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Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer

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1. ABSTRACT

Epidemiological data suggest that epigallocatechin-3-gallate (EGCG) possesses chemopreventive properties against cancer. In this study, we examined the molecular mechanisms of EGCG in human pancreatic cancer cells. EGCG caused growth arrest at G1 stage of cell cycle through regulation of cyclin D1, cdk4, cdk6, $p21^{/WAF1/CIP1}$ and $p27^{/KIP1}$, and induced apoptosis through generation of reactive oxygen species and activation of caspase-3 and caspase-9. EGCG inhibited expressions of Bcl-2 and Bcl-X_L and induced expressions of Bax, Bak, Bcl-X_S and PUMA. Mouse embryonic fibroblasts (MEFs) derived from Bax and Bak double knockout mice exhibited greater protection against EGCG-induced apoptosis than wild-type or single knockout MEFs. EGCG caused Bax activation in $p53^{-/-}$ MEFs, suggesting that EGCG can induce apoptosis in the absence of p53. Furthermore, the activities of Ras, Raf-1 and ERK1/2 were inhibited, whereas the activities of MEKK1, JNK1/2 and p38 MAP kinases were induced by EGCG. Inhibition of cRaf-1 or ERK enhanced EGCG-induced apoptosis, whereas inhibition of JNK or p38 MAP kinase inhibited EGCG-induced apoptosis. EGCG inhibited the activation of p90 ribosomal protein S6 kinase, and induced the activation of cJUN. Our results suggest that EGCG induces growth arrest and apoptosis through multiple mechanisms, and can be used for pancreatic cancer prevention.

2. INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States. This year, approximately 32,000 Americans will die from cancer of the pancreas. With an overall 5-year survival rate of 3% (1), pancreatic cancer has one of the poorest prognoses among all cancers (2). Aside from its silent nature and tendency for late discovery, pancreatic cancer also shows unusual resistance to chemotherapy and radiation. Only 20% of pancreatic cancer patients are eligible for surgical resection, which currently remains the only potentially curative therapy (3). The operations are very complex, and unless performed by surgeons specially trained and experienced in this procedure, they can be associated with very high rates of post-operative morbidity and mortality. Unfortunately, many cancers of the pancreas are not resectable at the time of diagnosis. There are limited treatment options available for this disease because chemo- and radio-therapies are largely ineffective, and metastatic disease frequently redevelops even after surgery. Therefore, there is an urgent need to discover novel and effective chemopreventive approaches for pancreatic cancer.

There is clearly a great need to improve our understanding of the fundamental nature of cancer of the

pancreas. Ductal cancer of the pancreas putatively evolves through multistage neoplastic transformation processes that are reflected in a series of histologically well-defined precursor lesions termed pancreatic intraepithelial neoplasias (PanINs) (4). On the molecular level, the interplay between different signaling pathways remains an area of active investigation. Mutations in the K-ras gene occur early, the inactivation of the $p16^{INK4A}$ gene at intermediate stages, and the inactivation of p53 and DPC/Smad4 at a relatively late stage (5, 6). On the tissue level, the cell type that gives rise to ductal adenocarcinoma is not well understood. Proposed cellular origins for pancreatic carcinoma include duct cells (4, 7), islet cells (8, 9), acinar cells (10-12), or rare undifferentiated precursor cells (13). Centroacinar cells have emerged as a candidate cell of origin based upon the persistent activation of the Notch pathway in these cells in adulthood (14). Although centroacinar cells constitute the terminal cells of the ductal system and contain ultrastructural features of ductal cells, the precise lineage of centroacinar cells has not yet been elucidated. These genetic alterations are found in pancreatic ductal adenocarcinoma (PDA), thus supporting the concept that PanINs are precursors of the invasive tumors. Recently developed transgenic (K-ras, Shh, HB-EGF, and TGFalpha) or knockout (PTEN) mice models (e.g.) provide new insights into the development of human pancreatic cancer. However, the strengths of these models for pancreatic cancer preventions have not been examined.

Pancreatic cancer becomes clinically apparent at late stages and it resists all forms of conventional chemotherapy and radiotherapy (1, 2). Therefore, understanding the pathogenesis of the preinvasive stage, and developing effective strategies to prevent pancreatic neoplasms are of paramount importance. A polyphenolic constituent, (-)-epigallocatechin-3-gallate (EGCG), is the major and most effective chemopreventive agent in green tea. EGCG has been shown to exert a growth-suppressive effect on human pancreatic cancer cells *in vitro* (15, 16). It induces cell cycle arrest and apoptosis in pancreatic cancer cells and thus holds great promise for development as a chemopreventive agent. Unfortunately, the intracellular mechanisms by which EGCG inhibits proliferation and induces apoptosis in pancreatic cancer cells are not fully understood.

The objective of this paper is to examine the molecular mechanisms by which EGCG regulates MAP kinase signaling pathway, disrupts mitochondrial homeostasis, inhibits growth, and induces apoptosis in pancreatic cancer cells. Our data show that EGCG inhibits cell proliferation through induction of cell cycle inhibitors $p21^{/WAF1/CIP1}$ and $p27^{/KIP1}$, and induces apoptosis through activation of JNK and p38, and inhibition of ERK MAP kinase pathways. Furthermore, EGCG regulates Bcl-2 family members, generates reactive oxygen species (ROS), and releases mitochondrial cytochrome c and Smac/DIABLO. Thus, EGCG may regulate pancreatic cancer growth through activation of multiple signaling pathways.

3. MATERIALS AND METHODS

3.1. Reagents

Antibodies against $p21^{/WAF1/CIP1}$, $p27^{/KIP1}$, Bcl-2, Bcl-X_L, Bax, Bak, MEKK1, Ras, cdk4, cdk6, cyclin D1 and beta-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Raf-1 antibody, N-acetylcysteine (NAC), Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay kit, inhibitors of cRaf-1 (5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone), ERK (PD098059), JNK (SP600125) and p38 (SB203580) MAP kinases, and caspase-3 and caspase-9 activity kits were purchased from EMD Biosciences (San Diego, CA). Antibodies against phospho ERK, phospho JNK, phospho p38, total JNK, total ERK, total p38, phospho cJUN and phospho p90 ribosomal protein S6 kinases (p90 RSK) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-cytochrome c and anti-Smac/DIABLO antibodies were purchased from BD Biosciences (San Diego, CA). Bax siRNA and control plasmids were purchased from BioVision, Inc. (Mountain View, CA). JC-1, and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) were purchased from Invitrogen / Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence (ECL) Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). EGCG was purchased from LKT Laboratories, Inc. (St. Paul, MN).

3.2. Cell Culture

PANC-1, MIA PaCa-2, Hs 766T and AsPC-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen) at 37oC in a humidified atmosphere of 95% air and 5% CO_2. For biochemical analyses, cells were collected by rinsing in phosphate buffered saline (PBS) thrice, scraping with a rubber policeman in 10-ml PBS, and then centrifuging at 700 g for 5 minutes. After removing the PBS, the cell pellets were either used immediately or stored at -80oC until use.

3.3. XTT Assay

Cells (1 X 10^4 in 200 microliters culture medium per well) were seeded in 96-well plate (flat bottom), treated with or without drugs and incubated for various time points at 37^0C and 5% CO_2. Before the end of the experiment, 50 microliters XTT labeling mixture [final concentration, 125 microM XTT (sodium 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) and 25 microM PMS (phenazine methosulphate)] per well was added and plates were incubated for further 4 h at 37^0C and 5% CO_2. The spectrophotometric absorbance of the sample was measured using a microtitre plate (ELISA) reader. The wavelength to measure absorbance of the formazon product was 450 nm, and the reference wavelength was 650 nm.

3.4. Transient transfection

Cells were plated in 60-mm dishes in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture at a density of 1 X 10^6 cells/dish. The next day

transfection mixtures were prepared. For siRNA experiment, PANC-1 cells were transiently transfected with plasmids expressing Bax siRNA (pGB-Bax siRNA) or control siRNA (pGB-control) in the presence of pCMV-LacZ (Invitrogen life technologies) vector expressing beta-galactosidase to control transfection efficiency. For each transfection, 2 micrograms of DNA was diluted into 50 microliters of medium without serum. After the addition of 3 microliters of LipofectAMINE (Invitrogen life technologies) into 50 microliters Opti-MEM medium, the transfection mixture was incubated for 10 min at room temperature. Cells were washed with serum-free medium, the transfection mixture was added, and cultures were incubated for 24 h. The next day, culture medium was replaced with fresh RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin mixture and EGCG was added for desired times. At the end of incubation, cells were washed with ice-cold PBS and lysed in RIPA buffer.

3.5. Western blot analysis

Cell pellets were lysed in RIPA buffer containing 1 X protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA). Cell lysates were electrophoresed in 12.5% SDS polyacrylamide gels and then transferred onto nitrocellulose membranes. After blotting in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies at 1:1,000 dilution in TBS-Tween 20 overnight at 4oC, and then secondary antibodies conjugated with horseradish peroxidase at 1:5,000 dilution in TBS-Tween 20 for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system.

3.6. Measurement of cell cycle and apoptosis

For cell cycle analysis, cells were harvested, fixed in 85% ethanol, and stained with 1 microgram/milliliter propidium iodide (PI) in phosphate-buffered saline with 0.5% Nonidet P-40 and RNase A. PI stained cells were analyzed using a Beckton Dickson FACStar flow cytometer. The cell cycle distribution was calculated using ModFit LT.

Apoptosis was measured by the terminal deoxynucleotidyl transferase-mediated nick end-labeling method which examines DNA strand breaks during apoptosis. Briefly, 1 x 10^5 cells were treated with EGCG at the indicated doses for various time points at 37oC. Thereafter, cells were washed with PBS, air-dried, fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells were incubated with reaction mixture for 60 minutes at 37oC. Stained cells were mounted and analyzed under a fluorescent Olympus microscope (Olympus America Inc, Melville, NY). Pictures were captured using a Photometrics Coolsnap CF color camera (Olympus) and SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI).

3.7. Caspase Activity Assay

Cells (3 x 10^4 per well) were seeded in a 96-well plate with 200 microliters culture medium. Approximately 16 h later, cells were treated with various doses of EGCG to induce apoptosis. Casapse-3 or caspase-9 activity was measured as per manufacturer's instructions (EMD Biosciences) with a fluorometer.

3.8. Cellular Fractionation

Cellular fractions were prepared as we described elsewhere (17). The protein concentrations were determined by Bradford method (Bio-Rad, Hercules, CA).

3.9. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by retention of JC-1 dye (Molecular Probes Inc., Eugene) as we described earlier (17, 18). In brief, EGCG treated cells (5 X 10^5) were loaded with JC-1 dye (1 microgram/ml) during the last 30 min of incubation at 37 ^{0}C in a 5% CO_2 incubator. Cells were washed in PBS twice. Fluorescence was monitored in a fluorometer using 570-nm excitation/595-nm emission for the J-aggregate of JC1. $\Delta\Psi_m$ was calculated as a ratio of the fluorescence of J-aggregate (aqueous phase) and monomer (membrane-bound) forms of JC1.

3.10. Measurement of reactive oxygen species

Cells were seeded in 96-well plates. After 16 h, cells were loaded with 5 microM CM-H_2DCFDA dye for 30 min, and treated with either EGCG (10 or 20 microM) or 0.05% DMSO for 0-360 min. Fluorescence was measured at excitation wavelength of 488 nm and emission wavelength of 515 nm using a fluorescence plate reader.

3.11. Bax Immunocytochemistry

Cells were grown on coverslips (Beckton Dickinson, Bedford, MA), washed in PBS, and fixed for 15 min in 4% paraformaldehyde. Cells were permeabilized in 0.1% Triton X-100, washed and blocked in 10% normal goat serum. Cells were incubated with anti-cytochrome c or anti-Bax antibody (1:200) for 18 h at 4 ^{0}C. Cells were then washed and incubated with fluorescently labeled secondary antibodies (1:200) along with DAPI (1 microgram/ml) for 1 h at room temperature. Cells were washed and coverslips were mounted using Vectashield (Vector Laboratories, Burlington, CA). Isotype-specific negative controls were included with each staining. Stained cells were mounted and visualized under a fluorescent Olympus microscope (Olympus America Inc.). Pictures were captured using a Photometrics Coolsnap CF color camera (Olympus) and SPOT software (Diagnostic Instruments Inc.).

3.12. Statistical analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one or two way ANOVA. Significant differences among groups were calculated at $P < 0.05$.

4. RESULTS

4.1. EGCG inhibits cell growth in human pancreatic cancer cell lines

We first examined the effects of EGCG on cell proliferation in four human pancreatic cancer cell lines by XTT assay. EGCG inhibited cell viability in all the four cell lines with varying sensitivity (Figure 1). PNAC-1 and MIA PaCa-2 cell lines were most sensitive, AsPC-1 cell line was moderately sensitive, and Hs 766T cell line was least sensitive.

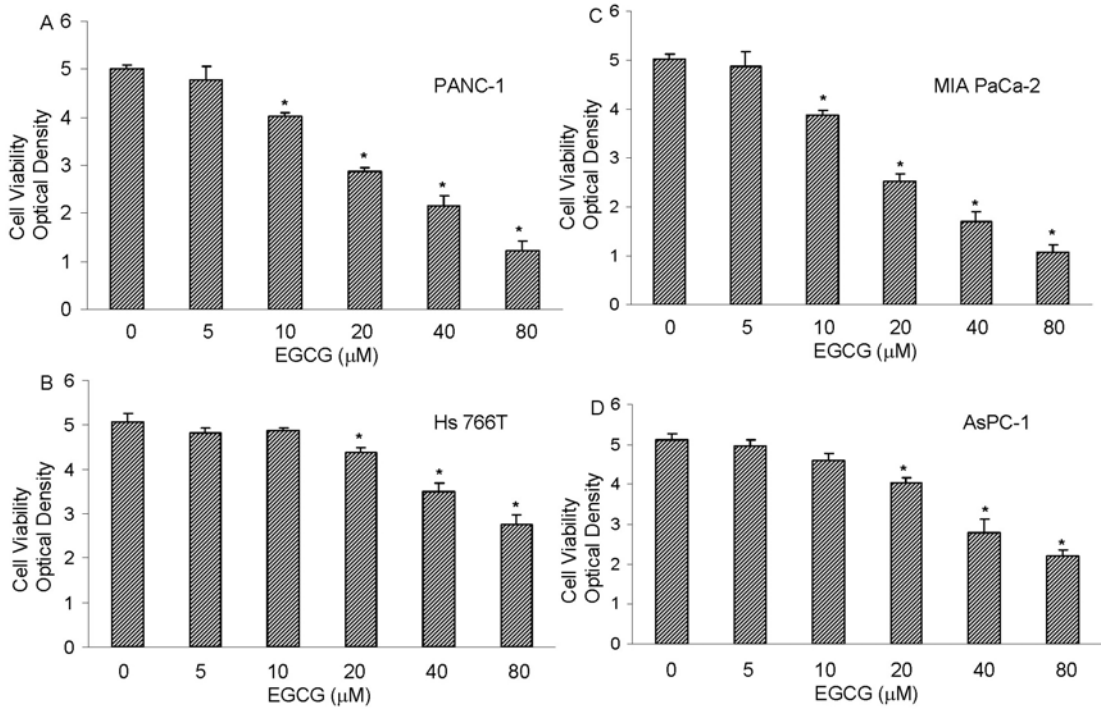

Figure 1. Effect of EGCG on viability of pancreatic cancer cells. (A-C), Pancreatic cancer (PNAC-1, MIA PaCa-2, Hs766T and AsPC-1) cells were treated with various doses of EGCG (0-80 μM) for 24 h. Cell viability was measured by XTT assay. Data represent the mean ± S.D. * = significantly different from respective controls, $P < 0.05$.

These data suggest that EGCG can be a viable chemopreventive agent for inhibiting pancreatic cancer cell proliferation.

4.2. EGCG induces caspase-3 activation in human pancreatic cancer cell lines

Most chemopreventive agents induce apoptosis through activation of caspase cascade. We therefore examined whether EGCG-induced apoptosis requires caspase-3 activation in pancreatic cancer cell lines (Figure 2). EGCG induced caspase-3 activation in PANC-1, MIA PaCa-2, Hs 766T and AsPC-1 cells. However, high doses (40 and 80 microM) of EGCG were required to activate caspase-3 in Hs 766T cells compared to other pancreatic cancer cell lines. These data suggest that EGCG induces apoptosis through caspase-3 activation and may engage the mitochondrial pathway of apoptosis.

4.3. EGCG regulates Bcl-2 family members

Bcl-2 family of proteins are known to regulate apoptosis in response to a wide variety of stress stimuli (19-21). Several studies including ours have demonstrated that the overexpression of Bcl-2 or Bcl-X_L causes cancer cells to resist chemotherapy (22-24). Therefore, we sought to examine the regulation of Bcl-2 family members by EGCG (Figure 3). EGCG inhibited expression of antiapoptotic Bcl-2 and Bcl-X_L, and induced expression of proapoptotic Bax, Bak, Bcl-X_S and PUMA in pancreatic cancer PANC-1 cells (Figure 3A). Induction of Bax, Bak and PUMA by EGCG suggests that these proteins may cause disruption of mitochondrial homeostasis.

The translocation of proapoptotic protein Bax from cytosol to mitochondria is essential for Bax and Bak oligomerization, and the subsequent release of mitochondrial proteins (17, 25, 26). In healthy cells, Bax is found in the cytoplasm, whereas in response to cellular stress it is activated and translocated to mitochondria. We therefore examined the activation / conformational change of Bax with a monoclonal antibody (6A7) which recognizes activated Bax by immunoprecipitation (Figure 3B). This antibody recognizes an epitope at the amino terminus of the protein, which becomes exposed only after a change in conformation of Bax. The immunoprecipated complex was then subjected to SDS-PAGE followed by immunoblotting using anti-Bax polyclonal antibody. The treatment of PANC-1 cells with EGCG resulted in activation of Bax and the formation of monomers and dimers. We confirmed these data with immunohistochemistry where the translocation of Bax to the mitochondria was examined (Figure 3C). The data revealed that in control cells Bax was mainly in the cytoplasm, as evident by distinct red (mitochondria) and green (Bax) colors. The treatment of PANC-1 cells with EGCG resulted in translocation of Bax to mitochondria (appearance of yellow mitochondria), suggesting that EGCG causes activation and translocation of Bax to mitochondria and this event may disrupt mitochondrial homeostasis.

We next examined the contribution of Bax in EGCG-induced apoptosis by inhibiting Bax expression using RNAi technology (Figure 3D). We have previously used this Bax siRNA expression vector which inhibited about 90% Bax expression (27). As before, EGCG induced

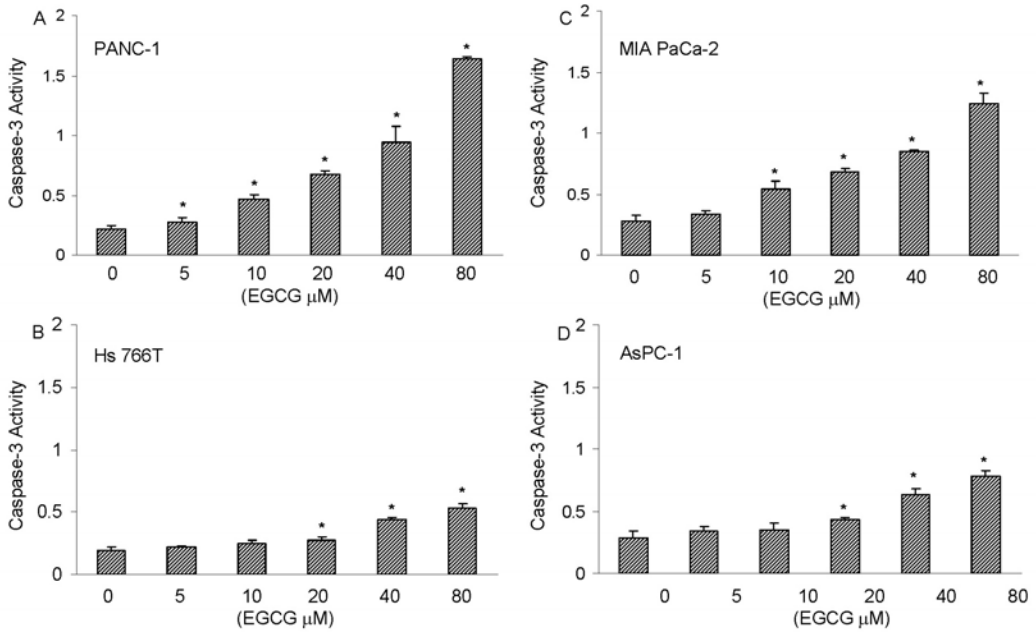

Figure 2. Effect of EGCG on caspase-3 activity. (A-C), Pancreatic cancer PANC-1, MIA PaCa-2, Hs 766T and AsPC-1 cells were treated with EGCG (0-80 μM) for 12 h and caspase-3 activity was measured as per manufacturer's instructions (EMD Biosciences). Data represent the mean ± S.D. * = significantly different from respective controls, $P < 0.05$.

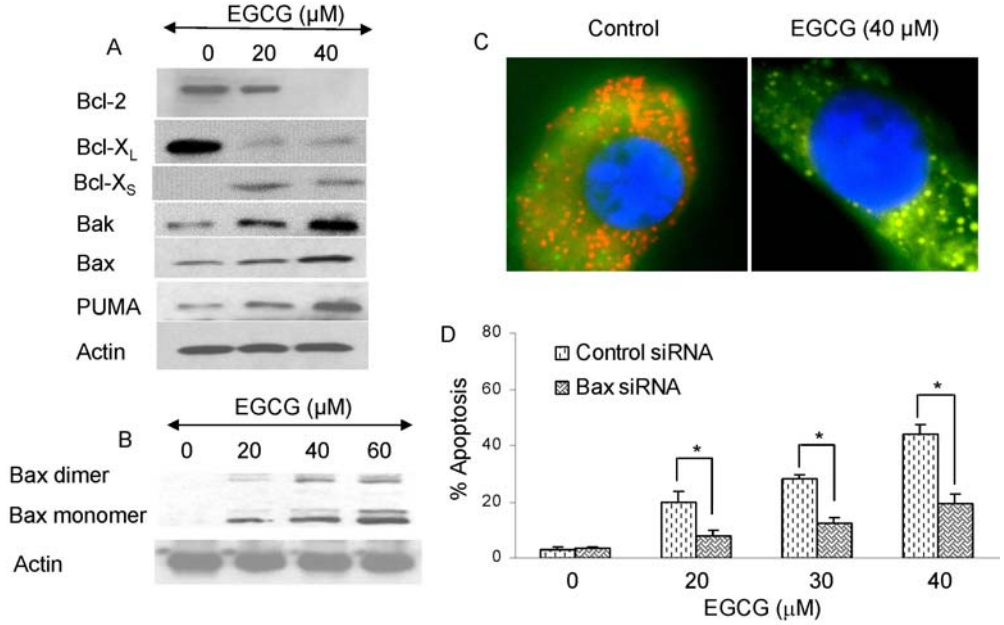

Figure 3. Effects of EGCG on Bcl-2 family members. (A), Pancreatic cancer PANC-1 cells were treated with EGCG (0-40 μM) for 48 h. The expression of Bcl-2, Bcl-X_L, Bcl-X_S, Bak, Bax and PUMA was measured by Western blotting. Anti β-actin antibody was used as a loading control. (B), Analysis of conformational change of Bax using lysates from PANC-1 cells treated with EGCG (0-60 μM) for 8 h. Bax was immunoprecipitated from equal amounts of lysates proteins using anti-Bax monoclonal antibody 6A7. Immunoprecipitated complexes were subjected to SDS-PAGE followed by immunoblotting using anti-Bax polyclonal antibody. (C), Immunohistochemistry for analysis of Bax localization. PANC-1 cells were treated for 8 h with DMSO or 40 μl EGCG. Cells were then stained with anti-Bax antibody (green fluorescence), Mitotracker Red (red fluorescence), and DAPI (blue fluorescence). Merged images are shown, which indicate yellow-orange staining of mitochondria in EGCG-treated PANC-1 cells due to merge of green and red fluorescence. Mitochondria in DMSO-treated control were stained red. (D), Effects of Bax inhibition by Bax siRNA on EGCG-induced apoptosis. PANC-1 cells were transiently transfected with either control plasmid or plasmid expressing Bax siRNA along with plasmid (pCMV-LacZ) encoding the β-galactosidase (β-*Gal*) enzyme. There was no difference in transfection efficiency between groups. Transfected cells were treated with or without EGCG (20 or 40 μM) for 48 h, and apoptosis was measured by TUNEL assay.

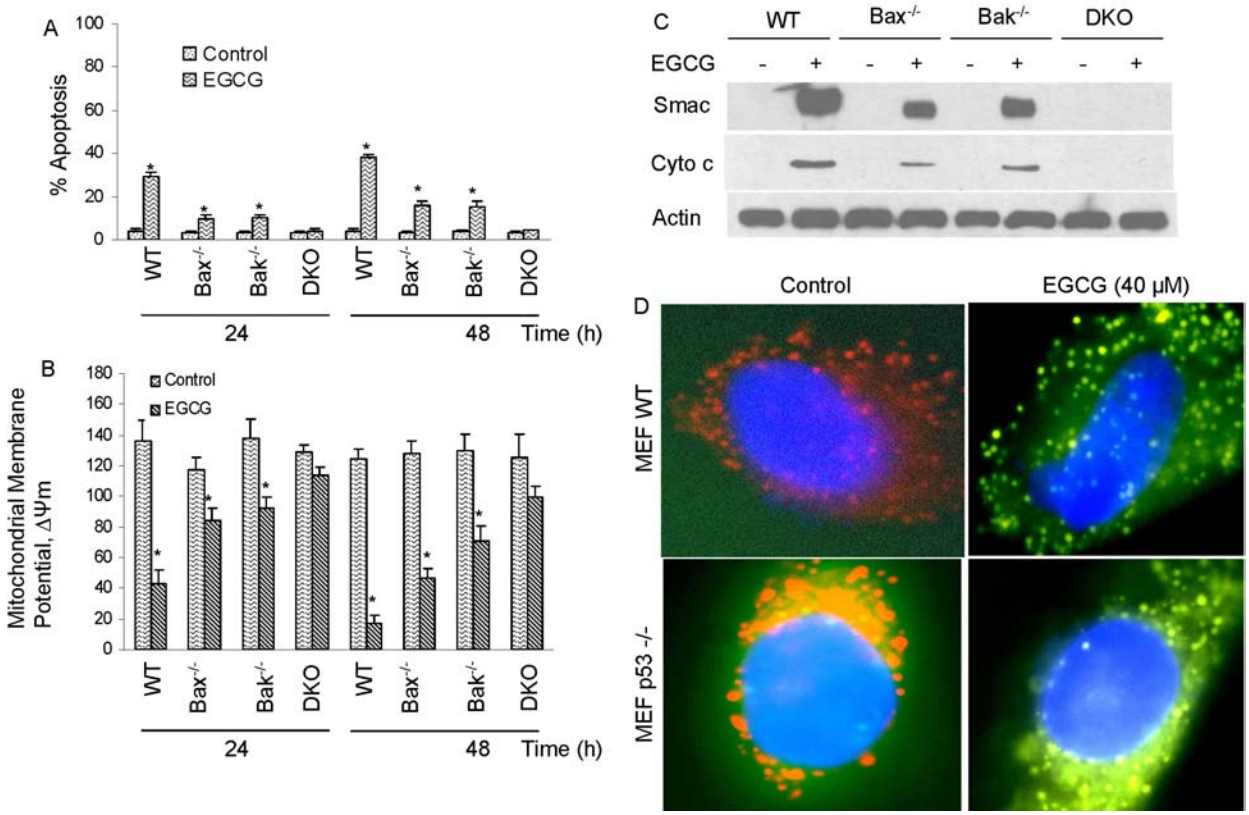

Figure 4. MEFs derived from Bax and Bak double knockout mice were resistant to EGCG-induced apoptosis. (A), Wild-type, Bax $^{-/-}$, Bak $^{-/-}$, and Bax$^{-/-}$ and Bak$^{-/-}$ double knockout MEFs were treated with or without 40 μM EGCG for 24 and 48 h, and apoptosis was measured by DAPI staining. Data represent mean ± SD. * = Significantly different from respective control. (B), Effects of EGCG on mitochondrial membrane potential. Wild-type, Bax $^{-/-}$, Bak $^{-/-}$, and Bax$^{-/-}$ and Bak$^{-/-}$ double knockout MEFs were treated with or without 40 μM EGCG for 24 and 48 h, and mitochondrial membrane potential was measured as described in Materials and Methods. Data represent mean ± SD. * = Significantly different from respective control. (C), EGCG treatment caused release of apoptogenic molecules from mitochondria to cytosol in Wild-type, Bax $^{-/-}$, and Bak $^{-/-}$ MEFs, but not in Bax$^{-/-}$ and Bak$^{-/-}$ double knockout MEFs. Immunoblotting of cytochrome c and Smac/DIABLO using cytosolic fractions from wild-type MEFs treated with 40 μM EGCG. (D), EGCG causes translocation of Bax in p53$^{-/-}$ mouse embryonic fibroblasts (MEF p53$^{-/-}$). Wild type MEF (MEF WT) and MEF p53$^{-/-}$ were treated for 8 h with DMSO or 40 μl EGCG. Cells were then stained with anti-Bax antibody (green fluorescence), mitotracker red (red fluorescence), and DAPI (blue fluorescence). Merged images are shown, which indicate yellow-orange staining of mitochondria in EGCG-treated MEFs due to merge of green and red fluorescence. Mitochondria in DMSO-treated control were stained red.

apoptosis in PANC-1 cells in a dose-dependent manner. Inhibition of Bax by siRNA inhibited more than 50 % Bax-induced apoptosis. These data suggest that Bax plays an important role in EGCG-induced apoptosis in pancreatic cancer cells.

4.4. Bax $^{-/-}$ and Bak $^{-/-}$ double knockout MEFs are resistant to apoptosis by EGCG

We and others have shown that Bax and Bak genes play a major role in apoptosis by regulating the release of mitochondrial proteins such as cytochrome c and Smac/DIABLO (17, 28). We also demonstrated that Bax$^{-/-}$ and Bak$^{-/-}$ double knockout MEFs were completely resistant to TRAIL-, curcumin- and resveratrol-induced apoptosis (17, 27, 29). To investigate the role of BAX and BAK in EGCG-induced apoptosis, MEFs from wild-type, BAX$^{-/-}$, BAK$^{-/-}$, or Bax- and Bak-deficient (double knockout) mice were used. As shown in Figure 4, EGCG induced apoptosis in wild type MEFs at 24 h; and these effects of EGCG on apoptosis were further enhanced at 48 h. EGCG induced apoptosis was significantly inhibited in Bax$^{-/-}$ MEFs or Bak$^{-/-}$ MEF compared to wild type MEF at 24 and 48 h. Interestingly, loss of Bax and Bak genes completely inhibited EGCG-induced apoptosis in Bax$^{-/-}$ and Bak$^{-/-}$ double knockout MEFs.

During apoptosis, engagement of the mitochondrial pathway involves the permeabilization of the outer mitochondrial membrane (OMM), which leads to the release of mitochondrial proteins such as cytochrome c and Smac/DIABLO (29, 30). OMM permeabilization depends on activation, translocation and oligomerization of multidomain Bcl-2 family proteins such as Bax or Bak. Thus, mitochondria can be activated by a variety of signals and can integrate them to trigger a process called

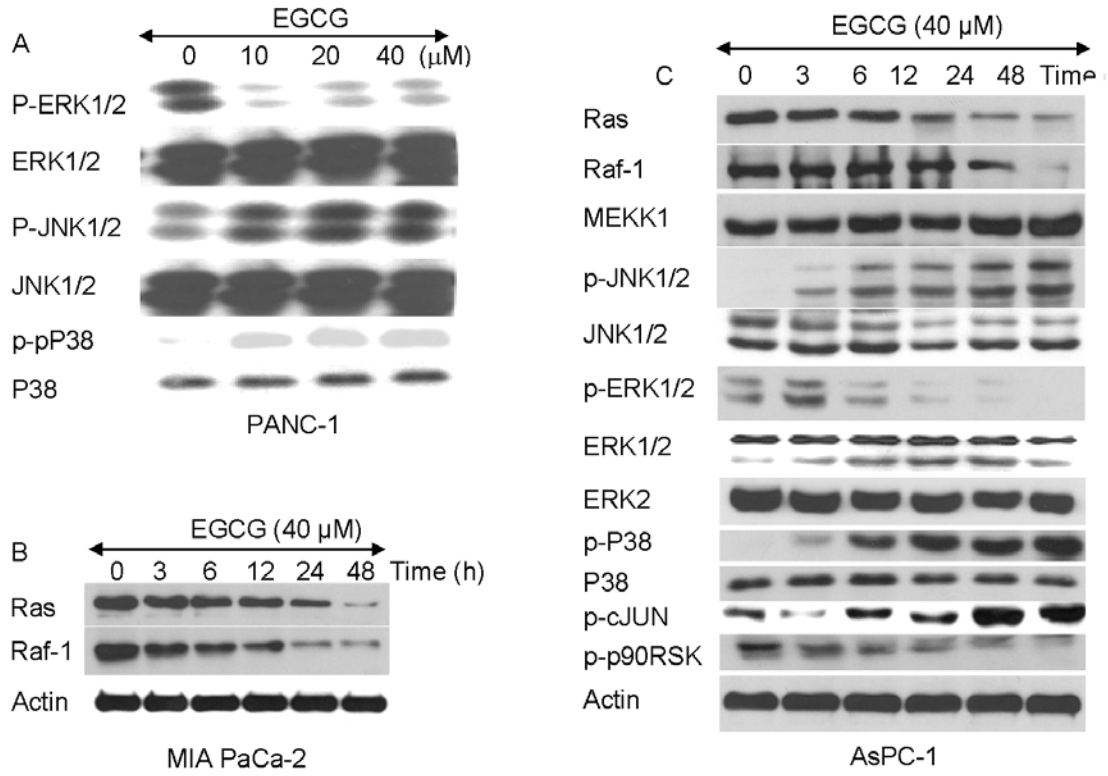

Figure 5. Effects of EGCG on MAP kinase pathway. (A), PANC-1 cells were treated with EGCG (0- 40 μM) for 12 h, and the expression of ERK1/2, JNK1/2 and p38 (phosphorylated and total) was measured by Western blot analysis. (B), MIA PaCa-2 cells were treated with EGCG (40 μM) for 0-48 h, and the expression of Ras and Raf-1 was measured by Western blot analysis. β-actin was used as a loading control. (C) AsPC-1 cells were treated with EGCG (40 μM) for 0-48 h, and the expression of Ras, Raf-1, MEKK1, JNK1/2 (phospho and total), ERK1/2 (phospho and total), p38 (phospho and total), phospho-JUN, and phospho-p90RSK was measured by Western blot analysis. β-actin was used as a loading control.

mitochondrial membrane permeabilization (MMP), which induces the ultimate events of apoptosis. We therefore assessed the loss of mitochondrial membrane potential in MEFs (Figure 4B). EGCG induced a massive drop in mitochondrial membrane potential in wild type MEF at 24 and 48 h. EGCG had little but significant effect in dropping mitochondrial membrane potential in $Bax^{-/-}$ or $Bak^{-/-}$ MEFs. By comparison, mitochondrial membrane potential did not change in DKO MEFs at 24 and 48 h.

Loss of membrane potential leads to opening of the permeability transition pore leaking the inner components (e.g. cytochrome c and Smac/DIABLO) into cytosol, which provide the executing signals for apoptosis. We therefore measured the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol in MEFs treated with EGCG (Figure 4C). Treatment of wild type MEFs with EGCG resulted in the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol. EGCG also caused the release of cytochrome c and Smac/DIABLO in $Bax^{-/-}$ and $Bak^{-/-}$ MEFs, but significantly lower than wild type MEFs. Interestingly, EGCG had no effect on the release of mitochondrial cytochrome c and Smac/DIABLO in DKO MEFs.

We have recently shown that mitochondrial p53 may, in part, be responsible for disrupting mitochondrial homeostasis in prostate cancer cells (31). We therefore examined whether translocation of Bax to mitochondria is dependent of p53 using MEF wild type and MEF $p53^{-/-}$. In DMSO treated wild type MEFs, the Bax staining was restricted to the cytosol (Figure 4D). On the other hand, the mitochondria in EGCG-treated MEFs were stained yellow-orange due to merge of green fluorescence (Bax immunostaining) and red fluorescence (mitotracker red staining), indicating translocation of Bax from cytosol to the mitochondria. Similar to wild type MEF, in untreated $p53^{-/-}$ MEFs, Bax was located in the cytoplasm. Interestingly, the translocation of Bax to mitochondria was also noticed in MEF $p53^{-/-}$. These data suggest that the deletion of $p53^{-/-}$ in MEFs had no effect on translocation of Bax to mitochondria, and EGCG induced apoptosis in the presence or absence of wild type p53.

4.5. EGCG regulates MAP kinase pathways

MAP kinase pathways regulate many cellular activities, which range from gene expression to mitosis, movement, metabolism, and apoptosis (32-36). We therefore examined the effects of EGCG on the expression of Ras, Raf-1, MEKK1 and activation/expression of

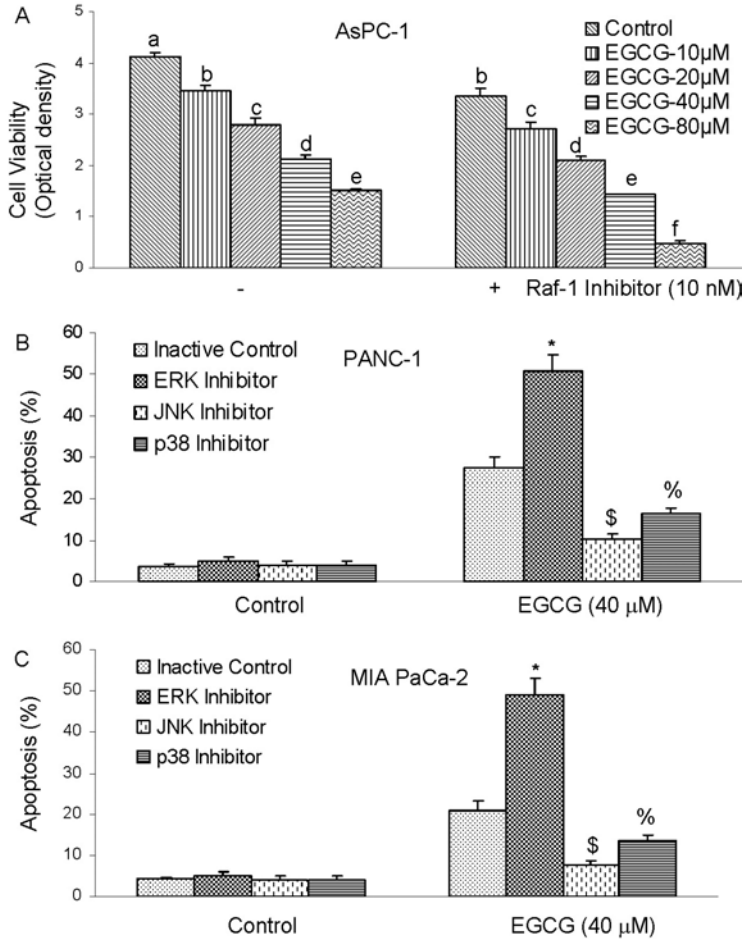

Figure 6. Regulation of MAP kinase pathway by inhibitors of Raf-1, ERK, JNK and p38 MAPK. (A), Effects of Raf-1 inhibitor on EGCG-induced cell viability. AsPC-1 cells were pretreated with cRaf-1 kinase inhibitor (10 nM) for 4 h, followed by treatment with EGCG (40 μM) for 48 h. Cell viability was measured by XTT assay. Data represent the mean ± S.D. a, b, c, d, e and f = significantly different from each other, $P < 0.05$. (B and C), Regulation of EGCG-induced apoptosis by inhibitors of MAP kinases. PANC-1 and MIA PaCa-2 cells were pretreated with inhibitors of ERK (5 μM), JNK (50 nM) or p38 (400 nM) for 4 h, followed by treatment with EGCG (40 μM) for 48 h. Cells were harvested and apoptosis was measured by TUNEL assay. Data represent the mean ± S.D. *, $ or % = significantly different from respective controls, $P < 0.05$.

ERK1/2, JNK1/2 and p38 MAP kinases (Figure 5). Treatment of PANC-1 cells with EGCG caused a decrease in ERK1/2 phosphorylation, and an increase in JNK1/2 and p38 phosphorylation (Figure 5A). Since Ras and Raf-1 act upstream of ERK, we examined to effect of EGCG on the activity/expression of Ras and Raf-1. EGCG inhibited the activity/expression of Ras and Raf-1 in MIA PaCa-2 cells (Figure 5B). Similarly, EGCG inhibited the activities / expressions of Ras, Raf-1 and ERK1/2, and induced activities/expression of MEKK1, JNK1/2 and p38 in AsPC-1 cells (Figure 5C). EGCG also inhibited the phosphorylation / activation of p90 RSK, and induced the phosphorylation / activation of cJUN, as measured by phospho-specific antibodies. These data suggest that EGCG inhibits growth and induces apoptosis through regulation of MAP kinase pathways.

In order to understand the roles of MAP kinases in antiproliferative effects of EGCG, we used specific inhibitors of Raf-1, ERK, JNK and p38 MAP kinases. As shown in Figure 6A, EGCG inhibited cell viability in AsPC-1 cells in a dose-dependent manner. Raf-1 inhibitor slightly inhibited cell viability. The inhibition of Raf-1 significantly enhanced the inhibitory effect of EGCG on cell viability. We next examined the effects of ERK, JNK or p38 inhibitors on EGCG-induced apoptosis (Figure 6B and C). ERK, JNK or p38 inhibitors alone had no effect on apoptosis of PANC-1 and MIA PaCa-2 cells (Figure 6C and D). Treatment of both pancreatic cancer cell lines with EGCG resulted in apoptosis. While ERK inhibitor enhanced the apoptosis-inducing potential of EGCG, inhibition of JNK interfered with EGCG-induced apoptosis. By comparison, p38 inhibitor had marginal effect on EGCG-induced apoptosis in PANC-1 and MIA PaCa-2 cell lines. These data suggest that EGCG mediates cell growth and apoptosis primarily through activation of JNK and inhibition of ERK pathways.

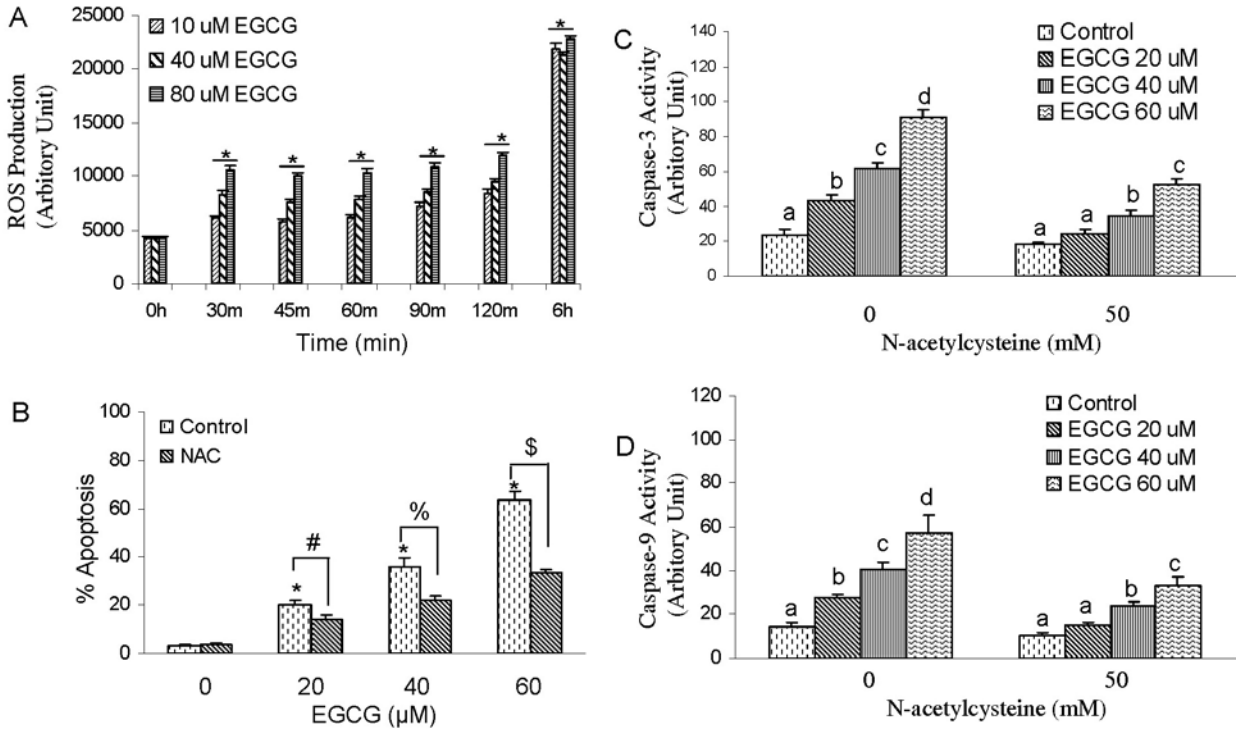

Figure 7. Involvement of reactive oxygen species in EGCG-induced caspase-3 and caspase-9 activities, and apoptosis. (A), Generation of ROS by EGCG. PANC-1 cells were seeded in 96-well plates, loaded with 5 μM CM-H_2DCFDA dye for 30 min, and treated with EGCG (10, 40 and 80 μM) for 0-360 min. Fluorescence was measured by a fluorometer as per manufacturer's instructions (EMD Biosciences / Molecular Probes). * = significantly different from respective controls, $P < 0.05$. (B), Inhibition of EGCG-induced apoptosis by N-acetylcysteine (NAC). PANC-1 cells were pretreated with or without 50 mM NAC for 2 h followed by treatment with EGCG (0, 20, 40 or 60 μM) for 48 h, and apoptosis was measured by TUNEL assay. * = significantly different from control; $, % or & = significantly different between groups, $P < 0.05$. (C), Inhibition of EGCG-induced caspase-3 activity by NAC. PANC-1 cells were pretreated with 50 mM NAC for 2 h followed by treatment with EGCG (0, 20, 40 or 60 μM) for 12 h, and caspase-3 activity was measured by a fluorometer as per manufacturer's instructions. a, b, c and d = significantly different from each other, $P < 0.05$. (D), Inhibition of EGCG-induced caspase-9 activity by NAC. PANC-1 cells were pretreated with or without 50 mM NAC for 2 h followed by treatment with EGCG (0, 20, 40 or 60 μM) for 12 h, and caspase-9 activity was measured by a fluorometer as per manufacturer's instructions. a, b, c and d = significantly different from each other, $P < 0.05$.

4.6. Generation of reactive oxygen species (ROS) by EGCG

It is well known that the generation of ROS by oxidative damage plays an important role in apoptosis (37, 38). We have shown that chemopreventive agents such as curcumin and resveratrol induce apoptosis in cancer cells through generation of ROS (27, 29, 31). We therefore examined whether EGCG-induced apoptosis is regulated by ROS. Treatment of PANC-1 cells with EGCG resulted in ROS production which increased at 30 min, and reached a maximum level at 360 min (Figure 7A). Pretreatment of PANC-1 cells with N-acetylcysteine (NAC) inhibited EGCG-induced apoptosis (Figure 7B). Since EGCG induced caspase-3 activity and apoptosis, we next examined whether generation of ROS was responsible for caspase-3 and caspase-9 activation. EGCG induced caspase-3 and caspase-9 activities in a dose-dependent manner (Figure 7C and 6D). Pretreatment of PANC-1 cells with NAC followed by EGCG treatment caused a significant inhibition of EGCG-induced caspase-3 (Figure 7C) and caspase-9 activities (Figure 7D). Together these results suggest that EGCG-induced apoptosis is also mediated through generation of ROS which, in turn, may activate cell-intrinsic pathway.

4.7. Effects of EGCG on cell cycle

Since EGCG induced apoptosis in pancreatic cancer cells, we sought to examine whether it also causes growth arrest through regulation of cell cycle proteins. As shown in Figure 8A, EGCG induced cell cycle arrest at G1 stage of the cell cycle with declined in S and G2 stages. Furthermore, EGCG induced the expressions of cell cycle inhibitors $p21^{/WAF1/CIP1}$ and $p27^{/KIP1}$, and inhibited the expressions of cyclin D1, cdk4 and cdk6 (Figure 8B). These data suggest that EGCG may induce apoptosis by causing growth arrest.

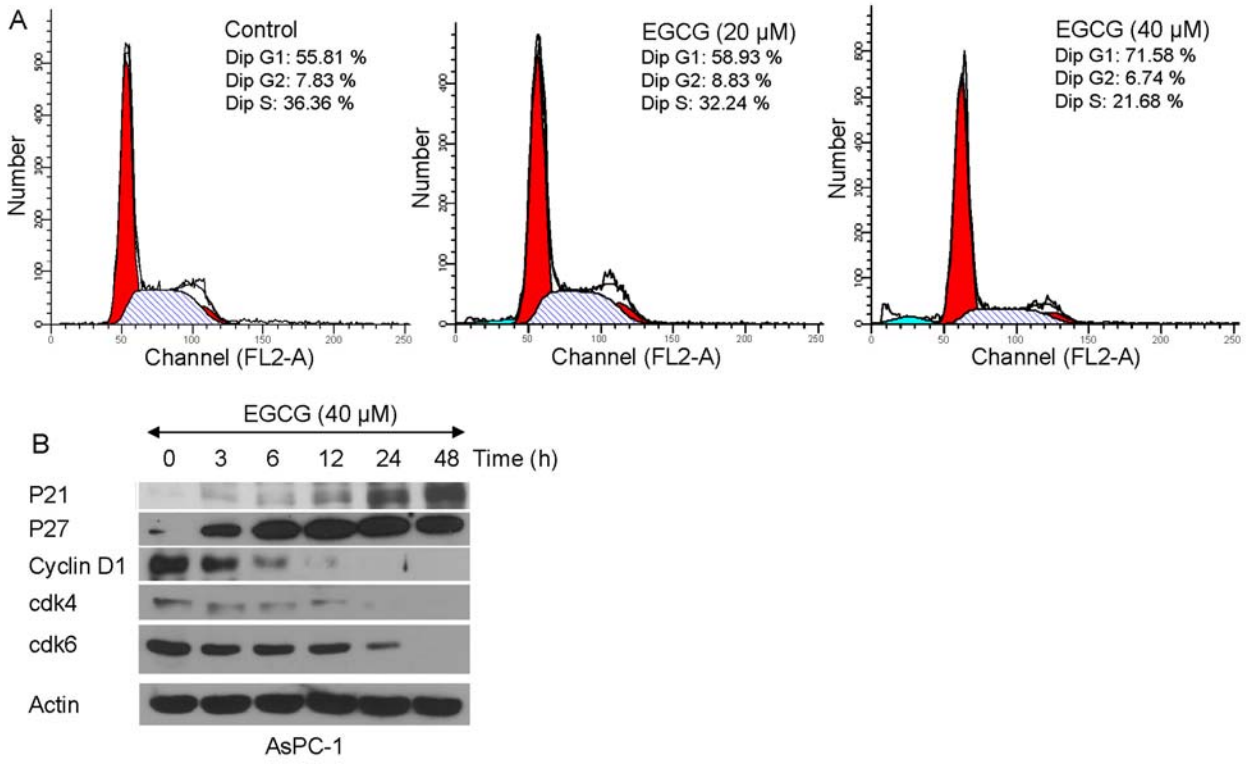

Figure 8. Effects of EGCG on cell cycle. (A), Effects of EGCG on cell cycle distribution. AsPC-1 cells were treated with EGCG (0, 20 or 40 μM) for 24 h. Cells were harvested, stained with propidium iodide and analyzed by flowcytometry. (B), Effects of EGCG on cell cycle regulatory proteins. AsPC-1 cells were treated with EGCG (40 μM) for 0-48 h, and the expressions of $p21^{/WAF1/CIP1}$, $p27^{/KIP1}$, cdk4, cdk6 and cyclin D1 were measured by Western blot analysis. Anti β-actin antibody was used as a loading control.

5. DISCUSSION

Our study has demonstrated that EGCG inhibited growth and induced apoptosis through multiple signaling pathways. *In vitro* studies have demonstrated that EGCG inhibited cell proliferation, induced apoptosis through caspase-3 and caspase-9 activation, induced proapoptotic Bax, Bak, Bcl-Xs and inhibited antiapoptotic Bcl-2 and Bcl-X_L. In addition, EGCG caused Bax oligomerization, generated reactive oxygen species (ROS), and depolarized mitochondrial membranes to facilitate cytochrome c and SMAC/DIABLO release into cytosol. These mitochondrial events are essential for cell-intrinsic pathway of apoptosis.

Antiproliferative action of EGCG on pancreatic carcinoma may also occur through cell cycle arrest (15, 16). The present study also demonstrated that EGCG caused growth arrest at G1 stage of cell cycle, and induced cell cycle inhibitors $p21^{/WAF1/CIP1}$ and $p27^{/KIP1}$, and inhibited the expressions of cyclin D1, cdk4 and cdk6. Similarly, EGCG has been shown to cause growth arrest at G1 stage of cell cycle by inducing $p21^{/WAF1}$ in prostate cancer cells (39, 40).

The Ras signal transduction pathway is complex with multiple intersections and bifurcations (41). Black and green tea extracts, GTP, and EGCG decreased the expression of the K-ras gene, and inhibited growth of pancreatic cancer cells (16). Ras activates three mitogen-activated protein kinases (MAPKs) including ERK, JNK, and p38 (41). Cells utilize the various Ras-mediated signal transduction pathways to regulate a plethora of phenotypes. Ras also can mediate responses to hypoxia via NFkappaB (42) and responses to a variety of environmental stresses via JNK (43-45), as well as apoptosis in response to FAS (46, 47), and tumor necrosis factor (48). Raf-1 contributes directly to ERK activation but not to JNK activation, whereas MEKK participated in JNK activation (44). In the current study, EGCG inhibited ERK1/2 and induced JNK1/2 and P38 MAP kinases in pancreatic cancer cells. EGCG also regulated upstream events, which was clear from its inhibitory effects on Ras and Raf-1, and stimulatory effect on MEKK1 in pancreatic cancer cells. Furthermore, the inhibition of ERK1/2 phosphorylation by EGCG was correlated with the inhibition of phosphorylation / activation of p90 ribosomal S6 kinase (p90 RSK). P90RSKs integrate upstream signals through two catalytic domains. Autophosphorylation of Ser386 by the regulatory C-terminal kinase domain (CTD) is thought to be essential for activation of the N-terminal kinase domain (NTD), which phosphorylates multiple downstream

targets. EGCG triggers apoptotic signal through the inhibition of Raf-MEK-ERK1/2-p90RSK, and activation of MEKK1-JNK1/2-JUN pathways. It has been shown that oxidative stress and activation of the JNK pathway induce the nucleocytoplasmic translocation of the pancreatic transcription factor Pdx-1, which leads to pancreatic beta-cell dysfunction (49). Similar to our findings, a recent report has shown that EGCG activates JNK in pancreatic carcinoma cells (15). In addition, Raf has MEK- and ERK-independent functions at the mitochondrial membrane. For example, mitochondrial localized Raf can phosphorylate Bad which results in its dissociation from the mitochondrial membrane (50, 51). Since Ras is highly activated in pancreatic cancer cells, the Ras/Raf/MEK/ERK pathway appears to be an attractive target for pancreatic cancer therapy and prevention.

The damage signals are transduced by the diverse 'BH3-only' proteins, distinguished by the BH3 domain used to engage their pro-survival relatives: Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1. This interaction ablates pro-survival function and allows activation of Bax and Bak, which commit the cell to apoptosis by permeabilizing the outer membrane of the mitochondrion. Certain BH3-only proteins (e.g. Bim, Puma) can engage all the pro-survival proteins, but others (e.g. Bad, Noxa) engage only subsets. Activation of Bax and Bak appears to require that the BH3-only proteins engage the multiple pro-survival proteins guarding Bax and Bak, rather than binding to the latter. The balance between the pro-survival proteins and pro-apoptotic proteins regulates tissue homeostasis, and either overexpression of a pro-survival family member or loss of a pro-apoptotic relative can be oncogenic. We have shown that EGCG inhibited Bcl-2, Bcl-X_L, and induced the expression of Bax, Bak, PUMA, and NOXA. Thus, the induction of proapoptotic members of the Bcl-2 family and inhibition of Bcl-2 and Bcl-X_L by EGCG, together with inhibition of ERK and activation of JNK and p38, could provide the required signals for the activation of Bax/Bak and the subsequent induction of apoptosis.

The activation of p53 can be compromised by up-regulation of negative regulators such as MDM-2 (52-54) or by defective positive effectors such as 14-3-3 or p14ARF (55, 56). Moreover, multiple proapoptotic (e.g. PUMA) or antiapoptotic members of the Bcl-2 family (e.g. Bcl-X_L, and Mcl-1) have been shown to act downstream of p53 to enhance or prevent the release from the mitochondrial cytochrome c, Smac/DIABLO, and AIF, respectively (57-60). Additionally, inhibition of caspases can result from the increased expression of several members of the inhibitors of apoptosis proteins (IAP) family, and/or by down-regulation of APAF-1, a cofactor of caspase-9 (61). A previous study in prostate cancer cells has demonstrated that EGCG activates growth arrest and apoptosis primarily via p53-dependent pathway that involves the function of both $p21^{/WAF1/CIP1}$ and Bax (39). Our data have shown that EGCG causes activation and translocation of Bax to the mitochondria in the absence of p53 in MEF $p53^{-/-}$, suggesting that EGCG is capable of inducing apoptosis in the absence of p53.

In conclusion, we have shown a potential of EGCG as a chemopreventive agent for pancreatic cancer. EGCG induces apoptosis through multiple mechanisms i.e. activation of caspases, regulation of Bcl-2 family members, generation of ROS, inhibition of Raf-1, ERK and upregulation of JNK and p38 MAP kinase pathways. Furthermore, EGCG caused growth arrest at G1 stage of cell cycle by regulating the expression of cell cycle inhibitors and cdks. The ability of EGCG to inhibit growth, and induce apoptosis in pancreatic cancer cells suggest that EGCG can be used in the management of pancreatic cancer prevention.

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