CHAPTER 4

REGULATION OF VEGF-A IN UVEAL MELANOMA

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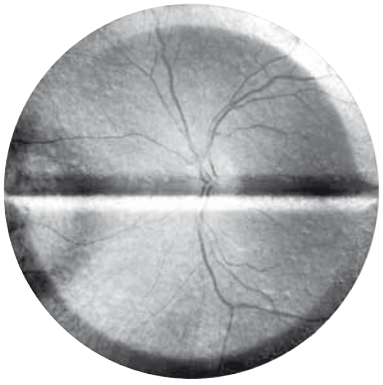

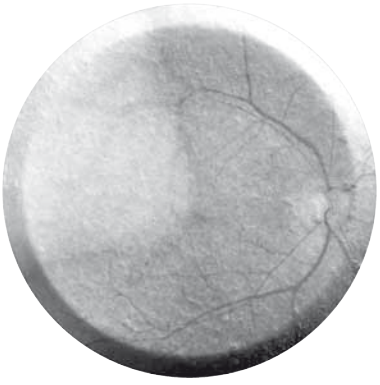

ABSTRACT

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. We examined regulation of the pro-angiogenic factor Vascular Endothelial Growth Factor (VEGF-A).

Material & Methods: Cell lines and primary tumours was 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 HIF-1α and VEGF-A in UM cell lines and primary tumour cultures. Hypoxia did not influence proliferation. VEGF-A expression in primary tumours was variable, demonstrating no correlation with specific histological markers or prognosis. However, VEGF-A levels were significantly raised in UM patients with metastases compared with those without metastases ($p<0.001$).

Conclusion: VEGF-A expression by UM cells is mainly controlled by hypoxia and involves the HIF-1α pathway, thus indicating an important role for the tumour cell environment. Metastases led to increased serum VEGF-A levels, indicating that VEGF-A may be involved in the growth of metastases.

INTRODUCTION

Uveal melanoma (UM), although rare, is the most common primary intraocular tumour, with an annual incidence of 0.7/100.000 in the adult Western population [1]. Angiogenesis is an important mediator in tumour progression, as a tumour depends on its blood supply for oxygen and nutrients. Without a functional vasculature, tumour growth cannot proceed beyond the limits of perfusion. The formation of a 'tumour-associated vasculature' is a process referred to as tumour angiogenesis.

Angiogenesis research has identified many pro- and anti-angiogenic factors that regulate tumour angiogenesis. One of the major groups of cytokines influencing 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 up-regulated in retinopathies of the eye that are associated with angiogenic proliferation [2]. Many tumours such as e.g. brain malignancies and breast cancer are known to produce VEGF-A [3,4]. In previous studies by Boyd and later by Missotten and others, it was demonstrated that VEGF-A concentrations were increased in the aqueous humour of eyes with uveal melanoma [5,6] Furthermore, in situ hybridization showed that both the tumour as well as 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 tumour 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 previously demonstrated that several UM cell lines express VEGF-A in vitro, indicating a possible role in UM angiogenesis and proliferation[7]. 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, 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 might 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 uveal melanoma cells.

We subsequently analyzed several UM tumour cell lines and primary UM tumour cell cultures for expression of VEGF-A under normoxic (20 % O_2) and hypoxic conditions (1 % O_2) to answer the question 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, tumour-associated vessels promote metastasis by facilitating tumour cell entry into the circulation[8]. Undeniably, this plays a role in uveal melanoma, which metastasises almost completely by the haematogenous route. Of all uveal

melanoma patients, up to 50% eventually die due to metastatic disease [9,10]. Several studies have shown that the presence of areas with a high local vascular density as well as ingrowth of tumour cells into the lumen of tumour blood vessels or into scleral vessels in UM is associated with poor survival[11-13]. Since VEGF-A plays a pivotal role in tumour angiogenesis, regardless of the way in which it is regulated, the presence of a high amount of this factor in the primary UM could possibly 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, the development of metastatic disease and UM-related death, and whether it discriminates between patients with and without metastases.

MATERIAL AND METHODS

UM cell lines

Nine UM cell lines were cultured under either normoxic (20% O_2) or hypoxic (1% O_2) conditions (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). Mel 202, Mel 285, Mel290, Mel270, 92.1 and OCM-1 were all primary tumour-derived cell lines, while OMM1, OMM2.3 and OMM2.5 were metastases-derived cell lines. OCM-1 was provided by dr. J. Kan-Mitchell (Karmanos Cancer Institute, Detroit, MI), Mel202, Mel270, Mel285, Mel290, OMM2.5 and OMM2.3 by dr. B. Ksander (Schepens Eye Research Institute, Boston, MA), OMM1 by dr. G.P.M. Luyten (Erasmus University Medical Center, Rotterdam, the Netherlands) and 92.1 was established in our laboratory [14-18]. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (RPMI) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (GIBCO, Life Technologies, Paisley, UK). The cells were passaged once or twice a week using trypsin (0.05%).

Patient tumour material and cell cultures

Informed consent was obtained prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Diagnosis was made based on histopathology of the tumour samples. Fresh tumour 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 tumour cell tissues were cultured in Amniochrome® Pro Medium (Lonza Group Ltd, Basel, Switzerland) and passaged maximally once or twice before exposing the cells to different conditions using a normal and a hypoxic chamber: normoxia (20% O_2, 5% CO_2, 37°C) or hypoxia (1% O_2, 5% CO_2, 37°C).

TABLE 1. Primer sequences of the genes studied in the qPCR assay

Primer	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Endogenous controls	RPS11	AAGCAGCCGACCATCTTTCA	CGGGAGCTTCTCCTTGCC
	β-ACTIN	CGGGACCTGACTGACTACCTC	CTCCTTAATGTCACGCACGATTT
Genes under study	VEGF-*A*	GCCCTTGCCTTGCTGCTCTACC	GTGATGATTCTGCCTCCTCCTTC

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

Quantitative PCR

Expression of VEGF-A mRNA expression was analyzed by reverse transcriptase-polymerase chain reaction (qPCR). RNA was isolated using an Rneasy® Mini Kit (Qiagen, Valencia, USA). RNA samples were stored at -80°C until further processing. Approximately 1 µg of RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). The 20µl solutions obtained this way were diluted by adding sterile water until the volume reached a total of 100 µl. Using 96-well plates, 2 µl of this solution was added to a 15 µl solution of iQ SYBR Green Supermix, forward and reverse primers (10µM solutions) for beta-actine (β-ACTIN), ribosomal protein S11 (RPS-11) 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, then 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds, then 95°C for 1 minute and 60°C for 1 minute. To correct the sample-to-sample variation when determining gene expression, an accepted method is to select a cellular housekeeping gene that serves as an endogenous control, against which the target gene expression levels can be normalized [19]. RPS-11 is a housekeeping gene that has recently been introduced to normalize gene expression in UM cells[7,20]. 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 using a Bürker counting chamber. In addition, cell proliferation was measured by mitochondrial function 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 uveal melanoma cells per well, filled with regular medium (control), and either placed in a normoxic (20% O_2, 5% CO_2, 37°C) or hypoxic (1% O_2, 5 % CO_2, 37°C) chamber (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). Absorbance was measured at 450nm (n=8) on a multiwell spectrophotometer (Perkin Elmer, Wellesley, MA).

Patient and controls for serum samples

Serum samples were obtained from 74 consecutive patients (38 males, 36 females) with the diagnosis intraocular UM, enucleated at the Leiden University Medical Center from 1992 to 2000. Serum was collected prior to enucleation, and was stored at -80°C until analysis. At that time, none of the patients had liver metastases on ultrasonographic images of the liver.

Tumours were classified histopathologically: in 59 out of 74 cases, the UM was located in the choroid (79.7%), in six cases in the ciliary body (8.1%), in nine cases in both the choroid and the ciliary body (12.2%). 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 male, 24 female), who voluntarily donated blood for this occasion. 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 Ethical Committee.

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

For the determination of lactic dehydrogenase (LD), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatise (AP) and γ-glutamyl transpeptidase (γGT), standardized tests were used (Roche Diagnostics, Mannheim, Germany). The detection limits and upper normal limits of these tests are mentioned in Table 2.

VEGF-A protein expression

VEGF-A protein concentrations in the supernatant of cell cultures were determined using commercial solid phase sandwich enzyme-linked immunosorbent assays (VEGF-A: human VEGF ELISA immunoassay Kit, Biosource, Camarillo, USA.). The VEGF-A concentration in sera of patients were measured using 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 upper normal limit 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 immunofluorescent assay [23,24]. Briefly, cells were grown to approximately 70% confluency in a microtiter plate (NUNC, Roberts, WI, USA) and either placed in a normoxic (20% O_2, 5% CO_2, 37°C) or hypoxic (1% O_2, 5 % CO_2, 37°C) chamber (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA) for 24 hours. For binding assays, medium was replaced

TABLE 2. Detection limits and UNL for serum tests

	Detection limit	Upper normal limit
LD, U/L	1	450
AP, U/L	1	120
ASAT, U/L	1	40
ALAT, U/L	1	45
γ-GT, U/L	1	F: 35 / M:50
VEGF-A, pg/ml,	5	600

U/L, unit per liter; F, female; M, male.

with Fixing Solution (3.7% formaldehyde in 1X PBS) for 20 minutes at room temperature to immediately fix the cells. Subsequently, cells were washed four times with Triton Washing Solution (1xPBS+0.1% TritonX-100). Cells were blocked in Odyssey Blocking Buffer for 1.5 hours, then incubated with HIF-1α antibody (Bethyl laboratories INC Montgomery, TX, USA)) (1:1000) for 2 hours. After washing the cells five times with Tween Washing Solution (1x PBS + 0.1% Tween-20), plates were incubated with the second antibody IRDye® 800CW (1:800) and for normalization of cell number, the 700nm-channel DRAQ5 (1:2000) and Sapphire700 (1:1000) were used (all three from LI-COR Biosciences, Lincoln NB, USA).

Washing steps were repeated and the plate was scanned on an Odyssey infrared scanner (LI-COR Biosciences, Lincoln NB, USA). Data were acquired using the scanner software, exported to Excel (Microsoft, Redmond, WA, USA), and analyzed by GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 sofware (SPSS Inc. Chicago, Illinois, USA). An independent t-test was performed 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 values and expression data between the tested groups were compared using non-parametric tests (Mann-Whitney or Kruskal-Wallis), because of small sample size and categorical scale. Overall survival were estimated by the Kaplan Meier method and log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

VEGF-A mRNA expression in UM cell lines and primary tumour 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 tumour 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 5.8-fold difference (normalized expression under hypoxia/ normalized expression under normoxia after 24 hours), compared to normoxic conditions (range; 1.5 to 9.8 fold difference, shown for six cell lines in Figure 1). Cell lines created from primary tumours and from metastases behaved similarly.

As cell lines may have been modified during culture, we also tested short-term cultured primary UM (Figure 2). These cultures also showed hypoxia-stimulated expression of VEGF-A, with an induction of 4.2-fold difference, compared to 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 tumour 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 (Figure 3).

Basic VEGF-A protein expression in the primary tumour cell cultures was significantly higher than seen 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 tumour cell cultures (hypoxia: median of 328 pg/ml) with a 2.7-fold difference (range 1.4-4.5-fold difference, Figure 4).

HIF-1α in-cell Western 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 experiment 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 4 (range 1.9-8.1) compared to normoxic conditions (Figure 5).

Cell proliferation and cell death

As hypoxia stimulates endothelial cell proliferation, we considered the option that hypoxia might be involved in stimulation of tumour growth and performed a viability assay to investigate this possibility. Surprisingly, though VEGF-A expression is induced under ischemic conditions, the WST-1 assay demonstrated a reduced cell proliferation rate under hypoxia in

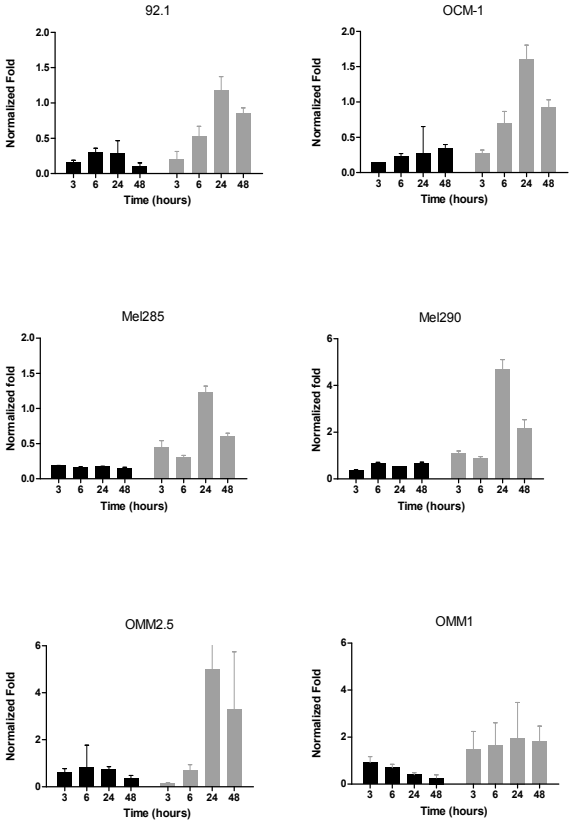

FIGURE 1. VEGF-A mRNA expression in UM cell lines.
The amount of VEGF-A mRNA expression was measured with qPCR in UM cell lines under normoxic (black) and hypoxic (gray) exposure after four different time intervals (3, 6, 24 and 48 hours). Normalized expression is shown with a maximum of 2.0- or 6.0-fold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

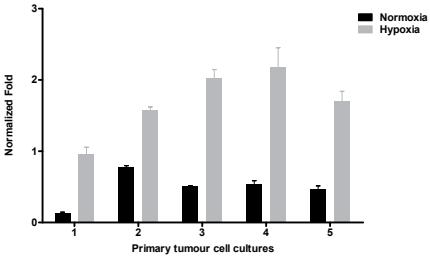

FIGURE 2. VEGF-A mRNA expression in primary UM cell cultures.
The amount of VEGF-A mRNA expression was measured with qPCR in primary UM cell cultures (cultures 1-5) under normoxic (black) and hypoxic (gray) conditions after 24 hours. Normalized expression is demonstrated.

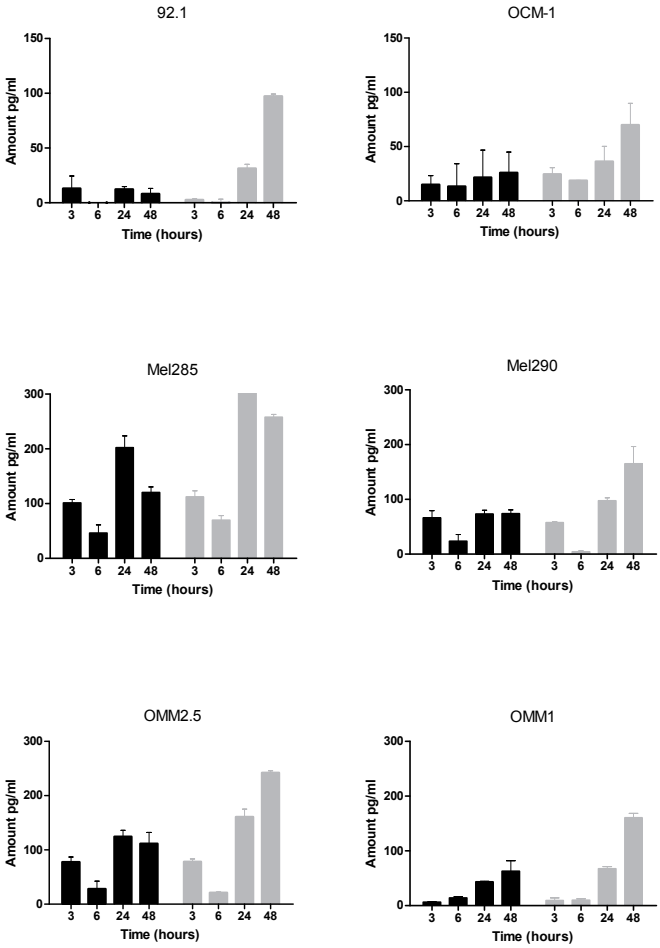

FIGURE 3. VEGF-A protein expression in UM cell lines.
The amount of VEGF-A protein expression measured with ELISA in supernatants of UM cell lines under normoxic (black) and hypoxic (gray) exposure after four different time intervals (3, 6, 24 and 48 hours). Expression is demonstrated in amount of protein (pg/ml) with a maximum of 150 or 300 pg/ml.

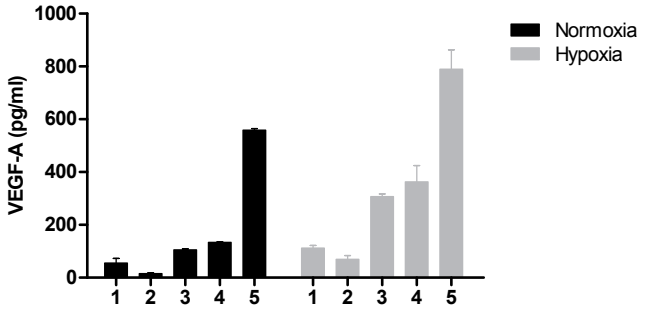

FIGURE 4. VEGF-A protein expression in primary UM cell cultures.
The amount of VEGF-A protein expression measured with ELISA in supernatants of primary UM cell cultures (cultures 1-5) under normoxic (black) and hypoxic (gray) exposure after 24 hours. Expression is demonstrated in amount of protein (pg/ml).

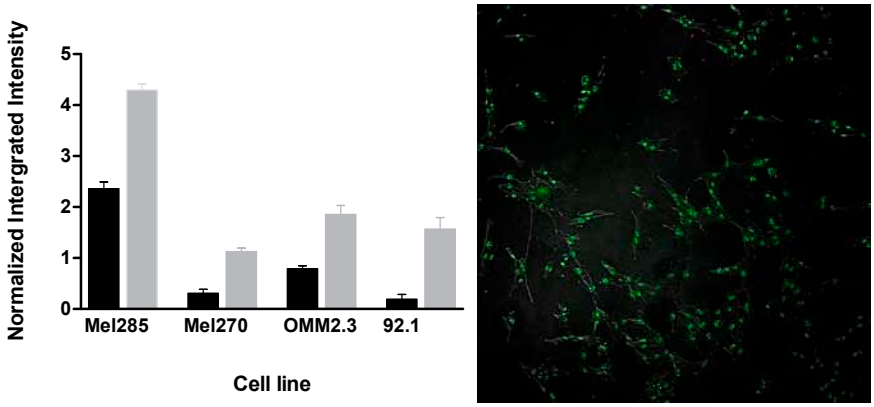

FIGURE 5. HIF-1α expression in UM cell lines.
Expression of HIF-1α was measured in UM cell lines using in-cell Western assay under normoxic (black) and hypoxic (gray) exposure during 24 hours. Expression is demonstrated in normalized integrated intensity ((HIF-1α/ cell count) ×100) (top). Image of HIF-1α staining in UM cell line Mel 270 captured by confocal microscopy (bottom).

all nine UM cell lines (Figure 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 three days in culture. After three 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 tumour tissue

We evaluated whether expression of VEGF-A had any prognostic value, by determining expression by qPCR analysis in 27 samples of primary UM tumour tissues and comparing the data to clinical parameters. Some samples showed almost no expression (0.04-fold

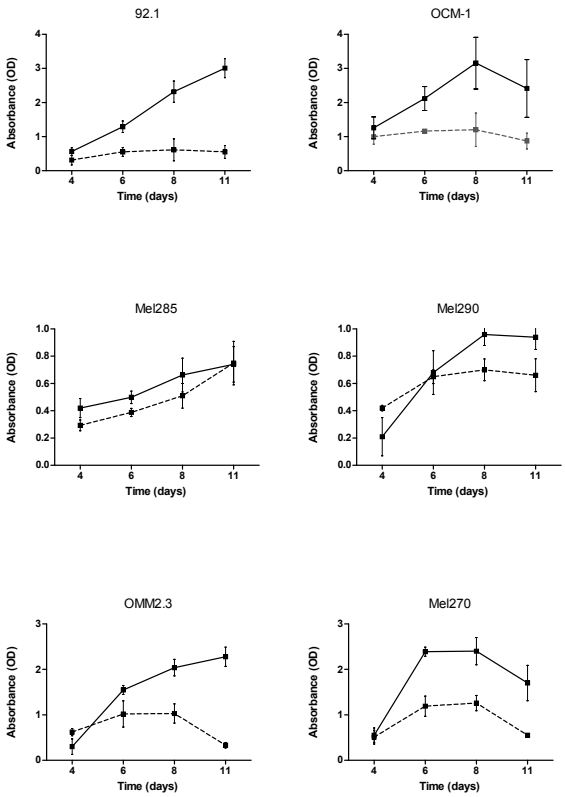

FIGURE 6. Cell proliferation of UM cell lines.
The proliferation rate of UM cell lines measured by WST-1 viability assay under normoxic (solid line) and hypoxic (dashed line) exposure after four different time intervals (4, 6, 8 and 11 days). Proliferation is measured by viability of the cells at each time point, and is expressed in absorbance (optical density, OD).

normalized mean), while others demonstrated up to approximately 10 times normalized expression (9.55-fold normalized mean). We looked for a possible association of VEGF-A expression with histopathological characteristics of the tumour, 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) could be demonstrated. Further analysis with the Kaplan Meier method

TABLE 3. VEGF-A mRNA expression determined in 27 primary UM samples with qPCR.

Variables		No. of cases (%)	median VEGF-A (normalized fold expression)	
Location				P†=0.152
	choroid	16 (59)	1.01	
	choroid + ciliary body	11 (40)	2.67	
Cell type				
	Mixed	18 (67)	1.79	P*=0.191
	Epithelioid	5 (19)	1.02	
	Spindle	4 (15)	4.06	
Bruch's membrane				P†=0.834
	Intact	6 (22)	2.41	
	Broken	17 (63)	1.91	
	not known	4 (15)	1.73	
Scleral ingrowth				P†=0.399
	No	3 (11)	1.70	
	Yes	23 (85)	2.50	
	Episcleral	1 (4)	0.49 (1 value)	
Tumour diameter				P†=0.457
	≤ 10 mm	8 (30)	1.45	
	> 10 mm	19 (70)	2.01	
Tumour prominence				P†=0.136
	≤ 5 mm	9 (33)	0.99	
	> 5 mm	18 (67)	2.58	

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

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

Previously, VEGF-A was shown to be present in the aqueous humour of enucleated eyes with uveal melanoma, and a high concentration was correlated with a bad prognosis [6]. 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, while this was 167 pg/ml in the serum of 50 control persons (Figure 7), which difference was not significant (P=0.28). We also compared VEGF-A levels with histopathological characteristics of the tumour. No significant relations were observed (data not shown).

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 and 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 upper normal limit (UNL, the maximum level of VEGF-A in healthy individuals, Table 4) with a median concentration of 351 pg/ml, while only 7 out of 74 (9%) melanoma patients without metastases and 4 out of 50 (8%) controls had an amount of VEGF-A above the UNL.

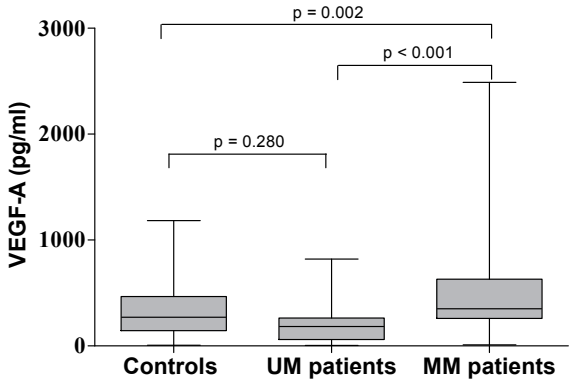

FIGURE 7. Concentration of serum VEGF-A in (metastatic) UM patients and controls.
Concentration of serum VEGF-A in the control group, and in patients with and without metastatic uveal melanoma. P-values (Mann-Whitney test) between the different groups are indicated in the graph. Each box shows the median, 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 upper normal limit (UNL) for sera from healthy control subjects and patients with UM with or without metastases.

Group	N > UNL. (%)	Median VEGF-A (pg/ml)	Range
Controls (n = 50)	4 (8)	168.6	5.3 – 846
Uveal melanoma patients (n=74)	7 (9)	183.2	4.8 – 821
Patients with metastatic melanoma (n=20)	6 (30)	351.2	11.1 –2490

The difference in VEGF-A concentration between patients with and without metastases was highly significant (Mann-Whitney, $p < 0.001$) (Figure 7).

DISCUSSION

Tumour angiogenesis is important for tumour growth and metastatic potential. Both microvessel density as well as 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, both with regard to the intra-ocular tumour as well as to 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 favoured [25,26].

The process of angiogenesis is initiated when cells within the tumour respond to hypoxia by increasing their production of pro-angiogenic factors. We studied several UM tumour cell lines and primary UM tumour cell cultures under normoxic and hypoxic environments to analyze whether expression of the pro-angiogenic factor VEGF-A is regulated by tumour microenvironmental conditions or other mechanisms. All cell lines and primary tumour cultures expressed VEGF-A, as described before [5,6,27]. Additionally, 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 pro-angiogenic factor VEGF-A in UM. HIF-1α is thought to be the most important initiator of hypoxia-induced VEGF-A expression [28]. We observed that also in UM, expression of HIF-1α was induced under hypoxic conditions. Still, accumulating evidence indicates that HIF-independent pathways can also control angiogenesis [26]. 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 known to also produce VEGF. As hypoxia is known to induce macrophage migration into the hypoxic areas, induction of VEGF by hypoxic tumour 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 under hypoxic conditions in comparison to UM cells in a normoxic environment. This indicates that VEGF-A protein does not independently increase uveal melanoma 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 melanoma model.

Apart from which mechanisms regulate VEGF-A in UM, several studies have observed VEGF expression to be correlated with development of experimental metastasis [30-32]. On the contrary, Sheidow et. al. found no correlation between VEGF immunoreactivity in uveal melanoma samples of enucleated eyes and the occurrence of metastatic disease [33] . We compared VEGF-A mRNA expression in 27 uveal melanoma 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 short follow-up, these findings may not be conclusive. That the primary tumour samples showed a great variation in VEGF-A mRNA expression, may be due to sampling bias, as some tumour 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 sections showed genetic heterogeneity [34]. Determination of VEGF-A mRNA expression in a sample obtained from primary uveal melanoma may therefore not be representative of the whole tumour due to this heterogeneous distribution. To avoid such a sampling bias, examination of the amount of VEGF-A protein in sera of UM patients can be an alternative. In several tumours, e.g. 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 metastases [35-37]. Until now, lactate dehydrogenase (LD) and alkaline phosphatase (AP) are the most indicative serum markers for metastatic disease in UM, in combination with liver ultrasonography [38,39]. Elevated serum osteopontin, melanoma-inhibitory activity (MIA) and S-100beta levels showed a correlation with metastatic UM to the liver in some studies [40,41]. However, serum markers that indicate micrometastases at an early stage would be clinically preferable. When we indeed determined the amount of VEGF-A in sera of UM patients, we observed that VEGF-A levels were not increased at the time of enucleation and that only 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. [42] . 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 prior to clinical recognition of UM metastases and whether this marker can be used for screening purposes.

In conclusion, we observe a basic as well as a hypoxia-induced expression of HIF-1α and VEGF-A mRNA and protein expression by UM cells. The basic expression is variable, especially in biopsies taken from UM in enucleated eyes, but in all cell lines and primary tumour UM cell cultures, VEGF-A expression was increased by hypoxia. Most likely, the variation between biopsies is due to different degrees of ischemia in different tumour 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 the use of VEGF-A inhibiting agents should therefore be considered.

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