Angiostatin Produced by Certain Primary Uveal Melanoma Cell Lines Impedes the Development of Liver Metastases

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Objectives: To evaluate the ability of human uveal melanomas to produce angiostatin in vitro and the effect of angiostatin on the development of liver metastases in vivo.

Methods: Human uveal melanoma cell lines (OCM1, OCM3, MEL202, MEL285, 92-1, OM431, and OMM1) were assayed for their ability to produce angiostatin in vitro by an angiostatin bioassay and by Western blot analysis. The OCM3 and OMM1 tumor cells were inoculated either in the posterior or the anterior segment of nude mice. One group of mice in each experiment underwent enucleation and hepatic metastases were assayed by histopathologic and liver function analysis.

Results: OCM1, OCM3, and 92-1 cell lines significantly inhibited bovine endothelial cell proliferation in vitro and generated 38-Kd angiostatin molecules. Enucleation of eyes containing OCM3 in the posterior segment resulted in a higher number of metastatic foci (26.5) in that group compared with the nonenucleated group of mice (11.17). After enucleation, elevated levels of serum aspartate transaminase and alanine aminotransferase were observed in mice bearing OCM3 in either anterior or posterior segments. The enucleation of eyes containing OMM1 (nonangiostatin–producing cells) had no significant effect on liver metastasis.

Conclusion: By removing a source of angiostatin, enucleation of melanoma-containing eyes may unwittingly exacerbate the metastatic potential of uveal melanomas.

Clinical Relevance: In certain circumstances, enucleation of melanoma-containing eyes may unwittingly exacerbate metastatic disease. The results also suggest that exogenous angiostatin may have potential therapeutic implications in the management of patients with primary intraocular melanomas.


VEAL MELANOMAS, including melanomas of the choroid and ciliary body, are the most common types of primary intraocular malignancy in adults. Uveal melanomas are successfully treated with enucleation, radiotherapy, transpupillary thermotherapy, laser photocoagulation, intravenous chemotherapy, immunotherapy, local tumor resection, or a combination of the above-mentioned treatments. There is a range in mortality rate depending on the size and location of the tumor. The mortality rates ranged between 29% and 58% depending on the tumor location. Additionally, mortality rates pertaining to the various sizes of tumors examined ranged from 15% to 66%. The liver is the most frequently affected organ in metastatic disease.

Encleulation of the tumor-bearing eye has been a standard treatment for many years, especially for larger tumors. In 1978, Zimmerman et al. compared the survival data of patients with uveal melanomas who had undergone enucleation vs observation. After a computational analysis of the survival data, they hypothesized that enucleation may promote dissemination of uveal melanoma cells and increase mortality. In a C57BL6 murine model, Niederkorn demonstrated that enucleation of the melanoma-containing eye coupled with immunologic impairment of the host by gamma irradiation resulted in a significant increase in the incidence and severity of pulmonary metastases in mice who underwent enucleation compared with control mice. In a feline model, it was demonstrated that enucleation of virally induced melanoma-containing eyes led to increased frequency and severity of new primary tumors, ie, fibrosarcomas, at extraocular sites. The “enucleation dilemma” was not without controversy. Siegel et al. reanalyzed the survival data...
MATERIALS AND METHODS

MELANOMA CELL LINES

Seven human uveal melanoma cell lines, designated OCM1, OCM3, MEL202, MEL285, 92-1, OM431, and OMM1, were used. OCM1 and OCM3 were provided by June Kahn-Mitchell, MD. MEL202 and MEL285 were provided by Bruce Ksander, PhD. 92-1 was provided by Martine J. Jager, MD. OM431 was provided by Daniel M. Albert, MD. OMM1, a subcutaneous metastasis originating from a uveal melanoma, was provided by Gregory P. Luyten, MD. OCM1, OCM3, and OM431 were cultured in Ham’s F-2 medium containing 10% heat inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% Heps buffer, and 1% antibiotic-antimycotic solution. MEL 202, MEL 285, and 92-1 were cultured in RPMI medium containing the same supplements. OMM1 cells were cultured in Dulbecco’s Modified Eagle Medium containing the same supplements described above.

ANGIOSTATIN BIOASSAY

A bioassay for measuring angiostatin was performed as described previously.14 Bovine capillary endothelial (EJG) cells were obtained from American Type Culture Collection (Rockville, Md) and were cultured in Dulbecco’s Modified Eagle Medium with 10% heat inactivated fetal calf serum. Bovine capillary endothelial cells were plated into gelatin-coated 24-well plates and incubated at 37°C (1 × 10^6 cells/well). The following day, 300 mL of melanoma cell supernatant (3 × 10^6 cells/3 mL) generated over 72 hours with or without 100 mg/mL of human plasminogen was added to each well. The cells were pulsed with 100 μCi of [3H]-thymidine and incubated for 24 hours. Incubation period allowed the EJG cells to incorporate [3H]-thymidine at amounts commensurate with their level of proliferation. The wells were washed with phosphate-buffered saline (PBS) and the contents were then solubilized with 10% sodium dodecyl sulfate and the radioactivity counted in a liquid scintillation counter. All tests were performed in triplicate.

Angiostatin is produced by proteolytic degradation of human plasminogen and is a potent inhibitor of capillary endothelial cell proliferation. Endothelial cells were incubated with and without plasminogen and assayed for proliferation. Bovine capillary endothelial cells were similarly incubated (in the absence of melanoma supernatants) as a control. Endothelial cell proliferation was compared with the control EJG cells with and without plasminogen. This facilitated a computational analysis of each experimental EJG cell proliferation as a percentage of the control EJG cell proliferation with and without plasminogen. The data are presented as % angiostatin activity = (% of control EJG cell proliferation in the presence of plasminogen) – (% of control EJG cell proliferation in the absence of plasminogen). The assays were repeated in 2 to 3 experiments. The data are presented as the average of these experiments.

WESTERN BLOT ANALYSIS

Melanoma supernatants were generated as described above. 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. Ten milligrams of proteins were loaded in each lane. Purified human angiotatin (provided by Gerald A. Soff, MD) was used as the positive control. The protein bands were then electrochemically transferred to a polyvinylidene membrane at 4°C and 0.2 amperes for 1 hour. The transfer buffer contained 25mM Tris, 192mM glycine, and 20% methanol.

RESULTS

All 7 human uveal melanoma cell lines were tested for their ability to produce angiostatin in the bioassay. As presented in the Table, only OCM1, 92-1, and OCM3 demonstrated significant angiostatin activity in vitro. The ability of these tumor cells to produce angiostatin was also confirmed by Western blot analysis. Incubation of human uveal melanoma cell lines was used to confirm the presence of angiostatin in vivo. Manschet et al11 recommended using a “no-touch” technique during early enucleation of the tumor-bearing eye coupled with preoperative radiation to prevent incidental dissemination of tumor cells during surgery.

With the enucleation debate as a backdrop, angiogenesis was discovered to play a key role in many physiologic processes. Angiogenesis is defined as the formation of new capillaries from existing blood vessels. Angiogenesis plays a critical role in reproduction, wound healing, bone repair, rheumatoid arthritis, ischemic heart disease, peripheral vascular disease, diabetic retinopathy, and tumor growth, proliferation, and metastasis.12,13 Numerous studies have demonstrated that highly vascularized tumors have a greater propensity for metastasis compared with their less vascularized counterparts.12 In 1994, the discovery of a novel antiangiogenic factor, angiostatin, significantly advanced the understanding of tumor biology.14 Angiostatin is a 38-Kd protein with more than 98% homology to an internal fragment of plasminogen.14 It is a specific inhibitor of endothelial cell proliferation and is isolated from serum generated only in the presence of the primary tumor. Tumor-induced cytokines and elastases promote the degradation of plasminogen to angiostatin, which is present in the circulation up to 5 days after removal of the primary tumor.14,15 Angiostatin produced in the presence of the primary tumor inhibited the development and proliferation of metastases.14 In addition, removal of the primary tumor or antibody-mediated depletion of angiostatin led to loss of the protective effect.14 Recent data indicate that human uveal melanomas produce antiangiogenic factors and that angiostatin treatment may reduce the number of metastases in a murine model.16,17 The present study assessed the ability of human uveal melanomas to produce angiostatin. In a murine model, we also analyzed the effect of enucleation of the melanoma-bearing eye on the development of hepatic metastases.
methanol. The membrane was then blocked for 1 hour with 5% casein in PBS and washed twice with fresh changes of PBS. The membrane was then incubated 1 hour in 1 mg/mL of monoclonal mouse anti-human kringle 1 to 3 fragment of plasminogen (VAP 230 L, Enzyme Research Laboratory, South Bend, Ind). The membrane was washed twice with PBS and incubated with 1:1000 dilution of horseradish peroxidase-conjugated goat antimouse immunoglobulin G. The membrane was developed using an electrochemiluminescence Western blotting kit. The membrane was placed in a film cassette and exposed to scientific imaging film. The film was then developed and the protein bands analyzed.

INTRACAMERAL TUMOR INOCULATION AND METASTASES

Intracameral tumor inoculation was performed as described previously. Briefly, nude mice were deeply anesthetized with 0.66 mg/kg of ketamine hydrochloride administered intramuscularly. Tumor cells (10⁶ cells/5 mL) were inoculated into the anterior chamber using a 0.1-ml Hamilton syringe (Hamilton Co, Reno, Nev) fitted with a 33-gauge glass needle. One group of mice underwent enucleation when the tumor occupied 75% of the anterior segment of the eye. Mice were euthanized on day 60 after enucleation. A second group of mice did not undergo enucleation but was euthanized on day 60 after the tumor volume occupied 75% of the anterior segment of the eye. A third group of mice was untreated and served as the control group.

POSTERIOR SEGMENT (PS) INOCULATION OF TUMOR CELLS

Posterior segment tumor inoculation was performed as described previously. Briefly, nude mice were deeply anesthetized as described. Using a 33-gauge glass needle, a tunnel was prepared from the cornea at the limbus, along the sclera and ciliary body, to the choroid, using a dissection microscope. Melanoma cells (10⁵ cells/2.5 µL) were injected into the PS. One group of mice was enucleated at day 26 while the second group of mice was observed. All mice were examined at day 74 following PS injection and euthanization.

ASSESSMENT OF HEPATIC METASTASES

Metastatic foci in the liver were analyzed by histopathologic examination. The livers were scored by microscopy as the mean number of metastases per 10 low-power fields screened in a blinded fashion by 3 independent observers.

ASPARTATE AMINOTRANSFERASE (AST) AND ALANINE AMINOTRANSFERASE (ALT) LEVELS

It has been determined that metastatic disease from human uveal melanomas can be effectively monitored by liver function assays. Metastatic tumor burden was also assessed by measuring serum AST and ALT levels in tumor-bearing mice and control mice on the day of necropsy. Serum AST and ALT levels were assessed on the Olympus AU400 Chemistry Analyzer (Olympus, Melvin, NY) and were represented as units per liter.

STATISTICAL ANALYSIS

For in vitro and in vivo (metastatic foci) data, statistical significance was calculated by a paired t test. P<.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Percentage of Angiostatin Activity*</th>
<th>Melanoma Cell Lines</th>
<th>Average Activity</th>
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</thead>
<tbody>
<tr>
<td>OCM1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>OCM3</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>92-1</td>
<td>33.7</td>
<td></td>
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<tr>
<td>MEL202</td>
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<td>MEL285</td>
<td>0</td>
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<tr>
<td>OCM3</td>
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<tr>
<td>OMM1</td>
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*Percentage of angiostatin activity = (% of the control EJ6 [bovine capillary endothelial cell proliferation in the absence of plasminogen] – (% of the control EJ6 cell proliferation in the presence of plasminogen). The data presented are representative of 2 to 3 experiments.

Figure 1. Production of angiostatin by melanoma cell supernatants. Melanoma cell supernatants, with or without plasminogen, were analyzed by Western blot. Angiostatin is generated in supernatants in the presence of plasminogen. MW indicates molecular weight marker; angiostatin, purified human angiostatin.

requirement of plasminogen as a precursor for angiostatin production was clearly demonstrated by the absence of angiostatin in tumor cell supernatants generated without any added plasminogen.

We then studied the effect of angiostatin production by the primary tumor on the development and proliferation of metastases. We used a murine metastasis model and OCM3 tumor cells to perform our analysis. OCM3 cells were selected because of their ability to produce a significant amount of angiostatin in vitro. Mice were injected in the PS with 2 × 10⁵ OCM3 cells on day 0. One group of mice (n=6) underwent enucleation at day 26 while the second group of mice (n=6) was observed. All mice were euthanized on day 74 following PS injection and tumor burden.
assessed by liver histopathologic analysis and serum AST and ALT levels. The enucleated group of mice had a significantly higher number of metastatic foci (26.50±6.64) compared with the nonenucleated tumor-bearing mice (11.17±3.77) (P=.04) (Figure 2). The enucleated group also had significantly elevated serum AST (P=.02) and ALT (P=.02) levels compared with the nonenucleated group (Figure 3). These data suggest that angiostatin produced by the primary uveal melanoma, ie, OCM3, inhibits the development of distant hepatic metastases and that enucleation may promote metastatic disease by removing the source of angiostatin production.

Melanomas occurring in the anterior segment of the eye are generally less malignant than PS melanomas. Therefore, experiments were performed to assess the effect of enucleation on the incidence of hepatic metastases arising from OCM3 implanted into the anterior segment of the eyes. To test this, 1×10⁶ OCM3 cells were injected intracameral into the anterior segment of nude mice. One group of mice (n=8) underwent enucleation when the tumor volume occupied 75% of the anterior chamber of the eye. The second group of mice (n=7) maintained their tumor-bearing eye, and the third group of mice served as controls (n=4). All mice were euthanized on day 60 and serum AST and ALT levels were assayed. As shown in Figure 4, the enucleated group of mice had significantly elevated serum AST (P=.05) and ALT (P=.04) levels compared with the nonenucleated and control groups.

The data thus far suggested that angiostatin produced by a primary intraocular melanoma such as OCM3 extended significant protection against the development of liver metastases. To test this hypothesis, we assessed the ability of a human uveal melanoma (OMM1), which does not produce angiostatin in the bioassay, to protect against the development of distant metastases. OMM1 cells (2×10⁶) were injected in the PS of nude mice. One group of mice (n=8) underwent enucleation on day 26 while the second group (n=8) was allowed to maintain the primary tumor. All mice were euthanized on day 74 and liver metastases were assessed histologically. There was no significant difference in the mean number of metastatic foci between the enucleated and nonenucleated groups of mice. The mean±SD number of liver foci in the enucleated group was 21.6±31.57 and in the nonenucleated group was 6.17±2.06 (P=.20).

Angiogenesis is a pleiotropic physiologic process that plays an essential role in tumor proliferation and metastasis. Multiple angiogenic and antiangiogenic stimuli have been identified as endogenous regulators of angiogenesis. Angiostatin is a 38-kd protein that is a degradation product of plasminogen and is produced in the presence of a primary tumor. Tumor-derived cytokines stimulate the release of metalloelastases from tumor-infiltrating macrophages. Macrophage-derived metalloelastases in turn promote the breakdown of serum plasminogen to angiostatin. Angiostatin produced in the presence of the primary tumor inhibits the growth and development of metastases. The ³H-thymidine bioassay is an in vitro assay of angiostatin production by certain tumors. Angiostatin is produced by proteolytic degradation of human plasminogen and is a potent inhibitor of capillary endothelial proliferation, a cleavage product of plasminogen.

We have previously shown that certain human uveal melanomas can produce antiangiogenic factors in vitro. A recent report has also demonstrated preliminary evidence that exogenous administration of angiostatin to animals bearing primary intraocular melanomas reduces the subsequent metastatic burden.

In this study, we have shown that 3 of the 9 human uveal melanoma cell lines studied (OCM1, OCM3, and 92-1) produce angiostatin in vitro. Using a murine model, we have also shown that removal of the OCM3 tumor-bearing eye (ie, the source of angiostatin) results in a significant increase in the incidence and severity of hepatic metastases as assayed by histopathologic and liver function analysis. The results were comparable after anterior or PS injection.
of the tumor. OMM1 is a human uveal melanoma cell line that does not produce angiostatin in vitro. There was no statistical difference in the incidence of hepatic metastases of the enucleated vs nonenucleated mice following PS injection of OMM1 tumor cells. It should be recognized that the OMM1 results do show a trend favoring higher metastases in the nonenucleated group of mice compared with the enucleated group. This result is consistent with our finding that OMM1 does not produce angiostatin and that enucleation may be an appropriate therapy for melanomas that do not produce angiostatin. The lack of a statistical difference between the 2 OMM1 groups may be related to the relatively small sample size. The exact mechanism by which angiostatin may impede the formation and propagation of metastases remains uncertain. It may affect the formation of the initial metastatic microfocus or may initiate a state of tumor dormancy in the liver. As a corollary to this hypothesis, enucleation of an angiostatin-producing uveal melanoma may perturb this delicate balance and facilitate the development of metastases. These results suggest that angiostatin produced by the primary intraocular tumor impedes the development of liver metastases. Our findings complement previous results that certain uveal melanoma cell lines produce angiostatin in vitro and that exogenous angiostatin reduces the metastatic burden in the B16L5S9 murine tumor model.17-24,25

This study has potential implications for future treatment protocols involving human uveal melanomas. The ability of the primary intraocular melanoma to produce angiostatin may be assayed by monitoring serum angiostatin levels. It may then be possible to reduce the metastatic burden and facilitate long-term survival by complementing current treatment strategies with exogenous administration of angiostatin. Further experiments need to be initiated to study the safety and efficacy of such therapeutic regimens.

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