SPANDIDOS PUBLICATIONS

Pomegranate extract inhibits the proliferation and viability of MMTV-Wnt-1 mouse mammary cancer stem cells *in vitro*

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Abstract. Pomegranate (*Punica granatum* L.) is known to possess anticancer activities. The effects of a standardized extract of pomegranate (PE) on a mouse mammary cancer cell line (designated WA4) derived from mouse MMTV-Wnt-1 mammary tumors were examined in this study. The WA4 cell line has been previously characterized as containing a majority of cells possessing stem cell characteristics. PE inhibited the proliferation of WA4 cells in a time- and concentration-dependent manner. This was due to an arrest of cell cycle progression in the G0/G1 phase. PE was also cytotoxic to quiescent WA4 cells in a concentration-dependent manner at concentrations >10 μg/ml. PE treatment of WA4 cells resulted in an increase in caspase-3 enzyme activity in a time- and concentration-dependent manner, indicating that the cytotoxic effect of PE was due to the induction of apoptosis. We tested the effect of several individual phytochemicals derived from PE on WA4 cells. Ellagic acid, ursolic acid and luteolin caused a time- and concentration-dependent reduction of cell proliferation and viability, suggesting that they contribute to the inhibitory effect of PE, while caffeic acid had no effect. Cancer stem cells, which are highly resistant to conventional chemotherapeutic agents, are thought to be the origin of both primary and secondary breast tumors, and thus are a critical target in both breast cancer therapy and prevention. These data suggest that PE, which is a proven and safe dietary supplement, has promise as an treatment against breast cancer by preventing proliferation of cancer stem cells.

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Abbreviations: CA, caffeic acid; DMSO, dimethylsulfoxamine; EA, ellagic acid; L, luteolin; MMTV, mouse mammary tumor virus; PBS, phosphate-buffered saline; PE, pomegranate extract; SRB, sulforhodamine B; UA, ursolic acid

Key words: pomegranate, mammary cancer stem cells, apoptosis, cell cycle arrest, caspase-3

Introduction

Breast cancer is the most common cancer in women. According to the American Cancer Society, breast cancer comprises 27% of all cancer cases and 15% of all cancer deaths among women in the United States in 2009 (1). Diets rich in fruits and vegetables are associated with a reduced risk of breast and other types of cancers (2,3); it is estimated that >20% of all cancers could be eliminated by consumption of fruits and vegetables (4). This has led to the hypothesis that plant-derived phytochemicals inhibit cancer initiation and progression by affecting the biochemical and molecular pathways relevant to carcinogenesis (4). Phytochemicals have all the characteristics desirable in chemopreventive agents: they are non-toxic, economical and readily available. Of particular interest is the fruit of the pomegranate tree (*Punica granatum* L.). Various parts of pomegranate has been used as medicine for a variety of ailments in many cultures since ancient times (5,6). Pomegranate is commonly consumed as fresh fruit and commercial pomegranate fruit juice, and is a rich source of flavanoids and tannins, which exhibit anti-oxidant, anti-proliferatative and anti-inflammatory activities all consistent with chemoprevention (5,6). Recent studies have demonstrated that pomegranate is a potent anti-carcinogenic agent that causes the induction of apoptosis and cell cycle arrest, and inhibits multiple signaling pathways, in cancer cell lines and animal models of breast, prostate, lung, colon, skin and blood cancers (4-7).

In the present study we examined the potential of a standardized extract of whole pomegranate fruit to inhibit mammary cancer cells *in vitro*. A cell line derived from the mouse mammary tumor virus (MMTV)-Wnt-1 transgenic mouse, a model known to be stem cell-driven (8), designated WA4, has been previously characterized as possessing a majority of cells containing stem-cell like characteristics (Smith *et al*, Am Assoc Cancer Res, Annual Meeting, abs. 3087, 2009; Smith *et al*, Aspen Cancer Conf., Aspen, CO, 2009; Smith *et al*, Am Assoc Cancer Res., abs. B66, 2009). Mammary cancer stem cells are thought to be the origin of mammary cancer in humans and in animal models (9,10). Although a few previous studies have demonstrated the inhibitory effects of pomegranate products on differentiated breast cancer cells (11), our study is the first to examine the effects of a standardized, commercially available and certified safe pomegranate extract (PE) specifically on a cancer stem cell line. We demonstrate that PE is a potent inhibitor of

mammary cancer stem cell proliferation and viability by inducing cell cycle arrest and apoptosis.

Materials and methods

Materials. Pomella®, an HPLC-standardized extract of pomegranate that retains the natural polyphenolic ratio of whole pomegranate fruit, was the kind gift of Verdure Sciences (Noblesville, IN). Ellagitannins (gallic acid, punicalagin α and punicalagin ß) comprises 37.5% of PE and ~2.7% is ellagic acid, as shown in the HPLC profile previously reported (12). Fetal bovine serum, glutamine, RPMI-1640 medium, trypsin/EDTA and phosphate-buffered saline (PBS) were obtained from HyClone (Logan, UT). Dimethyl sulfoxide (DMSO), ribonuclease, Tritin X-100, propidium iodide, sulforhodamine B (SRB), trichloroacetic acid, acetic acid and Tris-base buffer were purchased from Sigma Chemical (St. Louis, MO). The EnzChek Caspase-3 Assay kit was purchased from Invitrogen (Carlsbad, CA). Ellagic acid (EA), ursolic acid (UA), luteolin (L) and caffeic acid (CA) were obtained from LKT Laboratories (St. Paul, MN).

Mammary cancer stem cells. The WA4 cell line (the gift of Dr Stephen D. Hursting, Department of Nutritional Sciences, The University of Texas at Austin) was clonally-derived from a mammary tumor that arose spontaneously in an MMTV-Wnt-1 transgenic mouse. The WA4 cell line contains a majority of cells that display markers of stemness: these include the ability to form mammospheres in low-attachment conditions, high expression of CD44 and low expression of CD24, and enhanced tumorigenicity in xenographs (Smith *et al*, Am Assoc Cancer Res, Annual Meeting: abs. 3087, 2009; Smith *et al*, Aspen Cancer Conf., Aspen, CO, 2009; Smith *et al*, Am Assoc Cancer Res, Annual Meeting, abs. B66, 2009). WA4 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine and maintained in monolayer culture at 37˚C and 5% CO_2 in a humidified incubator. Cells was passaged twice weekly using 0.05% trypsin/0.02% EDTA.

Proliferation and cytotoxicity assays. WA4 cells were placed into 24-well plates at 10000 cells per well. For proliferation assays, WA4 cells were plated at 10000 cells/well in 24-well plates and treated 24 h later with DMSO (control) or the indicated concentrations of PE or phytochemical. For cytotoxicity experiments, 25000 cells/well were placed into 24-well plates and allowed to grow to confluence for 3 days before treatment was begun. Pomegrantate phytochemicals are known to interfere with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (13); therefore, proliferation and cytotoxicity were measured by SRB assay, which has been validated to accurately reflect viable cell number (14). Briefly, following incubation cells were fixed by adding 10% TCA for 60 min at 4˚C. The plates were rinsed with dH_2O, dried overnight, and stained with 0.4% SRB for 30 min at room temperature. Plates were washed with acetic acid to remove unbound dye, blotted dry and bound dye was solubilized by adding 1 ml 10 mM Tris base buffer. The optical density of dye was measured on a microplate reader at a wavelength of 530 nm. DMSO at the concentrations used had no effect on cell viability.

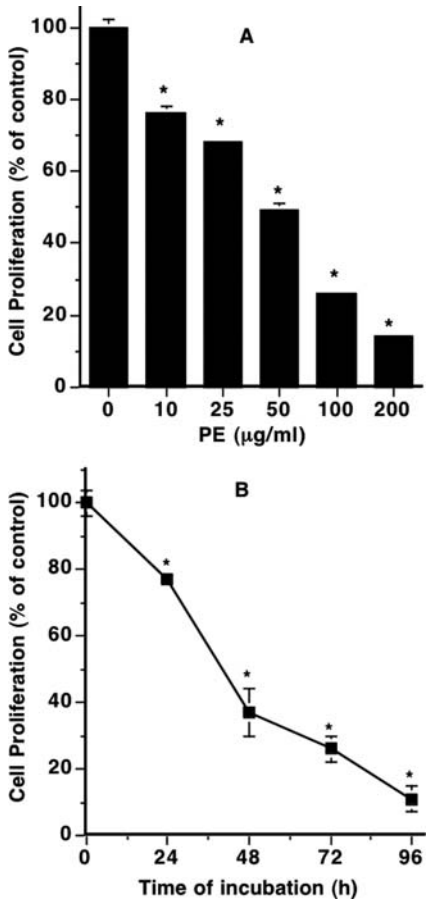

Figure 1. Effect of PE on the proliferation of WA4 cells. (A) Viable cell number was determined by SRB assay after treatment with DMSO (control) or the indicated concentrations of PE for 72 h. (B) WA4 cells were treated with DMSO (control, not shown on graph) or 100 µg/ml of PE for the times indicated and viable cell number was assayed by SRB assay. Results are shown as the means ± SE. *Significant difference between control and PE treated cells ($p<0.05$).

Assay for caspase-3 activity. The ability of PE to induce apoptosis was determined by assaying caspase-3 activity. Briefly, WA4 cells grown in 25 cm^2 flasks were harvested after treatment of DMSO or PE at the indicated concentrations and times. Cell lysate from $2x10^6$ cells was used for each reaction and assayed as directed. Florescence was measured in a microplate reader using excitation/emission at 485/530 nm after incubation with Z-DEVD-R110 (substrate) for 45 min.

Cell cycle analysis. To determine cell cycle distribution, confluent cells grew on 25 cm^2 flasks were treated with PE at 10 or 25 µg/ml in complete medium for 24 h. Briefly, each sample of $2x10^6$ cells were trypsinized, washed with cold PBS, fixed in 1% methanol-free formaldehyde for 20 min in ice. The cells were centrifuged at 300 x g for 10 min at 4˚C and resuspended in PBS. The cell suspension was added to 70% ice-cold ethanol overnight. Cells were pelleted and stained with 500 µl solution of 50 µg/ml propidium iodide, 0.1 mg/ml RNase and 0.05% Tritin X-100 for 45 min at 37˚C. Cell cycle analysis was performed immediately after staining with FACS-Calibur (BD, Franklin Lakes, NJ). A minimum of 10000 cells for each sample were used and cell cycle distribution was analyzed with Flowjo software.

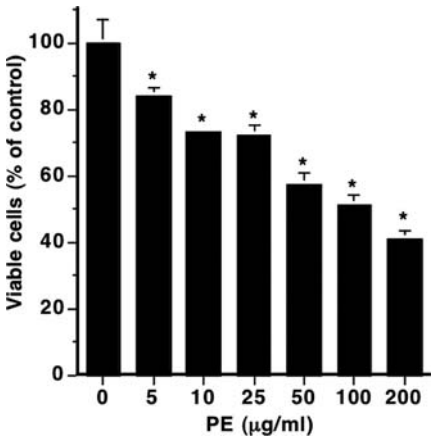

Figure 2. Effect of PE on viability of WA4 cells. Confluent WA4 cells were treated DMSO (control) or PE for 48 h and cell viability was determined by SRB assay. Results are shown as the means ± SE. *Significant difference between control and PE treated cells ($p<0.05$).

Statistical analysis. Data are presented as the mean of triplicate or quadruplicate determinations with standard error. Statistical analysis was performed to assess the differences between the means of untreated and treated samples using a two-tailed Student's t-test with SPSS statistical software. A p-value <0.05 was considered statistically significant.

Results

Effect of PE on WA4 cell proliferation and cell viability. As shown in Fig. 1A, the addition of as little as 10 μg of PE per ml of tissue culture medium significantly inhibited the proliferation of subconfluent WA4 cells. Higher concentrations caused a potent, concentration-dependent inhibition of cell proliferation with a half maximal inhibitory concentration (IC_{50}) of ~50 μg/ml. This inhibition occurred in a time-dependent manner (Fig. 1B). Treatment of confluent WA4 cells with PE caused a concentration-dependent decrease in cell viability at concentrations ≥10 μg/ml culture medium, with an IC_{50} of 200 μg/ml (Fig. 2).

Effects of pomegranate phytochemicals on WA4 cell proliferation and cell viability. The effect of individual pomegranate phytochemicals on proliferation and cytotoxicity of the WA4 cell line was determined. EA, UA and L caused a concentration- and time-dependent inhibition of proliferation of subconfluent cells with IC_{50} between 5 and 10 μM (Fig. 3). EA, UA and L were also cytotoxic to confluent cells (Fig. 4). UA was a significantly more potent inhibitor of both proliferation and viability than EA or L. CA, on the other hand, did not affect the proliferation or viability of WA4 cells.

Effect of PE on caspase-3 activity in WA4 cells. Incubation of WA4 cells with PE caused an activation of capase-3 enzyme activity in a concentration-dependent manner that was significant at ≥25 μg/ml (Fig. 5A). Incubation of WA4 cells with 100 μg/ml PE caused a time-dependent increase in caspase-3 activity that was significant at 12 of incubation and maximal at 48 h of incubation and resulted in a >3-fold increase in activity (Fig. 5B).

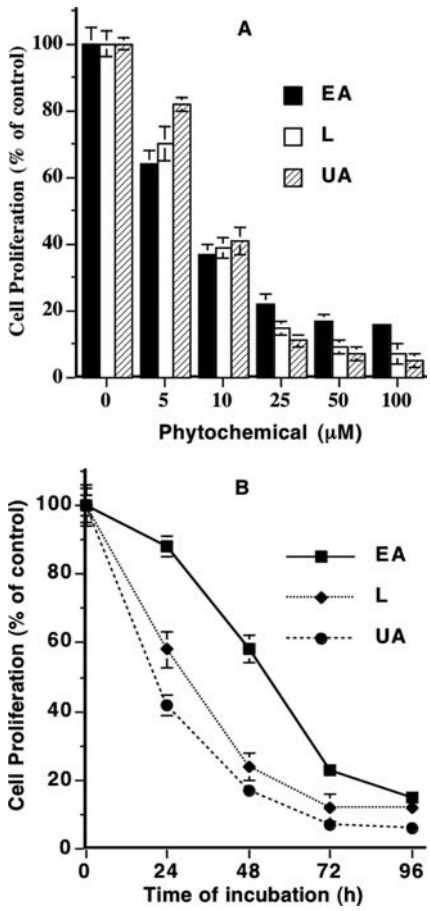

Figure 3. Effect of EA, L and UA on the proliferation of WA4 cells. (A) WA4 cells were treated with DMSO, EA, L or UA and proliferation was determined after 72 h. (B) Cells were treated with EA, L or UA at 25 μM and proliferation was determined. Results are shown as the means ± SE. EA, L and UA significantly inhibited cell proliferation at every concentration and time-point tested with the exception of EA at 24 h of incubation (B) ($p<0.05$).

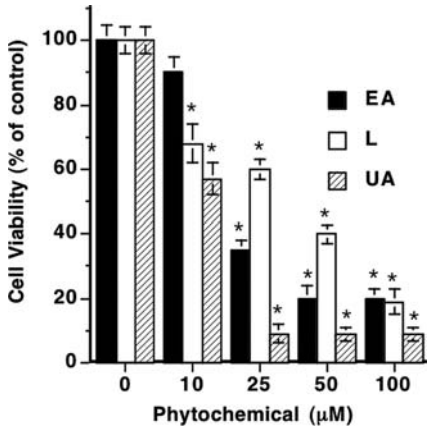

Figure 4. Effect of EA, L and UA on viability of WA4 cells. Cells were treated with DMSO (control) or phytochemical for 48 h and cytotoxicity was determined by SRB assay. Results are shown as the means ± SE. *Significant difference between control and the treated cells ($p<0.05$).

Effect of pomegranate extract on cell cycle arrest. To examine the effect of PE on cell cycle arrest on WA4 cells, cell cycle analysis was determined by flow cytometry after cell staining

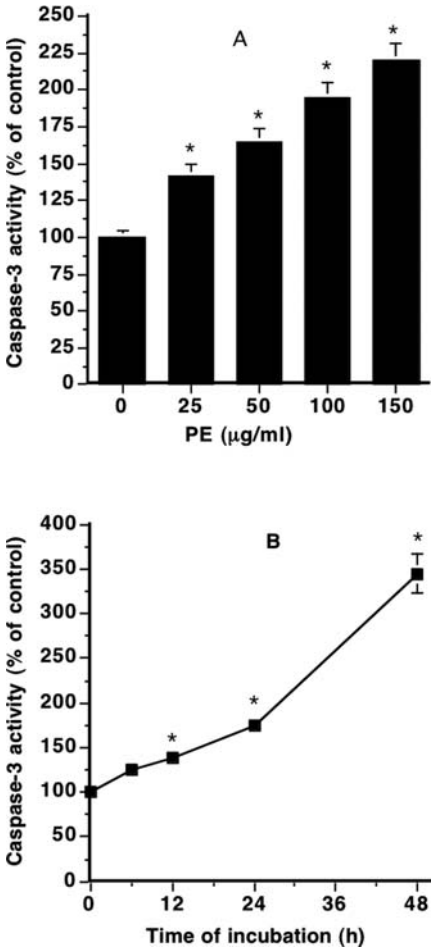

Figure 5. Effect of PE on caspase-3 activity in WA4 cells. (A) Cells were treated with DMSO (control) or PE for 24 h and caspase-3 activity was assayed as described in Materials and methods. (B) Cells were treated with 100 μg/ml PE for the times indicated and caspase-3 activity was determined. Results are shown as the means ± SE. *Significant difference between control and the treated cells ($p<0.05$).

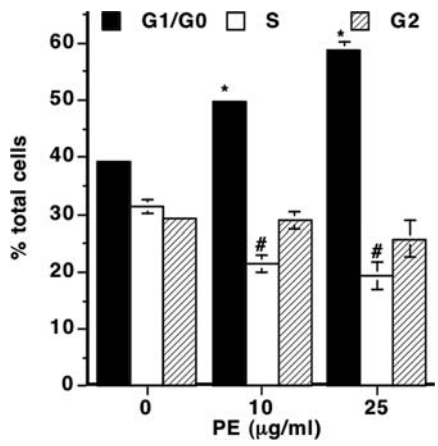

Figure 6. The effect of PE on WA4 cell cycle progression. WA4 cells were treated with PE at the indicated concentrations for 24 h. Cells were stained with PI and separated by flow cytometry, and analyzed by FlowJo software. Results are shown as the means ± SE. *,#Significant difference between control and the treated cells ($p<0.05$).

with PI. As shown in Fig. 6, cells treated with PE for 24 h caused a significant concentration-dependent increase in the number of cells in the G0/G1 phase and a reduction in cells in S phase.

Discussion

The stem cell hypothesis of cancer is based on the existence of a subset of cells in tumors with a high proliferative capacity and multipotency, the stem cell-like ability to produce all cell types found in tumors (9). Tumor heterogeneity arises not by the serial clonal expansion of individual cancer cells that have acquired new, advantageous mutations, but from cancer stem cells. The cancer stem cell hypothesis explains the ultimate failure of chemotherapy; tumors destroyed by chemotherapy may be repopulated by cancer stem cells, which are highly drug resistant through a variety of mechanisms (10). As such, successful chemotherapy and cancer prevention must target not only the differentiated tumor cells that make up the bulk of the tumor, but also cancer stem cells, and treatment may have to be long-term, since cancer stem cells are long-lived. A promising source of novel chemopreventive and chemotherapeutic agents are fruits and vegetables, which are associated with a reduced risk of breast cancer (4,14). Phytochemicals derived from fruits and vegetables have a proven ability to inhibit cancer cell growth *in vitro* and prevent the formation of tumors *in vivo* (4,6) and are well tolerated and safe, allowing for long-term dietary administration. However, since the cancer stem cell hypothesis has only recently been proposed, there are few studies on the effect of phytochemicals on cancer stem cells (16,17). We hypothesized that phytochemicals inhibit the growth and viability of mammary cancer stem cells. To test this hypothesis, in the present study we used a mammary cancer stem cell line, designated WA4, derived from tumors arising spontaneously in MMTV-Wnt-1 transgenic mice, as an *in vitro* model of cancer stem cells. Studies have suggested that mammary tumors arising in this model contain a proportion of tumor cells with cancer stem cell-like characteristics (8), and the ß-catenin pathway, which is activated by overexpression of Wnt-1, is important in governing self-renewal of cells, a hallmark of 'stemness'. The majority of the cells in the WA4 cell line displays many cancer stem-cell like characteristics, as described in Materials and methods. We chose PE as our test agent. PE is a standardized extract of pomegranate that is commercially available as a dietary supplement and is certified 'generally regarded as safe' by the Food and Drug Administration. The extract is standardized by HPLC to contain no less than 70% total polyphenolic compounds, and the final polyphenolic composition is similar to that of the pomengranate fruit (12). Pomegranate juice and seed oil have previously been shown to inhibit the proliferation and metastasis of various differentiated human breast cancer cell lines *in vitro* (11,18,19). Pomegranate extracts have also been shown to inhibit the proliferation of other cancer cell types *in vitro*, including lung, prostate, colon and oral cancer (7,20-23). However, the current study is, to the best of our knowledge, the first to examine the effect of PE on cancer stem cells.

As demonstrated in Fig. 1, PE proved to be a potent inhibitor of cancer stem cell proliferation, with an IC_{50} of only 50 μg/ml. Consistent with the inhibition of proliferation,

metry revealed that PE caused a significant cell cycle arrest, with the proportion of cells in G0/G1 phase significantly increased, while cells in S phase decreased (Fig. 6). PE was also cytotoxic to confluent cells that were not actively dividing at very low concentrations (Fig. 2), and the activation of caspase-3 activity suggests that its cytotoxicity was caused by the induction of apoptosis (Fig. 5). Thus, PE both arrests cell cycle progression and causes death of mammary cancer stem cells. PE polyphenols have been shown to be bioavailable in human pharmacokinetic studies (12). The relatively low concentrations needed to inhibit mammary cancer stem cells *in vitro* and the demonstrated bioavailability of PE suggest that PE may be an effective inhibitor of mammary tumor growth *in vivo*, a possibility that is currently being tested in our laboratory.

We attempted to identify active components in PE that inhibit mammary cancer stem cells. EA is a polyphenolic compound commonly thought to be the most potent of the active anti-oxidants of pomegranate (24-26). EA has been shown to have anti-proliferative activity against a variety of cancer cells (27). In our model system, EA inhibited both the proliferation of subconfluent mammary cancer stem cells (Fig. 3) and was cytotoxic to confluent cells (Fig. 4). However, it was not the only active pomegranate component. PE also contains L, a flavonoid of the flavone class, and UA, a triterpene. L and UA were more potent than EA in inhibiting the proliferation of the WA4 cell line (Fig. 3), and in inducing cytotoxicity (Fig. 4). The proportion of these components in PE is low (pEA constitutes <5% of PE) (12), and therefore there may be other active components present in PE. In addition, it has been postulated that the individual components in PE may exert synergistic action that is superior to that of individual compounds (6).

In summary, the present study demonstrates that PE is a potent inhibitor of mammary cancer stem cells *in vitro*. This is the first study to examine the potential of a dietary extract to target cancer stem cells. Further study will focus on the effects of PE on the Wnt-1 signaling pathway in WA4 cells and the efficacy of PE against mammary stem cells grown syngeneically *in vivo*.

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