 cells, the expression of several apoptosis-associated proteins was examined. The expression of mitochondrial Bcl-2 was decreased by treatment with EGCG (**Figure 5**). Western blot analysis also revealed that mitochondrial Bax protein increased after exposure of the hepatocarcinoma SMMC7721 cells to EGCG, together with a decrease in cytosolic Bax (**Figure 5**). These data indicated that EGCG-induced the loss of Bcl-2 from mitochondria and Bax translocation.

EGCG-induced Death Signaling Is Mediated through the Mitochondrial Pathway. Recent studies have suggested that mitochon-

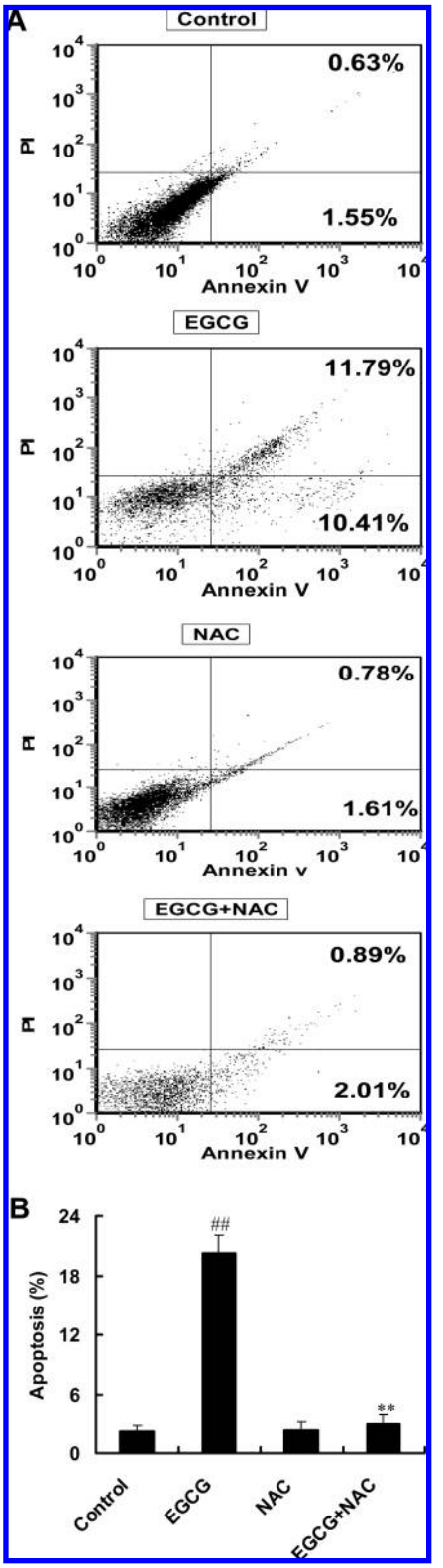

Figure 3. Effects of (−)-epigallocatechin-3-gallate (EGCG) on apoptosis in hepatocarcinoma SMMC7721 cells: (**A**) detection of apoptotic cells by Annexin V and PI double staining (cells were cultured with 22 μmol/L EGCG for 4 h with or without NAC, stained with Annexin V and PI labeling, and analyzed by flow cytometry); (**B**) column bar graph of apoptosis. (eight independent experiments were done, and all gave similar results; $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ vs EGCG group).

dria play an essential role in death signal transduction. Mitochondrial changes, including permeability transition pore

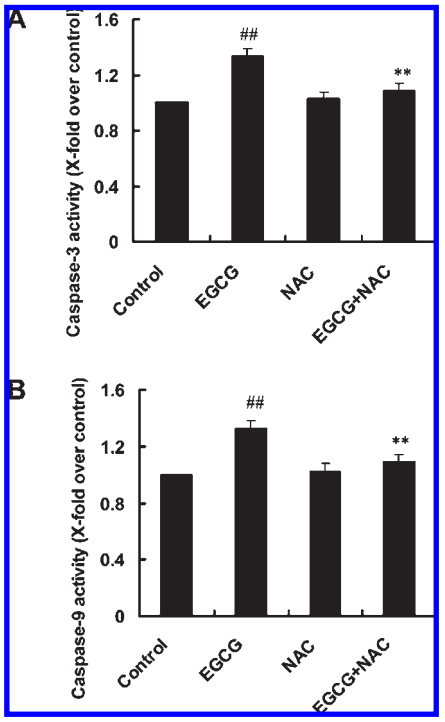

Figure 4. Effects of (−)-epigallocatechin-3-gallate (EGCG) on caspase activities in hepatocarcinoma SMMC7721 cells: quantitative analysis of caspase-3 activity (**A**) and caspase-9 activity (**B**). Caspase-3 and caspase-9 activities were measured using synthetic caspase substrates DEVD-ρNA and LEHD-ρNA. Eight independent experiments were done, and all gave similar results. $^{\#\#}P < 0.01$ vs control; $^{**}P < 0.01$ vs EGCG.

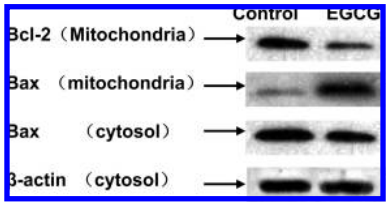

Figure 5. Effect of (−)-epigallocatechin-3-gallate (EGCG) on the expression of Bcl-2 family in hepatocarcinoma SMMC7721 cells. The cytosolic and mitochondrial proteins were analyzed by Western blotting with anti-β-actin, Bax, and Bcl-2 antibodies. Eight independent experiments were done, and all gave similar results.

opening and the collapse of the $\Delta\psi_m$, result in the release of cytochrome *c* into the cytosol, which subsequently causes apoptosis by the activation of caspases (*17*). After treatment with EGCG for 4 h, Rhodamine-123 staining in hepatocarcinoma SMMC7721 cells indicated an increase in the loss of $\Delta\psi_m$ (**Figure 6**). The loss of $\Delta\psi_m$ appeared in parallel with the activation of caspase-3 and caspase-9, as well as with apoptosis. In addition, EGCG induced a substantial release of mitochondrial apoptogenic protein (cytochrome *c*) from the mitochondria into the cytosol in hepatocarcinoma SMMC7721 cells (**Figure 7**). The loss of mitochondrial Bcl-2 and Bax translocation was also detected after EGCG treatment (**Figure 5**). These results suggested that mitochondrial dysfunction caused the release of cytochrome *c* into the cytosol; caspase-9 and caspase-3 were then activated, thereby propagating the death signal.

ROS Production Triggers EGCG-induced Apoptosis. Several investigators have reported that EGCG-induced apoptosis is often associated with the generation of ROS (*18*). To investigate the role of ROS in EGCG-induced apoptosis, we used antioxidant NAC

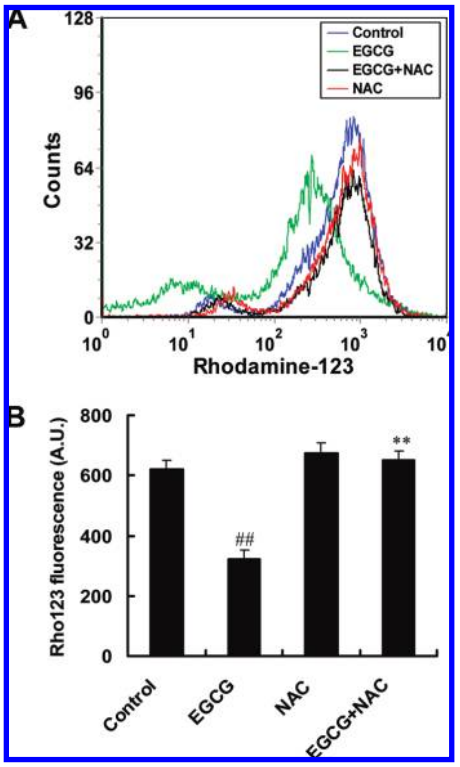

Figure 6. Effects of (−)-epigallocatechin-3-gallate (EGCG) on the loss of $\Delta\psi_m$ in hepatocarcinoma SMMC7721 cells: (**A**) flow cytometric analysis of $\Delta\psi_m$ as estimated by the Rhodamine-123 intensity (cells were cultured with 22 μmol/L EGCG for 4 h with or without NAC, and Rhodamine-123 fluorescence was analyzed by flow cytometry); (**B**) column bar graph of cell fluorescence for Rhodamine-123 (eight independent experiments were done, and all gave similar results; $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ vs EGCG group).

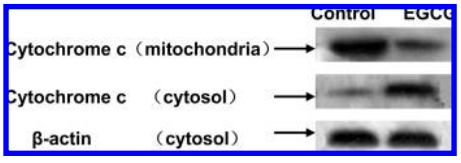

Figure 7. Effects of (−)-epigallocatechin-3-gallate (EGCG) on cytochrome *c* release in hepatocarcinoma SMMC7721 cells: Western blot analysis of cytochrome *c* release in EGCG-treated cells. The cytosolic and mitochondrial proteins were analyzed by Western blotting with anticytochrome *c* and β-actin antibodies. Eight independent experiments were done, and all gave similar results.

for further experiments. Treatment of hepatocarcinoma SMMC7721 cells with NAC (15 mmol/L) significantly blocked EGCG-induced apoptosis (**Figure 3**). We then analyzed the roduction of intracellular ROS in control and EGCG-treated cells. Oxidation of H_2DCFDA leads to an increase in the fluorescent product dichlorofluorescein and permits the quantification of relative levels of ROS. After treatment with EGCG for 4 h in hepatocarcinoma SMMC7721 cells, the results showed a dramatic increase of ROS generation, compared with control cells (**Figure 8**). Furthermore, treatment of hepatocarcinoma SMMC7721 cells with NAC blocked the generation of intracellular ROS and the loss of $\Delta\psi_m$ in EGCG-induced apoptosis (**Figures 6** and **8**). Furthermore, down-regulation of mitochondrial Bcl-2, Bax translocation, release of cytochrome *c*, and caspase activation after EGCG treatment were prevented by NAC cotreatment (**Figure 9**). Our data indicated that the modulation of

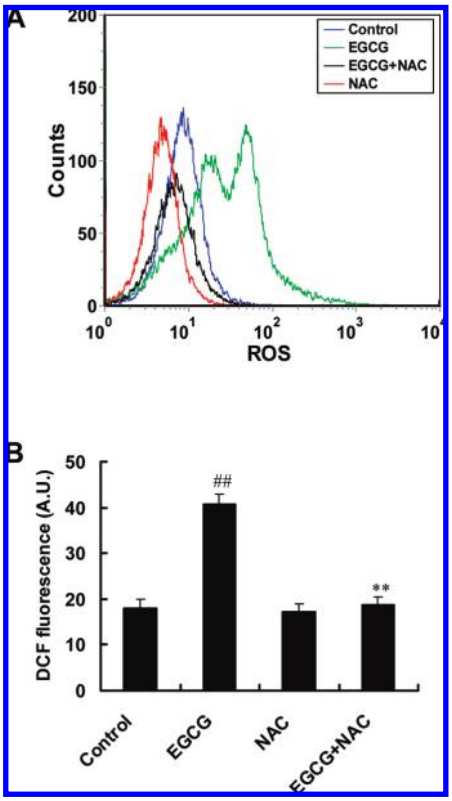

Figure 8. Effects of (−)-epigallocatechin-3-gallate (EGCG) on ROS generation in hepatocarcinoma SMMC7721 cells: (**A**) flow cytometric histograms of fluorescence of 2′,7′-dichlorofluorescein (DCF) in hepatocarcinoma SMMC7721 cells; (**B**) column bar graph of cell fluorescence for DCF (eight independent experiments were done, and all gave similar results; $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ vs EGCG group).

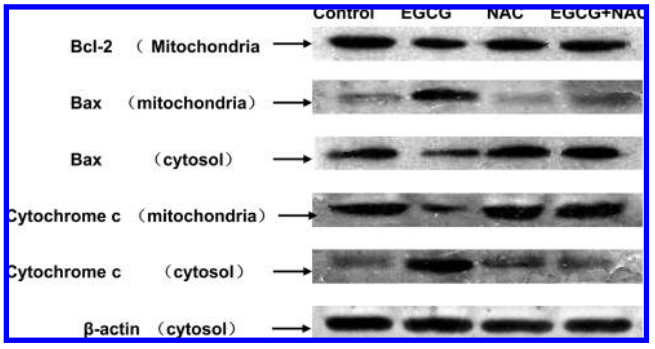

Figure 9. Effects of (−)-epigallocatechin-3-gallate (EGCG) on expression of the apoptosis-associated proteins in hepatocarcinoma SMMC7721 cells: expression of the various apoptosis-associated proteins in hepatocarcinoma SMMC7721 cells treated with EGCG with or without NAC. The cytosolic and mitochondrial proteins were analyzed by Western blotting with anti-β-actin, cytochrome *c*, Bax, and Bcl-2 antibodies. Eight independent experiments were done, and all gave similar results.

molecules involved in the redox system may determine the sensitivity of hepatocarcinoma SMMC7721 cells to EGCG.

DISCUSSION

Green tea is one of the most widely consumed beverages in the world. The water-extractable fraction of green tea contains several polyphenolic compounds known as catechins. Generally, a typical cup of green tea contains 100−150 mg of catechins, including 50% of EGCG, 15% of (−)-epigallocatechin, 15% of (−)-epicatechin-3-gallate, and 8% of (−)-epicatechin (*19*). Numerous studies have shown that green tea extract derived from the dried fresh leaves of the plant *Camellia sinensis* and one of its major constituents, EGCG, possess antiproliferative, antiangiogenic, antimetastatic, and pro-apoptotic effects in various in vitro and in vivo tumor models. These experimental studies together with several epidemiologic studies have suggested that green tea extract and EGCG, harbor strong anticancer and cancer preventive effects in numerous human cancers (*20*). However, the influence of EGCG on signaling molecules directly involved in apoptotic pathway has not been fully examined in hepatocellular carcinoma.

Cancer is described as a disease that involves excessive proliferation of cells and abandonment of their ability to die (*21*). Our investigation suggested that EGCG rapidly inhibited the cellular proliferation of hepatocarcinoma cell lines (BEL-7402 and SMMC7721) in a dose- and time-dependent manner, but the toxicity to normal liver cell line (L-02) was negligible. These data indicated that EGCG might be a novel effective and less toxic therapeutic strategy among patients with hepatocellular carcinoma. In hepatocarcinoma SMMC7721 cells, the activities of EGCG that inhibited the cellular growth significantly increased, compared with this in hepatocarcinoma BEL-7402 cells. Therefore, we adopted the hepatocarcinoma SMMC7721 cells for our further studies.

Normally, cells can kill themselves in a balanced process known as "apoptosis". It is becoming clear that too little cell suicide by apoptotic process can lead to a variety of cancers, including hepatocellular carcinoma (*22*). At present, two major pathways that link apoptosis have been identified: (a) intrinsic or mitochondrial and (b) extrinsic or death receptor-related (*23*). The intrinsic pathway involves the cell sensing stress that triggers the mitochondria-dependent processes, resulting in cytochrome *c* release and activation of caspase-9. Cytochrome *c* release is a crucial step in apoptosis induced by many death stimuli that are controlled by Bcl-2 family proteins. The Bcl-2 family, which possesses both anti- and pro-apoptotic members, constitutes a decisive checkpoint within the common portion of the cell death pathway (*24*). Bcl-2 can prevent ROS production and regulate the mitochondrial transitional pore opening by opposing the effect of Bax, thereby blocking cytochrome *c* release and inhibiting caspase activities (*25*). Moreover, the anti-apoptotic function of Bcl-2 is thought to be primarily derived from Bcl-2 presented in the mitochondria. Normally, Bax exists as a soluble monomer in cytosol or is loosely associated with mitochondria. However, upon apoptotic stimulation, Bax translocates to mitochondria, where it forms oligomers that are inserted into the outer mitochondrial membrane, and the mitochondria Bcl-2 decreases from mitochondria (*26*).

In this study, we showed that EGCG rapidly induced apoptotic cell death in hepatocarcinoma SMMC7721 cells in association with the down-regulation mitochondria Bcl-2, Bax translocation from the cytosol to mitochondria, loss of $\Delta\psi_m$, release of cytochrome *c* from mitochondria into the cytosol, and activation of caspase-3 and caspase-9. Our results, together with the results of previous studies, suggested that EGCG induced apoptosis by mitochondrial death pathways.

Elevation of intracellular ROS production was also shown during EGCG-induced apoptosis of hepatocarcinoma SMMC7721 cells. Various studies have shown that stress-induced changes in $\Delta\psi_m$ correlate with an increase in ROS and the release of mitochondrial cytochrome *c*. The role of ROS in mediating apoptosis in various cancer cells is well established (*27*). The generation of ROS has been linked to the release of cytochrome *c* from mitochondria to the cytosol during apoptosis (*28*). In the present study, an antioxidant, NAC, significantly blocked ROS

production, loss of $\Delta\psi_m$, caspase-3 and caspase-9 activation, and EGCG-induced apoptosis in hepatocarcinoma SMMC7721 cells. Previous studies have shown that NAC abrogated ROS generation and inhibited EGCG-mediated H_2O_2 generation. In addition, it has been reported that ROS directly down-regulates Bcl-2 and Bax translocation (*29*). Therefore, NAC protected the down-regulation of mitochondrial Bcl-2 and Bax translocation in EGCG-treated hepatocarcinoma SMMC7721 cells. These results suggested that ROS played an upstream important mediator during EGCG-induced apoptosis in hepatocarcinoma SMMC7721 cells.

Previous studies on the antioxidative property of EGCG have shown the trapping effect of ROS as well as the inhibitory effect of lipid peroxidation. However, after neutralizing the peroxyl or other radicals, EGCG itself could be converted to phenoxyl radical (*30*). In a recent investigation, the chemical property of EGCG as a potential pro-oxidant was highlighted by the blocking effects of GSH and NAC against EGCG-induced apoptosis (*31*). Oxidative stress has been suggested to be a mechanism by which anticancer drugs cause cell death (*32*). Apoptosis has been shown to be associated with the generation of ROS in several experimental models. Antioxidants and free radical scavengers are able to inhibit apoptosis induced by anticancer drugs. Moreover, some studies have suggested that ascorbic acid decreases cellular GSH levels and potential agent-mediated cell death in chemotherapy resistance (*33*). Consistent with previous results, it is possible that the combination of EGCG and anticancer drugs would enhance therapeutic activity and overcome drug resistance in hepatocellular carcinoma.

The present study provides unequivocal evidence for the first time that EGCG induces apoptosis of hepatocarcinoma SMMC7721 cells by mitochondrial pathways related to reactive oxygen species. EGCG is a natural compound and seems safer than popular chemotherapeutic agents. In particular, it might be useful in older or immunocompromised patients because of its safety and lack of known toxicity. These results support a role for EGCG as a promising anticancer drug lead and as a possible novel therapeutic agent to replace or augment the more cytotoxic agents currently used to treat patients with hepatocellular carcinoma.

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