Knowledge-based treatment in uveal melanoma

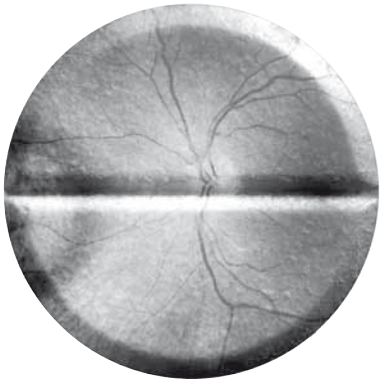
Filali, M. el

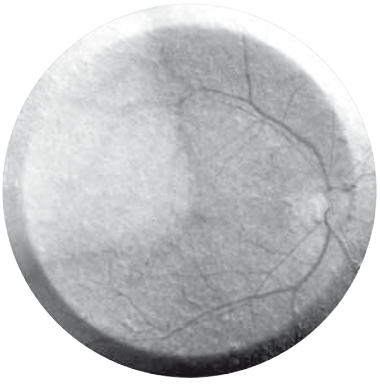
CHAPTER 3

ANTI-ANGIOGENIC THERAPY IN UVEAL MELANOMA

M. el Filali, P.A. van der Velden, M. J. Jager

Dev. Ophthalmol. 2012;49:117-36





ABSTRACT

For several decades, targeting of tumor- related vessels has been regarded as a potential anti-cancer therapy. Such anti-angiogenic therapy is based on the assumption that a tumor cannot grow beyond the limits of diffusion (about 1– 2 mm) of oxygen and nutrients from capillaries, unless angiogenesis takes place. Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis, regulating vasopermeability as well as the proliferation and migration of endothelial cells. In several types of cancer (colon carcinoma, soft tissue sarcomas and gastric cancer), serum VEGF levels are a marker for disease stage and an indicator of metastasis. VEGF levels are significantly elevated in uveal melanoma patients with metastatic disease compared to patients without metastases. Anti-angiogenic therapy, such as bevacizumab, is currently used for the treatment of metastases of several malignancies. Anti-angiogenic therapy has not yet been tested for the treatment of primary uveal melanoma or related metastatic disease. Clinicians, however, have a broad experience with anti-angiogenic agents in patients with uveal melanoma by treating the complications of radiation therapy. We will discuss tumor angiogenic processes and related molecular pathways in uveal melanoma. The role of VEGF and the potential use of current commercially and experimentally available anti-angiogenic drugs for the treatment of primary uveal melanoma and/or metastatic disease will be explained below.

The targeting of tumor-related vessels has been investigated for several decades. The fundamental belief that a tumor cannot grow beyond the limits of diffusion (about 1– 2 mm) of oxygen and nutrients from blood vessels has been advocated since the 1970s. Dr. J. Folkman has played a pivotal role, describing the molecular aspects of tumor angiogenesis and also predicting anti-angiogenic therapy. Furthermore, he demonstrated the importance of a potent tumor blood supply for the growth of metastases [1]. In uveal melanoma, metastasis occurs exclusively via the hematogenous route,emphasizing the importance of tumor vasculature [2].

Criscuolo et al.[3] were the first to describe the occurrence of vascular permeability increasing factor in malignant glioma, which we currently know as vascular endothelial growth factor (VEGF). The VEGF-A isotype, referred to as VEGF in this review, plays a key role in angiogenesis, regulating vasopermeability as well as the proliferation and migration of endothelial cells [4]. In several tumors (e.g. colon carcinoma, soft tissue sarcomas and gastric cancer), serum VEGF levels have been found to be a marker of disease stage and an indicator of metastasis [5-7]. In uveal melanoma, VEGF expression in sera of patients with a primary tumor, cannot predict survival. Nevertheless, serum VEGF levels are significantly higher in uveal melanoma patients with metastatic disease compared to patients without such spread [8,9].

In 2004, bevacizumab, the first angiogenesis inhibitor that targets VEGF, was developed, approved and licensed for intravenous infusion in the treatment of colorectal carcinoma [10]. Bevacizumab is also used for the treatment of metastases from several other malignancies, including renal and lung cancer [11,12] and is under investigation for other primary tumors (e.g. pancreas cancer and cutaneous melanoma) [13,14]. However, not all results are positive. It has been reported that VEGF inhibitors elicit tumor adaptation and increased lymphatic and distant metastasis in patients with pancreatic neuroendocrine carcinoma and glioblastoma-bearing mice [15]. In uveal melanoma, antiangiogenesis therapy has not yet been used for the treatment of primary uveal melanoma or related metastatic disease. Still, there has been extensive research into the effect of anti-angiogenic agents such as bevacizumab on uveal melanoma cells and animal models [16] (el Filali et al., submitted). Serendipitously, clinicians already have a broad experience with anti-angiogenic agents in patients with uveal melanoma as a result of treating the complications of radiation therapy of the primary tumor.

The role of VEGF as key mediator in tumor angiogenesis and as a main treatment target will be addressed as well as several other anti-angiogenic drugs for future treatment of primary uveal melanoma and/or metastatic disease.

TUMOR ANGIOGENESIS IN UVEAL MELANOMA

Several authors have investigated the role of blood vessels in uveal melanoma growth and metastasis.

Vascular Density

Microvessel density of uveal melanoma was studied by immunohistochemistry and has been found to correlate strongly with the risk of metastatic death [17]. Microvessel density was shown to be locally induced and not evenly distributed in the whole tumor [18,19]. In subsequent studies, specific 'hot spots' of vascular density have been shown to correlate with uveal melanoma- related metastatic death [20].

Extracellular Matrix Patterns and Vasculogenic Mimicry

Several extracellular matrix patterns have been described in uveal melanoma. When so-called closed loops and networks are present, they predict a worse 10- year probability of melanoma- specific survival (loops: 0.45 vs. 0.83; two- sided $p < 0.0001$, and networks: 0.41 vs. 0.72, two- sided $p < 0.0001$) [21]. Moreover, these patterns are also shown in uveal melanoma- related metastases and are described as 'vasculogenic mimicry'. This concept proposes the formation of fluid- conducting channels by tumor cells independent of local vascular outgrowth, without endothelium [22]. Vasculogenic mimicry has also been identified in several other malignancies and shown to be associated with aggressive tumor behavior [23]. The increased diffusion surface that these channels offer could allow continued growth of uveal melanoma.

Vasculature and Metastatic Disease

For metastases to occur, uveal melanoma cells must detach from the primary tumor and invade surrounding tissues to enter a nearby blood vessel, after which the cell can circulate systemically to a new location. A strong association has been observed between tumor cell ingrowth into blood vessels and extraocular extension, which is known to indicate a poor survival probability [24,25]. To form a metastatic tumor, the circulating malignant cells must exit the circulation and enter an organ, which in case of uveal melanoma is usually the liver [26,27]. The predominance of liver metastasis cannot be explained solely by blood circulation because the lungs are the first organ that uveal melanoma cells encounter. There must be a preferential microenvironment in which uveal melanoma cells proliferate more easily or quickly. Expression of insulin growth factor-1 receptor (IGF- 1R) in uveal melanoma offers a possible explanation for the bad prognosis [28]. IGF-1, the ligand for IGF-1R, leads to phosphorylation of IGF-1R, which in turn activates key signal molecules involved in cell proliferation [29]. IGF-1 is mainly produced by the liver and may explain the preferential growth of hepatic metastasis from uveal melanoma [29]. Besides a favorable microenvironment, the new location must provide a good blood supply. Interestingly, IGF-1 has been shown to stimulate secretion of VEGF in retinal pigment epithelial cells and possibly IGF-1 signaling is also involved in tumor angiogenesis in hepatic metastases from uveal melanoma [30].

Molecular Mediators of Angiogenesis

Uveal melanoma is characterized by slow progression and periods of dormancy, both of the primary tumor and of metastases. It has been suggested that this dormancy is associated with an avascular phase, in which a conversion to the angiogenic phenotype has yet to be established. This conversion, which is known as the 'angiogenic switch', is due to an alteration in the balance of inhibitory and stimulatory factors [31]. Folkman hypothesized that one important stimulatory factor, called tumor angiogenic factor, induces the tumor to convert to such an angiogenic phenotype. VEGF was later identified as one of the most potent tumor angiogenic factor molecules, which acts as the central mediator of tumor angiogenesis by regulating vasopermeability and the proliferation and migration of endothelial cells [4].

Another group of enzymes that has been implicated in tumor angiogenesis and the associated tissue remodeling is the family of metalloproteinases (MMPs) [32]. The major MMPs involved in tumor angiogenesis are MMP- 2, - 9, and - 14 [33]. The survival rate of patients with MMP- 2- and MMP- 9- positive uveal melanomas is worse than that of patients with MMP- 2- and MMP- 9- negative melanomas (31– 27 vs. 85%, $p < 0.05$) [34]. Epidermal growth factor (EGF) and its receptor (EGFR) also have an established role in tumorigenesis. EGF(R) is a potent proangiogenic factor able to induce migration of endothelial cells and regulate the production of angiogenic factors in tumor cells, such as VEGF, basic fibroblast growth factor and angiopoietin [35]. Other common angiogenic factors detected in tumors are platelet-derived growth factor, hepatocyte growth factor, and IGF family members [36]. In uveal melanoma, several of these proangiogenic factors have been analyzed. Boyd et al. [37], for example, demonstrated uveal melanoma cell expression of basic fibroblast growth factor at the protein level by immunohistochemistry and by RT- PCR in almost all tested samples (89%), especially around microvasculature. Expression of the receptors for hepatocyte growth factor and IGF are bad prognostic factors in uveal melanoma as described earlier [28].

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR

Structure

VEGF- α, also termed VEGF- A or VEGF, is a member of the VEGF platelet- derived growth factor family that also comprises placenta growth factor [38]. VEGF exists in a range of isoforms due to alternative splicing of the RNA: $VEGF_{121}$, $VEGF_{165}$ (the predominant form), $VEGF_{189}$, and $VEGF_{206}$ [39]. VEGF proteins are available to cells by at least two different mechanisms: (1) as freely diffusible proteins ($VEGF_{121}$, $VEGF_{165}$), or (2) after protease activation and cleavage of protein bound to heparin ($VEGF_{189}$, $VEGF_{206}$) [40].

The effects of VEGF are mainly mediated through binding to VEGF receptor 1 (Flt1) and VEGF receptor 2 (KDR), both of which are expressed on vascular endothelial cells as well as on tumor cells and on other cells in the tumor microenvironment [41]. Flt- 1 and KDR

are transmembrane tyrosine kinase receptors that become active upon ligand binding and thereby trigger signal transduction pathways that are involved in angiogenesis. VEGF receptor 3 (Flt- 4) is mainly involved in VEGF- C- and VEGF- D-mediated lymphangiogenesis [42].

Genetics

The human VEGF gene has been assigned to chromosome 6p21.3 [43]. Chromosome 6p gain in uveal melanoma has been reported in several studies and includes the VEGF locus [44,45]. A correlation between copy number changes in the 6p region and the expression of VEGF in uveal melanoma has not yet been established [46]. Moreover, abnormalities of chromosome 6 have been associated with a better survival in uveal melanoma patients, which seems to be in conflict with metastasis- promoting tumor angiogenesis [47]. Unfortunately, in this study, loss of 6q and gain of 6p were combined as one factor, thus limiting the evaluation of the role of 6p unclear.

Regulation

Several factors have been shown to participate in the regulation of VEGF expression. However, hypoxia is the best known factor and VEGF mRNA expression can be induced reversibly by exposure to low oxygen levels in many cell types [48]. The key regulator of hypoxia- induced VEGF is the transcription factor hypoxia- inducible factor (HIF)- 1α [49]. Under hypoxic conditions, HIF- 1α is stabilized and drives the expression of a large cluster of genes including VEGF and erythropoietin [50]. In tumors with significant necrosis, the expression of VEGF is mostly upregulated in the ischemic tumor cells adjacent to the necrotic areas [51].

Several cytokines or growth factors, such as EGF, platelet- derived growth factor, transforming growth factor β, interleukin 6, interleukin 1 and IGF-1, are also known to upregulate VEGF expression in several normal cells, including retinal pigment epithelial cells, and in tumor cells [4,30,52]. Uveal melanomas that overexpress one of these cytokines/growth factors or the receptors for these ligands might generate autocrine signaling that promotes tumor growth and tumor vascularization. Blocking IGFR with picropodophyllin in mice with induced choroidal neovascularization reduced VEGF levels and vessel formation [53]. In addition, picropodophyllin has been shown to inhibit uveal melanoma growth in vivo in uveal melanoma xenografts [54]. Furthermore, VEGF expression has been demonstrated to be increased in association with specific genetic events such as loss of tumor suppressor genes or activation of oncogenes. The von Hippel- Lindau tumor suppressor gene has been implicated in the regulation of VEGF gene expression [55]. Loss of von Hippel- Lindau protein function results in constitutive activation of HIF- 1α and thus VEGF expression [56,57].

Oncogenic mutations or amplification of *ras* and overexpression of v- *Src* have also been shown to upregulate VEGF [58]. Interestingly, we have demonstrated high Src activation in uveal melanoma that is associated with a constitutive activation of the mitogen- activated

protein kinase (MAPK) pathway and correlated with a bad prognosis [59] (el Filali et al., submitted).

Biological Function

VEGF is known to be involved in several different aspects of angiogenesis. After binding of VEGF to the VEGFR- 1 and - 2, several proteins are activated including focal adhesion kinase, PI3K and Src. These downstream kinases promote vascular permeability, endothelial cell proliferation, migration and survival [60]. Originally, VEGF was referred to as vascular permeability factor [61]. A rapid increase in vascular permeability occurs when the microvasculature is exposed acutely to any number of vascular permeabilizing factors, like VEGF, allowing the diffusion of trophic substances to adjacent tumor cells. VEGF promotes proliferation of endothelial cells through induction of the Raf- MEK- MAPK pathway and the formation of the endothelial lining of tumor vessels by attracting circulating endothelial cells. VEGF also activates focal adhesion kinase and the PI3- kinase- Akt pathway, inducing subsequent migration of endothelial cells expressing VEGFR- 2 [60]. In addition, VEGF is involved in cell survival (via PI3- kinase/Akt activation and antiapoptotic proteins) and monocyte activation, the description of which is beyond the scope of this chapter [62,63].

Expression and Implication of Vascular Endothelial Growth Factor in Uveal Melanoma

VEGF induction has been extensively demonstrated in a range of malignancies, including lung, breast, and gastrointestinal tract tumors [64-66]. In the eye, VEGF gene and protein expression are observed in ocular tissues, primarily in the retina and retinal pigment epithelium, and are particularly upregulated in retinopathies that are associated with angiogenic proliferation [67]. The first study that investigated VEGF gene expression in uveal melanoma applied RT- PCR to 7 uveal melanoma cell lines [68]. Subsequently, Sheidow et al. [69] showed VEGF immunostaining in uveal melanoma samples of enucleated eyes, but did not find any correlation between the occurrence of metastatic disease and the amount of VEGF expression in uveal melanoma tissue.

Using immunohistochemistry, Boyd et al. [70] showed only a moderate staining of VEGF in uveal melanoma samples (22%; n = 50). On the contrary, all uveal melanomas tested expressed VEGF mRNA (n = 20). Another publication by the same investigators describes elevated VEGF concentrations (up to 21.6 ng/ml) in vitreous and anterior chamber fluids of eyes with uveal melanoma compared to samples from healthy eyes (<0.96 ng/ml). Remarkably, the highest VEGF levels were found in fluids of eyes that had been treated with radiation [37]. In studies by Missotten et al. and others, elevated VEGF in the aqueous humor of eyes with uveal melanoma was confirmed and found to be correlated with largest basal tumor diameter and tumor height. In situ analysis further demonstrated that both the tumor cells as well as the retina cells express VEGF [71]. We further investigated the regulation of VEGF

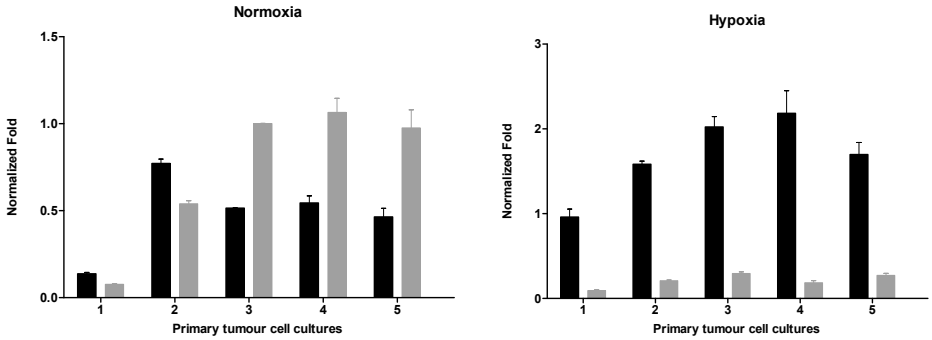


FIGURE 1. VEGF and TSP-1 mRNA expression in primary uveal melanoma cell cultures.
The amount of VEGF (black) and TSP-1 (gray) mRNA expression was measured with quantitative real- time RT- PCR in primary uveal melanoma cell cultures (cultures 1– 5) under normoxic (1% O2) and hypoxic (20% O2) conditions after 24 h. Expression is demonstrated in normalized fold.

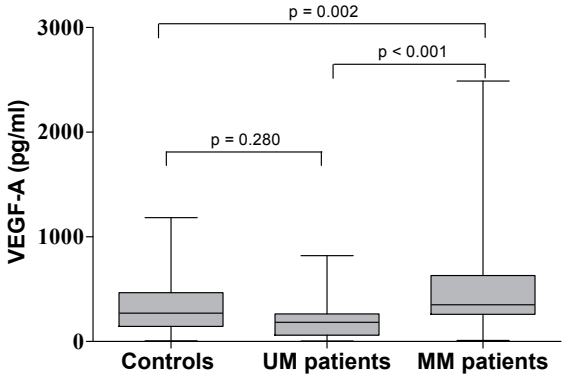


FIGURE 2. Concentration of serum VEGF- A in (metastatic) uveal melanoma patients and controls.
Concentration of serum VEGF- A in the control group (n = 50), and in patients with (n = 20) and without metastatic (n = 74) uveal melanoma. p values (Mann-Whitney test) between the different groups are indicated in the graph. Each box shows the median, quartiles (box length is the interquartile range) and whiskers represent the 90th and 10th percentiles (from el Filali et al. [9]). UM = Uveal melanoma; MM = metastatic melanoma.

in uveal melanoma and found that hypoxia massively induces HIF- 1α and VEGF in uveal melanoma cell lines and primary tumor cell cultures. On the contrary and as expected, TSP-1, an anti-angiogenic factor, was downregulated when uveal melanoma cells were exposed to ischemic conditions (fig. 1). VEGF expression in primary uveal melanoma samples (n = 27) was variable (range of expression 0.04– 9.55 normalized fold), and demonstrated no correlation with specific histological markers or prognosis. Upregulation of VEGF in uveal

melanoma cell lines, in response to hypoxia, did not increase cell proliferation [9]. The ability to modulate expression of VEGF by uveal melanoma cells may provide the tumor with the opportunity to initiate vascularization. Whether VEGF is essential for tumor angiogenesis should be analyzed in vivo, in a model resembling the tumor environment including paracrine signaling of endothelial cells.

In several tumors (e.g. colon carcinoma, soft tissue sarcomas and gastric cancer), serum VEGF levels have been found to be a marker of disease stage and an indicator of metastases [5,6,72]. Until recently, lactate dehydrogenase and alkaline phosphatase were the most indicative serum markers for metastatic disease in uveal melanoma, in combination with liver ultrasonography [73,74]. Elevated serum osteopontin, melanoma- inhibitory activity and S- 100β levels showed a correlation with metastatic uveal melanoma to the liver in some studies [75,76]. However, serum markers that indicate micrometastases at an early stage would be clinically preferable. In contrast to the immunohistochemical study of Sheidow et al. [69], several studies have observed VEGF expression in melanoma cell lines to be correlated with development of experimental metastasis [77,78]. In uveal melanoma, we found no difference in the amount of VEGF in sera of uveal melanoma patients compared to healthy people. However, VEGF levels are significantly raised in uveal melanoma patients with metastases compared with those without metastic disease ($p < 0.001$) (fig. 2). The same finding has recently been confirmed in other studies [79]. In addition, using a uveal melanoma mouse model, VEGF serum levels were increased in the presence of hepatic micro-metastases in hypoxic regions of the liver [80]. Also, Barak et al. demonstrated a significant increase of VEGF in sera of uveal melanoma patients after the occurrence of metastases; however, wide inter-patient variance prevents the use of a single VEGF serum level to be used as a marker for metastatic disease [8].

APPROVED ANTI-ANGIOGENIC TREATMENT IN CANCER

In the last two decades, most of the anticancer angiogenic treatments have focused on VEGF/VEGFR and EGF/EGFR, since these factors play such an important role in tumor angiogenesis. There are several other anti-angiogenic drugs that have been approved for the treatment of several different tumors (table 1). The four main methods used to block VEGF or any other angiogenic factors are:

1. neutralizing monoclonal antibodies against the factor or its receptor: bevacizumab, cetuximab, panitumumab, trastuzumab, ranibizumab;
2. small molecule tyrosine kinase inhibitors (TKIs) of receptors: sorafenib, sunitinib, erlotinib;
3. soluble receptors which act as decoy receptors: VEGF- Trap;
4. ribozymes which specifically target mRNA.

TABLE 1. Angiogenic inhibitors approved for tumor treatment

Drug	Method	Indications	Treatment
Bevacizumab (Avastin®)	Humanized anti-VEGF monoclonal antibody	Colorectal cancer, Non-small cell lung cancer, Advanced breast cancer	In combination with 5-FU-chemotherapy, carboplatin and paclitaxel
Sorafenib (Nexavar®)	Small molecule TK inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, and Raf-1.	Advanced renal cell carcinoma, Advanced hepatocellular carcinoma.	Monotherapy
Sunitinib (Sutent®)	Small molecule TK inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- β, and RET.	Gastrointestinal stromal tumor, advanced renal cell carcinoma	Monotherapy
Panitumumab (Vectibix®)	Humanized IgG2 anti-EGFR monoclonal antibody.	Metastatic colorectal cancer	Monotherapy after failed chemotherapy with fluoropyrimidine, oxaliplatin, and irinotecan.
Cetuximab (Erbitux®)	IgG1 anti-EGFR monoclonal antibody	Metastatic colorectal cancer, Head and neck cancer	Monotherapy and in combination with irinotecan and radiation
Erlotinib (Tarveca®)	Small molecule TK inhibitor of EGFR	Non-small cell lung cancer, Pancreatic cancer	Monotherapy after failed chemotherapy and combination with gemcitabine
Trastuzumab (Herceptin®)	Humanized IgG1 anti- HER-2 monoclonal antibody	Breast cancer	Monotherapy and in combination with doxorubicin, cyclophosphamide, and paclitaxel
Temsirolimus (Torisel®)	A small molecule inhibitor of mTOR and HIF-1α inhibitor	Advanced renal cell carcinoma	Monotherapy
Bortezomib (Velcade®)	Proteasome inhibitor; antiangiogenic (inhibition VEGF, IGF, Ang; mechanism unclear)	Multiple myeloma, Mantle cell lymphoma	Monotherapy after failed treatment
Thalidomide Thalomid®)	Immunomodulatoy, antiangiogenic properties; mechanism unclear	Multiple myeloma	In combination with dexamethasone

Due to the complexity of angiogenesis, as reviewed in the section on tumor angiogenesis, it is obvious that there may be several indirect ways to inhibit vessel growth besides the direct blocking of angiogenic factors. Temsirolimus (Torisel®), for instance, is an mTOR inhibitor that has direct antitumor activity by arresting cells in the G1 phase of the cell cycle and increasing apoptosis, but that also suppresses HIF- 1α transcription levels in tumor cells, thus reducing VEGF expression and angiogenesis [81]. Bortezomib (Velcade®) is a proteasome inhibitor that has been shown to inhibit VEGF, IGF-1, and angiopoietin by an unknown mechanism in multiple myeloma [82]. Thalidomide (Thalomid®) has been unpopular since the

1960s when its severe teratogenic effects were unknown and its use resulted in malformations of the extremities in unborn children of pregnant users. Nevertheless, thalidomide (or its derivative lenalidomide, introduced in 2004) has recently been shown to have potent anti-angiogenic properties, such as decreasing vascular density and successfully blocking angiogenic factors such as basic fibroblast growth factor, and VEGF and it is now under investigation for suppressing tumor angiogenesis [83]. Other approved and established drugs that have been found to exert anti-angiogenic activity include doxycycline and celecoxcib [84,85].

Additionally, multiple other agents targeting tumor angiogenesis in several different ways are still in (pre-)clinical investigation and should provide more treatment options in the future.

Anti- VEGF antibodies (bevacizumab) and TKIs (sunitinib and sorafinib) will be highlighted in the next section.

ANTI-ANGIOGENIC THERAPY IN UVEAL MELANOMA

At this time, no anti-angiogenic drugs are used clinically for the treatment of uveal melanoma or its metastases. Intravitreal use of bevacizumab in three cases of uveal melanoma who were wrongfully diagnosed as choroidal neovascularizations did not demonstrate inhibition of tumor growth [86].

With regard to the primary tumor, the current treatment includes enucleation, local resection and radiotherapy, either by brachytherapy (iodine or ruthenium), stereotactic or proton beam irradiation [87-90]. Radiotherapy achieves local tumor control in up to 97% of all treated eyes, and can therefore be regarded as being very effective [91,92]. One could therefore argue whether other therapies, such as anti-angiogenic drugs, are necessary. First of all, not all tumors can be irradiated: contraindications for irradiation are a tumor height of more than 10.0 mm (or 8.0 mm near the disk), a tumor diameter of more than 16.0 mm (although in the case of proton beam irradiation, larger tumors can be treated), when the tumor is not clearly defined by echography, is diffuse or multifocal, when there is neovascular or secondary glaucoma and in case of extrascleral extension [87,90]. In addition, radiation therapy can lead to radiation retinopathy, a delayed- onset complication characterized by retinal ischemia, neovascularization and leaking vessels [93,94]. Ultimately, radiation retinopathy can result in a severe decrease of visual acuity in the 'preserved' tumor-containing eye.

Anti-Angiogenic Treatment of Radiation Retinopathy

In eyes with uveal melanoma, bevacizumab is frequently utilized for the treatment of radiation retinopathy. Radiation retinopathy has been described in up to 63% of eyes after plaque radiation [95,96]. 'Off- label' use of intravitreal bevacizumab to treat macular edema and

neovascularization in radiation retinopathy demonstrates a decrease of macular edema and an improvement of visual acuity [97-99].

Other anti- VEGF agents besides bevacizumab have been widely used in ophthalmology this last decade in the treatment of age- related macular degeneration, diabetic macular edema and neovascular glaucoma. Pegaptanib (Macugen), an aptamer that only binds $VEGF_{165}$, was the first drug to receive approval for the treatment of macular degeneration. Although this drug is hardly used for any ocular pathology, one case study describes improved visual acuity after treatment with pegaptanib in a patient with proliferative radiation retinopathy [100]. Ranibizumab (Lucentis) is a recombinant humanized immunoglobulin monoclonal antibody fragment especially designed for intraocular use and is approved in many countries. The efficacy and safety of ranibizumab were evaluated in several randomized trials involving more than 1,000 patients with neovascular age- related macular degeneration and was shown to significantly maintain (90%) and improve (33%) visual acuity after 24 months [101]. In addition, treatment with ranibizumab also improved visual acuity in 4 of 5 patients with radiation maculopathy [102]. VEGF- Trap is a soluble protein that acts as a VEGF decoy receptor, and is currently undergoing phase 3 testing for age- related macular degeneration as well as for metastatic melanoma treatment [103].

Anti-Angiogenic Therapy for Uveal Melanoma Metastasis

Almost 50% of all uveal melanoma patients eventually develop metastatic disease, with the current 5- year uveal melanoma- related mortality ranging from 26 to 32% [27]. Life expectancy in the case of uveal melanoma- related metastatic disease ranges from 2 to 6 months since hardly any effective treatment is currently available; chemotherapy and local resection only prolong survival by a few months [104]. A number of anti-angiogenic agents may be of clinical use.

Bevacizumab

Yang et al. studied the effect of bevacizumab on the growth inhibition and number of hepatic micrometastases in an ocular melanoma mouse model, in which B16 melanoma cells were inoculated subchoroidally [16]. Bevacizumab was administered by intraperitoneal injection (starting dose: 50 or 250 μg/100 μl). Bevacizumab suppressed primary ocular melanoma growth and the formation of hepatic micrometastases in a dose- dependent manner ($p < 0.01$). In addition, bevacizumab significantly reduced the level of VEGF in the culture media of two human uveal melanoma cell lines.

In contrast, we found a rather unexpected effect of bevacizumab on uveal melanoma. Our mouse model consisted of B16 melanoma cells which were placed into the anterior chamber of the eye and bevacizumab (10 times the equivalent human dose, 20 μg/4 μl; equivalent human dose, 2 μg/4 μl) or a mock injection were given intraocularly. In vivo acceleration of intraocular tumor growth was observed in the eyes treated with bevacizumab, although it did

not influence B16 or uveal melanoma cell proliferation in vitro. Remarkably, bevacizumab did increase mRNA VEGF melanoma expression and HIF- 1α stabilization in vitro. This was especially seen under hypoxic conditions. Only after treatment with bevacizumab did we observe anterior chamber and tumor hemorrhages in murine eyes, emphasizing increased microvascular permeability, possibly due to induced VEGF expression. This 'pseudohypoxic' phenomenon has been described in other tumors and may be the consequence of a tumor adaptive or evasive response. It will be further elaborated in the following section (el Filali et al., submitted).

Sorafenib

Sorafenib, which inhibits VEGFR, has been tested in a xenograft model in which uveal melanoma cell line 92.1 was injected subcutaneously. Mangiameli et al. demonstrated inhibition of tumor growth ($p < 0.0035$) and fewer metastases after sorafenib treatment (33 vs. 60%) [105]. In patients with metastatic cutaneous melanoma, monotherapy with sorafenib has demonstrated hardly any antitumor activity [106]. Recently, the final results of a phase 3 trial, which compared treatment of metastatic (not including uveal) melanoma patients (n = 823) with carboplatin, paclitaxel and with either sorafenib (SCP) or a placebo (CP), did not demonstrate a difference in overall survival: the median overall survival for the SCP group was 11.1 months (95% CI 10.3– 12.3) and for the CP group 11.3 months (95% CI 9.7– 12.3) (ASCO meeting 2010, abstract number 8511).

Sunitinib

Sunitinib is another TKI which inhibits VEGFR [107]. There is not much preclinical evidence for antitumor activity in uveal melanoma. Still, a clinical benefit in advanced metastatic melanoma patients has recently been observed in a phase 2 trial analyzing the effect of sunitinib monotherapy. Three patients (8.3%) demonstrated a partial response, with a mean duration of 6.5 months. Nine had stable disease (25%), with a mean duration of 4.1 months (range: 3– 8.2 months), and 17 had progressive disease (47.2%) (ASCO meeting 2010, abstract number 8518). Although uveal melanoma is the most common intraocular tumor in adults (0.7/100,000 per year), it is still a relatively rare form of cancer. Conducting a good clinical trial in such a population is therefore quite challenging. There are ongoing and recruiting trials investigating bevacizumab, sorafenib and sunitinib as a single agent or in combination with other regimes. Most studies are focused on cutaneous metastatic melanoma. Fortunately, trials currently also include uveal melanoma patients and some of them even enroll only patients with ocular melanoma- related metastasis (clinicaltrials.gov). Hopefully this will give us some insight into current clinical anti-angiogenic treatments.

ADVERSE EFFECTS OF ANTI-ANGIOGENIC THERAPY

Vascular Endothelial Growth Factor Inhibitors

Clinical experience with predominantly bevacizumab has revealed that anti- VEGF therapy often prolongs overall survival of cancer patients by a few months, without really curing metastatic disease [108]. It has been proposed these past few years that VEGF inhibitors may actually promote tumorigenesis and metastatic dissemination on the long run [15,109]. Recently, the FDA has prohibited the use of bevacizumab (monotherapy) for metastatic breast cancer patients. When we observed tumor acceleration after treatment with bevacizumab in our mouse model, we also analyzed the effect on human uveal melanoma cells. In vitro treatment with bevacizumab induced VEGF mRNA expression in uveal melanoma cells. Moreover, we observed that this upregulation involved the HIF- 1α pathway (el Filali et al., submitted). VEGF inhibitors seem to elicit similar effects as described earlier for ischemic conditions that induce VEGF expression in uveal melanoma cells through the HIF- 1α pathway. The paradox of VEGF upregulation upon anti- VEGF treatment has been dubbed 'pseudohypoxia' and has been described before in other tumor studies. In mice bearing intracerebral glioma, it has been demonstrated that anti- VEGF treatment with pegaptanib (Macugen) increases GLUT- 1 expression (glucose transporter also upregulated by HIF- 1α) [51]. This 'pseudohypoxia' has also been shown to increase tumorigenesis in other types of cancer cells. Treatment of mice with pancreatic neuroendocrine tumors with anti-VEGFR also resulted in an initial response of tumor stasis followed by tumor recurrence. The relapsing tumor expressed higher levels of mRNAs of proangiogenic factors and demonstrated several hypoxic regions [110]. Furthermore, treated mice developed more invasive tumors and metastatic lesions, all characterized by hypoxic regions [15].

Ischemic conditions caused by anti- VEGF treatment can also lead to recruitment of various bone marrow- derived cells that have angiogenic capacities. Proangiogenic monocytes induce vessel growth by expression of several cytokines and angiogenic factors. In mice bearing glioblastoma multiforme tumors treated with bevacizumab, the stabilization of HIF- 1α has been demonstrated to promote angiogenesis by inducing recruitment of mature F4/80+ macrophages [111,112]. Additionally, a clinical study suggests that hypoxia determines survival outcome in patients treated with bevacizumab for glioblastoma multiforme [113]. Since it has previously been shown that malignant uveal melanoma tumors in patients with a poor survival contain many macrophages, this mechanism is especially relevant [114]. Moreover, we may be observing in our experiments resistance of the tumor cells, after an initial response phase, to adapt or evade therapy by inducing mechanisms that reduce dependence on neovascularization, leading to changed tumor proliferation. The 'pseudohypoxic' conditions could be responsible for selection of more malignant tumor cells, which are less sensitive to anti-angiogenic treatment and switch on other malignant pathways that result in proliferation, migration and invasion. Besides angiogenesis and vascular permeability, VEGF has

been shown to activate the ras/ raf pathway through activation of the tyrosine kinase VEGF receptors and the downstream MAPKs [115,116]. MAPK- driven proliferation has been shown to play an important role in uveal melanoma growth through upstream Src signaling [59].

Tyrosine Kinase Inhibitors

TKI side effects are related to their nonspecific nature [117]. In order to be able to predict treatment outcome, one should know the effect of TKIs on the different pathways and how these pathways may interact. Sorafenib treatment of cutaneous melanoma patients may have been disappointing because the combined effect on all inhibited kinases turned out to be negative for tumor inhibition [118]. Although the capacity of TKIs to target multiple kinases is interesting because of its wide application in several different malignancies, it also results in many 'off-target' effects demonstrated in several clinical trials. For example, hand- foot skin reactions, fatigue, stomatitis, diarrhea, hair color changes, myelosuppression, and thyroid dysfunction are frequently associated with TKI treatment. In addition, the low effectiveness of available TKIs requires higher doses. Unfortunately, higher doses are in turn associated with increased blockade of nontarget kinases due to low selectivity, again resulting in toxicity. The off- target effects of TKIs have also limited their use in combination with chemotherapeutic drugs due to overlapping toxicity profiles. Recently, treatment with sunitinib and sorafenib has been associated with cardiovascular toxicity as an adverse event [119]. These limitations have led to the development of more selective and potent anti- VEGFR TKIs [120].

CONCLUSION

Uveal melanoma remains a highly lethal tumor, which results in metastatic disease in almost 50% of all patients despite adequate primary tumor treatment. It is therefore important to investigate different treatment options to be used in curative or preventive therapy of uveal melanoma- related metastatic disease. Tumor angiogenesis has been demonstrated to be of great importance in tumor growth and dissemination. In addition, tumor vessel formation is also complex and extensive, involving various molecular mediators and pathways. Anti-angiogenic therapy has focused on VEGF, which has been implicated in uveal melanoma angiogenesis. Unfortunately, experimental and clinical trials using anti- VEGF monotherapy have been disappointing. In addition, VEGF inhibitors may actually promote tumorigenesis and metastatic dissemination. The key to effective treatment is good patient and tumor selection. The inhibition of protein activity by small molecules appears to be a promising approach for several types of malignancies. For example, imatinib has been analyzed for treatment of uveal melanoma- related metastases in a clinical trial, based on c- Kit overexpression and the in vitro response of cell lines with c- Kit expression to imatinib mesylate [121]. Treatment with imatinib mesylate did not result in improved survival, which may be due to absence of c- Kit

upregulation in the patients in the trial because patients had been treated irrespective of their c- Kit tumor status [122,123]. This could also be the case with anti-angiogenic therapies in which patients are treated irrespective of the angiogenic profile and VEGF/VEGFR expression of their uveal melanoma and/or metastases. We demonstrated that angiogenesis and especially VEGF expression can easily be modulated by the uveal melanoma cells themselves, either by tumor microenvironment or due to VEGF inhibitors. In addition, structures identified as vasculogenic mimicry may provide uveal melanoma with an alternative tumor circulation. Therefore, one could still question whether tumor angiogenesis and the angiogenic switch are necessary for uveal melanoma growth and malignant dissemination. They may merely be a consequence of tumor growth.

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