Angiogenesis **6**: 121–128, 2003.

Preliminary studies on the anti-angiogenic potential of pomegranate fractions *in vitro* and *in vivo*

Masakazu Toi[1], Hiroko Bando[1], Cheppail Ramachandran[2], Steven J. Melnick[2], Atsushi Imai[3], Rose S. Fife[4], Raymond Eric Carr[4], Tsutomu Oikawa[5] & Ephraim Philip Lansky[6]

[1]*Department of Surgery, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan;* [2]*Research Institute, Miami Children's Hospital, Miami, Florida, USA;* [3]*Department of Obstetrics and Gynecology, Gifu University, School of Medicine, Tsukasamachi, Gifu, Japan;* [4]*Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA;* [5]*Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan;* [6]*Rimonest Ltd, Horev Center, Haifa, Israel*

Received 11 December 2002; accepted in revised form 26 September 2003

Key words: angiogenesis, breast cancer, chemoprevention, MIF, *Punica granatum*, VEGF

Abstract

We previously showed pomegranate seed oil and fermented juice polyphenols to retard oxidation and prostaglandin synthesis, to inhibit breast cancer cell proliferation and invasion, and to promote breast cancer cell apoptosis. Here we evaluated the anti-angiogenic potential of these materials in several ways. We checked a possible effect on angiogenic regulation by measuring vascular endothelial growth factor (VEGF), interleukin-4 (IL-4) and migration inhibitory factor (MIF) in the conditioned media of estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells, or immortalized normal human breast epithelial cells (MCF-10A), grown in the presence or absence of pomegranate seed oil (SESCO) or fermented juice polyphenols (W). VEGF was strongly downregulated in MCF-10A and MCF-7, and MIF upregulated in MDA-MB-231, overall showing significant potential for downregulation of angiogenesis by pomegranate fractions. An anti-proliferative effect on angiogenic cells was shown in human umbilical vein endothelial cell (HUVEC) and in myometrial and amniotic fluid fibroblasts, and inhibition of HUVEC tubule formation demonstrated in an *in vitro* model employing glass carrier beads. Finally, we showed a significant decrease in new blood vessel formation using the chicken chorioallantoic membrane (CAM) model *in vivo*. 'In sum, these varied studies employing different models in different laboratories overall demonstrate for the first time an anti-angiogenic potential of pomegranate fractions, suggesting further *in vivo* and clinical investigations (for updates: info@rimonest.com).

Abbreviations: CAM – chick chorioallantoic membrane; CLA – conjugated linoleic acid; HUVEC – human umbilical vein endothelial cells; IL-4 – interleukin-4; MIF – migration inhibitory factor; N – non-saponified fraction of pomegranate seed oil; O – pomegranate seed oil polyphenols; P – pomegranate pericarp polyphenols; SEPO – Sde Eliahu pressed oil (cold-pressed pomegranate seed oil; SESCO – Sde Eliahu super critical oil (supercritical fluid extracted pomegranate seed oil); VEGF – vascular endothelial growth factor; W – wine (pomegranate fermented juice polyphenols)

Introduction

Angiogenesis is a critical process for the development and progression of cancer [1], and an important pharmacological target both for cancer prevention [2] and treatment [3]. In screening potential drug candidates against angiogenesis, a broad range of both *in vitro* and *in vivo* assessments provides the highest level of certainty for the potential drug's efficacy [4]. In this context, the present study was undertaken to test pomegranate fractions in multiple pharmacological settings pertinent to angiogenesis. Specifically, we looked at fibrocyte proliferation, production of vascular regulatory molecules by normal and cancerous breast cells, tubule formation and *in vivo* blood vessel formation in the chick embryo chorioallantoic membrane (CAM). Pomegranate (*Punica granatum*) is an ancient fruit of great medical interest. We previously showed extracts to exert suppressive and chemopreventive effects against breast cancer *in vitro* and in an animal organ culture model [5].

Correspondence to: Ephraim Philip Lansky, Rimonest Ltd, Horev Center, P.O. Box 9945, Haifa 34341, Israel. Tel: +972-4-8261477; Fax: +972-4-8110037; E-mail: info@rimonest.com

Pomegranate juice and peels are rich in estrogenic flavonoids like luteolin, which, elsewhere, have been shown to be anti-angiogenic [6, 7] or to inhibit factors which promote angiogenesis, e.g., bFGF (basic FGF) [8]. Furthermore, pomegranate seed oil is largely composed of a three-double-bond, conjugated linoleic acid (CLA), punicic acid [9]. The more common two-double-bond types of CLA found in bovine flesh and milk inhibits angiogenesis [10]. These grounds suggested that pomegranate might also possess anti-angiogenic activities.

Fibrocytes are important in angiogenesis because they lay its requisite intercellular infrastructure [11]. They also secrete extracellular matrix degrading enzymes (e.g., matrix metalloproteinase 9), which promote endothelial cell invasion, and secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibrocyte growth factor (bFGF) and the interleukins that, favor endothelial cell proliferation and/or tube cell formation, but very few anti-angiogenic factors [12]. For these reasons, we first examined the effect of the pomegranate fractions on fibrocyte proliferation. The fibrocytes studied were obtained under informed consent during caesarean sections from the pregnant myometrium and amniotic fluid. Angiogenesis is important during the rapid growth of the myometrium and amniotic membranes during pregnancy, which provide a laboratory-like setting for studying angiogenesis independent of the tumor environment [13].

Next, we turned our attention to some of those specific tumor-derived factors, that regulate angiogenesis. These included the aforementioned VEGF, interleukin 4 (IL-4) and migration inhibitory factor (MIF).

Angiogenesis is favored by upregulation of VEGF [14] and IL-4 [15], and downregulation of MIF [16]. We measured these factors in the conditioned media of normal and cancerous human breast cells grown in the presence and absence of the pomegranate fractions.

We also studied the direct effects of pomegranate fractions on endothelial cells, as these provide the lining for the new blood vessels themselves. We examined proliferation of human umbilical vein endothelial cells (HUVEC), and on tubule formation by HUVEC, an event recognized as necessary for angiogenesis [17].

Though *in vitro* tests provide the best opportunity to examine mechanisms, *in vivo* testing is also essential to study the angiogenic effect in the complex organismic environment. To address this requirement, we examined the effect of the pomegranate extracts also on new blood vessel formation in the chick embryo chorioallantoic membrane (CAM).

Materials and methods

Pomegranate extracts

Pomegranate extracts deriving from the 2000 crop of pomegranates of the 'Wonderful' cultivar, bio-organically grown on Kibbutz Sde Eliahu, Israel, were employed. These included pomegranate fermented juice (pomegranate 'wine') polyphenols (W), pomegranate pericarp polyphenols (P), cold-pressed pomegranate seed oil (SEPO), supercritical fluid extracted pomegranate seed oil (SESCO), pomegranate seed oil polyphenols (O), and unsaponified pomegranate seed oil fraction (N) all provided by Rimonest Ltd (Haifa, Israel).

Measurement of fibroblast proliferation using continuous and pulse-labeling

Human fibroblasts were derived from amniotic membrane and from pregnant myometrium from obstetrical patients, at the time of elective Caesarian section, who gave informed consent for the disposition of their surgically removed tissues. Approval from the Gifu University Institutional Review Board (IRB) was also obtained beforehand. The fibroblasts were seeded at confluence in plastic 24-well plates at 100,000 cell/cm^2. Monolayer cultures were preincubated in modified minimum essential medium (MEM) with 1% FBS for 2 days and were subjected to concurrent exposures of different pomegranate fractions (W, P, O, SEPO) at 37 °C. ^{3}H-thymidine uptake was examined by continuous labeling for 24 h or by using 2-h pulse labeling at 24 h with 1 μCi/well, and uptake was quenched by aspiration of the medium. The cells were washed with ice-cold 0.5% trichloroacetic acid, and the radioactivity was counted.

Growth of cells and measurement of VEGF, IL-4 and MIF in conditioned media

Three different human breast cell lines, estrogen-sensitive MCF-7 cancer cells, estrogen-resistant MDA-MB-231 cancer cells, and immortalized normal MCF-10A epithelial cells, were grown in culture at 37 °C for 24 h in humidified 5% CO_2 in air in the presence of the Rimonest individual pomegranate fractions W, P, SEPO and SESCO. After 72 h of growth, cultures were centrifuged to obtain the supernatants (conditioned media), and these were immediately packed on dry ice and subsequently analyzed for VEGF, MIF and IL-4 with the commercial R&D Elisa kits (R&D systems, Minneapolis, Minnesota) as previously described [18].

Growth of HUVEC and measurement of proliferation using the MTT assay

HUVECs were obtained from Cell Systems (Kirkland, Washington) and cultured in complete medium (MCDB-131 containing 10% fetal bovine serum (FBS; Moregate, Melbourne, Australia) containing 10 μg/ml endothelial cell growth supplement (Upstate, Lake Placid, New York), 10 ng/ml epidermal growth factor (Sigma, St. Louis, Missouri) and 10 μg/ml heparin (Sigma, St. Louis, Missouri).

HUVEC proliferation was determined using a 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay according to the manufacture's instructions (Promega, Tokyo, Japan). HUVECs (1×103 cell per 0.1 ml of growth medium) were dispensed within gelatin-coated 96-well culture plates (Iwaki, Tokyo, Japan). The cells were incubated at 37 °C for 4 h in a CO_2 incubator, and the medium was changed to that containing various concentrations of a test sample. After 72 h incubation, 100 μl of MTT reagent was added to each well. Following additional 4-h incubation at 37 °C, 10 μl of solubilization solution was added to dissolve the formazan crystals, and the absorbance was then measured at 570 nm using a MTP-120 microplate reader (Corona Co. Ltd, Ibaraki, Japan). Wells without cells were used as blanks and were subtracted as background from each sample. Results were expressed as percentage of control.

Assay for plasminogen activator activity

Plasminogen activator activity from HUVECs was determined as described previously [19]. In brief, HUVECs (9×105 cells/dish were cultured in gelatin-coated 35-mm dishes containing 2 ml of complete medium at 37 °C for 24 h in a humidified chamber under 5% CO_2, and then incubated in serum-free growth medium containing 0.1% bovine serum albumin in the presence or absence of a test sample (or medroxyprogesterone acetate) for 18 h. The medium was aspirated, and then the cells attached to dishes were washed twice with phosphate-buffered saline and then extracted with 0.5 ml of 0.5% Triton X-100 in phosphate-buffered saline. Plasminogen activator activity in the cell extracts was determined. Plasminogen activator activity was determined at 37 °C and pH 7.4 in 0.1 M Tris–HCl containing human plasminogen (Roche, Tokyo, Japan) and H-D-Val-Leu-Lys-p-nitroanilide (S-2251; Chromogenix, Molndal, Sweden). Protein concentrations were determined with bovine serum albumin as a standard according to the manufacturer's instructions (DC protein assay; Bio-Rad, Hercules, California).

Measurement of tubule formation in HUVEC

Stable complexes consisting of HUVEC adherent to microcarrier beads were established according to a previously described method, and tubule formation among the HUVEC assessed by visually counting the number of tubules extending from each bead [20]. The complexes were used in 12-well plates, and tubule formation stimulated with conditioned media from estrogen-resistant MDA-MB-435 breast cancer cells (grown in MEM, 5% FBS, 1% antibiotic-antimycotic solution, 1% sodium pyruvate, 2% MEM vitamin solution, non-essential amino acid solution, pH adjusted to 7.4), VEGF, and bFGF, all from Sigma, St. Louis, Missouri). The pomegranate fractions were added to the experimental wells following stimulation with the conditioned media, and tubule formation quantified daily by visual inspection at 10×.

Chick embryo CAM assay

The CAM assay was established as previously described [21–23]. Briefly, the CAMs of 5-day-old chick embryos were treated with ethylene–vinyl acetate copolymer 40 pellets containing, or not containing, pomegranate extracts W or P at 37 °C for 2 days in a humidified egg incubator, after which an appropriate volume of a 20% fat emulsion was injected into the choriolantois to show the vascular network better. The anti-angiogenic response was assessed as positive when the avascular zone exceeded 3 mm in diameter; only the frequency was monitored. This experiment was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science, and was carried out in accordance with the Guidelines for Animal Experiments of The Tokyo Metropolitan Institute of Medical Science.

Results

Suppression of fibroblast proliferation by pomegranate fractions

Figure 1 demonstrates the effect of pomegranate fractions W and P on proliferation of myometrial and amniotic fluid fibroblasts using continuous labeling. Pulse labeling generated a similar pattern (not shown). Pomegranate seed oil polyphenols (O) and cold pressed pomegranate seed oil (SEPO) exerted more modest inhibitory effects (not shown).

VEGF, IL-4 and MIF in conditioned media

Pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) caused downregulation of VEGF in MCF-7 estrogen-dependent breast cancer cells (Figure 2). A less pronounced downregulation

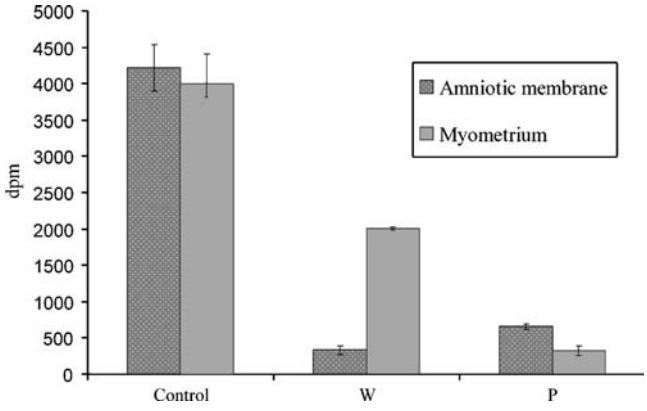

Figure 1. Effects of pomegranate fractions W and P (35 μg/ml) on proliferation of human myometrial and amniotic fluid fibrocytes. Samples obtained under informed consent.

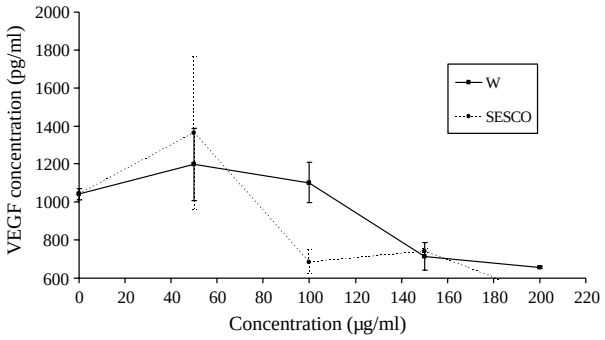

Figure 2. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-7 estrogen-sensitive human breast cancer cells.

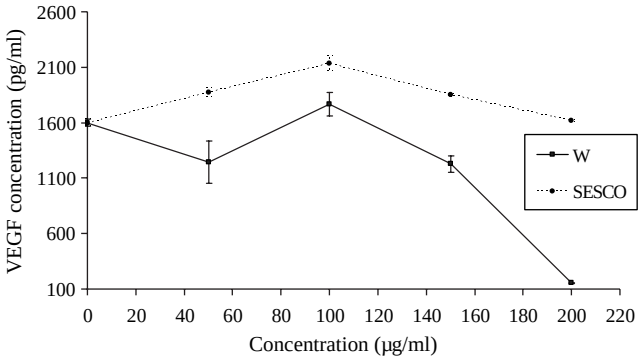

Figure 3. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MDA-MB-231 estrogen-resistant human breast cancer cells.

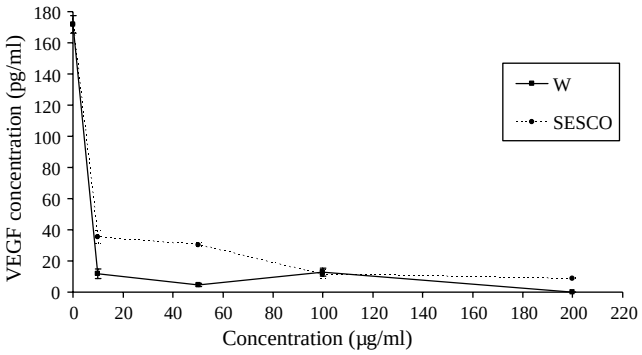

Figure 4. Effects of pomegranate seed oil (SESCO) and pomegranate fermented juice polyphenols (W) on VEGF expression by MCF-10 immortalized normal human breast epithelial cells.

trend was noted in MDA-MB-231 estrogen-resistant breast cancer cells (Figure 3). The most pronounced and sustained downregulation was seen in the MCF-10A immortalized normal breast epithelial cells (Figure 4). Pomegranate pericarp polyphenols (P) effected a down-regulation similar, but milder, to that seen for the fermented juice polyphenols (W) in all cases (data not shown). No difference in IL-4 expression was noted from controls in all cases. A dose-dependent upregulation of MIF was caused in MDA-MB-231 cells by both W and SESCO (Figure 5), and a brief upregulation by

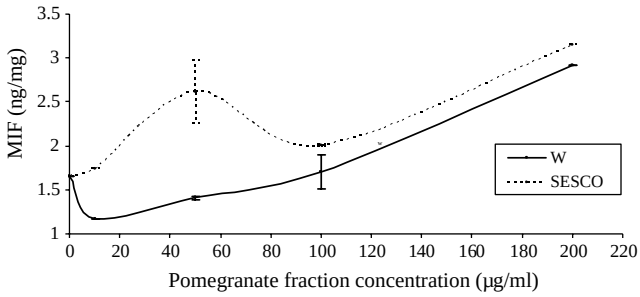

Figure 5. Effects of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on MIF expression in MB-MDA-231 estrogen-receptor-negative human breast cancer cells.

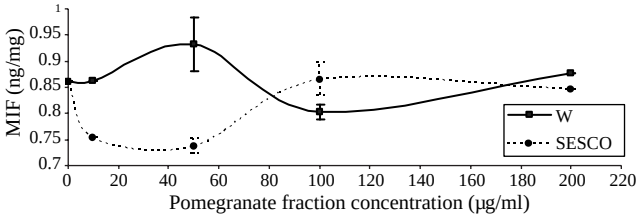

Figure 6. Effect of pomegranate fermented juice polyphenols (W) and pomegranate seed oil (SESCO) on MIF expression in MCF-10A immortalized normal human breast epithelial cells.

both fractions noted in MCF-10A at low dose only (Figure 6). The expression of MIF was not significantly affected by either fraction in MCF-7.

Proliferation of HUVEC

Figure 7 reveals a modest but significant inhibitory effect of W on HUVEC at even very low concentrations.

Plasminogen activator activity

Concentrations of W ranging from 10^{-4} to 10 μg/ml had no significant effect on plasminogen activator activity (data not shown).

Tubule formation by HUVEC

Figure 8 shows the effect of 10 μg/ml of different pomegranate fractions, or combinations, on suppression of tubule formation from HUVEC. Note there is a suggestion of an additive effect from the combination of W and O. A similar pattern was noted at 5 μg/ml (not shown).

Suppression of vascularization in chick embryo CAM

Figure 9A shows the suppression of vascularization in the CAM model by W, while P was without effect. Figure 9B demonstrates the same result graphically.

A simplified summary of the effects of all pomegranate extracts used in this study in all experimental settings is given in Table 1.

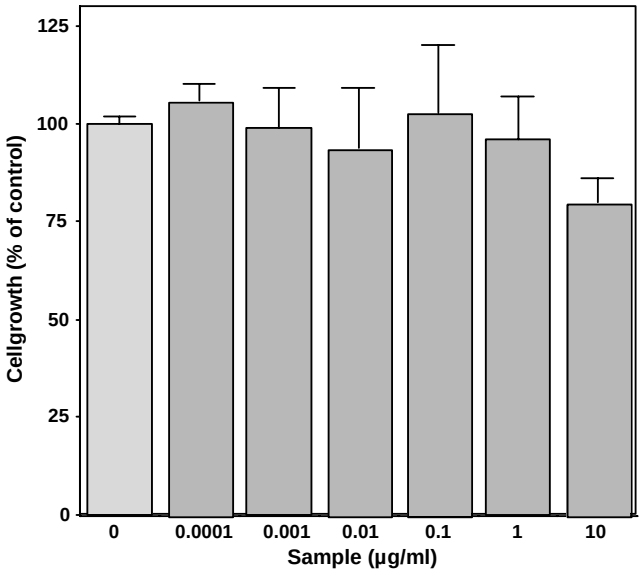

Figure 7. Effects of pomegranate fractions W and P on proliferation of HUVEC as measured by the MTT assay.

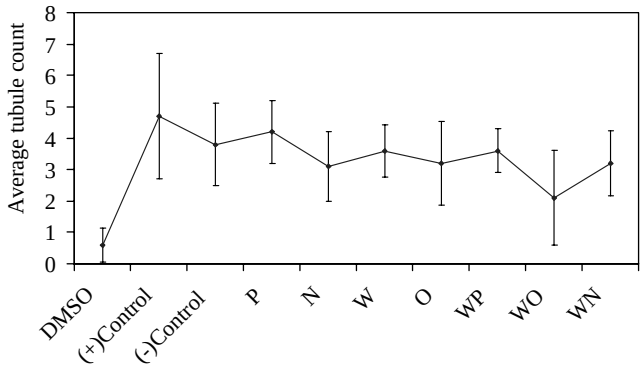

Figure 8. Effects of 10 µg/ml of selected pomegranate fractions and combinations on tubule formation in HUVEC that was stimulated by conditioned medium and soluble factors.

Discussion

Overall, this study helps to establish the potential of pomegranate fractions as potential adjuncts to both the chemoprevention and chemotherapy of breast cancer. The particularly striking suppression of VEGF in the most highly differentiated cell line, MCF-10A, is particularly suggestive of a role of these extracts in chemoprevention. That IL-4 was not affected in any cell line, helps to highlight the specificity of the effect of the extracts on VEGF.

Upregulation of MIF specifically in MDA-MB-231 is interesting in light of the seeming insensitivity of VEGF expression to the pomegranate fractions in this line. Thus, pomegranate fractions may possess

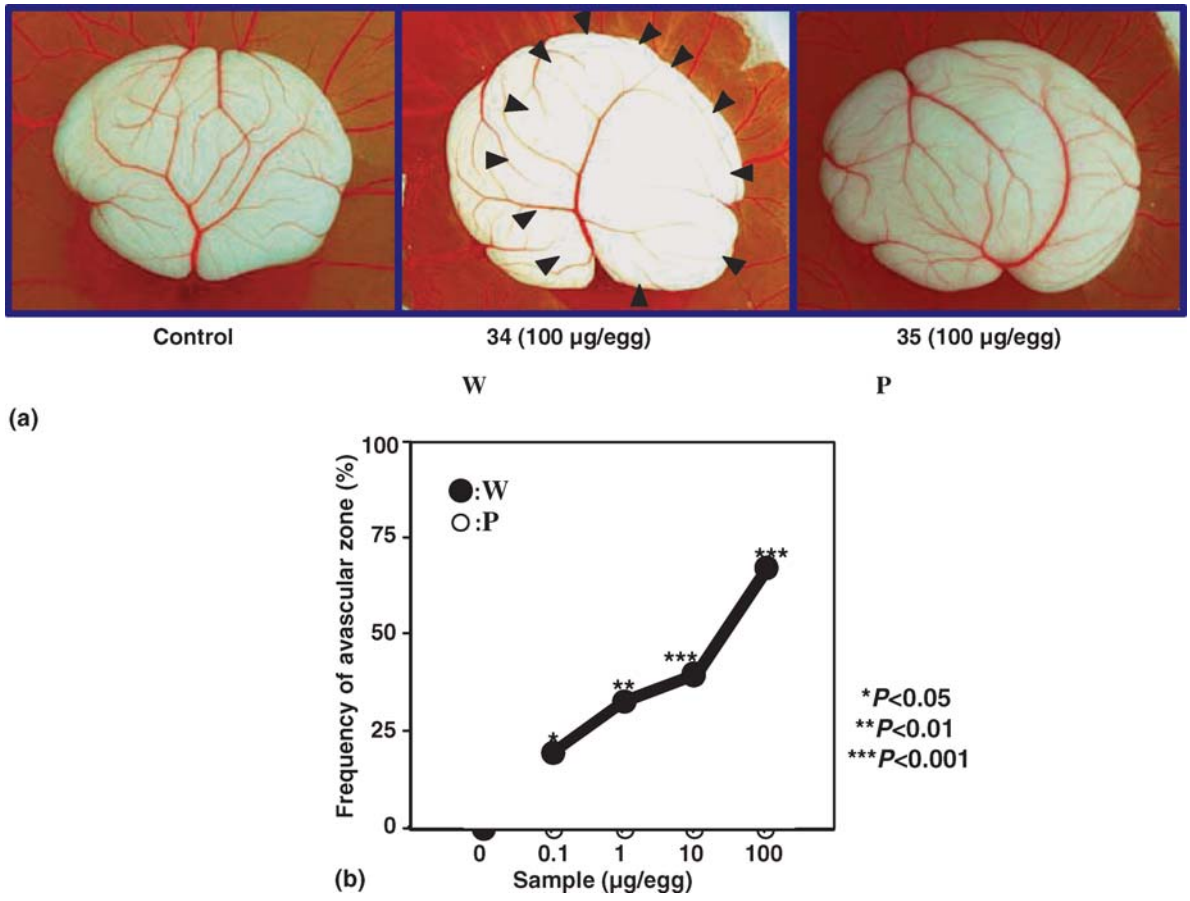

Figure 9. (A) Photomicrograph showing effect of pomegranate fraction W on neovascularization in chick embryo CAM assay. Fraction P was without effect. (B) Effect of pomegranate fraction W on neovascularization in chick embryo CAM. Fraction P was without effect.

Table 1. Summary of effects of pomegranate fractions on pharmacological parameters pertinent to angiogenesis.

Fraction	Test													
					VEGF downregulation			MIF upregulation			IL-4 upregulation			
	Myometrial fibrocyte proliferation inhibition	Amniotic fibrocyte proliferation inhibition	HUVEC proliferation inhibition	HUVEC tubule formation inhibition	MCF-7	MB-MDA-231	MCF-10-A	MCF-7	MB-MDA-231	MCF-10-A	MCF-7	MB-MDA-231	MCF-10-A	CAM angiogenesis inhibition *in vivo*
P	2	2	–	1	1	0	3	0	0	0	0	0	0	0
W	3	2	2	2	1	0	3	0	1	1	0	0	0	3
O	1	2	–	1	–	–	–	–	–	–	–	–	–	–
N	–	–	–	0	–	–	–	–	–	–	–	–	–	–
BOT	–	–	–	–	1	0	3	0	0	0	0	0	0	–
SEPO	1	2	–	–	1	0	2	0	0	0	0	0	0	–
SESCO	–	–	–	–	2	0	3	0	2	0	0	0	0	–
WP	–	–	–	1	–	–	–	–	–	–	–	–	–	–
WO	–	–	–	2	–	–	–	–	–	–	–	–	–	–
WN	–	–	–	1	–	–	–	–	–	–	–	–	–	–

P = pomegranate pericarp polyphenols; W = pomegranate fermented juice polyphenols; O = pomegranate seed oil polyphenols; N = non-sapoonified fraction of pomegranate seed oil; BOT = bottom fraction of pomegranate seed cake extract; SEPO = cold pressed pomegranate seed oil; SESCO = supercritical fluid extracted pomegranate seed oil; WP = 1:1 mixture of pomegranate fermented juice and pericarp polyphenols; WO = 1:1 mixture of pomegranate fermented juice and seed oil polyphenols; WN = 1:1 mixture of pomegranate fermented juice polyphenols and non-saponified fraction of pomegranate seed oil; HUVEC = human umbilical vein endothelial cells; VEGF = vascular endothelial growth factor; MIF = migration inhibitory factor; IL-4 = interleukin 4; CAM = chicken chorioallantoic membrane; MCF-7 = an estrogen receptor positive human breast cancer cell line; MB-MDA-231 = an estrogen receptor negative human breast cancer cell line; MCF-10A = an immortalized normal human breast epithelial cell line.

Scale: (3) = strong effect; (2) = moderate effect; (1) = mild effect; (0) = no significant effect; (–) = not tested.

anti-angiogenic effects which involve downregulation of an angiogenic promoter (VEGF) in some types of cancer cells (MCF-7), and upregulation of angiogenic suppressors (MIF) in other cell types (MDA-MB-231). Such multiple cytokine or chemokine targeting by the fractions is a desirable attribute for anticancer therapy [24].

Oxidative stress is an important trigger for angiogenesis [25, 26], and the mechanism for this trigger likely involves NFkappaB [27]. Significantly, NFkappaB is important in mediating the antioxidant activity of pomegranate fermented juice polyphenols [28]. Therefore, the downregulation of VEGF and upregulation of MIF might possibly involve anti-oxidative mechanisms.

Pomegranate fractions, especially the seed oil, are known to possess estrogenic activity [29]. Estrogens act on vascular endothelium [30] both through estrogen receptors [31] and *via* NFkappaB [32]. Thus, the effects here observed might also involve known patterns of estrogenic influence [33]. Specifically, the relative sensitivity of the estrogen receptor positive MCF-7 to downregulation of VEGF by the pomegranate fractions over the estrogen receptor negative MDA-MB-231 may partially be related to these mechanisms [34].

In addition to these arguments, pomegranate seed oil, as previously stated, consists largely of conjugated fatty acids similar to the CLA in bovine products. Recently, CLA has been shown to exert its anti-angiogenic activity possibly through blockade of another angiogenic promotor found in fibroblasts, i.e., bFGF [34]. The punicic acid in pomegranate seed oil might act likewise.

Furthermore, punicic acid inhibits prostaglandin formation [35]. Inhibition of prostaglandin biosynthesis by inhibiting cyclooxygenase also downregulates VEGF expression [36], and upregulates MIF expression [37]. In that the same pattern of VEGF downregulation and MIF upregulation was observed herein from pomegranate fractions suggests inhibition of cyclooxygenase and/or other eicosanoid enzymes as a likely mechanism.

Generally, the present study highlights anti-angiogenic potential for pomegranate extracts. This has been demonstrated herein from suppression of fibroblast growth, suppression of HUVEC growth and also HUVEC tubule formations. Angiogenic promoter molecules have been suppressed, and angiogenic inhibitory molecules promoted. Finally, inhibition of angiogenesis has been demonstrated *in vivo*. Undoubtedly, the results from the different pomegranate fractions, with their different chemistries, rely upon different and multiple mechanisms.

Such pharmacological redundancy is a valuable and an important attribute of the pomegranate that may reinforce its applicability as an inhibitor of angiogenesis, and as an anti-cancer agent. The present study is in no way meant to be seen as comprehensive, as it leaves many gaps. Most notably, not all the fractions were tested in all of the models. However, in sum, there are sufficient indications to indicate anti-angiogenic potential of these extracts. In other settings [38] we proved that the different fractions (e.g., P, W and SEPO or SESCO) acted synergistically to suppress prostate cancer proliferation and invasion, and also to suppress the activity of phospholipase A2. Conceivably, such kind of synergistic interaction may also apply to the activities of these extracts against angiogenesis.

In fact, the extracts employed in the present study are seen as components of a complex pomegranate drug in development for the prevention and/or treatment of breast and/or prostate cancer. The present preliminary studies lend credence to a more systematic examination of this agent in the context of angiogenesis.

Acknowledgements

The authors wish to thank the family of the late Jeffrey Marks for fermentation of the pomegranate juice, to Eli Merom for generosities in supplying the organically grown pomegranates, to Talia Livney for preparation of samples, and to Alexander Botvinnik for technical assistance in the preparation of the manuscript.

References

1. Eatock MM, Schatzlein A, Kaye SB. Tumour vasculature as a target for anticancer therapy. Cancer Treat Rev 2000; 26: 191–204.
2. Pfeffer U, Ferrari N, Morini M et al. Antiangiogenic activity of chemopreventive drugs. Int J Biol Markers 2003; 18: 70–4.
3. Scappaticci FA. The therapeutic potential of novel antiangiogenic therapies. Expert Opin Invest Drugs 2003; 12: 923–32.
4. Auerbach R, Lewis R, Shinners B et al. Angiogenesis assays: A critical overview. Clin Chem 2003; 49: 32–40.
5. Kim ND, Mehta R, Yu W et al. Chemopreventive and adjuvant therapeutic potential of pomegranate (*Punica granatum*) for human breast cancer. Breast Cancer Res Treat 2002; 71: 203–17.
6. Fotsis T, Pepper MS, Montesano R et al. Phytoestrogens and inhibition of angiogenesis. Baillieres Clin Endocrinol Metab 1998; 12: 649–66.
7. Le Marchand L. Cancer preventive effects of flavonoids – a review. Biomed Pharmacother 2002; 56: 296–301.
8. Sartippour MR, Heber D, Zhang L et al. Inhibition of fibroblast growth factors by green tea. Int J Oncol 2002; 21: 487–91.
9. Schubert SY, Lansky EP, Neeman I. Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. J Ethnopharmacol 1999; 66: 11–7.
10. Masso-Welch PA, Zangani D, Ip C et al. Inhibition of angiogenesis by the cancer chemopreventive agent conjugated linoleic acid. Cancer Res 2002; 62: 4383–9.
11. Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol 2002; 17: 599–621.
12. Hartlapp I, Abe R, Saeed RW et al. Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis *in vivo*. FASEB J 2001; 15: 2215–24.
13. Kapiteijn K, Koolwijk P, Van Der Weiden R et al. Steroids and cytokines in endometrial angiogenesis. Anticancer Res 2001; 21: 4231–42.
14. Toi M, Matsumoto T, Bando H. Vascular endothelial growth factor: Its prognostic, predictive, and therapeutic implications. Lancet Oncol 2001; 2: 667–73.
15. Nagai S, Toi M. Interleukin-4 and breast cancer. Breast Cancer 2000; 7: 181–6.
16. Bando H, Matsumoto G, Bando M et al. Expression of macrophage migration inhibitory factor in human breast cancer: Association with nodal spread. Jpn J Cancer Res 2002; 93: 389–96.

17. Imamura M. Extracellular matrix components and angiogenesis. Nippon Yakurigaku Zasshi 1996; 107: 153–60.
18. Ueno T, Toi M, Saji H et al. The role of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis and survival of human breast cancer. Clin Cancer Res 2000; 6: 3282–9.
19. Oikawa T, Sasaki T, Nakamur M et al. The proteasome is involved in angiogenesis. Biochem Biophys Res Commun 1998; 246: 243–8.
20. Fife RS, Sledge Jr GW, Sissons S et al. Effects of tetracyclines on angiogenesis *in vitro*. Cancer Lett 2000; 153: 75–8.
21. Oikawa T, Hirotani K, Nakamura O et al. A highly potent antiangiogenic activity of retinoids. Cancer Lett 1989; 48: 157–62.
22. Oikawa T, Hirotani K, Ogasawara H et al. Inhibition of angiogenesis by vitamin D3 analogues. Eur J Pharmacol 1990; 178: 247–50.
23. Nakamura M, Katsuki Y, Shibutani Y, Oikawa T. Dienogest, a synthetic steroid, suppresses both embryonic and tumor-cell-induced angiogenesis. Eur J Pharmacol 1999; 386: 33–40.
24. Pavlakovic H, Havers W, Schweigerer L. Multiple angiogenesis stimulators in a single malignancy: Implications for anti-angiogenic tumour therapy. Angiogenesis 2001; 4: 259–62.
25. Maulik N, Das D. Redox signaling in vascular angiogenesis (1,2). Free Radic Biol Med 2002; 33: 1047.
26. Leek RD, Talks KL, Pezzella F et al. Relation of hypoxia-inducible factor-2 alpha (HIF-2 alpha) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in human breast cancer. Cancer Res 2002; 62: 1326–9.
27. Sasaki H, Ray PS, Zhu L et al. Oxidative stress due to hypoxia/reoxygenation induces angiogenic factor VEGF in adult rat myocardium: Possible role of NFkappaB. Toxicology 2000; 155: 27–35.
28. Schubert SY, Neeman I, Resnick N. A novel mechanism for the inhibition of NFkB activation in vascular endothelial cells by natural antioxidants. FASEB J 2002; 16: 1931–3.
29. Sharaf A, Nigm SAR. The oestrogenic activity of pomegranate seed oil. J Endocrinol 1964; 29: 91–2.
30. Cid MC, Schnaper HW, Kleinman HK. Estrogens and the vascular endothelium. Ann N Y Acad Sci 2002; 966: 143–57.
31. Gargett CE, Zaitseva M, Bucak K et al. 17Beta-estradiol up-regulates vascular endothelial growth factor receptor-2 expression in human myometrial microvascular endothelial cells: Role of estrogen receptor-alpha and -beta. J Clin Endocrinol Metab 2002; 87: 4341–9.
32. Galea E, Santizo R, Feinstein DL et al. Estrogen inhibits NF kappa B-dependent inflammation in brain endothelium without interfering with I kappa B degradation. Neuroreport 2002; 13: 1469–72.
33. White RE. Estrogen and vascular function. Vasc Pharmacol 2002; 38: 73–80.
34. Takei H, Lee ES, Jordan VC. In vitro regulation of vascular endothelial growth factor by estrogens and antiestrogens in estrogen-receptor positive breast cancer. Breast Cancer 2002; 9: 39–42.
35. Nugteren DH, Christ-Hazelhof E. Naturally occurring conjugated octadecatrienoic acids are strong inhibitors of prostaglandin biosynthesis. Prostaglandins 1987; 33: 403–17.
36. Fukuda R, Kelly B, Semenza GL. Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. Cancer Res 2003; 63: 2330–4.
37. Meyer-Siegler K. COX-2 specific inhibitor, NS-398, increases macrophage migration inhibitory factor expression and induces neuroendocrine differentiation in C4-2b prostate cancer cells. Mol Med 2001; 7: 850–60.
38. Lansky EP, Jiang WG, Mo H et al. Therapeutic synergy in pomegranate. Poster presented at the 94th Annual Meeting of the American Association for Cancer Research, July 11–14, 2003, Washington, DC.