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Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells

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Polyphenols such as epigallocatechin-3-gallate (EGCG) from green tea extract can exert a growth-suppressive effect on human pancreatic cancer cells *in vitro*. In pursuit of our investigations to dissect the molecular mechanism of EGCG action on pancreatic cancer, we observed that the antiproliferative action of EGCG on pancreatic carcinoma is mediated through programmed cell death or apoptosis as evident from nuclear condensation, caspase-3 activation and poly-ADP ribose polymerase (PARP) cleavage. EGCG-induced apoptosis of pancreatic cancer cells is accompanied by growth arrest at an earlier phase of the cell cycle. In addition, EGCG invokes Bax oligomerization and depolarization of mitochondrial membranes to facilitate cytochrome *c* release into cytosol. EGCG-induced down-regulation of IAP family member X chromosome linked inhibitor of apoptosis protein (XIAP) might be helpful to facilitate cytochrome *c* mediated downstream caspase activation. On the other end, EGCG elicited the production of intracellular reactive oxygen species (ROS), as well as the c-Jun *N*-terminal kinase (JNK) activation in pancreatic carcinoma cells. Interestingly, inhibitor of JNK signaling pathway as well as antioxidant *N*-acetyl-L-cysteine (NAC) blocked EGCG-induced apoptosis. To summarize, our studies suggest that EGCG induces stress signals by damaging mitochondria and ROS-mediated JNK activation in MIA PaCa-2 pancreatic carcinoma cells.

Introduction

Green tea, a popular beverage consumed worldwide is known to have a cancer chemopreventive effect against a broad spectrum of cancer (1–3). Catechins are the key components of teas that exert antiproliferative properties. Among them, the most abundant in green tea extract, epigallocatechin-3-gallate (EGCG) has significant effects against tumorigenesis and tumor growth (3–9). Since green tea is non-toxic and it is effective in a wide range of organs, the worldwide interest in green tea as a cancer preventive agent for humans has increased. The chemopreventive effects of EGCG and other structurally related green tea polyphenols have been investigated using a variety of rodent tumor models. One of the most plausible mechanisms of its chemopreventive activity might be attributed to its ability to suppress the promotion of carcinogenesis in animals as well as in cultured cells (10–12). Apart from inhibiting tumor promotion, EGCG administered intraperitoneally (i.p.) and the green tea polyphenol fraction infused orally caused the regression of experimentally induced skin papillomas in mice (13,14). The i.p. injection of this polyphenol can retard the progression of tumors arising from human prostate cancer cells or mammary carcinoma cells in athymic (nude) mice (15). However, its effect was not promising on 7,12-dimethylbenz[a]anthracene-triggered carcinogenesis (16). The molecular mechanism of the antiproliferative action of EGCG in cancer cells has been, in part, attributed to the induction of a highly regulated cellular process called apoptosis (17–21).

The previous studies demonstrate the ability of EGCG to suppress growth and invasion of pancreatic cancer cells in a dose-dependent manner (22,23). It is quite interesting because the conventional chemotherapy or irradiation is quite ineffective in killing pancreatic cancer cells. Here, we performed experiments to determine whether the antiproliferative action of EGCG in pancreatic cancer cell lines is mediated through apoptosis. Our results reveal that EGCG can indeed promote apoptosis in pancreatic cancer through mitochondrial membrane depolarization and the activation of executioner caspases.

Abbreviations: DAPI, 4′, 6 diamidino-2-phenylindole dihydrochloride; EGCG, epigallocatechin-3-gallate; JNK, c-Jun *N*-terminal kinase; NAC, *N*-acetyl-L-cysteine; OMM, outer mitochondrial membrane; PARP, poly-ADP ribose polymerase; ROS, reactive oxygen species; XIAP, X-chromosome linked inhibitor of apoptosis protein.

Materials and methods

Cells and culture conditions

Human pancreatic cancer cell lines MIA PaCa-2, PANC-1, AsPC-1, BxPC-3, Hs 766T, SU86.86 and CFPAC-1 were grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin at 37°C in a 5% CO_2 humidified atmosphere.

Soft agar assay

A total of ~20 000 cells was resuspended in 0.35% noble agar (in RPMI supplemented with 10% FBS) and plated on 6-cm plates containing a solidified bottom layer (0.5% noble agar in growth medium). The plates were incubated in a humidified incubator at 37°C for 10–14 days. The plates were stained with 0.005% crystal violet. The colonies were counted by using an inverted microscope.

Analysis of cell death

Chromatin condensation was determined by 4′,6-diamidino-2-phenylindole (DAPI) fluorescence as described previously (24). A total of 5×10^6 cells (in triplicate plates) was cultured in the growth medium in the presence or absence of 0.1–0.2 mM EGCG for 24 h. After fixing and permeabilization, the cells were mounted in a fluid containing 2 μg/ml DAPI (Vector Laboratories, Burlingame, CA). A Nikon Eclipse E600 fluorescence microscope (Huntley, IL) was used to visualize the nuclear stain.

Poly-ADP ribose polymerase (PARP) cleavage analysis

Cells were treated with specified concentrations of EGCG for designated time periods. After treatment, the total cellular proteins were extracted as described previously (24,25). After normalization for the total protein content, the resulting lysate was subjected to SDS–PAGE and immunoblotting with

monoclonal against PARP (Pharmingen, San Diego, CA). Immunodetection was accomplished by enhanced chemiluminescence method (Amersham/Pharmacia Biotech, Piscataway, NJ).

Cell cycle analysis by flow cytometry

After the designated treatment period with 0.2 mM EGCG, cells were sorted by a fluorescence activated cell sorter at Ireland Cancer Center's core facility as described previously (25).

Mitochondrial membrane depolarization study by confocal microscopy

MIA PaCa-2 cells were grown in a 6-well tissue culture dish. The cells were either treated with 0.2 mM EGCG or untreated for 14 h. After treatment, the medium was replaced with serum-free medium containing 10 μg/ml JC-1, a potential-dependent J-1 aggregate forming lipophilic cation (Molecular Probes, Eugene, OR). Cells were incubated at 37°C for 10 min followed by washing with PBS. Immediately, the cells were visualized by a confocal laser-scanning microscope (Leica SP2, Bannockburn, IL). The monomer and J-aggregate forms were simultaneously excited by 488-nm argon-ion laser sources (26). Polarized mitochondria were marked by punctate orange-red fluorescence staining.

Determination of cytochrome c and oligomeric Bax by immunofluorescence microscopy

For cytochrome *c*, control and EGCG-exposed cells were fixed and permeabilized followed by immunostaining with mouse monoclonal cytochrome *c* antibody as primary and CY3 conjugated goat anti-mouse IgG as secondary. To detect oligomeric Bax, immunostaining was performed with mouse anti-Bax monoclonal antibody 6A7 (27) as primary. Slides were visualized under a Leica SP2 confocal microscope (25,28).

Immunocomplex kinase assay

Equal amount of protein from control and EGCG-treated MIA PaCa-2 cells were subjected to immunoprecipitation with anti-human c-Jun *N*-terminal kinase (JNK) antibody in lysis buffer containing 10 mM HEPES, pH 7.2, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% NP-40 and protease inhibitors. After overnight incubation at 4°C, the immunocomplex was trapped in Protein A-sepharose CL4B beads (Amersham-Pharmacia Biotech) by incubating for 2 h at 4°C. After washing, the kinase reaction for JNK was performed by incubating the immunoprecipitated proteins in 25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM DTT, 0.1 mM sodium orthovanadate, 10 μM ATP, 2 μl activated JNK (Calbiochem) and 10 μCi of [γ-^{32}P]ATP. Reactions were carried out at 30°C for 15 min. Kinase reactions were terminated by boiling with Lammeli's sample buffer followed by SDS–PAGE and autoradiography.

Measurement of reactive oxygen species (ROS)

Flow cytometric determination of ROS production was carried out as described previously (21). MIA PaCa-2 cells were collected by trypsinization and 200 μl of cell suspension (2×10^6 cells/ml) was mixed with 800 μl PBS. Cells were incubated with 10 μM 5-(and-6)-chloromethyl–2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (molecular probes) for 15 min, followed by the addition of EGCG or hydrogen peroxide. The incubation was continued for 20 min at 37°C. The oxidative burst (hydrogen peroxide) was detected using a FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively.

Results

Antiproliferative effect of EGCG by soft agar assay

The soft agar assay determines the ability of cells to grow in the absence of adhesion and adhesion-independent growth of the transformed cells reflects one of the few *in vitro* characteristics that can be compared with the malignant phenotype *in vivo* (29,30). For this purpose, single cell suspensions of MIA PaCa-2 cells were mixed with 0.35% agar in culture media in the presence or absence of EGCG. After layering the mixture on top of a solidified bottom agar for 3 weeks, the plates were stained with crystal violet and visualized under a phase contrast microscope. In contrast to the plates containing EGCG, the control plates that do not contain any EGCG exhibited colonies of a larger size (Figure 1). Moreover, EGCG can reduce the total number of colonies formed when compared with the control. Thus, the ability of EGCG to reduce the size as well as the number of total colonies formed

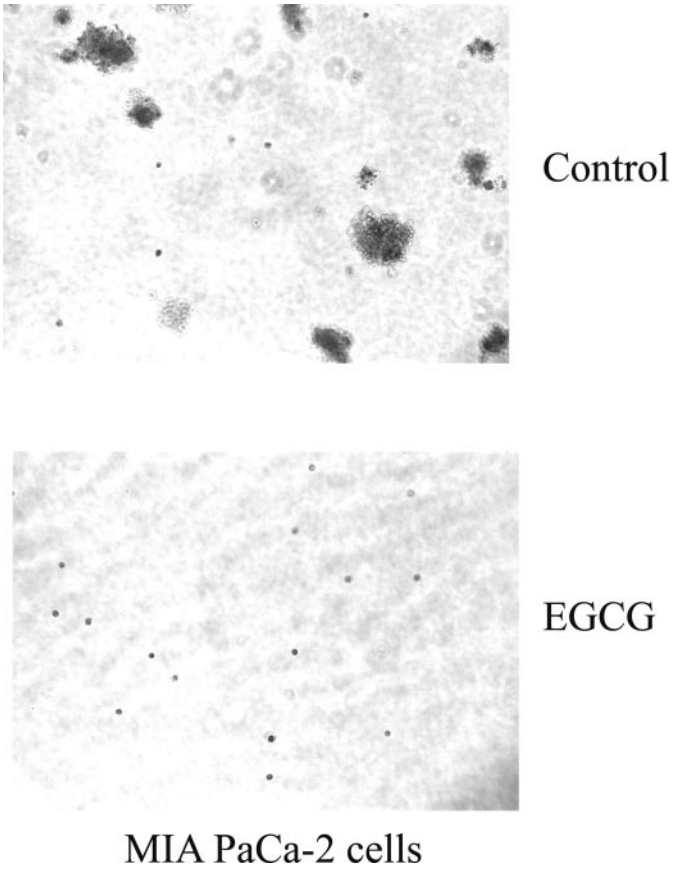

Fig. 1. Effect of EGCG on clonogenic cell survival of MIA PaCa-2 cells in soft agar. Single cell suspensions were mixed with 0.35% agar in culture medium with or without 0.2 mM EGCG. The mixture was layered on top of a solidified bottom agar. After 3 weeks, the plates were stained with crystal violet. Phase contrast microscopic images shown are representative of typical patterns most commonly observed in these experiments.

suggests that EGCG can exert antiproliferative activity on MIA PaCa-2 cells.

Apoptotic signaling in pancreatic cancer cells owing to EGCG exposure

We thought that the antiproliferative action of EGCG in pancreatic cancer might be mediated through the initiation of apoptotic cascade. Accordingly, we examined the effect of EGCG on seven human pancreatic cancer cell lines that exhibit aggressive clinical behavior. The cell lines under investigation were MIA PaCa-2, BxPC-3, SU86.86, AsPC-1, Hs 766T, CFPAC-1 and PANC-1. In order to determine whether EGCG can trigger any apoptotic effect in these cells, two different parameters were chosen: (a) PARP cleavage analysis and (b) DAPI staining. The member of caspase family of cysteine proteases, such as caspase-3, cleaves specific substrate to execute a programmed cell death. PARP is a 116-kDa nuclear protein that is specifically cleaved by caspase-3 into a signature 85-kDa fragment (25). In response to EGCG treatment, the extent of PARP cleavage differed from one cell line to another. Among them, MIA PaCa-2 cells demonstrated an elevated level of 85-kDa fragmented PARP (Figure 2, lane 2). On the other hand, BxPC-3 and SU86.86 cells were moderately responsive to EGCG-induced PARP cleavage (Figure 2, lanes 4 and 6). Both metastatic pancreatic carcinoma cell lines AsPC-1 and Hs 766T were apparently non-responsive

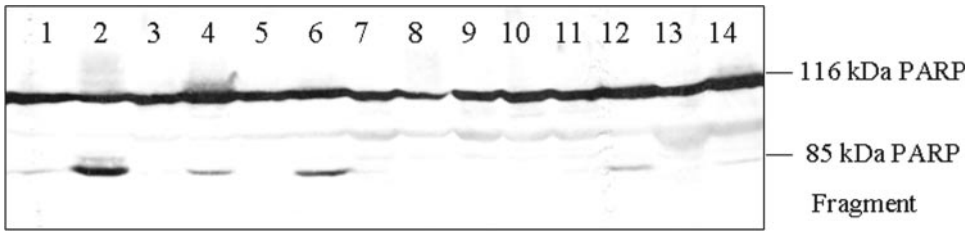

Fig. 2. EGCG-induced apoptosis in pancreatic cancer cells. Cells were exposed to 0.2 mM EGCG for 24 h followed by western blot analysis of cell lysate using monoclonal antibody against PARP. Lanes 1, 3, 5, 7, 9, 11 and 13, control; lanes 2, 4, 6, 8, 10, 12 and 14, EGCG-treated; lanes 1 and 2, MIA PaCa-2; lanes 3 and 4, BxPC-3; lanes 5 and 6, SU86.86; lanes 7 and 8, AsPC-1; lanes 9 and 10, Hs 766T; lanes 11 and 12, CFPAC-1; lanes 13 and 14, PANC-1.

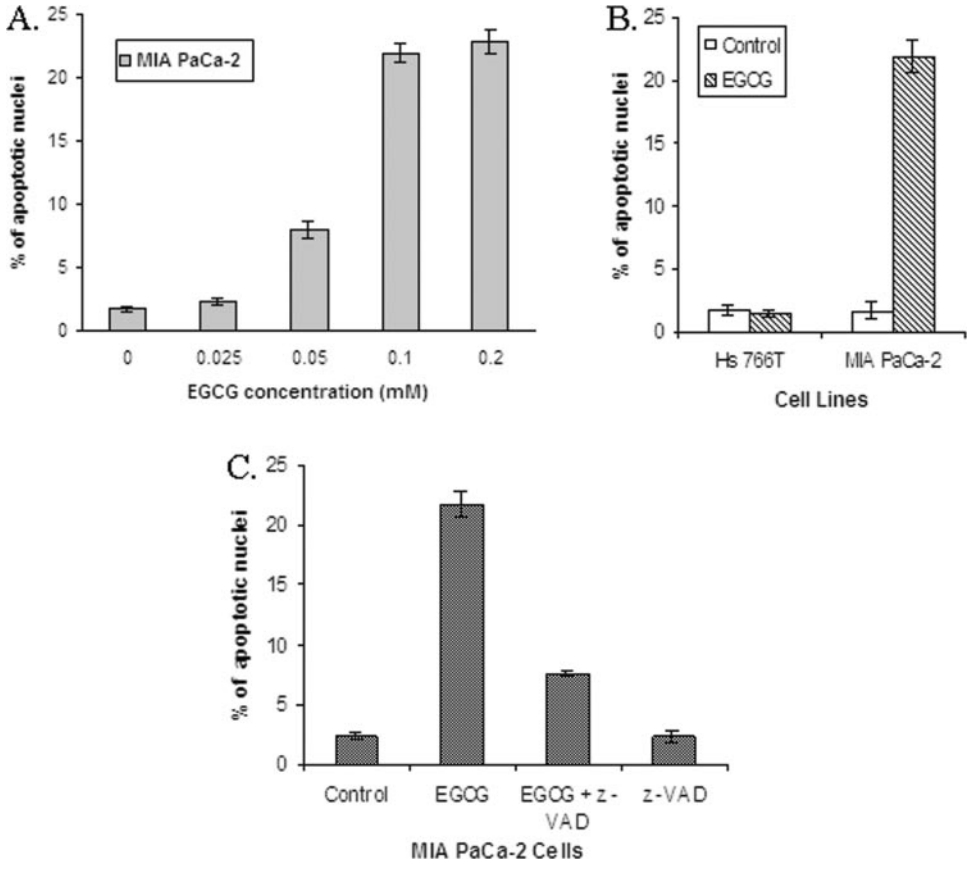

Fig. 3. EGCG can promote apoptosis in MIA PaCa-2 cells but not in metastatic pancreatic cancer cell line Hs 766T. Apoptotic cell death was visualized by staining with DNA group binding dye, DAPI. Percentage of apoptotic nuclei was determined by scoring 500 cells from randomly chosen fields. (**A**) Concentration-dependent apoptotic cell death in EGCG-exposed MIA PaCa-2 cells. (**B**) Metastatic pancreatic carcinoma cells Hs 766T do not exhibit apoptotic nuclei when challenged with 0.1 mM EGCG. (**C**) Caspase inhibitor z-VAD can significantly reduce 0.1 mM EGCG-induced apoptosis ($P < 0.001$). Cells were pre-treated with 500 nM z-VAD for 1 h followed by co-incubation with EGCG for 24 h. Error bars represent SD between counts of three independent experiments.

to EGCG because they exhibited little or no fragmented PARP (Figure 2, lanes 8 and 10). However, PANC-1 and CFPAC-1 cells isolated from ductal adenocarcinoma were moderately responsive to EGCG-triggered PARP cleavage (Figure 2, lanes 12 and 14). Next, we stained the control and EGCG-treated cells with a DNA group binding dye, DAPI (28,31). In correlation, MIA PaCa-2 cells, which demonstrated significant PARP cleavage, also had quite a large proportion of apoptotic nuclei in response to EGCG (Figure 3). At the same time, the metastatic pancreatic cancer cell line Hs 766T showed little response to EGCG as reflected by the negligible presence of chromatin condensation (Figure 3B). Even the increase in dose of EGCG to 0.2 mM was ineffective in triggering apoptosis in Hs 766T cells (data not shown). In contrast, the concentration curve demonstrates that EGCG as low as 0.1 mM concentration can induce apoptosis in MIA PaCa-2 cells (Figure 3A). Moreover, pre-treatment of MIA PaCa-2 cells with caspase inhibitor z-VAD blocked the EGCG-induced apoptosis (Figure 3C).

The cells susceptible to EGCG-induced apoptosis also showed activation of caspase-3 as evident by the decrease in the level of procaspase-3 (Figure 4A, lane 2). In general, caspase-3 exists as a precursor (inactivated) form that is cleaved to generate the active enzyme to act on several death substrates (PARP, laminin, etc.) to execute apoptosis. The significant decrease in the level of procaspase-3 was observed in EGCG-exposed MIA PaCa-2 cells, contrary to Hs 766T cells (Figure 4A, lane 2 versus lane 4). Notably, Hs 766T cells

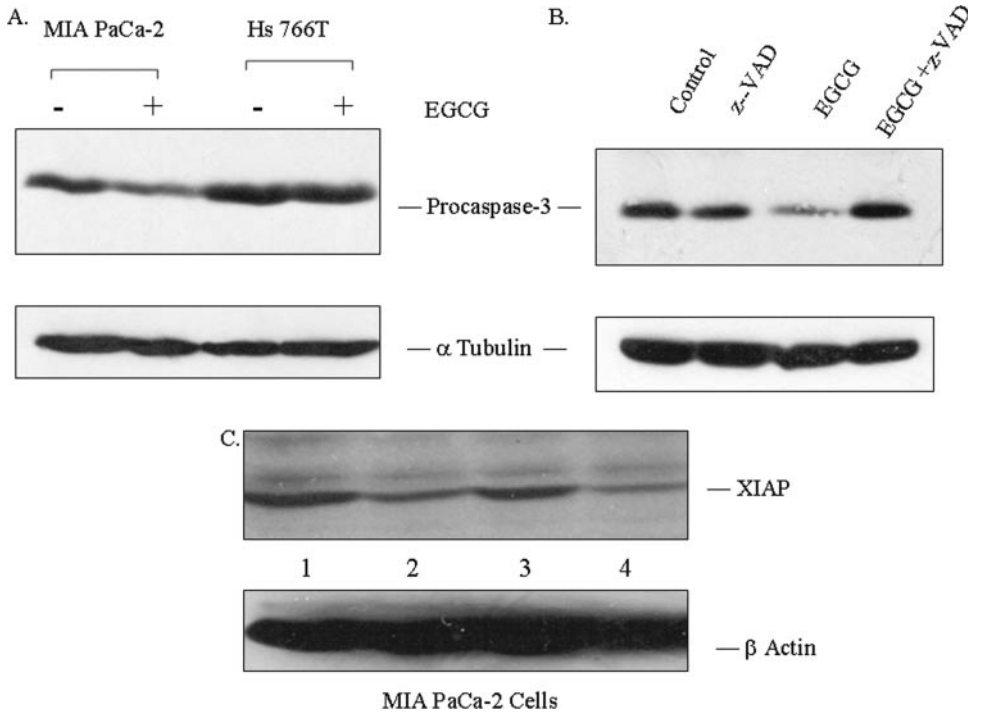

Fig. 4. Diminished procaspase-3 level following EGCG exposure can be restored by caspase inhibitor z-VAD. (**A**) Cell lysate from control and EGCG-exposed cells were subjected to immunoblot analysis with caspase-3 antibody. It is to be noted that the level of procaspase-3 in MIA PaCa-2 cells is decreased following EGCG treatment, whereas non-responsive Hs 766T cells are unaffected. (**B**) Effect of caspase inhibitor z-VAD on EGCG induced decrease of procaspase-3 level. MIA PaCa-2 Cells were pre-treated with 500 nM zVAD for 1 h followed by co-incubation with 0.1 mM EGCG for 24 h. (**C**) Western blot analysis with XIAP monoclonal antibody. Lane 1, 24 h control; lane 2, 0.1 mM EGCG for 24 h; lane 3, 48 h control; lane 4, 0.1 mM EGCG for 48 h. The same blot was probed with β-actin antibody.

are not responsive to EGCG-induced cell death (Figure 3B). Moreover, caspase inhibitor z-VAD can prevent the EGCG-triggered diminished level of procaspase-3 in MIA PaCa-2 cells (Figure 4B).

X chromosome linked inhibitor of apoptosis protein (XIAP) is known to be the most potent endogenous inhibitor of processed caspase-3, -7 and -9. Here, we were interested to examine whether EGCG treatment can inhibit the expression of antiapoptotic factor such as XIAP in pancreatic cancer cells. Immunoblot analysis using monoclonal antibody against XIAP clearly suggests the downregulation of XIAP protein in MIA PaCa-2 cells pre-treated with 0.1 mM EGCG for 24 or 48 h (Figure 4C). It is worth mentioning that XIAP protein remains unaltered in non-responsive Hs 766T cells following EGCG treatment (data not shown).

EGCG blocks taxol induced G2-M arrest of pancreatic cancer cells

EGCG is known to arrest epidermoid carcinoma cells A431 at a G0–G1 phase of cell cycle resulting in apoptotic cell death. Initially, by flow cytometric analysis, we were unable to differentiate the cell cycle distribution pattern between the control and EGCG-treated cells (Figure 5, Panels I and II). Taxol, a microtubule-disrupting agent, can arrest cells at the G2-M phase. We thought if EGCG can truly block cells at the G1 phase, its pre-treatment should prevent the cells entering into the mitotic phase following the post-treatment with taxol. As shown in Figure 5, Panel III, taxol can arrest pancreatic cancer cells CFPAC-1 at the G2-M phase of cell cycle in the absence of pre-treatment with EGCG. However, cells harbor at G1-S phase despite the presence of taxol if pre-treated with 0.2 mM EGCG (Figure 5, Panel IV). This is indicative of the ability of EGCG to retard cells at an early stage of the cell cycle.

Mitochondrial membrane depolarization by EGCG

Subsequently, we were interested to test whether a drop in mitochondrial membrane potential precedes the caspase-3 activation and subsequent cleavage of death substrate. The cationic dye JC-1 is useful for detection of the mitochondrial membrane depolarization occurring at the early stage of apoptosis (26,32). In living cells, JC-1 exhibits potential-dependent accumulation in mitochondria leading to the concentration-dependent formation of red fluorescent J-aggregates (26,32,33). The punctate fluorescent orange-red staining is indicative of the presence of polarized mitochondria. On depolarization, there is usually a reduction in orange-red staining. The red fluorescence is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape and density. JC-1 monomer binding with mitochondrial membrane results in green fluorescence and such interaction is independent of the membrane potential. Here, we stained the control and EGCG-treated MIA PaCa-2 cells with JC-1 and monitored the staining pattern by confocal laser scanning microscopy. As shown in Panel IV of Figure 6, EGCG-exposed cells dramatically reduced the formation of red-fluorescent J-aggregates indicative of mitochondrial membrane depolarization. Control cells showed punctate distribution of red fluorescence typical of live cells with polarized mitochondria (Figure 6, Panel I). The green fluorescence is noted in both the control and EGCG-treated cells owing to JC-1 monomer binding (Figure 6, Panels II and V). Nevertheless, the pattern of green fluorescence is apparently diffused

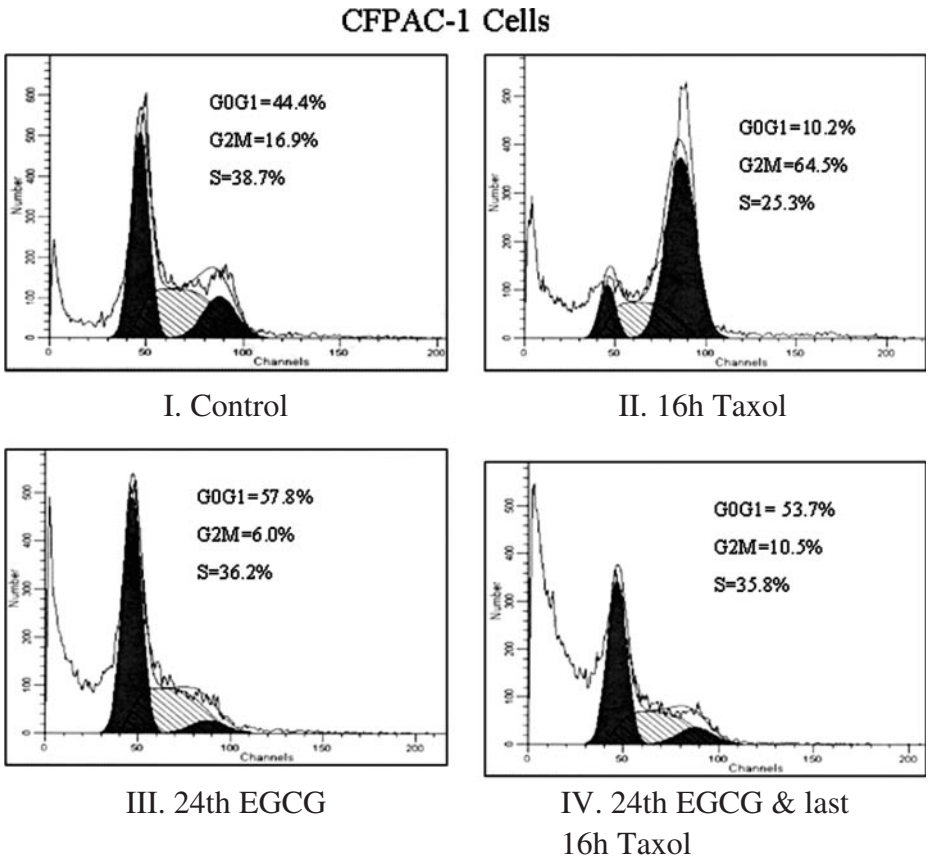

Fig. 5. Taxol-induced mitotic arrest of CFPAC-1 cells is blocked by pre-treatment with EGCG. CFPAC-1 cells were either treated with (I) vehicle, (II) 1 μM taxol for 16 h, (III) 0.2 mM EGCG for 24 h or (IV) 24 h EGCG (0.2 mM) with 1 μM taxol for the last 16 h. Cell cycle distribution was analyzed by flow cytometry.

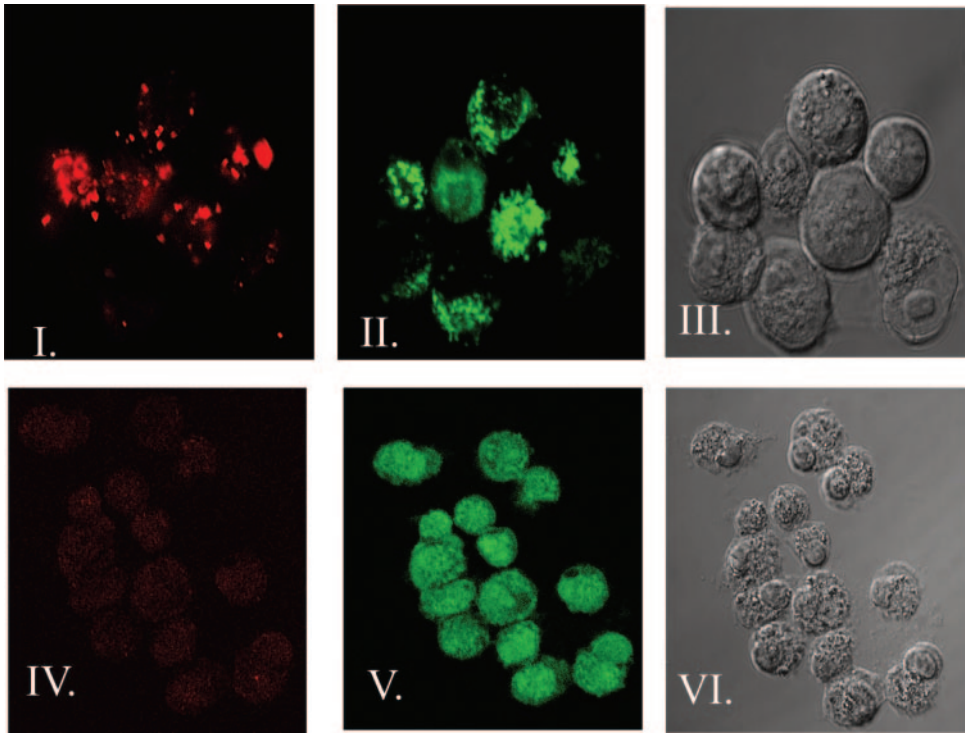

Fig. 6. Mitochondrial membrane depolarization by EGCG. Control and EGCG-treated MIA PaCa-2 cells were stained with JC-1 and the staining pattern was monitored by confocal laser scanning microscopy. For detection of J-aggregate form (red) and J-monomer alone (green), argon–krypton laser line was excited at 568 and 480 nm, respectively. Panels I, II and III represent control cells; panels IV, V and VI represent EGCG-treated cells. Panels III and VI represent differential interference contrast (DIC) images.

in EGCG-exposed apoptotic cells, contrary to the punctate distribution in untreated cells.

Release of cytochrome c in cytosol

Mitochondria are known to play a central role to elicit apoptosis in response to many triggers. EGCG-dependent apoptosis in MIA PaCa-2 cells was also observed to be accompanied by a drop in mitochondrial membrane potential (Figure 6). Studies have shown that a decrease in mitochondrial membrane potential because of permeability transition is an early event in apoptosis (34,35). The permeability transition also leads to the release of proteins, including cytochrome *c* from the mitochondrial intermembrane space and such proteins have been shown to cause apoptotic changes in isolated nuclei (36). Therefore, we speculated whether this EGCG-induced mitochondrial depolarization could release cytochrome *c* from the mitochondria to cytosol. A typical mitochondrial pattern (37,38) was seen by confocal microscopy in the control cells stained with monoclonal antibodies against cytochrome *c*. This pattern was altered when cells were exposed to EGCG: a diffused staining was clearly visible (Figure 7) implicating the release of cytochrome *c* from the mitochondria of EGCG-challenged pancreatic cancer cells (38).

Detection of oligomeric Bax in EGCG treated cells

The release of cytochrome *c* in cytosol (Figure 7) prompted us to determine the status of upstream regulators of mitochondrial membrane perturbations. Among the upstream regulators, the redistribution of soluble proapoptotic protein Bax from the cytosol to organelle appears to be important in promoting cell death (39). Bax then inserts itself into the outer mitochondrial membrane (OMM) where it oligomerizes and facilitates the release of cytochrome *c* from the intermembrane mitochondrial space (39). Upon EGCG exposure, we tested the oligomerization of Bax in the control and 0.1 mM EGCG-exposed MIA PaCa-2 cells by using a monoclonal antibody that specifically recognizes Bax oligomer (27). As shown in Figure 8A, Bax oligomer was present only in EGCG-treated MIA PaCa-2 cells, contrary to untreated control. The biochemical analysis also reveals the upregulation of Bax in EGCG-treated MIA PaCa-2 cells (Figure 8B) but not in EGCG challenged non-responsive metastatic pancreatic carcinoma cells (Hs 766T).

Effect of EGCG on the generation of intracellular ROS and apoptosis

The prevailing notion implicates intracellular ROS as signaling intermediates that are involved in signal transduction pathways of apoptosis. In addition, EGCG is also known to exhibit pro-oxidant properties (40,41). On this basis, we examined whether *N*-acetylcysteine (NAC), a distinct antioxidant or ROS scavenger, can exert any effect on EGCG-induced cell

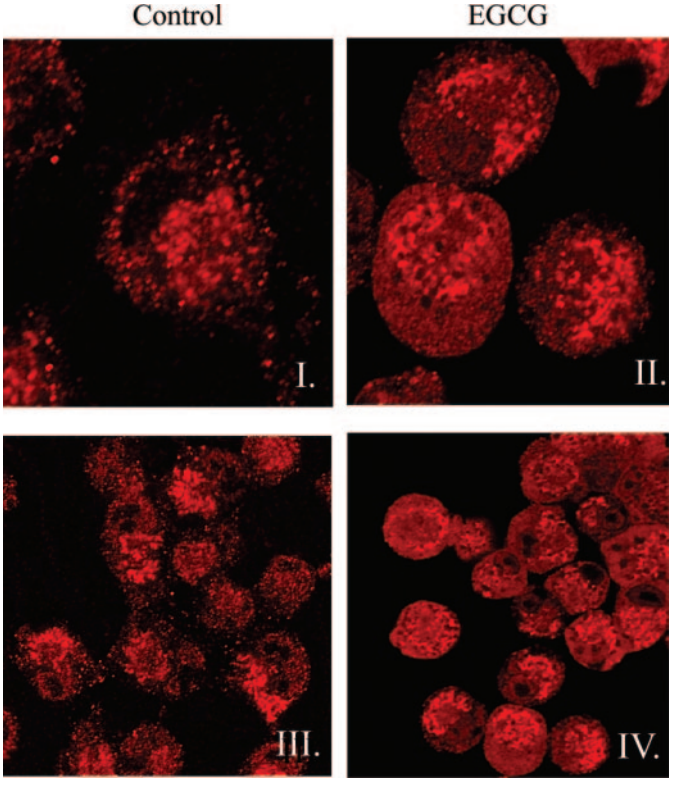

Fig. 7. EGCG-mediated cytochrome *c* release in MIA PaCa-2 cells. Immunofluorescence detection of cytochrome *c*. Panels I and III, control cells; Panels II and IV, EGCG-exposed cells; upper and lower panels represent images at higher and lower magnification, respectively.

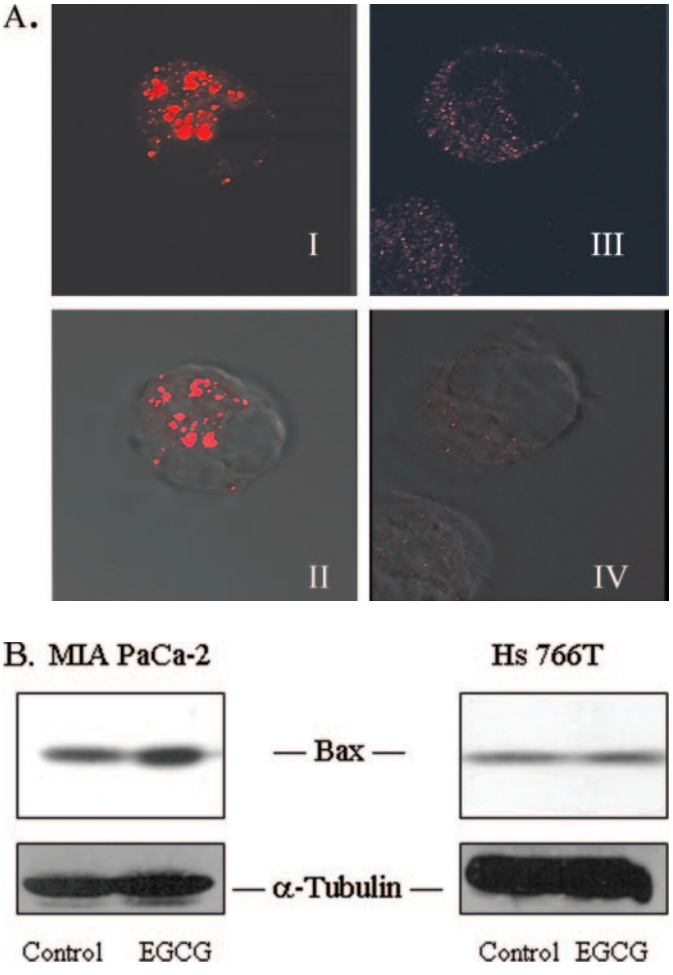

Fig. 8. Oligomerization and upregulation of proapoptotic protein Bax in EGCG-treated MIA PaCa-2 cells. (**A**) Immunofluorescence detection of oligomeric Bax. Panels I and II, 0.1 mM EGCG-treated MIA PaCa-2 cells; Panels III and IV, control cells. It is to be noted that the confocal micrographs indicate the presence of CY3 fluorescence only in EGCG-treated cells (I and II) in contrast to control (III and IV). DIC images (II and IV) confirm the presence of cells in both control and EGCG-treated slides. (**B**) Western blot of EGCG-treated MIA PaCa-2 and Hs 766T cellular extract against Bax polyclonal antibody.

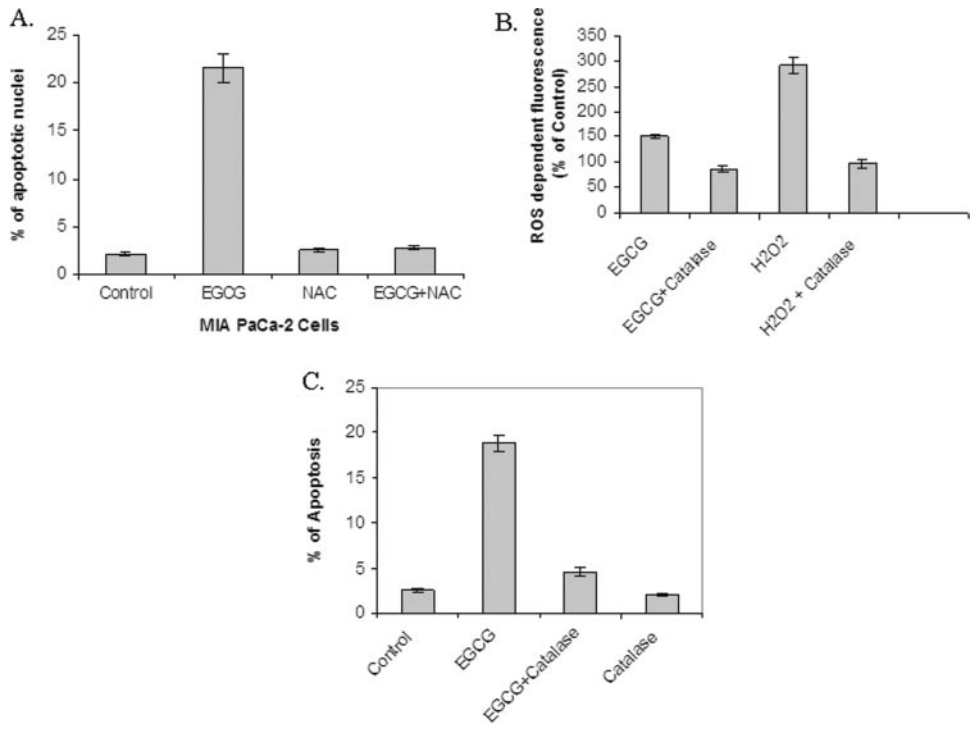

Fig. 9. Effect of EGCG on oxidative burst and apoptosis in MIA PaCa-2 cells. (**A**) Effect of antioxidant NAC was investigated by adding into the media 2 h before EGCG treatment. Assay of cell death was performed as described previously in Figure 3. Control, no treatment; EGCG, 0.1 mM EGCG for 24 h; NAC, 10 mM NAC for 2 h; EGCG + NAC, pre-treatment for 2 h with 10 mM NAC followed by 24-h exposure with 0.1 mM EGCG. Following designated treatment, percentage of apoptotic nuclei was determined by DAPI staining. Owing to pre-treatment with 10 mM NAC, a statistically significant difference was noted in apoptosis index ($P < 0.001$). **(B)** Estimation of hydrogen peroxide following 0.1 mM EGCG exposure. The hydrogen peroxide dependent fluorescence values in EGCG-treated cells were significantly higher than control ($P < 0.01$). 50 U/mL catalase can suppress hydrogen peroxide production in EGCG treated cells ($P < 0.01$). 30 μM hydrogen peroxide was used as a positive control. **(C)** Effect of catalase on EGCG mediated cell death. 50 U/ml catalase was added to the cell culture medium 5 min before the addition of 0.1 mM EGCG. Apoptosis was determined by DAPI staining following 24 h EGCG exposure. Pre-treatment with catalase can significantly block EGCG-induced apoptosis ($P < 0.001$).

death in MIA PaCa-2 cells. For this purpose, cells were subdivided into four different groups for treatment: (a) Control; (b) EGCG alone; (c) EGCG and NAC; (d) NAC alone, respectively. Following pre-treatment with 10 mM NAC, MIA PaCa-2 cells were subjected to 0.1 mM EGCG treatment for 24 h. As shown in Figure 9A, the pre-treatment of NAC can significantly abolish the EGCG-triggered cell death in MIA PaCa-2 cells.

Subsequently, the measurement of oxidative burst revealed ~50% increase in peroxide level following 0.1 mM EGCG exposure, which was attenuated by pre-treatment with 50 U/ml catalase (Figure 9B). It is to be noted that the pre-incubation of cells with the same concentration of catalase completely prevented the 0.1 mM EGCG-induced apoptosis in MIA PaCa-2 cells (Figure 9C).

EGCG-induced JNK activation

Since increased ROS levels were observed to exert the activation of stress kinase JNK (40,41), we were interested to assess the activation status of JNK by immunocomplex kinase assay in the control and EGCG-exposed MIA PaCa-2 cells. Figure 10A clearly demonstrates the EGCG-induced JNK activation in MIA PaCa-2 cells. In order to understand whether JNK activation is necessary for EGCG-mediated cell killing, we next examined whether the blocking of JNK activity exerts any effect on EGCG-induced apoptosis. As demonstrated in Figure 10B, co-incubation with 10 μM JNK inhibitor II rendered the MIA PaCa-2 cells refractory to EGCG-induced apoptosis.

Discussion

Cancer is described as a disease that involves excessive proliferation of cells and abandonment of their ability to die. 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 pancreatic cancer. Our investigation suggests that EGCG has antiproliferative action on pancreatic cancer cells and this antiproliferative action is mediated through programmed cell death or apoptosis. Although some studies (22,23) indicated the antiproliferative action of EGCG in pancreatic cancer, the mechanism of action was never investigated. More importantly, the present study unravels the involvement of mitochondria in EGCG-induced cell death of pancreatic cancer cells. The novelty of our studies is the differential response of different pancreatic cancer cell lines to tea polyphenol (Figure 2). Thus, it would be of value to develop biomarkers of resistance in the course of mechanistic studies and to develop a sense of resistant phenotype. Studies are in progress in the laboratory to understand the mechanism of differential sensitivity of pancreatic cancer cell lines to EGCG.

The results of our studies involving pancreatic cancer are quite concordant with the previous observation that implicates a direct inhibition of antiapoptotic Bcl-2 family proteins (1,2) except the necessity of higher concentration of EGCG in pancreatic cancer cells. The inhibition of Bcl-2 or Bcl-xL by EGCG might result in an upregulation of proapoptotic member

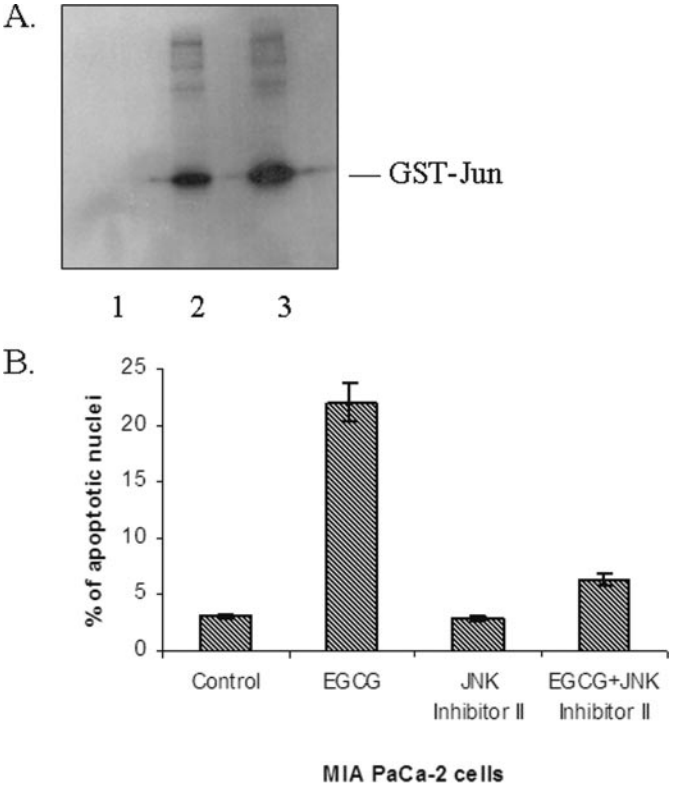

Fig. 10. JNK activation by EGCG and inhibition of EGCG-mediated cell death by JNK inhibitor II. (**A**) Immunocomplex kinase assay in EGCG-treated MIA PaCa-2 pancreatic cancer cells. JNK was immunoprecipitated from control cells and cells exposed to 0.1 mM EGCG for 24 h using antibody against JNK as described in Materials and methods. Lane 1, immunoprecipitation with lysis buffer control; lane 2, immunoprecipitation using lysate from untreated cells; lane 3, immunoprecipitation using lysate from EGCG-treated cells. (**B**) Effect of JNK inhibitor II on EGCG-induced apoptosis. MIA PaCa-2 cells were pre-treated with 10 μM JNK Inhibitor II (Calbiochem) followed by 24-h treatment with 0.1 mM EGCG. Apoptotic nuclei were visualized by DAPI staining. Approximately 500 cells were scored for each category. Results are means ± SD of three independent experiments. It is of note that EGCG-induced apoptosis significantly differ ($P < 0.001$) in JNK inhibitor II pre-treated and untreated cells.

Bax (Figure 8B). In addition, we noted an oligomerization of Bax along with cytochrome *c* release/change in the mitochondrial membrane potential in EGCG-treated pancreatic cancer cells (Figures 7 and 8). The requirement of high concentration of EGCG to suppress cell proliferation or to induce apoptosis in pancreatic cancer cells might be due to the differential cell permeability of EGCG or sequestering of EGCG by other proteins yet to be identified. A previous study by Takada *et al*. (23) demonstrates the suppression of growth inhibition, as well as invasion of pancreatic cancer cells, at 0.1–0.2 mM concentration of EGCG. Treatment with 0.2 mM EGCG resulted in a suppression of the growth of PANC-1 (15.4%) and MIA PaCa-2 (26.0%) cells. It is to be noted that 0.1 mM concentration of EGCG is often required to trigger stress signal in cancer cells.

The prevention of EGCG-mediated apoptosis by JNK inhibitor II and NAC suggests the involvement of ROS-mediated JNK activation in this pathway. The ROS comprise of singlet oxygen, hydroxyl radicals, superoxide, hydroperoxides and peroxides. In our studies, we have observed an increase in hydrogen peroxide level owing to EGCG exposure as noted in the case of lung tumor cell lines (21). Lei *et al*. (42) have indicated that JNK signaling is necessary for the stress-induced release of cytochrome *c* release and programmed cell death. JNK signaling and cytochrome *c* release may be interlinked to a pro-apoptotic member of Bcl-2 family because activated JNK is unable to evoke apoptosis in cells deficient of Bax. Our observation indicates that the concerted efforts of JNK activation and Bax oligomerization might play a pivotal role in the demise of pancreatic cancer cells.

At present, two major pathways that link apoptosis have been identified: (a) intrinsic or mitochondrial and (b) extrinsic or death receptor-related (43–45). The intrinsic pathway involves the cell sensing stress that triggers the mitochondria-dependent processes, resulting in cytochrome *c* release and activation of caspase-9. The extrinsic pathway is triggered by binding of legends such as TNF or FasL to their receptors, leading to the recruitment of adaptor proteins such as FADD and the subsequent activation of procaspase-8. As observed in the case of leukemic cells (46), EGCG-induced apoptosis in some type of cancer cells might be orchestrated by the cooperative effects of both 'extrinsic' and 'intrinsic' pathways.

To summarize, our data suggest that EGCG initiates the cell death process through cell cycle arrest at an earlier phase, as well as the oligomerization of pro-apoptotic regulator Bax, in pancreatic cancer. Perhaps, oligomeric Bax along with other pro-apoptotic members (Bak or Bid) form pores in the mitochondrial membrane to facilitate the release of apoptogenic factors from the mitochondria to cytosol. The permeabilization of the outer membrane is thought to be a major event in releasing proteins such as cytochrome *c* from the intermembrane space. Indeed, EGCG-induced apoptosis in pancreatic cancer cells is accompanied by the mitochondrial membrane depolarization and the release of cytochrome *c* from mitochondria into the cytosol. Cytochrome *c* is bound to the outer surface of the inner membrane phospholipids, primarily to cardiolipin molecules. An early event in the release of cytochrome *c* is its dissociation from the inner mitochondrial membrane, where it is bound to cardiolipin. Mitochondrial ROS can promote cytochrome *c* release to the cytosol by dissociating from cardiolipin (47). Mechanistically, it is possible that ROS generation in mitochondria targets membrane lipid cardiolipin to dissociate cytochrome *c*. In pancreatic cancer cells as observed here, EGCG-mediated ROS production might play such a role. In a variety of cell types, the apoptosis triggering effects of ROS were noted *in vitro* (48,49). Interestingly, a similar apoptosis induction by ROS was noted in *in vivo* animal studies (50,51). In earlier studies using the TgT_{121} transgenic brain tumor model, Salganik *et al*. (50) reported that dietary antioxidant depletion inhibited the de novo tumorigenesis owing to an enhanced tumor-ROS and apoptosis. Subsequently, using a de novo transgenic mouse mammary tumor model, Albright *et al*. (51) demonstrated that dietary antioxidant depletion significantly limits the size of primary mammary tumors. Both primary and metastatic tumor foci exhibited significantly increased, tumor-targeted, oxidative stress and apoptosis in mice fed on an antioxidant-depleted diet. These responses were specific to tumor tissues because no increases in ROS or apoptosis were observed in normal tissue (51).

The very poor prognosis and high mortality that pancreatic cancer patients face results, in part, from our current inability to both identify individuals at increased risk for this disease and detect neoplasms earlier. The elucidation of the mechanism by which existing chemopreventive agents decrease

pancreatic tumor growth should facilitate the establishment of efficacious regimens for the inhibition of human pancreatic carcinoma. Apoptosis in EGCG-exposed pancreatic cancer cells might hold future promise for deploying green tea as a chemopreventive agent. The development of a chemopreventive agent in regular diet is very promising for pancreatic cancer, since this spiteful form of human malignancy is often diagnosed very late. Thus, the induction of the cell death program by the polyphenol constituent of green tea might be helpful in evading the potential malignant outcome of genomic damage in pancreatic cancer.

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