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# Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells

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**Epigallocatechin-3-gallate (EGCG), a major component in green tea polyphenols, has been proven to suppress colonic tumorigenesis in animal models and epidemiological studies. As EGCG is retained in the gastrointestinal tract after oral administration, this pharmacokinetics property gives it the potential to function as a chemopreventive agent against colon cancer. In this study, human colorectal carcinoma HT-29 cells were treated with EGCG to examine the anti-proliferative and pro-apoptotic effects of EGCG, as well as the molecular mechanism underlying these effects. Cell viability assay, nuclear staining, DNA fragmentation, caspase assay, cytochrome *c* release, $DiOC_6(3)$ staining, mitogen-activated protein kinases (MAPK) phosphorylation and trypan blue exclusion assays, were utilized to dissect the signaling pathways induced by EGCG. After 36 h treatment, EGCG inhibited HT-29 cell growth with an $IC_{50}$ of ~100 μM. HT-29 cells treated with doses higher than 100 μM showed apparent nuclear condensation and fragmentation, which was confirmed by DNA laddering. Caspase-3 and -9 activation was detected after 12 h treatment, accompanied by mitochondrial transmembrane potential transition and cytochrome *c* release. Activation of MAPKs was detected as early signaling event elicited by EGCG. Inhibition of c-Jun N-terminal kinase (JNK) pathway showed the involvement of JNK in EGCG-induced cytochrome *c* release and cell death. EGCG-induced JNK activation was blocked by the antioxidants glutathione and *N*-acetyl-L-cysteine, suggesting that the cell death signaling was potentially triggered by oxidative stress. In summary, our results from this study suggest that in HT-29 human colon cancer cells (i) EGCG treatment causes damage to mitochondria, and (ii) JNK mediates EGCG-induced apoptotic cell death.**

## Introduction

Colon cancer is the second leading source of cancer-derived mortality in the US (1). Epidemiological studies have linked the cause of high incidence of colon cancer in Western society to the dietary custom (2). In Asian countries with relatively lower incidence of colon cancer, such as China, Japan and Korea, one of the prominent lifestyles is the daily consumption of green tea drinks by a large population. Chemically, the water-extractable fraction of green tea contains abundant polyphenolic compounds, in which epigallocatechin-3-gallate (EGCG) is the major constituent (>50% of polyphenolic fraction). After a common brewing procedure, 30–42% (w/w) of green tea can be dissolved in water. Therefore, a single cup of green tea can contain up to 200 mg of EGCG (3). The cancer prevention effect of green tea and EGCG against various tumors has been proven with numerous animal models (4,5). Human clinical trials, although inconclusive, also indicated some positive link between the drinking of green tea and the decrease of cancer incidence (6). In rodent models for determining the pharmacokinetics profile of green tea catechins, the highest tissue concentrations of EGCG have always been found in the intestine after either oral or i.v. administrations (7–9). Furthermore, substantial amounts of EGCG were present in human colon mucosa samples from the patients drinking tea 12 h before colorectal surgery (4). Because of this pharmacokinetic profile, as well as the high local concentration in intestinal microenvironment that come from direct contact of green tea drinks with colon mucosa, the study on potential effectiveness of EGCG on the inhibition of colorectal carcinogenesis has generated great interest. It has been reported that EGCG inhibited azoxymethane-induced colon tumorigenesis in the rat (10), and also drinking green tea blocked the formation of 1,2-dimethylhydrazine-induced colonic aberrant crypt foci, which is a typical precursor lesion of chemical-initiated colon cancer (11).

Previous studies have shown that the chemoprevention function of EGCG could be attributed to its properties as an antioxidant, phase II enzyme inducer, cell growth inhibitor or apoptosis inducer (3,12–16). Mechanistic studies of EGCGs inhibitory effect on cell proliferation have demonstrated the regulatory influence of EGCG on the levels and activities of transcription factor AP-1 (17,18), NF-κB (19–21), cell cycle-related protein p21 (22), PI3 kinase (23) and mitogen-activated protein kinases (MAPKs) (24,25). However, the influence of EGCG on signaling molecules directly involved in apoptotic pathway has not been fully examined. In this study, we compared the anti-proliferation effect of EGCG on normal colon epithelial cells and colon carcinoma cells, investigated the morphological changes after EGCG treatments, and determined the influence of EGCG on caspases, cytochrome *c*, mitochondria, as well as MAPKs. Additionally, through inhibition experiments, we showed that c-Jun N-terminal kinase (JNK) and oxidative stress play important roles in the EGCG-induced apoptosis in HT-29 human colon cancer cells.

**Abbreviations:** DAPI, diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EGCG, (–)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated protein kinase; GSH, reduced glutathione; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; NAC, *N*-acetyl-L-cysteine.

## Materials and methods

### *Cell culture*

Human colorectal cancer cells, HT-29 and human normal colon epithelial cells, FHC, were purchased from American Type Culture Collection

(ATCC). HT-29 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 100 U/ml penicillin and 100 mg/ml streptomycin. FHC cells were maintained in Ham's F12 medium (45%) and Dulbecco's modified Eagle's medium (45%) supplemented with 10% fetal bovine serum, 25 mM HEPES, 10 ng/ml cholera toxin, 5 μg/ml insulin, 5 μg/ml transferrin and 100 ng/ml hydrocortisone. Both HT-29 and FHC cells were maintained in low serum overnight in MEM containing 0.5% serum before chemical treatments.

*Reagents*

EGCG, dimethyl sulfoxide (DMSO), reduced glutathione (GSH), *N*-acetyl-L-cysteine (NAC), hydrogen peroxide ($H_2O_2$), catalase and diamidino-2-phenylindole (DAPI) were purchased from Sigma (St Louis, MO). Mouse anti-cytochrome *c* monoclonal antibody was purchased from Pharmingen (San Diego, CA). Rabbit anti-phospho-extracellular signal-regulated protein kinase (ERK)1/2, -JNK and -p38 polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Fluorogenic peptide substrates of caspase-3 (Ac-DEVD-MCA), caspase-9 (Ac-LEHD-MCA) and caspase-8 (Ac-IETD-MCA), were purchased from Peptides International (Louisville, KY). SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) were purchased from Calbiochem (La Jolla, CA). 3,3′-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was purchased from Molecular Probes (Eugene, OR).

*Cell viability assay (MTS assay)*

Cells were cultured in 96-well plates at a density of $10^4$ cells/well. Twenty-four hours later, cells were treated with a series of concentrations of EGCG (dissolved in DMSO, the final concentrations of which in culture medium was $<0.1\%$) for 36 h. Cell viability was assayed with CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI). Briefly, 20 μl of combined solution of a tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt], and an electron coupling reagent phenazine methosulfate (PMS), was added to each well. After incubation for 1 h at 37°C in a humidified 5% $CO_2$ atmosphere, the absorbance at 490 nm was measured on an enzyme-linked immunosorbent assay plate reader.

*Nuclear staining assay*

After treatments, HT-29 cells were scraped off the plates and collected by centrifugation at 1000 *g* for 10 min. Apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining. Briefly, cells were washed once with ice-cold phosphate-buffered saline and fixed methanol: acetic acid (3:1) solution for 30 min. Fixed cells were placed on slides and stained 1 μg/ml DAPI for 15 min. Nuclear morphology of cells was examined by a fluorescence microscopy.

*DNA fragmentation assay*

After treatment with EGCG, HT-29 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 12 000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed by agarose gel electrophoresis.

*Caspase activity assay*

After treatments, HT-29 cells were washed twice with ice-cold PBS and lysed in a hypotonic buffer containing 50 mM Tris–HCl (pH 7.4), 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothrietol (DTT) and 150 μg/ml digitonin. Cell lysates were homogenized by passing through a 23-G needle three times. Homogenates were kept on ice for 30 min, and then centrifuged at 12 000 *g* for 20 min at 4°C. The supernatants were collected and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA). The enzymatic activities of caspase-3, -9 and -8, were analyzed by using 200 μM Ac-DEVD-MCA, Ac-LEHD-MCA and Ac-IETD-MCA as fluorogenic substrates, respectively, in assay buffer (100 mM HEPES, 10% sucrose, 10 mM DTT and 0.1% CHAPS). The fluorescence intensity was measured on CytoFluor II fluorescence reader by setting 360 nm as excitation wavelength and 460 nm as emission wavelength.

*Preparation of cytosolic extracts and western blotting analysis of cytochrome c release*

HT-29 cells were washed twice with ice-cold PBS and scraped off the plates. Cells were collected by centrifugation at 500 *g* for 10 min. The cell pellet was resuspended in 500 μl of extraction buffer containing 210 mM mannitol, 70 mM sucrose, 20 mM HEPES–KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete Cocktail; Roche Molecular Biochemicals, Indianapolis, IN). After incubation on ice for 30 min, cells were homogenized with a glass Dounce and a B pestle. Homogenates were centrifuged at 12 000 *g* for 5 min at 4°C. Supernatants were collected and further centrifuged at 100 000 *g* for 30 min at 4°C to yield cytosol. Twenty-five micrograms of total protein, as determined by the Bradford method (Bio-Rad), was resolved on 15% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane using semi-dry transfer system (Fisher, Suwanee, GA). Membrane was blocked with 5% non-fat dry milk in TBS (20 mM Tris–HCl, 8 g/l NaCl, pH 7.4) for 1 h at room temperature, followed by incubation with 1 μg/ml primary monoclonal anti-cytochrome *c* antibody in TBS containing 3% non-fat milk overnight at 4°C. Membrane was washed three times with TBS and blotted with secondary antibody conjugated with horseradish peroxidase (1:10 000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. The protein was visualized by using ECL™ (Enhanced Chemiluminescence, Amersham, Piscataway, NJ) after washing three times in TBS.

*Measurement of mitochondrial membrane potential*

After EGCG treatments, HT-29 cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C. Then, cells were washed with ice-cold PBS and scraped off the plates in PBS. Cells were collected by centrifugation at 500 *g* for 10 min and resuspended in 500 μl of PBS containing 2% fetal bovine serum and 40 nM $DiOC_6(3)$. Fluorescence intensities of $DiOC_6(3)$ were analyzed on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) with excitation and emission settings of 484 and 500 nm, respectively. Propidium iodide was added to the sample to gate out dead cells before data collection.

*Western blotting to examine phosphorylation of JNK, ERK and p38*

After treatments, HT-29 cells were washed with ice-cold PBS and lysed with 500 μl of lysis buffer (10 mM Tris–HCl, pH 7.4, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 mM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton-X 100). The lysates were homogenized by passing through a 23-G needle three times, and kept in ice for 30 min. The homogenates were centrifuged at 12 000 *g* for 15 min at 4°C. The protein concentration of whole cell lysates was determined by Bio-Rad protein assay kit. An equal amount of protein was then resolved on a 10% SDS–polyacrylamide gel and transferred to PVDF membrane using semi-dry transfer system. The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature, then incubated overnight at 4°C with anti-phospho-MAPK primary antibody (1:1000 dilution, Cell Signaling Technology), which specifically recognized phosphorylated ERK1/2 (Thr202/Tyr204), JNK (Thr183/Tyr185) and p38 (Thr180/Tyr182), respectively. After hybridization with primary antibody, the membrane was washed with TBS for three times, then incubated with horseradish peroxidase-conjugated secondary antibody (1:10 000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature and washed with TBS three times. Final detection was performed with ECL reagents.

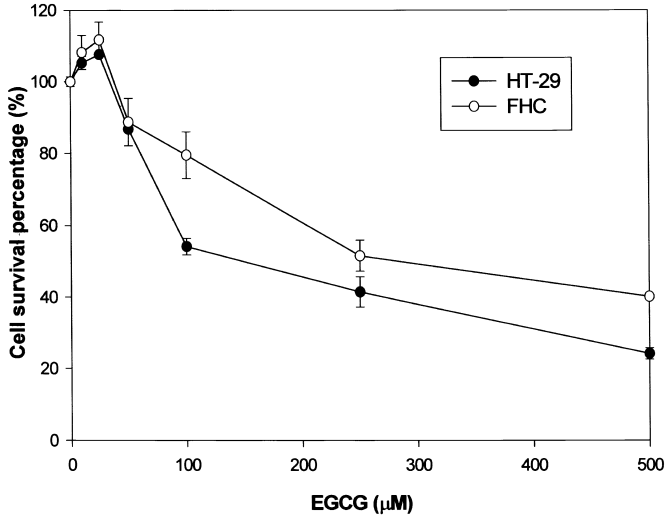


**Fig. 1.** Effect of EGCG on HT-29 and FHC cell viability. HT-29 and FHC cells were maintained in 96 well plates at a density of $10^4$ cells/well. Cells were treated with different doses of EGCG for 36 h. The MTS assay was performed with CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega). The percentage of viable cell was calculated as a ratio of $A_{490\ nm}$ of treated cells versus control cells (treated with 0.1% DMSO vehicle).

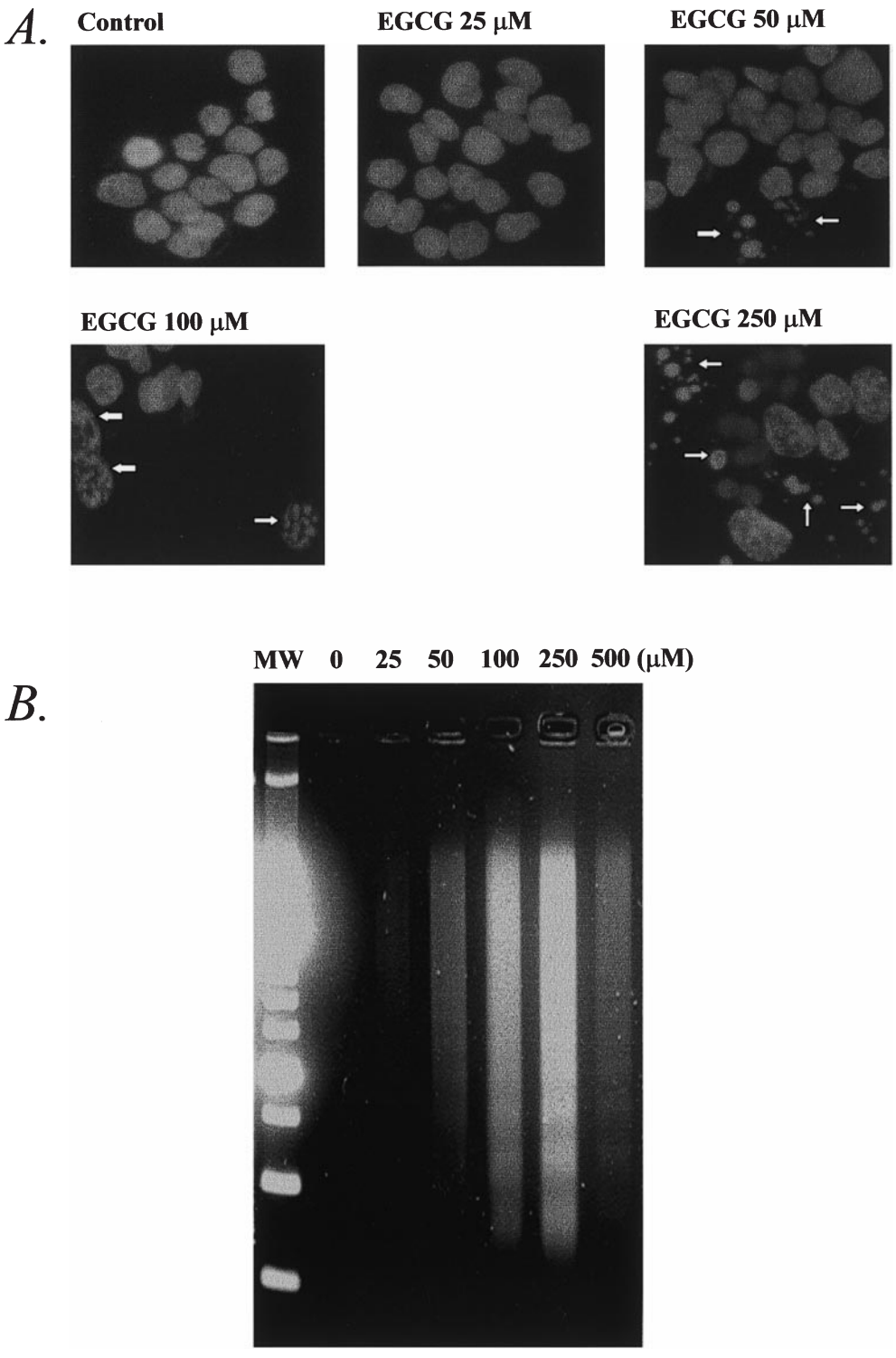


**Fig. 2.** Induction of apoptosis by EGCG in HT-29 cells. (**A**) Nuclear condensation. HT-29 cells were either treated with 0.1% DMSO as vehicle control or treated with different concentrations of EGCG for 48 h. Cells were harvested and washed with ice-cold PBS, followed by fixation in methanol–acetic acid (3:1) for 30 min. The fixed cells were incubated with 1 μg/ml DAPI, and nuclear staining was examined under a fluorescence microscopy. Arrows indicate the apoptotic cells with condensed and fragmented nuclei. (**B**) DNA laddering. Cells were treated with different concentrations of EGCG for 36 h. Cellular DNA was extracted and analyzed by agarose gel electrophoresis. Data shown are representative of three independent experiments.

*In vitro immunocomplex kinase assay for JNK activity*

Briefly, after treatments, HT-29 cells were washed twice with ice-cold PBS and harvested with lysis buffer. Kinase reactions were initiated by adding 30 μl of kinase assay buffer containing 10 μg of GST-c-Jun(1-79) fusion protein, 2 μCi of [$\gamma$-$^{32}$P]ATP and 20 μM ATP. After incubation for 30 min at 30°C, the reactions were terminated by adding 10 μl of 4× Laemmli buffer and by heating at 94°C for 5 min. The phosphorylation products were resolved in 10% SDS–polyacrylamide gel and visualized by autoradiography.

*Trypan blue staining*

After chemical treatments, floating and attached cells were harvested and combined for 10 min centrifugation at 1000 *g*. Cell pellets were resuspended

in PBS, and mixed with 0.4% Trypan Blue solution (Sigma) for 15 min. The number of cells was counted by using a hemocytometer. Unstained cells were read as viable cells; and stained blue cells as dead cells. The percentage of dead cells was calculated as the ratio between the number of stained cells and the total cell counts.

## Results

### *Antiproliferative effect of EGCG on colonic cells*

To assess the inhibitory effect of EGCG on cell growth, human HT-29 colorectal tumor cells and FHC normal colon epithelial cells, were exposed for 36 h to various concentrations of EGCG (from 10 to 500 μM), after which cell proliferation was measured by MTS assay. As shown in Figure 1, when treatment concentration was $>50$ μM, cell growth in both cell lines was arrested and cytotoxicity of EGCG became apparent. Noticeably, the inhibitory effect of EGCG against cell growth of HT-29 cells was much stronger than that of FHC cells. The effective concentration to inhibit 50% HT-29 cell growth ($IC_{50}$) was ~100 μM of EGCG. However, $IC_{50}$ for FHC was $>250$ μM. The reason for this difference between tumor and normal cell lines is not clear at this time, but the selectivity of EGCG against HT-29 cells led to the further study on the mechanism of EGCG-induced cell death in tumor cells. Moreover, interestingly, cell growth was slightly promoted in the low concentration range (10 and 25 μM).

### *EGCG-induced apoptotic cell death in HT-29*

To examine the effect of EGCG treatment on cell morphology and identify cell death pathway, HT-29 cells were stained with DAPI nuclear dye. Nuclear fragmentation and condensation implicating apoptotic cell death was increased dose-dependently (Figure 2A), which is consistent with an intensified DNA laddering pattern from cells undergoing the same treatments (Figure 2B). Therefore, EGCG-induced apoptosis on HT-29 cells in the concentration ranges from 50 to 250 μM. Treatments with higher concentrations mostly led to non-specific necrotic cell death.

### *EGCG-induced caspase activities*

As caspases play important roles in dismantling cellular infrastructure during apoptotic events, the influence of EGCG on the caspase activities was examined by using specific fluorogenic substrate for each caspase. First, the activity of caspase-3, which is a converging point of different caspase-dependent apoptosis pathways, was measured after the treatments of different doses of EGCG. As shown in Figure 3A, the induction of caspase-3 activity in HT-29 cells was consistent with the results shown in DAPI staining and DNA fragmentation experiments. 100 μM of EGCG led to ~8-fold induction of caspase-3 activity after 24 h treatment. As the major upstream signal transducers of caspase-3 are death receptor-related caspase-8 and mitochondria damage-related caspase-9, the time-course response from these caspases against 100 μM EGCG treatment were determined to elucidate the caspase-3 activation pathway. As shown in Figure 3B, the activation of caspase-3 was initiated around 12 h after treatments, which was relatively delayed compared with other chemical stress-induced caspase-3 activation. During the 24 h period, the activity of caspase-8 in HT-29 cells was unchanged, but the activity of caspase-9 was consistently increased. This implied the possible involvement of mitochondrial damage in EGCG-induced apoptosis.

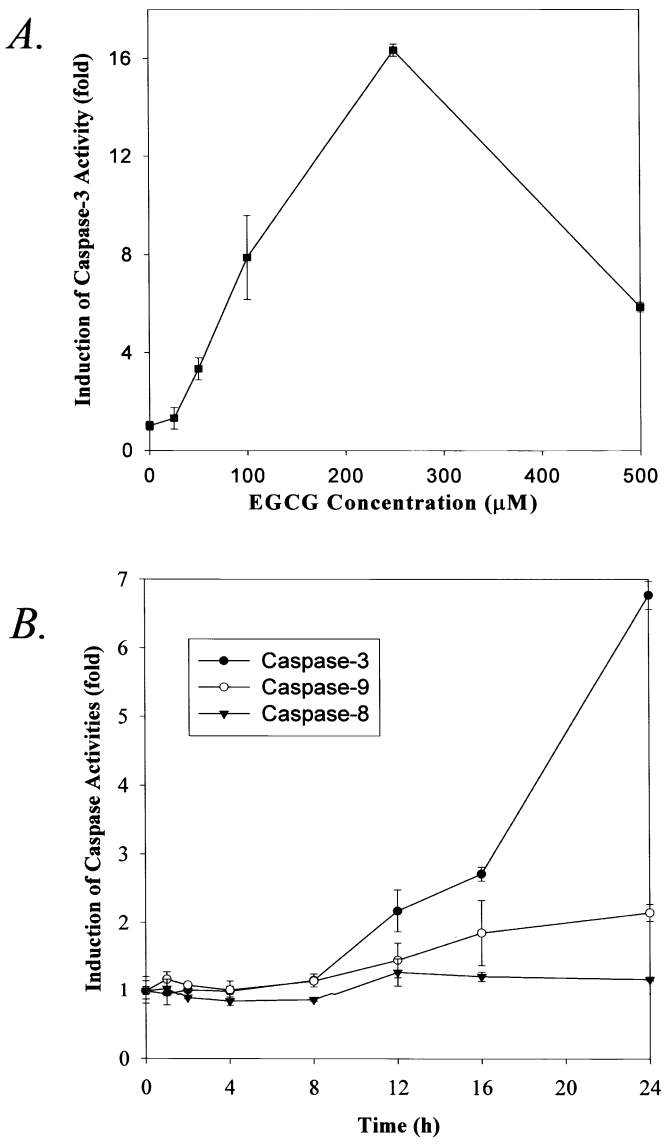


**Fig. 3.** Induction of caspase activity. (**A**) Dose-dependent activation of caspase-3. Following treatments with different concentrations of EGCG for 24 h, cells were harvested, and the caspase-3 activity was determined by incubation of 10 μg of total protein with fluorogenic substrate, Ac-DEVD-MCA, for 2 h at 37°C. The release of fluoroscence was monitored spectrofluorometrically (excitation = 360 nm; emission = 460 nm). (**B**) Kinetics of caspase-3, -8 and -9 activation. Cells were treated with 100 μM of EGCG for different time periods. Caspase-3, -9 and -8, were assayed with Ac-DEVD-MCA, Ac-LEHD-MCA and Ac-IETD-MCA fluorogenic substrates, respectively, for 2 h at 37°C. Enzyme activity is expressed as the fold of induction relative to untreated controls. Means and standard deviations are calculated based on the data from four independent experiments.

### *EGCG-induced cytochrome c release and mitochondrial membrane potential change*

To examine the possible role of mitochondria in the apoptotic death of HT-29 cells induced by EGCG, the existence of cytochrome *c* in cytosolic fraction of cell lysis was measured after EGCG treatments. As shown in Figure 4A, the release of cytochrome *c* from mitochondria was time- and dose-dependent. Twelve hours of 100 μM EGCG treatment led to a dramatically increased amount of cytochrome *c* leaking from mitochondrial membrane into cytosol in HT-29 cells. The influence of EGCG on mitochondrial membrane integrity was also measured by $DiOC_6(3)$ dye staining (Figure 4B),

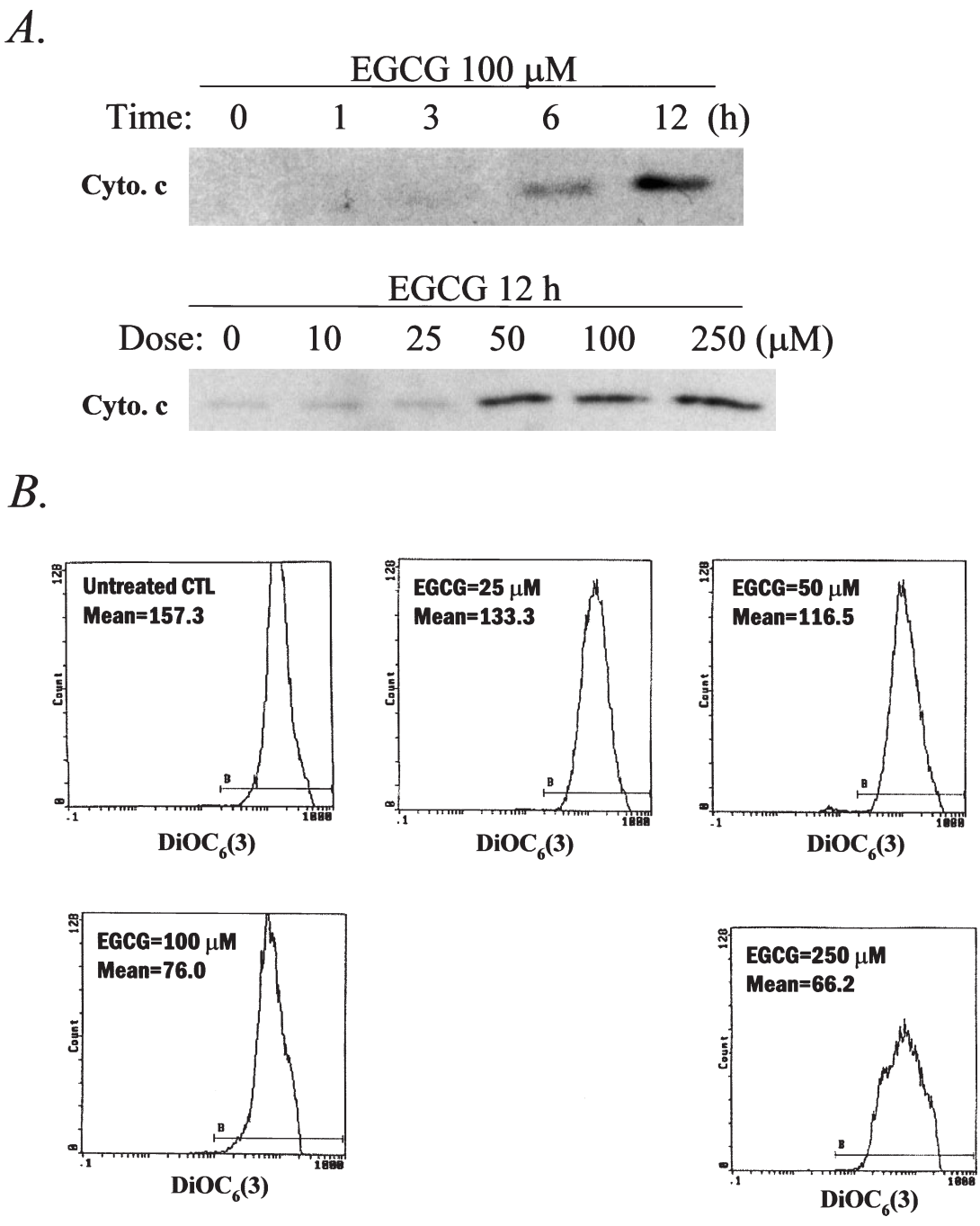


**Fig. 4.** Effect of EGCG on mitochondria. (**A**) Time-dependent and dose-dependent release of cytochrome *c*. After EGCG treatments, cytosolic extracts of HT-29 cells were prepared as described in the Materials and Methods. Twenty micrograms of cytosolic protein was analyzed by western blotting using anti-cytochrome *c* antibody. (**B**) Reduction of mitochondrial transmembrane potential ($\Delta\psi_m$). After HT-29 cells were treated with different concentrations of EGCG for 12 h, $DiOC_6(3)$ was added to the culture medium during the last 15 min of treatment at a final concentration of 40 nM. The fluorescence intensity of $DiOC_6(3)$ was analyzed by flow cytometry. Data shown are representative of three independent experiments.

which showed the decreased membrane potential ($\Delta\psi_m$) caused by the mitochondrial membrane permeability transition after EGCG treatments.

*EGCG-induced MAPK activation*

The activation of caspases and the release of cytochrome *c* from mitochondria in HT-29 cells were only observed after 6–12 h of 100 μM EGCG treatments. In order to identify some early signaling events induced by EGCG in HT-29 cells, the responses of MAPKs, which are a group of important signaling molecules sensitive and responsive to extracellular stimuli, were measured. As shown in Figure 5A, all of the three major MAPKs, JNK, ERK and p38, were phosphorylated and activated time-dependently in HT-29 cells after EGCG treatments. The activation of MAPKs was observed starting at ~5–15 min, and peaking at ~1 h. Additionally, the result obtained from the phosphorylation of JNK protein was corroborated by using *in vitro* kinase assay with GST-c-Jun as substrate. Moreover, the activation of these MAPKs was dose-dependent (Figure 5B). Comparing the EGCG concentrations for the activation of JNK and p38, the activation of ERK was initiated with relatively lower concentration (~10 μM).

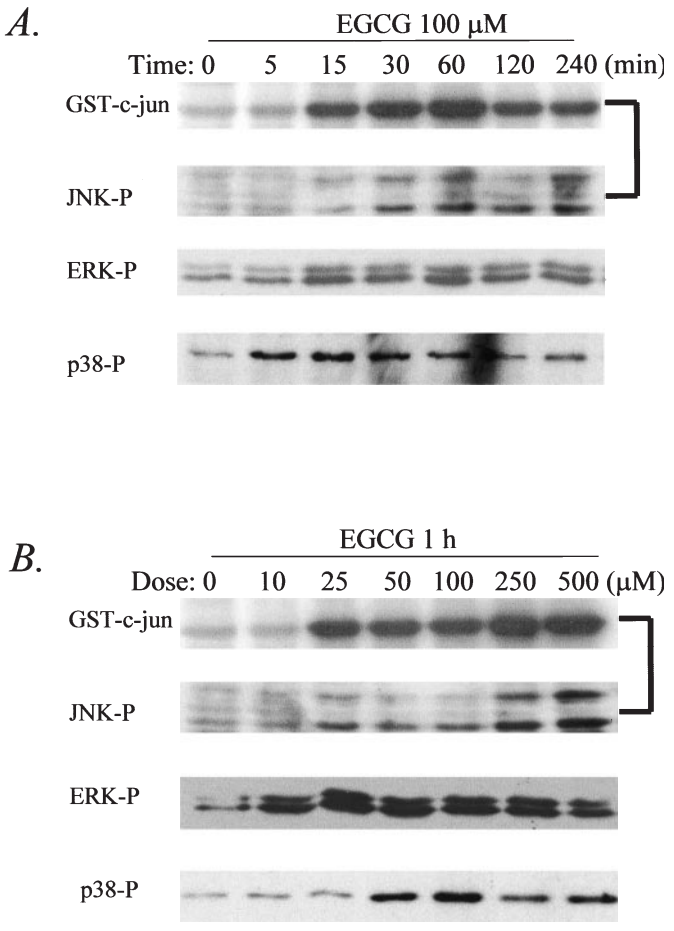


**Fig. 5.** MAP kinases activation by EGCG. (**A**) Time course of MAPK activities in HT-29 cells treated with 100 μM EGCG. (**B**) Dose–response of MAPK activities in HT-29 cells after 1 h EGCG treatment. After overnight serum starvation, cells were challenged and harvested following as described in the Materials and Methods. JNK kinase activity was measured by *in vitro* immunocomplex kinase assay using GST-c-Jun fusion protein as substrate. Phosphorylation of MAP kinases, JNK, ERK and p38, were detected by immunoblotting with respective phospho-MAPK antibodies. Data shown are representative of three independent experiments.

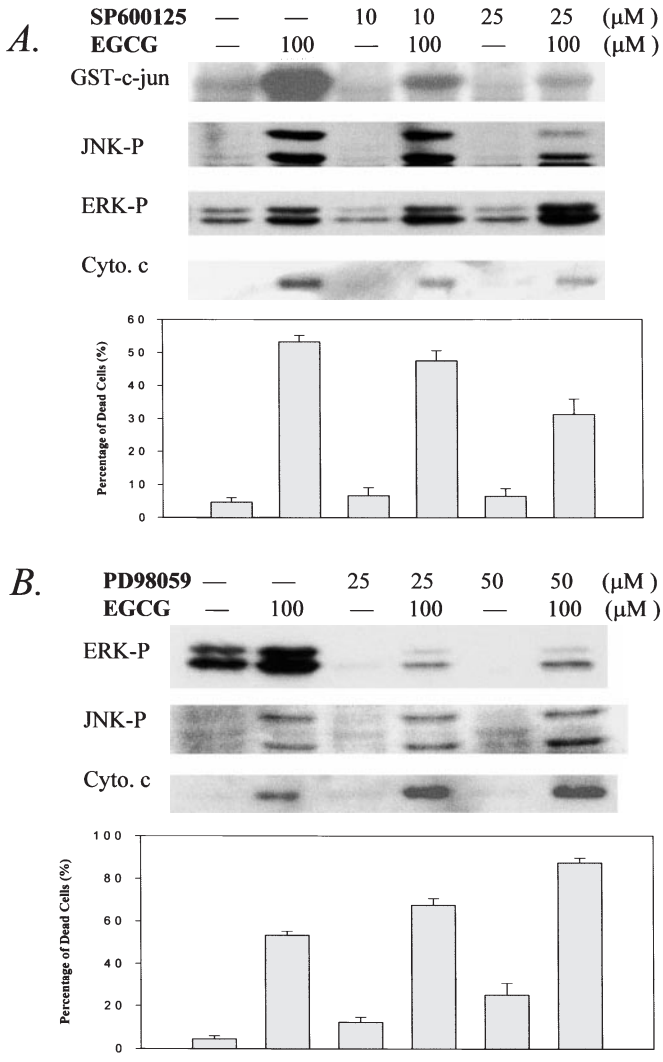


**Fig. 6.** Effect of inhibiting JNK and ERK pathways on EGCG-induced cell death. (**A**) Influence of SP600125 pre-treatment. (**B**) Influence of PD98059 pre-treatment. After overnight serum starvation, different concentrations of SP600125 or PD98059 compounds were added into the media 1 h before EGCG treatment. For measuring the activation of JNK and ERK, cell samples were harvested after 1 h of EGCG treatment. JNK kinase activity was measured by *in vitro* immunocomplex kinase assay using GST-c-Jun fusion protein as substrate. Phosphorylation of JNK and ERK was examined by immunoblotting with phospho-JNK and phospho-ERK antibodies. For measuring the amount of cytochrome *c* released from mitochondria, cytosolic fraction of HT-29 cells was prepared following as described in the Materials and Methods after 12 h of EGCG treatment, and examined by western blot with monoclonal cytochrome *c* antibody. Samples for cell death assay were harvested after 36 h of EGCG treatments. Suspended cells were stained by Trypan Blue. The percentage of dead cells was calculated as the ratio between the number of stained cells versus the total cell counts. Western blot data shown are representative of three independent experiments. Trypan Blue staining data are means and SD of four independent replicates.

*Role of JNK and ERK in EGCG-induced cell death*

The immediate and potent activation of JNK pathway led to the interest on the possible function of JNK in initiating apoptotic signals in HT-29 cells. A newly discovered JNK inhibitor, SP600125, was adopted to block this pathway (26). As shown in Figure 6A, pre-treatment of SP600125 can dramatically decrease JNK kinase activities and the phosphorylation of JNK1 and JNK2 induced by 1 h treatment of 100 μM EGCG. Interestingly, the decrease of JNK activation was accompanied with the simultaneous increase in ERK activation, especially in the pre-treatment of the cells with 25 μM SP600125. To elucidate the influence of inhibiting early JNK activation on late-stage cell death events, the cytosolic level of cytochrome *c* protein after 12 h EGCG treatment was also examined. SP600125 pre-treatment led to the decreased release of cytochrome *c* from mitochondrial membrane after EGCG treatment, which was consistent with the lower percentage of cell death in HT-29 samples co-treated with EGCG and SP600125, as compared with the cells treated only with EGCG (Figure 6A). Next, the role of ERK in EGCG-induced apoptosis was examined by using PD98059, a potent ERK inhibitor. As shown in Figure 6B, pre-treatment of PD98059 not only eliminated EGCG-induced ERK activation, but also abolished the basal level of activated ERK in HT-29 cells. At the same time, the phosphorylation of JNK was slightly increased in the co-treatment of 50 μM PD98059 and 100 μM EGCG. Cytochrome *c* release assay showed that PD98059 did not independently cause damage to mitochondria, but it robustly raised the release of cytochrome *c* induced by EGCG treatment (Figure 6B). Cell death assay indicated that, comparing the

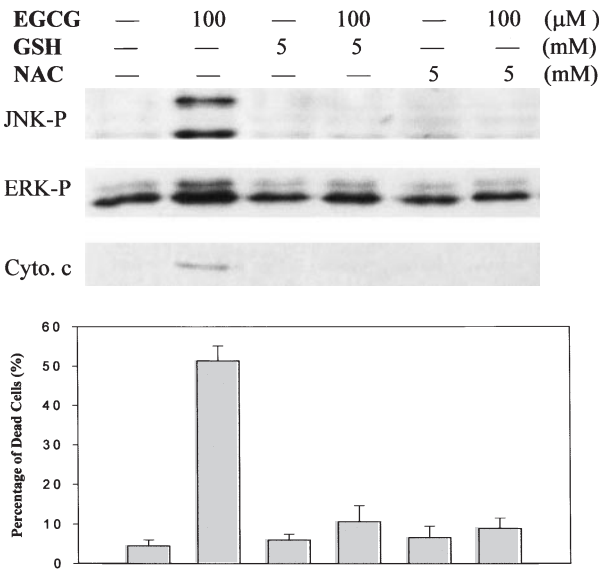


**Fig. 7.** Effect of thiol antioxidants on EGCG-induced intracellular signals and cell death. Reduced GSH (5 mM) or NAC was added into the media 1 h before 100 μM EGCG treatment. Measurement of the phosphorylation of JNK and ERK, as well as cytochrome *c* release and cell death assays, was performed as described in Figure 6.

respective controls, cell death was increased after PD98059 treatment alone as well as co-treatment with EGCG (Figure 6B). In contrast, SB203580, a p38 pathway inhibitor, had no major effect on cytochrome *c* release or cell death induced by EGCG (data not shown).

*Role of oxidative stress in EGCG-induced cell death*

EGCG not only can function as an antioxidant, but it possesses the chemical property of pro-oxidant. To test whether the generation of the apoptotic signals was related to oxidative stress after EGCG treatments, HT-29 cells were pre-treated with major intracellular antioxidants, GSH and NAC. As shown in Figure 7, the activation of JNK or ERK was eliminated by the GSH and NAC pre-treatments, which was followed by the abolishment of cytochrome *c* release. Also, cell death induced by EGCG in HT-29 cells was dramatically inhibited (Figure 7). These results indicated that oxidative stress is involved in EGCG-induced cell death. It has been shown that EGCG may induce the production of $H_2O_2$ in the culture media, which may be linked to the cytotoxic effect of chemical treatments (27,28). To identify whether the source of oxidative stress caused by EGCG in HT-29 cells is via the generation of $H_2O_2$, catalase, which catalyze $H_2O_2$ to water and oxygen, was added to the media before EGCG treatment. Surprisingly, the pre-treatment of catalase did not block the activation of JNK and ERK induced by EGCG, but had clear inhibitory effect on $H_2O_2$ treatment (Figure 8A). Examining the cell morphology under the microscope, the apparent protective effect from catalase can only be observed against $H_2O_2$ treatment, but not against EGCG treatment (Figure 8B).

## Discussion

Cancer chemoprevention is conducted through the administration of chemical or dietary components to interfere with the initiation, promotion and progression of tumors. According to the report of the Chemoprevention Working Group to the American Association of Cancer Research in 1999, one of the major strategies adopted in chemoprevention is to suppress the carcinogenic process after initiation (29). In this study, EGCG elicited the strong pro-apoptotic effect on carcinoma cells, and affected the proliferation of normal and tumor colon cells distinctively. The selectivity of cytotoxicity against cancerous cells is consistent with a previous report that showed EGCG had a pronounced growth inhibitory effect on SV40 virally transformed WI38 human fibroblasts but not on their normal counterparts (30). This observation infers that EGCG has the potential to function as a suppressing agent against the promotion and progression of adenoma and carcinoma cells. In comparison with other carcinoma cell lines, HT-29 is not the most sensitive cell line to EGCG treatments with respect to the growth inhibition and the induction of cell death (4). The reason for this observation may partially be attributed to the fact that EGCG is extensively metabolized to methylated and glucuronidated conjugates in HT-29 cells, and then actively pumped out of cells by multi-drug resistance-related proteins (MRPs) as shown in recent study about the uptake, biotransformation, and efflux of EGCG in HT-29 cells (31). Furthermore, 100 μM concentration of EGCG adopted in this study is within the range of EGCG concentration in the normal green tea beverage, which may reach up to 300 μM as calculated from the fact that a single cup of green tea drink may contain 150–200 mg EGCG (32,33).

Caspase-3, -8 and -9, which are members of the protease family, function as initiators and effectors in the apoptosis process. After proteolytic activation from procasapases, activated caspases can recognize the tetrapeptide motif in target proteins and specifically cleave the peptide chain after aspartic acid, which then leads to deregulation of protein activities and dismantling of the cytoskeletal and cellular structure (34). The activation of caspase-3 has been shown in human chondrosarcoma and cervical cancer cells treated with EGCG (25,35). It has been reported that EGCG can directly bind to Fas death receptor to initiate the caspase-8 activation and apoptosis (36). However, caspase-8 activity in HT-29 cells remained unchanged after EGCG treatments. Contrasting to caspase-8, the activity of caspase-9 in HT-29 cells had the similar induction pattern as caspase-3 activity after EGCG treatments. Therefore, it implied the involvement of mitochondrial damage in EGCG-induced apoptosis. This is the first report to show that EGCG can induce cytochrome *c* release and mitochondria membrane permeability transition. These observations are consistent with the previous report that EGCG can lead to the decrease of anti-apoptotic Bcl-2 and Bcl-X(L) proteins and the increase of pro-apoptotic Bax proteins (37).

The activation and initiation of direct apoptotic signaling is quite delayed in EGCG-treated HT-29 cells, as compared with the apoptotic events induced by Fas ligands or chemicals that specifically target mitochondria. In this study, we observed the early response from HT-29 cells through measuring the changes on MAPKs, among which JNK1/JNK2 and p38, are connected to stress-activated apoptosis, and ERK1/ERK2 are linked to cell proliferation and survival (38). After EGCG treatments, the phosphorylation of JNKs, ERKs and p38 are pronounced with minor differences in the extent and the kinetics of activation. This phenomenon is consistent with our previous observation on HeLa and HepG2 cells treated with green tea polyphenols (24,25), as well as the results in some recent reports about the effect of EGCG in breast cancer cells, macrophages and keratinocytes (39–41), but it is

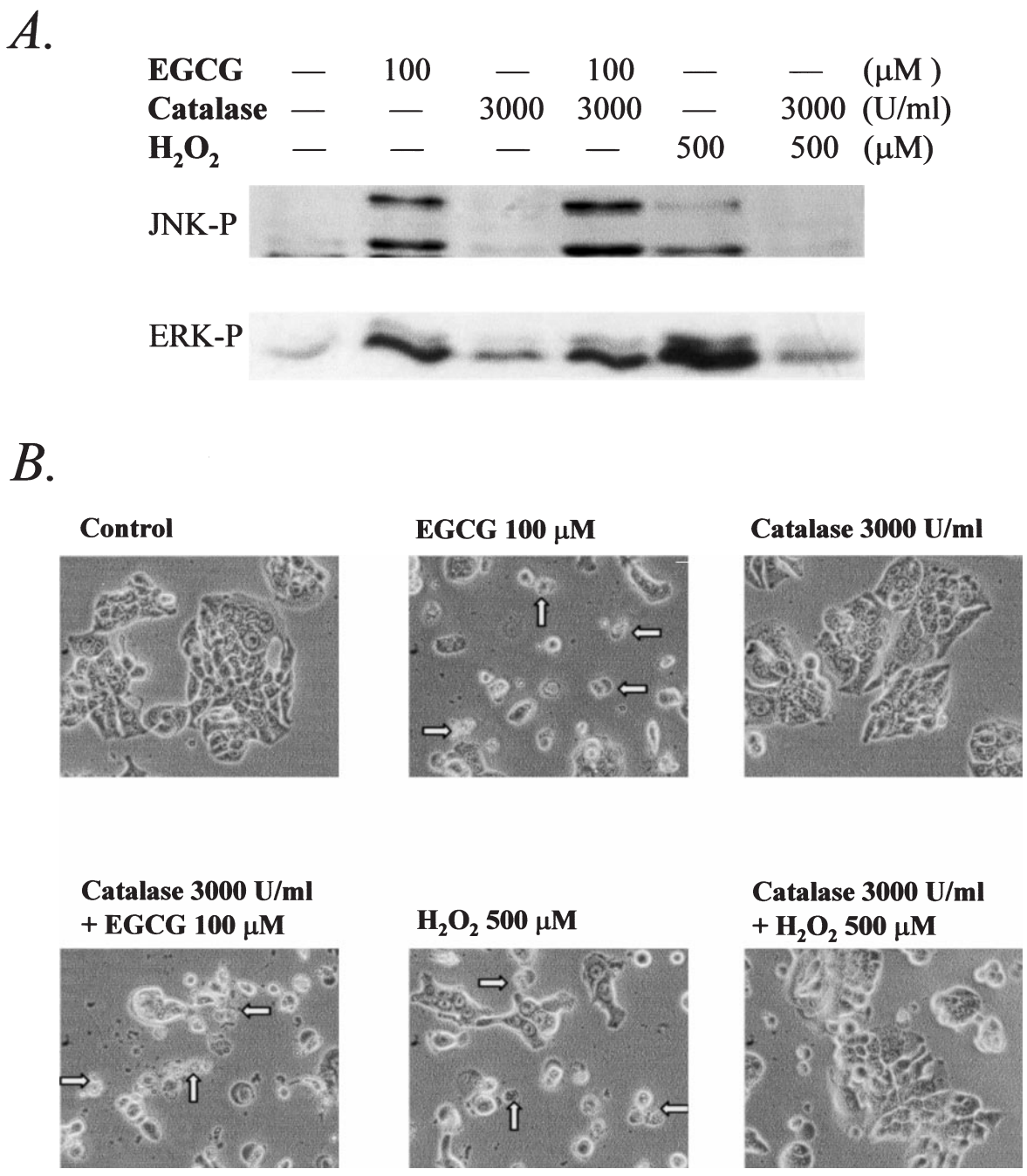


**Fig. 8.** Role of $H_2O_2$ in EGCG-induced oxidative stress. (**A**) Effect of catalase pre-treatment on EGCG- or $H_2O_2$-induced JNK and ERK activation. (**B**) Cell morphology after co-treatments of catalase with EGCG or $H_2O_2$. 3000 U/ml of catalase was added into the media 0.5 h before 100 μM EGCG or 500 μM $H_2O_2$ treatments. Phosphorylation of JNK and ERK was measured by western blot after 1 h of EGCG or $H_2O_2$ treatment. Cell morphology was observed under inverted microscopy (original magnification ×200) after 36 h of EGCG or $H_2O_2$ treatment. Arrows indicate the apoptotic cell bodies.

opposite to the conclusions with other publications, which showed the inhibitory effect of EGCG on the activation of MAPKs (42,43). The reason for this discrepancy is unclear, but may be attributed to the experimental designs as well as cell lines.

Recent studies by using target gene disruption have established that JNK signaling pathway is required for stress-induced release of mitochondrial cytochrome *c* and apoptosis (44). The intermediate signaling moiety between JNK activation and cytochrome *c* release may be linked to Bax protein, as activated JNK fails to induce apoptosis in cells deficient of members of the proapoptotic Bax subfamily of Bcl2-related proteins; as well as in JNK-deficient cells, stress signals fail to activate Bax, induce cytochrome *c* release and cause cell death (45). In our present study, pre-treatments of SP600125, a specific blocker for JNK pathway through reversible ATP-competitive inhibition, led to substantial decrease of cytochrome *c* release and cell death in response to EGCG treatment. This suggests that JNK pathway may play a pivotal role in EGCG-induced cell death. With regard to ERK pathway, noticeably, relatively lower EGCG concentrations were needed for ERK activation, as compared with the

concentrations for JNK and p38. This was consistent with the slight increment of cell proliferation in the treatments with 10 and 25 mM EGCG. Also, the application of PD98095 dramatically intensified the damage to HT-29 cells caused by EGCG. Both events highlighted the positive function of ERKs in cell proliferation and survival. The simultaneous sustained activation of all three major MAPKs, which have different and in some case opposite biological function, indicates that the balance and integration of MAPK pathways may modulate the commitment of cells to apoptosis or survival following external stimuli (46,47). In our study, co-treatments of JNK inhibitor and EGCG led to an increased ERK activation. Furthermore, ERK inhibitor and EGCG led to the increased JNK activation. This indicates that the cross-talk exists between these two pathways. It has been shown that there are extensive interactions among the upstream kinases of all three MAPKs (38). Future investigation about the influence of EGCG on these upstream elements will shed light on how those MAPKs are activated and how EGCG-initiated chemical signals are converted to apoptotic response.

Among all of the green tea phenolic compounds, EGCG is the most potent in terms of the bioactivity, and EGCG also contains the most hydroxyl functional groups in its chemical structure. Previous studies on the antioxidative property of EGCG have demonstrated both the trapping effect of reactive oxygen species (ROS) as well as the inhibitory effect of lipid peroxidation (48). However, after neutralizing the peroxyl and/or other radicals, EGCG itself could be converted to phenoxyl radical (49). In addition, under normal physiological pH condition, EGCG may undergo auto-oxidation to form dimers, accompanying with the generation of ROS intermediates (31,50). In the present study, the chemical property of EGCG as a potential pro-oxidant was highlighted by the blocking effects of GSH and NAC against EGCG-induced MAPK activation, cytochrome *c* release and cell death. It has been shown that EGCG may induce the production of hydrogen peroxide ($H_2O_2$) in the culture media (27,28). However, pre-treatment of catalase in our study failed to diminish EGCG-induced MAPK activation and apoptosis in HT-29 cells. This observation appears to be consistent with a previous report that the inhibition of β-catenin expression by EGCG may not be related to the generation of $H_2O_2$ (51). Further studies are required to examine whether other oxidizing radicals are involved in the initiation of stress signals generated by EGCG.

In conclusion, in this study, we examined the molecular mechanism of EGCG-induced apoptosis on HT-29 human colon adenocarcinoma cell line. EGCG treatments induce oxidative stress, thereafter activate stress signals, such as JNK pathway, which lead to the changes on mitochondrial membrane permeability and the release of cytochrome *c*. Subsequently, capsase-9 and -3 are activated, followed by DNA fragmentation and nuclear condensation. Collectively, these results imply that the pro-apoptotic effect of EGCG may in part contribute to its overall chemopreventive function against colonic carcinogenesis.

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## References

1. Potter,J.D., Slattery,M.L., Bostick,R.M. and Gapstur,S.M. (1993) Colon cancer: a review of the epidemiology. *Epidemiol. Rev.*, **15**, 499–545.
2. Bruce,W.R., Giacca,A. and Medline,A. (2000) Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 1271–1279.
3. Mukhtar,H. and Ahmad,N. (1999) Mechanism of cancer chemopreventive activity of green tea. *Proc. Soc. Exp. Biol. Med.*, **220**, 234–238.
4. Yang,C.S., Chung,J.Y., Yang,G., Chhabra,S.K. and Lee,M.J. (2000) Tea and tea polyphenols in cancer prevention. *J. Nutr.*, **130**, 472S–478S.
5. Steele,V.E., Bagheri,D., Balentine,D.A. *et al*. (1999) Preclinical efficacy studies of green and black tea extracts. *Proc. Soc. Exp. Biol. Med.*, **220**, 210–212.
6. Bushman,J.L. (1998) Green tea and cancer in humans: a review of the literature. *Nutr. Cancer*, **31**, 151–159.
7. Chen,L., Lee,M.J., Li,H. and Yang,C.S. (1997) Absorption, distribution, elimination of tea polyphenols in rats. *Drug Metab. Dispos.*, **25**, 1045–1050.
8. Kim,S., Lee,M.J., Hong,J., Li,C., Smith,T.J., Yang,G.Y., Seril,D.N. and Yang,C.S. (2000) Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutr. Cancer*, **37**, 41–48.
9. Suganuma,M., Okabe,S., Oniyama,M., Tada,Y., Ito,H. and Fujiki,H. (1998) Wide distribution of [3H](–)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis*, **19**, 1771–1776.
10. Yamane,T., Nakatani,H., Kikuoka,N., Matsumoto,H., Iwata,Y., Kitao,Y., Oya,K. and Takahashi,T. (1996) Inhibitory effects and toxicity of green tea polyphenols for gastrointestinal carcinogenesis. *Cancer*, **77**, 1662–1667.
11. Jia,X. and Han,C. (2001) Effects of green tea on colonic aberrant crypt foci and proliferative indexes in rats. *Nutr. Cancer*, **39**, 239–243.
12. Weisburger,J.H. (1999) Tea and health: the underlying mechanisms. *Proc. Soc. Exp. Biol. Med.*, **220**, 271–275.
13. Rice-Evans,C. (1999) Implications of the mechanisms of action of tea polyphenols as antioxidants *in vitro* for chemoprevention in humans. *Proc. Soc. Exp. Biol. Med.*, **220**, 262–266.
14. Chou,F.P., Chu,Y.C., Hsu,J.D., Chiang,H.C. and Wang,C.J. (2000) Specific induction of glutathione *S*-transferase GSTM2 subunit expression by epigallocatechin gallate in rat liver. *Biochem. Pharmacol.*, **60**, 643–650.
15. Yang,G.Y., Liao,J., Kim,K., Yurkow,E.J. and Yang,C.S. (1998) Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis*, **19**, 611–616.
16. Valcic,S., Timmermann,B.N., Alberts,D.S., Wachter,G.A., Krutzsch,M., Wymer,J. and Guillen,J.M. (1996) Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. *Anticancer Drugs*, **7**, 461–468.
17. Dong,Z., Ma,W., Huang,C. and Yang,C.S. (1997) Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (–)-epigallocatechin gallate and theaflavins. *Cancer Res.*, **57**, 4414–4419.
18. Barthelman,M., Bair,W.B. 3rd, Stickland,K.K., Chen,W., Timmermann,B.N., Valcic,S., Dong,Z. and Bowden,G.T. (1998) (–)-Epigallocatechin-3-gallate inhibition of ultraviolet B-induced AP-1 activity. *Carcinogenesis*, **19**, 2201–2204.
19. Nomura,M., Ma,W., Chen,N., Bode,A.M. and Dong,Z. (2000) Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced NF-kappaB activation by tea polyphenols, (–)-epigallocatechin gallate and theaflavins. *Carcinogenesis*, **21**, 1885–1890.
20. Yang,F., Oz,H.S., Barve,S., de Villiers,W.J., McClain,C.J. and Varilek,G.W. (2001) The green tea polyphenol (–)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. *Mol. Pharmacol.*, **60**, 528–533.
21. Ahmad,N., Gupta,S. and Mukhtar,H. (2000) Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch. Biochem. Biophys.*, **376**, 338–346.
22. Liberto,M. and Cobrinik,D. (2000) Growth factor-dependent induction of p21 (CIP1) by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett.*, **154**, 151–161.

23. Nomura,M., Kaji,A., He,Z., Ma,W.Y., Miyamoto,K., Yang,C.S. and Dong,Z. (2001) Inhibitory mechanisms of tea polyphenols on the ultraviolet B-activated phosphatidylinositol 3-kinase-dependent pathway. *J. Biol. Chem.*, **276**, 46624–46631.
24. Yu,R., Jiao,J.J., Duh,J.L., Gudehithlu,K., Tan,T.H. and Kong,A.N. (1997) Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant-responsive element-mediated phase II enzyme gene expression. *Carcinogenesis*, **18**, 451–456.
25. Chen,C., Yu,R., Owuor,E.D. and Kong,A.N. (2000) Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch. Pharm. Res.*, **23**, 605–612.
26. Bennett,B.L., Sasaki,D.T., Murray,B.W. *et al.* (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl Acad. Sci. USA*, **98**, 13681–13686.
27. Sakagami,H., Arakawa,H., Maeda,M., Satoh,K., Kadofuku,T., Fukuchi,K. and Gomi,K. (2001) Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants. *Anticancer Res.*, **21**, 2633–2641.
28. Yang,G.Y., Liao,J., Li,C., Chung,J., Yurkow,E.J., Ho,C.T. and Yang,C.S. (2000) Effect of black and green tea polyphenols on c-jun phosphorylation and H(2)O(2) production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. *Carcinogenesis*, **21**, 2035–2039.
29. AACR Chemoprevention Working Group (1999) Prevention of cancer in the next millennium: Report of the Chemoprevention Working Group to the American Association for Cancer Research. *Cancer Res.*, **59**, 4743–4758.
30. Chen,Z.P., Schell,J.B., Ho,C.T. and Chen,K.Y. (1998) Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.*, **129**, 173–179.
31. Hong,J., Lu,H., Meng,X., Ryu,J.H., Hara,Y. and Yang,C.S. (2002) Stability, cellular uptake, biotransformation and efflux of tea polyphenol (–)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Res.*, **62**, 7241–7246.
32. Lin,J.K., Liang,Y.C. and Lin-Shiau,S.Y. (1999) Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.*, **58**, 911–915.
33. Yang,C.S. and Wang,Z.Y. (1993) Tea and cancer. *J. Natl Cancer Inst.*, **85**, 1038–1049.
34. Thornberry,N.A. and Lazebnik,Y. (1998) Caspases: enemies within. *Science*, **281**, 1312–1316.
35. Islam,S., Islam,N., Kermode,T., Johnstone,B., Mukhtar,H., Moskowitz,R.W., Goldberg,V.M., Malemud,C.J. and Haqqi,T.M. (2000) Involvement of caspase-3 in epigallocatechin-3-gallate-mediated apoptosis of human chondrosarcoma cells. *Biochem. Biophys. Res. Commun.*, **270**, 793–797.
36. Hayakawa,S., Saeki,K., Sazuka,M., Suzuki,Y., Shoji,Y., Ohta,T., Kaji,K., Yuo,A. and Isemura,M. (2001) Apoptosis induction by epigallocatechin gallate involves its binding to Fas. *Biochem. Biophys. Res. Commun.*, **285**, 1102–1106.
37. Masuda,M., Suzui,M. and Weinstein,I.B. (2001) Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin. Cancer Res.*, **7**, 4220–4229.
38. Kyriakis,J.M. and Avruch,J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.*, **81**, 807–869.
39. Deguchi,H., Fujii,T., Nakagawa,S., Koga,T. and Shirouzu,K. (2002) Analysis of cell growth inhibitory effects of catechin through MAPK in human breast cancer cell line T47D. *Int. J. Oncol.*, **21**, 1301–1305.
40. Park,J.W., Choi,Y.J., Suh,S.I. and Kwon,T.K. (2001) Involvement of ERK and protein tyrosine phosphatase signaling pathways in EGCG-induced cyclooxygenase-2 expression in Raw 264.7 cells. *Biochem. Biophys. Res. Commun.*, **286**, 721–725.
41. Balasubramanian,S., Efimova,T. and Eckert,R.L. (2002) Green tea polyphenol stimulates a Ras, MEKK1, MEK3 and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J. Biol. Chem.*, **277**, 1828–1836.
42. Katiyar,S.K., Afaq,F., Azizuddin,K. and Mukhtar,H. (2001) Inhibition of UVB-induced oxidative stress-mediated phosphorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (–)-epigallocatechin-3-gallate. *Toxicol. Appl. Pharmacol.*, **176**, 110–117.
43. Chung,J.Y., Park,J.O., Phyu,H., Dong,Z. and Yang,C.S. (2001) Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (–)-epigallocatechin-3-gallate and theaflavin-3,3′-digallate. *FASEB J.*, **15**, 2022–2024.
44. Tournier,C., Hess,P., Yang,D.D., Xu,J., Turner,T.K., Nimnual,A., Bar-Sagi,D., Jones,S.N., Flavell,R.A. and Davis,R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*, **288**, 870–874.
45. Lei,K., Nimnual,A., Zong,W.X., Kennedy,N.J., Flavell,R.A., Thompson,C.B., Bar-Sagi,D. and Davis,R.J. (2002) The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH (2)-terminal kinase. *Mol. Cell. Biol.*, **22**, 4929–4942.
46. Boldt,S., Weidle,U.H. and Kolch,W. (2002) The role of MAPK pathways in the action of chemotherapeutic drugs. *Carcinogenesis*, **23**, 1831–1838.
47. Park,H.J., Kim,B.C., Kim,S.J. and Choi,K.S. (2002) Role of MAP kinases and their cross-talk in TGF-beta1-induced apoptosis in FaO rat hepatoma cell line. *Hepatology*, **35**, 1360–1371.
48. Wiseman,S.A., Balentine,D.A. and Frei,B. (1997) Antioxidants in tea. *Crit. Rev. Food Sci. Nutr.*, **37**, 705–718.
49. Valcic,S., Muders,A., Jacobsen,N.E., Liebler,D.C. and Timmermann,B.N. (1999) Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (–)-epigallocatechin gallate with peroxyl radicals. *Chem. Res. Toxicol.*, **12**, 382–386.
50. Yang,C.S., Maliakal,P. and Meng,X. (2002) Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol.*, **42**, 25–54.
51. Dashwood,W.M., Orner,G.A. and Dashwood,R.H. (2002) Inhibition of beta-catenin/Tcf activity by white tea, green tea and epigallocatechin-3-gallate (EGCG): minor contribution of H(2)O(2) at physiologically relevant EGCG concentrations. *Biochem. Biophys. Res. Commun.*, **296**, 584–588.

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