Regulation of VEGF-A in Uveal Melanoma

Mariam el Filali, Guy S. O. A. Missotten, Willem Maat, Long V. Ly, Gregorius P. M. Luyten, Pieter A. van der Velden, and Martine J. Jager

Purpose. Blood vessels are important constituents of intraocular uveal melanoma (UM), but whether angiogenesis is regulated by environmental factors such as ischemia or by genetic mechanisms is not known. This study was undertaken to examine the regulation of the proangiogenic factor vascular endothelial growth factor (VEGF-A).

Methods. Cell lines and primary tumors were tested for expression of VEGF-A, under normoxic and hypoxic conditions, using quantitative PCR, ELISA, WST-1 viability, and in-cell Western experiments. VEGF-A serum levels were determined by ELISA.

Results. Hypoxia induced expression of HIF-1α and VEGF-A in UM cell lines and primary tumor cultures, but it did not influence proliferation. VEGF-A expression in primary tumors was variable, demonstrating no correlation with specific histologic markers or prognosis. However, VEGF-A levels were significantly raised in UM patients with metastases compared with those without metastases (P < 0.001).

Conclusions. VEGF-A expression by UM cells is mainly controlled by hypoxia and involves the HIF-1α pathway, thus indicating an important role for the tumor cell environment. Metastases led to increased serum VEGF-A levels, indicating that VEGF-A may be involved in the growth of metastases. (Invest Ophthalmol Vis Sci. 2010;51:2329–2337) DOI:10.1167/iovs.09-4739

Uveal melanoma (UM), although rare, is the most common primary intraocular tumor, with an annual incidence of 0.7/100,000 in the adult Western population. Angiogenesis is an important mediator in tumor progression, as a tumor depends on its blood supply for oxygen and nutrients. Without a functional vasculature, tumor growth cannot proceed beyond the limits of perfusion. The formation of a tumor-associated vasculature is a process referred to as tumor angiogenesis.

Angiogenesis research has identified many pro- and antiangiogenic factors that regulate tumor angiogenesis. One of the major groups of cytokines that influence adult angiogenesis is the vascular endothelial growth factor family (VEGF). VEGF-A is known to cause increased vascular permeability, endothelial cell growth, angiogenesis, and monocyte activation. Expression of the VEGF-A gene and protein occurs in ocular tissues, especially the retina and retinal pigment epithelium and has been shown to be upregulated in retinopathies of the eye that are associated with angiogenic proliferation. Many tumors, such as brain malignancies and breast cancer, are known to produce VEGF-A. Boyd et al. and later Missotten et al. have demonstrated that VEGF-A concentrations were increased in the aqueous humor of eyes with UM. Furthermore, in situ hybridization showed that both the tumor and the retina were sources of VEGF-A.

However, it is still unknown whether VEGF-A in UM is induced by ischemic conditions or is produced continuously as part of tumor progression—for example, due to selection of specific UM cells that express a high amount of VEGF-A. In general, the key regulator of hypoxia-induced angiogenesis is the transcription factor hypoxia-inducible factor (HIF)-1α. We have demonstrated that several UM cell lines express VEGF-A in vitro, indicating a possible role in UM angiogenesis and proliferation. Accidentally, while performing in vitro experiments with UM cells in our laboratory, one UM cell culture was not harvested before (as intended), but after the weekend. During that time, the cells had proliferated and filled the culture well. Assessment of that culture showed a very high VEGF-A protein level. An explanation for this phenomenon may be lack of nutrients or lack of oxygen supply to the multilayered cells, and this led us to explore the effect of hypoxia on VEGF-A expression by UM cells.

We subsequently analyzed several UM tumor cell lines and primary UM tumor cell cultures for expression of VEGF-A under normoxic (20% O2) and hypoxic conditions (1% O2) to answer the question of whether the angiogenic factors are continuously turned on in UM, or whether these angiogenic factors are still under hypoxic, and thus environmental, regulation. Besides the supply of oxygen and nutrients, tumor-associated vessels promote metastasis by facilitating tumor cell entry into the circulation. Undeniably, this facilitation plays a role in UM, which metastasizes almost completely by the hematogenous route. Of all UM patients, up to 50% eventually die of metastatic disease. Several studies have shown that the presence of areas with a high local vascular density as well as ingrowth of tumor cells into the lumen of tumor blood vessels or into scleral vessels in UM is associated with poor survival. Since VEGF-A plays a pivotal role in tumor angiogenesis, regardless of the way in which it is regulated, the presence of a high amount of this factor in the primary UM could be used as a prognostic marker for the presence of UM and/or formation of metastatic disease.

We determined whether VEGF-A in the sera of patients is related to prognostic markers and the development of metastatic disease and UM-related death and whether it discriminates between patients with and without metastases.
**Table 1.** Primer Sequences of the Genes Studied in the qPCR Assay

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
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</thead>
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<tr>
<td>Endogenous controls</td>
<td>RPS11</td>
<td>AAGCAGCGGACGATCTTTCA</td>
<td>CGGGACGCTTCCATGATCAC</td>
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<tr>
<td></td>
<td>B-Actin</td>
<td>GGGGACGCTTCCATGATCAC</td>
<td>CTCTCTAAATTCAAGCAGG</td>
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<tr>
<td>Genes under study</td>
<td>VEGF-A</td>
<td>GCCCTTGCTTGCTGCTTCA</td>
<td>GTGATGATGATGCTCTCC</td>
</tr>
</tbody>
</table>

Primer sequences of the control and studied genes that were used in qPCR assays to determine levels of expression in UM cell lines.

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**Material and Methods**

**UM Cell Lines**

Nine UM cell lines were cultured under either normoxic (20% O2) or hypoxic (1% O2) conditions (HERAcell 240 CO2 Incubator; Thermo Fisher Scientific Inc., Pittsburgh, PA). Mel 202, Mel 285, Mel290, Mel270, 92.1, and OCM-1 are all primary tumor–derived cell lines, whereas OMM1, -2.3, and -2.5 are metastasis-derived cell lines. OCM-1 was provided by June Kan-Mitchell (Karmanos Cancer Institute, Detroit, MI); Mel202, Mel270, Mel285, Mel290, OMM2.5, and OMM2.3 by Bruce Ksander (Scheppens Eye Research Institute, Boston, MA); and OMM1 by one of the authors (GPML; Erasmus University Medical Center, Rotterdam, The Netherlands). The 92.1 line was established in our laboratory.

**Patient Tumor Tissue and Cell Cultures**

Informed consent was obtained before enucleation, and the study was performed according to the tenets of the Declaration of Helsinki. Diagnosis was made based on histopathology of the tumor samples. Fresh tumor tissue, obtained immediately after enucleation of the eye, was frozen in liquid nitrogen–cooled isopentane for cryopreservation (cryosections) or used to isolate RNA and to develop a cell culture.

Five primary tumor cell cultures were cultured (Ammiachrome Pro Medium; Lonza Group Ltd., Basel, Switzerland) and passed maximally once or twice before the cells were exposed to different conditions in a normoxic (20% O2, 5% CO2, 37°C) or a hypoxic (1% O2, 5% CO2, 37°C chamber.

**Quantitative PCR**

Expression of VEGF-A mRNA was analyzed by reverse transcription in combination with polymerase chain reaction (qPCR). RNA was isolated (RNaseasy Mini Kit; Qiagen, Valencia, CA), and the samples were stored at −80°C until further processing. Approximately 1 μg of RNA per sample was reverse transcribed with a cDNA synthesis kit (iScript; Bio-Rad, Hercules, CA). The 20-μL solutions were diluted by adding sterile water until the volume reached a total of 100 μL. To 96-well plates, 2 μL of this solution was added to a 15-μL solution of SYBR Green (iQ Supermix; Bio-Rad); forward and reverse primers (10-μM solutions) for β-actin, ribosomal protein S11 (RPS11), and VEGF-A (Table 1); and sterile water (volume ratio, respectively, 10:1:1:8).

A quantitative PCR analysis (IQ5 PCR System; Bio-Rad) of the samples for gene expression was then performed. The PCR reaction settings were 95°C for 3 minutes, 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds, and 95°C for 1 minute and 60°C for 1 minute. An accepted method of correcting the sample-to-sample variation when determining gene expression is to select a cellular housekeeping gene that serves as an endogenous control, against which the target gene expression levels can be normalized.19 RPS11 is a housekeeping gene that has recently been introduced to normalize gene expression in UM cells.20 β-Actin is a relatively stable cytoskeletal protein generally thought to be present at a constant level in cells, regardless of experimental treatment or technical procedure.21

**Cell Proliferation and Cytotoxicity**

Cell death was determined by trypan blue dye exclusion in a Bürker counting chamber. In addition, cell proliferation was measured by mitochondrial function by using the water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Indianapolis, IN), as previously described.22 In short, 96-well plates were filled with 1200 UM cells per well and regular medium (control), and placed in either a normoxic (20% O2, 5% CO2, 37°C) or a hypoxic (1% O2, 5% CO2, 37°C) chamber (HERAcell 240 CO2 Incubator; Thermo Fisher Scientific, Inc.). Absorbance was measured at 450 nm (n = 8) on a multiwell spectrophotometer (Perkin Elmer, Wellesley, MA).

**Patient and Control Subjects for Serum Samples**

Serum samples were obtained from 74 consecutive patients (38 men, 36 women) with diagnosed intraocular UM who had undergone enucleation at the Leiden University Medical Center from 1992 to 2000. Serum had been collected before enucleation and was stored at −80°C until analysis. At that time, none of the patients had liver metastases detected in ultrasonographic images of the liver.

The tumors were classified histopathologically. In 59 (79.7%) of 74 cases, the UM was located in the choroid, in 6 (8.1%) cases in the ciliary body, and in 9 (12.2%) cases in both the choroid and the ciliary body. Mean age at the time of enucleation was 66.0 years (SD 12.5). As a control, serum was obtained from 50 healthy individuals (26 men, 24 women), who voluntarily donated blood for this occasion. The mean age of the healthy individuals was 63.4 years (SD 12). Control sera were also frozen at −80°C until analysis. The study was performed according to the Declaration of Helsinki, and with the agreement of the local Medical Ethics Committee.

At the time of diagnosis of metastasis, 20 samples were collected. Fourteen patients had primarily been treated with ruthenium irradiation and six with enucleation. All patients had liver metastases, confirmed by liver ultrasonographic images. In 14 cases, liver metastases were confirmed by fine-needle aspiration biopsy (FNAB) and histopathology. The mean time between diagnosis and metastasis was 2.6 years (0.01–7.90 year). The mean time between development of clinical metastasis and melanoma-related death was 1.1 year (range, 0.21–3.20).

Standardized tests (Roche Diagnostics, Mannheim, Germany) were used for the determination of lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (AP), and yglutamyl transpeptidase (γGT). The detection limits and upper normal limits (UNLs) of these tests are shown in Table 2.

**Table 2.** Detection Limits and UNL for Serum Tests

<table>
<thead>
<tr>
<th>Test</th>
<th>Detection Limit</th>
<th>UNL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH, U/L</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>AP, U/L</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>ASAT, U/L</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ALAT, U/L</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>γGT, U/L</td>
<td>F:35/M:50</td>
<td></td>
</tr>
<tr>
<td>VEGF-A, pg/mL</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

U/L, unit per liter; F, female; M, male.
VEGF-A Protein Expression

VEGF-A protein concentrations in the supernatant of cell cultures were determined by using commercial solid-phase sandwich enzyme-linked immunosorbent assays (Human VEGF ELISA Immunoassay Kit; BioSource, Camarillo, CA). The VEGF-A concentrations in the sera of all patients were measured with the same assay. The lowest measurable concentration was 5 pg/mL with an intra-assay coefficient of variation (CV) of 4.7% and an inter-assay CV of 8.1%. The UNL is 600 pg/mL, according to the manufacturer (Table 2).

HIF-1α In-Cell Western Experiment

Phosphorylation and activation of HIF-1α was determined by an in-cell Western immunofluorescence assay. Briefly, cells were grown to approximately 70% confluence in a microtiter plate (NUNC, Roberts, WI) and either placed in a normoxic (20% O2, 5% CO2, 37°C) or hypoxic (1% O2, 5% CO2, 37°C) chamber (HERAcell 240 CO2 Incubator; Thermo Fisher Scientific Inc.) for 24 hours. For binding assays, the medium was replaced with fixing solution (3.7% formaldehyde in 1× PBS) for 20 minutes at room temperature to immediately fix the cells. Subsequently, the cells were washed four times with Triton washing solution (1× PBS + 0.1% Triton X-100). The cells were blocked in blocking buffer for 1.5 hours, then incubated with HIF-1α antibody (1:1000; Odyssey; Bethyl Laboratories Inc, Montgomery, TX) for 2 hours. After the cells were washed five times with Tween washing solution (1× PBS + 0.1% Tween-20), the plates were incubated with the second antibody (IRDye 800CW; 1:800) and for normalization of the number of cells, the 700-nm-channel DRAQ5 (1:2000) and Saphire700 (1:1000) were used (all three from LI-COR Biosciences, Lincoln, NE).

Washing steps were repeated, and the plate was scanned on an infrared scanner (Odyssey; LI-COR Biosciences). The data were acquired with the scanner software, exported to a spreadsheet (Excel; Microsoft, Redmond, WA), and analyzed (Prism; GraphPad Software, San Diego, CA).

Statistical Analysis

Statistical analysis was performed (SPSS, ver. 16.0; SPSS Inc., Chicago, IL) with an independent t-test, to detect a possible association of VEGF-A in primary UM samples (data normally distributed) and the presence of metastatic disease. Median concentrations of serum and expression data between the tested groups were compared by using nonparametric tests (Mann-Whitney or Kruskal-Wallis), because of small sample size and categorical scale. Overall survival was estimated by the Kaplan-Meier method and logrank test. P < 0.05 was considered statistically significant.

RESULTS

VEGF-A mRNA Expression in UM Cell Lines and Primary Tumor Cell Cultures

We analyzed whether VEGF-A is continuously secreted in UM or is regulated by hypoxia. VEGF-A mRNA was determined in nine UM cell lines and five primary UM tumor cell cultures. All cell lines expressed VEGF-A, but at variable levels. Ischemic conditions had a major time-dependent effect on VEGF-A expression in all nine cell lines with a VEGF-A induction of a 5.8-fold difference (normalized expression under hypoxia/normoxia after 24 hours), compared with normoxic conditions (range, 1.5- to 9.8-fold difference, shown for six cell lines in Fig. 1). Cell lines created from primary tumors and those from metastases behaved similarly.

As cell lines may have been modified during culture, we also tested short-term cultured primary UM (Fig. 2). These cultures also showed hypoxia-stimulated expression of VEGF-A, with an induction of 4.2-fold difference, compared with normoxia (range, 2.0–7.2-fold difference).

VEGF-A Protein Expression

To assess whether mRNA expression of UM cells resulted in the production of protein, we analyzed VEGF-A protein expression under either hypoxic or normoxic conditions in UM cell lines and primary tumor cell cultures using an ELISA on culture supernatant. All UM cell lines and cell cultures produced VEGF-A, but clear differences were observed in the amounts. Assessment of culture supernatant showed that UM cell lines under normoxia and hypoxia had a median VEGF-A protein expression (sum of amount of VEGF-A/four time points) of 41 pg/mL (range, 0–202 pg/mL) and 77 pg/mL (range, 0–418 pg/mL), respectively. Hypoxic conditions induced VEGF-A protein expression in the same time-dependent manner as observed for mRNA expression (Fig. 3).

Basic VEGF-A protein expression in the primary tumor cell cultures was significantly higher than that in the UM cell lines (normoxia: median of 174 pg/mL after 24 hours). As seen with UM cell lines, hypoxia induced VEGF-A protein expression in primary tumor cell cultures (hypoxia: median of 328 pg/mL) with a 2.7-fold difference (range, 1.4–4.5-fold difference, Fig. 4).

HIF-1α In-Cell Western Analysis on UM Cell Lines

The key regulator of VEGF-A under hypoxic circumstances is HIF-1α. To test whether the VEGF-A expression alterations are regulated by the HIF-1α pathway as expected under hypoxic circumstances, an in-cell Western analysis was performed on UM cell lines. Hypoxia induced expression of HIF-1α in all UM cell lines (mean HIF-1α expression under normoxia: 0.9 density light units; hypoxia: 2.2 density light units), with a mean increase by a factor of 4 (range, 1.9–8.1) compared with normoxic conditions (Fig. 5).

Cell Proliferation and Cell Death

As hypoxia stimulates endothelial cell proliferation, we considered the option that hypoxia might be involved in stimulation of tumor growth and performed a viability assay to investigate this possibility. Surprisingly, although VEGF-A expression is induced under ischemic conditions, the WST-1 assay demonstrated a reduced cell proliferation rate under hypoxia in all nine UM cell lines (Fig. 6). Hypoxia had no toxic effect on UM cells up to 11 days, as there were no differences in the percentages of dead cells between the different conditions measured by the Bürker counting chamber (data not shown).

Short-term cultures obtained from primary UM (n = 5) showed similar proliferation rates under either hypoxia or normoxia up to 3 days in culture. After 3 days, proliferation rates and toxicity varied considerably between cultures. However, no differences in proliferation rate between the different environments (normoxia and hypoxia) were observed (data not shown).

VEGF-A mRNA Expression in Primary Tumor Tissue

We evaluated whether expression of VEGF-A had any prognostic value, by determining expression by qPCR analysis in 27 samples of primary UM tumor tissues and comparing the data with clinical parameters. Some samples showed almost no expression (0.04-fold, normalized mean), whereas others demonstrated up to approximately 10 times normalized expression (9.55-fold normalized mean). We looked for a possible associ-
ation of VEGF-A expression with histopathologic characteristics of the tumor, but no association was observed (Table 3). An independent t-test analysis of VEGF-A and the presence of metastatic disease was performed. No association between VEGF-A expression and the occurrence of metastasis ($P = 0.14$) was demonstrated. Further analysis with the Kaplan-Meier method and log-rank test did not show a relation between VEGF-A expression and metastases-free survival ($P = 0.76$, mean follow-up of 57 months; range, 8–206 months).

**Prognostic Value of Serum VEGF-A at the Time of Enucleation**

VEGF-A has been shown to be present in the aqueous humor of enucleated eyes with UM, and a high concentration correlates with a bad prognosis. We investigated whether the level of VEGF-A in serum at the time of enucleation for UM was of prognostic significance. VEGF-A was measured in sera of 74 UM patients and showed a median value of 183 pg/mL, whereas the level was 167 pg/mL in the serum of 50 control persons (Fig. 7), a difference that was not significant ($P = 0.28$). We also compared VEGF-A levels with the histopathologic characteristics of the tumor. No significant relations were observed (data not shown).

**Figure 1.** VEGF-A mRNA expression in UM cell lines. The amount of VEGF-A mRNA expression was measured with qPCR in UM cell lines under normoxic (black) and hypoxic (gray) exposure after four different time intervals (3, 6, 24, and 48 hours). Normalized expression is shown with a maximum of 2.0- or 6.0-fold on the y-axis.

**Figure 2.** VEGF-A mRNA expression in primary UM cell cultures. The amount of VEGF-A mRNA expression was measured with quantitative PCR in primary UM cell cultures (cultures 1–5) under normoxic and hypoxic conditions after 24 hours. Normalized expression is demonstrated.
**Regulation of VEGF-A in Uveal Melanoma**

**VEGF-A in Serum of Patients with Metastatic Disease**

We also investigated the possibility of using VEGF-A serum levels to discriminate between UM patients, with or without metastasis. VEGF-A was measured in the serum of 20 patients with metastatic UM and compared to serum levels of 74 patients with UM without metastases and 50 controls. Six of 20 patients with metastases (30%) had a VEGF-A level above the UNL (the maximum level of VEGF-A in healthy individuals, Table 4), with a median concentration of 351 pg/mL, whereas only 7 (9%) out of 74 melanoma patients without metastases and 4 (8%) of 50 control subjects had an amount of VEGF-A above the UNL. The difference in the VEGF-A concentration between patients with and without metastasis was significant (Mann-Whitney, \( P < 0.001 \); Fig. 7).

**DISCUSSION**

Tumor angiogenesis is important for tumor growth and metastatic potential. Both microvessel density and specific extracellular matrix patterns contribute independently to prognosis and survival in patients with UM.\(^{11,12}\) The clinical course of many cases of UM is slow, and it has been suggested that dormancy is associated with an avascular phase. The conversion to proliferation could be associated with a change to an angiogenic phenotype, with regard to both the intraocular...
tumor and the metastases. This conversion, which is known as the angiogenic switch, is due to an alteration in the balance of inhibitory and stimulatory factors such that vessel growth is favored.25,26

The process of angiogenesis is initiated when cells within the tumor respond to hypoxia by increasing their production of proangiogenic factors. We studied several UM tumor cell lines and primary UM tumor cell cultures under normoxic and hypoxic environments to analyze whether expression of the proangiogenic factor VEGF-A is regulated by tumor microenvironmental conditions or other mechanisms. All cell lines and primary tumor cultures expressed VEGF-A, as described before.5,6,27 In addition, hypoxia had a major inducing effect on VEGF-A mRNA and protein expression. We can therefore conclude that ischemic conditions greatly contribute to the induction of the proangiogenic factor VEGF-A in UM. HIF-1α is thought to be the most important initiator of hypoxia-induced VEGF-A expression.28 We also observed in UM that expression of HIF-1α was induced under hypoxic conditions. Still, accumulating evidence indicates that HIF-independent pathways can also control angiogenesis.29 As many cell lines had a basic VEGF-A production, the two routes may be additive. In addition, VEGF-A may not only be produced by UM cells, but also by infiltrating cells. UMs often contain macrophages, and these are also known to produce VEGF. As hypoxia is known to induce macrophage migration into the hypoxic areas, induction of VEGF by hypoxic tumor cells may at the same time stimulate the influx of VEGF-producing macrophages.29 Moreover, it is known that numerous other HIF-1α target genes besides VEGF-A can modulate angiogenesis by promoting the mitogenic and migratory activities of endothelial cells.28

Although hypoxia considerably induced VEGF-A expression in cell lines and primary cell cultures, cell proliferation rates did not increase. Moreover, UM cell proliferation was significantly reduced in hypoxic conditions in comparison to UM cells in a normoxic environment. This finding indicates that VEGF-A protein does not independently increase UM growth through a possible feedback loop. Whether VEGF-A protein is essential for formation of new, functioning vasculature will be analyzed in a three-dimensional in vitro model and in a mouse UM model.

Apart from which mechanisms regulate VEGF-A in UM, several studies have observed VEGF expression to correlate with development of experimental metastasis.30–32 On the contrary, Sheidow et al.33 found no correlation between VEGF immunoreactivity in UM samples of enucleated eyes and the occurrence of metastatic disease. We compared VEGF-A mRNA expression in 27 UM samples with clinical and prognostic data for metastatic disease and found no relation (Table 3). Additional t-test and survival analysis of the data demonstrated no association between VEGF-A expression and formation of metastasis. Because of the small number of samples used and, in some cases, the short follow-up, these findings may not be conclusive. That the primary tumor samples showed a great variation in VEGF-A mRNA expression, may be due to sampling bias, as some tumor samples may have originated from ischemic regions and some of the samples from fully oxygenated areas. It has been demonstrated that analysis of UM paraffin-embedded sections showed genetic heterogeneity.34 Determination of VEGF-A mRNA expression in a sample obtained from primary UM may therefore not be representative of the whole tumor, due to this heterogeneous distribution. To avoid such a
sampling bias, examination of the amount of VEGF-A protein in sera of UM patients is an alternative. In several tumors—colon carcinoma, soft tissue sarcomas, and gastric cancer—serum VEGF-A levels have been found to be a marker of disease stage and an indicator of metastasis. Until now, lactate dehydrogenase (LDH) and alkaline phosphatase (AP) have been the most indicative serum markers for metastatic disease in UM, in combination with liver ultrasonography. Elevated serum osteopontin, melanoma-inhibitory activity (MIA), and S-100β levels showed a correlation with metastatic UM to the liver in some studies. However, serum markers that indicate micrometastases at an early stage would be clinically preferable. When we indeed determined the amount of VEGF-A in the sera of UM patients, we observed that VEGF-A levels were not increased at the time of enucleation and that only the patients with manifest metastases had high VEGF-A serum levels (Fig. 7). A similar study was recently presented with the same results by Barak et al. Thus, although we found no correlation between VEGF-A expression by the primary tumor and histologic parameters, VEGF-A levels were significantly higher in patients with metastatic disease. It may be that the size of the hypoxic areas (the small eye versus the large liver) influences the VEGF-A levels in the blood.

Further research is necessary to determine whether VEGF-A levels increase before clinical recognition of UM.

**Figure 6.** Cell proliferation of UM cell lines. The proliferation rate of UM cell lines measured by WST-1 viability assay under normoxic (solid line) and hypoxic (dashed line) exposure after four different time intervals (4, 6, 8, and 11 days). Proliferation is measured by viability of the cells at each time point and is expressed in absorbance (optical density, OD).
VEGF-A in the sera of patients with UM metastases suggests ischemia in the different tumor areas. The high amounts of expression were increased by hypoxia. Most likely, the variation in biopsy specimens taken from UM in enucleated eyes, but in all cell lines and primary tumor UM cell cultures, VEGF-A expression by UM cells. The basic expression is variable, especially in biopsy specimens taken from UM in enucleated eyes, and in all cell lines and primary tumor UM cell cultures, VEGF-A expression was increased by hypoxia. Most likely, the variation between the tissue specimens was due to different degrees of ischemia in the different tumor areas. The high amounts of VEGF-A in the sera of patients with UM metastases suggests that VEGF-A plays a role in the growth of metastases and that the use of VEGF-A inhibiting agents should therefore be considered.

**Acknowledgments**

The authors thank Mieke Versluis and Joke E. van den Berg-Leemreize for technical assistance.

**References**


**TABLE 3. VEGFA mRNA Expression Determined in 27 Primary UM Samples with qRT-PCR**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases n (%)</th>
<th>Median VEGF-A (Normalized n-Fold Expression)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Choroid</td>
<td>16 (59)</td>
<td>1.01</td>
<td>0.152*</td>
</tr>
<tr>
<td>Choroid + ciliary body</td>
<td>11 (40)</td>
<td>2.67</td>
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</tr>
<tr>
<td>Cell type</td>
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</tr>
<tr>
<td>Mixed</td>
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<td>1.79</td>
<td>0.191†</td>
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<tr>
<td>Epithelioid</td>
<td>5 (19)</td>
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<tr>
<td>Spindle</td>
<td>4 (15)</td>
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<tr>
<td>Bruch’s membrane</td>
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<td>Intact</td>
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<td>Broken</td>
<td>17 (65)</td>
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<td>1.73</td>
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<td>Scleral ingrowth</td>
<td></td>
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<tr>
<td>No</td>
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<td>Yes</td>
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<tr>
<td>≤5 mm</td>
<td>9 (33)</td>
<td>0.99</td>
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<tr>
<td>&gt;5 mm</td>
<td>18 (67)</td>
<td>2.58</td>
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</table>

Levels of expression were compared with histological tumor characteristics.
* Mann-Whitney U test.
† Kruskal-Wallis test.

**FIGURE 7.** Concentration of serum VEGF-A in (metastatic) UM patients and control subjects. Concentration of serum VEGF-A in the control group, and in patients with and without metastatic UM. P-values, Mann-Whitney test. Each box shows the median, quartiles (box length is the interquartile range) and whiskers represent the 90th and 10th percentiles.

**TABLE 4. Amount of VEGF-A in Sera above the Detection Limit and UNL for Sera from Healthy Control Subjects and Patients with UM, with or without Metastasis**

<table>
<thead>
<tr>
<th>Group</th>
<th>n &gt; UNL</th>
<th>Median VEGF-A (%)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects (n = 50)</td>
<td>4 (8)</td>
<td>168.6</td>
<td>5.3–846</td>
</tr>
<tr>
<td>UM patients (n = 74)</td>
<td>7 (9)</td>
<td>183.2</td>
<td>4.8–821</td>
</tr>
<tr>
<td>UM patients with metastasis (n = 20)</td>
<td>6 (30)</td>
<td>351.2</td>
<td>11.1–2490</td>
</tr>
</tbody>
</table>

that VEGF-A plays a role in the growth of metastases and that the use of VEGF-A inhibiting agents should therefore be considered.


