Vascular Endothelial Growth Factor A in Eyes With Uveal Melanoma

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Objectives: To determine the presence of vascular endothelial growth factor A (VEGF-A) in the aqueous humor of eyes with uveal melanoma and to identify its source.

Methods: The VEGF-A concentrations were determined in aqueous humor samples obtained after enucleation from 74 eyes with untreated uveal melanoma and from 8 eyes with treated uveal melanoma. Patient survival and clinical and histopathological tumor variables were compared. In situ hybridization, Western blot analysis, and enzyme-linked immunosorbent assay were used to determine expression of VEGF-A in tumor tissue and in overlying retina.

Results: Aqueous VEGF-A concentrations ranged from 18 to 826 pg/mL in 74 untreated eyes, while concentrations in 30 control eyes were significantly lower (median, 50.1 pg/mL) ($P < .001$). Concentrations in 8 treated eyes were much higher (median, 364 pg/mL). In situ hybridization on tissue sections and Western blot analysis and enzyme-linked immunosorbent assay on tissue extracts revealed VEGF-A in uveal melanoma tissue and in retinal tissue.

Conclusions: Uveal melanoma is associated with increased concentrations of VEGF-A in aqueous humor. Aqueous VEGF-A concentration correlates with largest basal tumor diameter and with the tumor height. In eyes with uveal melanoma, tumor and retinal tissues are sources of VEGF-A.

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Uveal melanoma is the most common primary intraocular neoplasm in adults, with an incidence in white populations of 0.8 cases per 100,000. Once metastases have formed, the prognosis for patients with uveal melanoma is poor: only 15% of patients survive the first year after diagnosis of distant metastases, with a median patient survival of 6 months. Therefore, the development of new treatment strategies for metastatic uveal melanoma is urgently needed.

Uveal melanoma disseminates hematogenously, as there is no major lymphatic drainage from the eye. Because growth of solid tumors and development of metastases are dependent on the formation of new blood vessels, antiangiogenesis treatment may help to prevent spread and subsequent growth of metastases. One of the major groups of cytokines influencing adult angiogenesis is the vascular endothelial growth factor (VEGF) family. Type A VEGF is secreted by various types of tumor cells and can cause increased vascular permeability, endothelial cell growth, angiogenesis, and monocyte activation and chemotaxis. Eyes with uveal melanoma may reveal aqueous flare, which is considered a sign of increased vascular permeability.

Expression of the VEGF-A gene and protein occurs in healthy ocular tissues, especially the retina, and has been shown to be up-regulated during neovascularization responses associated with proliferative retinopathies. Concentrations of VEGF-A have been found to be increased in the aqueous fluid of eyes that have formed intraocular neovascularization because of ischemic retinal diseases such as diabetic retinopathy, retinal vein occlusion, neovascular glaucoma, and radiation retinopathy, as well as in eyes with hemangiomas.

In a previous study, it was demonstrated that cell lines from uveal melanoma secrete several angiogenic factors, including VEGF-A. In addition, Boyd et al showed increased VEGF concentrations in aqueous fluid and vitreous of eyes with uveal melanoma.

To further understand the role of the VEGF family and angiogenesis in uveal melanoma, we determined VEGF-A concentrations in the aqueous humor of eyes with uveal melanoma. To identify its production source, the presence of VEGF-A was examined using 3 different techniques in uveal melanoma tissue and in the overlying retina.
PATIENTS, CLINICAL FINDINGS, AND AQUEOUS HUMOR SAMPLES

Aqueous humor samples from 82 consecutive eyes enucleated for uveal melanoma were tested for VEGF-A. The total group was divided into a group of 74 untreated eyes and a group of 8 eyes that had received local treatment before enucleation (1 eye had been treated with transpupillary thermotherapy, 5 eyes with ruthenium radiation [1 of which also underwent transpupillary thermotherapy twice], and 2 eyes with proton beam irradiation). Data regarding radiation retinopathy were collected from the patients’ medical records. Patients with diabetes mellitus were excluded from the study, as diabetes influences VEGF-A concentrations in aqueous humor.5–12,17

In the untreated group, the mean patient age at the time of enucleation was 63.8 years (age range, 28-87 years). Thirty-seven patients were male, and 37 were female. Aqueous humor samples were collected within 10 minutes after enucleation and were frozen at −80°C. To investigate whether enucleation in itself affected VEGF-A aqueous humor concentrations, aqueous humor samples of 3 patients were collected before and after enucleation.

The mean follow-up time was 64.2 months (range, 1-136 months). The 74 tumors were classified histopathologically according to cell type, tumor location, integrity of Bruch membrane, and pigmentation grade (Table 1). The largest basal tumor diameter and the tumor height were measured in millimeters, and the number of mitoses was counted in 15 high-power fields with a magnification of ×320. The mean ± SD largest basal tumor diameter was 11.1 ± 3.2 mm, and the mean ± SD tumor height was 5.6 ± 2.8 mm.

As control specimens, we used 30 samples of aqueous humor obtained from patients at the time of cataract surgery. Patients with diabetes mellitus or with other retinopathies were excluded from the control group. In 22 other patients with retinal detachment, aqueous humor samples were obtained at the time of surgery. The study was institutional review board–approved and adhered to the tenets of the Declaration of Helsinki.

IN SITU HYBRIDIZATION

In situ hybridization was performed as described previously on paraffin-embedded sections of 10 uveal melanomas. In brief, VEGF-A complementary DNA was cloned into pGEM3 plasmid (pGEM-3Zf[+]; Vector; Promega, Madison, Wis). The copy RNA probes were labeled with digoxigenin according to the manufacturer’s protocol (Boehringer-Mannheim Biochemica, Mannheim, Germany). The VEGF-A probes used were 5′-GCTCCGAAACATGACTGTTT-3′ (sense) and 5′-CCGATAATCTGATGTGAT-3′ (antisense). After pre-treatment, the tumor sections were hybridized with 10 ng of VEGF-A antisense riboprobe per slide overnight at 62°C. Subsequently, sections were washed and finally treated with 2 U/mL of ribonuclease T1 (Roche Products, Basel, Switzerland) in 2× SSC plus 1-mmol EDTA. Immunodetection of digoxigenin-labeled hybrids was performed using nitroblue tetrazolium as a chromogen and bicholylindolyl phosphate (Roche Products) as a coupling agent. Adjacent tumor slides, hybridized with VEGF-A sense riboprobes, were included as negative controls and did not show staining.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The VEGF-A concentrations in aqueous humor, tumor and retinal tissue homogenates, and cell line supernatants were measured using an enzyme-linked immunosorbent assay kit (BioSource International, Camarillo, Calif). The lowest measurable concentration was 4 pg/mL, with an intra-assay coefficient of variation of 4.7% and an interassay coefficient of variation of 8.1%. To analyze samples in duplicate and to simultaneously preserve the sample, ocular fluids were diluted at 1:2.

TUMOR AND RETINAL TISSUE HOMOGENATES

Tumor and retinal tissues were collected from 10 patients. Retinal tissue was obtained from areas adjacent to and overlying the tumor. Tissue was snap-frozen in liquid nitrogen within 10 minutes after enucleation. Thirty micrograms of tissue was lysed in 200 µL of radioimmunoprecipitation assay buffer (Roche Products). The sample was homogenized, and protease and phosphatase inhibitor was added. Insoluble fragments were removed by centrifugation at 13 000 rpm for 10 minutes, and the supernatant lysate was immediately frozen at −80°C.

WESTERN BLOT ANALYSIS

To determine the presence of VEGF-A in tumor and retinal tissues, Western blot analysis was performed on uveal melanoma tissue and on retinal tissue obtained from 10 patients. Tissue lysates (30 µg per lane) were separated by 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis. Proteins were immunodetected using the appropriate anti–VEGF-A monoclonal antibody (R&D Systems, Minneapolis, Minn). Protein bands were visualized using the SuperSignal chemiluminescent substrate kit (Pierce Chemical, Rockford, Ill). Semiquantitative estimates of relative protein levels were made using computerized densitometry (Scion Image for Windows; Scion Corporation, Frederick, Md).

STATISTICAL ANALYSIS

The distribution of aqueous humor VEGF-A samples was skewed. Therefore, in all statistical analyses, the natural logarithm of the VEGF-A concentration (indicated as LN VEGF-A)
was used to obtain the natural distribution that is needed for standard statistical tests. The Pearson product moment correlation was used to assess the correlation between LN VEGF-A and the largest basal tumor diameter and the tumor height. A melanoma-specific patient survival analysis was performed using the Kaplan-Meier method and the log-rank test. Cox proportional hazards regression analysis was used to establish a predictive model for patient survival. All statistical analyses were performed using the SPSS version 11.0 statistical software package (SPSS Inc, Chicago, Ill).

RESULTS

Our results demonstrated increased aqueous VEGF-A in eyes with uveal melanoma. Aqueous concentration of VEGF-A correlates with patient survival, largest basal tumor diameter, tumor height, and ciliary body involvement. Our results further showed that uveal melanoma cells and overlying retina produce VEGF-A.

Boyd\textsuperscript{19} described increased concentrations of VEGF-A in the aqueous humor of eyes with uveal melanoma but, based on varied immunohistochemical test results, concluded that the source of VEGF-A is not fully understood. Because VEGF-A is a leakage factor and a stimulant for new vessel formation, we hypothesize that larger tumors, characterized by increased vessel densities and vessel leakage, might produce higher concentrations of VEGF-A. Therefore, we set out to test VEGF-A concentrations in aqueous humor in a series of uveal melanomas.

VEGF-A IN AQUEOUS HUMOR

To test whether aqueous humor samples obtained after enucleation were representative of the in vivo setting, VEGF-A concentrations in 3 patients were determined in aqueous humor samples taken before and after enucleation. These concentrations differed by less than 5\%.
CLINICAL AND HISTOPATHOLOGICAL VARIABLES

Of 74 patients with uveal melanoma, 20 died of uveal melanoma during follow-up, 11 died of nonrelated causes, and 43 are still alive. We determined the VEGF-A concentrations in aqueous humor samples obtained from 30 control eyes at the time of cataract surgery. The median concentration was 50.1 pg/mL (range, 4.0-149 pg/mL).

Compared with the controls, in 74 eyes with uveal melanoma, the median VEGF-A concentration was significantly higher at 146.5 pg/mL (range, 18-826 pg/mL) (P < .001) (Figure 1A). None of the 74 eyes showed retinal neovascularization at the time of enucleation. The median VEGF-A concentration in the aqueous humor of 8 additional eyes with uveal melanoma that had undergone treatment in the past was 364 pg/mL (range, 79-3000 pg/mL). Three of these eyes (1 eye that had twice received transpupillary thermotherapy and once received ruthenium therapy, and 2 eyes that had been treated with proton beam irradiation) had developed radiation retinopathy and showed high VEGF-A concentrations (555, 1877.5, and 3000 pg/mL, respectively) (Table 2).

To assess why some eyes with previously untreated uveal melanomas produced more VEGF-A than others, we looked for associations between aqueous VEGF-A concentrations and clinical and histopathological variables. To perform statistical tests, the natural logarithms of VEGF-A values were used to obtain a normal distribution.

Using Pearson product moment correlation, significant positive correlations were found between LN VEGF-A and the largest basal tumor diameter (P < .001) (Figure 1B) and the tumor height (P = .001) (Figure 1C). The LN VEGF-A was compared with other histopathological variables as given in Table 1. There were no correlations between LN VEGF-A and the presence of necrosis (P = .90), breakdown of Bruch membrane (P = .40), number of mitoses (P = .87), pigmentation grade (P = .21), or cell type (P = .81). The mean LN VEGF-A of 4.7 in 51 choroidal tumors was significantly lower than the mean LN VEGF-A of 5.4 in 23 tumors with ciliary body involvement (P = .02).

Patient survival was studied in relation to LN VEGF-A. The patients were divided into 2 groups having values above or below the median LN VEGF-A of 4.93. Statistically significant lower patient survival was found in the group with higher LN VEGF-A compared with the group with lower LN VEGF-A (P < .05, log-rank test) (Figure 1D). Cox proportional hazards regression analysis showed a significant difference in patient survival (P = .04), with a β level of 0.78 and an SE of 0.38. This indicates that doubling of VEGF-A concentration is associated with an increased risk of dying of uveal melanoma of 2.174-20.78.

In univariate Cox proportional hazards regression analysis of melanoma-specific mortality, largest basal tumor diameter, LN VEGF-A, and cell type had the strongest relationships with patient survival (χ² = 5.3, χ² = 4.6, and χ² = 4.1, respectively) (Table 3). Multivariate analysis showed no significance for LN VEGF-A. Largest basal tumor diameter and cell type reached significance in a multivariate model.

VEGF-A PRODUCTION BY TUMOR CELLS BASED ON RESULTS OF IN SITU HYBRIDIZATION

Because an association was observed between tumor size and VEGF-A concentration in aqueous humor, it would be logical to assume that VEGF-A is produced by the tumor cells. Although it has been shown that uveal melanoma cell lines produce VEGF-A (16) (and I.C.N., unpublished data, 2005), results of immunohistochemical staining of uveal melanoma tissues have been contradictory. Therefore, we performed in situ hybridization of VEGF-A in 10 eyes with uveal melanoma. Intense expression of VEGF-A messenger RNA (mRNA) was found in the retinal pigment epithelium and in the inner nuclear layer, in ganglion cells, and in external limiting membrane. The staining was more

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Table 2. Eight Treated Eyes With Uveal Melanoma

<table>
<thead>
<tr>
<th>Eye No.</th>
<th>Therapy</th>
<th>VEGF-A Concentration, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ruthenium plaque</td>
<td>78.9</td>
</tr>
<tr>
<td>2</td>
<td>Ruthenium plaque</td>
<td>86.2</td>
</tr>
<tr>
<td>3</td>
<td>Ruthenium plaque</td>
<td>97.2</td>
</tr>
<tr>
<td>4</td>
<td>TTT</td>
<td>173.2</td>
</tr>
<tr>
<td>5</td>
<td>Ruthenium plaque</td>
<td>555.0</td>
</tr>
<tr>
<td>6</td>
<td>Proton beam irradiation</td>
<td>1058</td>
</tr>
<tr>
<td>7</td>
<td>TTT twice and ruthenium plaque</td>
<td>1877.5</td>
</tr>
<tr>
<td>8</td>
<td>Proton beam irradiation</td>
<td>3000</td>
</tr>
</tbody>
</table>

Abbreviations: VEGF-A, vascular endothelial growth factor A; TTT, transpupillary thermotherapy.

Table 3. Univariate Cox Proportional Hazards Regression Analysis of Melanoma-Specific Mortality

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient (SE)</th>
<th>Patient Survival Likelihood Ratio</th>
<th>P Value</th>
<th>Hazard Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largest basal tumor diameter</td>
<td>0.17 (0.08)</td>
<td>5.3</td>
<td>.02</td>
<td>1.19 (1.02-1.38)</td>
</tr>
<tr>
<td>LN VEGF-A</td>
<td>0.78 (0.38)</td>
<td>4.6</td>
<td>.04</td>
<td>2.17 (1.02-4.62)</td>
</tr>
<tr>
<td>Cell type</td>
<td>1.09 (0.58)</td>
<td>4.1</td>
<td>.06</td>
<td>2.96 (0.96-9.13)</td>
</tr>
<tr>
<td>Tumor height</td>
<td>0.19 (0.10)</td>
<td>4.0</td>
<td>.05</td>
<td>1.21 (1.00-1.08)</td>
</tr>
<tr>
<td>Age</td>
<td>0.04 (0.02)</td>
<td>4.0</td>
<td>.06</td>
<td>1.04 (0.99-1.08)</td>
</tr>
</tbody>
</table>

Abbreviation: LN VEGF-A, natural logarithm of the vascular endothelial growth factor A concentration.
intense in detached retina. There was diffuse VEGF-A expression throughout the tumors, especially around blood vessels (Figure 2).

**EXPRESSION OF VEGF-A IN TUMOR AND RETINAL TISSUES**

The in situ hybridization data showed that not only the tumor cells but also the overlying retina might be sources of VEGF-A. Therefore, we extended our study to determine VEGF-A protein levels in uveal melanoma and in adjacent retinal tissue using semiquantitative Western blot analysis and enzyme-linked immunosorbent assay.

Western blot analysis (Figure 3A) performed on retinal and tumor tissues from 10 eyes with uveal melanoma showed that retinal and tumor tissues contained VEGF-A. Relative protein levels showed that retinal tissue contained 1.62-fold more VEGF-A than tumor tissue. Enzyme-linked immunosorbent assay in the 10 samples showed expression of VEGF-A in all tumor and retinal tissues.
retinal tissues (Figure 3B). The mean ± SD levels of VEGF-A were 6.5 ± 1.5 ng/g of tumor tissue and 6.0 ± 1.7 ng/g of retinal tissue.

Because these expression data indicated that the cause of VEGF-A production might be retinal detachment, we determined VEGF-A concentrations in the aqueous humor of 22 eyes with retinal detachment. Aqueous humor samples were obtained during retinal detachment surgery. The median VEGF-A concentration was 43.5 pg/mL (range, 20-427 pg/mL). This is similar to that found in the cataract cases that were used as controls. Therefore, the increased concentrations of VEGF-A in the aqueous humor of eyes with uveal melanoma cannot be explained by concomitant retinal detachment. It is more likely that a combination of ischemia in the tumor and ischemia in the overlying retina causes induction of VEGF-A production in both tissues.

COMMENT

Our study demonstrated increased VEGF-A concentrations in the aqueous humor of eyes with uveal melanoma. In our sample, which was larger than that analyzed by Boyd et al,20 we compared VEGF-A concentrations and clinical and histopathological variables and observed an association between LN VEGF-A and largest basal tumor diameter, the tumor height, and ciliary body involvement. It is known that uveal melanoma cell lines produce VEGF-A in vitro, and our in situ hybridization results demonstrated VEGF-A expression in uveal melanoma.16 Tumor was not the sole source of VEGF-A production, as VEGF-A was also expressed in adjacent and overlying retina. In various types of tumors, including brain,21 breast,22 and colorectal23 tumors, VEGF-A is produced by the tumor. This was previously unsubstantiated for uveal melanoma, as the reported immunohistochemical protein expression of VEGF-A had varied notably among studies. Sheidow et al24 demonstrated expression of VEGF-A in 94% of uveal melanomas, although at low levels (staining was weak in 62% of tumors). Vinores et al25 found VEGF-A expression in only 26% of uveal melanomas. Other immunohistochemical studies20,26 found lower percentages. Our results and those of an in situ hybridization study by Stitt26 et al showed marked expression of VEGF-A mRNA, especially around blood vessels. Boyd et al20 reported the presence of VEGF-A mRNA in 100% of 20 tumors examined using reverse-transcriptase polymerase chain reaction.

Stitt et al26 suggested that the retina may be a source of VEGF-A, as they found increased production of VEGF-A mRNA in ganglion cells and inner nuclear layers of the retina, proximal and distal to tumor deposits, and this was confirmed by our study. Vinores et al25 reported expression of VEGF-A in eyes with uveal melanoma in retinas close to (46%) and distant from (24%) the melanoma. Kivela et al27 proposed the prognostic significance of the finding of retinal detachments in eyes with uveal melanoma, which correlated with increased tumor size and tumor vessel networking. The results of our in situ hybridization investigation suggest that the tumor and the retina are sources of aqueous VEGF-A, and we observed increased VEGF-A expression in detached retinal tissue. Because eyes with retinal detachment only did not show increased concentrations of aqueous VEGF-A compared with control eyes (P = .11), we conclude that retinal detachment alone was insufficient to induce the increased VEGF-A concentrations seen in eyes with uveal melanoma. Uveal melanoma may secrete factors that induce VEGF-A production in an autocrine fashion in the tumor and in the surrounding tissues. This is consistent with our finding that aqueous VEGF-A concentration was not an independent prognostic factor in our study but correlated with largest basal tumor diameter and with tumor height. This suggests that aqueous VEGF-A concentration, retinal distress or retinal detachment, and tumor size are interrelated factors.

The high VEGF-A concentrations observed in 8 pretreated eyes in our study are in accord with observations by Boyd et al14 that eyes with neovascularization after radiotherapy demonstrated higher aqueous VEGF-A concentrations. In our series, 3 pretreated eyes had radiation retinopathy, and these had the highest VEGF-A concentrations of any eyes tested. One eye that had previously undergone proton beam irradiation, without noticeable radiation retinopathy clinically, also had a high concentration of VEGF-A. Ocular neovascularization is a serious complication after irradiation and may lead to secondary enucleation. Our data suggest a potentially beneficial role for anti–VEGF-A therapy in these eyes to avoid neovascularization, neovascular glaucoma, and subsequent enucleation.

In conclusion, the correlations between aqueous VEGF-A concentration and largest basal tumor diameter, the tumor height, and increased VEGF-A expression by uveal melanoma cells suggest important mechanisms in uveal melanoma angiogenesis. Their possible application as targets for therapy warrants further study, especially in cases of radiation retinopathy.

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REFERENCES


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