Epigallocatechin Gallate Dose-Dependently Induces Apoptosis or Necrosis in Human MCF-7 Cells

YAN-DER HSUUW[a] AND WEN-HSIUNG CHAN[b]

[a]*Department of Life Science, National Pingtung University of Science and Technology, Pingtung, Taiwan 912*

[b]*Department of Bioscience Technology and Center for Nanotechnology, Chung Yuan Christian University, Chung Li, Taiwan 32023*

ABSTRACT: The catechins, a family of polyphenols found in tea, can evoke various responses, including cell death. However, the precise molecular mechanisms of these effects are unknown. Here, we demonstrate that treatment of human MCF-7 cells with 50 μM (-)-Epigallocatechin-3-gallate (EGCG), a catechin that is highly abundant in green tea, can induce apoptotic changes, including mitochondrial membrane potential changes and activation of c-Jun N-terminal kinase (JNK), caspase-9, and caspase-3. In contrast, higher concentrations of EGCG (100–400 μM) do not induce apoptosis, but rather trigger necrotic cell death in MCF-7 cells. Investigations of the possible mechanisms underlying these differences revealed that treatment with lower concentrations of EGCG (10–50 μM) directly increased intracellular oxidative stress, while higher concentrations (100–400 μM) did not. Immunoblotting revealed that treatment of MCF-7 cells with 10–50 μM EGCG caused increases in Bax protein levels and decreases in Bcl-2 protein levels, shifting the Bax–Bcl-2 ratio to favor apoptosis, while treatment with 100–400 μM EGCG had no such effect. Moreover, we observed a dose-dependent decrease in intracellular ATP levels in cells treated with high-dose EGCG. Blockade of reactive oxygen species (ROS) generation and ATP synthesis using antioxidants and ATP synthesis inhibitors revealed that ROS and ATP play important roles to switch cell death types with apoptosis or necrosis. Collectively, these results indicate for the first time that EGCG treatment has a dose-dependent effect on ROS generation and intracellular ATP levels in MCF-7 cells, leading to either apoptosis or necrosis, and that the apoptotic cascade involves JNK activation, Bax expression, mitochondrial membrane potential changes, and activation of caspase-9 and caspase-3.

KEYWORDS: catechin; EGCG; apoptosis; necrosis; oxidative stress

Address for correspondence: Wen-Hsiung Chan, Department of Bioscience Technology, Chung Yuan Christian University, Chung Li, Taiwan 32023. Voice: +886-3-2653515; fax: +886-3-2653599.
e-mail: whchan@cycu.edu.tw

Ann. N.Y. Acad. Sci. 1095: 428–440 (2007).
doi: 10.1196/annals.1397.046

INTRODUCTION

Apoptosis and necrosis are two distinct types of cell death that differ in terms of their biochemical and morphological characteristics, as well as their regulatory mechanisms.[1,2] Apoptosis (programmed cell death) plays an important role in the embryogenesis and homeostasis of multicellular organisms, and impairment of apoptotic function has been associated with several human diseases, including neurodegenerative disorders and cancer.[3] In contrast, necrosis (cell lysis) occurs in response to acute and nonphysiological injuries.[4] Previous reports have indicated that the magnitude of the initial insult, not the stimulus type, plays a critical role in prompting a cell to undergo either necrosis or apoptosis.[5,6]

The catechins, a family of polyphenols found in tea, have been demonstrated to have antioxidative, antiallergic, antimutagenic, and anticarcinogenic functions.[7–9] Recent reports have demonstrated that catechins can prevent cancer progress by inhibiting carcinogenesis, tumor growth, cancer cell invasion, and tumor angiogenesis.[10,11] (-)-Epigallocatechin-3-gallate (EGCG), a catechin that is highly abundant in green tea, induces cell apoptosis,[12] perhaps accounting for its antitumor effects.[13]

A number of studies have examined the regulatory mechanisms responsible for directing cells toward apoptosis or necrosis, and several lines of evidence have suggested that this "switch" is controlled by the intracellular levels of ATP and inactivation of caspases.[14] Specifically, high energy levels are required for execution of the apoptotic process, while necrosis can proceed in the presence of low ATP levels.[15,16] Thus, we herein examined the effects of high- or low-dose EGCG treatment on human MCF-7 cells.

Our results revealed for the first time that EGCG treatment has dose-dependent effects on MCF-7 cells, with low doses inducing apoptosis and high doses inducing necrosis. Furthermore, we showed that the two different cell death modes were associated with differences in reactive oxygen species (ROS) generation and ATP levels.

MATERIALS AND METHODS

Cell Culture and EGCG Treatment

Human breast cancer MCF-7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells ($\sim$5-6 $\times$ 10^6) were incubated in media containing various concentrations of EGCG for the indicated times. Cell lysates were collected, sonicated on ice for 3 $\times$ 10 s, and then centrifuged at 15,000 *g* for 20 min at 4°C. The resulting supernatants were used as the cell extracts.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test, a colorimetric assay that measures the percentage of cell survival, was performed as described in our previous report.[17] The results were analyzed by spectrophotometry using an ELISA reader at a wavelength of 570 nm.

Assessment of Apoptosis and Necrosis

Oligonucleosomal DNA fragmentation in apoptotic cells was measured using the Cell Death Detection ELISA^{plus} Kit (TUNEL Apoptosis Assay Kit) according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). The spectrophotometric data were obtained using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 405 nm. Cell apoptosis and necrosis were also assessed by propidium iodide (PI) and Hoechst 33342 staining, as described in our previous report.[18] As a second index of necrosis, we examined the lactate dehydrogenase (LDH) activity present in the culture medium, using an LDH ELISA Assay Kit (Promega, WI).

ROS Assay

ROS generation was measured using 2′-7′-dichlorofluorescein diacetate (DCFDA) dye and determined using a fluorescence ELISA reader (excitation 485 nm, emission 530 nm) as described in our previous study.[19] The results were expressed as arbitrary absorbance units/mg protein.

Detection of Mitochondrial Membrane Potential

Cells were plated and grown in 96-well plates for 24 h and then incubated with various concentrations of EGCG for an additional 6 h. The fluorescent dye, DiOC6(3) (20 nM), was then added to each well, and plates were incubated for 15 min. Fluorescence was measured with a plate spectrofluorometer (excitation: 485 nm; emission: 535 nm).

Caspase Activity Assays

Caspase-3 activity was measured using the Z-DEVD-AFC fluorogenic substrate (Calbiochem, San Diego, CA), as previously described.[18] Caspase-8 and caspase-9 activities were assayed using the Colorimetric Caspase-9 Assay Kit and the Fluorometric Caspase-8 Assay Kit (Calbiochem).

Immunoblots

Immunoblot analysis was carried out essentially as described in our previous study.[20]

ATP Level Analysis

The intracellular ATP level in each extract was determined by a bioluminescence assay using a liquid scintillation analyzer, according to the manufacturer's protocol (Molecular Probes, Eugene, OR).

Statistics

Data were analyzed using one-way analysis of variance (ANOVA), and differences were evaluated using a Student's *t*-test and ANOVA. P values < 0.05 were considered significant.

RESULTS

Effects of EGCG on MCF-7 Cells

MCF-7 cells were incubated with various doses of EGCG for 6 h, and cell viability was determined. Our results revealed that cell viability decreased dose-dependently in cells treated with 10 to 400 μM EGCG (FIG. 1 A). ELISA analysis of TUNEL-stained apoptotic cells revealed that 10–50 μM EGCG increased the apoptosis-associated parameter in a dose-dependent manner, but that the apoptosis-associated parameter decreased in cells exposed to EGCG concentrations of 100–400 μM (FIG. 1 B). Indeed, higher concentrations of EGCG (100–400 μM) induced a rapid shift from apoptotic to necrotic cell death over a relatively small range of EGCG concentrations (50–100 μM) (FIG. 1 C). These results were confirmed by an LDH release assay; the LDH activity in the culture medium sharply increased in cells treated with 100–400 μM EGCG (FIG. 1 D).

Low-Dose EGCG Treatment Induces ROS Generation and Mitochondrial Membrane Potential Changes

As ROS have been shown to promote cell injury,[21] we used DCFDA to examine ROS formation in EGCG-treated MCF-7 cells. We found that low doses of EGCG (10–50 μM) increased the intracellular ROS contents of

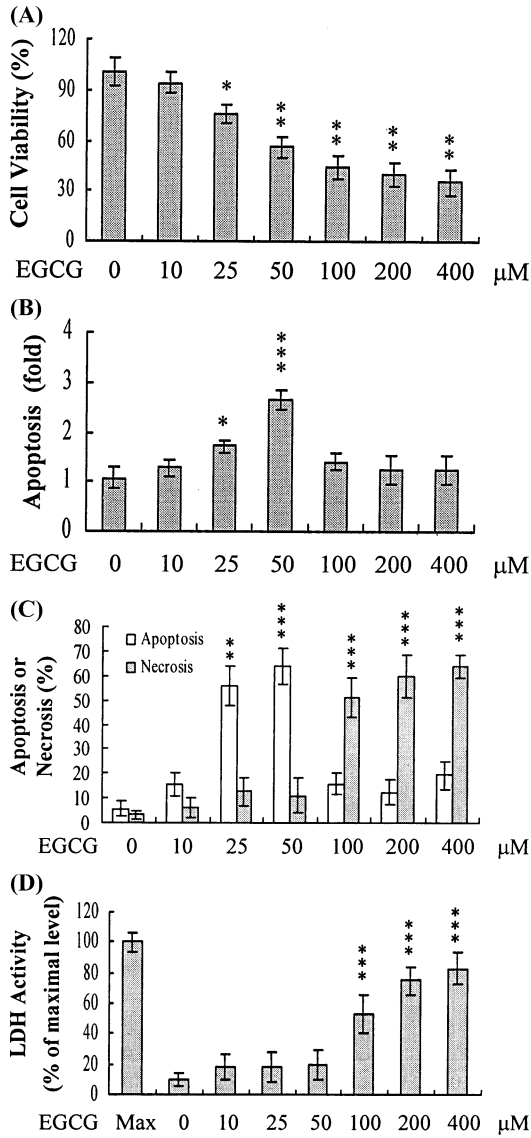

FIGURE 1. Effects of EGCG treatment on MCF-7 cells. MCF-7 cells were incubated with or without various concentrations of EGCG for 6 h. Cell viability was determined by MTT assay (**A**) and apoptosis was evaluated using the Cell Death Detection ELISA Kit (**B**), as described in the "Materials and Methods" section. (**C**) The percentages of apoptosis and necrosis were determined by PI and Hoechst 33342 staining. (**D**) Necrosis was further assessed by measurement of LDH activity in the culture medium. The data are expressed as a percentage of the maximal level (Max) of LDH activity determined after total cell lysis. Values are presented as mean ± SD of five determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P<0.001$ versus the control (EGCG-free) group.

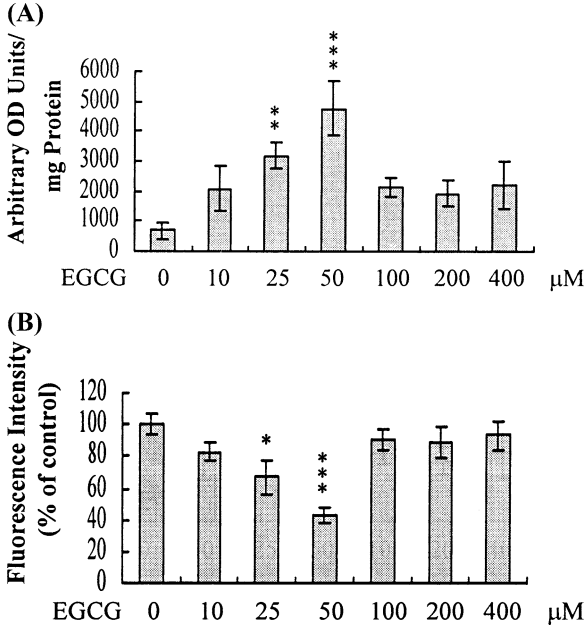

FIGURE 2. Dosage effect of EGCG on ROS generation and mitochondrial membrane potential changes. (**A**) MCF-7 cells were incubated with the indicated concentrations of EGCG for 6 h and ROS generation was assayed using DCFDA. (**B**) Cells were then incubated with 40 nM DiOC6(3) for 1 h, and analyzed by spectrofluorometry. Values are presented as mean ± SD of five determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control (EGCG-free) group.

MCF-7 cells, whereas no such effect was seen in response to higher concentrations (100–400 μM) of EGCG (FIG. 2 A). Since mitochondrial membrane potential changes have been directly associated with apoptosis,[22–24] we investigated the effect of EGCG on mitochondrial membrane potential in MCF-7 cells by measuring DiOC6(3) uptake. Low-dose (10–50 μM) EGCG treatment decreased DiOC6(3) uptake into the mitochondria, indicating a loss of mitochondrial membrane potential, whereas treatment with 100–400 μM EGCG had no such effect (FIG. 2 B). These results indicate that increased ROS generation and decreased mitochondrial membrane potential may be involved in low-dose EGCG-induced apoptosis but not high-dose EGCG-induced necrosis of MCF-7 cells.

Low-Dose EGCG Treatment Induces Activation of JNK, Caspase-9, and Caspase-3 in MCF-7 Cells

As the activations of c-Jun N-terminal kinase (JNK) and caspases have been directly associated with apoptosis,[20,25] we assessed the dose–response effect

of EGCG on these parameters. We found that treatment of MCF-7 cells with 10–50 μM EGCG led to activation of JNK, caspase-9, and capase-3 (FIG. 3 A–C), whereas treatment of cells with 100–400 μM EGCG had little effect on these apoptotic changes (FIG. 3 A–C). In contrast, caspase-8 was not activated following either treatment (FIG. 3 D), indicating that caspase-8 is not involved in EGCG-induced cell death in these cells. Collectively, these results indicate that in MCF-7 breast cancer cells, ROS generation, JNK activity, mitochondrial membrane potential changes, and activation of caspase-9 and caspase-3 are all correlated with low-dose EGCG-induced apoptosis, but not with high-dose EGCG-induced necrosis (FIGS. 2 and 3).

Dose-Dependent Effects of EGCG on Bax–Bcl-2 Expression and Intracellular ATP Levels

As the Fas receptor is known to stimulate apoptosis, we examined whether the Fas receptor was involved in EGCG-induced apoptosis or necrosis. Our result revealed no change in Fas expression levels following low- or high-dose EGCG treatment of MCF-7 cells (FIG. 4 A). Since the expression ratio of Bax versus Bcl-2 is significant for apoptosis determination,[26] we investigated whether EGCG induced apoptosis by modulating the expression of Bcl-2 family members. Our results revealed that low-dose EGCG (10–50 μM) treatment increased Bax protein levels and decreased Bcl-2 protein expression levels, whereas high-dose EGCG treatment had no such effect (FIG. 4 B). Densitometric analysis of the protein bands revealed that low-dose EGCG treatment increased the Bax–Bcl-2 ratio (favoring apoptosis), while high-dose EGCG treatment did not affect the Bax–Bcl-2 ratio (FIG. 4 C). Finally, since ATP levels are important mediators capable of switching the mode of cell death from apoptosis to necrosis,[16] we examined changes in ATP content following low- and high-dose EGCG treatment of MCF-7 cells. Our results revealed that intracellular ATP levels decreased in MCF-7 cells treated with doses of EGCG higher than 100 μM, but that no such effect was seen in cells treated with lower concentrations of EGCG (10–50 μM) (FIG. 4 D). These results collectively indicate that low-dose EGCG-induced apoptosis involves changes in the protein expression levels of Bcl-2 family members but not that of the Fas receptor, whereas high-dose EGCG-induced necrosis is associated with decreased ATP levels in MCF-7 cells.

ROS Generation and High ATP Levels are Required for Low-Dose EGCG-Induced Apoptosis

To further determine whether ROS generation and ATP levels control the dose–response switch mechanism between EGCG-induced apoptosis and

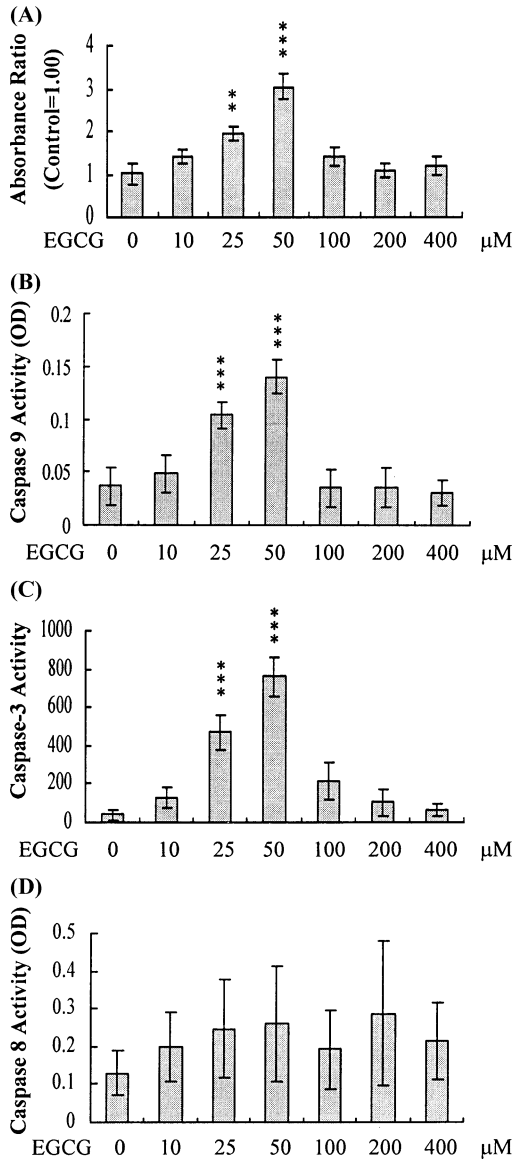

FIGURE 3. Dosage effect of EGCG on activation of JNK and caspases. MCF-7 cells were incubated with the indicated concentrations of EGCG for 6 h. (**A**) JNK/AP-1 activity was evaluated by ELISA detection of phosphorylated c-Jun. The results were expressed in relation to controls, which were arbitrarily set to 1.00. (**B**–**D**) Caspase-9, caspase-3, and caspase-8 activities were assayed using the Colorimetric or Fluorescence Assay Kit as described in the "Materials and Methods" section. Values are presented as mean ± SD of three to five determinations. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control (EGCG-free) group.

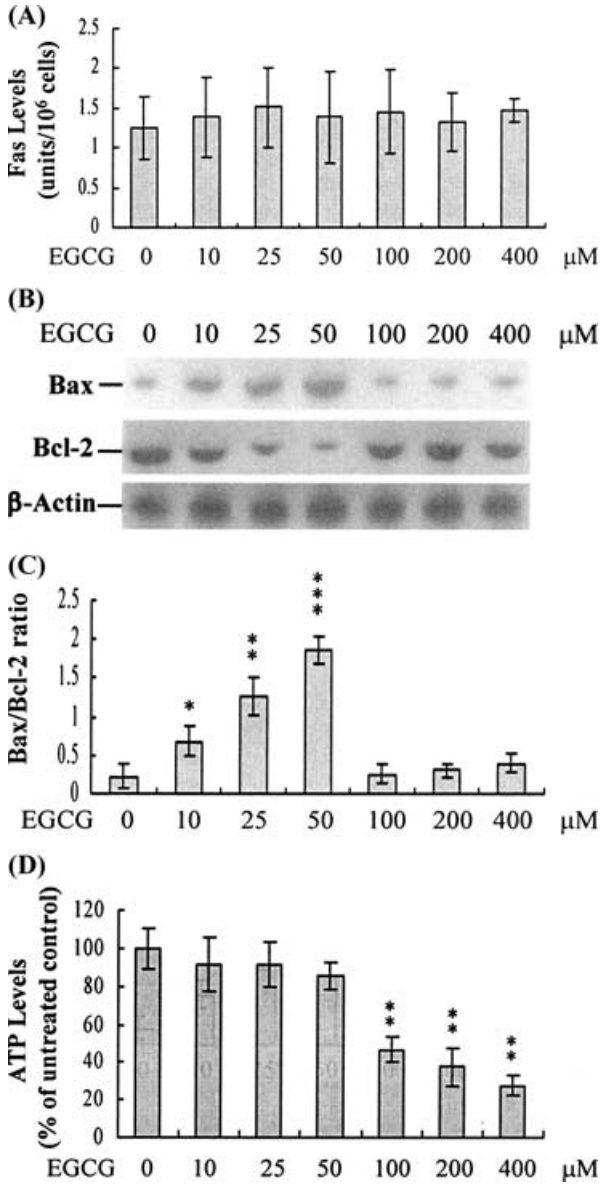

FIGURE 4. Dosage effect of EGCG on Fas expression, ratio of Bax–Bcl-2, and ATP levels in MCF-7 cells. MCF-7 cells were incubated with the indicated concentrations of EGCG for 6 h. (**A**) The Fas content in cell extracts was measured using the Fas/APO-1 ELISA Kit. (**B**) Bax and Bcl-2 protein levels were analyzed by immunoblotting. (**C**) Densitometry was used to calculate the Bax–Bcl-2 ratios. (**D**) Intracellular ATP levels were determined by a bioluminescence-based ELISA assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the control (EGCG-free) group.

necrosis, we examined the effects of two ROS scavengers (N-acetyl cysteine [NAC] and α-tocopherol) and antimycin A, an inhibitor of mitochondrial respiratory chain complex III, on MCF-7 cells treated with 50 μM EGCG. Our results showed that pretreatment with the antioxidants or antimycin A significantly attenuated intracellular ROS generation or decreased ATP levels, and that these effects were associated with reduced apoptosis in MCF-7 cells treated with 50 μM EGCG (FIG. 5 A–C). These findings indicate that ROS generation and ATP levels act as important controllers for low-dose EGCG-induced apoptosis.

DISCUSSION

Low doses of antimycin A, a specific inhibitor of the mitochondrial respiratory chain, were previously shown to kill rat fibroblast cells via apoptosis, whereas necrosis was increasingly favored as the intensity of the hypoxic insult increased.[27] Other studies have suggested that intracellular ATP levels could represent an important regulator for switching cells toward apoptosis or necrosis,[14,15,27] with necrosis favored under low ATP conditions in many model systems. Consistent with this notion, studies have shown that high ATP levels are essential for the process and progression of apoptosis.[28] Here, we showed that high and low doses of EGCG induce two distinct cell death programs in MCF-7 cells (FIG. 1 B–D).

To examine the possible molecular mechanisms by which EGCG induces apoptosis or necrosis, we examined ROS production, activation of JNK, mitochondrial membrane potential changes, and activation of caspase family members, all of which are known to be important signal mediators for cell death.[29,30] We observed significant ROS generation and mitochondrial membrane potential changes in MCF-7 cells treated with 10–50 μM EGCG, but not in those treated with 100–400 μM EGCG or in untreated controls (FIG. 2 A and B). Similar results were obtained when we tested activation of JNK, caspase-9, and caspase-3 (FIG. 3 A–C). In addition, when we examined the levels of Fas and Bax–Bcl-2 in EGCG-treated MCF-7 cells, we found that low-dose EGCG (10–50 μM) treatment increased the ratio of Bax–Bcl-2, while high-dose EGCG (100–400 μM) treatment had no such effect (FIG. 4 B). We further demonstrated that EGCG dose-dependently decreases intracellular ATP levels in MCF-7 cells (FIG. 4 D), and finally used ROS scavengers and an ATP synthesis inhibitor to show that ROS generation and high ATP levels are required for EGCG-induced apoptosis (FIG. 5). These results support the hypothesis that ATP can act as a switch to determine EGCG-induced ROS generation and subsequent apoptosis. In addition, our findings indicate that the necrosis induced by 100–400 μM EGCG did not involve signaling via pathways involving ROS generation, increased Bax–Bcl-2 ratios, mitochondrial membrane potential changes, or activation of JNK, caspase-9, and caspase-3. However, these

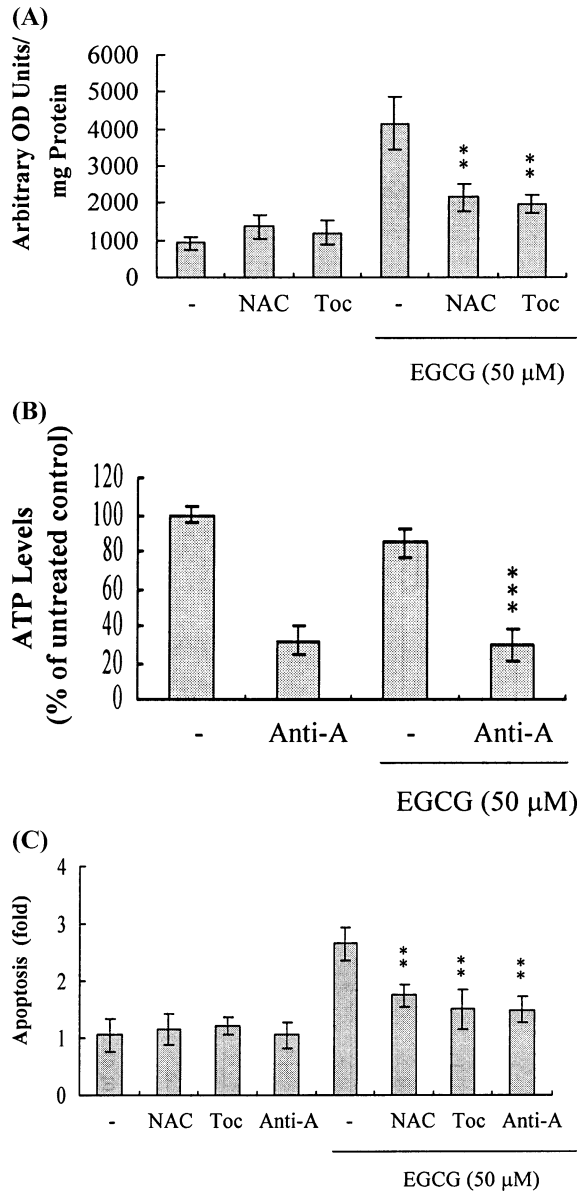

FIGURE 5. Effect of N-acetyl cysteine, α-tocopherol, and antimycin A on low-dose EGCG-induced apoptosis. Cells were incubated with N-acetyl cysteine (NAC; 2 mM), α-tocopherol (Toc; 300 μM), or antimycin A (anti-A; 300 μM) for 1 h and then treated with EGCG (50 μM) for another 6 h. ROS generation (**A**), intracellular ATP levels (**B**), and apoptosis (**C**) were measured as described. Values are presented as mean ± SD of three to five determinations. $^{**}P < 0.01$, $^{***}P < 0.001$ versus value of the "EGCG-treatment only" group.

biochemical changes were clearly associated with low-dose EGCG (10–50 μM)-induced apoptosis (FIGS. 2–4). Furthermore, our results show that apoptosis and necrosis have two independent pathways in the cell death processes during EGCG-treated MCF-7 cells.

In sum, our results show for the first time that the EGCG treatment dosage can determine the cell death mode in MCF-7 cells, and provide initial insights into the mechanisms involved in EGCG-induced apoptosis. Although future work will be required to fully define the precise regulatory mechanism, our findings suggest that ATP and ROS are likely to be important regulators of the cellular decision between the apoptotic and necrotic cell death pathways in EGCG-treated MCF-7 cells.

ACKNOWLEDGMENT

This work was supported by grants (NSC94-2320-B-033-003) from the National Science Council of Taiwan, ROC.

REFERENCES

1. MAJNO, G. & I. JORIS. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. Am. J. Pathol. **146:** 3–15.
2. SCHWARTZ, S.M. & M.R. BENNETT. 1995. Death by any other name. Am. J. Pathol. **147:** 229–234.
3. THOMPSON, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science **267:** 1456–1462.
4. ALISON, M.R. & C.E. SARRAF. 1994. Liver cell death: patterns and mechanisms. Gut **35:** 577–581.
5. BONFOCO, E. *et al*. 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. USA **92:** 7162–7166.
6. HAMPTON, M.B. & S. ORRENIUS. 1997. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. **414:** 552–556.
7. JANKUN, J., S.H. SELMAN, R. SWIERCZ & E. SKRZPYCZAK-JANKUN. 1997. Why drinking green tea could prevent cancer. Nature **387:** 561.
8. SANO, M. *et al*. 1999. Novel antiallergic catechin derivatives isolated from oolong tea. J. Agric. Food Chem. **47:** 1906–1910.
9. KAWASE, M. *et al*. 2000. Antioxidative activity of (-)-epigallocatechin-3-(3″-O-methyl)gallate isolated from fresh tea leaf and preliminary results on its biological activity. Biosci. Biotechnol. Biochem. **64:** 2218–2220.
10. FUJIKI, H. *et al*. 1992. Anticarcinogenic effects of (-)-epigallocatechin gallate. Prev. Med. **21:** 503–509.
11. VALCIC, S. *et al*. 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.

12. KAWAI, K. *et al*. 2005. Epigallocatechin gallate induces apoptosis of monocyte. J. Allergy Clin. Immunol. **115:** 186–191.
13. NAKAZATO, T. *et al*. 2005. Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. Clin. Cancer Res. **11:** 6040–6049.
14. EGUCHI, Y., S. SHIMIZU & Y. TSUJIMOTO. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res. **57:** 1835–1840.
15. RICHTER, C. *et al*. 1996. Control of apoptosis by the cellular ATP level. FEBS Lett. **378:** 107–110.
16. LEIST, M. *et al*. 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. **185:** 1481–1486.
17. CHAN, W.-H. 2005. Effect of resveratrol on high glucose-induced stress in human leukemia K562 cells. J. Cell. Biochem. **94:** 1267–1279.
18. HSUUW, Y.-D. *et al*. 2005. Curcumin prevents methylglyoxal-induced oxidative stress and apoptosis in mouse embryonic stem cells and blastocysts. J. Cell. Physiol. **205:** 379–386.
19. CHAN, W.-H. & H.-J. WU. 2004. Antiapoptotic effects of curcumin on photosensitized human epidermal carcinoma A431 cells. J. Cell. Biochem. **92:** 200–212.
20. CHAN, W.-H., J.-S. YU & S.-D. YANG. 1999. PAK2 is cleaved and activated during hyperosmotic shock-induced apoptosis via a caspase-dependent mechanism: evidence for the involvement of oxidative stress. J. Cell. Physiol. **178:** 397–408.
21. BUTTKE, T.M. & P.A. SANDSTROM. 1994. Oxidative stress as a mediator of apoptosis. Immunol. Today **15:** 7–10.
22. LI, P. *et al*. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/ caspase-9 complex initiates an apoptotic protease cascade. Cell **91:** 479–489.
23. ZOU, H. *et al*. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell **90:** 405–413.
24. WEBER, T. *et al*. 2003. Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agent with antineoplastic activity: comparison with receptor-mediated pro-apoptotic signaling. Biochemistry **42:** 4277–4291.
25. CHAN, W.-H., J.-S. YU & S.-D. YANG. 2000. Apoptotic signalling cascade in photosensitized human epidermal carcinoma A431 cells: involvement of singlet oxygen, c-Jun N-terminal kinase, caspase-3 and p21-activated kinase 2. Biochem. J. **351:** 221–232.
26. ANTONSSON, B. & J.-C. MARTINOU. 2000. The Bcl-2 protein family. Exp. Cell Res. **256:** 50–57.
27. FORMIGLI, L. *et al*. 2000. Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J. Cell. Physiol. **182:** 41–49.
28. KASS, G.E. *et al*. 1996. Chromatin condensation during apoptosis requires ATP. Biochem. J. **318:** 749–752.
29. ENARI, M. *et al*. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature **380:** 723–726.
30. CHAN, W.-H., C.-C. WU & J.-S. YU. 2003. Curcumin inhibits UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermoid carcinoma A431 cells. J. Cell. Biochem. **90:** 327–338.