

Anti-angiogenic property of edible berry in a model of hemangioma

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Abstract Hemangiomas represent a powerful model to study *in vivo* angiogenesis. Monocyte chemotactic protein 1 (MCP-1) is known to be responsible for recruiting macrophages to sites of infection or inflammation and facilitate angiogenesis. Recently we have demonstrated that edible berry extracts potently suppress inducible vascular endothelial growth factor expression and *in vitro* angiogenesis. Comparative analysis of several berry extracts led to the observation that wild blueberry and a berry mix were most effective. Our goal was to follow up on our findings with wild blueberry and the berry mix (OptiBerry). The present work rests on our current finding that these two berry powders significantly inhibit inducible MCP-1 expression in endothelioma cells. Therefore, we sought to examine the effects of wild blueberry and berry mix in an *in vivo* model of experimental angiogenesis. Reporter studies showed that the berry powders significantly inhibited basal MCP-1 transcription and inducible nuclear factor κ B transcription. Endothelioma cells pre-treated with berry powders showed diminished ability to form hemangioma. Histological analysis demonstrated markedly decreased infiltration of macrophages in hemangioma of treated mice compared to placebo-treated controls. The current results provide the first *in vivo* evidence substantiating the anti-angiogenic property of edible berries.

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1. Introduction

Hemangiomas are the most common tumor of infancy occurring in approximately 1:100 normal newborns, but in premature infants weighing less than 1000 g the incidence rises to 1:5 live births [1]. The hemangioma is characterized by rapid growth during the first year of life (proliferative phase) followed by a decline in growth (involutional phase) over the next 5–6 years, with complete regression of the lesion in 90% of affected individuals by age 9 (the involuted phase). The stimuli responsible for initiating, maintaining and inhibiting the endothelial cell growth in hemangiomas have not been identified. Although 90% of these lesions spontaneously

regress, 5% of hemangiomas cause serious tissue damage and approximately 1–2% of all hemangiomas are life threatening. Proliferating hemangiomas are highly angiogenic with urinary basic fibroblast growth factor levels present in the urine of affected individuals at 25–50-fold levels above normal controls [2]. Hemangiomas arise from clonal expansion of a single endothelial cell precursor [3]. The presence of macrophages is associated with proliferating hemangiomas, but not involuting hemangiomas [4], and the significance of this association remains unexplained.

The CC chemokine monocyte chemotactic protein 1 (MCP-1) is known to be responsible for recruiting macrophages to sites of infection or inflammation. In this way, MCP-1 could be viewed as a major accessory facilitating angiogenesis. Recently, a direct role of MCP-1 on angiogenesis has been also evident [5]. MCP-1 induced chemotaxis of human endothelial cells at nanomolar concentrations. This chemotactic response was inhibited by a monoclonal antibody to MCP-1. MCP-1 also induced the formation of blood vessels *in vivo*. Moreover, the direct effect of MCP-1 on angiogenesis was consistent with the expression of CCR2, the receptor for MCP-1, on endothelial cells. It was thus suggested that antagonists of MCP-1 should prove to be anti-angiogenic [5].

Nutrition is a major tool in health preservation and disease prevention. The therapeutic property of edible berries has been long known [6]. More recently, it has been observed that edible berries may have potent chemopreventive properties [7–11]. Berries are rich in anthocyanins, flavonoid glycosides responsible for the red, violet, purple and blue color of the fruit. Dietary consumption of anthocyanin has been shown to improve overall antioxidant defense status of human plasma [12]. On one hand, the search is on for specific medical drugs that would efficiently limit angiogenesis [13,14]. On the other hand, diet-based approaches to limit angiogenesis are being actively explored [7,8,15–20]. Proven safety for human use is a major merit that strengthens this latter approach.

While it is evident that consumption of a plant-based diet can prevent the development and progression of disorders associated with extensive neovascularization [15], the underlying mechanisms remain unclear. Vascular endothelial growth factor (VEGF)/vascular permeability factor plays a crucial role in angiogenesis. Recently we have demonstrated that edible berry extracts including OptiBerry potently suppress inducible VEGF expression and *in vitro* angiogenesis [21]. Analysis of several berry extracts led to the observation

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that wild blueberry and a berry mix were most effective in inhibiting inducible VEGF expression. These extracts also had the highest antioxidant function as determined by the oxygen radical absorbing capacity [21]. Our goal was to follow up on our findings with wild blueberry and berry mix. The present work rests on our current finding that these two berry extracts significantly inhibit inducible MCP-1 expression in endothelioma cells. Therefore, we sought to examine the effects of wild blueberry and berry mix (OptiBerry) in an *in vivo* model of experimental angiogenesis.

2. Materials and methods

2.1. Materials

Wild blueberry and berry powder mix (Mix 1 [21] OptiBerry IH141) were obtained from InterHealth Nutraceuticals (Benicia, CA, USA). Unless otherwise stated all other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

2.2. Cells and cell culture

Endothelioma (EOMA) cell line was originally derived from a spontaneously arising hemangioendothelioma in the 129/J strain [22]. To perform MCP-1 enzyme linked immunosorbent assays (ELISA), flow cytometer propidium iodide assays, MCP-1 and nuclear factor κ B (NF- κ B) luciferase assays, EOMA cells were seeded at 1.5×10^5 cells/ml density in 6 well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ as previously described [23]. Following 24 h incubation, the culture medium was replaced with fresh medium. Wild blueberry and berry mix powders were added at 50 μ g/ml (in dimethyl sulfoxide (DMSO)) as described previously [21]. Cells in the control group were treated with the same volume (0.01% v/v) of DMSO.

2.3. MCP-1 ELISA assay

Following 24 h of seeding of EOMA cells, the culture medium was replaced with fresh medium and the cells were treated with berry powder as described above for 12 or 24 h. After such treatment, cells were activated with tumor necrosis factor α (TNF α , 400 IU/ml) for 12 h. MCP-1 levels in cell-free culture media were measured using commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions as previously described [23]. Results were normalized to total protein concentration measured from cell lysates.

2.4. Luciferase reporter assay

EOMA cells were transiently transfected with the MCP-1-Luc or NF- κ B-Luc constructs using Effectene (Invitrogen) reagent according to the manufacturer's instructions. For MCP-1 luciferase assay, cells were transiently transfected with the MCP-1 distal enhancer plus proximal promoter construct attached to the luciferase encoding region as described previously [24]. After 24 h of transfection, cells were treated for 24 h with the respective berry powders as indicated in the figure legends. For NF- κ B-Luc assay, cells were activated with TNF α for 6 h as described above. Luciferase activity was determined using a commercial kit (Stratagene).

2.5. Cell viability assay flow cytometry

Cell membrane integrity was also monitored by flow cytometry (EPICS Elite or XL, Coulter) as a measure of cell viability. For this assay, the non-permeant DNA intercalating dye propidium iodide (Molecular Probes) was used. This dye is excluded by viable cells. A 15 mW powered argon ion laser was used for excitation at 488 nm, and the emission signal was collected at 575 nm as described earlier [25].

2.6. Generation of hemangiomas

There are two murine models of hemangioma. One model uses endothelial cells transformed with the oncogenic middle T antigen of the murine polyoma virus [26,27] and the other model uses endo-

thelial cells from a spontaneously arising hemangioendothelioma (a clinical subtype of hemangioma). Either cell line can be injected subcutaneously to generate hemangiomas. We have chosen to use the EOMA cell line derived from the spontaneously arising hemangioma for several reasons. The polyoma transformed cell lines have been reported to create tumors by recruiting host cells [28]. EOMA cells were derived from a 129 P/3 strain, which has an H-2^b MHC background, while the other cell line is derived from a B/6 \times DBA/2 F₂ hybrid, giving it a mixed H-2^b and H-2^d MHC background limiting its use *in vivo* to nude or SCID mice. The EOMA cell line has also been well characterized in the literature with regard to its derivation [22], expression of endothelial cell markers [29], ability to induce Kasabach–Merritt syndrome [30,31], production of endostatin [32–34] and response to angiostatin [35].

EOMA cells were prepared for injection as described previously [23]. The cells were grown in 175 cm² tissue culture flasks in the conditions mentioned above, and were trypsinized when they reached ~85% confluence. The cells were washed three times in large volumes (50 ml) of phosphate buffered saline, pH 7.4 (PBS), counted and resuspended in PBS at 5×10^7 cells/ml and kept on ice. The cell suspension was loaded into a 1 cc insulin syringe with 28 gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) to a final volume of 100 μ l per syringe. 129 P3 mice from Jackson Laboratories (Bar Harbor, ME, USA) are syngeneic with the EOMA cells. Eight week old mice received inhalation anesthesia and were injected subcutaneously with 100 μ l of cell suspension for a total dose of 5×10^6 cells. Tumor was harvested 1 week after the injection. After weighing and scaled digital photography, the samples were treated with OCT reagent for frozen sections. All animal protocols were approved by the Animal Institutional Laboratory Animal Care and Use Committee (ILACUC) of the Ohio State University, Columbus, OH, USA.

2.7. Histology

Freshly dissected, unfixed hemangioma specimens were snap-frozen and immediately cut into 10 μ m frozen sections. Endogenous peroxidase activity was blocked with 0.3% (v/v) H₂O₂ in Tris buffered saline (TBS). The slides were washed three times with TBS. Before addition of primary antibody, tissue non-specific binding was blocked using serum-free protein block (Dako, Glostrup, Denmark) for 15 min. After three washes in TBS, the slides were incubated for 60 min with rat anti-mouse CD11b (1:200 dilution; Chemicon International, Temecula, CA, USA). To confirm macrophage specificity of stains, other sections were incubated for 60 min with rat anti-mouse F4/80 (1:50 dilution; Serotec, Raleigh, NC, USA). Next, the slides were washed with TBS and blocked in avidin and biotin solutions (Dako) for 20 min each prior to the secondary antibody. Biotinylated rabbit anti-rat (Vector) secondary antibody in 3% mouse serum was incubated for 30 min. Slides were washed with TBS and incubated with streptavidin–horseradish peroxidase complex (Dako) for 15 min. After three washes, slides were incubated with substrate-chromogen solution (3,3'-diaminobenzidine, Dako) for 5 min and counterstained with Mayer hematoxylin for 3 min. The slides were then mounted with Gel Mount (Biomed). Digital images were obtained using a motorized Zeiss Axiovert 200 microscope fitted with color AxioCam camera and Axiovision 4.0 software.

2.8. Statistical analysis

In vitro data are reported as mean \pm S.D. of three experiments each conducted in triplicate. Comparisons between berry treated groups and control samples were done using independent samples *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

TNF α is a potent inducer of MCP-1 in EOMA cells. Pre-treatment of cells with both berry powders significantly decreased inducible MCP-1 expression. Two durations of pre-treatment, 12 h and 24 h, were tested. It was evident that the effect of berry powders on inducible MCP-1 expression did not change with increased duration of treatment. Inducible MCP-1 protein was significantly lower than the control group in both 12 and 24 h pre-treatment groups (Fig. 1). To verify

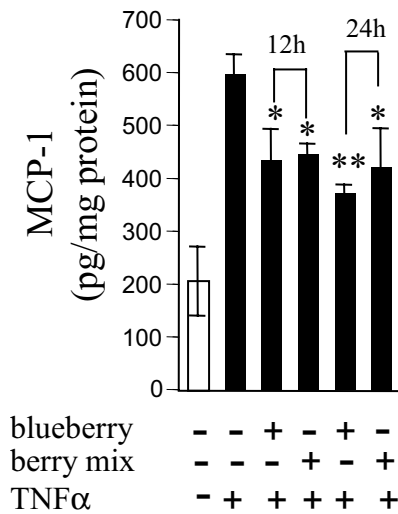


Fig. 1. MCP-1 protein expression. EOMA cells were treated with wild blueberry or berry mix powder for 12 or 24 h prior to TNFα treatment. Mean ± S.D. **P* < 0.05, ***P* < 0.01, lower compared to untreated cells for the effect of berry treatment on TNFα induced MCP-1 expression.

that the berry powders did not have any cytotoxic property at the concentrations used, cells were treated with the berry powders either at test concentrations or in excess. Flow cytometric analysis of cell membrane integrity showed that under standard culture conditions less than 5% of the total cells stained with propidium iodide indicating lost viability. When treated with berry powders at test concentrations, no further loss of cell viability was detected. These observations confirm our previous observation that these berry powders are not toxic to cells at the tested concentrations [21]. However, treatment of EOMA cells with a five-fold excess of berry powders induced cell death. Under these conditions, the proportion of dead cells in the suspension tripled to approximately 15% (Fig. 2). It is therefore reasonable to interpret that the reported effects of berry powders in this study are not related to general cytotoxicity of the powders. The observation that

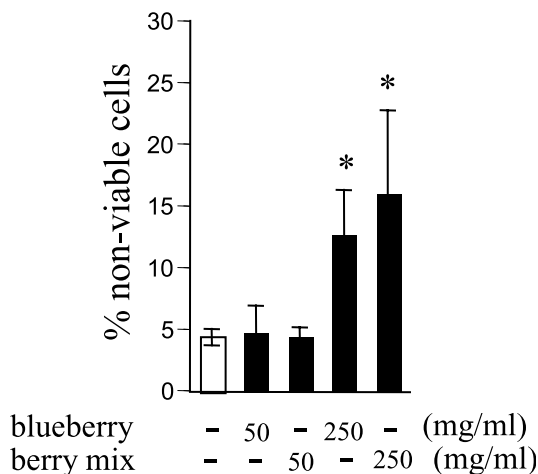


Fig. 2. Effect of berry powders on cell viability. Flow cytometric propidium exclusion assay for viability. Mean ± S.D. **P* < 0.05, significant toxic effect of berry powder treatment was seen at 250 μg/ml but not at 50 μg/ml.

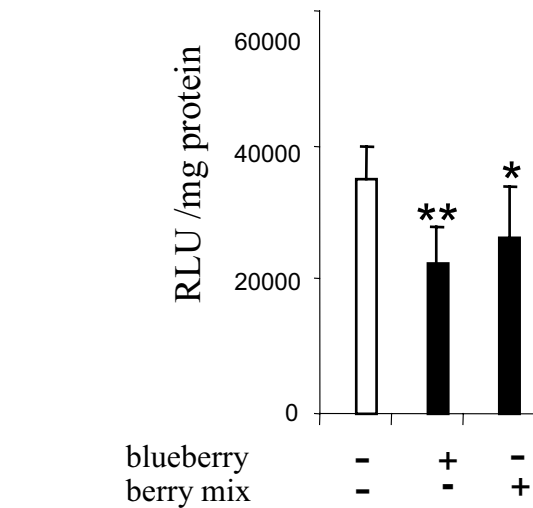


Fig. 3. MCP-1 reporter assay. MCP-1-Luc reporter constructs were transiently transfected in EOMA cells. After 24 h of transfection, the cells were treated with wild blueberry or berry mix powders for 24 h. Mean ± S.D. **P* < 0.05, ***P* < 0.01, lower compared to untreated cells for the effect of berry treatment.

high concentrations of berry powder induce cell death is consistent with the demonstrated pro-apoptotic effects of natural flavonoids [36].

Transfection of EOMA cells with a MCP-1 luciferase reporter construct resulted in high baseline luciferase activity suggesting elevated levels of basal MCP-1 transcription in this EOMA cells. Pre-treatment of cells with both berry powders significantly lowered basal MCP-1-luciferase reporter activity (Fig. 3). This observation suggests that the berry powders inhibited basal MCP-1 transcription in EOMA cells. MCP-1 transcription is mediated by several transcription factors among which NF-κB is a key player [37]. Therefore, we sought to examine whether inducible NF-κB transcription is regulated by the tested berry powders. As expected, TNFα

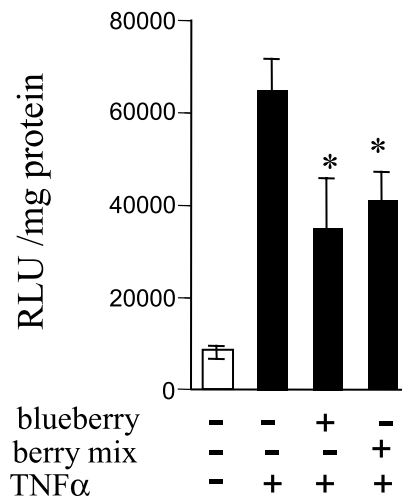


Fig. 4. NF-κB reporter assay. NF-κB-Luc reporter construct was transiently transfected in EOMA cells. After 24 h of transfection, the cells were treated with wild blueberry or berry mix powders for 24 h. Subsequently cells were activated using TNFα for 6 h. Mean ± S.D. **P* < 0.05, lower compared to untreated cells for the effect of berry treatment.

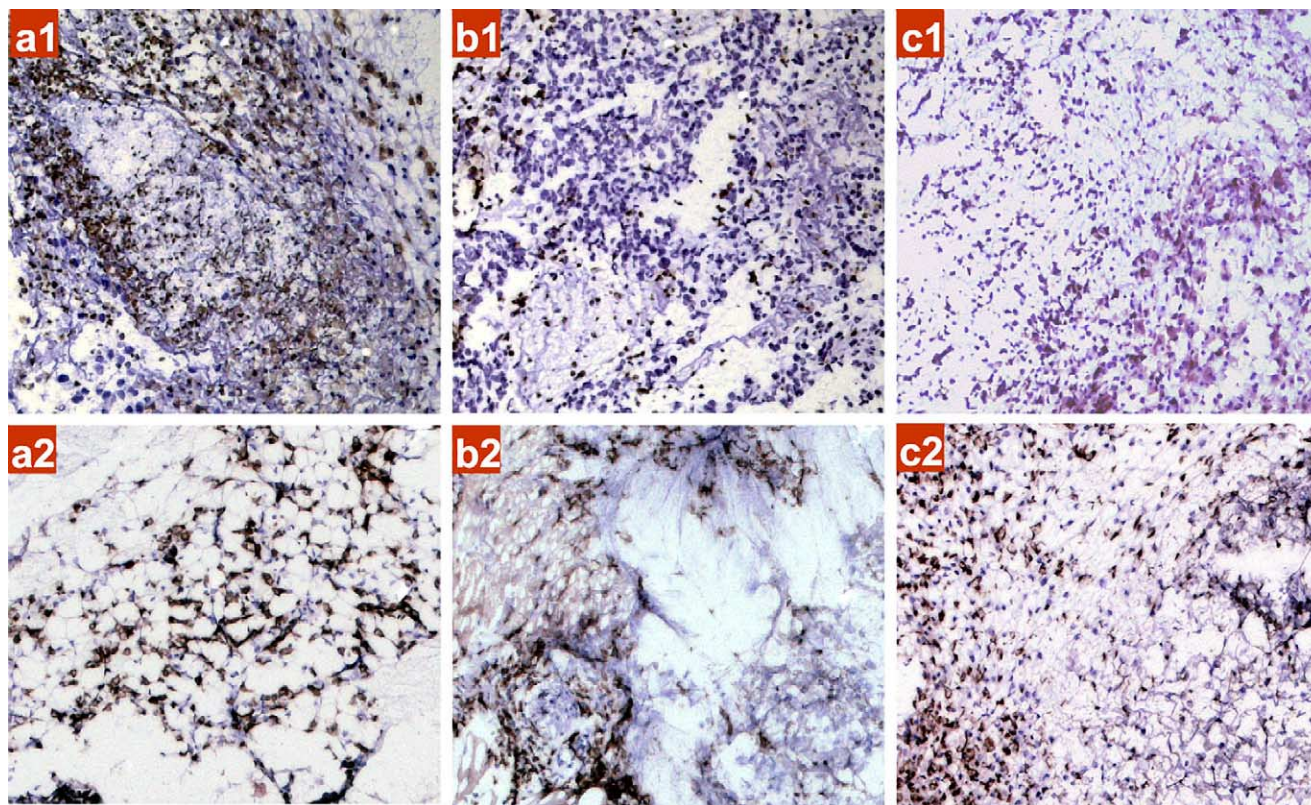


Fig. 5. Immunohistochemical localization of macrophages in hemangioma. Sections were collected 1 week after subcutaneous injection of either untreated (treated with DMSO vehicle at 0.01% v/v, a) or wild blue berry (b) or berry mix (c) treated EOMA cells to 129 P3 mice. Sections counterstained with Mayer hematoxylin for contrast. Dark (brown) spots represent macrophages. Representative samples shown. CD11b (top; a1–c1) or F4/80 (bottom; a2–c2) staining was performed to detect macrophages as described in Section 2. CD11b stains are CD3 and CD8 negative. 10×.

potently induced NF-κB transcription. Inducible NF-κB activity was significantly lower in EOMA cells pre-treated with berry extracts (Fig. 4). These results provide first evidence that inducible NF-κB in EOMA cells is subject to regulation by berry constituents.

Next, our goal was to test the functional significance of our findings in an in vivo setting. To accomplish this objective, EOMA cells were either treated or not with berry powders prior to being injected into mice. Subcutaneous injection of EOMA cells in mice results in the rapid formation of hemangioma in 100% of the cases. Histological examination of these hemangiomas revealed massive macrophage infiltration as detected by CD11b as well as F4/80 staining. As indicated in a later section, injection of EOMA cells pre-treated with berry powder did not result in hemangioma formation in all mice. In cases where a hemangioma was visible, histological analysis clearly demonstrated that macrophage infiltration in such hemangioma was markedly diminished (Fig. 5). This effect was consistently observed in all mice. A total of 36 mice were injected with EOMA cells in this study. As illustrated in Fig. 6, 100% of the controls, 47% of the wild blueberry treated group, and 92% of the berry mix treated group tested positive for the presence of a hemangioma. Of importance, although the treated group did test positive for the presence of hemangioma the average mass of such growth was below 50% of the average growth observed in the untreated control group. In the wild blueberry treated group, the absence of hemangioma in over 50% of the cases is indeed remarkable.

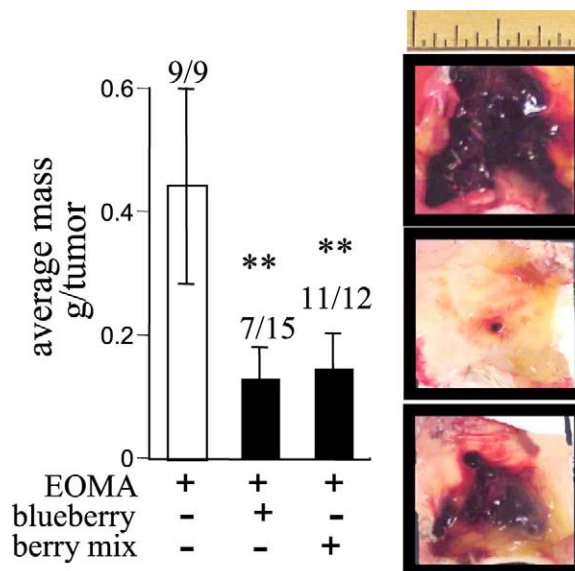


Fig. 6. Hemangioma mass, incidence and appearance. Data collected 1 week after subcutaneous injection of wild blueberry or berry mix treated or untreated (control) EOMA cells to 129 P3 mice. Mean ± S.D. ***P* < 0.01, lower mass compared to control group. See Section 2 for details. Photo: top, control; middle, blueberry treated, case of no hemangioma shown, injected cells seen at the center; bottom, berry mix treated. See bar graph for average mass of hemangioma. Scale = 1 inch.

4. Discussion

Folkman described hemangiomas as a relatively pure form of angiogenesis [1]. Injected EOMA cells proliferate to form blood vessel conduits that anastomose with the general circulation to draw blood into the hemangioma. This results in erythrocyte sequestration and rapid fall of hematocrit. Mice typically succumb to a disorder clinically referred to as Kasabach–Meritt syndrome [38] within 3–4 weeks of EOMA injection. In addition to its clinical relevance, the EOMA model of angiogenesis offers the following advantages as an experimental system. First, injection of EOMA cells results in tumor production in days with 100% efficiency resulting in quick reliable *in vivo* data generation. Second, EOMA cells can be pharmacologically or genetically treated *in vitro* to study the effects of variable manipulation on endothelial cell behavior *in vitro*. Finally, manipulating these cells *in vitro* followed by injection *in vivo* provides an efficient unique model to study the influence of tumor cell derived signals that regulate angiogenesis through the recruitment of host endothelial cells.

The ability of macrophages to influence angiogenesis has been recognized for many years beginning with experiments that showed cultured supernatants from macrophages could stimulate angiogenesis *in vitro* [39–41]. There is indirect evidence in clinical situations to support this notion. For example, wound healing is an angiogenic process that fails to occur without the assistance of macrophages [42]. A direct correlation has been documented between the presence of tumor associated macrophages and vascularity in breast cancer specimens and mortality [43–47], the implication being that the presence of macrophages facilitates the angiogenic processes of the tumor thereby promoting metastases. Macrophages have also been proposed to play a role in vasculogenic mimicry, the creation of non-endothelial lined channels emanating from tumors, such as melanoma, that contain red blood cells or shed tumor cells and are associated with aggressive tumor behavior and high mortality [48,49]. Despite the plethora of circumstantial evidence, the mechanisms and extent of macrophage involvement in the angiogenesis-dependent tumor proliferation process are not well defined.

Macrophages have been shown to produce many growth factors and cytokines known to participate in the regulation of angiogenesis [50–52] and Polverini and Leibovich have even shown that tumor associated macrophages are angiogenic [40]. Furthermore, our recent studies demonstrate that macrophage derived reactive species support angiogenesis [53–55]. Our findings are consistent with previous data showing that treatment of macrophage cultures with oxygen free radical scavengers substantially inhibits the angiogenic activity of conditioned media in rat corneal angiogenesis assays [50]. Activated macrophages co-cultured with bovine aortic endothelial cells (BAECs) grown on type I collagen gels are known to induce branching/sprouting behavior in the BAECs indicative of angiogenic behavior. This sprouting behavior was significantly inhibited by catalase suggesting an angiogenic role of H₂O₂ [56]. We have previously reported that hemangioma is characterized by footprints of oxidative stress suggesting involvement of elevated levels of reactive oxygen species [23]. More recently it has been shown that oxidants drive MCP-1 expression [57]. Indeed redox-sensitive transcription factors such as NF- κ B and AP-1 [58–60] regulate inducible MCP-1

expression [37,61]. Our results show that treatment of cells with both berry extracts, blueberry and OptiBerry, inhibits inducible NF- κ B transcription. Pharmacokinetic studies addressing the absorption of dietary berry constituents support the relevance of our findings [12,62]. Given that inducible NF- κ B activation is sensitive to antioxidants [58–60] and that the berry powders tested are known to be rich in antioxidant activity [21] it is plausible that the observed effect of berries on inducible NF- κ B activation and MCP-1 expression is dependent on their antioxidant property. Berry phenolics, especially anthocyanins, are primarily responsible for their antioxidant property [63].

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