

EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer

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1. ABSTRACT

We have shown that epigallocatechin-3-gallate (EGCG), a polyphenolic compound from green tea, inhibits growth and induces apoptosis in human pancreatic cancer cells. However, the preclinical potential of EGCG in a suitable mouse model has not been examined. In this study, we examined the molecular mechanisms by which EGCG inhibited growth, invasion, metastasis and angiogenesis of human pancreatic cancer cells in a xenograft model system. EGCG inhibited viability, capillary tube formation and migration of HUVEC, and these effects were further enhanced in the presence of an ERK inhibitor. *In vivo*, AsPC-1 xenografted tumors treated with EGCG showed significant reduction in volume, proliferation (Ki-67 and PCNA staining), angiogenesis (vWF, VEGF and CD31) and metastasis (MMP-2, MMP-7, MMP-9 and MMP-12) and induction in apoptosis (TUNEL), caspase-3 activity and growth arrest (p21^{WAF1}). EGCG also inhibited circulating endothelial growth factor receptor 2 (VEGF-R2) positive endothelial cells derived from xenografted mice. Tumor samples from EGCG treated mice showed significantly reduced ERK activity, and enhanced p38 and JNK activities. Overall, our data suggest that EGCG inhibits pancreatic cancer growth, invasion, metastasis and angiogenesis, and thus could be used for the management of pancreatic cancer prevention and treatment.

2. INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States with an overall 5-year survival rate of <5% (1). Its biology is characterized by the propensity of early and aggressive invasion and metastasis, such that <10% of patients have surgically respectable disease at the time of diagnosis. The poor prognosis of pancreatic cancer is related with late presentation, aggressive local invasion, early metastasis, and poor response to 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.

Cancer cell metastasis is a step-wise process that includes detachment of cells from the primary tumor, local proteolysis of the basement membrane, intravasation, survival of the circulation, arrest in distant organ, extravasation and invasion into the surrounding tissue and growth (3-5). Metastasis involves penetration of the extracellular matrix (ECM) and basement membrane, and requires the action of proteases. The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that are capable of degrading the components of

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the extracellular matrix (ECM) and are involved in tumor invasion (4, 5). Among the MMPs, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are key enzymes for degrading type IV collagen, a major component of the basement membrane (6, 7). Most MMPs are secreted as inactive proenzymes and their proteolytic activities are regulated by other proteases or inhibited by specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). It appears that the balance between MMP and TIMP levels is a critical determinant of the net proteolytic activity. The increased activities of MMP-2 and MMP-9 have been associated with increasing tumor metastases in various human cancers, suggesting an important functional role for these proteases in the metastatic process.

Angiogenesis is the development of new blood vessels from pre-existing vasculature to provide a nutritive blood supply and is indispensable for tumor growth and survival. Tumor angiogenesis plays a key role in the development of distant metastases (8, 9). The anti-angiogenic approach to antitumor treatment is thought not only to eradicate primary tumor tissues, but also to suppress tumor metastasis (10). A growing tumor may switch to the angiogenic phenotype, induce the formation of new capillaries, and start to invade the surrounding tissue. The “angiogenic switch” depends on a net balance of negative and positive angiogenic factors in the tumor. Thus, the angiogenic phenotype may result from the production of growth factors, such as vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), transforming growth factor alpha (TGF alpha) and basic fibroblast growth factor (bFGF), by tumor cells and/or the down-regulation of negative modulators, like thrombospondin-1, in tissues with a quiescent vasculature (11). Recent clinical trial suggested that pancreatic cancer is highly angiogenesis dependent and a high microvessel density within pancreatic tumors is a prognostic factor for early disease progression (12-15). Furthermore, clinical data indicate that expression of proangiogenic factors such as VEGF, EGF, and thymidine phosphorylase positively correlate with a high pancreatic tumor relapse rate and shorter patient survival (12-15).

Green tea, which is widely consumed in China, Japan and India, contains polyphenolic compounds, which account for 30% of the dry weight of the leaves. It has also been reported that the quantity of green tea consumed, plays an important role in reducing cancer risk and in delaying cancer outbreak and recurrence. A polyphenolic constituent, (-)-epigallocatechin-3-gallate (EGCG), is the major and most effective chemopreventive agent in green tea. Epidemiological studies revealed that the incidences of stomach and prostate cancers are the lowest in the world among a population that consumes green tea on a regular basis (16-20). EGCG and green tea polyphenols act as an antioxidant, antiproliferative, antitumor, and anti-angiogenic agent, and thus a novel candidate for chemoprevention (17, 19, 20). We and others have shown that EGCG inhibit growth and induces apoptosis in human pancreatic cancer cells *in vitro* through regulation of Bcl-2 family members and MAP kinase pathway, and generation of reactive oxygen species (20, 21), thus holds great promise for development as a chemopreventive agent.

Furthermore, the efficacy of EGCG for the prevention of pancreatic cancer has not yet been examined in a suitable animal model system.

Several diverse genetically engineered mouse models of pancreatic neoplasia have been developed. Recent studies utilizing transgenic and knockout mice have elucidated the genetic pathways of pancreatic cancer (22). Mutations in KRAS occur early in disease progression and present in greater than 90% of invasive carcinomas. Tumor suppressor gene mutations such as CDKN2A/INK4A (95%), TP53 (>75%), and DPC4/SMAD4 (~55%) have been associated with pancreatic cancer. Endogenous Kras^{G12D} expression can initiate pancreatic tumorigenesis and that the resultant preinvasive lesions progress to invasive and metastatic pancreatic ductal carcinoma (23). It has been shown that the concomitant Kras^{G12D} and Trp53^{R172H} expression results in accelerated development of PDA with histological, genetic and clinical features that closely recapitulate those of the human disease. Furthermore, the pancreatic cancer progression is accelerated when there is a deficiency of p16^{Ink4a} (24) or biallelic deletion of p16^{Ink4a}/p19^{Arf} (25), albeit with distinct clinical and histopathological features. We have recently shown that EGCG inhibits the activation / expression of Ras, Raf and ERK proteins and induces apoptosis in human pancreatic cancer cells (26). Since Ras/Raf/MAP kinase pathway in crucial for pancreatic carcinogenesis, inhibition of this pathway by EGCG has a great significance for pancreatic cancer prevention and treatment.

The purpose of this study was to determine whether EGCG inhibited tumor growth, angiogenesis, and metastasis of pancreatic cancer in a xenograft model of athymic nude mice. Our data showed that EGCG inhibited tumor growth, angiogenesis and metastasis in AsPC-1 xenografts in nude mice through regulation of multiple signaling pathways, and has a great potential for pancreatic cancer prevention and treatment.

3. MATERIALS AND METHODS

3.1. Reagents

EGCG was purchased from LKT Laboratories, Inc. (St. Paul, MN). Antibodies against p21^{WAF1/CIP1}, VEGF, VEGF-R2, vWF, and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ERK inhibitor, antibodies against PCNA, Ki-67, MMP-2, MMP-7, MMP-9 and MMP-12, and Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay kit were purchased from EMD Biosciences (San Diego, CA). Antibodies against phospho ERK, phospho JNK, phospho p38, total JNK, total ERK and total p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Alkaline phosphatase (AP) and Hydrogen Peroxide (HRP) polymer-AEC chromagen substrate kits were purchased from Lab Vision Corporation (Fremont, CA).

3.2. Cell Culture and cell survival assay

AsPC-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum

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(FBS) and 1% antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD) and maintained in endothelial cell growth factor medium-2 (EGM2 MV SingleQuots, Clonetics) supplemented with 5% FBS. The effect of EGCG on HUVEC viability was determined by trypan blue dye exclusion assay. Stock solutions of the EGCG were prepared in DMSO and diluted with complete medium, and an equal volume of DMSO (final concentration, 0.05%) was added to the controls.

3.3. Capillary tube formation assay

Matrigel (100 µl) was added to wells of a 96-well culture plate and allowed to polymerize for 1 h at 37 °C. To examine the effects of EGCG on *in vitro* angiogenesis, subconfluent HUVECs were resuspended in complete medium and added to Matrigel containing wells (1 X 10⁴ cells / well), and exposed to various concentrations of EGCG (20, 40 and 60 µM) or DMSO (control). The plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ (27). Capillary tube formation was assessed after 24 h by counting the total number of capillary like tubular structures from three randomly chosen fields using an inverted microscope.

3.4. *In vitro* cell migration (invasion) assay

Migration of HUVEC or AsPC-1 cells was assessed using Transwell Boyden chamber (Corning, Acton, MA) containing a polycarbonated filter with a pore size of 8-µM. HUVECs or AsPC-1 (4 X 10⁴ cells in 0.2 ml) cells in complete medium was mixed with desired concentration of EGCG or DMSO (control), and the cell suspension was added to the upper chamber. The lower chamber contained 0.6 ml of complete medium with the same concentration of EGCG or DMSO. Migration through the membrane was determined after 24 h of incubation at 37°C. Cells remaining on the topside of the transwell membrane were removed using a cotton swab. The membrane was washed with ice-cold PBS. Cells that had migrated to the underside were fixed with 90% ethanol and stained with hematoxylin and eosin. Cell migration was quantified by counting the number of cells per field in five random fields.

3.5. *In vivo* studies

Athymic nude mice (Balb c nu/nu, 4-6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). AsPC-1 cells (2 X 10⁶ cells as a 50% suspension in matrigel, Becton Dickinson, Bedford, MA) in a final volume of 0.1 ml were injected subcutaneously at right flank of nude mice. When the average tumor volume reached about 100 mm³, mice were randomized into four groups of 7 mice/group, and the following treatment protocol was implemented. Group 1, vehicle control, 0.1 ml normal saline administered orally everyday five days a week lasting throughout the duration of experiment. Group 2, EGCG (60 mg/kg, in 0.1 ml normal saline) administered orally everyday five days a week lasting throughout the duration of experiment; Group 3, EGCG (80 mg/kg, in 0.1 ml normal saline) administered orally everyday five days a week lasting throughout the

duration of experiment; and Group 4, EGCG (100 mg/kg, in 0.1 ml normal saline) administered orally everyday five days a week lasting throughout the duration of experiment. The whole body weight of mice was taken weekly during the experimental period to assess toxicity of the treatments. Mice were housed under pathogen-free conditions and maintained on a 12 h light/12 h dark cycle, with food and water supplied *ad libitum*. Tumor volume was calculated using the equation: (volume=length x width x depth x 0.5236 mm³). Experiments were performed under IACUC's approved protocol.

3.6. Immunohistochemistry

Immunohistochemistry was performed as described earlier (28, 29). In brief, tumor tissues were collected on week 6, excised and fixed with 10% formalin, embedded in paraffin and sectioned. Tissue sections were stained with primary antibodies against active caspase-3, Ki-67, PCNA, p21^{WAF1/CIP1}, vWF, CD31, VEGF, MMP-2, MMP-7, MMP-9 and MMP-12 or TUNEL reaction mixture. For immunohistochemistry, sections were fixed in cold 100% acetone for 3 min, air-dried, and incubated with various primary antibodies at room temperature for 4 h. Subsequently, slides were washed three times in PBS and incubated with secondary antibody at room temperature for 1 h. Finally, alkaline phosphatase or hydrogen peroxide polymer-AEC chromagen substrate kits were used as per manufacturer' instructions (Lab Vision Corporation). After washing with PBS, Vectashield (Vector Laboratories) mounting medium was applied and sections were coverslipped and imaged. In each experimental group, the numbers of CD31, and VEGF positive tumor cells were counted in 7 fields in a blinded fashion.

3.7. TUNEL assay.

For TUNEL staining, paraffin-embedded tissue slides were dewaxed and rehydrated by heating at 60°C followed by washing in xylene and rehydration through a graded series of ethanol and double distilled water. Tissue sections were incubated for 20 min at 37°C with proteinase K working solution (15 µg/ml in 10 mM Tris/HCl, pH 7.4) followed by rinsing twice with PBS. After air-drying the slides, 50 µl TUNEL reaction mixture was added on sample and covered with lid, and slides were incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Slides were washed thrice with PBS, air-dried, mounted and visualized with a microscope. All sections were coded and observed by an independent investigator.

3.8. Measurement of circulating endothelial cells

Five hundred microliters of whole blood was collected from mice in EDTA or heparin using 1 ml microcentrifuge tubes and examined for the number of circulating endothelial cells. After plasma was separated, 300 µl of DMEM supplemented with 10% FBS were added to the tube. Red blood cells were removed with RBC lysis solution and the mixture was placed on 8-chambered slides. After 6 hr, incubation that allowed endothelial cells to attach to the slide, the attached cells were stained with anti-VEGFR2 antibody. The VEGFR2-positive cells were counted under the microscope in 7 fields.

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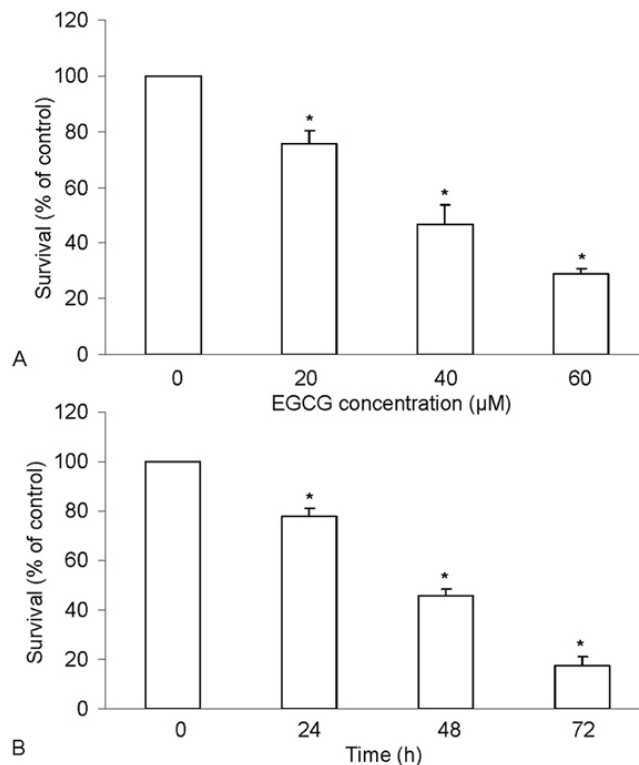


Figure 1. EGCG inhibits viability of human umbilical vein endothelial cells (HUVECs). (A), HUVECs were seeded in 12-well plates and treated with various concentration of EGCG for 48 h. Cells were harvested and viability was determined by trypan blue staining. Data represent mean \pm SD. * = significantly different from control, $P < 0.05$. (B), HUVECs were seeded in 12-well plates and treated with 40 μM of EGCG for various time points (0-72 h). Cells were harvested and viability was determined by trypan blue dye exclusion assay. Data represent mean \pm SD. * = significantly different from control, $P < 0.05$.

3.9. Statistical analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one or two way ANOVA. The non-parametric Mann-Whitney U test was performed to assess the difference of tumor volume between control and treatment group. To assess the difference between two groups under multiple conditions, one-way ANOVA followed by Bonferoni's multiple comparison tests were performed using PRISM statistical analysis software (GrafiPad Software, Inc., San Diego, CA). Significant differences among groups were calculated at $P < 0.05$.

4. RESULTS

4.1. EGCG inhibits viability of HUVEC

To test the hypothesis that EGCG may inhibit angiogenesis, initially we determined the effects of EGCG on survival of HUVEC by trypan blue exclusion assay. As shown in Figure 1, EGCG inhibited the viability of HUVEC in a dose- and time-dependent manner. These data suggest that EGCG may play a role in angiogenesis.

4.2. EGCG inhibits capillary tube formation by blocking ERK activity

Because EGCG treatment significantly decreased HUVEC viability, we sought to examine whether EGCG

inhibited *in vitro* angiogenesis. We explored this possibility by determining the effect of EGCG treatment on formation of capillary tube formation by HUVEC on growth factor-reduced matrigel, which is well-accepted technique to measure *in vitro* angiogenesis. The data revealed that EGCG inhibited capillary tube formation in a dose-dependent manner (Figure 2A). We next examined the involvement of ERK in capillary tube formation by using a specific inhibitor of ERK pathway. As shown in Figure 2B, EGCG and ERK inhibitor alone inhibited capillary tube formation, and this effect of EGCG was further enhanced in the presence of ERK inhibitor. These results indicated that EGCG treatment inhibited capillary tube formation by HUVEC, and ERK is a critical regulator of angiogenesis.

4.3. EGCG inhibits migration of HUVEC and AsPC-1 cells

Next we determined the effect of EGCG treatment on invasion potential (migration) of HUVEC and AsPC-1 cells using a modified Boyden Chamber assay. In DMSO-treated controls, a large fraction of HUVEC migrated to the bottom face of the membrane (Figure 3A and B). Treatment of chambers with EGCG resulted in inhibition of migration of HUVECs in a dose-dependent manner. Similarly, EGCG inhibited AsPC-1 cell migration in Boyden Chamber assay (Figure 3C and D). ERK

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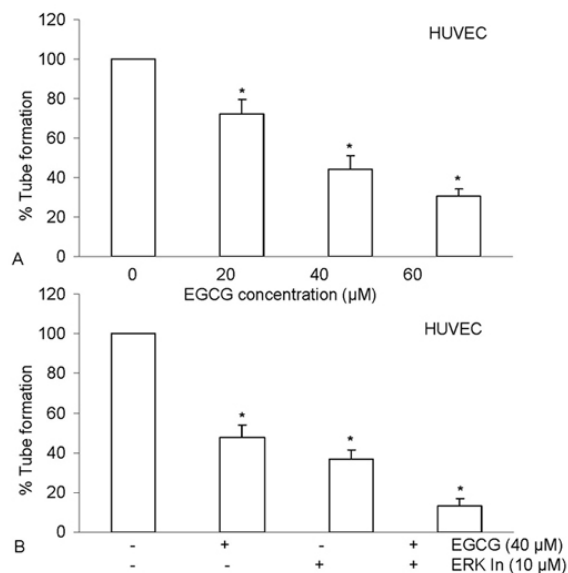


Figure 2. EGCG inhibits capillary tube formation. (A), HUVECs were seeded in 24-well plates containing matrigel, and treated with various concentrations of EGCG for 24 h. Capillary tubes were counted under a microscope. Data represent mean \pm SD. * = significantly different from control, $P < 0.05$. (B), HUVECs were seeded in 24-well plates containing matrigel. Cells were pretreated with ERK inhibitor (10 μ M) for 3 h, followed by treatment with EGCG (40 μ M) for 24 h. Capillary tubes were counted under a microscope. Data represent mean \pm SD. * = significantly different from control, $P < 0.05$.

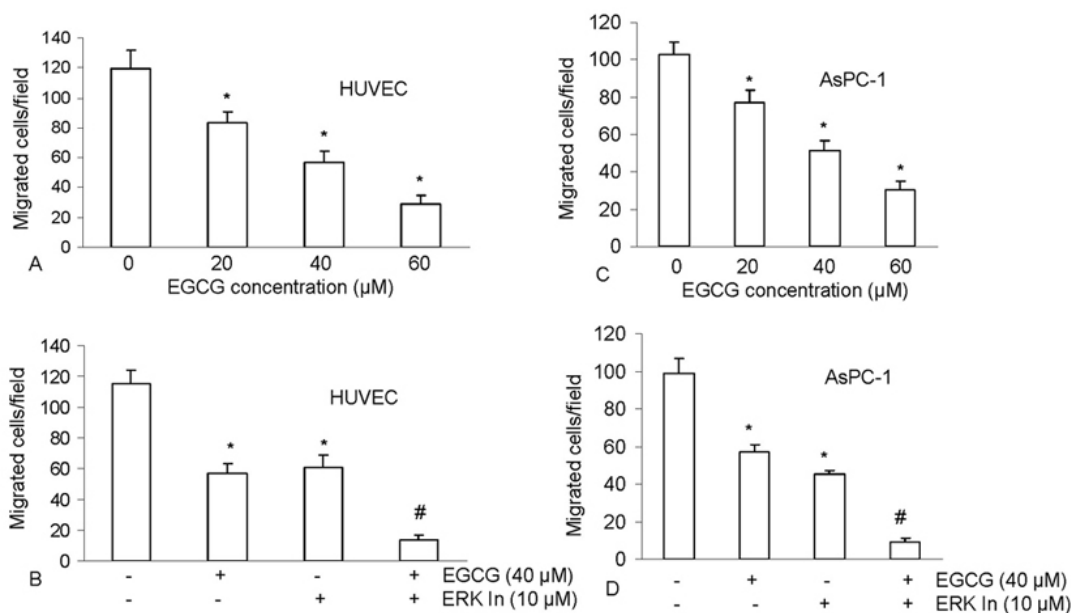


Figure 3. EGCG inhibits migration of HUVEC and AsPC-1 cells. Migration of HUVEC and AsPC-1 cells was assessed using Transwell Boyden chamber containing a polycarbonated filter. (A), HUVECs (4×10^4 cells) were treated with various concentrations of EGCG (20, 40 and 60 μ M) or DMSO (control). Migration through the membrane was determined after 24 h of incubation at 37°C. Cells that had migrated to the lower chamber were fixed with 90% ethanol, stained with hematoxylin and eosin, quantified by counting the number of cells under a microscope. Data represent mean \pm SD. * = significantly different from control, $P < 0.05$. (B), HUVECs (4×10^4 cells) were pretreated with ERK inhibitor (10 μ M) for 3 h, followed by treatment with EGCG (40 μ M) or DMSO (control) for 24 h at 37°C. Cells migrated to the lower chamber were fixed, stained and quantified. (C), AsPC-1 cells (4×10^4 cells) were treated with various concentrations of EGCG (20, 40 and 60 μ M) or DMSO (control) for 24 h at 37°C. Cells migrated to the lower chamber were fixed, stained, and quantified. (D), AsPC-1 cells were pretreated with ERK inhibitor (10 μ M) for 3 h, followed by treatment with EGCG (40 μ M) or DMSO (control) for 24 h at 37°C. Cells migrated to the lower chamber were fixed, stained, and quantified.

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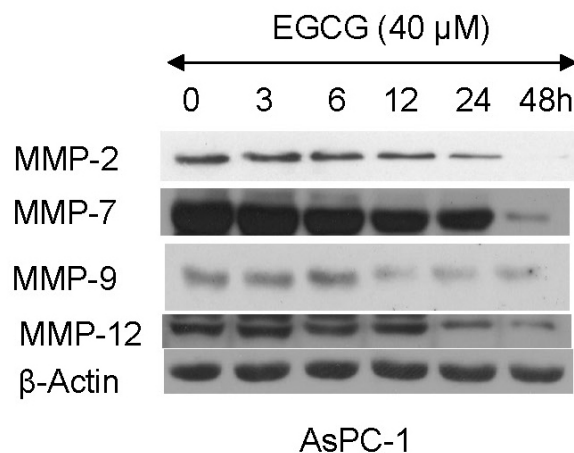


Figure 4. Effects of EGCG on matrix metalloproteinases *in vitro*. AsPC-1 cells were treated with EGCG (40 μM) for various time points (0-48 h). At the end of incubation period, cells were harvested and the expression of MMP-2, MMP-7, MMP-9, and MMP-12 was measured by Western blot analysis. Anti β-actin antibody was used as a loading control.

inhibitor inhibited migration of HUVEC and AsPC-1 cells, and the inhibitory effects of EGCG on cell migration were further enhanced in the presence of ERK inhibitor (Figure 3 B and D). These results indicated that the migration by HUVECs and AsPC-1 cells was inhibited by EGCG, and ERK is an important regulator of cell migration.

4.4. EGCG inhibits matrix metalloproteinase *in vitro*.

Elevated expression of matrix metalloproteinases (MMPs) is associated with increased metastatic potential in many tumor cells (30). We therefore examined whether EGCG regulates the expression of MMP-2, MMP-7, MMP-9 and MMP-12 (Figure 4). EGCG inhibited the expression of MMP-2, MMP-7, MMP-9 and MMP-12 in AsPC-1 cells. These data suggest that EGCG can regulate pancreatic cancer metastasis by inhibiting MMPs.

4.5. EGCG inhibits growth of pancreatic tumor cells xenografted in nude mice

We have recently demonstrated that EGCG inhibited growth of pancreatic cancer cells *in vitro* (26). We therefore sought to examine the effects of EGCG on AsPC-1 xenografts in nude mice (Figure 5). EGCG inhibited the growth of AsPC-1 xenografts in a dose-dependent manner (Figure 5A and B). A group of mice receiving 100 mg/kg EGCG never had tumor larger than the initial volume (100 mm³). We have also observed a dramatic reduction in tumor and spleen sizes from mice treated with EGCG (Figure 5C). This is perhaps reduction in infiltration of tumor cells into spleens in EGCG-treated mice. These data strongly suggest that EGCG is highly effective in suppressing the growth of pancreatic cancer *in vivo*.

4.6. EGCG induces apoptosis in tumor tissues

In order to examine the mechanisms by which EGCG inhibits growth of pancreatic tumor in nude mice,

we examined the effects of EGCG on apoptosis, and caspase-3 activity (Figure 6). Examination of tumor tissues demonstrated that EGCG induced apoptosis in xenografted tumors in a dose-dependent manner. EGCG treatment also resulted in caspase-3 activation in tumor tissues derived from AsPC-1 xenografted nude mice (Figure 6B). These data suggest that EGCG inhibited growth of established tumors by inducing apoptosis through caspase-3 activation.

4.7. EGCG inhibits proliferation and causes growth arrest in tumor tissues

We next examined the molecular mechanisms by which EGCG inhibited tumor growth in xenografted nude mice. Examination of tumor tissues by immunohistochemistry demonstrated that EGCG inhibited staining of Ki-67 and PCNA, and caused growth arrest by inducing p21^{CIP1/WAF1} expression (Figure 7). Ki-67 and PCNA are used as markers of cell proliferation. Overall, these data suggest that EGCG inhibits pancreatic tumor growth *in vivo* by inhibiting cell proliferation and inducing growth arrest.

4.8. EGCG inhibits ERK1/2 activity and induced JNK1/2 activity

We have recently shown that EGCG inhibits Ras, Raf and ERK activation, and induces p38 and JNK activation in pancreatic cancer cells (26). We therefore examined whether EGCG induces similar pattern in MAP kinase activity *in vivo*. The activation of ERK, JNK and p38 MAP kinase was examined by immunohistochemistry using phospho-specific antibodies. As shown in Figure 9, EGCG inhibited the activation of ERK and induced the activation JNK MAP kinases. EGCG also induced a slight but significant activation of p38 MAP kinase. These data suggest that the regulation of MAP kinase pathway by EGCG is an important event for pancreatic cancer prevention.

4.9. EGCG inhibits angiogenesis

Whether regression in tumor growth by EGCG was due to inhibition of angiogenesis, we analyzed the markers of angiogenesis by immunohistochemistry in tumor samples. Examination of tumor tissues by immunohistochemistry demonstrated that EGCG inhibited the expression of vWF in tumor tissues (Figure 9A). Control mice demonstrating increased rate of tumor growth had increased numbers of CD31-positive blood vessels and VEGF positive tumor cells compared to EGCG treated mice (Figure 9B and C). Several laboratories, including ours, have demonstrated that increases in the circulating vascular endothelial growth factor receptor 2 (VEGF-R2)-positive endothelial cells correlate directly with increase in tumor angiogenesis and can serve as *in vivo* indicators of tumor angiogenesis (31-33). As expected, control mice had increased circulating VEGF-R2-positive endothelial cells compared to EGCG treated mice (Figure 9D). Thus, these data demonstrate that EGCG attenuated tumor growth by inhibiting angiogenesis.

4.10. EGCG inhibits metastasis

Elevated expression of matrix metalloproteinases (MMPs) is associated with increased metastatic potential in many tumor cells (34, 35). We therefore sought to examine

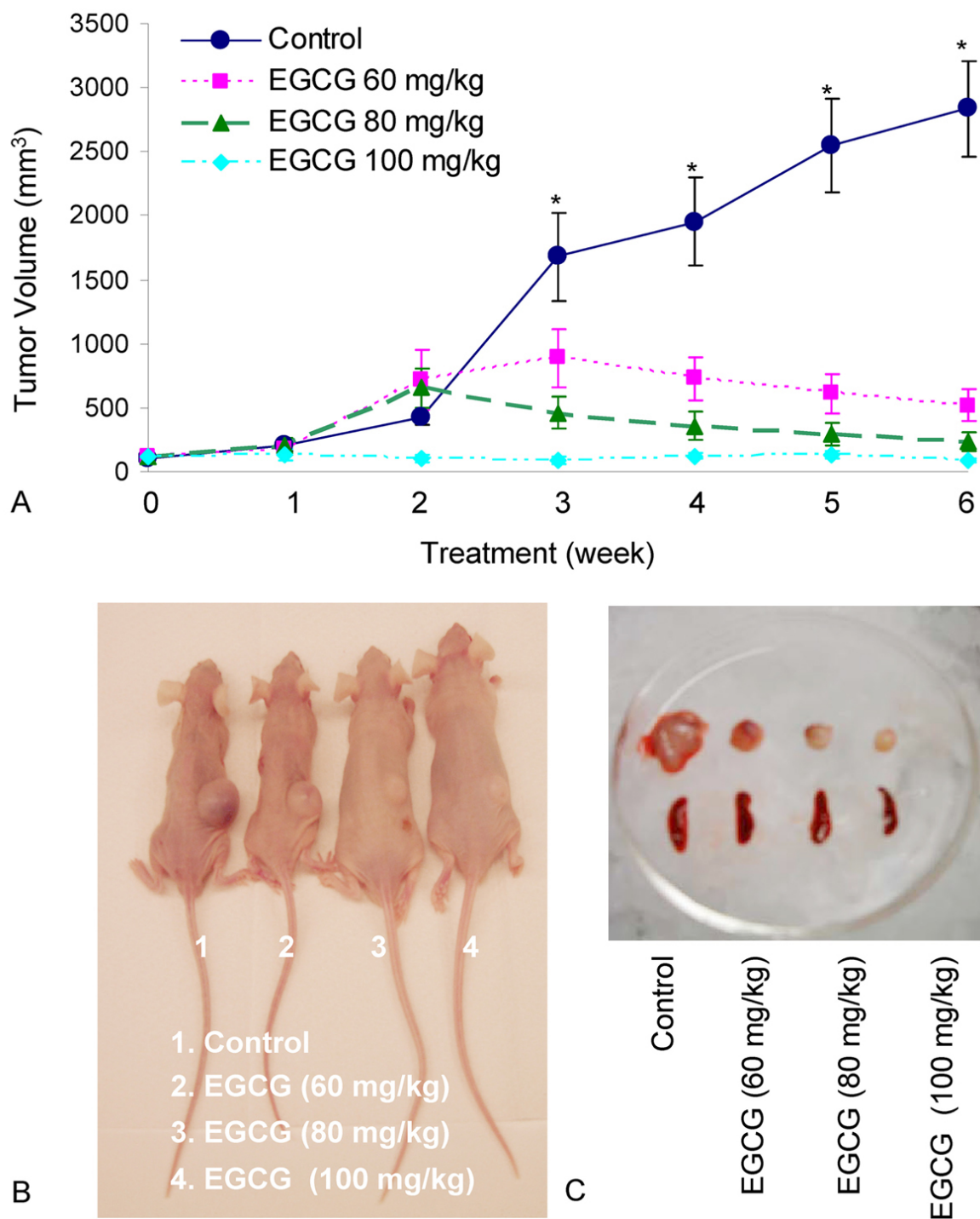


Figure 5. Effects of EGCG on AsPC-1 xenografts in nude mice. (A), Effects of EGCG on tumor growth. AsPC-1 cells (2×10^6 cells as a 50% suspension in Matrigel, Becton Dickinson, Bedford, MA, in a final volume of 0.1 ml) were injected subcutaneously into the right flank of balb c nude mice. After tumor formation (about 100 mm³), mice (7 per group) were treated (gavage) with vehicle control (0.1 ml normal saline by oral feeding), EGCG (60 mg/kg), EGCG (80 mg/kg) and EGCG (100 mg/kg) everyday 5 days per week throughout the duration of the experiment. Data represent mean \pm S.E. * = significantly different from treatment groups, $P < 0.05$. (B), Photographs of nude mice bearing AsPC-1 xenografts. 1 = control, 2 = EGCG (60 mg/kg), 3 = EGCG (80 mg/kg), and 4 = EGCG (100 mg/kg). (C), Photographs of tumors and spleens derived from control and EGCG treated mice at week 6.

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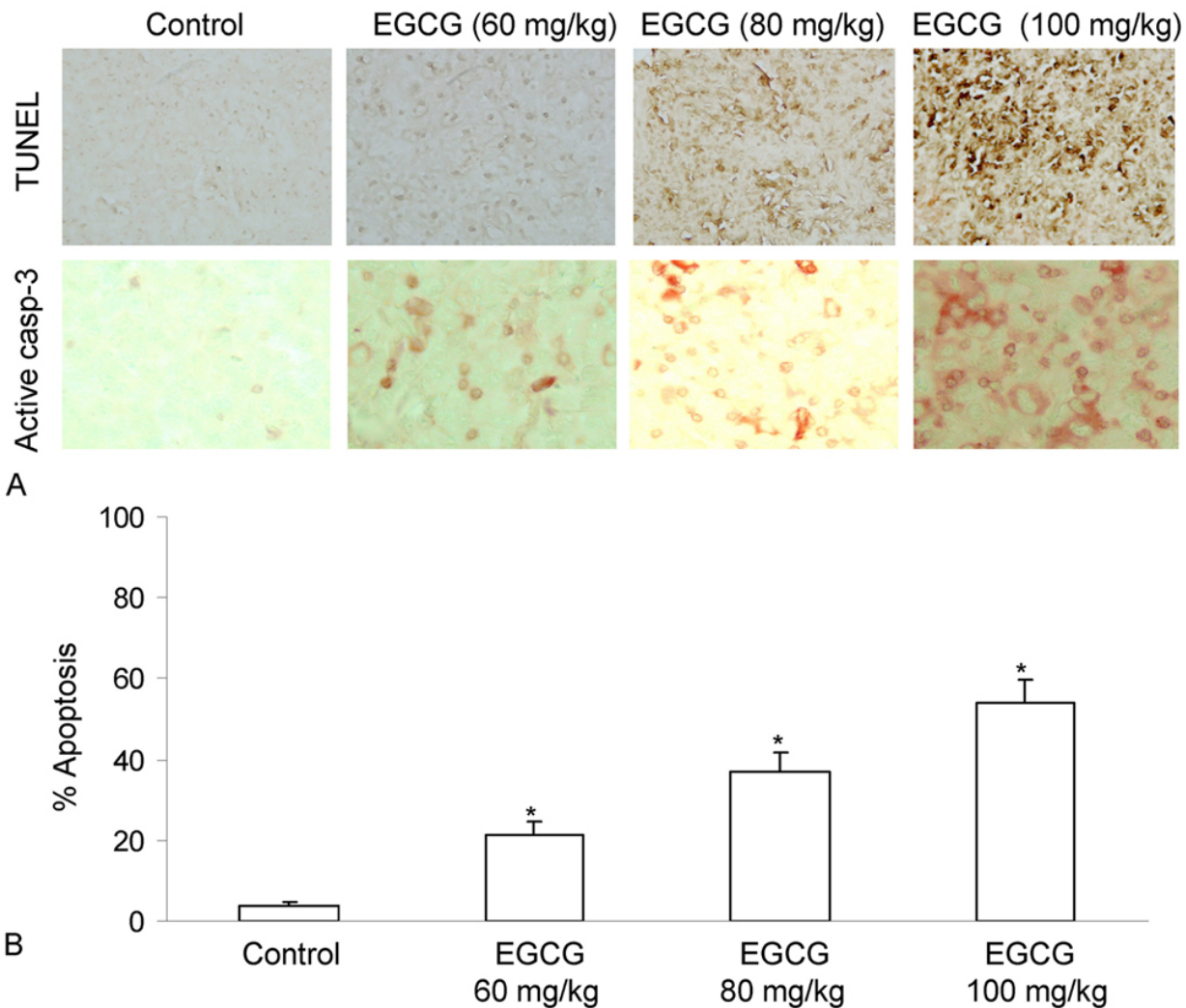


Figure 6. Effects of EGCG on apoptosis in AsPC-1 pancreatic tumors derived from xenografted nude mice. Tumor samples were collected at week 6. Paraffin sections (5 μ m thick) were mounted on slides and dried. Sections were deparaffinized in xylene followed by treatment with a graded series of alcohol (100%, 95%, and 80% ethanol/double-distilled water (v/v) and rehydrated in PBS (pH 7.5), treated with pepsin for 15 min at 37^o C, and washed with PBS. (A), TUNEL staining was performed as per manufacturer's instructions (Promega). Slides were also stained with active caspase-3 antibody. (B), TUNEL stained cells were counted for apoptotic index. Data represent mean \pm SD. * = significantly different from the control ($P < 0.05$).

the effects of EGCG on MMP-2, MMP-7, MMP-9, and MMP-12 in tumor tissues derived from xenografted nude mice. EGCG inhibited expression of MMP-2, MMP-7, MMP-9 and MMP-12 in tumor tissues (Figure 10). These data suggest that EGCG can inhibit pancreatic cancer progression by inhibiting metastasis.

DISCUSSION

To the best of our knowledge, this is the first study to examine the effects of EGCG on pancreatic cancer xenografts in nude mice. We have recently demonstrated that EGCG inhibited growth and induced apoptosis through multiple signaling pathways (26). *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 (26). EGCG also caused mitochondrial dysfunction as indicated by Bax oligomerization, generation of reactive oxygen species (ROS), depolarization of mitochondrial membranes, and release of cytochrome c and SMAC/DIABLO from mitochondria. Our AsPC-1 xenograft studies revealed that EGCG inhibited tumor cell proliferation, metastasis, invasion and angiogenesis, and induced apoptosis and growth arrest in tumor cells.

The Ras signal transduction pathway is complex with multiple intersections and bifurcations (36). Black and green tea extracts, GTP, and EGCG decreased the expression of the K-ras gene, and inhibited growth of pancreatic cancer cells (20). Ras activates three mitogen-

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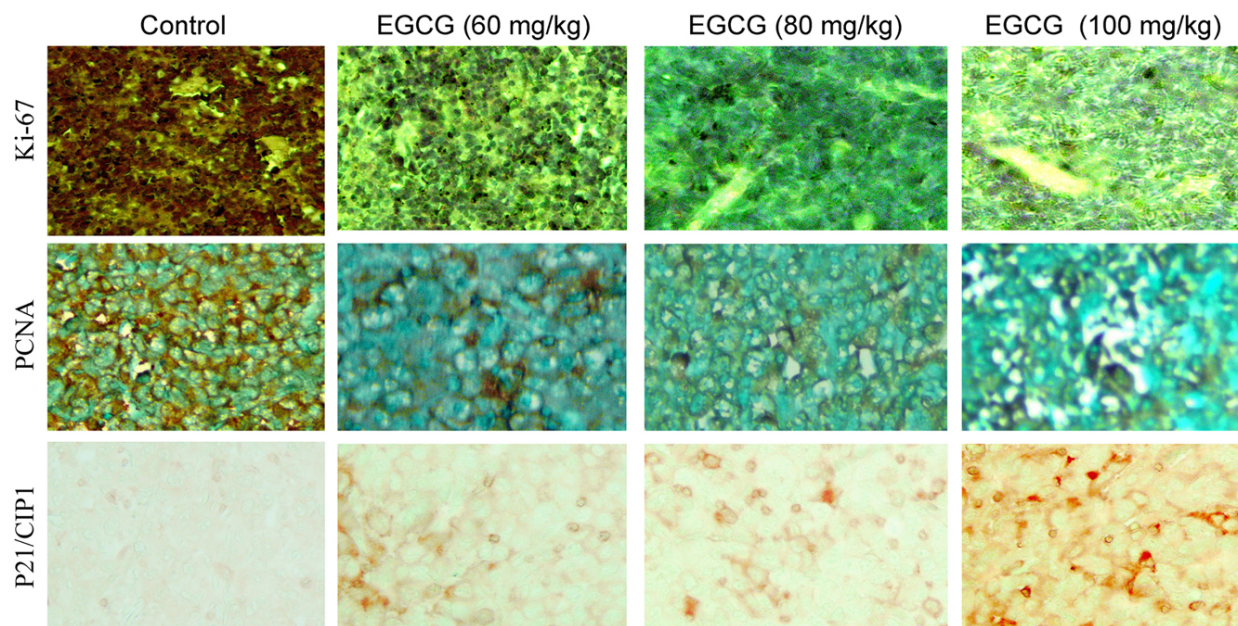


Figure 7. Effects of EGCG on molecular markers of proliferation and cell cycle in tumor tissues derived from AsPC-1 xenografted nude mice. Tumor samples were collected at week 6 from the experiment described above. Immunohistochemical procedures for detection of Ki-67, PCNA, and p21^{WAF1/CIP1} were performed as described in materials and methods.

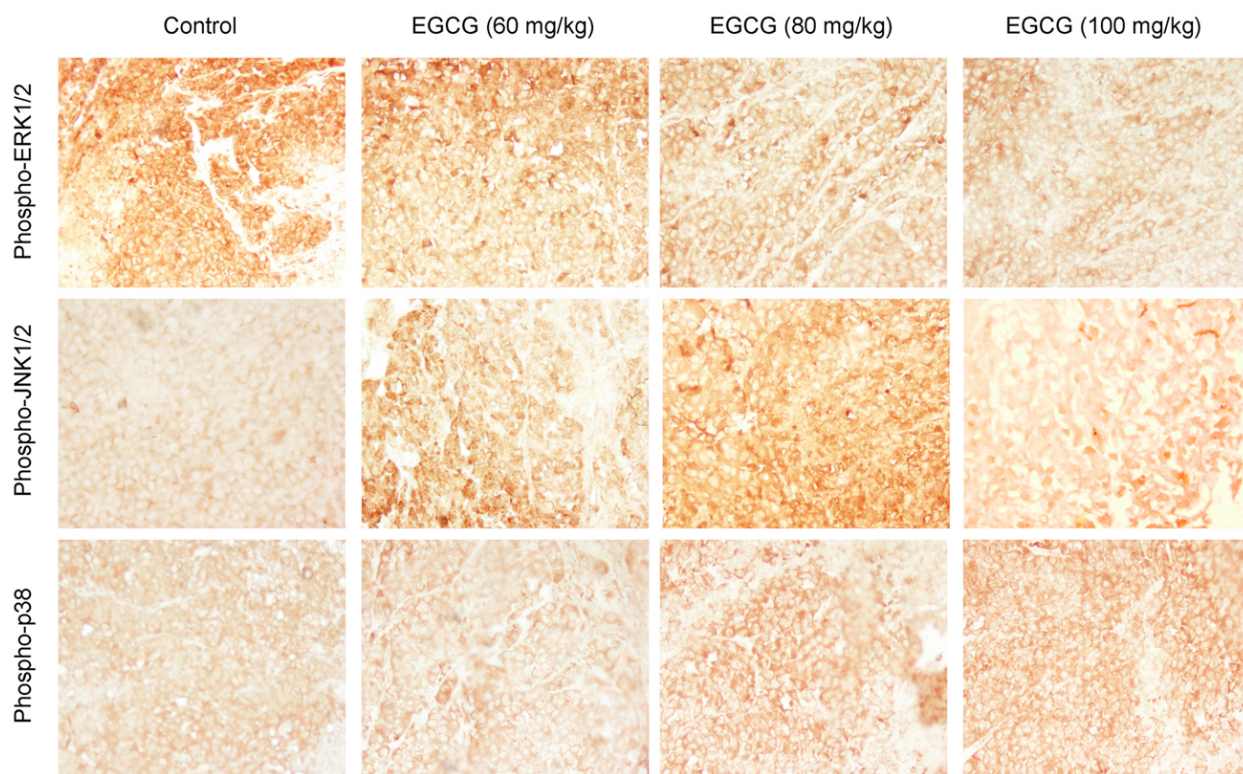


Figure 8. Effects of EGCG on MAP kinase activity in tumor tissues derived from AsPC-1 xenografted nude mice. Tumor samples were collected at week 6 from the experiment described above. Immunohistochemical procedures for detection of phospho-ERK1/2, phospho-JNK1/2 and phospho-p38 were performed as described in materials and methods.

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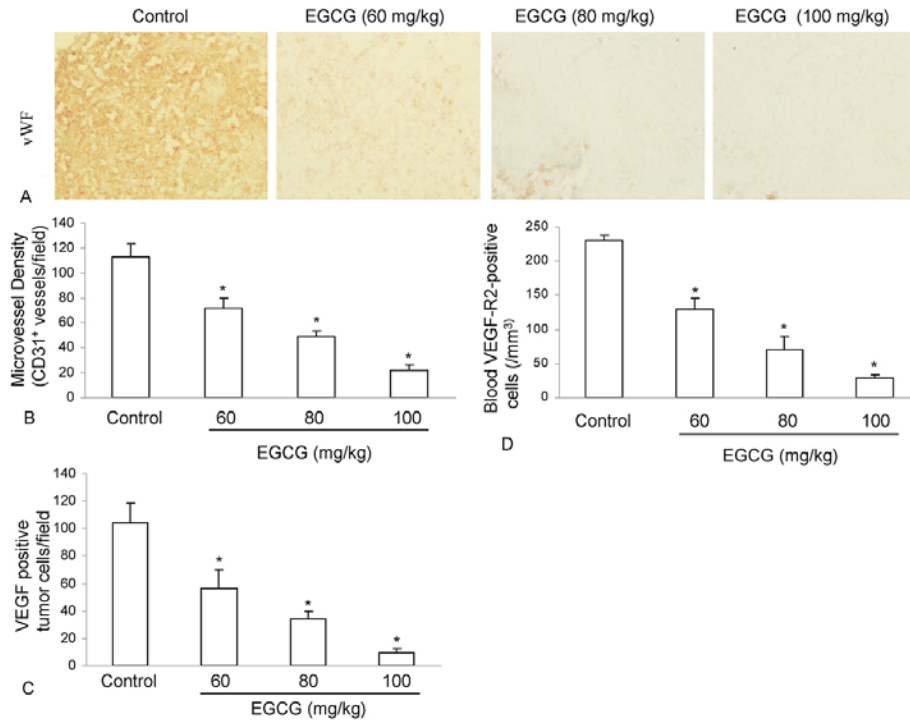


Figure 9. Effects of EGCG on angiogenesis. (A), Immunohistochemical procedures for detection of vWF in tumor tissues at week 6. (B), Blood vessel quantification in tumors derived at week 6. Sections from tumor tissue were stained with anti-CD31 antibody, and the number of CD31-positive blood vessels was counted. The results are shown as the mean \pm SD. * = significantly different from control, $P < 0.05$. (C), Quantification of VEGF positive cells in tumor tissues derived at week 6. Sections from tumor tissue were stained with anti-VEGF antibody, and the number of VEGF-positive cells were counted. (D), VEGF receptor 2 (VEGF-R2)-positive circulating endothelial cells in mice at week 6. The blood cells from peripheral blood attached to the slide were stained with anti-VEGF-R2 antibody, and the number of positive cells was counted under a microscope.

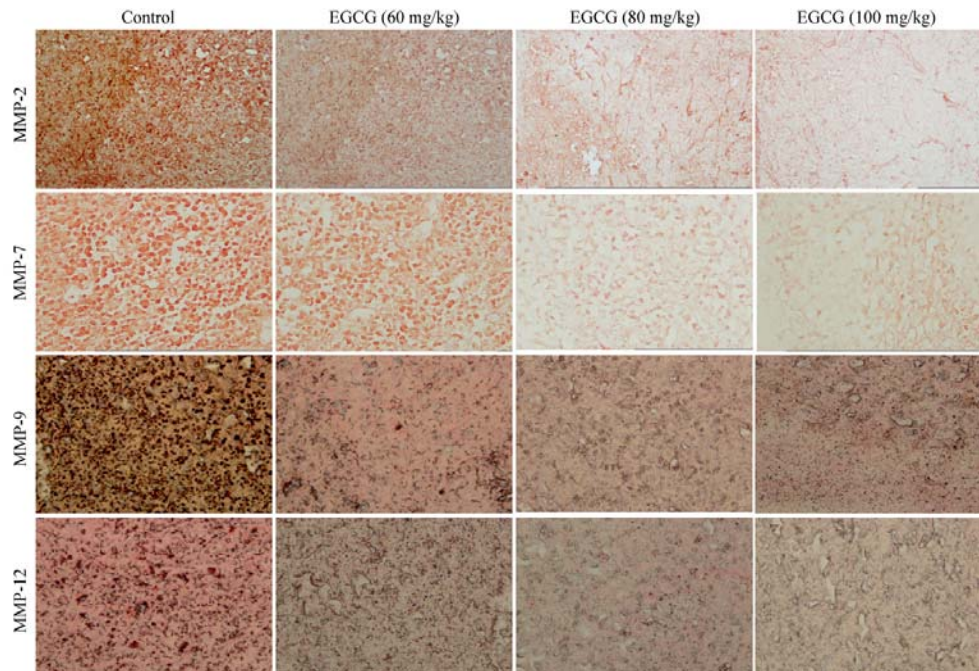


Figure 10. Effects of EGCG on markers of metastasis in AsPC-1 xenografted nude mice. Tumor samples were collected on week 6 from the experiment described above. Immunohistochemical procedures for detection of MMP-2, MMP-7, MMP-9 and MMP-12 were performed as described in materials and methods.

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activated protein kinases (MAPKs) including ERK, JNK, and p38 (36). Cells utilize the various Ras-mediated signal transduction pathways to regulate a plethora of phenotypes. Ras also can mediate responses to hypoxia via NfκB (37) and responses to a variety of environmental stresses via JNK (38-40), as well as apoptosis in response to FAS (41, 42), and tumor necrosis factor (43). Raf-1 contributes directly to ERK activation but not to JNK activation, whereas MEKK participated in JNK activation (39). We and others have shown that EGCG inhibited ERK and activated JNK and P38 MAP kinase pathways in pancreatic cancer cells *in vitro* (21, 26). Similarly, the present study demonstrated that EGCG inhibited ERK and induced JNK and P38 MAP kinases in AsPC-1 xenografts. Our previous studies have also demonstrated that EGCG inhibited Ras and Raf-1 expression in pancreatic cancer cells (26). Thus, *in vivo* data obtained in the present study validates the findings of our recently conducted *in vitro* studies. The pancreatic β-cell dysfunction has been demonstrated in response to oxidative stress and activation of the JNK pathway which induce the nucleocytoplasmic translocation of the pancreatic transcription factor Pdx-1 (44). Since Ras is highly activated in pancreatic cancer cells, the inhibition of Ras/Raf/MEK/ERK pathway by EGCG appears to be an attractive strategy for pancreatic cancer therapy and prevention.

In the present study, we have shown that EGCG inhibited growth of AsPC-1 xenografts in nude mice through multiple mechanisms. The inhibition of tumor growth was associated with a decrease in tumor cell proliferation (Ki-67 and PCNA staining), an increase in growth arrest (induction of p21^{WAF1}), induction of caspase-3 activation and apoptosis (TUNEL staining), and inhibition of angiogenesis (vWF, VEGF and CD31) and metastasis (MMP-2, -7, -9 and -12). EGCG also inhibited number of VEGF-R2 positive circulating endothelial cells derived from xenografted nude mice. EGCG inhibited cell migration and capillary tube formation, and these beneficial effects of EGCG were further enhanced in the presence of ERK MAP kinase inhibitor, pointing a positive role of ERK in angiogenesis and metastasis. Thus, EGCG is useful for not only inhibiting tumor growth but also angiogenesis, invasion and metastasis.

Several oncogenes and their intracellular protein products such as v-raf, fos, src, v-ras, K-ras, and v-yes induce the up-regulation of angiogenic factors like VEGF and PDGF and increase the production of cytokines and proteolytic enzymes (45-50). Our data demonstrates that EGCG inhibits several genes related to invasion, metastasis and angiogenesis. Therefore, agents such as EGCG that regulate proteins involved in tumor growth, metastasis and angiogenesis might be good candidate for cancer therapy and prevention.

In conclusion, here, we have shown a potential of EGCG as a chemopreventive agent for pancreatic cancer. Our studies strongly suggest that EGCG can modulate the expression of genes known to play a role in the cancer progression, invasion, metastasis and angiogenesis and,

therefore, may be potential agents for chemoprevention against pancreatic cancer. A recent study from our laboratory has demonstrated that 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 (26). Furthermore, EGCG can also inhibit pancreatic tumor cell invasion and/or metastasis by regulating MMPs, and angiogenesis by regulating VEGF and its receptors. The doses of EGCG (60-80 mg/kg) used in the current animal studies is quite relevant to that in human subjects. The ability of EGCG to inhibit growth, angiogenesis and metastasis, and induce apoptosis of cancer cells *in vitro* and *in vivo*, suggest that EGCG can be used in the management of pancreatic cancer prevention. Studies in our laboratory are underway to examine the effects of EGCG on an orthotopic and a Kras transgenic mice models of pancreatic cancer. Further clinical studies are necessary to confirm our findings in patients with pancreatic cancer.

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