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Pomegranate (*Punica granatum*) Fruit Extract Suppresses Cancer Progression and Tumor Angiogenesis of Pancreatic and Colon Cancer in Chick Chorioallantoic Membrane Model

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ABSTRACT

Pomegranate fruit extract contains many polyphenols and flavonoids of diverse biological importance including anticancer potential. In cancer, the angiogenesis process facilitates solid cancer growth and metastasis. Here, the antiangiogenic effect of pomegranate fruit extract against human pancreatic cancer (Suit-2) and colon (colo205) cell lines in the chick chorioallantoic membrane (CAM) model was studied along with the effect of pomegranate fruit extract on fibroblast growth factor (FGF2). Pomegranate fruit extract significantly reduced the tumor weight and hemoglobin content in CAM models of pancreatic Suit-2 and colon colo205.

Abbreviation: EDTA: ethylene diamine tetraacetate; PBS: phosphate buffered saline; DMEM: Dulbecco's Modified Eagle's medium; ELISA: enzyme linked immunosorbent assay; CAM model: chick chorioallantoic membrane model; ANOVA: analysis of variance

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Introduction

Pancreatic cancer is a malignant disease and represents the fourth leading cause of cancer death in the US, where it is predicted to become the second most frequent cause of cancer-related death by 2030 (1). The incidence rate of pancreatic cancer in both sexes increases with age, therefore, it can be defined as a disease of elderly populations (2).

Histological, morphological, and genetic changes are associated with colon cancer development that accumulate over time (3). Nowadays, colon cancer is the fourth most deadly cancer worldwide with about 900,000 deaths annually. Besides an ageing population and dietary habits, risk factors like obesity, lack of physical exercise, and smoking increase the risk of colorectal cancer (4).

Tumor angiogenesis has been thought to be a valid target for many solid tumors (5). Because angiogenesis is an important factor in tumor progression, inhibition of angiogenesis can lead to tumor growth inhibition (6). Hypoxia-inducing factor-1 (HIF-1) can be expressed in tumors often under hypoxic conditions (7). As a result, HIF-1 induces the expression of endothelial growth factor (VEGF), epidermal growth, fibroblast growth factor (FGF), and hepatocyte growth factor and thus promotes hypervascularization (8). FGF signaling regulates several processes including angiogenesis. Therefore, agents targeting FGF signaling potentially target both the VEGF and FGF pathways and inhibit angiogenesis (9).

Pomegranate (*Punica granatum*) is a natural product that has been shown to be anti-cancerous due to its large concentration of polyphenols, including ellagitannins, ellagic acid, and other flavonoids like quercetin, kaempferol, and luteolin glycosides (10). After patients with colorectal cancer consumed pomegranate extract at 900 mg/day for 15 days, their colon tissues were found to have significantly high levels of ellagic acid and derivatives, suggesting a potential prevention role by pomegranates against colorectal cancer (11). Furthermore, punicalagin and ellagic acid induced significant inhibition of benzo[a]pyrene-induced DNA adducts (12). Pomegranate induced its anticancer effect through cell-cycle arrest, induction of apoptosis, and inhibition of angiogenesis and metastasis (13).

Numerous cancer studies have investigated antiangiogenesis in either in vitro or in vivo models

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(14–17). The current study investigated the antiangiogenic effect of pomegranate against pancreatic and colon cancer cells using the chick chorioallantoic membrane (CAM) model.

Materials and Methods

Materials

Human pancreatic cancer (Suit-2) and colon (colo205) cell lines were obtained from ATCC (Manassas, VA, USA). Cell culture reagents and hemoglobin (Hb) standard, Drabkin's reagent, and other common reagents were purchased from Sigma (St. Louis, MO, USA). Matrigel was purchased from BD Bioscience (San Jose, CA, USA).

POMx is a pomegranate (*P. granatum*) derivative of fruits (Pom Wonderable, Los Angeles, CA, USA). POMx capsule contains polyphenolics of about 753 mg that are expressed as mg gallic acid equivalents (18).

Cells and Cell Culture

Cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Cells were cultured at 37 °C with a humidifier atmosphere of 5% CO_2 to sub-confluence and treated with 0.25% (w/v) trypsin/EDTA to effect cell release from culture flask. After washing cells with culture medium, they were suspended in DMEM (free of phenol red and fetal bovine serum) and counted.

Chick Chorioallantoic Membrane (CAM) Cancer Implant Model

Tumor Growth in the CAM Cancer Implant Model

Seven-day old chick embryos were purchased from Spafas, Inc. (Preston, CT, USA) and incubated at 37 °C with 55% relative humidity. A hypodermic needle was used to make a small hole in the shell at the air sac and a second hole was made on the broadside of the egg, directly over an avascular portion of the embryonic membrane that was identified by candling. A false air sac was created beneath the second hole by the application of negative pressure at the first hole, causing the CAM to separate from the shell. A window, approximately 1.0 cm^2 was made in the shell over the dropped CAM using a small craft grinding wheel (Dremel, Racine, WI, USA), allowing direct access to the underlying CAM.

The pancreatic (Suit-2) and colon cancer (colo205) cells in exponential growth phase were harvested using 0.25% trypsin-EDTA, washed and suspended in medium. Only suspensions of single cells with a viability exceeding 95% were inoculated. Approximately 1×10^6 cells in 30 μl of medium were mixed with the same volume (30 μl) of Matrigel® (BD Bioscience) and implanted on the chorioallantoic membrane. Matrigel containing different treatments were also inoculated to determine their effect on tumor growth and tumor angiogenesis at day 7 after tumor cell implantation. Three sets of CAM experiments were done, all using the Matrigel and PBS as the control as described next.

In experiment 1, pancreatic cancer cells (all in Matrigel) were treated with PBS, pancreatic cancer (Suit-2) + pomegranate (5 µg/CAM), pancreatic cancer (Suit-2) + pomegranate (10 µg/CAM), and pancreatic cancer (Suit-2) + pomegranate (20 µg/CAM). In experiment 2, the treatment groups (all in Matrigel) were PBS, DMSO, colon cancer (colo205) + pomegranate (20 µg/CAM), and colon cancer (colo205) + resveratrol (a promising anticancer drug, 20 µg/CAM). In experiment 3, CAMs were inoculated with PBS, FGF2 (40 ng/CAM), and FGF2 (40 ng/CAM) + pomegranate (20 µg/CAM), all in Matrigel.

Hemoglobin (Hb) content of tumor masses was determined with Drabkin's reagent. Data represent mean tumor weight (mg) and tumor Hb (mg/ml) ± SEM per treatment group ($n = 5$–8 per group). Tumor mass Hb content was measured as described previously Yalcin et al. (19) to index vascularity of the tumor. For this purpose, each tumor mass was placed into a 0.5 ml tube of double-distilled H_2O and homogenized for 5–10 min. The samples were then centrifuged at 3,000 *g* for 10 min, and the supernatants were collected for Hb determination. Fifty microliters of supernatant were mixed with 50 μl Drabkin's reagent and allowed to sit at room temperature for 15–30 min; 100 μl of this mixture was then placed in a 96-well plate, and absorbance was measured at 540 nm with a Microplate Manager ELISA reader (BioRad Laboratories, Hercules, CA). Hb concentration (mg/ml) was determined by comparison with a standard curve.

CAM Growth Factors-Mediated Angiogenesis Assay

The antiangiogenesis potency of pomegranate was examined in the CAM of angiogenesis using 10-day-old chick embryos (20–22). FGF2 (40 ng, dissolved in PBS) was used as a standard pro-angiogenic agent to induce new blood vessel branches on the CAM. Sterile 1.0 cm diameter disks of #1 filter paper were pretreated with 3 mg/mL cortisone acetate and air dried under sterile conditions. PBS (control) and FGF2 with pomegranate (20 µg/CAM) were then applied to the pretreated disks and dried. The disks were placed on

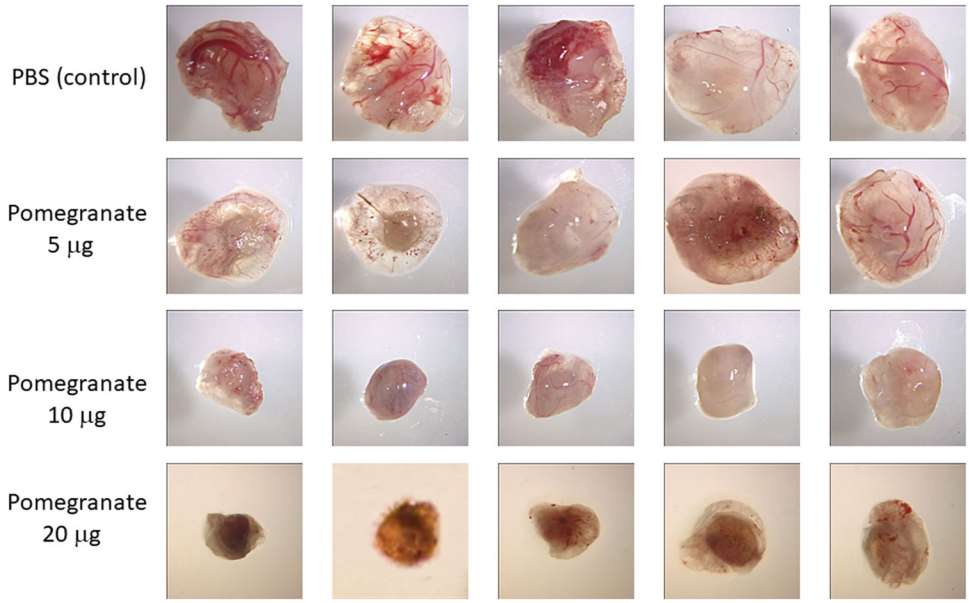

Figure 1. Dose–response reduction of pancreatic tumor weight as a function of pomegranate dose in CAM tumor model.

growing CAMs in an area between preexisting vessels. After incubation at 37 °C with 55% relative humidity for 3 days, the CAM tissue directly beneath each filter disk was resected from the control and treated CAM samples. Tissues were washed three times with PBS, placed in 35-mm Petri dishes and examined under an SV6 stereomicroscope (Karl Zeiss, Thornwood, NY) at 50× magnification. Digital images of the CAM sections were collected using a 3-CCD color video camera system and analyzed with Image-Pro software (Media Cybernetics, Silver Spring, MD, USA). The numbers of vessel branch points contained in a circular region equal to the area of each filter disk were counted. One image was counted in each CAM preparation, and findings from 5 to 8 CAM preparations/group were used for each treatment condition.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA and comparing the mean ± SEM of branch points from each experimental group with its respective control group. Statistical differences approaching $P < 0.05$ were considered statistically significant.

Results

Effect of Pomegranate on Pancreatic Tumor Weight and Hemoglobin Concentration

The effect of different pomegranate extract concentrations (5, 10, and 20 µg/ml) on the morphology, tumor weight, and Hb concentration are presented in Figures 1–3. Compared with control, pomegranate extract significantly decreased tumor weight ($P < 0.05$) at 5–10 µg/ml and $P < 0.01$ at 20 µg/ml.

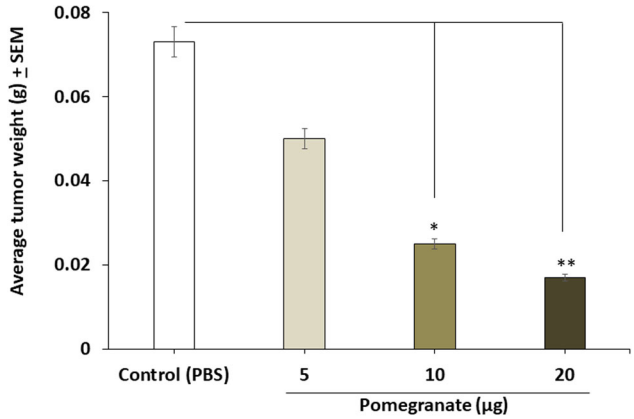

Figure 2. Effect of pomegranate extract on pancreatic tumor growth (g) in CAM tumor model. $n = 5$-8, $^{*}P < 0.05$; $^{**}P < 0.01$.

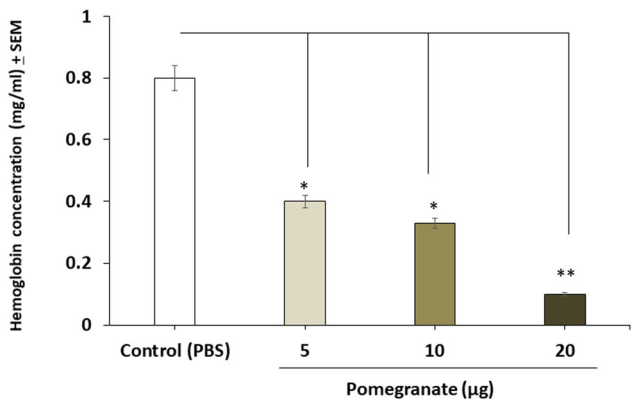

Figure 3. Effect of pomegranate extract on pancreatic tumor-mediated angiogenesis (hemoglobin concentration, mg/ml) in CAM tumor model. $n = 5$–8, $^{*}P < 0.05$; $^{**}P < 0.01$.

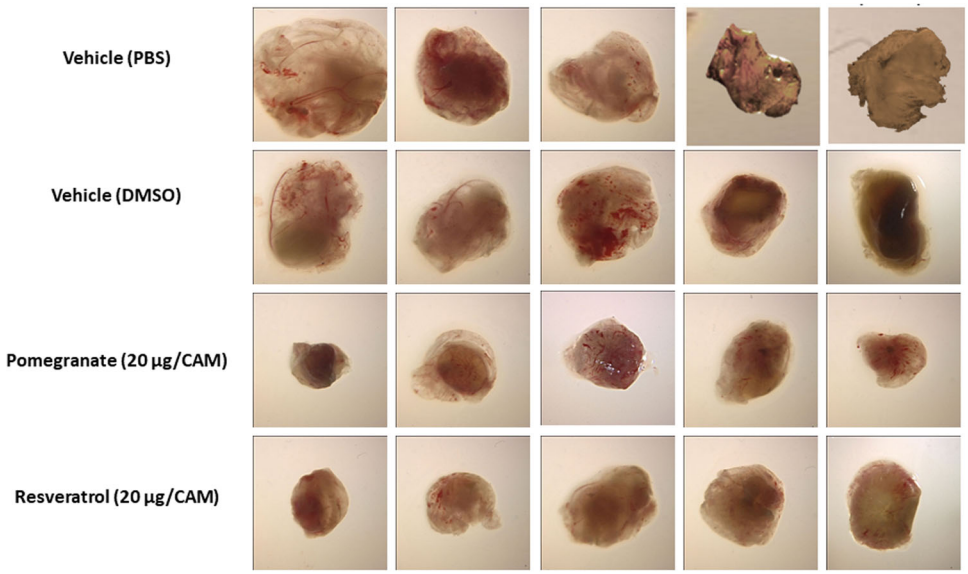

Figure 4. Anti-carcinogenic effect of pomegranate and resveratrol on colo205 tumors in CAM tumor model.

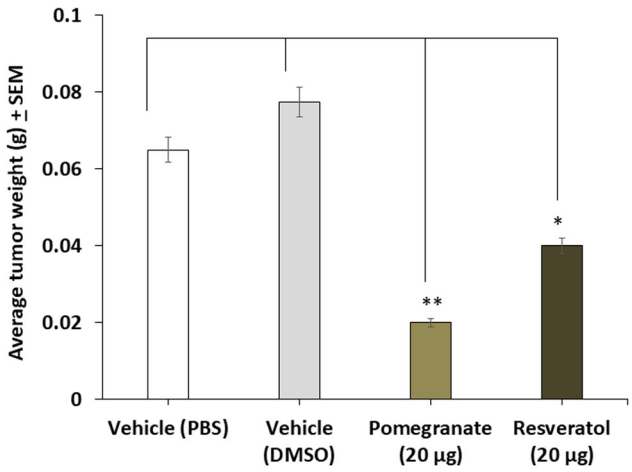

Figure 5. Reduction of colon cancer (colo205) tumor weight by pomegranate or resveratrol in CAM tumor model. $n = 5–8$, $^{*}P < 0.05$; $^{**}P < 0.01$.

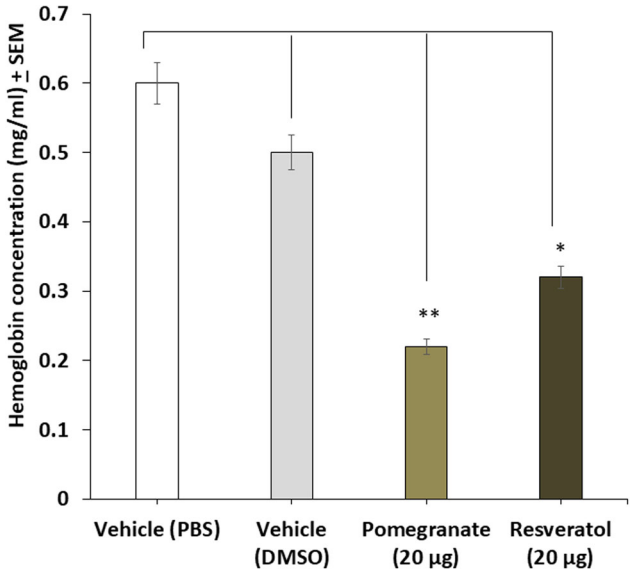

Figure 6. Reduction of colon (colo205) tumor weight by pomegranate or resveratrol in CAM tumor model. $n = 5–8$, $^{*}P < 0.05$; $^{**}P < 0.01$.

Hb concentrations were significantly decreased in CAM groups treated with 5 µg/ml ($P < 0.05$), 10 µg/ml ($P < 0.05$), and 20 µg/ml ($P < 0.01$) pomegranate extract.

Effect of Pomegranate on Colon Tumor Weight and Hemoglobin Concentration

Data illustrated in Figure 4 show the morphological reduction in the tumor of colo205 cancer size due to pomegranate extract (20 µg/ml) in comparison with control and resveratrol-treated (20 µg/ml) CAM.

Pomegranate extract at 20 µg/ml significantly decreased ($P < 0.01$) tumor weight in comparison with positive control group (Figure 5). In comparison with the resveratrol-treated group, pomegranate also induced significant reduction in tumor size ($P < 0.05$).

Results shown in Figure 6 revealed a significant decrease in Hb concentrations in pomegranate ($P < 0.01$) and resveratrol-treated ($P < 0.05$) groups compared with positive control CAM. Also, pomegranate significantly decreased ($P < 0.05$) the Hb concentration in comparison with resveratrol treated.

Effect of Pomegranate on FGF2-Induced Angiogenesis

Results shown in Figures 7 and 8 are the effect of FGF2 (40 ng/ml) and pomegranate extract (20 µg/ml) on the average blood vessel branch count in CAMs.

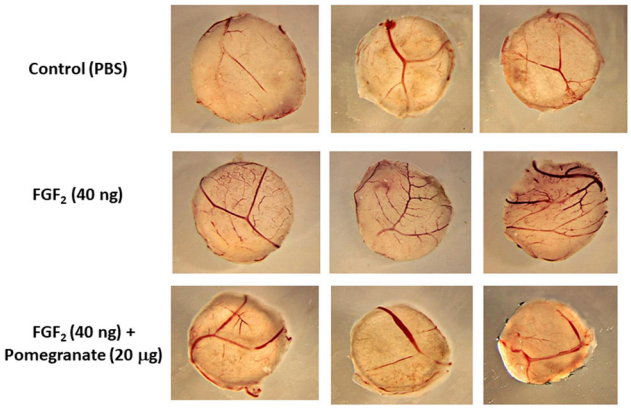

Figure 7. Effect of pomegranate on FGF2-mediated angiogenesis in CAM tumor model.

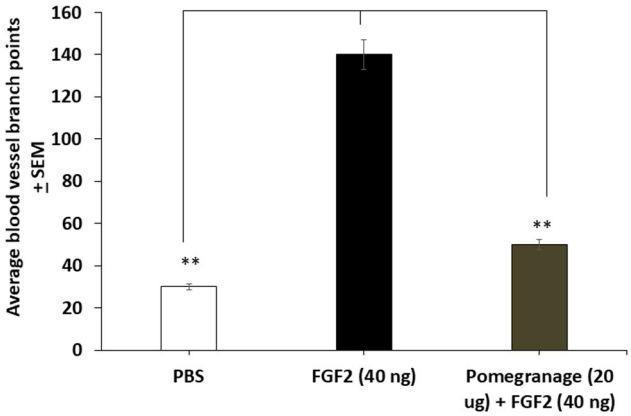

Figure 8. Reduction of hemoglobin content in colon (colo205) tumors by pomegranate CAM tumor model. $n = 5–8$, $^{**}P < 0.01$.

FGF2 significantly increased the average blood vessel branch count compared with control CAM, while pomegranate-treated CAM significantly decreased the average blood vessel branch count compared with FGF2-treated CAM.

Discussion

Angiogenesis is a process that is pivotal for cancer progression (23). Therefore, many studies have investigated the antiangiogenesis effect of various synthetic and natural products (24,25). Pomegranate is a natural product with an anticancer effect against different cancer types including breast (26), colorectal (27), pancreatic (28), prostate (29), lung (30), ovarian (31), and liver (32) cancers.

In the present study, pomegranate extract significantly decreased the tumor weight and Hb concentrations of CAM-implanted pancreatic and colon cancers. These results indicated the anticancer effect of pomegranate, especially through attenuation of angiogenesis. Sartippour et al. (15) revealed that the ellagitannin-rich pomegranate extract induced antiangiogenetic effect on human prostate cancer cells (LNCaP) through induction of HIF-1α and VEGF. Also, pomegranate seed oil or fermented juice polyphenols downregulated VEGF in MCF-7 breast cancer cells and upregulated migration inhibitory factor (MIF) in MDA-MB-231 triple negative breast cancer cells (14). Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice through downregulation of nuclear factor-kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR), and VEGF (17). Ellagic acid, a constituent of pomegranate, induced a significant decrease in VEGF level in LNCaP at concentrations of 25 and 50 μM (33). Also, ellagic acid reduced VEGF receptor type 2 (VEGFR-2) expression in human bladder cancer cells (34). Moreover, ellagic acid inhibited MDA-MB-231 cancer growth and VEGFR-2 expression in a breast cancer xenograft study (35).

Overexpression of FGF2 and its receptors has a wide range of effects in various cancers and many human tumor cell lines (36) including cellular proliferation, cellular invasiveness, increased angiogenesis, and enhanced metastasis (37). In the present study pomegranate extract defeated the angiogenetic effect of FGF2. Therefore, pomegranate extract is a promising natural product that can effectively control tumor angiogenesis.

Conclusion

Pomegranate extract attenuated the angiogenesis of pancreatic (Suit-2) and colon (colo205) cancer cells through significant reduction in tumor weight and small blood vessels' count in the CAM model. In addition, pomegranate extract successfully reduced angiogenesis in the CAM that was induced by FGF2. Therefore, we can conclude that pomegranate and its active constituents can reduce cancer progression and angiogenesis in the CAM model.

Disclosure Statement

The authors declare no conflict of interest.

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