Low dose adjuvant angiostatin decreases hepatic micrometastasis in murine ocular melanoma model

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Purpose: To investigate the effect of different doses of adjuvant angiostatin affecting hepatic micrometastasis in a murine model of metastatic ocular melanoma.

Methods: Angiostatin and plasminogen expression was detected in three murine melanoma cell lines (Queens, B16F10, and B16LS9). The three cell lines were heterotopically inoculated into the posterior compartment (PC) of the right eyes of C57BL/6 mice. After enucleation, the mice were given injections of 100 µl PBS and low dose (0.1 µg/µl) or high dose (0.3 µg/µl) murine recombinant angiostatin every day for 14 days after enucleation. The mice were sacrificed at 21 days post-enucleation and hepatic micrometastases were counted. In vitro migration/invasion assays were performed with low (0.1 µg/µl) and high (50 µg/µl) concentration angiostatin supplementation. Quantitative RT-PCR detected mRNA and Western analysis determined protein expression of VEGF for all cell lines. Evaluation of TdT mediated dUTP nick end labeling (TUNEL) and MIB1 immunostaining of the micrometastases determined apoptosis and proliferation ratios.

Results: There was a decrease in micrometastasis in the low dose group for Queens (p<0.05), B16F10 (p<0.05), and B16LS9 melanoma (p<0.01) cell lines. Two of the cell lines (B16F10 and B16LS9) elucidated plasminogen and were able to cleave plasminogen into K1-K4 (angiostatin). There was a decrease in the in vitro migration and invasion after supplementation with low concentration compared to high concentration angiostatin (p<0.01). VEGF mRNA and protein expression decreased in all cell lines in low concentration angiostatin, with the greatest decrease in B16LS9 cells (p<0.05). Apoptosis ratios were increased (p<0.01) and proliferation ratios were decreased (p<0.01) in hepatic micrometastases after treatment with low dose angiostatin.

Conclusions: There were significantly fewer micrometastases in treated compared to controls with low dose compared to high dose angiostatin. This treatment results in apoptosis in the micrometastases. The mechanism appears to be related an anti-migratory effect and altered VEGF expression by melanoma cells.

Evaluation of survival rates in patients following enucleation for uveal melanoma compared to those simply observed (i.e., no enucleation) showed increased mortality in the enucleation group at two years (the Zimmerman-McLean-Foster effect) [1, 2]. This phenomenon was experimentally reproduced when irradiated C57BL/6 mice with implanted intraocular melanoma were shown to have increased metastasis after enucleation compared to non-enucleated mice [3]. We have confirmed this finding and found that there is an increase in metastasis in experimental posterior compartment compared to anterior chamber intraocular melanoma [4].

There is considerable evidence that tumorigenesis in humans is a multistep process reflecting genetic alterations that drive the progressive transformation of cells into malignant derivatives, similar to an evolutionary process [5]. One of these steps is the sustained angiogenesis of primary and metastatic malignant neoplasms [5]. Uveal melanoma metastasizes hematogenously, most often to the liver [6]. Experimental murine intraocular melanoma becomes vascularized [7] and metastasizes hematogenously to the liver, forming micrometastases of size less than 100 µm in diameter [8] that have the potential to grow and become vascularized metastases of size greater than 100 µm diameter [9]. We studied the anti-metastatic effect of angiostatin in a murine ocular melanoma model. We found that there were fewer micrometastases after low dose (0.1 µg/µl) compared to high dose (0.3 µg/µl) angiostatin. In vitro studies showed decreased melanoma migration/invasion (p<0.01) and decreased VEGF mRNA production (p<0.03) after low compared to high concentration supplemental angiostatin. This corresponded with apoptosis rates in the micrometastases.

METHODS

Cell lines: Queens and B16F10 melanoma cells (courtesy J. Niederkorn, University of Texas Southwestern, Dallas, TX) are lines of B16 melanoma cells that were obtained after serial passages in tissue culture [10, 11]. B16LS9 cells (courtesy D. Rusciano, Friedrich Miescher Institut, Basel, Switzerland) express high levels of c-met and metastasize to the liver [12]. All three cell lines are of murine cutaneous melanoma origin. The characteristics for each cell line including the number of passages is described elsewhere [10-12].

Mice and tumors: All experiments were conducted in accordance with the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals. Twelve week old female C57BL/6 mice were used (Jackson Laboratories, Bar Harbor, ME).
Harbor, ME). Frozen cells were thawed and resuspended in 15 ml of minimum essential medium (MEM) supplemented with fetal calf serum L-glutamine and sodium bicarbonate. The cell suspensions were centrifuged and the pellet was washed and resuspended in 15 ml of supplemental MEM. The suspension was placed in a 75 cm² tissue culture flask (T-75, Becton Dickinson, Franklin Lakes, NJ) in a carbon dioxide incubator (Kendro, Asheville, NC) at 37 °C and grown to confluence in 3 to 5 days. The cells were trypsinized, aliquoted, and washed 3 times in 5 ml of Hanks balanced salt solution. An aliquot of 10 µl of suspension was placed in a hemocytometer (AO, Buffalo, NY) to calculate the concentration of melanoma cells.

Posterior compartment inoculation: Aliquots of 2x10⁶ cells/µl were inoculated into the posterior compartment (PC) of the right eyes using a method previously described [13]. The mice were anesthetized with intramuscular (IM) ketamine hydrochloride 0.66/mg/kg and the tip of a 10 µl glass syringe with a blunt metal needle (Hamilton, Reno, NV) was introduced via a trans-corneal tunnel that had been prepared with a 30 gauge needle. A 5.0 µl suspension of cells was inoculated into the posterior compartment of the right eye with no tumor cell reflux. The right eye was enucleated 7 days after inoculation.

Assessment of hepatic micrometastasis: The right eyes were 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 extracellular extension of melanoma were excluded. The mice were euthanized 28 days after inoculation (21 days after enucleation) and necropsies were performed. The livers were grossly examined, submitted in 4% neutral buffered formaldehyde, and processed for light microscopic examination. Three sections through the centers of the livers were microscopically examined (Olympus BX41, Tokyo, Japan) for the presence of micrometastases (<100 µm diameter) and the average number of micrometastases per section was determined. This has been shown to be a reliable and reproducible method for detecting hepatic micrometastasis in our murine model [14].

Angiostatin: Murine angiostatin was utilized (BioExpress, Kaysville, UT). Murine angiostatin is expressed as an mFc-mAngiostatin fusion protein [15]. Polymerase chain reactions (PCRs) were used to adapt the cDNAs of murine angiostatin based on previous studies of angiostatin treatment of Lewis Lung carcinoma [16]. Control mice for each cell line/angiostatin dose received 100 µl IM PBS daily for 14 days starting day 1 after enucleation. The mice were sacrificed at 21 days after enucleation, which was 7 days after discontinuing angiostatin. Each experiment was performed in duplicate.

Apoposis and proliferation ratios and rates: Apoptosis in liver sections was detected by TUNEL (TdR mediated dUTP nick end labeling) analysis. Cryopreserved or paraffin embedded liver sections were routinely processed and the procedure was used according to the protocol of the in situ cell death detection kit (Roche, Mannheim, Germany). For MIB1 staining, paraffin embedded liver sections were cut (4 µm) and mounted on ProbeOn™ slides (Fisher Scientific, Pittsburgh, PA). The sections were dried, deparaffinized, and hydrated with xylene and graded ethanol. They were then washed with Tris buffered saline with 0.05% Tween 20 (TBST). Heat-fixed slides were placed in a target retrieval solution at 95 °C for 20 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water. The slides were washed with TBST, incubated with biotin and avidin block (DAKO, Carpinteria, CA), washed with TBST, and incubated for 1 h at room temperature with anti-mouse Ki67 (cell cycle protein) clone TEC-3 (DAKO, A/S, Denmark) 1:50 with Tris-HCl. The slides were washed 3 times, incubated with 1:200 secondary biotinylated rabbit anti-rat immunoglobulin (DAKO, A/S) for 30 min at room temperature, and washed 3 times. The slides were incubated in dilute streptavidin-peroxidase (DAKO) 1:300 and incubated for 30 min. They were washed 3 times, incubated with AEC (Sigma, St. Louis, MO) for 5-10 min, then counterstained with hematoxylin, aquamounted, and cloverslipped.

The TUNEL (apoptosis) and MIB1 (proliferation) ratios were determined for each group by counting the number and determining the percentage of TUNEL or MIB1 positive cells/total cells in micrometastases in adjacent serial sections of the livers as previously described [17].

Angiostatin bioassay: An angiostatin bioassay was performed as previously described [18]. Briefly, bovine capillary endothelial (EJ) cells were obtained (courtesy J. Niederkorn) and cultured in MEM with 10% heat inactivated fetal calf serum. The EJ cells were plated into gelatin coated 24 well plates and incubated at 37 °C (1x10⁵ cells/well). After one day, 300 ml of melanoma cell supernatant (supernatant from 5x10⁶ cells/3 µl) generated over 72 h with or without 100 mg/ml of human glu-plasminogen was added to each well. The cells were pulsed at 100 µCi of H-thymidine for 24 h. The wells were washed with PBS, the contents were solubilized with 10% sodium dodecyl sulfate and the radioactivity was
counted in a liquid scintillation counter (Wallac, 1450 MicroBeta TRILUXE, Turku, Finland). The angiotatin activity (as a percentage) was computed as the difference between the percentage of control of EJG cell proliferation in the absence and presence of plasminogen. The data are the average of 2 to 3 experiments.

**Plasminogen:** Reverse transcription-polymerase chain reaction (RT-PCR) for mouse plasminogen was performed. Before total RNA was extracted from mouse uveal melanoma cells using RNA-Bee™ RNA isolation solvent (Tel-test, Friendswood, TX), cells were cultured in complete RPMI1640 without serum overnight. The RNA samples were quantified by UV-1601 model UV-visible spectrophotometry (Shimadzu, Norcross, GA). RT-PCR was performed using the QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA) on the PTC-200 Peltier Thermal Cycler (MJ Research, Inc. Watertown, MA), mouse plasminogen accession number BC057186. The sequences of the primers were 5'-AGG TGT CTC GGA CTG TTT GG-3' and 5'-CCT GGA CA T GGT TCC TCT GT-3'. The reaction components included 1 ng total RNA from melanoma cells, Qiaqen OneStep RT-PCR buffer, dNTP mix, 0.6 µM the pair of primer, and QIAGEN OneStep RT PCR enzyme mix. Total reaction volume was 50 µl. The RT-PCR cycle parameters were: 52 °C 30 min, 95 °C 15 min and 40 cycles at 94 °C for 30 s, 59.8 °C for 40 s and 72 °C for 40 s, 72 °C 10 min. The PCR products were detected by 2% agarose gel electrophoresis. The image was developed by Eagle Eye™ II Still Video System (Stratagene, La Jolla, CA).

**Real Time (RT) PCR for VEGF mRNA:** The murine melanoma cell lines (Queens, B16F10, and B16LS9) were subcultured into 6 well plates. After reaching confluence, the medium was changed, and the cells were exposed to serum free RPMI1640 with 0.1 µg/µl and 50 µg/µl murine angiotatin at 5% CO₂, 37 °C for 6 h. Total RNA was extracted from the melanoma cells treated with different concentrations of angiotatin as described above using RNA-Bee™ RNA isolation solvent (Tel-test). The RNA samples were quantified by a UV-1601 model UV-visible spectrophotometer (Shimadzu). One step real time RT-PCR was performed using the QuantiTect™ SYBR® green RT-PCR kit (QIAGEN) with a thermocycler (iCycler, Bio-Rad, Hercules, CA). The primers were designed by Primer Express software (Applied Biosystems, Foster City, CA). The primers for detection of mouse VEGF mRNA were 5'-CGC GAG TCT GTG TTT TTG CA-3' and 5'-CAG AGC GGA GAA AGC A TT TGT -3'. 10 ng of total RNA from melanoma cells were mixed with 2 µl QuantiTect SYBR green RT-PCR master mix containing HotStarTaq DNA Polymerase, Tris-HCl, KCl, (NH₄)₂SO₄, 5 mM MgCl₂, dNTP mix, SYBR Green 1 and ROX, 0.3 µM of each specific primer, and 0.25 µl QuantiTect RT mixed to a volume of 20 µl. The following PCR cycle parameters were used: hot-start polymerase activation for 15 min at 95 °C and denaturation 94 °C 35 s, annealing 58.5 °C 30 s, extension 72 °C 20 s, total 45 cycles. All of the samples from the same cell line were run in the same 96 well plate. Detection of the fluorescence product was performed during all of the cycles. To confirm specificity, the PCR products from each pair of primers were analyzed by agarose gel electrophoresis and visualized under UV light.

**Figure 1.** B16F10 and B16LS9 melanoma cells produce plasminogen and cleave plasminogen into angiotatin. A: The angiotatin bioassay compared to the control shows Queens 0%, B16F10 57%, and B16LS9 127%. B: Western analysis shows angiotatin protein (50 kDa) produced by B16F10 and B16LS9 cell lines. C: RT-PCR shows mRNA for plasminogen (220 bp) produced by B16F10 and B16LS9 cell lines (lanes 8 and 9, respectively). There is no plasminogen mRNA expressed from Queens cells (lane 7). A 100 bp molecular ruler is present in lane 6.
ers were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. The quantification data were analyzed with the thermocycler system software (iCycleriQ, Bio-Rad).

**Western blot analysis:** Serum free melanoma supernatants were obtained as described above. For angiostatin, Queens, B16F10, and B16LS9 cell lines were studied. The same cell lines and metastatic B16LS9 cultured from a hepatic metastasis were evaluated for VEGF. VEGF was measured in B16LS9 and metastatic B16LS9 cells supplemented with low (0.1 µg/µl) or high (50 µg/µl) concentration angiostatin. Samples were electrophoresed under nonreducing conditions on 12% polyacrylamide gels in a tris-glycine running buffer and analyzed separately by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each lane was loaded with an equal volume of protein. Purified angiostatin or murine vascular endothelium were used as positive controls. The protein bands were then electrophoretically transferred to a polyvinylidene membrane at 4 °C and 0.2 amperes for 1 h. The transfer buffer contained 24 mM Tris, 192 mM glycine, and 20% methanol. The membrane was then blocked for 1 h with 5% casein in PBS and washed twice with fresh changes of PBS. The membrane was then incubated for 1 h in a 1:1000 1 mg/ml of monoclonal rabbit anti-human angiostatin antiserum (specific for kringle 1-4) for angiostatin (Alpha Diagnostic International, Inc., San Antonio, TX), or rabbit polyclonal anti-VEGF (Chemicon, Temecula, CA) for vascular endothelial growth factor (VEGF). The membrane was washed twice with PBS and incubated with 1:10,000 dilution of horseradish peroxidase conjugated goat-antirabbit immunoglobulin G. The membrane was developed using an electrochemiluminescence Western blotting kit (Pierce, Rockford, IL). The membrane was placed in a film cassette and exposed to scientific imaging film. The film was then developed and the protein bands were analyzed.

**Migration assay:** After Queens, B16F10, and B16LS9 melanoma cells were treated with 50 µg/µl angiostatin or 0.1 µg/µl angiostatin for 16 h, the medium was exchanged to the 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, German) at 0 h, 24 h, and 48 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 or 48 h) from the initial width of the injury line (at 0 h).

**Invasion assay:** The in vitro invasion assay was performed with a 96 well collagen-based cell invasion assay kit (Chemicon) and read at 480/520 nm in Quant Spectra MAX 190. After Queens, B16F10, and B16LS9 melanoma cell were treated with 50 µg/µl angiostatin or 0.1 µg/µl angiostatin 16 h, 2x10^5 cells were placed into an invasion chamber consisting of a 96 well collagen-based plate. The cells were incubated for 24 h at 37 °C in a 5% CO₂ incubator. The cells and media were discarded from the top of the insert, and the invasion chamber plate was placed onto the new 96 well feeder tray containing 150 µl of warm cell detachment solution in the wells and incubated for 30 min at 37 °C. Dye Solution (50 µl) was added to each well of the feeder tray containing 150 µl of cell detachment solution with the cells that invaded through the collagen coated membrane and incubated for 15 min at room temperature. The mixture (100 µl) was transferred to a new 96 well plate for measurement of fluorescence.

**Statistical analyses:** All statistical analyses were performed with SAS (version 8.0; SAS Institute, Cary, NC). Values reported in the tables are mean±standard error of the mean (SEM). For in vivo (metastatic foci) data, statistical testing used a paired Student’s t-test. The numbers of melanoma cells in the migration assay were compared between control, low, and high concentration angiostatin groups with the Mann-Whitney U test. The apoptotic (TUNEL+) and proliferation (MIB1+) ratios were compared between groups with the χ² test.

**RESULTS**

The angiostatin bioactivity assay in increasing order of activity was Queens (0% of control), B16F10 (57% of control), and B16LS9 (127% of control, Figure 1A). Western blot analysis corresponded to the bioassay, with no angiostatin detected for Queens and increasing levels of angiostatin produced by B16F10 and B16LS9 cell lines (Figure 1B). B16F10 and B16LS9 cells, but not Queens cells, expressed mRNA for plas-

<table>
<thead>
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<th>Group</th>
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<th>B16F10</th>
<th>B16LS9</th>
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<td>0.0258937</td>
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There is no decrease in the number of hepatic micrometastases in high dose angiostatin (0.3 µg/µl) treated mice compared to controls.

<table>
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<th>Cell lines</th>
<th>Queens</th>
<th>B16F10</th>
<th>B16LS9</th>
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<td>p value</td>
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<td>0.036977</td>
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There is a significant decrease in the number of hepatic micrometastases in low dose angiostatin (0.1 µg/µl) treated mice compared to controls.
Figure 2. Low dose angiostatin treatment causes increase in apoptosis:proliferation ratio in melanoma micrometastasis. A: TUNEL analysis shows significantly more apoptosis in hepatic micrometastasis in low dose angiostatin (0.1 µg/µl) treated mice compared to PBS treated controls. B: MIB1 (anti-Ki67) analysis shows significantly more proliferation in hepatic micrometastasis in PBS treated control compared to low dose angiostatin (0.1 µg/µl) treated mice. Taken together, A and B show increased apoptosis:proliferation ratio after low dose angiostatin treatment. C: Micrometastatic hepatic melanoma (between arrows) in PBS treated control immunostained for TUNEL (100x). D: Micrometastatic hepatic melanoma (between arrows) after low dose angiostatin treatment immunostained for TUNEL (100x). E: Immunostain for MIB1 (anti-Ki67) shows proliferation in hepatic micrometastatic melanoma (between arrows) in PBS treated control (100x). F: Immunostain for MIB1 shows lack of proliferation in hepatic micrometastatic melanoma (between arrows) after low dose angiostatin treatment (100x). Brown indicates positive reaction product. Bar represents 50 µm. Panels C-F show the B16LS9 cell line.
minogen (Figure 1C). Intraocular melanoma grew and was confined in the eye in 95% of each group.

There was no effect of high dose (0.3 µg/µl) angiostatin on decreasing the number of hepatic micrometastasis (Table 1). There were significantly fewer hepatic micrometastases with low dose (0.1 µg/µl) angiostatin for Queens, B16F10 (p<0.05), and B16LS9 (p<0.01 cells, Table 2). Absolute numbers of micrometastases cannot be compared among cell lines, as separate cell lines were thawed, grown to confluence, and used for the separate experiments. The experiments were designed to determine differences in absolute numbers in treated compared to control groups for a given cell line treated with a given concentration of angiostatin, not to compare the absolute number of micrometastases among cell lines [17].

Apoptosis ratios were significantly increased and proliferation ratios were significantly decreased in micrometastases in low dose angiostatin treatment groups compared with controls for all three cell lines (p<0.01, Figure 2). The migration assay showed significantly decreased migration in all three cell lines after low concentration compared to high concentration angiostatin supplementation (p<0.01, Table 3). Additionally, there was significantly decreased invasion in all three cell lines after low concentration angiostatin supplementation (p<0.01) and increased migration after high concentration angiostatin (Table 4).

VEGF mRNA was decreased with low compared to high concentration angiostatin supplementation in the media, which was significant for B16LS9 and metastatic B16LS9 cells (p<0.05, Table 5). VEGF protein was expressed by all cell lines and was lower after low concentration compared to high concentration angiostatin supplementation in B16LS9 cells (Figure 3).

| TABLE 3. MIGRATION DISTANCE WITH 0.1 µg/µl OR 50 µg/µl ANGIOSTATIN |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Groups                      | Time                        | Queens                      | B16F10                      | B16LS9                      |
| PBS                         | 24 h                       | 279.76±38.67                | 286.25±44.29                | 714.21±113.52               |
|                             | 48 h                       | 551.37±40.12                | 710.72±28.97                | 839.93±65.97                |
| Angiostatin                 | 24 h                       | 168.33±27.75**              | 211.53±37.02**              | 197.50±104.36**             |
| 0.1 µg/µl                   | 48 h                       | 697.11±15.78**              | 545.75±21.63**              | 777.93±45.86**              |
| Angiostatin                 | 24 h                       | 509.03±40.57**              | 410.12±50.31**              | 538.07±182.47**             |
| 50 µg/µl                    | 48 h                       | 641.68±53.02**              | 620.62±90.65**              | 900.42±49.57**              |

There is a significant reduction in the in vitro migration of Queens, B16F10, and B16LS9 melanoma cells cultured in low concentration (0.1 µg/µl) compared to high concentration (50 µg/µl) angiostatin. Asterisks mark a significant difference between the migration distance for the angiostatin 0.1 µg/µl group and the angiostatin 50 µg/µl group (** for p<0.01, * for p<0.05; unpaired Student’s t-test). Sharps mark a significant difference between the migration distance for one of the angiostatin groups and control group (# for p<0.01, # for p<0.05; unpaired Student’s t-test).

| TABLE 4. INVASION RELATIVE FLUORESCENCE UNITS IN MOUSE MELANOMA CELL LINES WITH LOW CONCENTRATION OR HIGH CONCENTRATION ANGIOSTATIN |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Group                       | Cell lines                  |
|                             | Queens                      | B16F10                      | B16LS9                      |
| PBS                         | 2.23±0.06                   | 1.81±0.003                  | 2.04±0.02                   |
| Angiostatin                 | 0.1 µg/µl                   | 1.51±0.05**##               | 1.36±0.10**##               | 1.37±0.23**##               |
| Angiostatin                 | 50 µg/µl                    | 2.58±0.23**##               | 3.33±0.83##                 | 3.42±0.20##                |

There is a significant reduction in the in vitro invasion of Queens, B16F10, and B16LS9 melanoma cells cultured in low concentration (0.1 µg/µl) compared to high concentration (50 µg/µl) angiostatin. Asterisks mark a significant difference between the invasion relative fluorescence units for the angiostatin 0.1 µg/µl group and the angiostatin 50 µg/µl group (** for p<0.01; unpaired Student’s t-test). Sharps mark a significant difference between the invasion RFU for one of the angiostatin groups and control group (# for p<0.01, # for p<0.05; unpaired Student’s t-test).

VEGF mRNA was decreased with low concentration compared to high concentration angiostatin supplementation in the media, which was significant for B16LS9 and metastatic B16LS9 cells.

Figure 3. Queens, B16F10, B16LS9, and metastatic B16LS9 melanoma cells produce VEGF. A: Western analysis shows that Queens, B16F10, B16LS9, and metastatic B16LS9 cell lines all produce VEGF (38 kDa). B: Western analysis shows that B16LS9 cells produce a higher level of VEGF when grown with high concentration (50 µg/µl) compared to low concentration (0.1 µg/µl) in vitro supplemental angiostatin. ME labels murine endothelium positive control.
DISCUSSION

One of the steps during tumor progression is sustained angiogenesis of the primary neoplasm [5]. Vascular formation in primary uveal melanoma is the subject of much interest [19,20]. It appears that channels in primary uveal melanoma include vessels incorporated from the uvea, mosaic vessels, angiogenic vessels, and forms of vasculogenic mimicry including extravascular matrix patterns and melanoma lined tubes [21,22]. Angiogenesis in uveal melanoma may develop adjacent to highly aggressive tumor cell populations but most likely develops within less aggressive portions of the melanoma [22]. The angiogenic cytokine vascular endothelial growth factor (VEGF), in particular VEGF-C, is expressed in uveal melanoma [23,24]. VEGF leads to endothelial cell proliferation, migration and tube formation [25].

Uveal melanoma metastasizes virtually exclusively hematogenously [26], predominantly to the liver [6], where it forms clinically undetectable micrometastases that may remain “dormant” for years [27]. These micrometastases measure up to approximately 100 µm in diameter and have the potential to grow and become vascularized [28,29]. This has been recapitulated in an in vivo model of metastatic ocular melanoma [8,9]. The micrometastases remain dormant when there is a balance between proliferation and apoptotic signaling [25,30]. An alteration in this balance favoring proliferation results in tumor growth. The shift toward proliferation may involve an “angiogenic switch”, with stimulations of up- and downregulation of pro- and anti-angiogenic molecules, respectively [25,31,32]. There is evidence that this is the case in metastatic uveal melanoma to the liver, which has a higher microvascular density than the primary uveal melanoma [29]. Our experiments support this concept, as metastatic melanoma cultured from the liver expressed higher levels of VEGF than the primary cell line (Figure 3). Previous studies have shown that highly metastatic human uveal melanoma cell lines have dedifferentiated to an embryonal-like phenotype [33] and mimic vascular channels by forming melanoma lined tubes [34]. Thus, metastatic uveal melanoma exhibits phenotypic characteristics similar to vascular endothelium. Low dose angiostatin may affect similar parameters of migration in endothelial cells and melanoma cells exhibiting vasculogenic mimicry.

In addition to pro-angiogenic cytokines (i.e., VEGF), uveal melanoma cells express anti-angiogenic cytokines. Apte and coworkers demonstrated 38 kDa angiostatin molecule production by human uveal melanoma cell lines, as the melanoma cells secrete a matrix metalloproteinase that cleaves plasminogen [18]. Our laboratory confirmed those findings [35] and demonstrated angiostatin production in two of three murine melanoma cell lines used in this study. Importantly, we found that the melanoma cells themselves produce plasminogen and the matrix metalloproteinase that cleaves the first four kringle domains of plasminogen (angiostatin). These findings help explain the Zimmerman-McLean-Foster effect [1,36]. A subset of uveal melanomas may produce angiostatin. A low serum concentration of angiostatin maintains micrometastases in a dormant state. Removal of the primary melanoma eliminates the low serum concentration of angiostatin and the micrometastases progress. Apte and coworkers demonstrated this effect by showing that removal of experimental ocular melanomas producing angiostatin resulted in increased metastasis [9]. Our experiments provide further evidence in that administration of systemic low dose angiostatin after removal of experimental ocular melanoma results in decreased metastasis.

Angiostatin, a 38 kDa internal fragment of plasminogen, is an anti-angiogenic endothelial cell inhibitor [37]. Angiostatin

Figure 4. Mechanism of angiostatin effect on micrometastatic melanoma. Proposed mechanism of action of angiostatin on hepatic micrometastatic melanoma. Melanoma cells synthesize plasminogen and a matrix metalloproteinase that cleaves plasminogen kringle 1-4 (angiostatin) from kringle 5. Angiostatin binds with plasminogen and forms a ternary complex (P*), thus limiting melanoma migration. Also, angiostatin blocks VEGF induced downregulation of caveolin-1, thus causing cell cycle arrest and apoptosis.
is made by cancer mediated conversion of plasminogen when the first four kringles (K1-4) are cleaved via a tumor induced cytokine/elastase mechanism [38]. Angiostatin blocks VEGF induced downregulation of caveolin-1 and causes cell cycle arrest with apoptosis of endothelial cells [39]. Angiostatin also blocks plasminogen enhanced invasion of tissue plasminogen activator (TPA) producing melanoma cells likely by TPA binding of angiostatin and plasminogen, forming a ternary complex (Figure 4) [40].

There was an in vitro anti-migration/anti-invasion effect of low concentration angiostatin in our study in all three cell lines, whereas high concentration angiostatin induced increased invasion. Low concentration angiostatin had its greatest effect in vitro decrease of VEGF production on B16L9 cells. The potential reason for failure of current angiostatin trials [41] is dose escalation of angiostatin and that low dose angiostatin might be more useful than high dose angiostatin. The final pathway is apoptosis of the micrometastasis. Apoptosis may be a direct effect of angiostatin or due to increased susceptibility of the intrahepatic micrometastasis to intrahepatic NK cell lysis due to decreased melanoma migration [17].

Our laboratory recently demonstrated that a potent anti-angiogenic cytokine, pigment epithelium derived factor (PEDF), is constitutively produced by human uveal melanoma cells and the same murine melanoma cell lines used in this study [42]. Preliminary studies show that a critical range of angiostatin concentrations in vitro result in melanoma PEDF/VEGF ratios favoring stabilization (dormancy) of the micrometastasis [42]. This range may be related to the in vivo differences between high and low concentration angiostatin in our current study, as angiostatin changes the PEDF/VEGF ratio both in vitro and in situ in micrometastases evaluated at necropsy (7 days after angiostatin treatment) [42]. This change in favor of PEDF appears to decrease the micrometastatic proliferation rate via nuclear transcription factors. The occurrence of micrometastases is not reduced; micrometastases that have occurred involute. Shifting toward an anti-angiogenic environment results in apoptosis and toward an angiogenic environment results in proliferation, similar to the final common pathway in our current study. These processes appear to be complex and possible mechanisms range from alterations in systemic angiostatin produced by the primary melanoma [18] to PEDF, VEGF, and hepatocyte growth factor scatter factor (HGF/SF) interactions between the micrometastasis, hepatic sinusoidal endothelium Kupfer cells, and hepatocytes [43].

ACKNOWLEDGEMENTS
Supported by NIH NEI R01EY13165; Dr. Grossniklaus is a recipient of the Research to Prevent Blindness Physician Scientist Award. We thank Jack Arbiser, MD, PhD for his critical review of this manuscript.

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