Angiostatin decreases cell migration and vascular endothelium growth factor (VEGF) to pigment epithelium derived factor (PEDF) RNA ratio in vitro and in a murine ocular melanoma model

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Purpose: Our previous experiments have shown that low dose angiostatin results in decreased hepatic micrometastasis in a mouse model of uveal melanoma. The purpose of these experiments is to evaluate the effect of angiostatin on in vitro migration of melanoma cells and to explore the in vivo mechanism of angiostatin in our model.

Methods: For in vitro studies, quantitative RT-PCR was used to detect VEGF and PEDF mRNA in mouse B16LS9 melanoma cells and Mel290 human uveal melanoma cells with or without supplemental 0.1 µg/ml murine or human recombinant angiostatin. A wound healing assay was used to measure cellular migration in these two groups of cells. For the in vivo mechanism, aliquots of tissue culture B16LS9 cells treated with or without 0.1 µg/ml murine angiostatin were heterotopically inoculated into the posterior compartments of the right eyes of C57BL/6 mice. Frozen hepatic tissue was prepared and stained with hematoxylin using an RNase-free technique. Hepatic micrometastatic uveal melanoma cells were obtained by laser capture microdissection (LCM). Levels of VEGF and PEDF mRNA were detected by real time RTPCR in the hepatic micrometastases.

Results: After in vitro treatment of the cell lines with angiostatin, the ratio of VEGF/PEDF mRNA significantly decreased in the B16LS9 (0.88±0.11 [mean±standard deviation] versus 2.70±0.15 in the control group; p=0.00006) and Mel290 (0.12±0.02 versus 0.68±0.04 in the control group; p=0.00346). However, the absolute VEGF mRNA and PEDF mRNA did not significantly change (p>0.08 for both cell lines). The migration assay showed significantly decreased migration at 24 h and 48 h after angiostatin treatment for both B16LS9 (p<0.01) and Mel290 (p<0.01) cell lines. For the in vivo experiments, pretreatment with angiostatin resulted in a decreased VEGF/PEDF mRNA ratio in B16LS9 cells compared to controls (0.0274±0.0070 versus 0.1726±0.0313; p=0.0014). Additionally, there was significantly increased PEDF mRNA (2.14±0.12 versus 0.30±0.05 in the control group; p=0.00002) in the liver metastases after pretreatment with angiostatin.

Conclusions: Angiostatin inhibits the migration of melanoma cells in vitro. Angiostatin significantly decreases the ratio of VEGF/PEDF mRNA level in vitro and in hepatic micrometastatic melanoma cells. Angiostatin increases PEDF mRNA in melanoma metastases.

Choroidal and ciliary body melanomas often metastasize to the liver [1,2]. Despite treatment of the primary tumor, approximately 40% of patients with uveal melanoma die within five years after diagnosis [3]. Death typically occurs within months after metastases are detected clinically [2], which spread hematogenously, most often to the liver. No effective treatment has been found to date for metastatic choroidal melanoma [1]. Uveal melanoma growth and metastasis requires concomitant growth of new blood vessels [4], which are stimulated by angiogenic factors [5,6], including vascular endothelial growth factor (VEGF) [6]. Melanoma cells secrete VEGF [7] and VEGF promotes tumor cell growth [8,9].

Angiogenesis is regulated by both stimulatory and inhibitory molecules [10]. A shift in the angiogenesis balance between activators and inhibitors to favor enhanced angiogenesis is thought to represent a key step in the progression and metastasis of malignant neoplasms [6,10]. Recent studies have identified that pigment epithelium-derived factor (PEDF) inhibits angiogenesis in the eye [11,12]. Our laboratory has shown that supplemental high dose (0.3 µg/ml) angiostatin increased the number of micrometastases whereas low dose (0.1 µg/ml) angiostatin significantly reduced the number of micrometastases.

Interestingly, VEGF and PEDF not only have pro-angiogenic and anti-angiogenic properties, but also they exhibit trophic and involutional effects on tumor cells, including cutaneous melanoma [13]. VEGF and PEDF production are inversely related [14]. VEGF has a trophic, proliferating, migratory effect and PEDF has an apoptotic, anti-proliferative, anti-migratory effect on melanoma cells [15]. Our laboratory has shown that uveal melanoma cells secrete VEGF, PEDF, and angiostatin [7]. Uveal melanoma may spread to the liver and form micrometastases that have the potential to grow and kill the host [16-18]. Our hypothesis is that supplemental low dose angiostatin alters the VEGF and/or PEDF mRNA levels in our model is such a way as to favor micrometastatic “dormancy” via decreasing VEGF, raising PEDF [7], or both. The following experiments were designed to test this hypothesis.
METHODS

**Cell cultures:** Murine B16LS9 melanoma cells [18] and Mel290 human uveal melanoma cells (courtesy of BR Ksander, PhD, Schepens Eye Institute, Boston, MA) were cultured at 37 °C in a 5% CO₂ incubator. The cells were grown in complete culture medium which included RPMI-1640 with HEPES, L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate solution, 1% MEM Vitamin Solution (GIBCO, Grand Island, NY), and a 1% Anti-biotic-Antimycotic Solution containing 100 units/ml Penicillin G, 250 ng/ml Amphotericin B, and 100 μg/ml Streptomycin solution (GIBCO). When cells grew to 80% of confluence, they were washed 3 times with Hank’s balanced salt solution, changed to fresh complete medium with or without 0.1 μg/ml murine or human recombinant angiostatin (BioExpress, Kaysville, UT), and incubated overnight at 37 °C, 5% CO₂. The dosage of the supplemental angiostatin was based on our previous experiments [7]. Angiostatin (plasminogen K1-4) is expressed as an mFc-mAngiostatin fusion protein. Polymerase chain reactions (PCRs) were used to adapt the cDNAs of angiostatin for expression in the pdsCs-mFc(D4) vector [19]. The forward and reverse primers are published [20]. Angiostatin was used since the melanoma cell lines used express angiostatin [7] and when plasminogen is cleaved to form angiostatin (K1-4), the other cleaved product, K5, stimulates production of PEDF [21]. The cells were trypsinized and expanded for use in all experiments.

**Mouse uveal melanoma model:** Eight-week-old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted according to the declaration of Helsinki and Guiding Principles in the Care and Use of Animals and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Aliquots of 5x10⁵ cells/2.5 μl were inoculated into the choroid of the right eyes using a trans-scleral technique that allows the inoculated cells to remain in the eye. The mice were anesthetized and a tunnel was prepared from the limbus within the sclera to the choroid with a 30 gauge needle under the guidance of a dissection microscope. The tip of a 10 ml glass syringe with a blunt metal needle (Hamilton, Reno, NV) was introduced into the choroid through the needle track, and no cells were inoculated until the needle tip was inside the eye. A 2.5 μl suspension of cells was inoculated. No tumor cell reflux occurred, and the subconjunctival space remained free of tumor cells. The right eyes were enucleated 7 days after inoculation. B16LS9 cells were cultured with or without 0.1 μg/ml murine angiostatin (BioExpress) prior to inoculation and the mice did not receive angiostatin by intramuscular injection. This B16LS9 model resembles highly aggressive metastatic human uveal melanoma cells that B16LS9 cells express c-met and are NK cell sensitive [18]. Additionally, there are no reproducible non-immune suppressed animal models of metastatic human uveal melanoma to the liver [22]. The mice were sacrificed at 21 days after enucleation. For laser capture microdissection (LCM), the lasers were collected from the two groups: mice inoculated with B16LS9 cells pretreated with 0.1 μg/ml murine angiostatin, and mice inoculated with untreated B16LS9 cells.

**Tissue collection and staining:** For assessment of hepatic micrometastases, the right eyes were obtained after enucleation and routinely processed for light microscopic examination. Serial 5 μm thick sections were stained with hematoxylin and eosin and evaluated for the presence and location of melanoma. Mice with eyes without melanoma growth or with extracocular extension of melanoma were excluded. The mice were euthanized 28 days after inoculation (21 days after enucleation) and necropsies were performed. The eyes and livers were grossly examined, submitted in 4% neutral buffered formalin, and processed for light microscopic examination. Three sections through the center of the liver were microscopically examined (Olympus BX41, Tokyo, Japan) for the presence of micrometastases (<100 μm diameter). This has been shown to be a reliable and reproducible method for detecting hepatic micrometastasis in our murine model.

For LCM, a clean surface was prepared with Rnase Away before tissue collection and staining. The livers were cut into small pieces and frozen in molds (SAKURA, Torrance, CA) filled with optimum cutting temperature compound (SAKURA) on dry ice. The samples were stored at -80 °C. Sections of frozen hepatic tissue (10 μm) were mounted on non-adhesive glass slides. The Histogen Frozen Section Staining kit (Arcturus, Mountain View, CA) was used for staining. The slides were placed in the following solutions: 75% ethanol for 30 s, distilled water for 30 s, HistoGene staining solution (hematoxylin) for 45 s, distilled water for 30 s, 75% ethanol for 30 s, 95% ethanol for 30 s, 100% ethanol for 30 s, xylene for 5 min. The slides were dried for 5 min and placed in a desiccator.

**Laser capture microdissection (LCM):** The PixCell IIe Laser Capture Microdissection system (Arcturus) was used to microdissect metastatic melanoma cells from the hepatic tissue sections. Prior to LCM, a field with micrometastases was microscopically identified. A CapSure HS LCM Cap (Arcturus) was placed on the micrometastatic area. The following settings were used: 7.5 μm laser spot size, 4.4 mA current, 100 mW power, and 750-950 ms pulse duration. The laser

| Table 1. B16LS9 VEGF and PEDF mRNA Levels after Supplemental Angiostatin |
|-------------------------|------------------|------------------|------------------|
| **Groups**              | **VEGF/PEDF**    | **VEGF/β-actin** | **PEDF/β-actin** |
| Angiostatin             | 0.8751±0.1116    | 0.132±0.0232     | 0.151±0.0459     |
| PBS                     | 2.6994±0.1453    | 0.2275±0.1089    | 0.085±0.0428     |
| p value                 | 0.00006          | 0.2176           | 0.1420           |

In vitro the level of VEGF and PEDF mRNA (mean±SEM) was determined by real time RT-PCR. When B16LS9 cells were grown in vitro in the presence of the supplemental angiostatin the level VEGF and PEDF mRNA and ratio of VEGF to PEDF mRNA level (mean±standard deviation) was less than when they were grown in media alone. VEGF and PEDF mRNA values are normalized to β-actin according to Pfaffl’s mathematical model for relative quantification in real time PCR [23].
spots were controlled to select the micrometastases without disturbing surrounding hepatic tissue. Complete capture was defined as capture of more than 90% of the tissue within the laser-activated capture area without transfer of any tissue outside the capture area. After LCM, the cap was removed. RNA buffer was immediately loaded and the cap was covered with a microcentrifuge tube.

**RNA extraction from cells obtained by LCM:** Total RNA of LCM obtained cells was extracted using the PicoPure®TM RNA isolation kit (Arcturus) according to instructions of the manufacturer. The procedure may be found at the Arcturus website.

**RNA extraction from cultured cells:** Total RNA was extracted from the melanoma cells treated with or without angiostatin using RNA-Bee™. The RNA isolation solvent was added to each sample, incubated at 42°C for 5 min, then chilled to 4°C for at least one min. The first Strand Master Mix (7 µl) and the first Strand Enzyme Mix (2 µl) were added, incubated at 42°C for 45 min, and then chilled to 4°C for at least one min. The first Strand Nuclease Mix (2 µl) was added to the sample, mixed and incubated at 37°C for 20 min, with continued incubation at 95°C for 5 min, then chilled to 4°C for at least one min. For performing the second strand synthesis in one round of amplification, 1 µl of Primer A at 65°C for 5 min, then chilled to 4°C for at least one min. The first Strand Master Mix (7 ß-actin primers were 5'-AAG TGT GAC GTT GAC TGC AAA ATG TGC-3' and 5'-CCT GGC ACC CAG ACT TCA GCA AGA-3'. The first Strand Master Mix (7 µl) was added to the second Strand Synthesis sample tube, mixed and pipetted into the purification column, To bind cDNA to the column, the sample was centrifuged at 100 g for 2 min and immediately followed by a centrifugation at 10,000 g for 30 s to remove flow-through. DNA Binding Buffer (200 µl) was added to the second Strand Synthesis sample tube, mixed and pipetted into the purification column. To bind cDNA to the column, the sample was centrifuged at 100 g for 2 min and immediately followed by a centrifugation at 10,000 g for 30 s to remove flow-through. DNA Elution Buffer (16 µl) was added onto the column membranes, incubated for 1 min at room temperature, centrifuged at 1,000 g for 1 min, and followed immediately at 16,000 g for 1 min. The elution containing DNA in the microcentrifuge tube was retained for further processing. For in vitro transcription, IVT Buffer (8 µl), IVT Master Mix (12 µl) and IVT Enzyme Mix (4 µl) were added to each sample, incubated at 42°C for 3 h, cooled to 4°C, incubated with Dnase Mix (2 µl) at 37°C for 15 min, and cooled to 4°C. The optical density of the RNA samples was measured at 260 nm and 280 nm by the UV-1601 model UV-visible spectrophotometer (Shimadzu, Norcross, GA).

**Real time RT-PCR:** One step real time RT-PCR was performed using QuantiTect™ SYBR® green RT-PCR kit (QIAGEN, Valencia, CA) on a thermocycler (iCycler, Bio-Rad, Hercules, CA). The primers were designed by Primer Express software (Applied Biosystems, Foster City, CA). The GenBank accession numbers of mouse VEGF and PEDF are NM_009505 and NM_011340, respectively. The primers for detection of mouse VEGF mRNA were 5’-CGC GAG TCT GTC TTT TTT CA-3’ and 5’-CAG AGC GGA GAA AGC ATT TGT-3’. The primers for detection of mouse caspin (PEDF) mRNA were 5’-TGC TGT CTT GGG TGC ATT GG-3’ and 5’-GTT GCA GCC TGG TAC TAC-3’. The primers for detection of mouse PEDF mRNA were 5’-TAT GAC CTG TAC CCG GTG CGA TCA T GA-3’ and 5’-CCA CAC TGA GAG GAC ACA GGA GC-3’. β-Actin was selected as the internal control reference gene. The sequences of mouse β-actin primers were 5’-AAG TGT GAC GTT GAC ATC CGT AA-3’ and 5’-TGC CTG GGT ACA TGG TGG TAC TA-3’, and human β-actin primers were 5’-CCT GGC ACC CAG CAC AAT G-3’ and 5’-CGC CGA TCC ACA CGG AGT AC-3’. The procedure was the same as previously performed [7].

The real time RT-PCR was repeated 3 times for each sample. The micrometastatic cells that were extracted to obtain RNA samples were captured by LCM from different livers from animals in each of the two groups (supplemental angioatin cultured B16LS9, n=15; no supplemental angioatin cultured B16LS9, n=15). Each cap captured 5 to 20 hepatic micrometastases depending on the cell number and size of the micrometastasis in the same livers. The levels of VEGF and PEDF mRNA and the ratio of VEGF to PEDF were calculated by Pfaffl’s methods [23]. The VEGF and PEDF values were normalized to β-actin according to Pfaffl’s mathematical model for relative quantification in real time PCR. For the studied genes, Western blot analysis was not performed.

### Table 2. *Mel290* VEGF and PEDF mRNA levels after supplemental angiostatin

<table>
<thead>
<tr>
<th>Groups</th>
<th>VEGF/PEDF</th>
<th>VEGF/β-actin</th>
<th>PEDF/β-actin</th>
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<tr>
<td>Angiostatin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>0.1222±0.0237</td>
<td>0.0269±0.0056</td>
<td>0.2200±0.1131</td>
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<td>Human β-actin</td>
<td>0.681±0.0401</td>
<td>0.0484±0.0770</td>
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<tr>
<td>P value</td>
<td>0.00346</td>
<td>0.08717</td>
<td>0.2068</td>
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</table>

In vitro the level of VEGF and PEDF mRNA (mean±SEM) was determined by real time RT-PCR. When Mel290 cells were grown in vitro in the presence of the supplemental angiostatin the level VEGF and PEDF mRNA and ratio of VEGF to PEDF mRNA level (mean±standard deviation) was less than when they were grown in media alone. VEGF and PEDF mRNA values are normalized to β-actin according to Pfaffl’s mathematical model for relative quantification in real time PCR [23].
based on previous data that showed correlation between mRNA expression and protein level for the cell lines tested [7].

**Wound healing assay:** After murine B16LS9 or human Mel290 melanoma cells were treated with 0.1 µg/ml murine or human angiostatin or PBS for 16 h, the medium was exchanged to complete medium without serum. The cells were injured in a linear fashion with a 1 ml sterilized pipette tip. Digital images were obtained with a Zeiss Axiovert 200 M inverted microscope (Oberkochen, Germany) at 0 h and 24 h. The width of injury lines were measured using Openlab Quantity Software version 3.0.8 (Improvision Ltd., Warwick, UK). The migration distance was computed by subtracting the width of the injury line (at 24 h) from the initial width of the injury line (at 0 h).

**Statistical analysis:** All statistical analyses were performed with SAS (version 8.0; SAS Institute, Cary, NC). Values reported are mean±standard deviation (SD). A Student’s t-test was used in all statistical tests.

## RESULTS

There was no significant difference (p>0.05) in the level of VEGF or PEDF mRNA between B16LS9 or Mel290 cells cultured with or without 0.1 µg/ml angiostatin (Table 1, Table 2). However, the ratio of VEGF/PEDF was significantly lower in B16LS9 and Mel290 cells cultured in the presence of 0.1 µg/ml angiostatin than in cultured B16LS9 cells without treatment (p<0.001; Figure 1). In the wound healing assay, the migration distance of cultured B16LS9 cells treated with 0.1 µg/ml angiostatin (197.5±104.36 µm) was significantly less than that in cells treated with vehicle (PBS; 714±113; p<0.01; Figure 1). Additionally, the migration distance of cultured Mel290 cells treated with 0.1 µg/ml angiostatin (110.59±33.18 µm) was significantly less than that in cells treated with vehicle (PBS; 459.55±47.23; p<0.01; Figure 2).

Hepatic micrometastatic melanoma was isolated by LCM (Figure 3). There was no apparent morphologic difference of the micrometastases between the 0.1 µg/ml angiostatin cultured versus the PBS cultured group. PEDF mRNA levels in micrometastatic melanoma cells derived from B16LS9 cells pretreated with 0.1 µg/ml angiostatin prior to inoculation were 7 fold higher than those in melanoma cells derived from vehicle pretreated cells (p<0.001, Table 2). In these cells, there was no significant difference (p>0.05) in the level of VEGF mRNA (Table 2), but the VEGF/PEDF mRNA ratio in melanoma cells derived from angiostatin pretreated cells was only 16% of that in melanoma cells derived from the control, vehicle pretreated cells (p<0.01; Table 3).

## DISCUSSION

The angiogenesis inhibitor angiostatin has been shown to inhibit the growth and metastasis of a variety of murine and human tumors in vivo [7,10,24,25]. Angiostatin may directly reduce the tumor size. Studies have shown that angiostatin inhibits ovarian cancer growth to a greater degree than another angiogenesis inhibitor, endostatin. After 2 weeks of treatment with angiostatin, a mean tumor volume of 200 mm³ was observed compared to a mean tumor volume of 362 mm³ in endostatin-treated mice, whereas the mean tumor volume of control mice was 589 mm³. Tumor growth was significantly reduced during the entire treatment period with angiostatin [25]. Lannutti, et al. [26] reported the effectiveness of human angiostatin administration in a mouse hemangioendothelioma model. Human angiostatin was administered to mice with subcutaneous hemangioendothelioma and associated disseminated intravascular coagulopathy. Angiostatin significantly reduced tumor volume, increased survival, and prevented thrombocytopenia and anemia in comparison to untreated controls. Apoptosis of tumor cells was induced by angiostatin. Angiostatin also mediated the suppression of metastasis in a Lewis lung carcinoma model [18]. Gene transfer of a cDNA coding for mouse angiostatin into murine T241 fibrosarcoma cells suppresses primary and metastatic tumor growth in vivo [27]. Implantation of stable clones expressing mouse angiostatin in C57BL/6 mice inhibits primary tumor growth by an average of 77%. After removal of primary tumors, pulmonary micrometastases in approximately 70% of mice remained in a microscopic dormant and avascular state. The tu-

![Figure 1. B16LS9 migration distance after supplemental angiostatin.](image1.png)

In vitro migration was determined by wound healing assay. When B16LS9 cells were grown in vitro in the presence of supplemental angiostatin, migration was less (197.5±104.36 µm) than when they were grown in media alone (714.21±113.52 µm). The values are shown as mean±standard deviation.

![Figure 2. Mel290 migration distance after supplemental angiostatin.](image2.png)

In vitro migration was determined by wound healing assay. When Mel 290 cells were grown in vitro in the presence of supplemental angiostatin, migration was less (110.59±33.18 µm) than when they were grown in media alone (459.55±47.23 µm). The values are shown as mean±standard deviation.
mor cells in the dormant micrometastases exhibited a high rate of apoptosis balanced by a high proliferation rate [26].

Our laboratory has developed a murine model of uveal melanoma that forms hepatic micrometastases [22,28]. We have previously shown that low dose angiostatin decreases the number of micrometastases, reduces the number of proliferating melanoma cells in the micrometastasis, and reduces invasiveness in mouse melanoma cell lines [7]. Measurements of the micrometastatic melanoma in that study showed no difference in the mean diameters of the micrometastasis between the angiostatin treated (18.93±4.48 µm) and control (19.98±4.02 µm) groups. In the current study, the wound healing assay showed that angiostatin dramatically decreases the migration of cultured B16Ls9 and Mel290 cells. Previous studies have shown that tumor invasiveness is directly reduced by angiostatin blocking plasminogen binding to its cellular receptor CD26 [29] or indirectly inhibiting the proliferation and migration of tumor cells by inducing apoptosis in endothelial cells [30,31]. Therefore, angiostatin inhibits the proliferation, invasion and migration of the primary tumor and metastatic tumor cell by a variety of mechanisms.

Tumor angiogenesis starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, encode for proteins that encourage growth of new blood vessels. Angiogenesis is regulated by both activator and inhibitor molecules. Folkman theorized that tumors lay dormant yet viable, unable to grow beyond 2 to 3 mm³ in size in the absence of neovascularization [32]. Progressive growth of tumors has been shown to be angiogenesis-dependent in various systems. The balance between activators and inhibitors of tumor angiogenesis is a potential target of anti-cancer therapies. There are different isoforms of VEGF, many of which have distinct pro-angiogenic properties, whereas others may

![Figure 3. Laser capture microdissection (LCM) of hepatic micrometastatic melanoma. A: Hepatic micrometastasis in a mouse hepatic frozen section prior to LCM. B: Laser spots outline the micrometastasis. The micrometastasis cells are inside the border. The normal hepatocytes outside the border are not disturbed. C: After the laser spot outline the micrometastasis, the tissue is removed by the cap. Approximately 90% of the cells in the micrometastasis are captured.](http://www.molvis.org/molvis/v12/a60/)

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be anti-angiogenic [33,34], this may be related to a switch in splice variants resulting in pro- versus anti-angiogenic isoforms of VEGF-A [34]. Balance between isoforms with different activities may fine-tune the angiogenic process. Moreover, PEDF, usually considered to have anti-angiogenic properties, may be pro-angiogenic in certain situations [35,36]. The mechanisms for these different behaviors are related to the PDE 34-mer peptide (residues 24-57) and the 44-mer peptide (residues 58-101) acting via the c-jun-NH2-kinase versus an alternate pathway [37]. The effects of PEDF are dependent on target cell age and type (e.g., presence/absence of receptors, cytoplasmic signals, glycosaminoglycan production, pH).

Angiostatin may shift the balance between the activators and inhibitors of tumor angiogenesis, inhibiting the growth of micrometastatic melanoma. Choroidal neovascularization (CNV) is also a proliferate pathologic process with angiogenesis [38]. The retinal pigment epithelium (RPE) contains PEDF in differentiated and non-differentiated RPE cells during oxidative stress. Their gene expression data found that, under physiological conditions, a critical balance between PEDF and VEGF exists, and PEDF may counteract the angiogenic potential of VEGF. Under oxidative stress, PEDF decreases, thus disrupting this balance. A similar mechanism appears to be the case in our model as the absolute level of PEDF mRNA in hepatic micrometastases increased in the presence of angiostatin, thus decreasing the VEGF/PEDF ratio. The findings in our current study suggest that the angiogenesis regulators PEDF and VEGF exist in a dynamic relationship in the maintenance of melanoma growth. When melanoma cells were treated with angiostatin, angiostatin decreased the VEGF/PEDF ratio, disturbing the balance between VEGF and PEDF, and allowed the melanoma cells to remain in a dormant state, where apoptosis balanced proliferation [7]. In addition to changes in the PEDF production while VEGF remains stable [40], changes in VEGF production while PEDF remains stable [41] correspond to a shift in the VEGF/PEDF ratio (balance) thus resulting in vascular endothelial proliferation involution or dormancy. Our results suggest a similar mechanism in metastatic uveal melanoma. A decreased VEGF/PEDF mRNA ratio and increased micrometastatic melanoma PEDF mRNA level are associated with altering the progression of hepatic micrometastatic melanoma.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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**Table 3. Micrometastatic B16LS9 VEGF and PEDF mRNA Levels After Supplemental Angiostatin**

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<th>VEGF/β-actin</th>
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<td>Angiostatin</td>
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In vivo the level of VEGF and PEDF mRNA (mean±SEM) in hepatic micrometastases was determined by real time RT-PCR. B16LS9 cells were grown in vitro in the presence of the supplemental angiostatin prior to implantation, the level VEGF and PEDF mRNA and ratio of VEGF to PEDF mRNA level (mean±standard deviation) in hepatic micrometastases was less than when they were grown in media alone. VEGF and PEDF mRNA values are normalized to β-actin according to Pfaffl’s mathematical model for relative quantification in real time PCR [23].


