Bevacizumab Suppression of Establishment of Micrometastases in Experimental Ocular Melanoma

Hua Yang, Martine J. Jager, and Hans E. Grossniklaus

PURPOSE. This study was undertaken to determine whether anti-vascular endothelial growth factor (VEGF) therapy inhibits growth of primary uveal melanoma and spread of its hepatic micrometastases.

METHODS. The human uveal melanoma cell lines Mel290 and Mel 270, HUVECs, mouse B16LS9 melanoma cells, and mouse vascular endothelial cells were separately cultured or co-cultured and incubated with bevacizumab or IgG1. The level of VEGF protein in the culture medium was measured by ELISA. In vitro angiogenesis and invasion assays were performed under bevacizumab or IgG1 treatment. Mel290 or B16LS9 cells were inoculated into NU/NU or C57Bl/6 mouse eyes which were enucleated after 7 days. The sizes of the intraocular tumors were determined. Time and dosage experiments were performed by using 50 or 250 μg bevacizumab starting at day 1 or 4 after inoculation. Hepatic micrometastases were enumerated. Proliferation, apoptosis, and angiogenesis markers were detected in the ocular tumor by immunofluorescence staining.

RESULTS. Bevacizumab significantly reduced the level of VEGF in the culture media from human uveal melanoma cells, mouse melanoma cells, and co-cultured cells. It also inhibited cell tube formation and decreased in vitro invasion of tumor cells. In the mouse model, bevacizumab suppressed primary ocular melanoma growth and the formation of hepatic micrometastases in a dose-dependent manner. Furthermore, immunohistochemical staining showed decreased Ki67 and unchanged caspase 3 expression after treatment with bevacizumab.


Branchytherapy, local resection, and enucleation are current treatments for primary uveal melanoma. The goals of treatment of primary uveal melanoma are to prevent further growth of the tumor, preserve vision, achieve a cosmetically acceptable result, and, if possible, reduce the risk of metastasis. Although these therapies have achieved many of these goals, mortality from metastatic disease has remained unchanged for decades.1,2

The formation of new blood vessels (angiogenesis) is a feature of growth and invasion in primary neoplasms and their metastases. Angiogenesis occurs in uveal melanoma and its metastases.3,4 Vascular endothelial growth factor (VEGF) plays a central role in tumor development including potentiating cell proliferation, survival, migration, and angiogenesis.5–7 VEGF is a member of the platelet-derived growth factor family of structurally related mitogens and has four isoforms: VEGF-A, -B, -C, and -D.8–10 VEGF-A is the most important isoform with regard to angiogenesis. VEGF is a homodimeric glycoprotein with a molecular mass of approximately 45 kDa. VEGF-A induces angiogenesis via a direct effect on endothelial cells.9–12 In vitro experiments using microvascular endothelial cells grown on the surface of three-dimensional collagen gels have shown that VEGF induces cellular invasion of the underlying matrix with formation of capillary-like tubules.13

VEGF-mediated angiogenesis is essential for tumor growth. Without an adequate vascular supply, solid tumors are only able to grow to approximately 1- to 2-mm diameter spheres (~10^6 cells), primarily because of a lack of oxygen and nutrients.14,15 Increasing concentrations of VEGF in a tumor results in blood vessels that are structurally different from normal, mature blood vessels.16 These disordered, abnormal tumor vessels are critical for invasation and extravasation of tumor cells during the metastatic process. The vascular endothelium marker CD31 is used to identify the vascular density of the primary tumor and that value correlates with metastasis. Previous studies in mouse models of breast carcinoma and fibrosarcoma have shown that a neutralizing anti-VEGF monoclonal antibody blocks the interaction of VEGF-A with its receptor, VEGF-R1, resulting in a reduction in the size of the primary tumor and the number of micrometastases.17,18

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that contains human framework regions, ~95% human and 7% murine protein sequence,19 and the complementarity-determining regions of a murine antibody that binds to VEGF (Genentech, San Francisco, CA). Bevacizumab binds to and inactivates all isoforms of VEGF, thus inhibiting angiogenesis, tumor proliferation, and growth.19,20 Since tumor cell lines often overexpress VEGF, bevacizumab’s activity reflects a key targeting strategy for cancer therapy.19–22 Recently, bevacizumab has been shown to control metastatic colorectal carcinoma, metastatic breast carcinoma, and non–small cell lung carcinoma.23–26 However, a potential role of bevacizumab therapy in uveal melanoma has not been demonstrated. Uveal melanoma becomes vascularized, with metastasis that is virtually exclusively hematogenous, thus making it a potential target for antiangiogenic therapy. The purpose of this study was to determine whether anti-VEGF therapy inhibits the growth of primary uveal melanoma and its micrometastases.

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Materials and Methods

Cells and Treatment

The following cell lines were used: human uveal melanoma Mel 290 and Mel 270 cells (courtesy of Bruce Ksander, Schepens Eye Institute, Boston, MA); mouse melanoma B16L59 (courtesy of Dario Ruciano, Friedrich Miescher Institute, Basel Switzerland); HUVECs and mouse vascular endothelial cells (Lifeline Cell Technology, Walkersville, MD); and human uveal melanocytes (courtesy of Dan-Ning Hu, The New York Eye and Ear Infirmary). The tumor cells were cultured at 37°C in a 5% CO₂ incubator in complete culture medium (RPMI-1640 with HEPES and l-glutamine. 10% fetal bovine serum 1% nonessential amino acids, 1% sodium pyruvate solution, 1% MEM vitamin solution [Invitrogen-Gibco, Grand Island, NY], 1% antibiotic-antimycotic solution containing 100 U/ml penicillin G, 250 ng/ml amphotericin B, and 100 µg/ml streptomycin solution [Invitrogen-Gibco]). The culture medium for vascular endothelium was obtained from Lifeline Cell Technology. The culture methods for human uveal melanocytes and generation of Mel290-GFP have been published.²⁷,²⁸

VEGF ELISA Assay

A portion (1 x 10⁵) of Mel 290, Mel 270, B16L59, HUVECs, and mouse vascular endothelial cells were separately plated in a six-well plate. Human uveal melanoma and mouse melanoma cells were also co-cultured with the HUVECs and mouse vascular endothelial cells, respectively. All cultures were performed in triplicate. When the cells reached 90% confluence at the third day after they were seeded, the media were changed to complete culture media with 10 or 100 µg/ml bevacizumab, or an equal amount of IgG1. The cell culture media were collected at 72 hours after treatment in complete culture medium in 5% CO₂ at 37°C. The concentration of VEGF in the supernatants was measured with an ELISA kit (Quantikine; R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

In Vitro Angiogenesis Assay

HUVECs or Mel290-GFP cells were suspended in complete culture medium with 100 µg/ml bevacizumab or an equal amount of IgG1 and were seeded on growth factor-containing gel (ECMatrix; Millipore, Billerica, MA) in a 24-well-plate, incubated in 5% CO₂ at 37°C for 10 hours, and inspected for tube formation with an inverted light microscope (Olympus, Tokyo, Japan). The pattern/value association criteria for tube formation are: 0, individual cells, well separated; 1, cells beginning to migrate and align themselves; 2, capillary tubes visible without spraying; 3, sprouting of new capillary tubes; 4, closed polygons beginning to form; and 5 complex meshlike structures developing. Three random fields per well were examined at 40× magnification, and the values were averaged.²⁹

Invasion Assay

The in vitro invasion assay was performed with a 96-well collagen-based cell-invasion assay kit (Chemicon, Temecula, CA) and read on a spectrophotometer at 480/520 nm (µQuant Spectra MAX 190; Bio-Tek Instruments, Inc. Winoski, VT). After the Mel 290 and B16L59 cells were treated with 10 or 100 µg/ml bevacizumab or an equal amount of IgG1 for 24 hours, 2 x 10³ cells were placed into an invasion chamber consisting of a 96-well, collagen-based plate. The cells were incubated for 24 hours at 37°C in 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 minutes at 37°C. Dye solution (50 µl) was added to each well of the feeder tray containing the detachment solution and the cells that invaded through the collagen-coated membrane and incubated for 15 minutes at room temperature. The mixture (100 µl) was transferred to a new 96-well plate for measurement of fluorescence.

Animal Experiments

A mouse uveal melanoma model was used as previously described. Ten-week-old female C57BL/6 or NU/NU mice were used (Charles River, Wilmington, MA). All experiments were conducted in accordance with the Guiding Principles in the Care and Use of Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Aliquots of 1 x 10⁴ of Mel290 or 5 x 10⁶ cells of B16L59/2.5 µL were inoculated into the choroid of the right murine eye with a transscleral 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 ½-gauge needle under the guidance of a dissection microscope. The tip of a 10-µL glass syringe with a blunt metal needle (Hamilton, Reno, NV) was introduced into the choroid through the needle track; 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 inoculated eyes were enucleated after 1 week. Livers were collected 4 weeks after inoculation, and the dose was repeated twice per week for 4 or 6 weeks. Mice in a dosage experiment were divided into three groups, with the groups receiving 50 or 250 µg IP bevacizumab or an equal volume of PBS starting on day 1 or 4. In a longitudinal experiment, the mice were divided into 10 groups, with half of them receiving 250 µg IP bevacizumab twice per week and half receiving PBS. All were killed, with hepatic tissue collection at 1, 2, 3, 4, or 6 weeks after the intraocular inoculation of melanoma cells. There were 10 mice in each group. Nine eyes and all livers were fixed in 10% formalin and embedded in paraffin. The eyes were cut into 7-µm serial sections and stained with hematoxylin/eosin. Ten sections with the largest tumor area in each eye were photographed at 40× magnification (DP10; Olympus). The sizes of the intraocular tumors were determined with Image J software (Image Processing Analysis in Java, ver. 1.42; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, available at http://rsb.info.nih.gov/ij/index.html). Hepatic micrometastases were microscopically determined in the three central sections of the liver and enumerated as previously described.³⁰ The animal experiments are outlined in Table 1.

Immunofluorescence Staining

Frozen sections of one enucleated mouse eye from each group were obtained from the in vivo experiments. Immunofluorescence staining for intratumoral vessels with anti-CD31 conjugated with FITC (BD-PharMingen, San Jose, CA), cellular proliferation with anti-Ki67 (Novocastra Laboratories, Newcastle, UK), and apoptosis with anti-caspase 3 (Abcam, Cambridge, MA), was followed by incubation with a secondary antibody, counterstaining with propidium iodide (Vector Laboratories, Burlingame, CA), mounting with mounting medium, and coverslipping. In the in vitro experiment, after treatment of Mel 290 cells with 100 µg/ml bevacizumab, IgG1 (negative treatment control), or staurosporine (positive treatment control) for 48 hours, Mel 290 cells were stained with anti-Ki67 or anti-caspase 3. In the in vivo experiment, after treatment with bevacizumab or PBS (negative treatment control), tumors in the enucleated eyes were stained with anti-Ki67 or anti-caspase 3. The quantitative assay for proliferation and apoptosis was performed with an image-analysis program (Image-Pro Plus 6; Media Cybernetics, Bethesda, MD). The software automatically counted the stained nuclei. The mean vascular density was determined by measuring the total vessel length or counting the number of CD31-positive capillaries per high-power field using 40× magnification and the image analysis program.
RESULTS

VEGF in Human Uveal Melanoma and Mouse Melanoma Cell Lines

Since uveal melanoma becomes vascularized in the eye and its metastases become vascularized in the liver, we studied VEGF expression and suppression with bevacizumab in melanoma and vascular endothelial cells lines cultured separately and co-cultured. Both 10 and 100 μg/mL bevacizumab decreased the level of VEGF secreted by human uveal melanoma cell lines and HUVECs, compared with IgG1 control cells. There were significant differences in the VEGF levels between the 10 or 100 μg/mL bevacizumab and IgG1 control cells, and between the 10 and 100 μg/mL bevacizumab treatment groups in both uveal melanoma cell lines and HUVECs (t-test, P < 0.01). Treatment with 100 μg/mL bevacizumab caused a more pronounced decrease in VEGF than treatment with 10 μg/mL bevacizumab (Fig. 1A). Analysis of ELISA results showed that bevacizumab decreased VEGF 84.30%, 95.52%, and 30.91% in the culture media of Mel270 and Mel290 cells and HUVECs, respectively. Mel290 was the most sensitive cell line to bevacizumab. The levels of VEGF were significantly increased when tumor cells were co-cultured with vascular endothelium. The levels of VEGF in co-culture media were 6.22 and 10.39 times

Table 1. Outline of Animal Experiments

<table>
<thead>
<tr>
<th>Experiment/Groups</th>
<th>Day 1 Ocular Inoculation</th>
<th>Day 4* Intraperitoneal Injection</th>
<th>Day 8 Enucleation</th>
<th>Day 29† Necropsy</th>
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<td>Dosage experiment</td>
<td>Group 1 BI6LS9 Bevacizumab 250 μg/100 μL</td>
<td>Yes</td>
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<td>Group 2 BI6LS9 Bevacizumab 50 μg/100 μL</td>
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<td>Yes</td>
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<td>Timing experiment</td>
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<td>Group 2 Mel 290 Bevacizumab 250 μg/100 μL</td>
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<tr>
<td>Group 3 Mel 290 PBS 100 μL (day 1)</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
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<tr>
<td>Group 4 Mel 290 PBS 100 μL</td>
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<td>Longitudinal experiment‡</td>
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<td>Group 3 Mel 290 Bevacizumab 250 μg/100 μL</td>
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<td>Group 5 Mel 290 Bevacizumab 250 μg/100 μL</td>
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<td>Yes (day 43)</td>
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n = 10 mice in each group.
* Intraperitoneal injection was performed on day 4 unless otherwise indicated.
† Hepatic tissue was obtained on day 29 unless otherwise indicated.
‡ All treated groups in this experiment had a PBS control group.

Figure 1. VEGF expression after in vitro treatment with bevacizumab. (A) Human uveal melanoma cell lines and HUVECs expressed from 150 to 500 pg/mL VEGF, with a significant decrease after treatment with 10 or 100 μg/mL bevacizumab and IgG1 control cells, and between the 10 and 100 μg/mL bevacizumab treatment groups in both uveal melanoma cell lines and HUVECs (t-test, P < 0.01). Treatment with 100 μg/mL bevacizumab caused a more pronounced decrease in VEGF than treatment with 10 μg/mL bevacizumab (Fig. 1A). Analysis of ELISA results showed that bevacizumab decreased VEGF 84.30%, 95.52%, and 30.91% in the culture media of Mel270 and Mel290 cells and HUVECs, respectively. Mel290 was the most sensitive cell line to bevacizumab. The levels of VEGF were significantly increased when tumor cells were co-cultured with vascular endothelium. The levels of VEGF in co-culture media were 6.22 and 10.39 times
greater than those in medium from tumor cells only, for Mel270 and Mel290, respectively. VEGF was reduced in Mel270 and Mel290 cells co-cultured with HUVECs. There were significant differences in the VEGF levels between the 10 and 100 μg/mL bevacizumab treatment groups and control cells and between the 10 and 100 μg/mL bevacizumab treatment groups for both uveal melanoma cell line co-cultured with HUVECs (t-test, P < 0.01, Fig. 1B). A high concentration of bevacizumab was more effective than a low concentration on reducing VEGF in tumor cells and HUVECs. VEGF secretion was slightly suppressed with 10 μg/mL bevacizumab (P = 0.0046) and greatly reduced with 100 μg/mL (P = 0.0091) in B16L59 cells, compared with the IgG1 control treatment. Treatment with 10 and 100 μg/mL bevacizumab resulted in 7.44% and 19.18% decreases, respectively, in VEGF in tumors in both the PBS control and bevacizumab-treated groups.

**Effect of Bevacizumab on Tumor Growth**

Systemic treatment with bevacizumab reduced the size of the intraocular tumor in our mouse model (Fig. 3). In the dosage experiment, the size of the intraocular melanoma in the mice treated with 250 μg μg bevacizumab was smaller than that in the mice treated with 50 μg, with significant differences between either group and the PBS-treated control mice (P < 0.01) and between the 250- and 50-μg treated groups (P < 0.01, Figs. 3A and 3B). In the timing experiment, the size of the intraocular tumor was smaller in the group where treatment had started on day 1 versus the group that was started on treatment on day 4 after intraocular inoculation of melanoma cells (Fig. 3C). Both in vitro and in vivo treatment with bevacizumab led to decreased nuclear expression of Ki67 (Fig. 4). Image analysis (Image-Pro Plus 6; Media Cybernetics) showed that in the in vitro experiment, 53% and 0% of nuclei stained for Ki67 after treatment with IgG1 and bevacizumab, respectively. In the in vivo experiment, 98% and 11% of nuclei stained for Ki67 in the PBS control and bevacizumab treatment groups, respectively. Bevacizumab did not change the expression of the apoptotic enzyme caspase 3 in uveal melanoma cells in vitro or in vivo (Fig. 5). Image analysis of the in vitro experiment showed that 6%, 4%, and 74% of nuclei stained for caspase 3 in the IgG1, bevacizumab, and staurosporine groups, respectively. In the in vivo experiment, there was no staining for caspase 3 in the tumors in both the PBS control and bevacizumab-treated groups.

**Effect of Bevacizumab on Angiogenesis In Vivo**

In our mouse model, the intraocular melanomas in mice treated with 50 or 250 μg IP bevacizumab showed a decreased CD31-vascular density compared with those in the control animals (12%, 1%, and 26%, respectively; Fig. 2C).

**Effect of Bevacizumab on Micrometastases**

The in vitro invasion assay showed significantly less invasion of both Mel 290 cells and B16L59 cells treated with bevacizumab, with 100 μg/mL bevacizumab being more effective than 10 μg/mL. Invasion of Mel290 cells decreased by 56% and 91% for the OD units after treatment with 10 and 100 μg/mL bevacizumab (0.1063 ± 0.0142 and 0.0877 ± 0.0053, respectively), compared with IgG treatment (0.2399 ± 0.0184, P < 0.001). In B16L59 cells compared with the IgG control (0.3604 ± 0.0198), 10 and 100 μg/mL bevacizumab reduced invasion by 15% and 27% (0.3064 ± 0.0166, 0.2626 ± 0.0112, P < 0.01), respectively. The human uveal melanoma cell line Mel290 was much more sensitive to bevacizumab than was the mouse melanoma cell line B16L59. The number of hepatic
micrometastases in the control group and the 50-μg and 250-μg bevacizumab groups were 194 ± 86, 108 ± 27, and 63 ± 23, respectively. There were significantly fewer micro-
metastases in our mouse ocular melanoma model after treatment with either 50 or 250 μg bevacizumab, (P = 0.03 and P = 0.01, respectively), compared with the number in the control.

**FIGURE 3.** Bevacizumab inhibited intraocular melanoma growth. Groups with IP injections of (A) 250 or (B) 50 μg bevacizumab starting at day 4 after inoculation had smaller intraocular melanomas than did the PBS-treated control group (C). The 50- and 250-μg IP bevacizumab groups, both starting on day 4 after inoculation, exhibited decreased intraocular tumor size compared with that in the PBS-treated control (D). The size of the intraocular melanoma was significantly smaller in the day-1 and -4 groups compared with the control, and the tumors in the day-1 groups were significantly smaller than those in the day-4 groups (E). Each group contained 10 mice.

**FIGURE 4.** Bevacizumab decreased Ki67 in human uveal melanoma and mouse melanoma cells. Melanoma cells grown in vitro and treated with 100 μg/mL bevacizumab for 48 hours showed inhibition of nuclear Ki67. (A) Top: IgG1-treated Mel290 cells showed 53% of nuclei stained. Bottom: bevacizumab-treated Mel 290 cells showed no stained nuclei. (B) Top: PBS-treated B16L89 tumor showed 98% of nuclei stained. Bottom: bevacizumab-treated B16L89 tumor showed 11% of nuclei stained.
group. There was a significant decrease in the number of micrometastases in the 250-μg/mL– compared with the 50-μg/ 

mL–treated groups (P = 0.002). In the longitudinal experiment, treatment with 250 μg bevacizumab resulted in control of the number of micrometastases at all intervals (1, 2, 3, 4, and 6 weeks) after inoculation. The treatment effect continued longitudinally, with significantly fewer micrometastases in mice treated with 250 μg/mL bevacizumab than in the control animals at 1, 2, 3, 4, and 6 weeks after inoculation.

DISCUSSION

VEGF is overexpressed in many human neoplasms, including uveal melanoma.6,31,32 This expression is associated with tumor size, necrosis and intraocular angiogenesis.6,31,32 New blood vessels that grow within the tumor secondary to VEGF expression are structurally and functionally irregular, as they exhibit dead ends, disordered blood flow, and increased permeability. These irregularities in blood flow lead to further tumor hypoxia and subsequent increases in VEGF production.33 Our study confirms previous studies showing that uveal melanoma cells overexpress VEGF mRNA.3,4

Angiogenesis is a critical process in tumor growth and development. VEGF is a specific stimulator of vascular endothelial cell proliferation and tumor angiogenesis. VEGF is produced in response to various cellular and environmental stimuli. We found that human uveal melanoma cells and human vascular endothelial cells spontaneously secrete VEGF when placed in culture. When melanoma cells were cocultured with vascular endothelium, VEGF production significantly increased 5- to 10-fold. The results of our in vitro angiogenesis assay showed that the total length of tubes formed from uveal melanoma cells co-cultured with vascular endothelium was two times longer than when using vascular endothelium alone. High serum levels of VEGF may be a marker in metastatic disease.34,35 Melanoma cell lines exhibit variable sensitivities to anti-VEGF antibodies (i.e., bevacizumab), with Mel 290 being the most sensitive cell line we tested.

VEGF exhibits trophic effects on the growth and progression of neoplasia. Several studies have shown a correlation between increased VEGF expression and tumor growth.19,20,36,37 Recent studies have indicated that bevacizumab treatment results in a 25% to 95% dose-dependent inhibition of tumor growth in mouse and rat models.19,38 In our study, bevacizumab gave a dose-dependent reduction of the size of the murine intraocular melanoma. Our study also showed that this was not due to a primary effect on melanoma cells, supporting the results of a prior study.39 Our results showed that bevacizumab caused decreased proliferation of vascular endothelium in vivo. We found that bevacizumab had no effect on inducing apoptosis in the melanoma cells and that the reduction in tumor size was probably due to reduced intratumoral endothelial cell proliferation and angiogenesis, leading to a shrinking effect on tumor volume. The in vivo timing experiment in this study identified that the early use of bevacizumab did not cause apoptosis in melanoma cells. (A) Treatment of Mel 290 cells with IgG1 (top, 6% nuclear staining) or bevacizumab (middle, 4% nuclear staining) did not result in increased expression of caspase 3, compared with staurosporine-treated positive control cells (bottom, 74% nuclear staining). (B) Similarly, intraocular melanoma in the PBS control (top) and the bevacizumab-treated mice (bottom) both showed a lack of expression of caspase 3, with no nuclear staining detected in either group.

FIGURE 5.
Bevacizumab Suppresses Experimental Ocular Melanoma

In summary, our experimental findings show that VEGF inhibition causes the reduction of ocular melanoma proliferation, angiogenesis, and invasion, resulting in suppression of establishment of micrometastases. Anti-VEGF therapy is a potential strategy for the treatment of metastatic uveal melanoma.

References


