KNOWLEDGE-BASED TREATMENT IN UVEAL MELANOMA

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PROEFSCHRIFT

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Mariam el Filali
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in 1982

Promotiecommissie

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Leid je leven en wees ofwel een geleerde, ofwel een student, ofwel iemand die aandachtig luistert (naar religieuze kennis) ofwel iemand die houdt van (kennis en de geleerden), maar wees nooit het vijfde (een onwetende), anders zal je in het verval terechtkomen.

Ali Ibn Abi Taalib - de vierde rechtgeleerde Kalief (Radiya Allaho ‘Anho)

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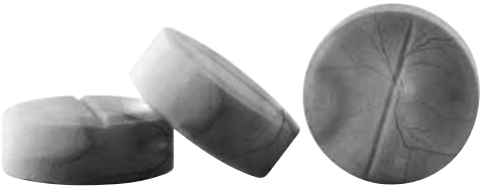
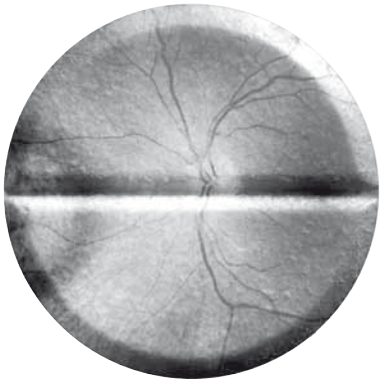


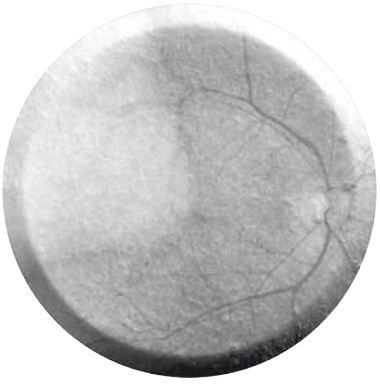
PART I

BACKGROUND OF UVEAL MELANOMA

CHAPTER 1

GENERAL INTRODUCTION





AIMS OF THIS THESIS

This thesis describes studies that all strive towards 'Knowledge-based treatment of uveal melanoma' and involves three different yet related topics.

The first part of this thesis analyzes the role of the tumor microenvironment and of malignant development of blood vessels in uveal melanoma and potential treatments.

The second part explores future treatment options in uveal melanoma by development and identification of specific targets for treatment.

The third part focuses on the evaluation of potential cancer treatments and explores different molecular mechanisms that play a role in uveal melanoma growth and dissemination.

UVEAL MELANOMA

Ocular melanoma accounts for only 5% of all melanomas and arises in four ocular tissues, namely the uveal tract, conjunctiva, eyelid, and orbit. Uveal melanoma (UM) is the most common of the ocular melanomas (85 %) with an annual incidence of seven cases per million adults per year in Caucasian populations, and develops in the uvea which consist of the iris, ciliary body and choroid. Most melanocytic tumors of the uveal tract arise in the choroid (80 %), which is one of the most capillary-rich tissues of the body [1]. Symptoms of a uveal melanoma include blurred vision, flashing lights and seeing shadows. Often, there are no symptoms, and therefore 30 % of the tumors is only discovered during routine examination [2].

Although much progress has been made in the last decades in the local treatment of uveal melanoma, prognosis is still poor. Uveal melanoma often is a deadly disease, with a 5-year mortality ranging from 26% to 32% [3]. Uveal melanoma spreads hematogeneously, mainly to the liver [4]. In case of metastases, life expectancy is merely a few months (2-6 months) [5].

Mechanisms involved in uveal melanoma development are largely unknown. Uveal melanoma is approximately 20 to 30 times more common in white people compared to black and Asian people [6]. In addition, light skin color and light iris color are established risk factors [7,8]. Still, unlike cutaneous sun-related malignancies, ultraviolet light exposure does not appear to be a consistent riskfactor in uveal melanoma [9]

Other known risk factors related to survival are histologic cell type, tumor diameter, tumor location, age, and gender [10]. Loss of chromosome 3 is one of the most significant predictors for uveal melanoma-related death [11,12]. In addition, concurrent loss of chromosome 1p and 6q and additional copies of 8q and 6p have also been shown to be potentially implicated in uveal melanoma survival [13,14]. Furthermore, a gene expression-based molecular classification has been described [15,16]. Tumors with a class 2 molecular "signature" are at high risk for the development of metastatic disease compared to tumors with a class 1 "signature".

TREATMENT OF METASTATIC AND PRIMARY UVEAL MELANOMA

Regarding metastatic uveal melanoma, current available treatments include chemotherapy, intra-hepatic arterial liver perfusion, chemoembolization and local resection [17-19]. Although some individual successes have been described using these treatments, unfortunately, the overall survival can as yet only be prolonged with a few months [20].

In case of primary UM, several treatment options are available. For tumors with a prominence of more than 10 mm (or 8.0 mm near the disc), enucleation is often the first choice of treatment. Small to medium-sized tumors with a prominence of less than 10 mm can be treated with proton beam therapy, stereotactic irradiation, or (plaque) brachytherapy, occasionally in combination with transpupillary thermo therapy (sandwich therapy) [21-23,23-25]. Radiation therapy results in destruction of the intraocular tumor, either through direct damage to a cell's DNA, which blocks cell division, or through damage to capillary endothelium, affecting the tumor's blood supply [26-28]. Brachytherapy is a highly successful treatment, achieving local tumor control of UM in 90-97% of the cases [29,30]. Unfortunately, radiation therapy is also associated with a range of complications, including hemorrhages into the tumor or the vitreous, cataract, optic neuropathy, and most commonly, radiation retinopathy.

RADIATION RETINOPATHY

Radiation retinopathy is a slowly-progressive, delayed-onset disease of retinal blood vessels due to changes in the structure and permeability of retinal vessels [31-33], and has been described to occur in up to 63% of eyes after plaque radiation treatment [34-37]. On average, the onset of radiation retinopathy occurs at 26 months after radioactive plaque therapy [38].

Ophthalmoscopic and fluorescein-angiographic findings characteristic of radiation retinopathy include macular changes such as macular edema, capillary non-perfusion, cotton wool spots, capillary telangiectasia, retinal neovascularization, micro aneurysms, retinal hemorrhages, intraretinal exudation, and neuronal changes: disc edema, disc pallor, optic atrophy, and neovascularization of the disk [31] (Figure 1).

Clinically, these signs are often identical to the findings seen in diabetic retinopathy. The threshold for radiation retinopathy depends on the total dose, the volume of irradiated retina, the fractionation scheme, and individual factors. In general, a greater total dose results in earlier, more severe and more pronounced radiation retinopathy [39]. In spite of radiation dose reduction during the last decade, vision loss after therapy remains extensive, particularly vision loss due to macular edema. In one follow-up study, out of 31 uveal melanoma patients with a moderate to good vision ($\geq$ 20/60 or 0.3) before radiation treatment, 5 years later, only 12 showed preservation of vision [21].

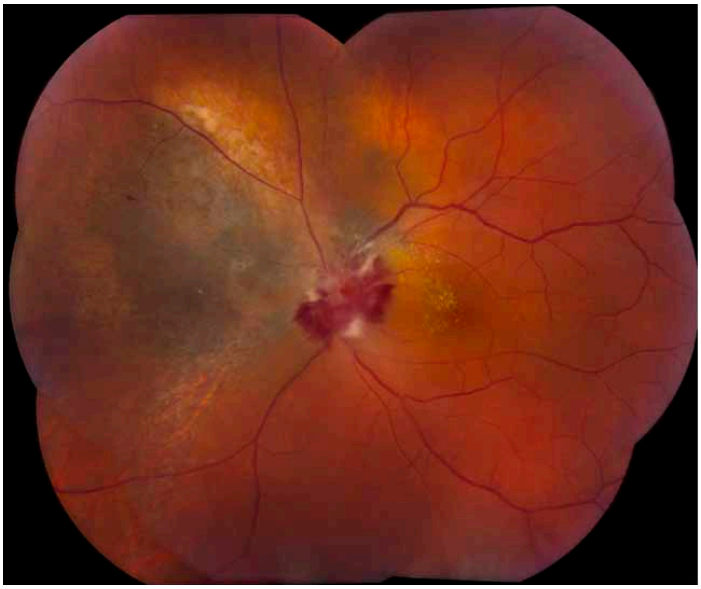


FIGURE 1. Radiation retinopathy due to Ruthenium brachytherapy of a choroidal melanoma.

ANGIOGENESIS AND ANTI-ANGIOGENIC THERAPY

Retinal ischemia and leaking vessels are the two most relevant factors in radiation retinopathy. It has been observed that 55% of eyes, that underwent enucleation after plaque radiation for treatment failure, demonstrate vascular anomalies [34]. As yet, there is no effective treatment to reverse visual loss from ischemic maculopathy due to capillary nonperfusion [31]. However, several anti-angiogenic agents have been used to resolve macular edema and prevent neovascularization. Corticosteroids such as triamcinolone acetonide (TA) have been shown to improve vision for a few months in patients with macular edema associated with diabetic retinopathy [40-43] and in patients with exudative age-related macular degeneration (ARMD) [44]. In addition, a recent study reported a temporary positive effect of intravitreal TA injections in a group of 31 patients with radiation maculopathy [45]. Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that has a crucial role in the formation of new vessels and most likely contributes to the pathogenesis of radiation retinopathy. Hence anti-angiogenic treatments that target VEGF, such as bevacizumab and ranibizumab, are also used for radiation retinopathy. Anti-angiogenic treatments are further discussed in (*Chapter 3*)

Angiogenesis is an important process in both radiation retinopathy and tumor progression. Besides the supply of oxygen and nutrients, tumor-associated vessels promote metastasis by facilitating tumor cell entry into the circulation [46]. Unquestionably, this phenomenon plays a role in uveal melanoma, which metastasizes almost completely hematogenously [4,5]. Several studies have shown that in uveal melanoma a high vascular density and ingrowth of tumor

cells into the lumen of tumor blood vessels or into a scleral vessel is associated with poor survival [47-49]. Furthermore, uveal melanoma cells are capable of forming a second microcirculation consisting of "looping" patterns of extracellular matrix independent of angiogenesis, called vasculogenic mimicry [50]. The potential to target tumor vessels has been investigated for these past decades in several malignancies and mainly focuses on the key mediator of angiogenesis, which is VEGF. Tumor angiogenesis, the role of VEGF, and anti-angiogenic therapy in cancer and particularly uveal melanoma is further reviewed in *Chapter 3*.

TARGETED THERAPY

Cancer growth depends on many different mechanisms, such as cell proliferation, angiogenesis etc. as described earlier. The combination of different treatments will probably render tumor therapy successful in the future. However, two main obstacles have to be overcome; a specific and effective drug-delivery, and early detection of (micro) metastases. In uveal melanoma, the general accepted hypothesis is that the tumor remains dormant in the bone marrow and liver until becoming clinically detectable in the liver [51]. Identification and inhibition of such micrometastases will prevent the occurrence of 'full-blown' metastatic disease and possibly improve survival in uveal melanoma patients. Effective drug-delivery that induces apoptosis and targets angiogenesis and cell signalling through uveal melanoma-specific ligands would be beneficial.

Other approaches may be immunological. Exploration of (cutaneous) melanoma-derived autoantigens and cell surface receptors has revealed potential targets in uveal melanoma. Currently, the most specific uveal melanoma markers include S-100 (specific for a protein derived from bovine brain cross-reacting with melanoma and melanocytes), HBM45 (specific for gp100) and A103, (recognizes the Melan-A/Mart-1 protein) [52-54]. Sadly, most of these receptors do not exhibit specificity for uveal melanoma and are also expressed by melanocytes or possibly other neural crest-derived cells, and treatment could cause significant side-effects.

PROLIFERATION PATHWAY

What do we know regarding the development and growth of a primary uveal melanoma? Although cutaneous melanoma and uveal melanoma share the same embryonic origin, no similarities can be found regarding mutations that regulate proliferation and cause loss of cell cycle control. Activation of the MAPK pathway in cutaneous melanoma occurs for instance through mutations of the NRAS and BRAF genes (60% of cases) [55,56]. Only BRAF

mutations have been demonstrated in uveal melanoma, though relatively uncommon and are therefore less relevant in this disease [57].

Recently, mutations in uveal melanoma tissue were identified in the GNAQ and GNA11 genes which are located on chromosome 9 and 19, respectively. GNAQ and GNA11 encode for Gαq-type subunits of the heterotrimeric G-protein. Mutations of glutamine encoding codon 209 and (less frequently) R183 can result in constitutive G-protein activation which mediates intracellular signals and activates the RAS-RAF-MEK-ERK or the classical mitogen-activated protein kinase (MAPK) pathway [58]. MAPK activation is crucial in the development of melanocytic neoplasia and constitutive activation of this pathway has been associated with many different types of cancer [59,60]. Mutations in the GNAQ gene were shown to be common in primary UM (45%) and in UM metastases (22%) [61]. GNA11 mutations were present in 32% of primary UM and 57% of UM metastases [62,63]. Cellular components that couple GNAQ/GNA11 to MAPK signaling are most likely the effectors IP3, Src tyrosine kinase [64] and PKC [65].

SRC AND KINASE INHIBITORS

Src is one of the longest known proto-oncogenes and is named after its viral counterpart v-Src, which was first described by Peyton Rous almost 100 years ago [66,67]. Since then, expression and involvement of Src has been demonstrated in several malignancies implicating four 'hallmarks' of cancer; proliferation, angiogenesis, cell survival and migration [66,68,69].

Increased Src signalling correlates with decreased E-cadherin expression and decreased cell-cell adhesion [70,71]. In addition, downstream substrates of Src seem to act largely in parallel to increase cell proliferation and survival [67]. Furthermore, Src activation is associated with increased expression of proangiogenic cytokines such as VEGF, and thus tumor angiogenesis [72,73].

Interestingly, several commercially-available pharmacologic agents are able to inhibit Src. Dasatinib for instance is a small-molecule inhibitor taken orally that has been demonstrated to inhibit proliferation in several malignancies in vitro [74,75] (Figure 2). Like most tyrosine kinase inhibitors, dasatinib also variably inhibits other (receptor) tyrosine kinases such as, BCR-ABL, c-KIT, PDGFR, and ephrin A2 [74,76].

OUTLINE

The first part of this thesis, *Chapter 2 and 3*, describes the literature regarding tumor angiogenesis, the role of vascular endothelial growth factor as key mediator of vessel growth, and the potential of anti-angiogenic therapies that are approved for treatment of several

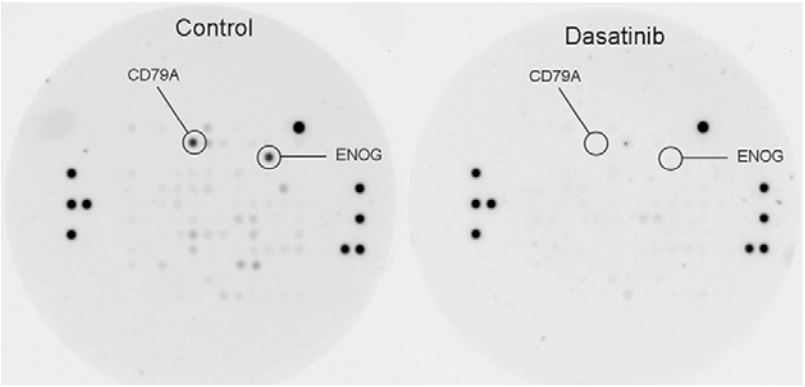


FIGURE 2. Dasatinib treatment (right) inhibits Src kinase-related lysate activity in metastatic uveal tissue analyzed with a Pamgene tyrosine kinase array.

malignancies or may be used in the future. Implications and studies involving uveal melanoma are highlighted in particular. In the second part, *Chapter 4,* tumor angiogenesis in uveal melanoma is experimentally analyzed. We demonstrate the involvement of hypoxia and the HIF-1α pathway in the induction of VEGF expression in uveal melanoma cell lines and cultures. In addition, we describe the association of high VEGF expression in sera of patients and the presence of uveal melanoma related metastases. In *Chapter 5*, we analyze the effect of two frequently used anti-angiogenic intraocular drugs for the treatment of radiation retinopathy caused by irradiation of uveal melanoma. Since local recurrences develop in some cases, the possibility that there are still living uveal melanoma cells present in eyes treated with radiotherapy cannot be excluded [77]. We found that triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth in vitro, and therefore most likely will not give rise to recurrences. On the contrary, we demonstrate in *Chapter 6,* that bevacizumab induces intraocular tumor growth in mice. Although bevacizumab did not stimulate UM cell proliferation in vitro, VEGF expression via the HIF-1α pathway was induced, resulting in a 'pseudohypoxic' condition. This phenomenon has been described in other tumor types and may be the consequence of tumor adaptive or evasive resistance.

Part III describes the search for specific uveal melanoma ligands to be used for targeted therapy or early identification of micrometastases. Somatostatin receptor subtype 2 (SSTR2) for instance is expressed in several tumors, and synthetic radiolabelled antagonists like octreotide and octreotate are already being used as diagnostic or therapeutic agents for gastroenteropancreatic and neuroendocrine tumors [78-81]. Although uveal melanoma cells and somatostatin-producing cells both originate from the neural crest, a low expression of SSTR2 was found in primary uveal melanoma specimens and in uveal melanoma cell lines. Furthermore, SSTR2 expression was not associated with tumor-free survival or any known prognostic factor *(Chapter 7).*

On the contrary, we describe in *Chapter 8* the identification of uveal melanoma-specific peptides (UMAPs) by in vitro panning using phage peptide libraries. Additionally, synthetic constructed peptides were shown to successfully internalize targeted UM cells.

In the final chapters of this thesis, the findings of all experiments are summarized and future implications and treatment options are discussed.

Finally, part IV describes the implication of the classical mitogen-activated protein kinase (MAPK) pathway in uveal melanoma. In *Chapter 9*, we identify Src as a determinant of ERK1/2 activation and show that Src expression and kinase activity together with ERK1/2 activation is reduced in UM metastases cell lines compared to primary UM cells. In addition, we demonstrate in *Chapter 10* the inhibition of MAPK activation via Src using the Src kinase inhibitor Dasatinib. Growth arrest was achieved in 60% of the tested UM cultures and the potential of growth inhibition may be predicted by MAPK and Src kinase activity. Furthermore, the sensitive cell cultures predominantly displayed monosomy 3 and, in a large set of UM samples, Src expression level was significantly associated with monosomy 3.

In summary, knowledge of side effects of current intraocular treatments prevents recurrences and may protect the uveal melanoma-containing eye, and hence *preserve vision.*

Pre-clinical evaluation of treatment efficacy and safety based on tumor physiology renders the possibility of new treatment options to treat and/or *prevent metastases.*

REFERENCE LIST

1. Singh, A. D., Bergman, L., and Seregard, S. *Uveal melanoma: epidemiologic aspects.* Ophthalmol.Clin. North Am. 2005. 18:75-84, viii.
2. Damato, B. *Detection of uveal melanoma by optometrists in the United Kingdom.* Ophthalmic Physiol Opt. 2001. 21:268-271.
3. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
4. Augsburger, J. J., Correa, Z. M., Freire, J., and Brady, L. W. *Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy.* Ophthalmology 1998. 105:1670-1678.
5. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
6. Stang, A., Parkin, D. M., Ferlay, J., and Jockel, K. H. *International uveal melanoma incidence trends in view of a decreasing proportion of morphological verification.* Int.J.Cancer 2005. 114:114-123.
7. Gallagher, R. P., Elwood, J. M., Rootman, J., Spinelli, J. J., Hill, G. B., Threlfall, W. J., and Birdsell, J. M. *Risk factors for ocular melanoma: Western Canada Melanoma Study.* J.Natl.Cancer Inst. 1985. 74:775-778.
8. Holly, E. A., Aston, D. A., Char, D. H., Kristiansen, J. J., and Ahn, D. K. *Uveal melanoma in relation to ultraviolet light exposure and host factors.* Cancer Res. 1990. 50:5773-5777.
9. Schmidt-Pokrzywniak, A., Jockel, K. H., Bornfeld, N., Sauerwein, W., and Stang, A. *Positive interaction between light iris color and ultraviolet radiation in relation to the risk of uveal melanoma: a case-control study.* Ophthalmology 2009. 116:340-348.
10. Mooy, C. M. and de Jong, P. T. *Prognostic parameters in uveal melanoma: a review.* Surv.Ophthalmol. 1996. 41:215-228.
11. Prescher, G., Bornfeld, N., Hirche, H., Horsthemke, B., Jockel, K. H., and Becher, R. *Prognostic implications of monosomy 3 in uveal melanoma.* Lancet 1996. 347:1222-1225.
12. Sisley, K., Rennie, I. G., Parsons, M. A., Jacques, R., Hammond, D. W., Bell, S. M., Potter, A. M., and Rees, R. C. *Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis.* Genes Chromosomes.Cancer 1997. 19:22-28.
13. Kilic, E., Naus, N. C., van Gils, W., Klaver, C. C., van Til, M. E., Verbiest, M. M., Stijnen, T., Mooy, C. M., Paridaens, D., Beverloo, H. B., Luyten, G. P., and de Klein, A. *Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients.* Invest Ophthalmol.Vis.Sci. 2005. 46:2253-2257.
14. Aalto, Y., Eriksson, L., Seregard, S., Larsson, O., and Knuutila, S. *Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2001. 42:313-317.
15. Onken, M. D., Worley, L. A., Ehlers, J. P., and Harbour, J. W. *Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death.* Cancer Res. 2004. 64:7205-7209.
16. Tschentscher, F., Husing, J., Holter, T., Kruse, E., Dresen, I. G., Jockel, K. H., Anastassiou, G., Schilling, H., Bornfeld, N., Horsthemke, B., Lohmann, D. R., and Zeschnigk, M. *Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities.* Cancer Res. 2003. 63:2578-2584.
17. Leyvraz, S., Spataro, V., Bauer, J., Pampallona, S., Salmon, R., Dorval, T., Meuli, R., Gillet, M., Lejeune, F., and Zografos, L. *Treatment of ocular melanoma metastatic to the liver by hepatic arterial chemotherapy.* J.Clin.Oncol. 1997. 15:2589-2595.

18. Mavligit, G. M., Charnsangavej, C., Carrasco, C. H., Patt, Y. Z., Benjamin, R. S., and Wallace, S. *Regression of ocular melanoma metastatic to the liver after hepatic arterial chemoembolization with cisplatin and polyvinyl sponge.* JAMA 1988. 260:974-976.
19. Becker, J. C., Terheyden, P., Kampgen, E., Wagner, S., Neumann, C., Schadendorf, D., Steinmann, A., Wittenberg, G., Lieb, W., and Brocker, E. B. *Treatment of disseminated ocular melanoma with sequential fotemustine, interferon alpha, and interleukin 2.* Br.J.Cancer 2002. 87:840-845.
20. Augsburger, J. J., Correa, Z. M., and Shaikh, A. H. *Effectiveness of treatments for metastatic uveal melanoma.* Am.J.Ophthalmol. 2009. 148:119-127.
21. Bartlema, Y. M., Oosterhuis, J. A., Journee-De Korver, J. G., Tjho-Heslinga, R. E., and Keunen, J. E. *Combined plaque radiotherapy and transpupillary thermotherapy in choroidal melanoma: 5 years' experience.* Br.J.Ophthalmol. 2003. 87:1370-1373.
22. Journee-de Korver, J. G., Oosterhuis, J. A., Kakebeeke-Kemme, H. M., and de Wolff-Rouendaal, D. *Transpupillary thermotherapy (TTT) by infrared irradiation of choroidal melanoma.* Doc.Ophthalmol. 1992. 82:185-191.
23. Melia, B. M., Abramson, D. H., Albert, D. M., Boldt, H. C., Earle, J. D., Hanson, W. F., Montague, P., Moy, C. S., Schachat, A. P., Simpson, E. R., Straatsma, B. R., Vine, A. K., and Weingeist, T. A. *Collaborative ocular melanoma study (COMS) randomized trial of I-125 brachytherapy for medium choroidal melanoma. I. Visual acuity after 3 years COMS report no. 16.* Ophthalmology 2001. 108:348-366.
24. Gragoudas, E. S., Lane, A. M., Munzenrider, J., Egan, K. M., and Li, W. *Long-term risk of local failure after proton therapy for choroidal/ciliary body melanoma.* Trans.Am.Ophthalmol.Soc. 2002. 100:43-48.
25. Gragoudas, E. S., Goitein, M., Verhey, L., Munzenreider, J., Suit, H. D., and Koehler, A. *Proton beam irradiation. An alternative to enucleation for intraocular melanomas.* Ophthalmology 1980. 87:571-581.
26. Gragoudas, E. S., Goitein, M., Verhey, L., Munzenreider, J., Suit, H. D., and Koehler, A. *Proton beam irradiation. An alternative to enucleation for intraocular melanomas.* Ophthalmology 1980. 87:571-581.
27. Char, D. H., Castro, J. R., Quivey, J. M., Phillips, T. L., Irvine, A. R., Stone, R. D., and Kroll, S. *Uveal melanoma radiation. 125I brachytherapy versus helium ion irradiation.* Ophthalmology 1989. 96:1708-1715.
28. Goodman, D. F., Char, D. H., Crawford, J. B., Stone, R. D., and Castro, J. R. *Uveal melanoma necrosis after helium ion therapy.* Am.J.Ophthalmol. 1986. 101:643-645.
29. Shields, C. L., Cater, J., Shields, J. A., Chao, A., Krema, H., Materin, M., and Brady, L. W. *Combined plaque radiotherapy and transpupillary thermotherapy for choroidal melanoma: tumor control and treatment complications in 270 consecutive patients.* Arch.Ophthalmol. 2002. 120:933-940.
30. Jampol, L. M., Moy, C. S., Murray, T. G., Reynolds, S. M., Albert, D. M., Schachat, A. P., Diddie, K. R., Engstrom, R. E., Jr., Finger, P. T., Hovland, K. R., Joffe, L., Olsen, K. R., and Wells, C. G. *The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma: IV. Local treatment failure and enucleation in the first 5 years after brachytherapy. COMS report no. 19.* Ophthalmology 2002. 109:2197-2206.
31. Guyer, D. R., Mukai, S., Egan, K. M., Seddon, J. M., Walsh, S. M., and Gragoudas, E. S. *Radiation maculopathy after proton beam irradiation for choroidal melanoma.* Ophthalmology 1992. 99:1278-1285.
32. Archer, D. B. and Gardiner, T. A. *Ionizing radiation and the retina.* Curr.Opin.Ophthalmol. 1994. 5:59-65.
33. Archer, D. B., Amoaku, W. M., and Gardiner, T. A. *Radiation retinopathy--clinical, histopathological, ultrastructural and experimental correlations.* Eye (Lond) 1991. 5 (Pt 2):239-251.

34. Sia, S., Harper, C., McAllister, I., and Perry, A. *Iodine-I25 episcleral plaque therapy in uveal melanoma.* Clin.Experiment.Ophthalmol. 2000. 28:409-413.
35. Stack, R., Elder, M., Abdelaal, A., Hidajat, R., and Clemett, R. *New Zealand experience of I125 brachytherapy for choroidal melanoma.* Clin.Experiment.Ophthalmol. 2005. 33:490-494.
36. Jensen, A. W., Petersen, I. A., Kline, R. W., Stafford, S. L., Schomberg, P. J., and Robertson, D. M. *Radiation complications and tumor control after 125I plaque brachytherapy for ocular melanoma.* Int.J.Radiat.Oncol.Biol.Phys. 2005. 63:101-108.
37. Jones, R., Gore, E., Mieler, W., Murray, K., Gillin, M., Albano, K., and Erickson, B. *Posttreatment visual acuity in patients treated with episcleral plaque therapy for choroidal melanomas: dose and dose rate effects.* Int.J.Radiat.Oncol.Biol.Phys. 2002. 52:989-995.
38. Finger, P. T. and Kurli, M. *Laser photocoagulation for radiation retinopathy after ophthalmic plaque radiation therapy.* Br.J.Ophthalmol. 2005. 89:730-738.
39. Harris, J. R. and Levene, M. B. *Visual complications following irradiation for pituitary adenomas and craniopharyngiomas.* Radiology 1976. 120:167-171.
40. Ferrara, N., Gerber, H. P., and LeCouter, J. *The biology of VEGF and its receptors.* Nat.Med. 2003. 9:669-676.
41. Er, H. and Yilmaz, H. *Intravitreal cortisone injection for refractory diffuse diabetic macular edema.* Ophthalmologica 2005. 219:394-400.
42. Patelli, F., Fasolino, G., Radice, P., Russo, S., Zumbo, G., DI Tizio, F. M., Frisone, G., and Marchi, S. *Time course of changes in retinal thickness and visual acuity after intravitreal triamcinolone acetonide for diffuse diabetic macular edema with and without previous macular laser treatment.* Retina 2005. 25:840-845.
43. Sorensen, T. L., Haamann, P., Villumsen, J., and Larsen, M. *Intravitreal triamcinolone for macular oedema: efficacy in relation to aetiology.* Acta Ophthalmol.Scand. 2005. 83:67-70.
44. Jonas, J. B., Spandau, U. H., Kamppeter, B. A., and Harder, B. *Follow-up after intravitreal triamcinolone acetonide for exudative age-related macular degeneration.* Eye 2007. 21:387-394.
45. Shields, C. L., Demirci, H., Dai, V., Marr, B. P., Mashayekhi, A., Materin, M. A., Manquez, M. E., and Shields, J. A. *Intravitreal triamcinolone acetonide for radiation maculopathy after plaque radiotherapy for choroidal melanoma.* Retina 2005. 25:868-874.
46. Papetti, M. and Herman, I. M. *Mechanisms of normal and tumor-derived angiogenesis.* Am.J.Physiol Cell Physiol 2002. 282:C947-C970.
47. Makitie, T., Summanen, P., Tarkkanen, A., and Kivela, T. *Microvascular density in predicting survival of patients with choroidal and ciliary body melanoma.* Invest Ophthalmol.Vis.Sci. 1999. 40:2471-2480.
48. Overkleeft, E. N., Zuidervaart, W., Hurks, H. M., Eilers, P. H., Wolff-Rouendaal, D., and Jager, M. J. *Prognostic value of the disodium phosphate 32P uptake test in uveal melanoma: a long-term study.* Arch.Ophthalmol. 2003. 121:1398-1403.
49. Foss, A. J., Alexander, R. A., Jefferies, L. W., Hungerford, J. L., Harris, A. L., and Lightman, S. *Microvessel count predicts survival in uveal melanoma.* Cancer Res. 1996. 56:2900-2903.
50. Folberg, R., Pe'er, J., Gruman, L. M., Woolson, R. F., Jeng, G., Montague, P. R., Moninger, T. O., Yi, H., and Moore, K. C. *The morphologic characteristics of tumor blood vessels as a marker of tumor progression in primary human uveal melanoma: a matched case-control study.* Hum.Pathol. 1992. 23:1298-1305.
51. Eide, N., Faye, R. S., Hoifodt, H. K., Overgaard, R., Jebsen, P., Kvalheim, G., and Fodstad, O. *Immunomagnetic detection of micrometastatic cells in bone marrow in uveal melanoma patients.* Acta Ophthalmol. 2009. 87:830-836.

52. Stefansson, K., Wollmann, R., and Jerkovic, M. *S-100 protein in soft-tissue tumors derived from Schwann cells and melanocytes.* Am.J.Pathol. 1982. 106:261-268.
53. Adema, G. J., de Boer, A. J., van 't, H. R., Denijn, M., Ruiter, D. J., Vogel, A. M., and Figdor, C. G. *Melanocyte lineage-specific antigens recognized by monoclonal antibodies NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA.* Am.J.Pathol. 1993. 143:1579-1585.
54. Heegaard, S., Jensen, O. A., and Prause, J. U. *Immunohistochemical diagnosis of malignant melanoma of the conjunctiva and uvea: comparison of the novel antibody against melan-A with S100 protein and HMB-45.* Melanoma Res. 2000. 10:350-354.
55. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. *Mutations of the BRAF gene in human cancer.* Nature 2002. 417:949-954.
56. van, E. A., Zerp, S., van der Flier, S., Kruse-Wolters, M., Vacca, A., Ruiter, D. J., and Schrier, P. *Analysis of N-ras mutations in human cutaneous melanoma: tumor heterogeneity detected by polymerase chain reaction/single-stranded conformation polymorphism analysis.* Recent Results Cancer Res. 1995. 139:57-67.
57. Maat, W., Kilic, E., Luyten, G. P., de, K. A., Jager, M. J., Gruis, N. A., and van der Velden, P. A. *Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2008. 49:23-27.
58. Hubbard, K. B. and Hepler, J. R. *Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins.* Cell Signal. 2006. 18:135-150.
59. Goding, C. R. *Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage.* Genes Dev. 2000. 14:1712-1728.
60. Reddy, K. B., Nabha, S. M., and Atanaskova, N. *Role of MAP kinase in tumor progression and invasion.* Cancer Metastasis Rev. 2003. 22:395-403.
61. Van Raamsdonk, C. D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J. M., Simpson, E. M., Barsh, G. S., and Bastian, B. C. *Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi.* Nature. 2008.
62. Van Raamsdonk, C. D., Griewank, K. G., Crosby, M. B., Garrido, M. C., Vemula, S., Wiesner, T., Obenauf, A. C., Wackernagel, W., Green, G., Bouvier, N., Sozen, M. M., Baimukanova, G., Roy, R., Heguy, A., Dolgalev, I., Khanin, R., Busam, K., Speicher, M. R., O'Brien, J., and Bastian, B. C. *Mutations in GNA11 in uveal melanoma.* N.Engl.J.Med. 2010. 363:2191-2199.
63. Fisher, D. E., Barnhill, R., Hodi, F. S., Herlyn, M., Merlino, G., Medrano, E., Bastian, B., Landi, T. M., and Sosman, J. *Melanoma from bench to bedside: meeting report from the 6th international melanoma congress.* Pigment Cell Melanoma Res. 2010. 23:14-26.
64. Cramer, H., Schmenger, K., Heinrich, K., Horstmeyer, A., Boning, H., Breit, A., Piiper, A., Lundstrom, K., Muller-Esterl, W., and Schroeder, C. *Coupling of endothelin receptors to the ERK/MAP kinase pathway. Roles of palmitoylation and G(alpha)q.* Eur.J.Biochem. 2001. 268:5449-5459.
65. Patel, M., Smyth, E., Chapman, P. B., Wolchok, J. D., Schwartz, G. K., Abramson, D. H., and Carvajal, R. D. *Therapeutic implications of the emerging molecular biology of uveal melanoma.* Clin.Cancer Res. 2011. 17:2087-2100.
66. Yeatman, T. J. *A renaissance for SRC.* Nat.Rev.Cancer 2004. 4:470-480.

67. Martin, G. S. *The hunting of the Src.* Nat.Rev.Mol.Cell Biol. 2001. 2:467-475.
68. Hanahan, D. and Weinberg, R. A. *The hallmarks of cancer.* Cell 2000. 100:57-70.
69. Alvarez, R. H., Kantarjian, H. M., and Cortes, J. E. *The role of Src in solid and hematologic malignancies: development of new-generation Src inhibitors.* Cancer 2006. 107:1918-1929.
70. Irby, R. B. and Yeatman, T. J. *Increased Src activity disrupts cadherin/catenin-mediated homotypic adhesion in human colon cancer and transformed rodent cells.* Cancer Res. 2002. 62:2669-2674.
71. Nam, J. S., Ino, Y., Sakamoto, M., and Hirohashi, S. *Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis.* Clin. Cancer Res. 2002. 8:2430-2436.
72. Kanda, S., Miyata, Y., Kanetake, H., and Smithgall, T. E. *Non-receptor protein-tyrosine kinases as molecular targets for antiangiogenic therapy (Review).* Int.J.Mol.Med. 2007. 20:113-121.
73. Mukhopadhyay, D., Tsiokas, L., Zhou, X. M., Foster, D., Brugge, J. S., and Sukhatme, V. P. *Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation.* Nature 1995. 375:577-581.
74. Chang, Q., Jorgensen, C., Pawson, T., and Hedley, D. W. *Effects of dasatinib on EphA2 receptor tyrosine kinase activity and downstream signalling in pancreatic cancer.* Br.J.Cancer 2008. 99:1074-1082.
75. Lombardo, L. J., Lee, F. Y., Chen, P., Norris, D., Barrish, J. C., Behnia, K., Castaneda, S., Cornelius, L. A., Das, J., Doweyko, A. M., Fairchild, C., Hunt, J. T., Inigo, I., Johnston, K., Kamath, A., Kan, D., Klei, H., Marathe, P., Pang, S., Peterson, R., Pitt, S., Schieven, G. L., Schmidt, R. J., Tokarski, J., Wen, M. L., Wityak, J., and Borzilleri, R. M. *Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays.* J.Med.Chem. 2004. 47:6658-6661.
76. Harbour, J. W., Char, D. H., Kroll, S., Quivey, J. M., and Castro, J. *Metastatic risk for distinct patterns of postirradiation local recurrence of posterior uveal melanoma.* Ophthalmology 1997. 104:1785-1792.
77. Pe'er, J., Stefani, F. H., Seregard, S., Kivela, T., Lommatzsch, P., Prause, J. U., Sobottka, B., Damato, B., and Chowers, I. *Cell proliferation activity in posterior uveal melanoma after Ru-106 brachytherapy: an EORTC ocular oncology group study.* Br.J.Ophthalmol. 2001. 85:1208-1212.
78. de, J. M., Breeman, W. A., Valkema, R., Bernard, B. F., and Krenning, E. P. *Combination radionuclide therapy using 177Lu- and 90Y-labeled somatostatin analogs.* J.Nucl.Med. 2005. 46 Suppl 1:13S-17S.
79. Krenning, E. P., de, J. M., Kooij, P. P., Breeman, W. A., Bakker, W. H., de Herder, W. W., Van Eijck, C. H., Kwekkeboom, D. J., Jamar, F., Pauwels, S., and Valkema, R. *Radiolabelled somatostatin analogue(s) for peptide receptor scintigraphy and radionuclide therapy.* Ann.Oncol. 1999. 10 Suppl 2:S23-S29.
80. Krenning, E. P., Valkema, R., Kwekkeboom, D. J., de Herder, W. W., Van Eijck, C. H., de, J. M., Pauwels, S., and Reubi, J. C. *Molecular imaging as in vivo molecular pathology for gastroenteropancreatic neuroendocrine tumors: implications for follow-up after therapy.* J.Nucl.Med. 2005. 46 Suppl 1:76S-82S.
81. Kwekkeboom, D. J., Mueller-Brand, J., Paganelli, G., Anthony, L. B., Pauwels, S., Kvols, L. K., O'dorisio, T. M., Valkema, R., Bodei, L., Chinol, M., Maecke, H. R., and Krenning, E. P. *Overview of results of peptide receptor radionuclide therapy with 3 radiolabeled somatostatin analogs.* J.Nucl.Med. 2005. 46 Suppl 1:62S-66S.

CHAPTER 2

MACROPHAGES IN UVEAL MELANOMA AND IN EXPERIMENTAL OCULAR TUMOR MODELS: FRIENDS OR FOES?

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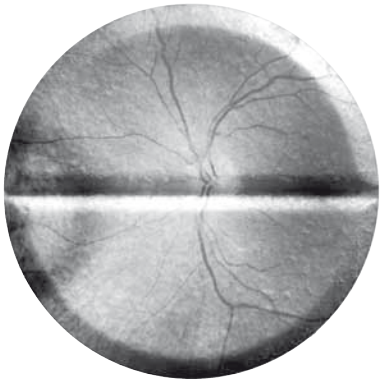

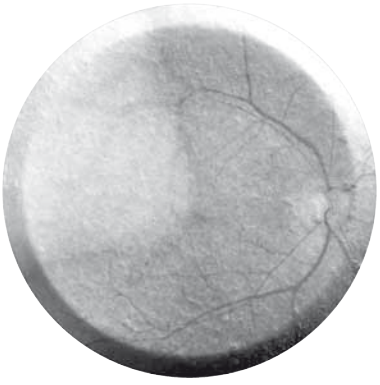

2.1 UVEAL MELANOMA AND ANGIOGENESIS

Tumor cells may leave the enclosure of the eye by intravascular spreading, by outgrowth through the trabecular meshwork or by migrating along blood vessels or nerves. Seddon et al. determined that in uveal melanoma the 10-year mortality without extraocular extension was 31%, and with extraocular extension 75% [1]. Recent studies looked at tumor cell ingrowth in blood vessels within the tumor, and in the sclera, and observed a strong correlation between any ingrowth of tumor cells into blood vessels and poor survival [2,3]. Extraocular extension was correlated with the grade of malignancy of the tumor itself, such as the presence of monosomy 3. The presence of intravascular tumor cells was strongly correlated to intrascleral invasion. These data indicate the important role of blood vessels in the clinical course of uveal melanoma.

The amount of blood vessels is also related to prognosis: when looking at areas with a high MVD, one can identify so-called "hot spots" of vascular density [4-6], which in some studies showed a correlation with death. Mäkitie et al. studied vessel density in a group of 134 uveal melanomas, using the CD34 monoclonal antibody to identify blood vessels: a high MVD was associated with the presence of epithelioid cells, a high largest basal tumor diameter and tumor height, and a decreased survival [7]. In addition, a high MVD was associated with an increased density of TAM [8].

Turning on angiogenesis, known as the angiogenic switch, is an important early event in tumor growth, including uveal melanoma. Angiogenesis often occurs as a response to intra-tumoral hypoxia, which leads to an upregulation of the transcription factor HIF-1α and expression of its target gene VEGF. In many types of cancer, intra-tumoral hypoxia, overexpression of HIF-1α, and increased microvascular density are found to be associated with tumor progression and poor prognosis [9,10]. Fluorescein angiography shows that the formation of new vessels is an early feature that discriminates between uveal melanoma and choroidal nevi. The main uveal melanoma blood vessels are connected to the choroidal and not the retinal vasculature, and fluorescein angiography may thus show a dual circulation. Uveal melanoma blood vessels may lack endothelial cells, and their walls are extremely thin while their lining often shows disruptions when tumor cells penetrate into the vessel lumen [11].The presence of new vessels is critical for tumor growth, as diffusion of oxygen and nutrients becomes limited when tumor size increases [12].Tumor blood vessels are considered to arise as an outgrowth of the local vascular bed (angiogenesis). But what determines the growth of new blood vessels in uveal melanoma?

2.2. VEGF AND REGULATION OF ANGIOGENESIS

VEGF is one of the most important angiogenic factors, with a potent vasopermeability function. Uveal melanoma cell lines produce VEGF as well as Platelet-Derived Growth Factor (PDGF) [13,14]. VEGF is present in the vitreous and aqueous of uveal melanoma-containing eyes, and has prognostic value [15-17]. While increased levels of VEGF have been reported in uveal melanoma eyes, with significant VEGF production by stromal cells and tumor cells. However, immunohistochemical studies do not show a universal picture: some studies using immunohistochemistry observed VEGF expression in at least 25% of uveal melanomas [18-21], while others reported a lack of VEGF expression in uveal melanoma [22-24]. The variable expression patterns of VEGF protein and mRNA reported in uveal melanoma [15,16] most likely reflect variations in fixation, tissue-processing techniques and antibody specificity (for example, to VEGF splice variants). Epigenetic mechanisms may also be involved in VEGF overexpression in ocular melanomas [19].

Missotten and others observed a correlation between VEGF levels in the aqueous humor and an increased basal tumor diameter, as well as with ciliary body involvement [177]. Patients with uveal melanoma metastases often have increased levels of VEGF in their serum (Fig 1) [25].

While tumor cells may be the source of VEGF, other ocular sources of VEGF may include retinal Müller cells or neurons overlying the tumor, RPE cells disrupted during tumor growth, or macrophages inside or outside the tumor (see below). Vinores and others observed that

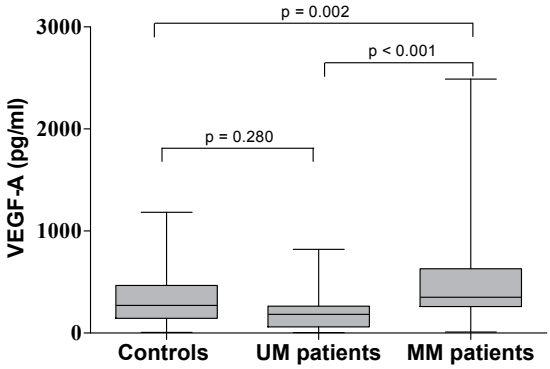

FIGURE 1. Concentration of serum VEGF-A in (metastatic) UM patients and controls.
Concentration of serum VEGF-A in the control group, and in patients with and without metastatic uveal melanoma. P-values (Mann–Whitney test) between the different groups are indicated in the graph. Each box shows the median, quartiles (box length is the interquartile range) and whiskers represent the 90th and 10th percentiles. Reproduced with permission from "Regulation of VEGF-A in uveal melanoma", by El Filali et al. (2010), via Copyright Clearance Center.

eyes containing a uveal melanoma expressed VEGF in ganglion cells, in vessel walls within the inner retina, and in the RPE, ciliary body and iris [20]. Analysis of frozen tumor sections revealed expression of VEGF mRNA in quite a large number of tumors, while short-term cultured primary uveal melanoma cells or uveal melanoma cell lines often expressed and produced VEGF (Fig. 2) [25,26]. Exposure of cultured tumor cells to hypoxia greatly induced the production of VEGF, and this may also play an important role in tumor angiogenesis. Similar to the situation in other solid tumors, outgrowth of uveal melanoma combined with limited oxygen diffusion probably leads to local areas of tumor ischemia, and thus hypoxia. This hypoxia subsequently induces HIF-1α, which stimulates VEGF production by oxygen-deprived tumor cells and tumor-associated macrophages. Ocular studies using a retinal laser photocoagulation model showed that macrophages directly stimulate angiogenesis, since a reduction of choroidal neovascularisation took place when macrophages were removed prior to laser treatment [27].

A similar situation may occur in uveal melanoma metastases, which may remain dormant for many years. It may well be that dormancy depends on the (temporary) lack of vascularisation in metastases. Preventing angiogenesis may be a useful therapy in this phase of the disease [28]. What triggers the metastatic cells to become active, often many years after enucleation of the melanoma-containing eye, remains elusive, but it may be that local macrophage accumulation plays a stimulatory role.

That hypoxia may influence malignant tumor-cell behaviour is well-illustrated by in vitro work by Victor et al., using the MUM2B uveal melanoma cell line. Exposure of these cells to

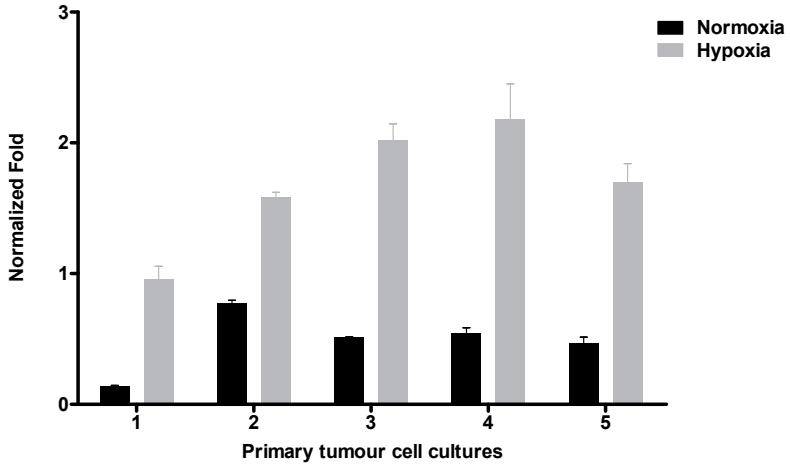

FIGURE 2. VEGF-A mRNA expression in primary UM cell cultures following exposure to hypoxia. The amount of VEGF-A mRNA expression was measured with quantitative real-time RT-PCR in primary UM cell cultures (cultures 1–5) under normoxic (black) and hypoxic (gray) conditions after 24 h. Expression is demonstrated in normalized fold. Reproduced with permission from "Regulation of VEGF-A in uveal melanoma", by El Filali et al. (2010) via Copyright Clearance Center.

an hypoxic environment induced several changes in RNA expression including upregulation of CXCR4, angiopoietin-related protein, pyrovate dehydrokinase 1, integrin β8 and others, and increased tumor-cell migration, invasion and adhesion [29]. Cells transfected with siRNA directed against HIF-1α did not show an increase in any of these characteristics, suggesting that HIF-1α-mediated processes were involved.

2.3 UVEAL MELANOMA AND MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that can degrade most extracellular (ECM) components. Secreted and transmembrane MMPs play a major role in tumor invasion and metastasis, and are important in angiogenesis, inflammation, apoptosis, cell-surface protein-shedding, release and activation of ECM-sequestered growth factors such as Transforming Growth Factor (TGF)-β and Fibroblast Growth Factor (FGF)-2, and signal transduction [30,31]. MMPs are generally constitutively expressed at low levels, and production is tightly regulated at the level of transcription as well as post-transcription by cytokines, growth factors and hormones, and by changes in cell–cell and cell–ECM interactions. MMPs are mostly secreted in latent (proMMP) forms and activated in the extracellular space by serine proteases such as plasmin, urokinase plasminogen activator (uPA) or by other MMPs, including membrane-type (MT)-MMPs. EMMPRIN (CD147, extracellular MMP inducing protein) can induce MMP production by stromal cells such as fibroblasts and endothelial cells, and can regulate VEGF and MMP production in tumor angiogenesis [32]. Tissue Inhibitors of MMPs (TIMPs) may modulate MMP activity, and regulate cell proliferation, angiogenesis, and apoptosis.

In uveal melanoma, MMPs have been implicated in both angiogenesis and vasculogenic mimicry due to their ability to degrade vascular basement membranes as well as to activate growth factors such as VEGF and TGFβ [33-36]. MMP-2, -9 and MT1-MMP have been reported to facilitate tumor angiogenesis, cell migration, and invasion [30,37,38]. Consistent with these observations, (latent and active) heterogeneous MMP-1, -2, -9 and MT1-MMP expression has been observed in uveal melanomas, including on the tumor vasculature [39] (Fig. 3).

In primary uveal melanoma, the presence of VEGF-A and MMP-9 is associated with metastasis formation [40]. Heterogeneous EMMPRIN expression was observed on primary uveal melanomas, in association with MMP-expressing fibroblasts, particularly at tumor edges (Fig. 4). Melanomas with a mixed and epithelioid morphology generally expressed higher levels of EMMPRIN, assessed with immunohistochemistry. In vitro studies showed that EMMPRIN-expressing uveal melanoma cells co-cultured with choroidal fibroblasts can induce fibroblast MMP-2 production and activation.

In uveal melanoma, immunohistochemistry, gene array, and in vitro studies collectively show an important role for MMPs in primary and metastatic tumors, and an association

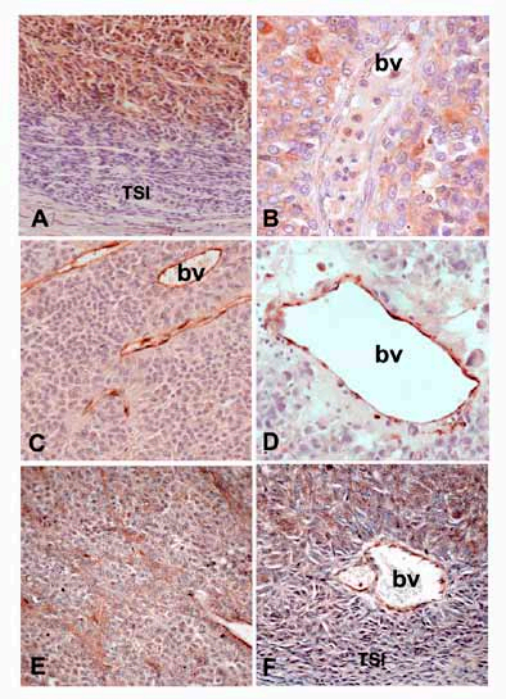

FIGURE 3. Immunoperoxidase labelling of MMP-1, -2 and -9 in uveal melanoma. A and B. Tumor cells immunolabelled for MMP-1, although not at the tumor-sceral interface (TSI). **B.** Tumor cells are also seen within a blood vessel (bv) **C and D.** Blood vessels (bv) show strong MMP-2 immunostaining, with little or no MMP-2 immunostaining of tumor cells. E and F. MMP-9 immunostaining of tumor cells and vasculature within uveal melanoma. As seen in E, the pattern of MMP-9 immunostaining appears similar to the extravascular matrix patterns seen with PAS staining. An MMP-9 positive intravascular leukocyte is also visible in F. (TSI – tumor-scleral interface; bv – blood vessel; hematoxylin counterstain).

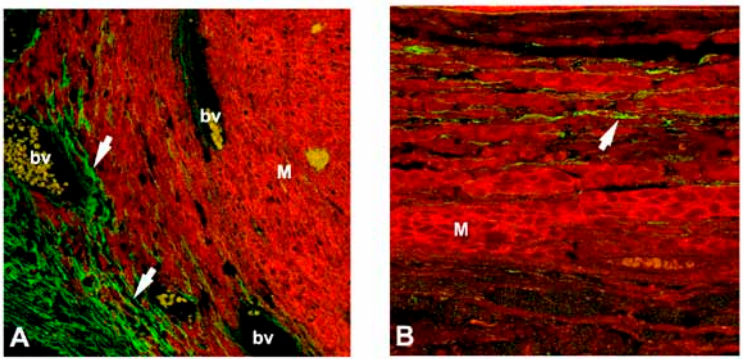

FIGURE 4. Co-immunolabelling for MMP-2 and EMMPRIN (CD147) in uveal melanoma, visualized using confocal microscopy. A and B.
Stromal cells (presumptive fibroblasts; arrowheads) near the tumour edge show strong MMP-2 immunostaining (green). Tumor cells (M) display distinct cell-surface EMMPRIN immunostaining (red); (bv – blood vessel).

with tumor invasion and vasculogenic mimicry [33,39,41]. MMPs can mediate formation of the laminin-rich matrix via cleavage of laminin 5γ2 to form pro-migratory fragments [42,43]. Blocking MMP activity in vitro using chemically-modified tetracyclines inhibits laminin 5γ cleavage, and downregulates MMP-2, -9, MT1-MMP, VEGF-C, VE-cadherin and TIE-1, inhibiting vasculogenic mimicry [43]. Gene-expression profiling of different phases of oncogenesis in uveal melanoma shows TIMP-3 (an MMP-inhibitor) downregulation during progression from melanocyte to metastasis [41]. TIMP-3 immunoreactivity was also decreased in more aggressive, epithelioid/mixed uveal melanomas [39]. Clinically, uveal melanoma expression of MMP-2 and -9 is associated with a higher incidence of metastatic disease, and patients with tumors expressing low levels of the MMP inhibitors TIMP-1 and -2 showed a worse survival [44,45]. As not only tumor cells but also macrophages can produce MMPs, inflammatory cytokines and VEGF, they too are important for the (further) induction of local inflammation as well as angiogenesis.

2.4 ANGIOGENESIS AND TREATMENT

Anti-angiogenic therapy is currently being trialed for many types of cancer. With regard to uveal melanoma, several potential areas for the use of anti-angiogenic therapy can be identified: growth inhibition of an intraocular melanoma, growth inhibition of metastases, and treatment of side effects of ocular irradiation, i.e. radiation retinopathy. As treatment of intraocular tumors with different types of irradiation leads not only to inhibition of tumor growth but also to shrinkage of the tumor, additional treatment with angiogenesis inhibitors may not be needed. On the other hand, one might consider treatments with fewer side effects than irradiation, and that might include a combination of anti-angiogenic agents with local chemotherapy or immunotherapy. Attacking blood vessels too early may not be such a good idea, as this might stop the local distribution of chemotherapy. Treating suspicious large nevi with anti-angiogenic treatments might be an option, as an early change to malignancy includes the development of intra-tumoral blood vessels. However, closing blood vessels may have an unwanted effect related to reduced oxygen distribution and local hypoxia, selecting outgrowth of tumor cells that are more resistant to hypoxia and that have become less sensitive to irradiation. In addition, temporary vessel closure may be followed by local hypoxia, resulting in more vessel growth instead of less. Finally, primary uveal melanoma and metastases not only contain blood vessels but also extravascular channels. This additional tumor microcirculation (vasculogenic mimicry) provides a route for alternative fluid movement and perfusion within solid tumors, and in vitro, does not appear to be targeted by agents specific for vascular endothelial cells such as endostatin [46].

With regard to metastasis, one looks for therapies that may stimulate tumor-cell dormancy. Using anti-angiogenic therapies may well be indicated when the tumors are still small and

have not developed their full vascularisation. We do not know whether systemic treatment with angiogenic inhibitors will help patients who have clinically significant liver metastases. El Filali and others observed that patients with uveal melanoma metastases often have increased levels of serum VEGF (Fig. 5) [25]. The first results from experimental studies of VEGF inhibitors to prevent the growth of metastases in mice are promising [47]. Using bevacizumab, metastases outgrowth in the liver could be partially inhibited in a mouse model, using B16 cells as well as human uveal melanoma cells. As combination treatments of anti-angiogenic and chemotherapy are nowadays recognized treatments of various types of cancer metastases, the first trials for the treatment of uveal melanoma metastases will probably soon be underway. It is unfortunate, that growth inhibition by anti-angiogenic agents is often only temporarily, and combination therapies should be sought.

The third area where anti-angiogenic agents can be applied is in the treatment of radiation retinopathy, which may occur after irradiation of an intraocular or orbital tumor. Radiation retinopathy is a complication of radiation therapy and intraocular VEGF-A expression was shown to be particularly high in eyes developing secondary iris and retinal neovascularisation after receiving local radiotherapy [15,17,48]. Several case histories or small series of cases have already been reported, with variable results.

REFERENCE LIST

1. Seddon, J. M., Albert, D. M., Lavin, P. T., and Robinson, N. *A prognostic factor study of disease-free interval and survival following enucleation for uveal melanoma.* Arch.Ophthalmol. 1983. 101:1894-1899.
2. Coupland, S. E., Campbell, I., and Damato, B. *Routes of extraocular extension of uveal melanoma: risk factors and influence on survival probability.* Ophthalmology 2008. 115:1778-1785.
3. Ly, L., Odish, O., de Wolff-Rouendaal, D., Missotten, G., Luyten, G., and Jager, M. *Intravascular presence of tumor cells as prognostic parameter in uveal melanoma: a 35-year survey.* Invest Ophthalmol. Vis.Sci. 2009.
4. Foss, A. J., Alexander, R. A., Jefferies, L. W., Hungerford, J. L., Harris, A. L., and Lightman, S. *Microvessel count predicts survival in uveal melanoma.* Cancer Res. 1996. 56:2900-2903.
5. Lane, A. M., Egan, K. M., Yang, J., Saornil, M. A., Alroy, J., Albert, D., and Gragoudas, E. S. *An evaluation of tumour vascularity as a prognostic indicator in uveal melanoma.* Melanoma Res. 1997. 7:237-242.
6. Schaling, D. F. and Pauwels, E. K. *The clinical role of immunoscintigraphy for the detection of ocular melanoma.* Q.J.Nucl.Med. 1996. 40:335-340.
7. Makitie, T., Summanen, P., Tarkkanen, A., and Kivela, T. *Microvascular density in predicting survival of patients with choroidal and ciliary body melanoma.* Invest Ophthalmol.Vis.Sci. 1999. 40:2471-2480.
8. Toivonen, P., Makitie, T., Kujala, E., and Kivela, T. *Microcirculation and tumor-infiltrating macrophages in choroidal and ciliary body melanoma and corresponding metastases.* Invest Ophthalmol.Vis.Sci. 2004. 45:1-6.
9. Hanahan, D. and Folkman, J. *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.* Cell 1996. 86:353-364.
10. Semenza, G. L. *HIF-1 and tumor progression: pathophysiology and therapeutics.* Trends Mol.Med. 2002. 8:S62-S67.
11. Radnot, M. and Antal, M. *Vessels of intraocular malignant melanomas.* Am.J.Ophthalmol. 1979. 88:472-478.
12. Holmgren, L., O'Reilly, M. S., and Folkman, J. *Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression.* Nat.Med. 1995. 1:149-153.
13. Ijland, S. A., Jager, M. J., Heijdra, B. M., Westphal, J. R., and Peek, R. *Expression of angiogenic and immunosuppressive factors by uveal melanoma cell lines.* Melanoma Res. 1999. 9:445-450.
14. Westphal, J. R., Van't Hullenaar, R., Peek, R., Willems, R. W., Crickard, K., Crickard, U., Askaa, J., Clemmensen, I., Ruiter, D. J., and De Waal, R. M. *Angiogenic balance in human melanoma: expression of VEGF, bFGF, IL-8, PDGF and angiostatin in relation to vascular density of xenografts in vivo.* Int.J.Cancer 2000. 86:768-776.
15. Boyd, S. R., Tan, D., Bunce, C., Gittos, A., Neale, M. H., Hungerford, J. L., Charnock-Jones, S., and Cree, I. A. *Vascular endothelial growth factor is elevated in ocular fluids of eyes harbouring uveal melanoma: identification of a potential therapeutic window.* Br.J.Ophthalmol. 2002. 86:448-452.
16. Boyd, S. R., Tan, D. S., de Souza, L., Neale, M. H., Myatt, N. E., Alexander, R. A., Robb, M., Hungerford, J. L., and Cree, I. A. *Uveal melanomas express vascular endothelial growth factor and basic fibroblast growth factor and support endothelial cell growth.* Br.J.Ophthalmol. 2002. 86:440-447.
17. Missotten, G. S., Notting, I. C., Schlingemann, R. O., Zijlmans, H. J., Lau, C., Eilers, P. H., Keunen, J. E., and Jager, M. J. *Vascular endothelial growth factor a in eyes with uveal melanoma.* Arch.Ophthalmol. 2006. 124:1428-1434.

18. Sheidow, T. G., Hooper, P. L., Crukley, C., Young, J., and Heathcote, J. G. *Expression of vascular endothelial growth factor in uveal melanoma and its correlation with metastasis.* Br.J.Ophthalmol. 2000. 84:750-756.
19. Abdel-Rahman, M. H., Craig, E. L., Davidorf, F. H., and Eng, C. *Expression of vascular endothelial growth factor in uveal melanoma is independent of 6p21-region copy number.* Clin.Cancer Res. 2005. 11:73-78.
20. Vinores, S. A., Kuchle, M., Mahlow, J., Chiu, C., Green, W. R., and Campochiaro, P. A. *Blood-ocular barrier breakdown in eyes with ocular melanoma. A potential role for vascular endothelial growth factor/vascular permeability factor.* Am.J.Pathol. 1995. 147:1289-1297.
21. Stitt, A. W., Simpson, D. A., Boocock, C., Gardiner, T. A., Murphy, G. M., and Archer, D. B. *Expression of vascular endothelial growth factor (VEGF) and its receptors is regulated in eyes with intra-ocular tumours.* J.Pathol. 1998. 186:306-312.
22. Pe'er, J., Shweiki, D., Itin, A., Hemo, I., Gnessin, H., and Keshet, E. *Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases.* Lab Invest 1995. 72:638-645.
23. Kvanta, A., Steen, B., and Seregard, S. *Expression of vascular endothelial growth factor (VEGF) in retinoblastoma but not in posterior uveal melanoma.* Exp.Eye Res. 1996. 63:511-518.
24. Clarijs, R., Schalkwijk, L., Ruiter, D. J., and De Waal, R. M. *Lack of lymphangiogenesis despite coexpression of VEGF-C and its receptor Flt-4 in uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2001. 42:1422-1428.
25. el, Filali. M., Missotten, G. S., Maat, W., Ly, L. V., Luyten, G. P., van, d., V, and Jager, M. J. *Regulation of VEGF-A in uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2009.
26. el, Filali. M., Homminga, I., Maat, W., van, d., V, and Jager, M. J. *Triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth.* Mol.Vis. 2008. 14:1752-1759.
27. Sakurai, E., Anand, A., Ambati, B. K., van, R. N., and Ambati, J. *Macrophage depletion inhibits experimental choroidal neovascularization.* Invest Ophthalmol.Vis.Sci. 2003. 44:3578-3585.
28. Jager, M. J. *Angiogenesis in uveal melanoma.* Ophthalmic Res. 2006. 38:248-250.
29. Victor, N., Ivy, A., Jiang, B. H., and Agani, F. H. *Involvement of HIF-1 in invasion of Mum2B uveal melanoma cells.* Clin.Exp.Metastasis 2006. 23:87-96.
30. Stamenkovic, I. *Matrix metalloproteinases in tumor invasion and metastasis.* Semin.Cancer Biol. 2000. 10:415-433.
31. Overall, C. M. and Kleifeld, O. *Towards third generation matrix metalloproteinase inhibitors for cancer therapy.* Br.J.Cancer 2006. 94:941-946.
32. Bougatef, F., Quemener, C., Kellouche, S., Naimi, B., Podgorniak, M. P., Millot, G., Gabison, E. E., Calvo, F., Dosquet, C., Lebbe, C., Menashi, S., and Mourah, S. *EMMPRIN promotes angiogenesis through hypoxia-inducible factor-2alpha-mediated regulation of soluble VEGF isoforms and their receptor VEGFR-2.* Blood 2009. 114:5547-5556.
33. Seftor, R. E., Seftor, E. A., Koshikawa, N., Meltzer, P. S., Gardner, L. M., Bilban, M., Stetler-Stevenson, W. G., Quaranta, V., and Hendrix, M. J. *Cooperative interactions of laminin 5 gamma2 chain, matrix metalloproteinase-2, and membrane type-1-matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma.* Cancer Res. 2001. 61:6322-6327.
34. Iida, J. and McCarthy, J. B. *Expression of collagenase-1 (MMP-1) promotes melanoma growth through the generation of active transforming growth factor-beta.* Melanoma Res. 2007. 17:205-213.
35. Raffetto, J. D. and Khalil, R. A. *Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease.* Biochem.Pharmacol. 2008. 75:346-359.

36. Ebrahem, Q., Chaurasia, S. S., Vasanji, A., Qi, J. H., Klenotic, P. A., Cutler, A., Asosingh, K., Erzurum, S., and Anand-Apte, B. *Cross-talk between vascular endothelial growth factor and matrix metalloproteinases in the induction of neovascularization in vivo.* Am.J.Pathol. 2010. 176:496-503.
37. Hofmann, U. B., Westphal, J. R., Van Kraats, A. A., Ruiter, D. J., and Van Muijen, G. N. *Expression of integrin alpha(v)beta(3) correlates with activation of membrane-type matrix metalloproteinase-1 (MT1-MMP) and matrix metalloproteinase-2 (MMP-2) in human melanoma cells in vitro and in vivo.* Int.J.Cancer 2000. 87:12-19.
38. Egeblad, M. and Werb, Z. *New functions for the matrix metalloproteinases in cancer progression.* Nat. Rev.Cancer 2002. 2:161-174.
39. Lai, K., Conway, R. M., Crouch, R., Jager, M. J., and Madigan, M. C. *Expression and distribution of MMPs and TIMPs in human uveal melanoma.* Exp.Eye Res. 2008. 86:936-941.
40. Sahin, A., Kiratli, H., Soylemezoglu, F., Tezel, G. G., Bilgic, S., and Saracbasi, O. *Expression of vascular endothelial growth factor-A, matrix metalloproteinase-9, and extravascular matrix patterns and their correlations with clinicopathologic parameters in posterior uveal melanomas.* Jpn.J.Ophthalmol. 2007. 51:325-331.
41. van der Velden, P. A., Zuidervaart, W., Hurks, M. H., Pavey, S., Ksander, B. R., Krijgsman, E., Frants, R. R., Tensen, C. P., Willemze, R., Jager, M. J., and Gruis, N. A. *Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development.* Int.J.Cancer 2003. 106:472-479.
42. Hendrix, M. J., Seftor, E. A., Seftor, R. E., Gardner, L. M., Boldt, H. C., Meyer, M., Pe'er, J., and Folberg, R. *Biologic determinants of uveal melanoma metastatic phenotype: role of intermediate filaments as predictive markers.* Lab Invest 1998. 78:153-163.
43. Hendrix, M. J., Seftor, E. A., Hess, A. R., and Seftor, R. E. *Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma.* Nat.Rev.Cancer 2003. 3:411-421.
44. Vaisanen, A., Kallioinen, M., von, D. K., Laatikainen, L., Hoyhtya, M., and Turpeenniemi-Hujanen, T. *Matrix metalloproteinase-2 (MMP-2) immunoreactive protein--a new prognostic marker in uveal melanoma?* J.Pathol. 1999. 188:56-62.
45. El Shabrawi, Y., Ardjomand, N., Radner, H., and Ardjomand, N. *MMP-9 is predominantly expressed in epithelioid and not spindle cell uveal melanoma.* J.Pathol. 2001. 194:201-206.
46. van der Schaft, D. W., Seftor, R. E., Seftor, E. A., Hess, A. R., Gruman, L. M., Kirschmann, D. A., Yokoyama, Y., Griffioen, A. W., and Hendrix, M. J. *Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells.* J.Natl.Cancer Inst. 2004. 96:1473-1477.
47. Yang, H., Jager, M. J., and Grossniklaus, H. E. *Bevacizumab suppression of establishment of micrometastases in experimental ocular melanoma.* Invest Ophthalmol.Vis.Sci. 2010. 51:2835-2842.
48. Boyd, S. R., Gittos, A., Richter, M., Hungerford, J. L., Errington, R. D., and Cree, I. A. *Proton beam therapy and iris neovascularisation in uveal melanoma.* Eye (Lond) 2006. 20:832-836.

CHAPTER 3

ANTI-ANGIOGENIC THERAPY IN UVEAL MELANOMA

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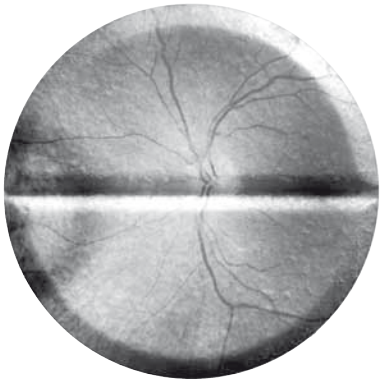
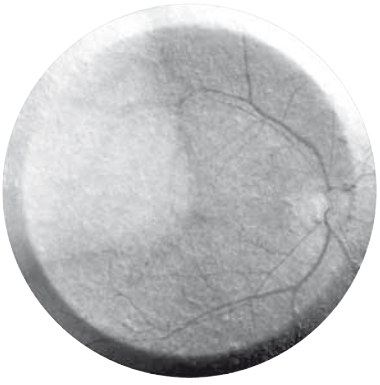




ABSTRACT

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

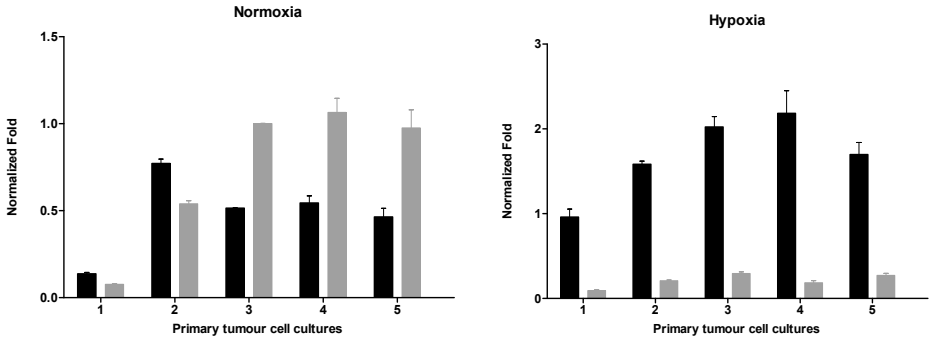


FIGURE 1. VEGF and TSP-1 mRNA expression in primary uveal melanoma cell cultures.
The amount of VEGF (black) and TSP-1 (gray) mRNA expression was measured with quantitative real- time RT- PCR in primary uveal melanoma cell cultures (cultures 1– 5) under normoxic (1% O2) and hypoxic (20% O2) conditions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

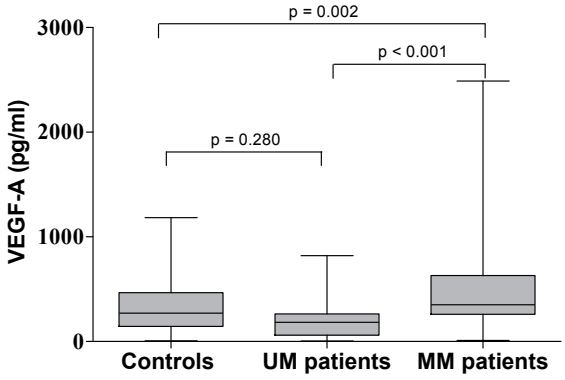


FIGURE 2. Concentration of serum VEGF- A in (metastatic) uveal melanoma patients and controls.
Concentration of serum VEGF- A in the control group (n = 50), and in patients with (n = 20) and without metastatic (n = 74) uveal melanoma. p values (Mann-Whitney test) between the different groups are indicated 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.

REFERENCE LIST

1. Holmgren L, O'Reilly MS, Folkman J: Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1995;1:149-153.
2. Papetti M, Herman IM: Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 2002;282:C947-C970.
3. Criscuolo GR, Merrill MJ, Oldfield EH: Further characterization of malignant glioma-derived vascular permeability factor. J Neurosurg 1988;69:254-262.
4. Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. Nat Med 2003;9:669-676.
5. Karayiannakis AJ, Syrigos KN, Polychronidis A, Zbar A, Kouraklis G, Simopoulos C, Karatzas G: Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery. Ann Surg 2002;236:37-42.
6. Celen O, Kahraman I, Yildirim E, Berberoglu U: Correlation of vascular endothelial growth factor (VEGF) and CEA with clinicopathological variables in colorectal cancer patients. Neoplasma 2004;51:293-299.
7. Hayes AJ, Mostyn-Jones A, Koban MU, A'Hern R, Burton P, Thomas JM: Serum vascular endothelial growth factor as a tumour marker in soft tissue sarcoma. Br J Surg 2004;91:242-247.
8. Barak V, Pe EJ, Kalickman I, Frenkel S: VEGF as a Biomarker for Metastatic Uveal Melanoma in Humans. Curr Eye Res 2011.
9. el Filali M, Missotten GS, Maat W, Ly LV, Luyten GP, van d, V, Jager MJ: Regulation of VEGF-A in uveal melanoma. Invest Ophthalmol Vis Sci 2009.
10. Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E: Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 2003;21:60-65.
11. Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA: A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 2003;349:427-434.
12. Cobleigh MA, Langmuir VK, Sledge GW, Miller KD, Haney L, Novotny WF, Reimann JD, Vassel A: A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. Semin Oncol 2003;30:117-124.
13. Varker KA, Biber JE, Kefauver C, Jensen R, Lehman A, Young D, Wu H, Lesinski GB, Kendra K, Chen HX, Walker MJ, Carson WE, III: A randomized phase 2 trial of bevacizumab with or without daily low-dose interferon alfa-2b in metastatic malignant melanoma. Ann Surg Oncol 2007;14:2367-2376.
14. Van CE, Vervenne WL, Bennouna J, Humblet Y, Gill S, Van Laethem JL, Verslype C, Scheithauer W, Shang A, Cosaert J, Moore MJ: Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. J Clin Oncol 2009;27:2231-2237.
15. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O: Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 2009;15:220-231.
16. Yang H, Jager MJ, Grossniklaus HE: Bevacizumab suppression of establishment of micrometastases in experimental ocular melanoma. Invest Ophthalmol Vis Sci 2010;51:2835-2842.
17. Foss AJ, Alexander RA, Jefferies LW, Hungerford JL, Harris AL, Lightman S: Microvessel count predicts survival in uveal melanoma. Cancer Res 1996;56:2900-2903.
18. Lane AM, Egan KM, Yang J, Saornil MA, Alroy J, Albert D, Gragoudas ES: An evaluation of tumour vascularity as a prognostic indicator in uveal melanoma. Melanoma Res 1997;7:237-242.

19. Foss AJ, Alexander RA, Hungerford JL, Harris AL, Cree IA, Lightman S: Reassessment of the PAS patterns in uveal melanoma. Br J Ophthalmol 1997;81:240-246.
20. Makitie T, Summanen P, Tarkkanen A, Kivela T: Microvascular density in predicting survival of patients with choroidal and ciliary body melanoma. Invest Ophthalmol Vis Sci 1999;40:2471-2480.
21. Makitie T, Summanen P, Tarkkanen A, Kivela T: Microvascular loops and networks as prognostic indicators in choroidal and ciliary body melanomas. J Natl Cancer Inst 1999;91:359-367.
22. Folberg R, Hendrix MJ, Maniotis AJ: Vasculogenic mimicry and tumor angiogenesis. Am J Pathol 2000;156:361-381.
23. Shirakawa K, Tsuda H, Heike Y, Kato K, Asada R, Inomata M, Sasaki H, Kasumi F, Yoshimoto M, Iwanaga T, Konishi F, Terada M, Wakasugi H: Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. Cancer Res 2001;61:445-451.
24. Coupland SE, Campbell I, Damato B: Routes of extraocular extension of uveal melanoma: risk factors and influence on survival probability. Ophthalmology 2008;115:1778-1785.
25. Ly LV, Odish OF, Wolff-Rouendaal D, Missotten GS, Luyten GP, Jager MJ: Intravascular presence of tumor cells as prognostic parameter in uveal melanoma: a 35-year survey. Invest Ophthalmol Vis Sci 2010;51:658-665.
26. Augsburger JJ, Correa ZM, Freire J, Brady LW: Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy. Ophthalmology 1998;105:1670-1678.
27. Kujala E, Makitie T, Kivela T: Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol Vis Sci 2003;44:4651-4659.
28. Economou MA, All-Ericsson C, Bykov V, Girnita L, Bartolazzi A, Larsson O, Seregard S: Receptors for the liver synthesized growth factors IGF-1 and HGF/SF in uveal melanoma: intercorrelation and prognostic implications. Invest Ophthalmol Vis Sci 2005;46:4372-4375.
29. Baserga R: The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res 1995;55:249-252.
30. Slomiany MG, Rosenzweig SA: IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 alpha expression and activity in retinal pigment epithelial cell line D407. Invest Ophthalmol Vis Sci 2004;45:2838-2847.
31. Bergers G, Benjamin LE: Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401-410.
32. Handsley MM, Edwards DR: Metalloproteinases and their inhibitors in tumor angiogenesis. Int J Cancer 2005;115:849-860.
33. Littlepage LE, Sternlicht MD, Rougier N, Phillips J, Gallo E, Yu Y, Williams K, Brenot A, Gordon JI, Werb Z: Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression. Cancer Res 2010;70:2224-2234.
34. El Shabrawi Y, Ardjomand N, Radner H, Ardjomand N: MMP-9 is predominantly expressed in epithelioid and not spindle cell uveal melanoma. J Pathol 2001;194:201-206.
35. De Luca A, Carotenuto A, Rachiglio A, Gallo M, Maiello MR, Aldinucci D, Pinto A, Normanno N: The role of the EGFR signaling in tumor microenvironment. J Cell Physiol 2008;214:559-567.
36. Ferrara N: The role of VEGF in the regulation of physiological and pathological angiogenesis. EXS 2005;209-231.
37. Boyd SR, Tan D, Bunce C, Gittos A, Neale MH, Hungerford JL, Charnock-Jones S, Cree IA: Vascular endothelial growth factor is elevated in ocular fluids of eyes harbouring uveal melanoma: identification of a potential therapeutic window. Br J Ophthalmol 2002;86:448-452.
38. Ferrara N, Davis-Smyth T: The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4-25.

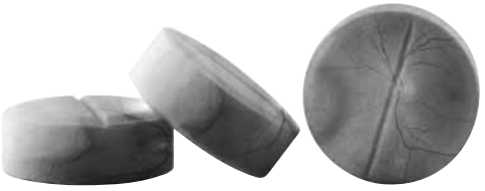
39. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 1991;5:1806-1814.
40. Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, Ferrara N: Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. J Biol Chem 1996;271:5638-5646.
41. Weigand M, Hantel P, Kreienberg R, Waltenberger J: Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. Angiogenesis 2005;8:197-204.
42. Ferrara N: Vascular endothelial growth factor as a target for anticancer therapy. Oncologist 2004;9 Suppl 1:2-10.
43. Vincenti V, Cassano C, Rocchi M, Persico G: Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 1996;93:1493-1495.
44. Prescher G, Bornfeld N, Becher R: Nonrandom chromosomal abnormalities in primary uveal melanoma. J Natl Cancer Inst 1990;82:1765-1769.
45. Naus NC, van Drunen E, de Klein A, Luyten GP, Paridaens DA, Alers JC, Ksander BR, Beverloo HB, Slater RM: Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. Genes Chromosomes Cancer 2001;30:267-273.
46. Abdel-Rahman MH, Craig EL, Davidorf FH, Eng C: Expression of vascular endothelial growth factor in uveal melanoma is independent of 6p21-region copy number. Clin Cancer Res 2005;11:73-78.
47. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE: Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. Cancer 1998;83:354-359.
48. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843-845.
49. Wang GL, Semenza GL: Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;270:1230-1237.
50. Mizukami Y, Kohgo Y, Chung DC: Hypoxia inducible factor-1 independent pathways in tumor angiogenesis. Clin Cancer Res 2007;13:5670-5674.
51. Verhoeff JJ, Stalpers LJ, Claes A, Hovinga KE, Musters GD, Peter VW, Richel DJ, Leenders WP, van Furth WR: Tumour control by whole brain irradiation of anti-VEGF-treated mice bearing intracerebral glioma. Eur J Cancer 2009;45:3074-3080.
52. Shibuya M: Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. Cell Struct Funct 2001;26:25-35.
53. Economou MA, Wu J, Vasilcanu D, Rosengren L, All-Ericsson C, van dP, I, Menu E, Girnita L, Axelson M, Larsson O, Seregard S, Kvanta A: Inhibition of VEGF secretion and experimental choroidal neovascularization by picropodophyllin (PPP), an inhibitor of the insulin-like growth factor-1 receptor. Invest Ophthalmol Vis Sci 2008;49:2620-2626.
54. Economou MA, Andersson S, Vasilcanu D, All-Ericsson C, Menu E, Girnita A, Girnita L, Axelson M, Seregard S, Larsson O: Oral picropodophyllin (PPP) is well tolerated in vivo and inhibits IGF-1R expression and growth of uveal melanoma. Invest Ophthalmol Vis Sci 2008;49:2337-2342.
55. Siemeister G, Weindel K, Mohrs K, Barleon B, Martiny-Baron G, Marme D: Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. Cancer Res 1996;56:2299-2301.
56. Rankin EB, Rha J, Unger TL, Wu CH, Shutt HP, Johnson RS, Simon MC, Keith B, Haase VH: Hypoxia-inducible factor-2 regulates vascular tumorigenesis in mice. Oncogene 2008;27:5354-5358.

57. Haase VH, Glickman JN, Socolovsky M, Jaenisch R: Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A 2001;98:1583-1588.
58. Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, Kerbel RS: Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res 1995;55:4575-4580.
59. Maat W, el FM, rks-Mulder A, Luyten GP, Gruis NA, Desjardins L, Boender P, Jager MJ, van d, V: Episodic Src activation in uveal melanoma revealed by kinase activity profiling. Br J Cancer 2009;101:312-319.
60. Takahashi H, Shibuya M: The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) 2005;109:227-241.
61. Bates DO, Harper SJ: Regulation of vascular permeability by vascular endothelial growth factors. Vascul Pharmacol 2002;39:225-237.
62. Qi JH, Matsumoto T, Huang K, Olausson K, Christofferson R, Claesson-Welsh L: Phosphoinositide 3 kinase is critical for survival, mitogenesis and migration but not for differentiation of endothelial cells. Angiogenesis 1999;3:371-380.
63. Jager MJ, Ly LV, El Filali M, Madigan MC: Macrophages in uveal melanoma and in experimental ocular tumor models: Friends or foes? Prog Retin Eye Res 2011;30:129-146.
64. Volm M, Koomagi R, Mattern J, Stammler G: Angiogenic growth factors and their receptors in non-small cell lung carcinomas and their relationships to drug response in vitro. Anticancer Res 1997;17:99-103.
65. Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR, Dvorak HF: Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 1993;53:4727-4735.
66. Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP: Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res 1996;56:2013-2016.
67. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO: Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 2003;22:1-29.
68. Ijland SA, Jager MJ, Heijdra BM, Westphal JR, Peek R: Expression of angiogenic and immunosuppressive factors by uveal melanoma cell lines. Melanoma Res 1999;9:445-450.
69. Sheidow TG, Hooper PL, Crukley C, Young J, Heathcote JG: Expression of vascular endothelial growth factor in uveal melanoma and its correlation with metastasis. Br J Ophthalmol 2000;84:750-756.
70. Boyd SR, Tan DS, de Souza L, Neale MH, Myatt NE, Alexander RA, Robb M, Hungerford JL, Cree IA: Uveal melanomas express vascular endothelial growth factor and basic fibroblast growth factor and support endothelial cell growth. Br J Ophthalmol 2002;86:440-447.
71. Missotten GS, Notting IC, Schlingemann RO, Zijlmans HJ, Lau C, Eilers PH, Keunen JE, Jager MJ: Vascular endothelial growth factor a in eyes with uveal melanoma. Arch Ophthalmol 2006;124:1428-1434.
72. Hayes AJ, Mostyn-Jones A, Koban MU, A'Hern R, Burton P, Thomas JM: Serum vascular endothelial growth factor as a tumour marker in soft tissue sarcoma. Br J Surg 2004;91:242-247.
73. Eskelin S, Pyrhonen S, Summanen P, Prause JU, Kivela T: Screening for metastatic malignant melanoma of the uvea revisited. Cancer 1999;85:1151-1159.
74. Kaiserman I, Amer R, Pe'er J: Liver function tests in metastatic uveal melanoma. Am J Ophthalmol 2004;137:236-243.
75. Barak V, Frenkel S, Kalickman I, Maniotis AJ, Folberg R, Pe'er J: Serum markers to detect metastatic uveal melanoma. Anticancer Res 2007;27:1897-1900.

76. Missotten GS, Korse CM, van Dehn C, Linders TC, Keunen JE, Jager MJ, Bonfrer JM: S-100B protein and melanoma inhibitory activity protein in uveal melanoma screening. A comparison with liver function tests. Tumour Biol 2007;28:63-69.
77. Potgens AJ, Lubsen NH, van Altena MC, Schoenmakers JG, Ruiter DJ, de Waal RM: Vascular permeability factor expression influences tumor angiogenesis in human melanoma lines xenografted to nude mice. Am J Pathol 1995;146:197-209.
78. Claffey KP, Brown LF, del Aguila LF, Tognazzi K, Yeo KT, Manseau EJ, Dvorak HF: Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. Cancer Res 1996;56:172-181.
79. Sahin A, Kiratli H, Soylemezoglu F, Tezel GG, Bilgic S, Saracbasi O: Expression of vascular endothelial growth factor-A, matrix metalloproteinase-9, and extravascular matrix patterns and their correlations with clinicopathologic parameters in posterior uveal melanomas. Jpn J Ophthalmol 2007;51:325-331.
80. Crosby MB, Yang H, Gao W, Zhang L, Grossniklaus HE: Serum vascular endothelial growth factor (VEGF) levels correlate with number and location of micrometastases in a murine model of uveal melanoma. Br J Ophthalmol 2011;95:112-117.
81. Land SC, Tee AR: Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J Biol Chem 2007;282:20534-20543.
82. Roccaro AM, Hideshima T, Raje N, Kumar S, Ishitsuka K, Yasui H, Shiraishi N, Ribatti D, Nico B, Vacca A, Dammacco F, Richardson PG, Anderson KC: Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. Cancer Res 2006;66:184-191.
83. Kenyon BM, Browne F, D'Amato RJ: Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization. Exp Eye Res 1997;64:971-978.
84. Kalas W, Gilpin S, Yu JL, May L, Krchnakova H, Bornstein P, Rak J: Restoration of thrombospondin 1 expression in tumor cells harbouring mutant ras oncogene by treatment with low doses of doxycycline. Biochem Biophys Res Commun 2003;310:109-114.
85. Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K: Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 2000;60:1306-1311.
86. Lima BR, Schoenfield LR, Singh AD: The impact of intravitreal bevacizumab therapy on choroidal melanoma. Am J Ophthalmol 2011;151:323-328.
87. Melia BM, Abramson DH, Albert DM, Boldt HC, Earle JD, Hanson WF, Montague P, Moy CS, Schachat AP, Simpson ER, Straatsma BR, Vine AK, Weingeist TA: Collaborative ocular melanoma study (COMS) randomized trial of I-125 brachytherapy for medium choroidal melanoma. I. Visual acuity after 3 years COMS report no. 16. Ophthalmology 2001;108:348-366.
88. Shields CL, Shields JA, Cater J, Gunduz K, Miyamoto C, Micaily B, Brady LW: Plaque radiotherapy for uveal melanoma: long-term visual outcome in 1106 consecutive patients. Arch Ophthalmol 2000;118:1219-1228.
89. ener-West M, Earle JD, Fine SL, Hawkins BS, Moy CS, Reynolds SM, Schachat AP, Straatsma BR: The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma, III: initial mortality findings. COMS Report No. 18. Arch Ophthalmol 2001;119:969-982.
90. Gragoudas ES, Lane AM, Munzenrider J, Egan KM, Li W: Long-term risk of local failure after proton therapy for choroidal/ciliary body melanoma. Trans Am Ophthalmol Soc 2002;100:43-48.
91. Shields CL, Cater J, Shields JA, Chao A, Krema H, Materin M, Brady LW: Combined plaque radiotherapy and transpupillary thermotherapy for choroidal melanoma: tumor control and treatment complications in 270 consecutive patients. Arch Ophthalmol 2002;120:933-940.

92. Jampol LM, Moy CS, Murray TG, Reynolds SM, Albert DM, Schachat AP, Diddie KR, Engstrom RE, Jr., Finger PT, Hovland KR, Joffe L, Olsen KR, Wells CG: The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma: IV. Local treatment failure and enucleation in the first 5 years after brachytherapy. COMS report no. 19. Ophthalmology 2002;109:2197-2206.
93. Archer DB, Gardiner TA: Ionizing radiation and the retina. Curr Opin Ophthalmol 1994;5:59-65.
94. Guyer DR, Mukai S, Egan KM, Seddon JM, Walsh SM, Gragoudas ES: Radiation maculopathy after proton beam irradiation for choroidal melanoma. Ophthalmology 1992;99:1278-1285.
95. Sia S, Harper C, McAllister I, Perry A: Iodine-I25 episcleral plaque therapy in uveal melanoma. Clin Experiment Ophthalmol 2000;28:409-413.
96. Jensen AW, Petersen IA, Kline RW, Stafford SL, Schomberg PJ, Robertson DM: Radiation complications and tumor control after 125I plaque brachytherapy for ocular melanoma. Int J Radiat Oncol Biol Phys 2005;63:101-108.
97. Mason JO, III, Albert MA, Jr., Persaud TO, Vail RS: Intravitreal bevacizumab treatment for radiation macular edema after plaque radiotherapy for choroidal melanoma. Retina 2007;27:903-907.
98. Finger PT: Radiation retinopathy is treatable with anti-vascular endothelial growth factor bevacizumab (Avastin). Int J Radiat Oncol Biol Phys 2008;70:974-977.
99. Gupta A, Muecke JS: Treatment of radiation maculopathy with intravitreal injection of bevacizumab (Avastin). Retina 2008;28:964-968.
100. Querques G, Prascina F, Iaculli C, Delle NN: Intravitreal pegaptanib sodium (Macugen) for radiation retinopathy following episcleral plaque radiotherapy. Acta Ophthalmol 2008;86:700-701.
101. Narayanan R, Kuppermann BD, Jones C, Kirkpatrick P: Ranibizumab. Nat Rev Drug Discov 2006;5:815-816.
102. Finger PT, Chin KJ: Intravitreous ranibizumab (lucentis) for radiation maculopathy. Arch Ophthalmol 2010;128:249-252.
103. Fukasawa M, Korc M: Vascular endothelial growth factor-trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. Clin Cancer Res 2004;10:3327-3332.
104. Augsburger JJ, Correa ZM, Shaikh AH: Effectiveness of treatments for metastatic uveal melanoma. Am J Ophthalmol 2009;148:119-127.
105. Mangiameli DP, Blansfield JA, Kachala S, Lorang D, Schafer PH, Muller GW, Stirling DI, Libutti SK: Combination therapy targeting the tumor microenvironment is effective in a model of human ocular melanoma. J Transl Med 2007;5:38.
106. Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye S, Marais R, Gibbens I, Hackett S, James M, Schuchter LM, Nathanson KL, Xia C, Simantov R, Schwartz B, Poulin-Costello M, O'Dwyer PJ, Ratain MJ: Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. Br J Cancer 2006;95:581-586.
107. Chow LQ, Eckhardt SG: Sunitinib: from rational design to clinical efficacy. J Clin Oncol 2007;25:884-896.
108. Kerbel RS: Tumor angiogenesis. N Engl J Med 2008;358:2039-2049.
109. Ebos JM, Lee CR, Bogdanovic E, Alami J, Van Slyke P, Francia G, Xu P, Mutsaers AJ, Dumont DJ, Kerbel RS: Vascular endothelial growth factor-mediated decrease in plasma soluble vascular endothelial growth factor receptor-2 levels as a surrogate biomarker for tumor growth. Cancer Res 2008;68:521-529.
110. Casanovas O, Hicklin DJ, Bergers G, Hanahan D: Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 2005;8:299-309.

111. Aghi M, Cohen KS, Klein RJ, Scadden DT, Chiocca EA: Tumor stromal-derived factor-1 recruits vascular progenitors to mitotic neovasculature, where microenvironment influences their differentiated phenotypes. Cancer Res 2006;66:9054-9064.
112. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z, Bergers G: HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 2008;13:206-220.
113. Sathornsumetee S, Cao Y, Marcello JE, Herndon JE, McLendon RE, Desjardins A, Friedman HS, Dewhirst MW, Vredenburgh JJ, Rich JN: Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan. J Clin Oncol 2008;26:271-278.
114. Bronkhorst IH, Ly LV, Jordanova ES, Vrolijk H, Versluis M, Luyten GP, Jager MJ: Detection of M2 macrophages in uveal melanoma and relation with survival. Invest Ophthalmol Vis Sci 2010.
115. D'Angelo G, Struman I, Martial J, Weiner RI: Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. Proc Natl Acad Sci U S A 1995;92:6374-6378.
116. Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M: Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. J Biol Chem 1998;273:4220-4226.
117. Shepard DR, Garcia JA: Toxicity associated with the long-term use of targeted therapies in patients with advanced renal cell carcinoma. Expert Rev Anticancer Ther 2009;9:795-805.
118. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Vigers G, Morales T, Aliagas I, Liu B, Sideris S, Hoeflich KP, Jaiswal BS, Seshagiri S, Koeppen H, Belvin M, Friedman LS, Malek S: RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 2010.
119. Boehm S, Rothermundt C, Hess D, Joerger M: Antiangiogenic drugs in oncology: a focus on drug safety and the elderly - a mini-review. Gerontology 2010;56:303-309.
120. Ansari J, Glaholm J, McMenemin R, James ND, Hussain SA: Recent advances and future directions in the management of metastatic renal cell carcinoma. Anticancer Agents Med Chem 2010;10:225-235.
121. Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z, Maurage CA, Auclair C, Mascarelli F: Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. J Biol Chem 2004;279:31769-31779.
122. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL: Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399-401.
123. Lindauer M, Hochhaus A: Dasatinib. Recent Results Cancer Res 2010;184:83-102.



PART II

PHYSIOLOGIC AND PHARMACOLOGIC EFFECTS REGARDING TUMOR-ANGIOGENESIS IN UVEAL MELANOMA

CHAPTER 4

REGULATION OF VEGF-A IN UVEAL MELANOMA

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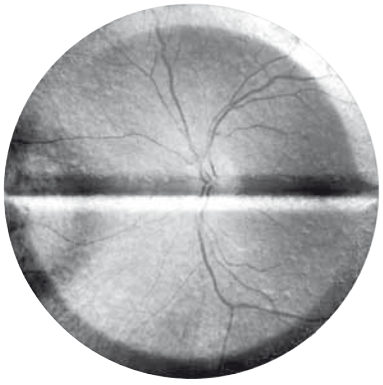

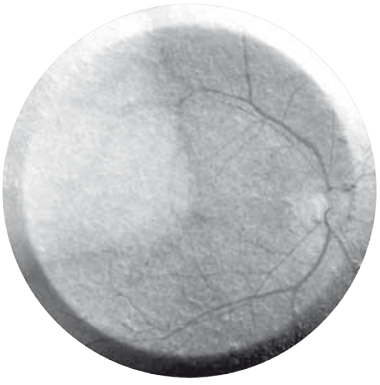

ABSTRACT

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

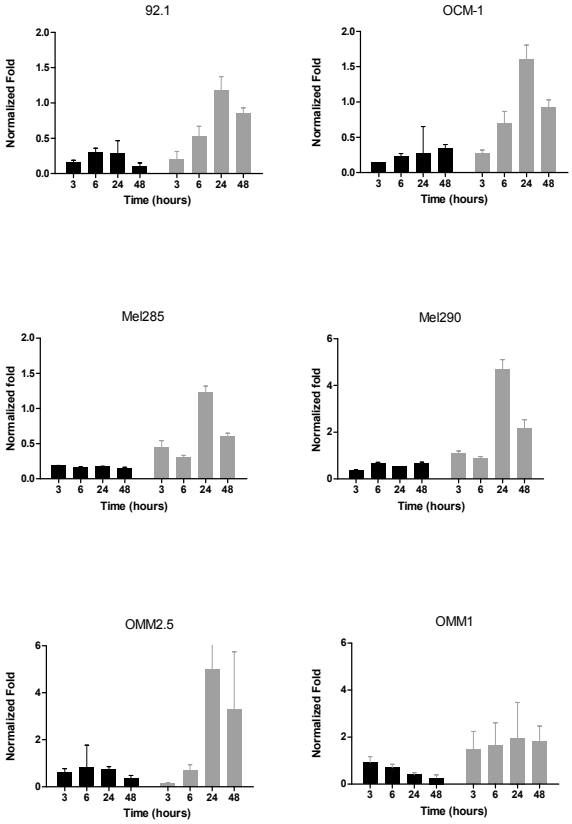

FIGURE 1. VEGF-A mRNA expression in UM cell lines.
The amount of VEGF-A mRNA expression was measured with qPCR in UM cell lines under normoxic (black) and hypoxic (gray) exposure after four different time intervals (3, 6, 24 and 48 hours). Normalized expression is shown with a maximum of 2.0- or 6.0-fold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

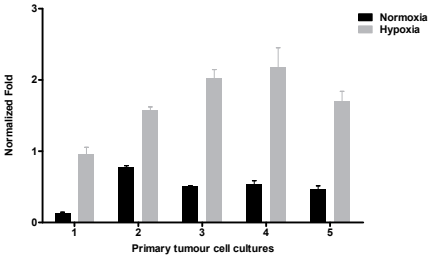

FIGURE 2. VEGF-A mRNA expression in primary UM cell cultures.
The amount of VEGF-A mRNA expression was measured with qPCR in primary UM cell cultures (cultures 1-5) under normoxic (black) and hypoxic (gray) conditions after 24 hours. Normalized expression is demonstrated.

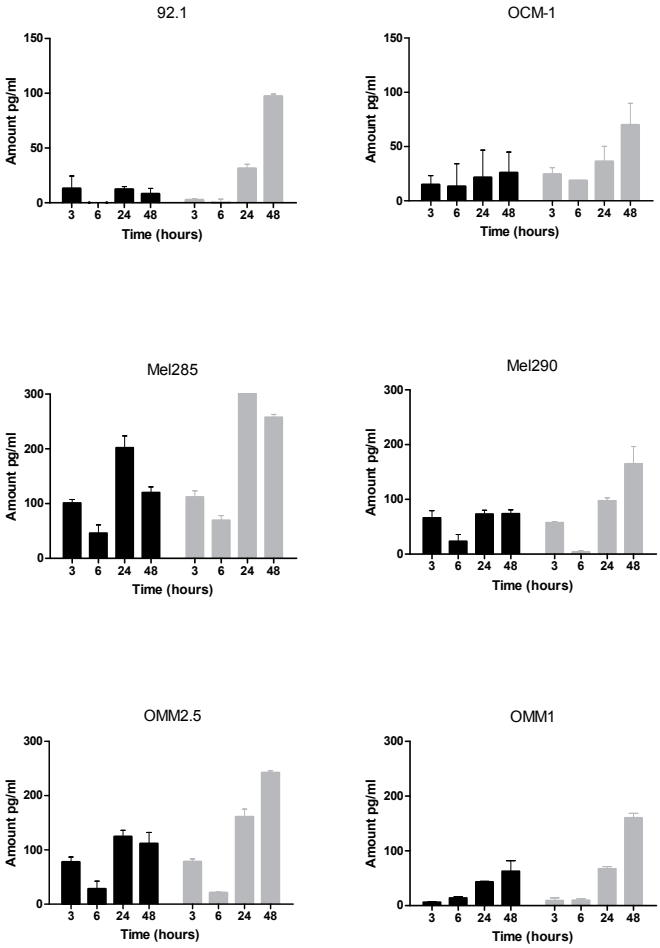

FIGURE 3. VEGF-A protein expression in UM cell lines.
The amount of VEGF-A protein expression measured with ELISA in supernatants of UM cell lines under normoxic (black) and hypoxic (gray) exposure after four different time intervals (3, 6, 24 and 48 hours). Expression is demonstrated in amount of protein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

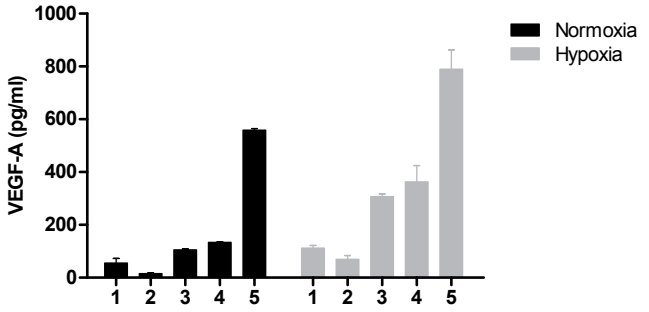

FIGURE 4. VEGF-A protein expression in primary UM cell cultures.
The amount of VEGF-A protein expression measured with ELISA in supernatants of primary UM cell cultures (cultures 1-5) under normoxic (black) and hypoxic (gray) exposure after 24 hours. Expression is demonstrated in amount of protein (pg/ml).

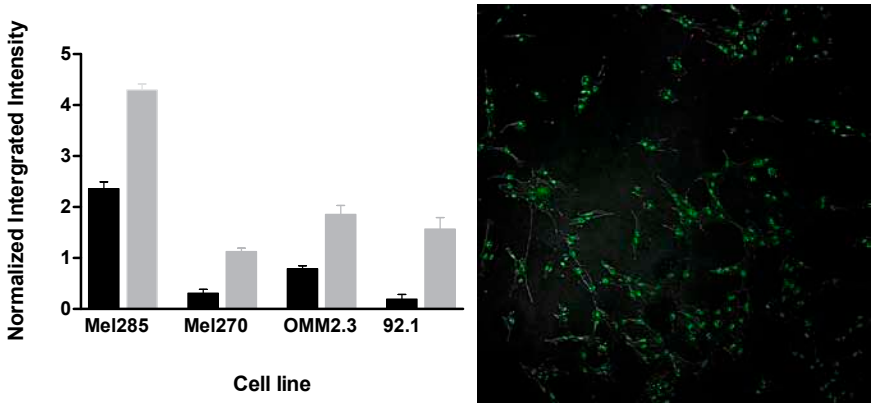

FIGURE 5. HIF-1α expression in UM cell lines.
Expression of HIF-1α was measured in UM cell lines using in-cell Western assay under normoxic (black) and hypoxic (gray) exposure during 24 hours. Expression is demonstrated in normalized integrated intensity ((HIF-1α/ cell count) ×100) (top). Image of HIF-1α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

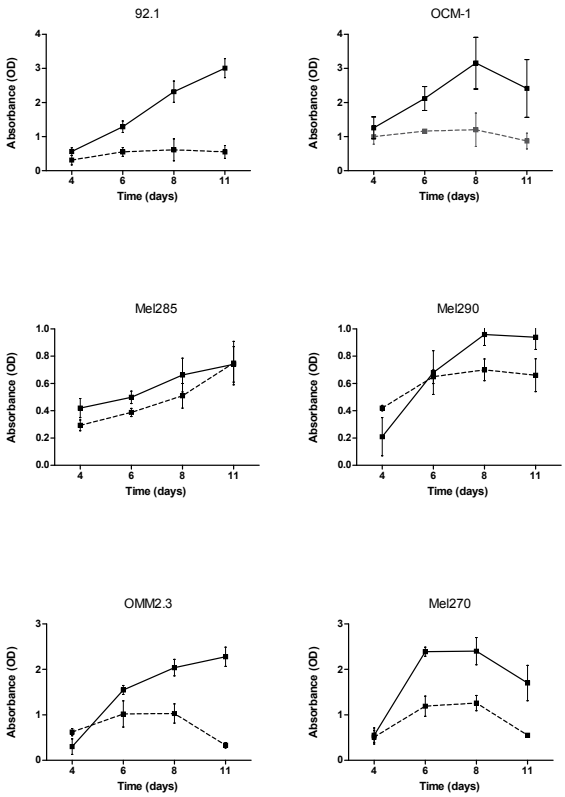

FIGURE 6. Cell proliferation of UM cell lines.
The proliferation rate of UM cell lines measured by WST-1 viability assay under normoxic (solid line) and hypoxic (dashed line) exposure after four different time intervals (4, 6, 8 and 11 days). Proliferation is measured by viability of the cells at each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

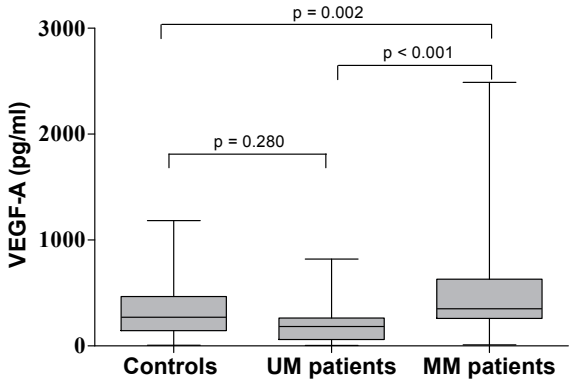

FIGURE 7. Concentration of serum VEGF-A in (metastatic) UM patients and controls.
Concentration of serum VEGF-A in the control group, and in patients with and without metastatic uveal melanoma. P-values (Mann-Whitney test) between the different groups are indicated in the graph. Each box shows the median, 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.

ACKNOWLEDGEMENTS

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REFERENCE LIST

1. Egan, K. M., Seddon, J. M., Glynn, R. J., Gragoudas, E. S., and Albert, D. M. *Epidemiologic aspects of uveal melanoma.* Surv.Ophthalmol. 1988. 32:239-251.
2. Witmer, A. N., Vrensen, G. F., Van Noorden, C. J., and Schlingemann, R. O. *Vascular endothelial growth factors and angiogenesis in eye disease.* Prog.Retin.Eye Res. 2003. 22:1-29.
3. Gasparini, G. *Prognostic value of vascular endothelial growth factor in breast cancer.* Oncologist. 2000. 5 Suppl 1:37-44.
4. Machein, M. R. and Plate, K. H. *Role of VEGF in developmental angiogenesis and in tumor angiogenesis in the brain.* Cancer Treat.Res. 2004. 117:191-218.
5. Boyd, S. R., Tan, D., Bunce, C., Gittos, A., Neale, M. H., Hungerford, J. L., Charnock-Jones, S., and Cree, I. A. *Vascular endothelial growth factor is elevated in ocular fluids of eyes harbouring uveal melanoma: identification of a potential therapeutic window.* Br.J.Ophthalmol. 2002. 86:448-452.
6. Missotten, G. S., Notting, I. C., Schlingemann, R. O., Zijlmans, H. J., Lau, C., Eilers, P. H., Keunen, J. E., and Jager, M. J. *Vascular endothelial growth factor a in eyes with uveal melanoma.* Arch.Ophthalmol. 2006. 124:1428-1434.
7. el, Filali M., Homminga, I., Maat, W., van, d., V, and Jager, M. J. *Triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth.* Mol.Vis. 2008. 14:1752-1759.
8. Papetti, M. and Herman, I. M. *Mechanisms of normal and tumor-derived angiogenesis.* Am.J.Physiol Cell Physiol 2002. 282:C947-C970.
9. Augsburger, J. J., Correa, Z. M., Freire, J., and Brady, L. W. *Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy.* Ophthalmology 1998. 105:1670-1678.
10. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
11. Foss, A. J., Alexander, R. A., Jefferies, L. W., Hungerford, J. L., Harris, A. L., and Lightman, S. *Microvessel count predicts survival in uveal melanoma.* Cancer Res. 1996. 56:2900-2903.
12. Makitie, T., Summanen, P., Tarkkanen, A., and Kivela, T. *Microvascular density in predicting survival of patients with choroidal and ciliary body melanoma.* Invest Ophthalmol.Vis.Sci. 1999. 40:2471-2480.
13. Ly, L., Odish, O., de Wolff-Rouendaal, D., Missotten, G., Luyten, G., and Jager, M. *Intravascular presence of tumor cells as prognostic parameter in uveal melanoma: a 35-year survey.* Invest Ophthalmol. Vis.Sci. 2009.
14. Chen, P. W., Murray, T. G., Salgaller, M. L., and Ksander, B. R. *Expression of MAGE genes in ocular melanoma cell lines.* J.Immunother. 1997. 20:265-275.
15. De Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer 1995. 62:155-161.
16. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
17. Luyten, G. P., Naus, N. C., Mooy, C. M., Hagemeijer, A., Kan-Mitchell, J., van, D. E., Vuzevski, V., de Jong, P. T., and Luider, T. M. *Establishment and characterization of primary and metastatic uveal melanoma cell lines.* Int.J.Cancer 1996. 66:380-387.
18. Verbik, D. J., Murray, T. G., Tran, J. M., and Ksander, B. R. *Melanomas that develop within the eye inhibit lymphocyte proliferation.* Int.J.Cancer 1997. 73:470-478.
19. Bustin, S. A. *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.* J.Mol.Endocrinol. 2000. 25:169-193.

20. Zuidervaart, W., van, d., V, Hurks, M. H., van Nieuwpoort, F. A., Out-Luiting, C. J., Singh, A. D., Frants, R. R., Jager, M. J., and Gruis, N. A. *Gene expression profiling identifies tumour markers potentially playing a role in uveal melanoma development.* Br.J.Cancer 2003. 89:1914-1919.
21. Gilsbach, R., Kouta, M., Bonisch, H., and Bruss, M. *Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data.* Biotechniques 2006. 40:173-177.
22. Narayanan, R., Kenney, M. C., Kamjoo, S., Trinh, T. H., Seigel, G. M., Resende, G. P., and Kuppermann, B. D. *Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells.* Invest Ophthalmol.Vis.Sci. 2005. 46:304-309.
23. Chen, H., Kovar, J., Sissons, S., Cox, K., Matter, W., Chadwell, F., Luan, P., Vlahos, C. J., Schutz-Geschwender, A., and Olive, D. M. *A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy.* Anal.Biochem. 2005. 338:136-142.
24. Kovar, J. L., Johnson, M. A., Volcheck, W. M., Chen, J., and Simpson, M. A. *Hyaluronidase expression induces prostate tumor metastasis in an orthotopic mouse model.* Am.J.Pathol. 2006. 169:1415-1426.
25. Hanahan, D. and Weinberg, R. A. *The hallmarks of cancer.* Cell 2000. 100:57-70.
26. Bergers, G. and Benjamin, L. E. *Tumorigenesis and the angiogenic switch.* Nat.Rev.Cancer 2003. 3:401-410.
27. Ijland, S. A., Jager, M. J., Heijdra, B. M., Westphal, J. R., and Peek, R. *Expression of angiogenic and immunosuppressive factors by uveal melanoma cell lines.* Melanoma Res. 1999. 9:445-450.
28. Mizukami, Y., Kohgo, Y., and Chung, D. C. *Hypoxia inducible factor-1 independent pathways in tumor angiogenesis.* Clin.Cancer Res. 2007. 13:5670-5674.
29. Barleon, B., Sozzani, S., Zhou, D., Weich, H. A., Mantovani, A., and Marme, D. *Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1.* Blood 1996. 87:3336-3343.
30. Potgens, A. J., Lubsen, N. H., van Altena, M. C., Schoenmakers, J. G., Ruiter, D. J., and de Waal, R. M. *Vascular permeability factor expression influences tumor angiogenesis in human melanoma lines xenografted to nude mice.* Am.J.Pathol. 1995. 146:197-209.
31. Claffey, K. P., Brown, L. F., del Aguila, L. F., Tognazzi, K., Yeo, K. T., Manseau, E. J., and Dvorak, H. F. *Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis.* Cancer Res. 1996. 56:172-181.
32. Westphal, J. R., van't Hullenaar, R. G., van der Laak, J. A., Cornelissen, I. M., Schalkwijk, L. J., van Muijen, G. N., Wesseling, P., de Wilde, P. C., Ruiter, D. J., and de Waal, R. M. *Vascular density in melanoma xenografts correlates with vascular permeability factor expression but not with metastatic potential.* Br.J.Cancer 1997. 76:561-570.
33. Sheidow, T. G., Hooper, P. L., Crukley, C., Young, J., and Heathcote, J. G. *Expression of vascular endothelial growth factor in uveal melanoma and its correlation with metastasis.* Br.J.Ophthalmol. 2000. 84:750-756.
34. Maat, W., Jordanova, E. S., van Zelderen-Bhola, S. L., Barthen, E. R., Wessels, H. W., Schalij-Delfos, N. E., and Jager, M. J. *The heterogeneous distribution of monosomy 3 in uveal melanomas: implications for prognostication based on fine-needle aspiration biopsies.* Arch.Pathol.Lab Med. 2007. 131:91-96.
35. Karayiannakis, A. J., Syrigos, K. N., Polychronidis, A., Zbar, A., Kouraklis, G., Simopoulos, C., and Karatzas, G. *Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery.* Ann.Surg. 2002. 236:37-42.
36. Hayes, A. J., Mostyn-Jones, A., Koban, M. U., A'Hern, R., Burton, P., and Thomas, J. M. *Serum vascular endothelial growth factor as a tumour marker in soft tissue sarcoma.* Br.J.Surg. 2004. 91:242-247.

37. Celen, O., Kahraman, I., Yildirim, E., and Berberoglu, U. *Correlation of vascular endothelial growth factor (VEGF) and CEA with clinicopathological variables in colorectal cancer patients.* Neoplasma 2004. 51:293-299.
38. Eskelin, S., Pyrhonen, S., Summanen, P., Prause, J. U., and Kivela, T. *Screening for metastatic malignant melanoma of the uvea revisited.* Cancer 1999. 85:1151-1159.
39. Kaiserman, I., Amer, R., and Pe'er, J. *Liver function tests in metastatic uveal melanoma.* Am.J.Ophthalmol. 2004. 137:236-243.
40. Barak, V., Frenkel, S., Kalickman, I., Maniotis, A. J., Folberg, R., and Pe'er, J. *Serum markers to detect metastatic uveal melanoma.* Anticancer Res. 2007. 27:1897-1900.
41. Missotten, G. S., Korse, C. M., van Dehn, C., Linders, T. C., Keunen, J. E., Jager, M. J., and Bonfrer, J. M. *S-100B protein and melanoma inhibitory activity protein in uveal melanoma screening. A comparison with liver function tests.* Tumour.Biol. 2007. 28:63-69.
42. Barak V, Frenkel S, Kalickman I, Pe'erJ. VEGF serum levels as a new marker for metastic uveal melanoma. Presented at the International Congress of Ocular Oncology, Cambridge, UK; 2009.

CHAPTER 5

BEVACIZUMAB AND INTRAOCULAR TUMORS: AN INTRIGUING PARADOX

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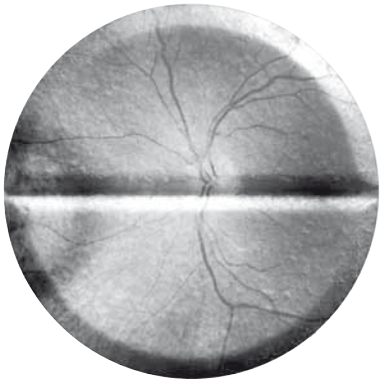

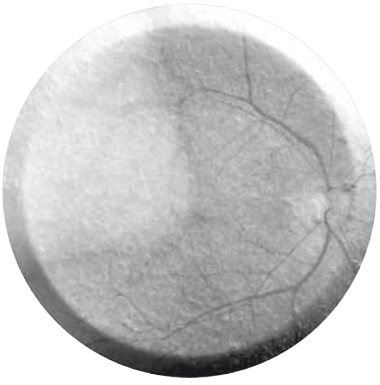

ABSTRACT

Purpose. Bevacizumab, a humanized monoclonal antibody to Vascular Endothelial Growth Factor-A (VEGF-A), was originally developed as an anti-tumor treatment. In ocular oncology, it is being used to treat macular edema due to radiation retinopathy, but it may also be useful for the treatment of primary uveal melanoma (UM) or its metastases. We determined the effect of bevacizumab on the growth of B16F10 cells inside the eye and on B16F10 and UM cells cultured in vitro.

Methods. B16F10 melanoma cells were placed into the anterior chamber of the eye of C57Bl/6 mice and tumor growth was monitored after injection of different doses of bevacizumab or mock injection. In addition, the effect of bevacizumab on in vitro growth of B16F10 and human UM cells and on the expression of VEGF-A, GLUT-1, and HIF-1α was evaluated.

Results. Following intraocular injection of bevacizumab into murine B16 tumor-containing eyes, an acceleration of tumor growth was observed, with the occurrence of anterior chamber hemorrhages. Bevacizumab did not affect proliferation of B16F10 cells in vitro, while it inhibited UM cell proliferation. Expression analysis demonstrated that addition of bevacizumab under hypoxic conditions induced VEGF-A, GLUT-1 and HIF-1α in B16F10 cells as well as in UM cell lines and two of four primary UM tumor cultures.

Conclusion. In contrast with expectations, intraocular injection of bevacizumab stimulated B16F10 melanoma growth in murine eyes. In vitro exposure of B16 and human UM cells to bevacizumab led to paradoxical VEGF-A upregulation. The use of VEGF inhibitors for treatment of macular edema (due to radiation retinopathy) after irradiation of UM should be considered carefully, because of the possible adverse effects on residual UM cells.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular tumor in adults with an annual incidence of 7-10 cases per million per year. Current treatments of UM depend on several clinical factors and include enucleation, radiotherapy (plaque, proton beam or stereotactic irradiation), transpupillary thermotherapy (TTT) and local resection [1-7]. Radiotherapy using a radioactive plaque is a highly successful therapy, achieving local tumor control of UM in up to 97% of treated cases [8-10]. However, radiation therapy may lead to radiation retinopathy, a slowly progressive, delayed-onset disease of retinal blood vessels characterized by retinal ischemia, neovascularization and leaking vessels [11-13]. Radiation retinopathy has been described in up to 63% of eyes after plaque radiation treatment [14-17]. Vascular endothelial growth factor (VEGF)-A, a strong pro-angiogenic factor, most likely contributes to its pathogenesis: it has been shown that VEGF-A can be produced by retinal tissue as well as hypoxic UM cells [18,19]. Intravitreal bevacizumab (a humanized monoclonal antibody to VEGF-A) has been used to treat numerous ophthalmologic disorders involving ischemia and neovascularization, including radiation retinopathy. Several studies demonstrate a decrease of macular edema in radiation retinopathy and improvement of visual acuity after intravitreal bevacizumab treatment [20-26].

Another indication for using bevacizumab might be the treatment of the tumor itself and its metastases. Bevacizumab has been approved for treatment of metastases of several malignancies, including colorectal, renal, and lung cancers [27-29], and is still under investigation for numerous other primary tumors and metastatic disease, e.g. of breast and pancreas cancer and cutaneous melanoma [30-32]. Despite the good primary tumor control achieved by current treatment options, estimates of 5-year UM-related mortality range from 26% to 32% [33,34], and up to 50% of all UM patients may eventually die due to metastatic disease [35,36].

Blood vessels in primary UM can facilitate tumor cell entry into the circulation, resulting in metastatic disease [37]. Yang et al. studied the systemic treatment of metastatic disease of UM with bevacizumab in mice and demonstrated a reduction in the number of metastases [38]. There are no studies describing a possible treatment with intravitreal VEGF inhibitors for primary uveal melanoma. It has been proposed that patients who develop clinical metastases from UM often harbour micrometastases for years which will most likely resemble the primary UM cell genotype [39]. Also, following radiation therapy of an intraocular melanoma, viable UM cells may remain, and these might be influenced by intraocular treatment with intravitreal bevacizumab. While bevacizumab might be a useful drug to attack uveal melanoma, several studies have been published describing unexpected effects of bevacizumab on tumor cells, resulting in tumor recurrences and therapy resistance [40,41]. We therefore investigated the effect of bevacizumab on intraocular tumor growth of the murine B16F10 melanoma cell line in an in vivo mouse model In addition, we analyzed bevacizumab's effect

on the proliferation of this B16F10 cell line, on UM cell lines in vitro, and on primary UM cell cultures [42].

MATERIAL AND METHODS

In vivo experiments

Male C57BL/6jico mice, 8 weeks old, were obtained from Charles River (Lille, France). The mice were housed under Specific Pathogen Free (SPF) conditions and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory animals, NIH guidelines on laboratory animal welfare, and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All research protocols were approved by the local Committee for Animal Welfare (DEC), LUMC, Leiden, The Netherlands. B16F10 melanoma cells were injected into the anterior chamber of the mice as described previously [43]. In short, mice were anesthesized intraperitoneally using Xylazine (Rompun 2%, Bayer, Leverkussen, Germany) and Ketamine Hydrochloride (Aescoket; Aesculaap bv, Boxtel, The Netherlands; ratio 1:1). A sterile 30 G needle was used to make a paracentesis at the corneoscleral junction, parallel and anterior to the iris. A fused silica capillary (200 µm outer diameter (OD), 100 µm inner diameter (ID) was fitted into a union (VALCO, Vici AG international, Schenkon, Switzerland). The capillary and the union were mounted onto a 0.1 ml Hamilton syringe. The capillary, loaded with B16F10 melanoma cells ($2.5.10^4$ cells/ 4 µL (approximate volume of anterior chamber of murine eye), was inserted through the paracentesis in the cornea, and the tumor cells were deposited into the anterior chamber.

Mice were separated into three groups (n=7 per group) and the experiment was performed twice; the first group received a low dose of bevacizumab (equivalent human dose; 2µg/ 4 µl), the second group received a high dose (10 times the equivalent human dose; 20 µg/ 4 µl) of bevacizumab; a control group was injected with a mock injection with 4 µl phosphate-buffered saline (PBS).

The eyes were examined three times a week using a dissecting microscope to monitor tumor growth. Tumor volume was recorded as the percentage of anterior chamber occupied with tumor. Mice were sacrificed by cervical dislocation when the tumor occupied 80-100% of the anterior chamber. Afterwards, all murine eyes were fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin. Serial sections of 4µm were prepared, mounted on glass slides and stained with hematoxylin and eosin. The number of vessels and their location were evaluated in every tumor-containing eye of one experiments (n=7 per group).

Cell lines and primary tumor cell cultures

The B16F10 melanoma cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, CA), supplemented with 10% fetal calf serum (FCS), glutamine and 2% penicillin/ streptomycin. Cells were incubated at (37°C, 5 % CO_2). When cultures showed 70% confluency, the cells were harvested and used for inoculation or in vitro experiments.

Two UM cell lines and four primary UM cell cultures were cultured under either normoxic (20% O_2) or hypoxic (1% $O_{2;}$ during experiments) conditions (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). Mel285 is a primary tumor-derived cell line, while OMM2.3 is a metastasis-derived cell line. Mel285, and OMM2.3 were provided by dr. B. Ksander (Schepens Eye Research Institute, Boston, MA) [44,45]. Four primary cell cultures were established in our laboratory. UM cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) 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%). Fresh tumor tissues, obtained immediately after enucleation of the eye, were cultured in Amniochrome® Pro Medium (Lonza Group Ltd, Basel, Switzerland) and passaged maximally once or twice before experiments.

Cell proliferation

Cell proliferation was measured by mitochondrial function using the water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Indianapolis, IN, USA), as previously described [46]. In short, 96-well plates were filled with 1200 cells per well, filled with regular medium (control) or bevacizumab solutions (three doses, see 'bevacizumab treatment' on the next page), 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 to mimic in vivo ischemia (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). Proliferation was measured on days 1, 2, and 3 (B16F10) or 1, 3, and 6 (human cell lines and primary cultures) by adding 10ul WST-reagent to 8 wells (8 wells without reagent for background absorbance). Absorbance was measured at 450nm (n=8) on a multiwell spectrophotometer (Perkin lmer, Wellesley, MA).

Quantitative PCR

To analyze VEGF-A mRNA expression after treatment with bevacizumab, reverse transcriptase in combination with quantitative PCR experiments were performed, as described before [19,47]. In short, RNA was isolated using an Rneasy® Mini Kit (Qiagen, Valencia, USA) and RNA samples were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Using 96-well plates, a solution of sample cDNA, iQ SYBR Green Supermix, forward and reverse primers for beta-actine (β-ACTIN), ribosomal protein S11 (RPS-11), VEGF-A and sterile water was prepared. Primer sequences were used as described previously and are summarized in Table 1 [19,48]. A quantitative analysis of the samples was then performed for gene expression by qPCR in a IQ5 PCR system (Bio-Rad, Hercules CA, USA).

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
	GLUT-1	CCCGCTTCCTGCTCATCAACC	GCCGACTCTCTTCCTTCATCTCC

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

The PCR reaction settings were 95°C for 3 minutes, 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds, followed by 95°C for 1 minute, and 60°C for 1 minute. To correct the sample-to-sample variation, several cellular housekeeping genes were selected using Genorm software (Center for Medical Genetics, Ghent, Belgium), and these served as an endogenous control against which the target-gene expression levels were normalized (normalized expression of treated cells/ normalized expression of control cells after 24 hours) [47-49].

VEGF-A protein expression

The VEGF-A protein concentration was measured in the supernatant of the B16F10 cell culture using a commercial sandwich enzyme-linked immunosorbent assay (mouse VEGF-A ELISA, Arcus Biologicals, Modena, Italy). The lowest measurable concentration was 4.5 pg/ml.

HIF-1α in-cell Western experiment

To determine if bevacizumab influences VEGF-A expression in UM cells through the HIF-1α pathway, phosphorylation and activation of HIF-1α were analysed using an in-cell Western immunofluorescent assay as described before [19,50,51]. Briefly, 96-well plates were filled with 600 melanoma cells per well, filled with regular medium (control) or bevacizumab solutions (three doses, see 'bevacizumab treatment' on the next page), and placed in a hypoxic (1% O_2, 5 % CO_2, 37°C) chamber (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA) for 24 hours, to mimic hypoxic in vivo conditions and stimulate VEGF-A expression. Cells were fixed and permeabilized for 1.5 hours before incubation with HIF-1α antibody (Bethyl laboratories INC Montgomery, TX, USA (1:1000 for 2 hours)) in Odyssey Blocking Buffer. After washing, plates were incubated with the secondary antibody Goat anti-Rabbit IRDye® 800CW (1:800); for determining the cell number, DRAQ5 (1:2000) and Sapphire700 (1:1000) were used (all three from LI-COR Biosciences, Lincoln NB, USA). The plate was scanned on an Odyssey infrared scanner, HIF-1α at the 800nm channel and DRAQ5 at the 700nm channel (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).

Bevacizumab treatment

Several bevacizumab (Avastin®, Roche Pharma, Germany) solutions were used to investigate treatment options in vitro and in vivo.

In vitro uveal melanoma cells

To treat UM cell cultures, bevacizumab was dissolved in medium to obtain solutions of 1.25 mg/ 5.46 ml (generally used intraocular human dose), 12.5 mg/5.46 ml (10 times the normal dose) or 25 mg/5.46 ml (20 times the normal dose) per well. As control, we used culture medium.

In vivo mice experiments

Equivalent mouse doses were calculated according to human and mouse intraocular volume: as 1.25 mg bevacizumab is used to inject the human eye with a mean volume of 5.46 ml, we used 2µg (first group; equivalent human dose) or 20 µg (second group 10 times equivalent human dose) to inject a murine eye with a mean volume of 9.60 µl. The third group received a mock injection with PBS as previously described. Injections were performed on days 2, 6, and 10 after tumor cell inoculation (not all mice received an injection on day ten since some of them were already sacrificed).

Statistical analysis

Statistical analysis was performed with Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA). Overall survival of mice was estimated by the Kaplan-Meier method and log-rank test. One-way ANOVA and the Bonferonni test were used to analyze for significant differencences in cell proliferation and HIF-1α activation of treated and untreated cells. $P < 0.05$ was considered statistically significant.

RESULTS

Intraocular tumor growth in a B16F10 eye tumor mouse model

As VEGF-A inhibitors are being used to treat many different types of cancer metastases, we wondered whether they can be used to inhibit intraocular tumor growth. After intraocular inoculation of B16F10 melanoma cells into the anterior chamber of C57Bl/6 mice, two different doses of bevacizumab or medium were injected intraocularly on days 2, 6 and 10. Each group consisted of seven mice, and the experiment was performed twice. A summary of the results of the two experiments is shown in Figure 1. Whereas inhibition of tumor and vessel growth was expected, an acceleration of intraocular tumor growth was observed in eyes treated with both doses of bevacizumab. Treated mice had to be sacrificed earlier because the tumor started to protrude through the cornea. During the experiment, several

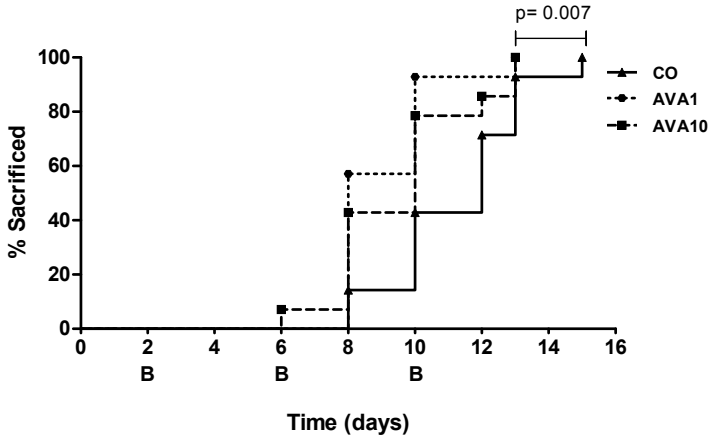

FIGURE 1. Acceleration of intraocular tumor growth in mice after treatment with bevacizumab.
After placement of B16F10 melanoma cells in the eye of C57Bl/6 mice, bevacizumab was injected intraocularly to try to inhibit intraocular tumor growth. Following three bevacizumab injections on days 2, 6 and 10, a significant acceleration of intraocular tumor growth occurred compared to the control group (AVA1 versus control p = 0.007, and AVA10 versus control p = 0.06). Growth was recorded as the percentage of anterior chamber occupied with tumor, and mice were sacrificed when the tumor occupied 80-100% of the anterior chamber. The curves are the pooled data from two experiments, with 14 (two times 7) mice in each of the three groups.
AVA1= equivalent human dose: 2 µg/ 4 µl; AVA10= 10 times the equivalent human dose: 20 µg/ 4 µl; CO= control group: 4 µl mock PBS injection.

anterior chamber and tumor hemorrhages were observed in the eyes that had been treated with bevacizumab but not in untreated eyes (Figure 2).

B16F10 murine melanoma cell line: in vitro experiments

In vitro cell proliferation

As the in vivo results showed the opposite effects of what was expected, we studied the effect of bevacizumab on tumor cell proliferation under normoxia and hypoxia. Addition of bevacizumab to B16F10 cells in vitro did not effect proliferation of tumor cells cultured in normoxic conditions, while a dose-dependent decrease in proliferation was noticed after treatment with bevacizumab under normoxic conditions (Figure 3).

In vitro VEGF-A mRNA expression

As intraocular and intratumoral hemorrhages were visible in eyes receiving bevacizumab, we wondered whether the anti-VEGF-A treatment with bevacizumab had a paradoxical stimulatory effect on VEGF-A gene expression. Indeed, bevacizumab induced VEGF-A mRNA expression in a dose-dependent manner when cells were cultured under hypoxic

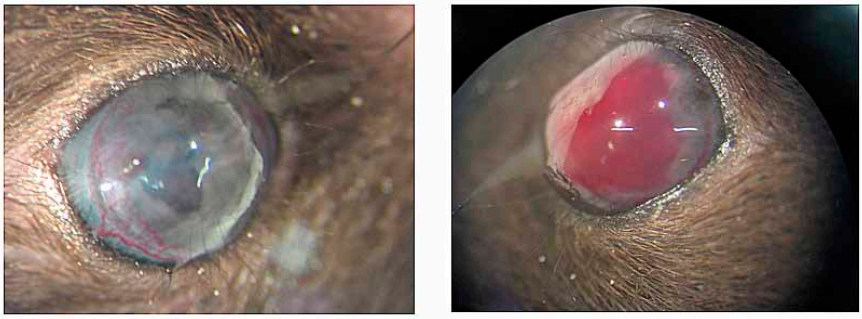

FIGURE 2. Anterior chamber hemorrhages in murine eyes treated with bevacizumab.
Anterior chamber hemorrhage as seen in a murine tumor eye treated with bevacizumab (right) in contrast to an untreated eye (left).

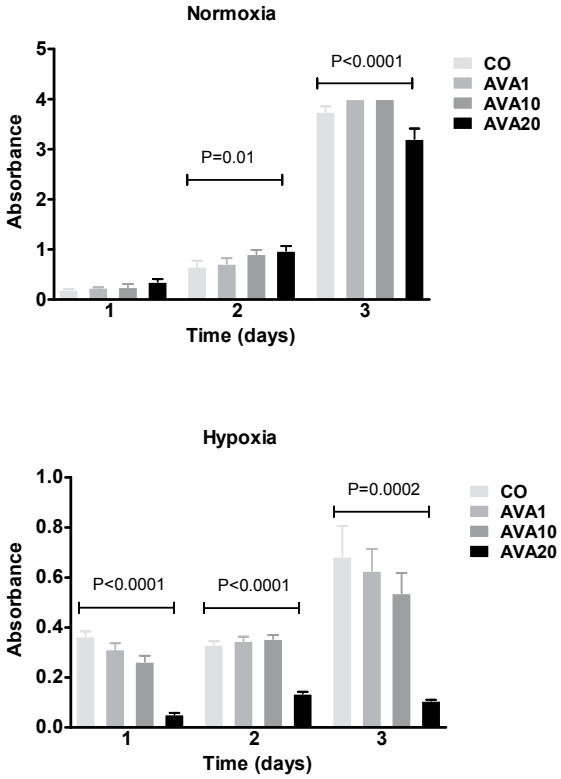

FIGURE 3. A high dose of bevacizumab inhibits proliferation of B16F10 cells under hypoxic conditions.
Bevacizumab was added to B16F10 cells in vitro under normoxic (top) and hypoxic (bottom) circumstances and proliferation was measured by water-soluble tetrazolium salt assay on days one, two and three after the addition of bevacizumab. Cell density is expressed in absorbance (optical density). A high dose of bevacizumab inhibited cell growth under hypoxic conditions

conditions. Under normoxia, baseline VEGF-A expression is relatively high but not affected by bevacizumab treatment (Figure 4).

In contrast to mRNA expression, in vitro treatment of B16F10 cells with the human equivalent dose of bevacizumab led to a lower VEGF protein expression after 3 or 6 days of culture (Figure 4). We hypothesize that the high concentration of bevacizumab effectively bound the produced VEGF-A, thus making it unavailable for binding in the immuno assay.

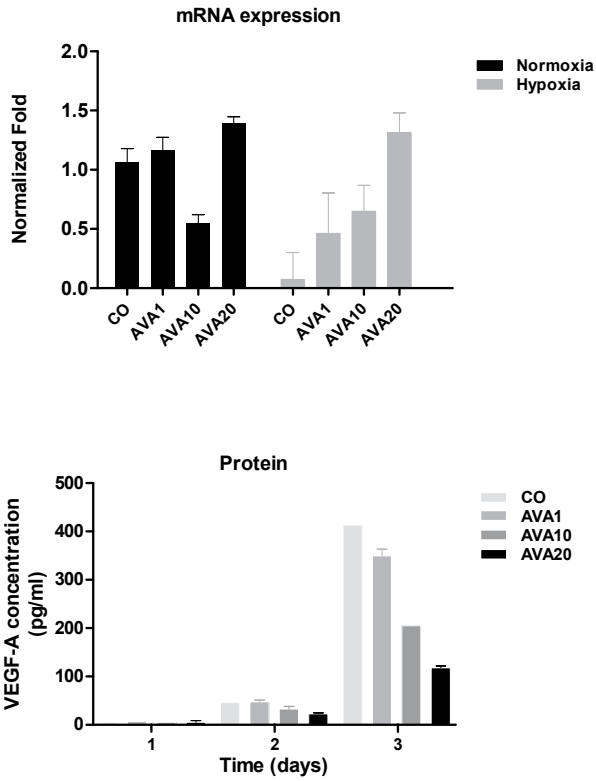

FIGURE 4. Bevacizumab induces vascular endothelial growth factor A mRNA expression in B16F10 cells under hypoxic conditions.

The effect of bevacizumab on VEGF-A expression in B16F10 cells was determined using qPCR analysis (top) and ELISA (bottom). The amount of VEGF-A mRNA expression was measured with qPCR under normoxic (black) and hypoxic (gray) conditions after 24 hours with different doses bevacizumab treatment. Expression is demonstrated in normalized fold. VEGF-A mRNA expression increased in a dose-dependent manner after treatment with bevacizumab, when cells were cultured under hypoxic conditions.

An ELISA was used to measure the amount of VEGF-A protein in the supernatant of UM cell lines after hypoxic exposure for 24 hours. Shown is the amount of VEGF-A protein (pg/ml). Paradoxically, the amount of produced protein is reduced in the presence of bevacizumab.

AVA1= equivalent human dose: 1.25 mg/5.46 ml; AVA10= 10 times the equivalent human dose: 12.5 mg/ 5.46 ml; AVA20= 20 times the equivalent human dose: 25 mg/ 5.46 ml; CO= control group: culture medium.

Histologic and in vivo observation of tumor-inoculated murine eyes

Based on the in vitro results, the acceleration of tumor growth in vivo could not be due to a direct proliferative effect, but possibly due to formation of new vessels after induction of VEGF-A. After sacrificing the mice, all eyes were enucleated and stained with haematoxylin and eosin to evaluate the amount of vessels and their location in the tumor. No apparent differences were present in the amount of vessels in the presence or absence of bevacizumab treatment (data not shown).

Involvement of HIF-1α pathway

As the new working hypothesis was that the observed intraocular tumor growth after bevacizumab was due to an increased production of VEGF-A, one might expect an upregulation of the pathways involved in VEGF-A induction. To investigate whether the increased VEGF-A mRNA expression after exposure to bevacizumab involved HIF-1α, an in-cell Western assay was performed. Under already hypoxic conditions, treatment with 10 times the human equivalent of bevacizumab induced an increase of HIF-1α protein in B16F10 melanoma cells in comparison to the control (Figure 5).

To further confirm involvement of HIF-1α, GLUT-1 expression was measured as a determinant of HIF-1α transcriptional activity. Gene expression analysis showed an induction of

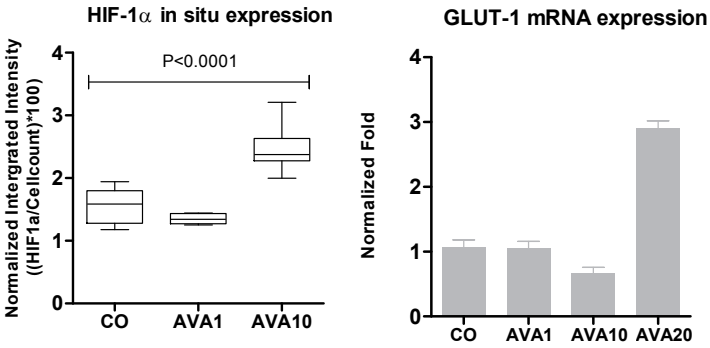

FIGURE 5. Bevacizumab induces HIF-1α activation and GLUT-1 mRNA expression in B16F10 cells under hypoxic conditions.

Expression of HIF-1α was measured in B16F10 cells using an in-cell Western assay after cell exposure to hypoxic conditions for 24 hours. Expression is demonstrated in normalized integrated intensity ((HIF-1α/ cell count)*100). A significant induction of HIF-1α was observed when comparing the AVA10 treated cells with the control (p=0.03) (top).

qPCR analysis shows an induction of GLUT-1 expression in B16F10 cells treated with the high dose of bevacizumab (AVA20) in comparison to no treatment. Expression is demonstrated in normalized fold (bottom).

AVA1= equivalent human dose: 1.25 mg/ 5.46 ml; AVA10= 10 times the equivalent human dose: 12.5 mg/ 5.46 ml; AVA20= 20 times the equivalent human dose: 25 mg/ 5.46 ml; CO= control group: culture medium.

GLUT-1 expression in B16F10 cells treated with bevacizumab in comparison to control, but only when treated with the highest dose (Figure 5).

Validation in human UM cell lines and primary cultures

In vitro uveal melanoma cell proliferation

In order to determine whether the mechanism observed in murine B16F10 cells was relevant to the human situation, the in vitro tests were repeated using human UM cell lines and primary cell cultures. Following treatment with bevacizumab, human UM cell lines showed a dose-dependent decrease of proliferation under normoxic as well as hypoxic culture conditions (Figure 6). Four primary tumor cell cultures displayed a greater variability: in

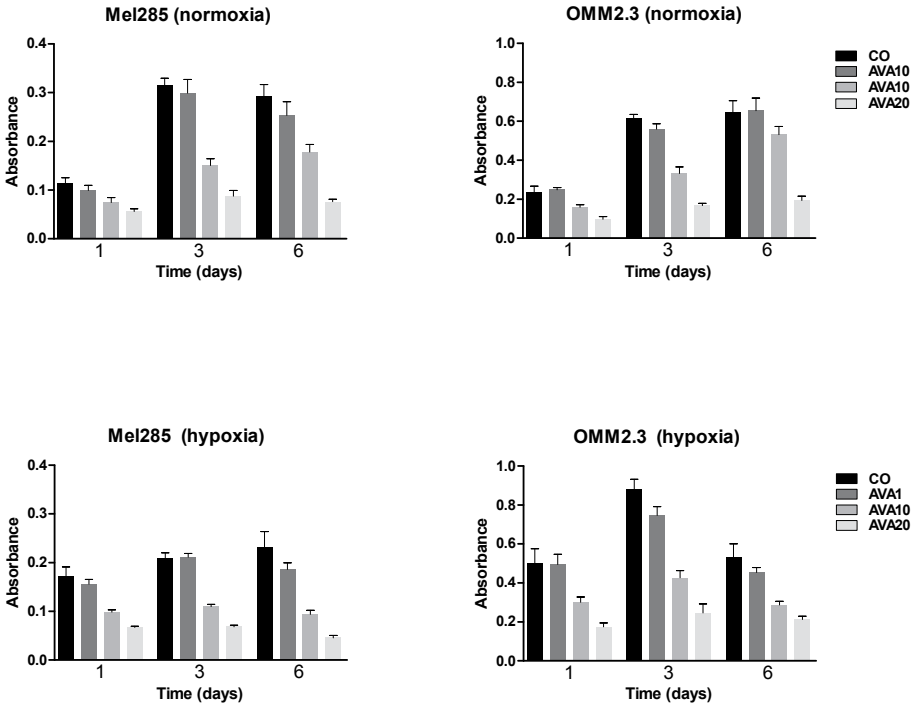

FIGURE 6. Bevacizumab inhibits proliferation of uveal melanoma cell lines in vitro.
Proliferation of UM cell lines was measured by WST-1 assay after exposure to three different doses of bevacizumab during one, three or six days, under normoxic and hypoxic conditions. Cell density is expressed as absorbance (optical density, OD). A dose-dependent decrease of proliferation after treatment with bevacizumab occurs under hypoxic as well as normoxic conditions. (Mel 285 normoxia day 1 p=0.0012, all other cellines/treatments p<0.0001). AVA1= equivalent human dose: 1.25 mg/ 5.46 ml; AVA10= 10 times the equivalent human dose: 12.5 mg/ 5.46 ml; AVA20= 20 times the equivalent human dose: 25 mg/ 5.46 ml; CO= control group: culture medium.

two cultures inhibition of proliferation was observed while two other cell cultures appeared resistant and did not show an effect (data not shown).

In vitro VEGF-A mRNA expression after bevacizumab treatment
Treatment of two UM cell lines with bevacizumab under hypoxic conditions led to an induction of VEGF-A mRNA (Figure 7). Exposure of UM cells to bevacizumab at normal oxygen levels showed the same trend, but was less substantial (data not shown).

In primary UM cell cultures, the effect of bevacizumab on proliferation had been variable: in the two cell cultures which had shown reduced proliferation, bevacizumab induced VEGF-A expression (Figure 8) with a maximum fold increase of 3.36 and 2.48 (cultures two and three). The two other cultures which continued to proliferate in the presence of bevacizumab showed a high VEGF-A expression that was not affected by treatment.

In vitro GLUT-1 mRNA expression after bevacizumab treatment under hypoxic conditions
To confirm involvement of HIF-1α in the response to bevacizumab, GLUT-1 expression was analyzed. An induction of GLUT-1 expression was present following treatment of UM cell lines with bevacizumab (Figure 7). GLUT-1 expression in primary cell cultures was significantly correlated with decrease in VEGF-A expression (correlation coefficient 0.974, P= 0.0002. Figure 8).

DISCUSSION

Anti-VEGF agents have proven to be potent therapeutic agents in ophthalmology for the treatment of Age-related Macular Degeneration (AMD), diabetic macular edema and neovascular glaucoma [47,48,52-54]. In UM, bevacizumab (Avastin®) is especially interesting because of a potential dual use: it is currently used in uveal melanoma patients to treat macular edema due to radiation retinopathy but may potentially be used to treat the primary uveal melanoma (UM) or its metastases.

We set out to determine the effect of intraocular injections of bevacizumab as a treatment for intraocular tumors in mice. Unexpectedly, an acceleration of intraocular tumor growth in response to anti-VEGF-A occurred. Previously, Yang et al. had shown a decrease of tumor size in the eye and a reduction in the number of metastases when bevacizumab was given systemically [38]. The difference in outcome may be due to a concentration effect, or it may be that direct contact with the vessel wall is important: in Yang's study, bevacizumab reached the tumor through the intravascular route, while we treated the tumor locally.

There has been some discussion concerning whether humanized bevacizumab can effectively block and neutralize murine VEGF [55]. The sequences of human $VEGF_{165}$ protein

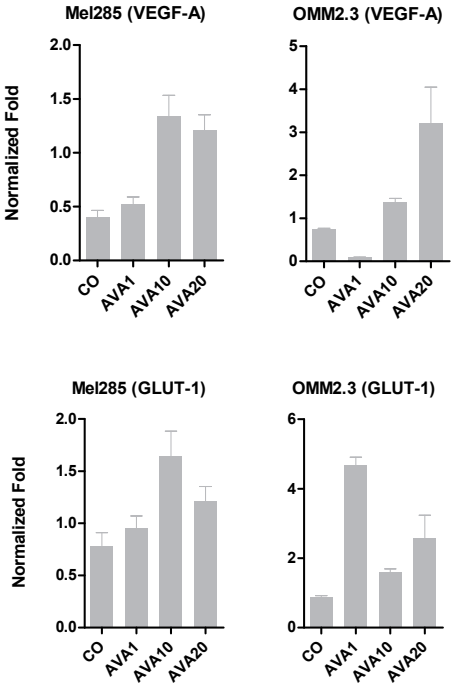

FIGURE 7. Bevacizumab induces VEGF-A and GLUT-1 mRNA expression in uveal melanoma cell lines.
mRNA expression was measured with qPCR after exposure to different doses of bevacizumab under hypoxic conditions for 24 hour. Expression is shown in normalized fold. Treatment with bevacizumab induced VEGF-A and GLUT-1 mRNA.
AVA1= equivalent human dose: 1.25 mg/ 5.46 ml; AVA10= 10 times the equivalent human dose: 12.5 mg/ 5.46 ml; AVA20= 20 times the equivalent human dose: 25 mg/ 5.46 ml; CO= control group: culture medium.

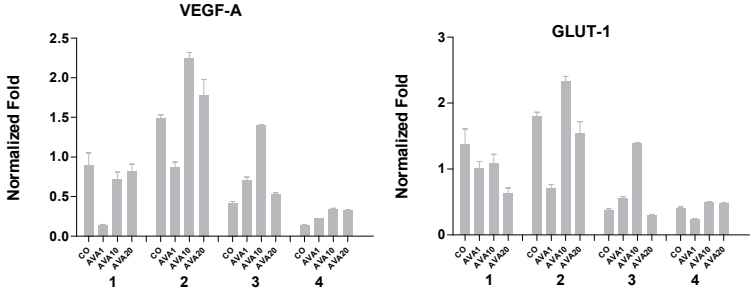

FIGURE 8. mRNA expression of VEGF-A differs in primary uveal melanoma cell cultures treated with bevacizumab but correlates with GLUT-1 mRNA expression.
mRNA expression was measured with qPCR under hypoxic conditions and after 24 hours of different doses bevacizumab treatment. Expression is demonstrated in normalized fold. Two cell cultures show a clear induction of VEGF-A expression after treatment with bevacizumab (culture two and three). GLUT-1 expression in UM cell cultures corresponds to the VEGF-A expression (bottom); correlation coefficient 0.974, P= 0.0002.
AVA1= equivalent human dose: 1.25 mg/ 5.46 ml; AVA10= 10 times the equivalent human dose: 12.5 mg/ 5.46 ml; AVA20= 20 times the equivalent human dose: 25 mg/ 5.46 ml; CO= control group: culture medium.

and mouse $VEGF_{164}$ protein are 86% similar [56]. In addition, Western blot analysis and ELISA analysis by Bock et al demonstrated that a high dose of bevacizumab can bind human as well as murine VEGF [57,58]. Additionally, we confirmed the effects noticed in the murine cells with human UM cell lines and short term fresh UM cultures: bevacizumab induced an upregulation of VEGF-A mRNA expression in B16F10 as well as human UM cells. This was especially true when the cells were cultured under hypoxic conditions, suggesting that a certain level of ischemia is needed to induce the VEGF-stimulating effect of bevacizumab.

We observed a relatively high VEGF-A expression under normoxic conditions in the B16F10 melanoma cells which suggests some kind of HIF-1α stabilization. This could involve the von Hippel-Lindau tumor suppressor (pVHL) that regulates the stability of Hypoxia-Inducible Factor (HIF)-1α. Loss of pVHL function results in constitutive activation of HIF-1α and thus VEGF-A expression [59-60]. Nonetheless, the observed induction of HIF-1α as well as GLUT-1 expression in response to hypoxia suggests that HIF-1α regulation is still intact.

One possible explanation for the in vivo tumor acceleration of tumor growth could be vessel formation after VEGF-A induction. Surprisingly, we detected no difference in the number of vessels between the different treatment groups. We did, however, perceive several anterior chamber and tumor hemorrhages in murine eyes treated with bevacizumab that may be a result of the VEGF-A induction. Originally, VEGF-A was referred to as vascular permeability factor (VPF) [61]. A rapid increase in vascular permeability occurs when the microvasculature is exposed acutely to any number of vascular permeabilizing factors, like VEGF-A, allowing for the diffusion of trophic substances to adjacent tumor cells [62]. An alternative explanation may be that mice were monitored for macroscopic tumor growth for merely eleven days, which may be too short for vessels to form. Moreover, the intraocular tumor is limited by the size of the anterior chamber of 2 mm which is about the limit to which cells can be supplied with oxygen and energy through the diffusion of trophic substances.

In our in vitro experiments, anti-VEGF-A therapy increased the amount of VEGF-A, mimicking hypoxic circumstances, and possibly inducing 'pseudohypoxia'. This phenomenon has been described before in other tumors. Verhoeff et al. for example describe that VEGF inhibition by local deposition of pegaptanib decreased tumor hypoxia (GLUT-1 positive tumor cells) in murine intracerebral glioma [63]. This 'pseudohypoxia' subsequently can activate alternative pro-angiogenic signaling circuits, as was noticed in a murine pancreatic neuroendocrine cancer model. In this model, treatment with an antibody that specifically blocked VEGFR signaling caused an initial response of tumor stasis followed by tumor recurrence afterwards. The relapsing tumor expressed higher levels of mRNAs of pro-angiogenic factors, and tumor relapse was preceded by hypoxic regions in the tumors in the response phase [40]. Additionally, more invasion and metastasis was implicated as a response to this anti-angiogenic treatment, while histological analysis demonstrated a more invasive phenotype and an increase of lymph node and liver metastasis in treated mice.

Recently, a novel set of isoforms has been described, the "VEGFxxxb" isoforms, which have the same length as the classical ones, because exon 8 (present in all the formerly known isoforms) is substituted by an alternatively-spliced exon of the same size (exon 8b). Several reports have demonstrated that VEGF165b may have anti-angiogenic properties [64,65]. On the other hand, it has been suggested that these isoforms may act as VEGF receptor agonists [66,67]. We did not study the possible contribution of VEGF165b.

With regard to the results in primary cell cultures we noticed inter-individual differences, as not all cultures showed an increase in VEGF-A or GLUT-1 expression after treatment with bevacizumab. This may be a reflection of molecular differences between tumors which may activate different biochemical pathways. We may be observing adaptive or evasive 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 to alternative malignant pathways that result in proliferation, migration and invasion.

Ischemic conditions caused by anti-VEGF treatment can also lead for instance to recruitment of various bone marrow-derived cells that have angiogenic capacities. Pro-angiogenic monocytes induce vessel growth by expression of several cytokines and angiogenic factors. Research in glioblastoma multiforme has demonstrated HIF-1α to promote angiogenesis by inducing recruitment of mature F4/80+ macrophages in mice treated with bevacizumab. Additionally, a clinical study suggests that hypoxia determines survival outcome in patients treated with bevacizumab for glioblastoma multiforme [68-70]. Since it has been shown previously that malignant UM tumors in patients with a poor survival have a lot of macrophages in their tumor, this mechanism is especially relevant [71].

In conclusion, we observe an acceleration of UM growth after treatment with bevacizumab in mice. We further demonstrate a 'pseudohypoxic' effect, through induction of HIF-1α and VEGF-A expression, in hypoxic UM cell lines and cultures after treatment with bevacizumab. This phenomenon has been described in other tumor types and maybe the consequence of tumor adaptive or evasive resistance. The use of bevacizumab for treatment of macular edema (due to radiation retinopathy) after irradiation of UM should be considered carefully.

REFERENCE LIST

1. Shields CL, Shields JA, Gunduz K, Freire JE, Mercado G. Radiation therapy for uveal malignant melanoma. Ophthalmic Surg Lasers. 1998;29:397-409.
2. Shields CL, Shields JA, Cater J, et al. Plaque radiotherapy for uveal melanoma: long-term visual outcome in 1106 consecutive patients. Arch Ophthalmol. 2000;118:1219-1228.
3. Melia BM, Abramson DH, Albert DM, et al. Collaborative ocular melanoma study (COMS) randomized trial of I-125 brachytherapy for medium choroidal melanoma. I. Visual acuity after 3 years COMS report no. 16. Ophthalmology. 2001;108:348-366.
4. Ener-West M, Earle JD, Fine SL, et al. The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma, III: initial mortality findings. COMS Report No. 18. Arch Ophthalmol. 2001;119:969-982.
5. Gragoudas ES, Goitein M, Verhey L, Munzenreider J, Suit HD, Koehler A. Proton beam irradiation. An alternative to enucleation for intraocular melanomas. Ophthalmology. 1980;87:571-581.
6. Gragoudas ES, Lane AM, Munzenrider J, Egan KM, Li W. Long-term risk of local failure after proton therapy for choroidal/ciliary body melanoma. Trans Am Ophthalmol Soc. 2002;100:43-48.
7. Journee-de Korver JG, Oosterhuis JA, Kakebeeke-Kemme HM, de Wolff-Rouendaal D. Transpupillary thermotherapy (TTT) by infrared irradiation of choroidal melanoma. Doc Ophthalmol. 1992;82:185-191.
8. Shields JA, Shields CL, Eagle RC, Jr., Lieb WE, Stern S. Malignant melanoma associated with melanocytoma of the optic disc. Ophthalmology. 1990;97:225-230.
9. Shields CL, Cater J, Shields JA, et al. Combined plaque radiotherapy and transpupillary thermotherapy for choroidal melanoma: tumor control and treatment complications in 270 consecutive patients. Arch Ophthalmol. 2002;120:933-940.
10. Jampol LM, Moy CS, Murray TG, et al. The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma: IV. Local treatment failure and enucleation in the first 5 years after brachytherapy. COMS report no. 19. Ophthalmology. 2002;109:2197-2206.
11. Guyer DR, Mukai S, Egan KM, Seddon JM, Walsh SM, Gragoudas ES. Radiation maculopathy after proton beam irradiation for choroidal melanoma. Ophthalmology. 1992;99:1278-1285.
12. Archer DB, Gardiner TA. Ionizing radiation and the retina. Curr Opin Ophthalmol. 1994;5:59-65.
13. Archer DB, Amoaku WM, Gardiner TA. Radiation retinopathy--clinical, histopathological, ultrastructural and experimental correlations. Eye (Lond). 1991;5 (Pt 2):239-251.
14. Stack R, Elder M, Abdelaal A, Hidajat R, Clemett R. New Zealand experience of I125 brachytherapy for choroidal melanoma. Clin Experiment Ophthalmol. 2005;33:490-494.
15. Jensen AW, Petersen IA, Kline RW, Stafford SL, Schomberg PJ, Robertson DM. Radiation complications and tumor control after 125I plaque brachytherapy for ocular melanoma. Int J Radiat Oncol Biol Phys. 2005;63:101-108.
16. Jones R, Gore E, Mieler W, et al. Posttreatment visual acuity in patients treated with episcleral plaque therapy for choroidal melanomas: dose and dose rate effects. Int J Radiat Oncol Biol Phys. 2002;52:989-995.
17. Sia S, Harper C, McAllister I, Perry A. Iodine-I25 episcleral plaque therapy in uveal melanoma. Clin Experiment Ophthalmol. 2000;28:409-413.
18. Missotten GS, Notting IC, Schlingemann RO, et al. Vascular endothelial growth factor a in eyes with uveal melanoma. Arch Ophthalmol. 2006;124:1428-1434.
19. El Filali M, Missotten GS, Maat W, et al. Regulation of VEGF-A in uveal melanoma. Invest Ophthalmol Vis Sci. 2009.

20. Mason JO, III, Albert MA, Jr., Persaud TO, Vail RS. Intravitreal bevacizumab treatment for radiation macular edema after plaque radiotherapy for choroidal melanoma. Retina. 2007;27:903-907.
21. Finger PT. Radiation retinopathy is treatable with anti-vascular endothelial growth factor bevacizumab (Avastin). Int J Radiat Oncol Biol Phys. 2008;70:974-977.
22. Finger PT, Chin K. Anti-vascular endothelial growth factor bevacizumab (avastin) for radiation retinopathy. Arch Ophthalmol. 2007;125:751-756.
23. Ziemssen F, Voelker M, Altpeter E, Bartz-Schmidt KU, Gelisken F. Intravitreal bevacizumab treatment of radiation maculopathy due to brachytherapy in choroidal melanoma. Acta Ophthalmol Scand. 2007;85:579-580.
24. Arriola-Villalobos P, Donate-Lopez J, Calvo-Gonzalez C, Reche-Frutos J, ejandre-Alba N, az-Valle D. Intravitreal bevacizumab (Avastin) for radiation retinopathy neovascularization. Acta Ophthalmol. 2008;86:115-116.
25. Solano JM, Bakri SJ, Pulido JS. Regression of radiation-induced macular edema after systemic bevacizumab. Can J Ophthalmol. 2007;42:748-749.
26. Gupta A, Muecke JS. Treatment of radiation maculopathy with intravitreal injection of bevacizumab (Avastin). Retina. 2008;28:964-968.
27. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol. 2003;21:60-65.
28. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med. 2003;349:427-434.
29. Cobleigh MA, Langmuir VK, Sledge GW, et al. A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. Semin Oncol. 2003;30:117-124.
30. Reck M, von PJ, Zatloukal P, et al. Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAil. J Clin Oncol. 2009;27:1227-1234.
31. Varker KA, Biber JE, Kefauver C, et al. A randomized phase 2 trial of bevacizumab with or without daily low-dose interferon alfa-2b in metastatic malignant melanoma. Ann Surg Oncol. 2007;14:2367-2376.
32. Van CE, Vervenne WL, Bennouna J, et al. Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. J Clin Oncol. 2009;27:2231-2237.
33. Bergman L, Seregard S, Nilsson B, Lundell G, Ringborg U, Ragnarsson-Olding B. Uveal melanoma survival in Sweden from 1960 to 1998. Invest Ophthalmol Vis Sci. 2003;44:3282-3287.
34. Singh AD, Topham A. Survival rates with uveal melanoma in the United States: 1973-1997. Ophthalmology. 2003;110:962-965.
35. Augsburger JJ, Correa ZM, Freire J, Brady LW. Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy. Ophthalmology. 1998;105:1670-1678.
36. Kujala E, Makitie T, Kivela T. Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol Vis Sci. 2003;44:4651-4659.
37. Papetti M, Herman IM. Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol. 2002;282:C947-C970.
38. Yang H, Jager MJ, Grossniklaus HE. Bevacizumab suppression of establishment of micrometastases in experimental ocular melanoma. Invest Ophthalmol Vis Sci. 2010;51:2835-2842.
39. Eskelin S, Pyrhonen S, Summanen P, Prause JU, Kivela T. Screening for metastatic malignant melanoma of the uvea revisited. Cancer. 1999;85:1151-1159.

40. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell. 2005;8:299-309.
41. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15:220-231.
42. Mueckler M, Caruso C, Baldwin SA, et al. Sequence and structure of a human glucose transporter. Science. 1985;229:941-945.
43. Boonman ZF, Schurmans LR, van RN, Melief CJ, Toes RE, Jager MJ. Macrophages are vital in spontaneous intraocular tumor eradication. Invest Ophthalmol Vis Sci. 2006;47:2959-2965.
44. Chen PW, Murray TG, Salgaller ML, Ksander BR. Expression of MAGE genes in ocular melanoma cell lines. J Immunother. 1997;20:265-275.
45. De Waard-Siebinga I, Blom DJ, Griffioen M, et al. Establishment and characterization of an uveal-melanoma cell line. Int J Cancer. 1995;62:155-161.
46. El Filali M, Homminga I, Maat W, van der Velden P, Jager MJ. Triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth. Mol Vis. 2008;14:1752-1759.
47. Nguyen QD, Shah SM, Khwaja AA, et al. Two-year outcomes of the ranibizumab for edema of the mAcula in diabetes (READ-2) study. Ophthalmology. 2010;117:2146-2151.
48. Abraham P, Yue H, Wilson L. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER study year 2. Am J Ophthalmol. 2010;150:315-324.
49. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2000;25:169-193.
50. Chen H, Kovar J, Sissons S, et al. A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. Anal Biochem. 2005;338:136-142.
51. Kovar JL, Johnson MA, Volcheck WM, Chen J, Simpson MA. Hyaluronidase expression induces prostate tumor metastasis in an orthotopic mouse model. Am J Pathol. 2006;169:1415-1426.
52. D'Amico DJ, Masonson HN, Patel M, et al. Pegaptanib sodium for neovascular age-related macular degeneration: two-year safety results of the two prospective, multicenter, controlled clinical trials. Ophthalmology. 2006;113:992-1001.
53. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med. 2006;355:1419-1431.
54. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med. 2006;355:1432-1444.
55. Ferrara,N.; Mass,R.D.; Campa,C .; Kim,R.Targeting VEGF-A to treat cancer and age-related macular degeneration.. Annu.Rev.Med. 2007;58:491-504.
56. Sharma,H.S.; Tang,Z.H.; Gho,B.C. et al. Nucleotide sequence and expression of the porcine vascular endothelial growth factor. Biochim.Biophys.Acta. 1995;1260:235-238.
57. Bock,F.; Onderka,J.; Dietrich,T.; Bachmann,B.; Kruse,F.E.; Paschke,M.; Zahn,G.; Cursiefen,C. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. Invest Ophthalmol.Vis.Sci. 2007;48:2545-2552.
58. Yang H., Jager M.J., Grossniklaus H.E.. Author Response: Bevacizumab Suppression of Establishment of Micrometastases in Experimental Ocular Melanoma. IOVS. 2010; 51:6906-6907
59. Haase VH, Glickman JN, Socolovsky M, Jaenisch R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci U S A. 2001;98:1583-1588.
60. Rankin EB, Rha J, Unger TL, et al. Hypoxia-inducible factor-2 regulates vascular tumorigenesis in mice. Oncogene. 2008;27:5354-5358.

61. Criscuolo GR, Merrill MJ, Oldfield EH. Further characterization of malignant glioma-derived vascular permeability factor. J Neurosurg. 1988;69:254-262.
62. Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. Vascul Pharmacol. 2002;39:225-237.
63. Verhoeff JJ, Stalpers LJ, Claes A, et al. Tumor control by whole brain irradiation of anti-VEGF-treated mice bearing intracerebral glioma. Eur J Cancer. 2009;45:3074-3080.
64. Woolard J, Wang WY, Bevan HS, Qiu Y, Morbidelli L, Pritchard-Jones RO, Cui TG, Sugiono M, Waine E, Perrin R, Foster R, Digby-Bell J, Shields JD, Whittles CE, Mushens RE, Gillatt DA, Ziche M, Harper SJ, Bates DO. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res. 2004; 64(21):7822-35
65. Rennel ES, Varey AH, Churchill AJ, Wheatley ER, Stewart L, Mather S, Bates DO, Harper SJ. VEGF(121) b, a new member of the VEGF(xxx)b family of VEGF-A splice isoforms, inhibits neovascularisation and tumor growth in vivo. Br J Cancer. 2009 Oct 6;101(7):1183-93.
66. Kawamura H, Li X, Harper SJ, Bates DO, Claesson-Welsh L.Vascular endothelial growth factor (VEGF)-A165b is a weak in vitro agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. Cancer Res. 2008 Jun 15;68(12):4683-92.
67. Glass CA, Harper SJ, Bates DO The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. J Physiol. 2006 Apr 1;572(Pt 1):243-57.
68. Aghi M, Cohen KS, Klein RJ, Scadden DT, Chiocca EA. Tumor stromal-derived factor-1 recruits vascular progenitors to mitotic neovasculature, where microenvironment influences their differentiated phenotypes. Cancer Res. 2006;66:9054-9064.
69. Sathornsumetee S, Cao Y, Marcello JE, et al. Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan. J Clin Oncol. 2008;26:271-278.
70. Du R, Lu KV, Petritsch C, et al. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell. 2008;13:206-220.
71. Bronkhorst IH, Ly LV, Jordanova ES, et al. Detection of M2 macrophages in uveal melanoma and relation with survival. Invest Ophthalmol Vis Sci. 2010.

CHAPTER 6

TRIAMCINOLONE ACETONIDE AND ANECORTAVE ACETATE DO NOT STIMULATE UVEAL MELANOMA CELL GROWTH

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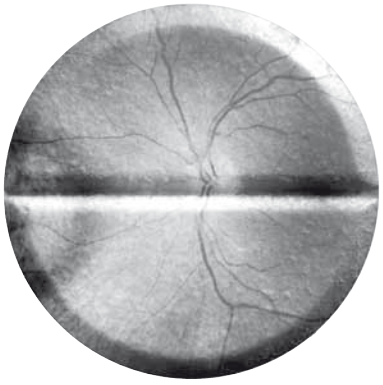
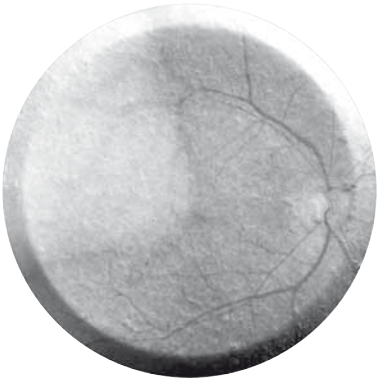




ABSTRACT

Purpose: Radiotherapy-induced radiation retinopathy can develop in over 40 % of eyes treated for uveal melanoma. Triamcinolone acetonide (TA) and anecortave acetate (AA) can be used to treat radiation retinopathy. Whether TA or AA has any effect on potentially still viable uveal melanoma cells in the choroid after radiotherapy is unknown. We therefore studied the effect of these drugs on the proliferation of uveal melanoma cell lines in vitro. Furthermore, as these drugs are supposed to counteract vascular leakage, we determined their effect on the expression and production of the pro-angiogenic vascular endothelial growth factor (VEGF-A), the anti-angiogenic pigment epithelium derived factor (PEDF) and thrombospondin (TSP-1) in uveal melanoma cells.

Method: Three uveal melanoma cell lines were treated in vitro with TA or AA. Cell proliferation was measured by counting cells and using the WST-1 assay. VEGF-A and PEDF production was measured by ELISA and intracellular expression of angiogenic-associated genes including VEGF-A, PEDF, and TSP-1 was determined by real time quantitative RT-PCR.

Results: We found no effect of TA or AA on tumor cell growth or production of VEGF-A and PEDF in any of the three uveal melanoma cell lines tested. Regarding expression as measured by RT-PCR, TA had an inhibiting effect on TSP-1 in only one cell line, and no effect on VEGF-A or PEDF. AA showed a similar lack of effect.

Conclusion: Since TA and AA do not stimulate uveal melanoma cell growth, it seems to be save using these drugs to treat radiation retinopathy after irradiation for uveal melanoma. Though, additional experiments using more cell lines or primary tumor cell cultures are needed to validate this conclusion. Furthermore, the results of our study suggest that TA does not exert its anti-leakage effect through down-regulation of VEGF-A or up regulation of TSP-1 or PEDF in uveal melanoma cell lines; it may be that TA and AA influences these pro- and anti-angiogenic factors only under hypoxic circumstances, and this will need further investigation.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular tumor in adults, with an incidence of approximately 5-7 per 1.000.000 in Caucasian populations [1-4]. The 5-year tumor-related survival rate is about 70-80% [5,6] , and eventually about 50% of patients will die from the disease [7,8] . Because the eye lacks lymphatic vessels, the route of dissemination is almost exclusively hematogenous. The most common site of metastasis formation is the liver [9,10]. Predictors of survival for UM patients are identified in histologic cell type, tumor diameter, tumor location, age, gender [11] and cytogenetic parameters. Loss of chromosome 3 is one of the most significant predictors for uveal melanoma-related deaths [12,13]. In case of a primary UM, different treatments are available. Enucleation remains a common treatment for large tumors. Small and medium-sized tumors with a prominence < 8 mm can be treated with proton beam therapy, local resection or radiation [14,15]. Radiotherapy is often combined with transpupillary thermotherapy, which may also allow treatment of larger tumors [16,17]. Radiotherapy, however, not only damages uveal melanoma cells, but also normal healthy cells located in the immediate surrounding of the tumor. Capillary endothelial cells are especially sensitive to radiation and presumably die directly or later by mitotic death [18]. Capillary endothelial cell loss causes changes in the structure and permeability of the vessels in the affected area. This leads to an occlusive microangiopathy, clinically known as radiation retinopathy. A variety of sight-threatening manifestations are associated with radiation retinopathy, such as macular edema and neovascularization [19]. The onset of radiation retinopathy occurs on average 26 months after radioactive plaque therapy [20]. The cause of this delay is not quite clear, though duration of the endothelial cell cycle and delayed mitotic cell death might be an explanation [18]. After one year, approximately 5% of patients treated by radioactive plaque has developed radiation retinopathy and after five years this percentage has risen to about 40% [21].

At this moment, there is no established effective treatment for radiation retinopathy and its complications, although laser photocoagulation has shown some beneficial effect [20]. A first report showed that anti-angiogenic agents such as Avastin™ have a beneficial effect [22].

Triamcinolone acetonide (TA) is a glucocorticoid that has already been shown to improve vision for a few months in patients with macular edema associated with diabetic retinopathy [23,24,24,25] and in patients with exudative age-related macular degeneration (ARMD) [26]. Although the causal mechanisms underlying these diseases differ, diabetic retinopathy, exudative ARMD and radiation retinopathy share the same sight-threatening complications, such as macular edema and neovascularization. A recent study reported a temporary positive effect of intravitreal TA injections in a group of 31 patients with radiation maculopathy [27].

While TA might be a suitable drug to treat complications of radiation retinopathy, its effect on uveal melanoma cells is unknown. The possibility that there are living uveal melanoma cells present in the choroid of eyes treated with radiotherapy cannot be excluded [28]; this is supported by the fact that local recurrences develop [29]. We therefore set out to study the

effect of TA on the proliferation of uveal melanoma cells before using this drug in patients with radiation retinopathy. To our knowledge, this has not been investigated previously.

TA [30-32] has been shown to have an anti-angiogenic effect, though the mechanism through which this effect comes about is not clear. The extent of tumor angiogenesis is determined by the balance between pro-angiogenic and anti-angiogenic molecules, released by both tumor cells and surrounding cells [33]. The formation of new vessels is in the first place a process involving endothelial cells; tumor cells that lack a sufficient blood supply are most likely the incentive of angiogenesis. VEGF-A plays an important role in angiogenesis, regulating vasopermeability and the proliferation and migration of endothelial cells [34]. VEGF-A seems also to be the key mediator in ocular vessel diseases as well as in tumor angiogenesis [35].

We also studied two inhibitors of angiogenesis, pigment epithelium-derived factor (PEDF) and thrombospondin-1 (TSP-1). Various studies have documented that PEDF inhibits angiogenesis in the eye [36,37] , whereas TSP-1 has been shown to inhibit cutaneous melanoma progression by suppressing tumor vessel formation [38].

One of the disadvantages of TA, however, is that it causes ocular hypertension in about 30% of the cases [39] and may lead to glaucomatous damage. A new steroid-derived substance, anecortave acetate (AA), an angiostatic cortisone, has been developed to be devoid of corticosteroid side effects such as ocular hypertension [40]. Hence, AA may be a good alternative to TA. A first study of AA administered in a juxtascleral depot to treat subfoveal choroidal neovascularization in age-related macular degeneration showed good results in the prevention of further vessel development [41]. We therefore analyzed the effect of both TA as well as AA on proliferation, expression and production of pro- and anti-angiogenic factors.

MATERIAL AND METHODS

Cell lines

Three very well described cell lines were selected on the basis of known differences in VEGF-A production (low, medium, high) to perform our experiments: OCM-1, Mel 285 and 92-1. Cell line 92-1 was established in our laboratory [42]. Cell line OCM-1 was kindly provided by Dr. J. Kan-Mitchell [43]. Mel 285 was a generous gift of Dr. B.R. Ksander [44]. Cell lines Mel 285 and 92-1 were cultured in RPMI 1640 (GIBCO, Life Technologies, Breda, The Netherlands) to which 10% fetal calf serum, 2% glutamine (GIBCO) and 2% penicilline/streptomycine (GIBCO) were added. OCM-1 was cultured in DMEM (GIBCO) with 10% fetal calf serum and 2% penicilline/streptomycine (GIBCO). Cultures were passaged every 3-4 days.

Triamcinolone acetonide and AL-4940 preparations

We used triamcinolone acetonide T6501 (Sigma-ALDRICH, St Louis, USA). The recommended intravitreal dose of TA is 4 mg (0.1 ml) [27]. The dose to which the uveal melanoma

cells are expected to be exposed in vivo after injection of TA is 1 mg/ml. Unfortunately, due to massive TA crystal deposits the maximal tolerated dose in vitro was 0,04 mg/ml. 10 mg TA was suspended in 230 µl methanol, producing a 100 mM stock suspension. This stock was serially diluted with medium to concentrations of 10 µM and 100 µM. Extra methanol was added to the 10 µM suspension to obtain a concentration of 0.1% methanol, similar to the 100µM suspension. As control, we used solutions of 0.1% methanol in medium. We used AL-4940-06 (9(11)-Dehydrocortisol, Alcon Laboratories, Forth Worth, USA) instead of AA, as in vivo AA is rapidly deacetylated into AL-4940 and AL-4940 is therefore the predominantly present active form [45]. A 10mM stock solution was prepared dissolving 5.4 mg AL-4940 in 1.6 ml dimethyl sulfoxide (DMSO). This stock was serially diluted with medium to concentrations of 0.1, 1.0 and 10 µM. A solution of 0.1% DMSO was used as control.

Triamcinolone acetonide and AL-4940 administration

Growth assays were performed in triplicate. For each assay, 64 wells per cell line were filled with $1 \cdot 10^4$ uveal melanoma cells per well in 1 ml medium, using 24-well- plates. Cells were allowed to settle for 24 hours. After 24 hours, the regular medium was replaced by 1 ml of one of the following media: 1) RPMI/DMEM (control), 2) RPMI/DMEM with methanol (second control), 3) 10 µM TA suspension, 4) 100 µM TA suspension; for the AL-4940 assay: 1) RPMI (control), 2) RPMI with DMSO (second control), 3) 0.1 µM AL-4940 solution, 4) 1.0 µM AL-4940 solution, 5) 10 µM AL-4940 solution. Cells were cultured in an incubator at 37° C and 5.0 % CO_2.

Cell proliferation and cytotoxicity

Four, six, eight, and ten days after medium replacement, cells were counted or replaced with fresh specific media. Cells from four wells of each cell line at one time point were pooled to obtain a large enough number of cells to obtain a reliable count. 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 WST-1 assay (Roche Diagnostics, Indianapolis, IN), as previously described [46]. In short, 96-well plates were filled with 1250 uveal melanoma cells per well and either filled with regular medium (control) or 100µM TA suspension. Absorbance was measured at 450nm (n=8) on a multiwell spectrophotometer (Perkin Elmer, Wellesley, MA).

VEGF-A and PEDF production

In the supernatant obtained on day eight, VEGF-A and PEDF concentrations were determined using commercial solid phase sandwich enzyme-linked immunosorbent assay kits (VEGF-A: human VEGF ELISA immunoassay Kit, Biosource, Camarillo, USA. PEDF: PEDF Sandwich ELISA Antigen Detection Kit, BioProducts MD, Middletown, USA).

Real-Time Quantitative RT-PCR analysis

Several different gene mRNA expressions were analyzed by real time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). RNA was isolated on day 8 in one TA assay, using an Rneasy® Mini Kit (Qiagen, Valencia, USA). RNA samples were stored at -80°C until further processing, when 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 up till 100 µl. In 96-wells, 2 µl of this solution was added to a 15 µl solution of iQ SYBR Green Supermix, forward and reverse primers (10µM solutions) for VEGF-A, PEDF, TSP-1, β-ACTIN, HRPT or RPS-11 and sterile water (volume ratio respectively 10:1:1:8). The primers for all genes under study were designed with the Primer Express software (PE Applied Biosystems) (Table 1) [47]. A quantitative analysis of the samples was then performed for all genes by real time quantitative RT-PCR in a MyiQ iCycler real-time PCR system (Bio-Rad, Hercules CA). 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 [48]. RPS-11 (ribosomal protein S11) is a housekeeping gene that has been recently used to normalize gene expression in uveal melanoma cells [47]. Beta-Actin (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 [49]. Hypoxanthine-guanine phosphoribosyltransferase (HPRT), an enzyme in purine metabolism, is reported as a constitutively expressed housekeeping gene [49,50].

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. With a control assigned Relative Quantity for any sample for all genes is calculated as follows: Relative Quantity $_{\text{sample (Gene x)}} = E_{\text{Gene x}}^{\text{(CT (Control) – CT (sample))}}$. Where E=Efficiency of primer/ (probe) set, C_T $^{\text{(Control)}}$ = Average C_T for the sample which has been assigned as a control and C_T $^{\text{(sample)}}$ = Average C_T for the sample. This is referred to as normalized expression [51].

TABLE 1. Primer sequences of the genes studied in the RT-PCR assay

Primer		Forward primer (5'-3')	Reverse primer (5'-3')
Endogenous controls	RPS11	AAGCAGCCGACCATCTTTCA	CGGGAGCTTCTCCTTGCC
	HRPT	CGAGATGTGATGAAGGAGATGG	GCAGGTCAGCAAAGAATTTATAGC
	B-ACTIN	CGGGACCTGACTGACTACCTC	CTCCTTAATGTCACGCACGATTT
Genes under study	VEGF-A	GCCCTTGCCTTGCTGCTCTACC	GTGATGATTCTGCCTCCTCCTTC
	PEDF	AGCATTCTCCTTCTCGGTGTGG	CCTCACGGTCCTCTCTTCATC
	TSP-1	AGGTCTTCAGCGTGGTGT	ACAAACAGGGTGATGCTCTTCC

Statistical Analysis

Data are expressed as the mean ± SD. Student's t-test was used to determine whether there were statistically significant differences between treatment groups determined in the cell viability assay. $P < 0.05$ was considered statistically significant.

RESULTS

Influence of TA and AA on uveal melanoma cell proliferation

TA and AA may be potential drugs to treat radiation retinopathy. However harmful side effects like proliferation of uveal melanoma cells have to be ruled out.

Treatment of uveal melanoma cells with TA did not induce cell death as measured by the Bürker counting chamber (data not shown). The WST-1 assay demonstrated no significant effect of TA treatment on cell proliferation of the uveal melanoma cell lines 92.1, OCM-1 and Mel285 ($P=0.755$, 0.844 and 0.487, respectively) (Figure 1). AA showed similar results. TA and AA had no toxic effect on uveal melanoma cells, as there were no differences in the percentages of dead cells between the different treatments (data not shown).

VEGF-A and PEDF production

To investigate any direct pro- or anti-angiogenic effects of TA and AA on uveal melanoma cells, we studied the influence of these drugs on VEGF-A and PEDF production of three uveal melanoma cell lines using an ELISA on culture supernatant. Since cell numbers per well differed considerably between some treatments and experiments, we corrected for the number of cells by dividing the concentration of VEGF-A or PEDF measured in the supernatant by the amount of cells present in the same well. All cell lines produced VEGF-A and PEDF, but clear differences were observed in the amounts: OCM-1 produced the highest levels of VEGF-A (50 pg/ml/10^4cells). Cell line 92-1 produced approximately one fifth of this amount (10 pg/ml/10^4cells), while cell line Mel 285 produced the lowest amount of VEGF-A (approximately 5 pg/ml/10^4cells).

Cell line 92-1 produced large amounts of PEDF (2.000 pg/ml/10^4cells), around 100 times the amount produced by cell line OCM-1 (20 pg/ml/10^4cells). Mel 285 produced very little PEDF (3 pg/ml/10^4cells). Addition of TA or AA to the cell cultures had no effect on either VEGF-A or PEDF production (Figure 2a and 2b).

VEGF-A, PEDF and TSP-1 expression

We determined by real time quantitative RT-PCR whether TA or AA had any effect on several genes in uveal melanoma cell lines that are associated with angiogenesis. VEGF-A was expressed in all cell lines at variable levels. TA treatment had no major (> 1.0 time fold difference) effect on VEGF-A expression. On the contrary, TA gave a dose-dependent reduction

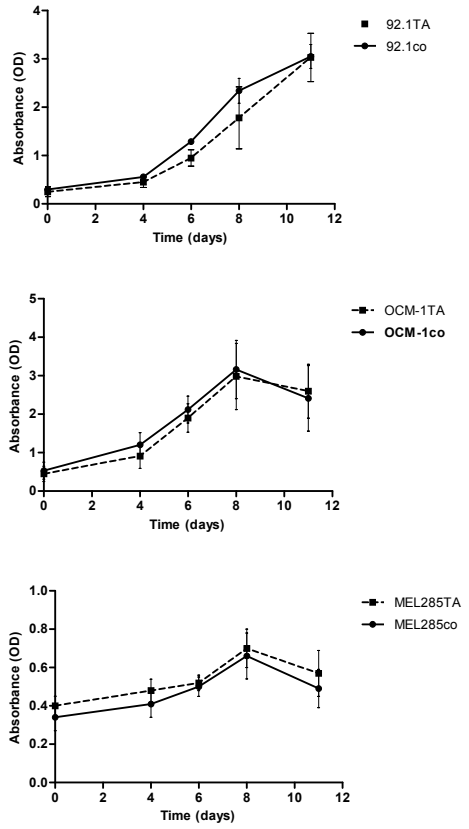
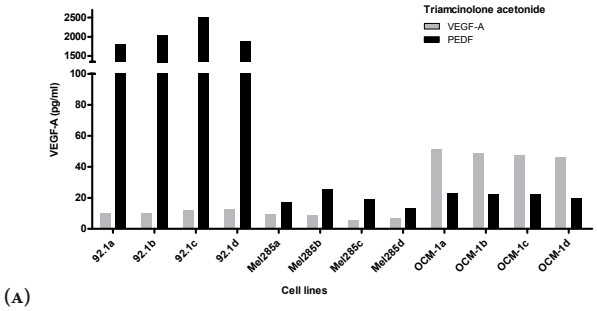


FIGURE 1. The effect of TA on uveal melanoma cell proliferation.
Cells were treated with four different suspensions; a) RPMI/DMEM (control), b) RPMI/DMEM with methanol (second control), c) 10 mM TA suspension, d) 100 mM TA suspension. Cell proliferation was determined by a WST-1 assay. No substantial difference is shown between TA treatment and controls in proliferation in the uveal melanoma cell lines 92.1, OCM-1 and Mel285 (P = 0.755, 0.844 and 0.487, respectively)

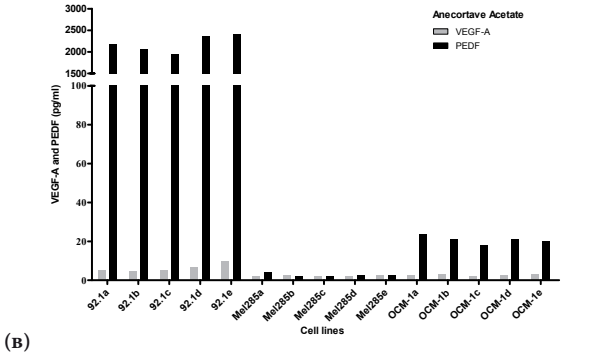
of TSP-1 expression in Mel285 (difference in times the fold= 1.5-2.9) (Figure 3). TSP-1 was hardly expressed in 92.1. AA had no significant effect either on VEGF-A or TSP-1 expression (Figure 3). PEDF is very highly expressed in cell line 92-1 and only to a minimal extend in the other cell lines. PEDF expression in cell line 92-1 was not effected by TA or AA.

DISCUSSION

Our results showed no apparent stimulating or inhibiting effect of TA on uveal melanoma cell proliferation in the concentrations used in this study [27]. The effect of TA on uveal melanoma cell lines had not yet been investigated, but the effect of TA on other cell types has been the object of some studies. For instance, TA inhibited the growth of retinal pigment epithelium



(A)



(B)

FIGURE 2A AND 2B. The effect of TA and AA on production of VEGF-A and PEDF.
Cells were treated with four/five different suspensions; for TA experiments: a) RPMI/DMEM (control), b) RPMI/DMEM with methanol (second control), c) 10 mM TA suspension, d) 100 mM TA suspension; for AA experiments: a) RPMI (control), b) RPMI with DMSO (second control), c)) 0.1 mM AL-4940 solution, d) 1.0 mM AL-4940 solution, e) 10 mM AL-4940 solution. All cell lines produced VEGF-A and PEDF, but clear differences were observed in the amounts: OCM-1 produced the highest levels of VEGF-A, while cell line Mel 285 produced the lowest amount of VEGF-A. Cell line 92-1 produced large amounts of PEDF (>2000 pg/ml). Mel 285 produced very little PEDF Addition of TA or AA to the cell cultures had no effect on either VEGF-A or PEDF production.

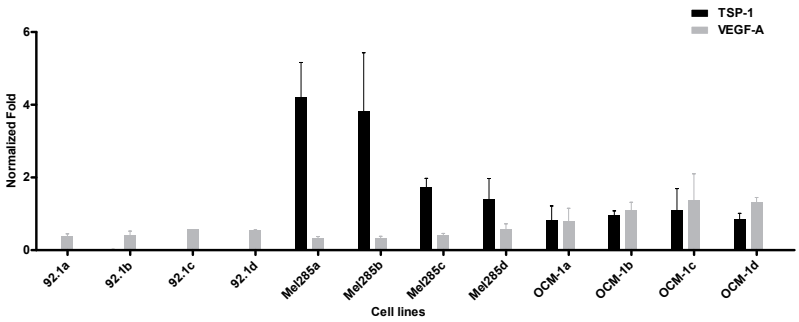


FIGURE 3. The effect of TA on expression of VEGF-A and TSP-1.
Cells were treated with four different suspensions; a) RPMI/DMEM (control), b) RPMI/DMEM with methanol (second control), c) 10 mM TA suspension, d) 100 mM TA suspension. VEGF-A was expressed in all cell lines at variable levels. TA treatment had no major (> 1.0 fold) effect on VEGF-A expression. TSP-1 was not expressed in 92.1. Mel 285 showed a decreased TSP-1 expression after treatment with TA.

(ARPE 19) in concentrations ranging from 1 μM to 2,3 mM, but stimulated growth at a concentration of 1 nM [52,53]. Another study, using bovine retinal endothelial cells, found that TA at a concentration of 115 μM had no effect on growth, concentrations ranging from 230 μM to 4,6 mM had an inhibiting effect and concentrations of 6,8 mM and higher were cytotoxic [54]. Likewise, in vitro incubation of retinal pigment epithelial cells (RPE) with corticosteroids induced a specific and dose-dependent reduction of cell viability. These toxic events were not associated with the anti-inflammatory activity of these compounds but depended on the hydro-solubility of their formulation [55].

While previous studies on RPE cells demonstrated an inhibition of VEGF production and expression by TA, we found no effect of TA on VEGF-A production or expression in uveal melanoma cells. For instance, TA reduced VEGF expression of retinal pigment epithelial (ARPE19) cells under oxidative stress [53] and inhibited cobalt-stimulated VEGF production in Müller cells, probably by a destabilization of VEGF mRNA [56]. The lack of inhibition of TA on VEGF-A production and expression in our study could perhaps be explained by the absence of specific stimuli that would up-regulate VEGF-A production and expression, such as oxidative stress or cobalt administration. In the study by Sears et al [56], only cobalt-stimulated VEGF production was inhibited by TA, while basal VEGF production was not affected. Similarly, another study showed no alteration in VEGF expression by TA in a rat retina when no special stress was applied [57].We are currently performing experiments to determine the effect of TA and other drugs on uveal melanoma cells under stressful conditions, such as hypoxia.

PEDF inhibits angiogenesis by inducing apoptosis in endothelial cells that try to form new vessels [58,59]. Besides the separate levels of VEGF-A and PEDF, the balance between the two seems to be decisive in whether or not angiogenesis takes place [60]. Yang et al has previously demonstrated expression of PEDF in uveal melanoma cell lines. Furthermore, they demonstrated an inhibitory effect of angiostatin on the ratio of VEGF/PEDF mRNA levels in vitro [59]. Likewise, Mel 285, 92-1, and OCM-1 all produced PEDF in our experiments. We observed no effect of TA on the production of PEDF by any of the uveal melanoma cell lines. Therefore, stimulation of production of PEDF by uveal melanoma cells seems not to be the anti-angiogenic working mechanism of this drug under normal circumstances. The high expression of PEDF by cell line 92-1 was unexpected, since PEDF has been shown to work as a tumor growth inhibitor through anti-angiogenic mechanisms [61-63] and cell line 92-1 was derived from a highly malignant tumor [42]. However, there are reports that melanocytic tumors have a relatively high expression of PEDF [64]. Also, PEDF might have a biphasic activity, inhibiting angiogenesis in at normal amounts, but stimulating angiogenesis at high concentrations [65].

The balance between VEGF-A and PEDF as described earlier, seems also to be influenced by thrombospondin (TSP-1). TSP-1 inhibits endothelial cell migration and proliferation and also induces apoptosis [66]. In cell line Mel 285, TSP-1 expression was about 2-fold inhibited in cells grown in TA compared to the control cells. Previous reports on the effect

of TA on the expression of TSP-1 could not be found, though some effects of other corticosteroids have been reported. Hydrocortisone up-regulated TSP-1 expression in a glioma cell line [67] and dexamethason increased TSP-1 expression in a murine trofoblast-like (MC_3T_3-E1) cell culture [68]. The results of Mel 285 do not agree with this, and may indicate an unexpected effect of TA. TSP-1 expression of cell line 92-1 was very low, possibly due to inactivation of this gene in the same manner as described for hypermethylated p16(INK4a) [69].

Parallel experiments with AA, to eliminate any possible side effects of the glucocortoid fraction, showed similar results as with TA. There seems to be no differential effect of this angiostatic cortisone compared to TA on potentially still viable uveal melanoma cells in the choroid after radiotherapy; both drugs have hardly any effect at all.

The risk of an increase in local recurrences of uveal melanoma by TA or AA in the treatment of radiation retinopathy by direct stimulation of uveal melanoma cell seems to be low. Though one has to realize that we based these conclusions on a restricted number of cell lines and extrapolation to the in vivo situation is by definition limited. In addition, the clinical dose of TA could not be investigated due to massive crystal deposits. Perhaps a certain threshold has to be reached before TA can demonstrate any effect.

Nonetheless, our results suggest that TA does not exert any anti-angiogenic effect through influencing basal VEGF-A, PEDF, TSP-1 production or expression in uveal melanoma cells. Though, influence of hypoxia and stimulation of these cell lines by VEGF or FGF2 or by anti-angiogenic factors is very well possible, and is under investigation.

Furthermore, there are many other pro- and anti-angiogenic factors that could be altered by TA. Plasminogen activator inhibitor type-1 (PAI-1) for instance, has been implicated in metastasis formation and tumor-associated angiogenesis in uveal melanoma [70], and would be an interesting candidate for further studies. These factors will need further investigation.

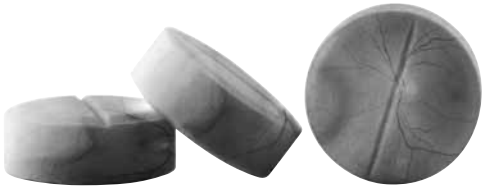
REFERENCE LIST

1. Egan, K. M., Seddon, J. M., Glynn, R. J., Gragoudas, E. S., and Albert, D. M. *Epidemiologic aspects of uveal melanoma.* Surv.Ophthalmol. 1988. 32:239-251.
2. Singh, A. D. and Topham, A. *Incidence of uveal melanoma in the United States: 1973-1997.* Ophthalmology 2003. 110:956-961.
3. Hu, D. N., Yu, G. P., McCormick, S. A., Schneider, S., and Finger, P. T. *Population-based incidence of uveal melanoma in various races and ethnic groups.* Am.J.Ophthalmol. 2005. 140:612-617.
4. Virgili, G., Gatta, G., Ciccolallo, L., Capocaccia, R., Biggeri, A., Crocetti, E., Lutz, J. M., and Paci, E. *Incidence of Uveal Melanoma in Europe.* Ophthalmology 2007.
5. Bergman, L., Seregard, S., Nilsson, B., Lundell, G., Ringborg, U., and Ragnarsson-Olding, B. *Uveal melanoma survival in Sweden from 1960 to 1998.* Invest Ophthalmol.Vis.Sci. 2003. 44:3282-3287.
6. Singh, A. D. and Topham, A. *Survival rates with uveal melanoma in the United States: 1973-1997.* Ophthalmology 2003. 110:962-965.
7. Augsburger, J. J., Correa, Z. M., Freire, J., and Brady, L. W. *Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy.* Ophthalmology 1998. 105:1670-1678.
8. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
9. Kath, R., Hayungs, J., Bornfeld, N., Sauerwein, W., Hoffken, K., and Seeber, S. *Prognosis and treatment of disseminated uveal melanoma.* Cancer 1993. 72:2219-2223.
10. Rietschel, P., Panageas, K. S., Hanlon, C., Patel, A., Abramson, D. H., and Chapman, P. B. *Variates of survival in metastatic uveal melanoma.* J.Clin.Oncol. 2005. 23:8076-8080.
11. Mooy, C. M. and De Jong, P. T. *Prognostic parameters in uveal melanoma: a review.* Surv.Ophthalmol. 1996. 41:215-228.
12. Prescher, G., Bornfeld, N., Hirche, H., Horsthemke, B., Jockel, K. H., and Becher, R. *Prognostic implications of monosomy 3 in uveal melanoma.* Lancet 1996. 347:1222-1225.
13. Sisley, K., Rennie, I. G., Parsons, M. A., Jacques, R., Hammond, D. W., Bell, S. M., Potter, A. M., and Rees, R. C. *Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis.* Genes Chromosomes.Cancer 1997. 19:22-28.
14. Damato, B. *Developments in the management of uveal melanoma.* Clin.Experiment.Ophthalmol. 2004. 32:639-647.
15. Potter, R., Janssen, K., Prott, F. J., Widder, J., Haverkamp, U., Busse, H., and Muller, R. P. *Ruthenium-106 eye plaque brachytherapy in the conservative treatment of uveal melanoma: evaluation of 175 patients treated with 150 Gy from 1981-1989.* Front Radiat.Ther.Oncol. 1997. 30:143-149.
16. Bartlema, Y. M., Oosterhuis, J. A., Journee-De Korver, J. G., Tjho-Heslinga, R. E., and Keunen, J. E. *Combined plaque radiotherapy and transpupillary thermotherapy in choroidal melanoma: 5 years' experience.* Br.J.Ophthalmol. 2003. 87:1370-1373.
17. Desjardins, L., Lumbroso-Le Rouic, L., Levy-Gabriel, C., Dendale, R., Delacroix, S., Nauraye, C., Esteve, M., Plancher, C., and Asselain, B. *Combined proton beam radiotherapy and transpupillary thermotherapy for large uveal melanomas: a randomized study of 151 patients.* Ophthalmic Res. 2006. 38:255-260.
18. Archer, D. B., Amoaku, W. M., and Gardiner, T. A. *Radiation retinopathy--clinical, histopathological, ultrastructural and experimental correlations.* Eye 1991. 5 (Pt 2):239-251.
19. Zamber, R. W. and Kinyoun, J. L. *Radiation retinopathy.* West J.Med. 1992. 157:530-533.

20. Finger, P. T. and Kurli, M. *Laser photocoagulation for radiation retinopathy after ophthalmic plaque radiation therapy.* Br.J.Ophthalmol. 2005. 89:730-738.
21. Gunduz, K., Shields, C. L., Shields, J. A., Cater, J., Freire, J. E., and Brady, L. W. *Radiation retinopathy following plaque radiotherapy for posterior uveal melanoma.* Arch.Ophthalmol. 1999. 117:609-614.
22. Finger, P. T. and Chin, K. *Anti-vascular endothelial growth factor bevacizumab (avastin) for radiation retinopathy.* Arch.Ophthalmol. 2007. 125:751-756.
23. Er, H. and Yilmaz, H. *Intravitreal cortisone injection for refractory diffuse diabetic macular edema.* Ophthalmologica 2005. 219:394-400.
24. Patelli, F., Fasolino, G., Radice, P., Russo, S., Zumbo, G., DI Tizio, F. M., Frisone, G., and Marchi, S. *Time course of changes in retinal thickness and visual acuity after intravitreal triamcinolone acetonide for diffuse diabetic macular edema with and without previous macular laser treatment.* Retina 2005. 25:840-845.
25. Sorensen, T. L., Haamann, P., Villumsen, J., and Larsen, M. *Intravitreal triamcinolone for macular oedema: efficacy in relation to aetiology.* Acta Ophthalmol.Scand. 2005. 83:67-70.
26. Jonas, J. B., Spandau, U. H., Kamppeter, B. A., and Harder, B. *Follow-up after intravitreal triamcinolone acetonide for exudative age-related macular degeneration.* Eye 2007. 21:387-394.
27. Shields, C. L., Demirci, H., Dai, V., Marr, B. P., Mashayekhi, A., Materin, M. A., Manquez, M. E., and Shields, J. A. *Intravitreal triamcinolone acetonide for radiation maculopathy after plaque radiotherapy for choroidal melanoma.* Retina 2005. 25:868-874.
28. Pe'er, J., Stefani, F. H., Seregard, S., Kivela, T., Lommatzsch, P., Prause, J. U., Sobottka, B., Damato, B., and Chowers, I. *Cell proliferation activity in posterior uveal melanoma after Ru-106 brachytherapy: an EORTC ocular oncology group study.* Br.J.Ophthalmol. 2001. 85:1208-1212.
29. Harbour, J. W., Char, D. H., Kroll, S., Quivey, J. M., and Castro, J. *Metastatic risk for distinct patterns of postirradiation local recurrence of posterior uveal melanoma.* Ophthalmology 1997. 104:1785-1792.
30. Murata, M., Shimizu, S., Horiuchi, S., and Taira, M. *Inhibitory effect of triamcinolone acetonide on corneal neovascularization.* Graefes Arch.Clin.Exp.Ophthalmol. 2006. 244:205-209.
31. Hashimoto, I., Nakanishi, H., Shono, Y., Toda, M., Tsuda, H., and Arase, S. *Angiostatic effects of corticosteroid on wound healing of the rabbit ear.* J.Med.Invest 2002. 49:61-66.
32. Antoszyk, A. N., Gottlieb, J. L., Machemer, R., and Hatchell, D. L. *The effects of intravitreal triamcinolone acetonide on experimental pre-retinal neovascularization.* Graefes Arch.Clin.Exp.Ophthalmol. 1993. 231:34-40.
33. Folkman, J. *The role of angiogenesis in tumor growth.* Semin.Cancer Biol. 1992. 3:65-71.
34. Ferrara, N., Gerber, H. P., and LeCouter, J. *The biology of VEGF and its receptors.* Nat.Med. 2003. 9:669-676.
35. Carmeliet, P. *VEGF as a key mediator of angiogenesis in cancer.* Oncology 2005. 69 Suppl 3:4-10.
36. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouck, N. P. *Pigment epithelium-derived factor: a potent inhibitor of angiogenesis.* Science 1999. 285:245-248.
37. Matsuoka, M., Ogata, N., Otsuji, T., Nishimura, T., Takahashi, K., and Matsumura, M. *Expression of pigment epithelium derived factor and vascular endothelial growth factor in choroidal neovascular membranes and polypoidal choroidal vasculopathy.* Br.J.Ophthalmol. 2004. 88:809-815.
38. Trotter, M. J., Colwell, R., and Tron, V. A. *Thrombospondin-1 and cutaneous melanoma.* J.Cutan.Med. Surg. 2003. 7:136-141.
39. Jaissle, G. B., Szurman, P., and Bartz-Schmidt, K. U. *[Ocular side effects and complications of intravitreal triamcinolone acetonide injection].* Ophthalmologe 2004. 101:121-128.

40. Clark, A. F., Mellon, J., Li, X. Y., Ma, D., Leher, H., Apte, R., Alizadeh, H., Hegde, S., McLenaghan, A., Mayhew, E., D'Orazio, T. J., and Niederkorn, J. Y. *Inhibition of intraocular tumor growth by topical application of the angiostatic steroid anecortave acetate.* Invest Ophthalmol.Vis.Sci. 1999. 40:2158-2162.
41. Schmidt-Erfurth, U., Michels, S., Michels, R., and Aue, A. *Anecortave acetate for the treatment of subfoveal choroidal neovascularization secondary to age-related macular degeneration.* Eur.J.Ophthalmol. 2005. 15:482-485.
42. Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer 1995. 62:155-161.
43. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
44. Nareyeck, G., Zeschnigk, M., Prescher, G., Lohmann, D. R., and Anastassiou, G. *Establishment and characterization of two uveal melanoma cell lines derived from tumors with loss of one chromosome 3.* Exp.Eye Res. 2006. 83:858-864.
45. Clark, A. F. *AL-3789: a novel ophthalmic angiostatic steroid.* Expert.Opin.Investig.Drugs 1997. 6:1867-1877.
46. Narayanan, R., Kenney, M. C., Kamjoo, S., Trinh, T. H., Seigel, G. M., Resende, G. P., and Kuppermann, B. D. *Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells.* Invest Ophthalmol.Vis.Sci. 2005. 46:304-309.
47. Zuidervaart, W., van der Velden, P. A., Hurks, M. H., van Nieuwpoort, F. A., Out-Luiting, C. J., Singh, A. D., Frants, R. R., Jager, M. J., and Gruis, N. A. *Gene expression profiling identifies tumour markers potentially playing a role in uveal melanoma development.* Br.J.Cancer 2003. 89:1914-1919.
48. Bustin, S. A. *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.* J.Mol.Endocrinol. 2000. 25:169-193.
49. Gilsbach, R., Kouta, M., Bonisch, H., and Bruss, M. *Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data.* Biotechniques 2006. 40:173-177.
50. de Kok, J. B., Roelofs, R. W., Giesendorf, B. A., Pennings, J. L., Waas, E. T., Feuth, T., Swinkels, D. W., and Span, P. N. *Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes.* Lab Invest 2005. 85:154-159.
51. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome Biol. 2007. 8:R19.
52. Yeung, C. K., Chan, K. P., Chan, C. K., Pang, C. P., and Lam, D. S. *Cytotoxicity of triamcinolone on cultured human retinal pigment epithelial cells: comparison with dexamethasone and hydrocortisone.* Jpn.J.Ophthalmol. 2004. 48:236-242.
53. Matsuda, S., Gomi, F., Oshima, Y., Tohyama, M., and Tano, Y. *Vascular endothelial growth factor reduced and connective tissue growth factor induced by triamcinolone in ARPE19 cells under oxidative stress.* Invest Ophthalmol.Vis.Sci. 2005. 46:1062-1068.
54. Spandau, U. H., Sauder, G., Schubert, U., Hammes, H. P., and Jonas, J. B. *Effect of triamcinolone acetonide on proliferation of retinal endothelial cells in vitro and in vivo.* Br.J.Ophthalmol. 2005. 89:745-747.
55. Valamanesh, F., Torriglia, A., Savoldelli, M., Gandolphe, C., Jeanny, J. C., BenEzra, D., and Behar-Cohen, F. *Glucocorticoids induce retinal toxicity through mechanisms mainly associated with paraptosis.* Mol.Vis. 2007. 13:1746-1757.
56. Sears, J. E. and Hoppe, G. *Triamcinolone acetonide destabilizes VEGF mRNA in Muller cells under continuous cobalt stimulation.* Invest Ophthalmol.Vis.Sci. 2005. 46:4336-4341.

57. Gao, H., Qiao, X., Gao, R., Mieler, W. F., McPherson, A. R., and Holz, E. R. *Intravitreal triamcinolone does not alter basal vascular endothelial growth factor mRNA expression in rat retina.* Vision Res. 2004. 44:349-356.
58. Bouck, N. *PEDF: anti-angiogenic guardian of ocular function.* Trends Mol.Med. 2002. 8:330-334.
59. Yang, H., Xu, Z., Iuvone, P. M., and Grossniklaus, H. E. *Angiostatin decreases cell migration and vascular endothelium growth factor (VEGF) to pigment epithelium derived factor (PEDF) RNA ratio in vitro and in a murine ocular melanoma model.* Mol.Vis. 2006. 12:511-517.
60. Tong, J. P. and Yao, Y. F. *Contribution of VEGF and PEDF to choroidal angiogenesis: a need for balanced expressions.* Clin.Biochem. 2006. 39:267-276.
61. Hosomichi, J., Yasui, N., Koide, T., Soma, K., and Morita, I. *Involvement of the collagen I-binding motif in the anti-angiogenic activity of pigment epithelium-derived factor.* Biochem.Biophys.Res.Commun. 2005. 335:756-761.
62. Streck, C. J., Zhang, Y., Zhou, J., Ng, C., Nathwani, A. C., and Davidoff, A. M. *Adeno-associated virus vector-mediated delivery of pigment epithelium-derived factor restricts neuroblastoma angiogenesis and growth.* J.Pediatr.Surg. 2005. 40:236-243.
63. Garcia, M., Fernandez-Garcia, N. I., Rivas, V., Carretero, M., Escamez, M. J., Gonzalez-Martin, A., Medrano, E. E., Volpert, O., Jorcano, J. L., Jimenez, B., Larcher, F., and Del Rio, M. *Inhibition of xenografted human melanoma growth and prevention of metastasis development by dual antiangiogenic/antitumor activities of pigment epithelium-derived factor.* Cancer Res. 2004. 64:5632-5642.
64. Tsuru, M., Arima, N., Toyozumi, Y., and Kato, S. *Pigment epithelium-derived factor as a new diagnostic marker for melanocytic tumors.* Kurume Med.J. 2005. 52:81-87.
65. Apte, R. S., Barreiro, R. A., Duh, E., Volpert, O., and Ferguson, T. A. *Stimulation of neovascularization by the anti-angiogenic factor PEDF.* Invest Ophthalmol.Vis.Sci. 2004. 45:4491-4497.
66. Ren, B., Yee, K. O., Lawler, J., and Khosravi-Far, R. *Regulation of tumor angiogenesis by thrombospondin-1.* Biochim.Biophys.Acta 2006. 1765:178-188.
67. Vedoy, C. G. and Sogayar, M. C. *Isolation and characterization of genes associated with the anti-tumor activity of glucocorticoids.* Brain Res.Mol.Brain Res. 2002. 106:57-69.
68. Leclerc, N., Luppen, C. A., Ho, V. V., Nagpal, S., Hacia, J. G., Smith, E., and Frenkel, B. *Gene expression profiling of glucocorticoid-inhibited osteoblasts.* J.Mol.Endocrinol. 2004. 33:175-193.
69. van der Velden, P. A., Metzelaar-Blok, J. A., Bergman, W., Monique, H., Hurks, H., Frants, R. R., Gruis, N. A., and Jager, M. J. *Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma.* Cancer Res. 2001. 61:5303-5306.
70. Ma, D., Gerard, R. D., Li, X. Y., Alizadeh, H., and Niederkorn, J. Y. *Inhibition of metastasis of intraocular melanomas by adenovirus-mediated gene transfer of plasminogen activator inhibitor type 1 (PAI-1) in an athymic mouse model.* Blood 1997. 90:2738-2746.



PART III

TUMOR-SPECIFIC TARGETING OF UVEAL MELANOMA

CHAPTER 7

EXPRESSION OF THE SST RECEPTOR 2 IN UVEAL MELANOMA IS NOT A PROGNOSTIC MARKER

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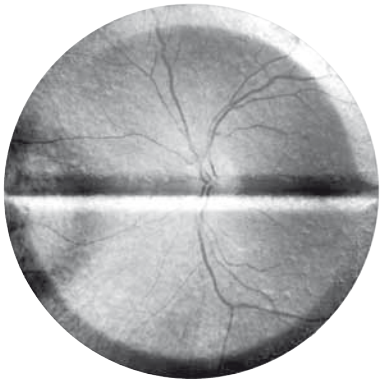

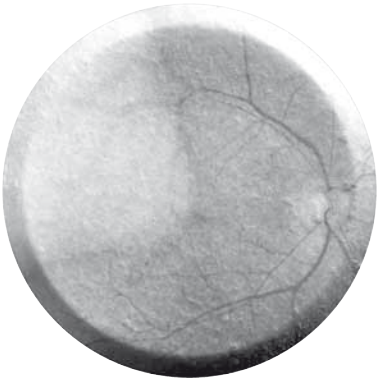

ABSTRACT

Introduction. Uveal melanoma (UM) cells and neurohormone-producing cells both originate from the neural crest. Somatostatin receptors subtype 2 (SSTR2) are over-expressed in several tumours, often from neuroendocrine origin, and synthetic antagonists like octreotide and octreotate are being used as diagnostic or therapeutic agents. We investigated the SSTR2 expression in uveal melanoma, and determined whether this expression was related to prognosis of the disease.

Material and Methods. UM cell lines and fresh primary UM samples were tested for SSTR2 expression by autoradiography (AR) using 125I-Tyr3-octreotate. Furthermore, UM cell lines were analyzed for SSTR2 mRNA expression with quantitative PCR.

Results. Using AR, cell surface SSTR2 expression was demonstrated in two metastatic cell lines, but no expression was detected in three cell lines derived from primary tumours. However, all primary and metastatic cell lines showed mRNA expression levels for SSTR2 using quantitative PCR. Only three of 14 primary UM demonstrated moderate SSTR2 expression, and this expression was not significantly associated with tumour-free survival or any tested prognostic factor.

Conclusion. Based on the rare and low expression of SSTR2 found in primary UM specimens and in UM cell lines, we conclude that SSTR2 is not widely expressed in uveal melanomas. Furthermore, SSTR2 expression was not associated with tumour-free survival and prognostic factors. Therefore SSTR2 is not suited as prognostic marker or therapeutic target in UM.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular tumour, with an annual incidence of 0.7/100.000 in the Western population [1]. Although less than 2% of the patients have clinically detectable metastasis at presentation, 50% of all patients die due to metastatic disease. The median survival after the diagnosis of metastasis is extremely poor. Predictors of survival for UM patients have been identified in histologic cell type, tumour diameter, tumour location, age, gender [2,3] and cytogenetic parameters. Loss of chromosome 3 is one of the most significant predictors for uveal melanoma-related deaths [4-6].

Most of the uveal melanomas are treated by radiotherapy, thus material for histopathologic and cytogenetic examination has to be obtained by Fine Needle Aspiration Biopsy (FNAB) for example. Shields et al. showed that FNAB provides adequate DNA for genetic analysis of uveal melanoma using a microsatellite assay [7]. Early and long-term complications following intraocular FNAB are rare [8]. Nevertheless, one suspected clinical case of extrascleral recurrence has been recently reported after diagnostic intraocular transscleral FNAB [9]. Therefore concern about tumour seeding due to this diagnostic procedure still exist [10], and FNAB is consequently not used routinely yet.

Conversely, neurohormone receptors as markers could be detected non-invasive and safe using scintigraphy, without the need to take a biopsy.

During embryogenesis, neural crest cells migrate to the diencephalon and to the uvea, where they give rise to pigmented melanocytes. Neural crest cells are able to produce neurohormones like somatostatin (SST). SST inhibits the release of growth hormone and thyroid-stimulating hormone. Its actions are mediated by specific G protein-coupled receptors, which are located in specific target cells of the gastrointestinal tract, the peripheral nervous system and several blood vessels [11].

Moreover, SST could be involved in the inhibition of tumour growth [12,13]. SST receptors (SSTR) have been detected in human neuroendocrine tumours [14-16], human lung tumours such as bronchial carcinoids [17] and gastro-entero-pancreatic tumours like insulinomas, gastrinomas and ileal carcinoids [18]. SST analogues, like octreotide and octreotate, can be radiolabelled with radionuclides via a chelator and are currently being used in the diagnosis (^{111}In) or therapy (^{90}Y or ^{177}Lu) of patients suffering from SSTR-expressing tumours [19-22].

Due to the common origin of SST and UM cells, a relation or interaction may be found. A relation between eye tissue and neurohormones has already been explored in several studies [23-25]. In a previous study, Ardjomand et al. concluded that expression of SST2 receptors (SSTR2) in uveal melanomas is correlated with a better ad vitam prognosis of the patients [26]. Our aim was to further investigate the expression of SSTR2 in uveal melanoma, in order to identify specific membrane receptors for diagnostic imaging and therapeutic targeting. Primary specimens and uveal melanoma cell lines, derived from primary or metastatic uveal melanomas were analyzed using in vitro AR and quantitative real-time RT-PCR techniques.

MATERIAL AND METHODS

Patient and tumour material

Informed consent was obtained prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki.

Fresh tumor tissue was obtained within 1 hour after enucleation, according to a standardized protocol. An incision was made through the tumor, leaving the optic nerve intact. A sample was taken from the side opposite the optic nerve and divided in three; one part was processed for fluorescent in situ hybridization (FISH), one part was fixed in 4 % paraformaldehyde overnight and the last part, as well as control tissues (rat brain, rat pancreas), were stored in liquid nitrogen for cryopreservation. Five-micrometer paraffin section were cut on a microtome (Microm HM 335^{E}), and put on uncoated slides (Menzel Superfrost) humidified with sterile water for haematoxylin-eosin (HE) and periodic acid-Schiff (PAS) staining. Five-micrometer cryosections of frozen tumour samples and control tissues were cut on a cryostat (Jung CM3000; Leica, Meyer Instruments, Inc. Houston, USA), and mounted on coated slides. Subsequently, the slides were air-dried and stored at –80°C and processed as below (see under 'autoradiography'). Conventional histopathologic examination was performed on all tumors and confirmed the origin of each one.

Cell lines

Mel 202, 92.1 and OCM-1, primary tumour-derived cell lines, were used as a model for human primary uveal melanoma. OMM1 and OMM2.3, skin and liver metastases-derived cell lines, were used as a model for human uveal melanoma metastasis. Rpe1 is a normal retinal pigment epithelium-derived cell line. OCM-1 was provided by dr. J. Kan-Mitchell, Mel202 and OMM2.3 by dr. B. Ksander, 92.1 by dr. M.J. Jager and OMM1 and Rpe1 were established in our laboratory [27-31]. The SSTR-expressing CA20948 cell line originated from the solid CA20948 rat pancreatic tumour and served as positive control for autoradiography experiments since ocreotate detect both rodent and human SSTR2, of which the homology is more than 90% [19,32]. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) 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%) or trypsin+EDTA (0.02%). Cells were centrifuged at 1,000 rpm for 10 min in DMEM, resuspended in 0.1 M DPBS and used to prepare cytospins on coated slides (Menzel Superfrost Plus Menzel-Gläser, Braunschweig, Germany) humidified with DPBS using a cytofuge (Nordic, Tilburg, The Netherlands).

Fluorescent In Situ Hybridization Analysis

Dual color FISH was performed on uncultured tumor tissue, by using centromeres, locus-specific cosmids, P1, or YAC as probes for chromosomes 1, 3, 6, and 8, respectively, as

described previously [33]. Seven probes were used: p1-79 (mapped to chromosome band 1p36), P 3.5 (centromere 3), YAC 827D3 (3q24), cos85 (6p21), and cos52 (6q23) (all from Yusuke Nakamura, Tokyo, Japan) and D8Z2 (centromere 8) and ETO (8q22). The probes were validated on normal peripheral blood cell metaphase spreads, and 10 metaphases were analyzed for each probe. Cutoff limits were less than 3%. The concentration for centromeric probes was 5 ng per slide; for cosmid, P1, and YAC probes, 50 to 75 ng per slide was used. After hybridization and washing, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in anti-fade medium (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, CA). Signals were counted in 300 interphase nuclei, according to the criteria of Hopman et al. [34]. Scoring for deletion (>15% of the nuclei with one signal) or amplification (>10% of the nuclei with three or more signals) was adapted from the available literature [35].

Autoradiography

^{125}I-DOTA-Tyr^{3}-octreotate (BioSynthema, St Louis Mo, USA), that has a high affinity binding to SSTR2, was used for the autoradiography study [36]. The autoradiographic experiments were performed on cryosections and cytospins using 10^{-9}M or 10^{-10}M ^{125}I-DOTA-Tyr^{3}-octreotate, with or without a blockade of 10^{-6}M unlabelled octreotide (Novartis, Basel, Switzerland), to investigate SSTR2 specific binding as described in literature [14,37]. After a 1 h incubation at room temperature and rinsing with Tris buffer to remove the non-bound radioactive octreotate, dried cryosection and cytospin slides were exposed to phosphor-imaging screens (Packard Instruments Co., Meriden, USA) in X-ray cassettes. After 24-72 hours, the screens were read using a Cyclone phosphor imager and analyzed with OptiQuant 03.00 image processing system (Packard Instruments Co., Groningen, The Netherlands). Binding of ^{125}I-DOTA-Tyr^{3}-octreotate to cytospins and cryosections was expressed in digital light units (DLU)/mm2. Net DLU/mm^{2} represents binding of ^{125}I-DOTA-Tyr^{3}-octreotate (specific binding) minus non-specific binding in adjacent sections incubated with ^{125}I-DOTA-Tyr^{3}-octreotate plus 10^{-6}M octreotide. Net DLU/mm^{2} was considered positive when > 5000 DLU/mm^{2}.

Quantitative real-time RT-PCR

SSTR2 mRNA expression was analyzed by real time-reverse transcriptase–polymerase chain reaction (real time RT-PCR). 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 up till 100 µl. In 96-wells, 2 µl of this solution was added to a 15 µl solution of iQ SYBR Green Supermix, forward and reverse primers (10µM solutions) for SSTR2 or RPS-11 (control gene) and sterile water (volume ratio respectively 10:1:1:8). The primers had the following sequences: SSTR2 forward 5'- TGCTGGGTCTGCCTTTCTTGG – 3', SSTR2 reverse 5' – AGAAGATGCTGGTGAACTGATTGATG – 3', RPS-11 forward 5' – aagcagccgaccatctttca

– 3', RPS-11 reverse 5' – cgggagcttctccttgcc – 3' [38]. A quantitative analysis of the samples was then performed for SSTR2 and RPS-11 (control gene) expression by real time-PCR in a MyiQ iCycler real-time PCR system (Bio-Rad, Hercules CA). 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 [39]. RPS-11 (ribosomal protein S11) is a housekeeping gene that has recently been introduced to normalize gene expression in uveal melanoma cells [40].

RESULTS

Patients and tumour material

In total, primary uveal melanomas from 14 patients (10 male and 4 female, with a mean age of 63 ± 10.1 years) were included in the study. Histopathologic analysis of HE and PAS stained paraffin sections was performed on all primary uveal melanomas. The mean tumour diameter was 12.6 ± 2.8 mm and their mean prominence was 8.5 ± 2.0 mm. Epithelioid cells were found in 10 of 14 cases, while in seven cases vascular loops and/or networks were detected. Furthermore, FISH analysis of all samples was performed; monosomy 3 as well as loss of chromosome 1p was found in 10 of 14 cases and gain of chromosome 8q was present in 7 of 14 cases. After a mean follow-up period of 36.1 ± 13.0 months, five patients had developed distant metastases (Table 1).

Autoradiography

Fresh primary uveal melanoma samples were tested for expression of SSTR2 by AR using ^{125}I-Tyr^{3}-ocreotate. In three of the in total 14 primary uveal melanomas a positive albeit weak binding was observed (Table 2). Statistical analysis with the Kaplan Meier method and log-rank test did not show any significant relation between SSTR2 expression and tumour-free survival (p=0,76). In the univariate and multivariate Cox-regression analysis, SSTR2 expression was not significantly associated with tumour-free survival or other important prognostic factors (cell type, vascular patterns, tumour diameter, prominence, monosomy 3, loss of chromosome 1p and gain of chromosome 8q).

Uveal melanoma cell lines were tested for expression of SSTR2 by autoradiography using ^{125}I-Tyr^{3}-ocreotate. All cell lines were tested in duplicate.

The positive control rat pancreatic tumour cell line (CA20948) showed a strong binding. Cell line 92.1 showed very low binding while none of the other primary UM derived cell lines (Mel202, OCM-1) or normal cell line (Rpe1) showed specific receptor binding. Both metastatic melanoma cell lines (OMM1, OMM2.3) showed high binding (Fig. 1, Table 3).

TABLE 1 Clinical features of included patients

Sex (male/female)	10/4
Age (mean ± SD , years)	63.0 ± 10.1
Diameter (mean ± SD, mm)	12.6 ± 2.8
Prominence (mean ± SD, mm)	8.5 ± 2.0
Cell type (epitheliod/non-epitheliod)	10/4
Vascular loops and/or networks (yes/no)	7/7
Pigmentation (yes/no)	8/6
Metastasis (yes/no)	5/9
Monosomy 3 (yes/no)	10/4
Loss of 1p (yes/no)	10/4
Gain of 8q (yes/no)	7/7
Follow-up (mean ± SD, months)	36.1 ± 13.0

TABLE 2 Autoradiography results of primary tumor samples

	METASTASIS[1]	AUTORADIOGRAPHY[2] ^{125}I-TYR^3-OCREOTATE (SST_2)
1	-	++
2	-	+
3	+	+
4	-	-
5	-	-
6	-	-
7	+	-
8	-	-
9	+	-
10	+	-
11	+	-
12	-	-
13	-	-
14	-	-
Control	NA[3]	++++

[1] Metastasis present: +, No metastasis:-

[2] Netto Digital Light Units/mm^2: <5000: –, 5000-10000: +, 10000-50000: ++, 50000-100000: +++, >100000:++++

[3] Not assessed

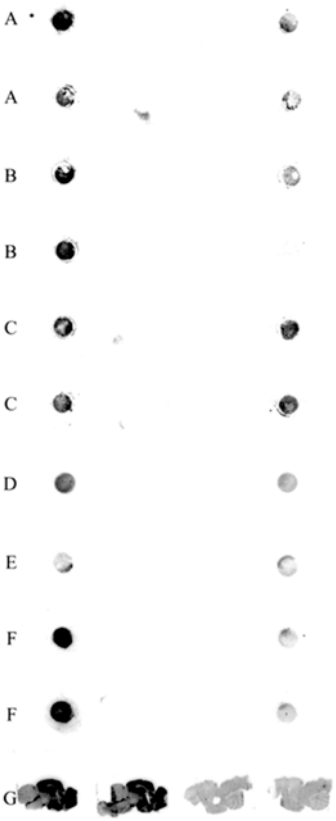

FIGURE 1. Autoradiogram of cytospins with binding to SSTR2
1ste column: incubated cytospins
2de column: negative controls
OMM2.3, B. OMM1, C. Mel202, D. OCM-1, E. Rpe 1, F. CA20948, G. Rat brain.
(a few cell lines are shown in duplicate)

TABLE 3 Autoradiography results of uveal melanoma cell lines

	Cell line	Autoradiography[1] 125I-Tyr3-Octreotate (SSTR2)
Primary	92.1	+
	Mel202	-
	OCM-1	-
Metastatic	OMM1	++
	OMM2.3	++
Normal Rpe2	Rpe1	-
Control	CA20948 [3]	++++

[1] Netto Digital Light Units/mm^2: <5000: –, 5000-10000: +, 10000-50000: ++, 50000-100000: +++, >100000:++++
[2] Normal retinal pigment epithelium cell line
[3] Control cell line from the solid CA20948 rat pancreatic tumor

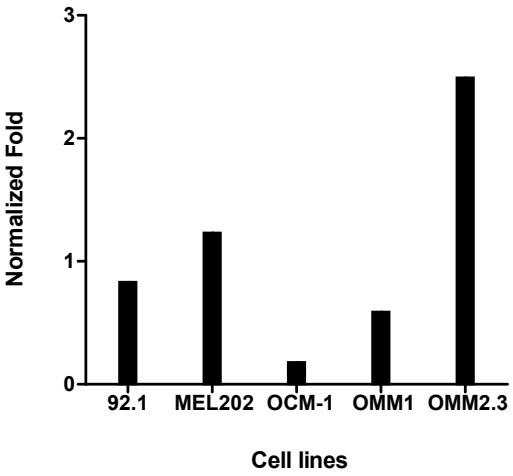

FIGURE 2. Expression of SSTR2 in uveal melanoma cell lines.
All five UM cell lines tested expressed SSTR2 mRNA. The expression levels were normalized to the housekeeping gene RPS-11. OMM2.3 shows a two to four times higher expression level of SSTR2 mRNA compared to OMM1, 92.1 and MEL202. Primary UM cell line OCM-1 had a very low expression level, and can therefore not be compared to the other UM cell lines.

Real-time quantitative RT-PCR in cell lines

Expression of SSTR2 mRNA in UM cell lines was evaluated by RT-PCR, with normalization of the expression levels to the housekeeping gene RPS-11. All five UM cell lines tested expressed SSTR2 mRNA, but the level of expression was quite variable. OMM 2.3 showed a two to four times higher expression level of SSTR2 mRNA compared to the other metastases derived UM cell line OMM1, and the primary UM cell lines 92.1 and Mel 202. The other tested primary UM cell line OCM-1 showed a very low SSTR2 mRNA expression level (Fig. 2).

DISCUSSION

Expression of somatostatin receptors in neuroendocrine tumours has been extensively investigated and led to the development of clinically relevant diagnostic and therapeutic strategies. Based on common embryonic origin, uveal melanoma could also be a candidate for such strategies, as many patients die from metastasized UM.

In this study, SSTR2 expression was studied in 14 primary UM tumour samples and several different uveal melanoma cell lines, either derived from primary or from metastized UM tumour samples. Primary tumours were tested at the protein level using in vitro autoradiography, cell lines were additionally analyzed on mRNA level. In only three primary specimens

moderate SSTR2 expression was demonstrated. One of these was a metastasized melanoma, while four other samples of metastasized UM did not express SSTR2.

Furthermore the relation between SSTR2 expression, tumour-free survival and prognostic factors was studied, although the number of samples we used was too small to perform reliable statistical analysis. With the data obtained thus far, no significant correlation between SSTR2 expression and tumour-free survival or any other important prognostic parameter could be found. Conversely, Ardjomand et al. detected by using immunohistochemistry on paraffin embedded UM tissue, that SSTR2 was expressed in nearly all of the 25 tested samples. When the prognosis of these 25 patients was compared with the expression level of SSTR2 in uveal melanoma tissue, a positive correlation was found between high SSTR2 and a better ad vitam prognosis [26].

Since Ardjomand et. al used a different approach and techniques to determine SSTR2 expression, it is difficult to compare our results. An explanation for the differences could be that we used cryosections instead of paraffin sections, in antibody versus peptide targeting. Furthermore, in immunohistochemistry it is possible to discriminate positive staining in individual cells by using antibodies for specific subtypes of somatostatin receptors. In our AR experiments, we analyzed tissue cryosections and a peptide analogue that can be applied in vivo for PET or SPECT imaging, after labelling with an appropriate radionuclide. In AR a certain threshold had to be reached before tissue could be identified as positive; however this threshold is low due to the high sensitivity of the phosphor imaging read out system. For in vivo imaging this threshold would be much higher. Most of the tumours tested by Ardjomand et. al contained between 11 and 80% of SSTR2 expressing tumour cells. If the percentage of SSTR2 expressing tumour cells in our tested specimens was within this range, these should have been detected using in vitro autoradiography. Furthermore, Ardjomand et. al also argued the diagnostic value of SSTR expression, since in only two of four patients with UM in their study the affected eye could be visualized by ocreotide scintigraphy.

Besides primary UM samples, UM derived cell lines, originating either from primary or from metastasized UM tumours, were tested for SSTR2 expression. By using the in vitro AR technique with cytospins of UM cell line cells, it was found that three primary melanoma cell lines showed almost no specific binding of ^{125}I-Tyr^{3}-octreotate, whereas both metastatic cell lines showed strong binding. This implied that expression of SSTR2 at the protein level could be associated with a bad prognosis and development of metastasis. This clear SSTR2 expression of the metastatic UM cell line OMM2.3 correlated with a four times higher expression level of SSTR2 mRNA compared to the primary UM line 92.1 in quantitative real-time RT-PCR. The primary UM cell line Mel 202 also showed a moderately high SSTR2 mRNA expression, but apparently this mRNA was not transcribed to protein. Apparently the correlation between SSTR2 expression at mRNA and protein level seems to be good in cell lines derived from metastasized UM, but less in lines from primary UM. Furthermore, there is a discrepancy between the SSTR2 expression in cell lines compared to that in primary

and metastized uveal melanoma specimens, in which only a small percentage of low SSTR2 expressing samples was found. Expression of high SSTR2 levels might be favourable for deriving an in vitro growing cell line from a UM biopsy. Thus cell lines might not be really representative for in vivo circumstances [41].

Considering the low SSTR2 expression of the primary UM samples, we conclude that imaging with somatostatin analogues to perform scintigraphy is not feasible in uveal melanoma patients.

Although FNAB has been shown to accurately demonstrate genetic and histologic prognostic factors in the obtained tissue [7], finding another tumour-specific (neurohormone) receptor that can be used as an in vivo target for diagnosis (and therapy) is still an attractive, non-invasive option to improve the diagnosis of (metastasized) UM using specific radiolabelled peptide analogues. Metastasis is the single leading cause of death of patients with UM. Kaplan-Meier estimates of 5-year melanoma-related mortality range from 26% to 32% [42]. The first site of metastasis is the liver in approximately 90% of patients, but later spread to the lungs, bone, and skin occurs frequently [43-45]. It remains unknown to which extent current treatments for metastasis actually prolong survival compared to no treatment at all [42,46]. It is proposed that patients who develop clinical metastases from uveal melanoma often harbour micro metastases for years [47]. Targeting tumour-specific receptors might be used to treat these (micro) metastases in future when specific analogues are radiolabelled with therapeutic β-emitting radionuclides like ^{177}Lu.

Further investigation targeting other neural-crest derived hormone receptors may thus reveal new options. Several studies have shown that vasoactive intestinal peptide (VIP) and pituitary adenylate-cyclase-activating polypeptide (PACAP) have tumour-growth promoting activities in breast cancer and neuroblastoma for example. Furthermore, VIP and PACAP antagonists demonstrated growth-inhibitory properties [48-51]. VIP receptors have been found to be ubiquitous expressed in all ocular tissues, with highest concentrations occurring in the choroid of several different mammals [23]. Most uveal melanomas are strongly pigmented. Alpha-melanocyte-stimulating hormone is primarily responsible for the regulation of pigmentation and could therefore be linked to uveal melanoma [52].

In conclusion, additional exploration of neurohormone receptors is needed to identify a specific membrane receptor to be used in diagnostic imaging and therapeutic targeting.

REFERENCE LIST

1. Egan, K. M., Seddon, J. M., Glynn, R. J., Gragoudas, E. S., and Albert, D. M. *Epidemiologic aspects of uveal melanoma.* Surv.Ophthalmol. 1988. 32:239-251.
2. Mooy, C. M. and De Jong, P. T. *Prognostic parameters in uveal melanoma: a review.* Surv.Ophthalmol. 1996. 41:215-228.
3. Mudhar, H. S., Parsons, M. A., Sisley, K., Rundle, P., Singh, A., and Rennie, I. G. *A critical appraisal of the prognostic and predictive factors for uveal malignant melanoma.* Histopathology 2004. 45:1-12.
4. Prescher, G., Bornfeld, N., Hirche, H., Horsthemke, B., Jockel, K. H., and Becher, R. *Prognostic implications of monosomy 3 in uveal melanoma.* Lancet 1996. 347:1222-1225.
5. Sisley, K., Rennie, I. G., Parsons, M. A., Jacques, R., Hammond, D. W., Bell, S. M., Potter, A. M., and Rees, R. C. *Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis.* Genes Chromosomes.Cancer 1997. 19:22-28.
6. Damato, B., Duke, C., Coupland, S. E., Hiscott, P., Smith, P. A., Campbell, I., Douglas, A., and Howard, P. *Cytogenetics of uveal melanoma: a 7-year clinical experience.* Ophthalmology 2007. 114:1925-1931.
7. Shields, C. L., Ganguly, A., Materin, M. A., Teixeira, L., Mashayekhi, A., Swanson, L. A., Marr, B. P., and Shields, J. A. *Chromosome 3 analysis of uveal melanoma using fine-needle aspiration biopsy at the time of plaque radiotherapy in 140 consecutive cases: the Deborah Iverson, MD, Lectureship.* Arch. Ophthalmol. 2007. 125:1017-1024.
8. Midena, E., Bonaldi, L., Parrozzani, R., Radin, P. P., Boccassini, B., and Vujosevic, S. *In vivo monosomy 3 detection of posterior uveal melanoma: 3-year follow-up.* Graefes Arch.Clin.Exp.Ophthalmol. 2007.
9. Caminal, J. M., Sanz, S., Carreras, M., Catala, I., Arruga, J., and Roca, G. *Epibulbar seeding at the site of a transvitreal fine-needle aspiration biopsy.* Arch.Ophthalmol. 2006. 124:587-589.
10. Kvanta, A., Seregard, S., Kopp, E. D., All-Ericsson, C., Landau, I., and Berglin, L. *Choroidal biopsies for intraocular tumors of indeterminate origin.* Am.J.Ophthalmol. 2005. 140:1002-1006.
11. Johnson, L. R. *Regulation of gastrointestinal mucosal growth.* Physiol Rev. 1988. 68:456-502.
12. Patel, Y. C. *Somatostatin and its receptor family.* Front Neuroendocrinol. 1999. 20:157-198.
13. Benali, N., Ferjoux, G., Puente, E., Buscail, L., and Susini, C. *Somatostatin receptors.* Digestion 2000. 62 Suppl 1:27-32.
14. Reubi, J. C., Krenning, E., Lamberts, S. W., and Kvols, L. *In vitro detection of somatostatin receptors in human tumors.* Digestion 1993. 54 Suppl 1:76-83.
15. Reubi, J. C., Kappeler, A., Waser, B., Laissue, J., Hipkin, R. W., and Schonbrunn, A. *Immunohistochemical localization of somatostatin receptors sst2A in human tumors.* Am.J.Pathol. 1998. 153:233-245.
16. Liu, Q., Reubi, J. C., Wang, Y., Knoll, B. J., and Schonbrunn, A. *In vivo phosphorylation of the somatostatin 2A receptor in human tumors.* J.Clin.Endocrinol.Metab 2003. 88:6073-6079.
17. Papotti, M., Croce, S., Bello, M., Bongiovanni, M., Allia, E., Schindler, M., and Bussolati, G. *Expression of somatostatin receptor types 2, 3 and 5 in biopsies and surgical specimens of human lung tumours. Correlation with preoperative octreotide scintigraphy.* Virchows Arch. 2001. 439:787-797.
18. Reubi, J. C. and Waser, B. *Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for in vivo multireceptor tumour targeting.* Eur.J.Nucl.Med.Mol.Imaging 2003. 30:781-793.
19. de Jong, M., Breeman, W. A., Valkema, R., Bernard, B. F., and Krenning, E. P. *Combination radionuclide therapy using 177Lu- and 90Y-labeled somatostatin analogs.* J.Nucl.Med. 2005. 46 Suppl 1:13S-17S.
20. Krenning, E. P., de Jong, M., Kooij, P. P., Breeman, W. A., Bakker, W. H., de Herder, W. W., van Eijck, C. H., Kwekkeboom, D. J., Jamar, F., Pauwels, S., and Valkema, R. *Radiolabelled somatostatin analogue(s) for peptide receptor scintigraphy and radionuclide therapy.* Ann.Oncol. 1999. 10 Suppl 2:S23-S29.

21. Krenning, E. P., Valkema, R., Kwekkeboom, D. J., de Herder, W. W., van Eijck, C. H., de Jong, M., Pauwels, S., and Reubi, J. C. *Molecular imaging as in vivo molecular pathology for gastroenteropancreatic neuroendocrine tumors: implications for follow-up after therapy.* J.Nucl.Med. 2005. 46 Suppl 1:76S-82S.
22. Kwekkeboom, D. J., Mueller-Brand, J., Paganelli, G., Anthony, L. B., Pauwels, S., Kvols, L. K., O'dorisio, T. M., Valkema, R., Bodei, L., Chinol, M., Maecke, H. R., and Krenning, E. P. *Overview of results of peptide receptor radionuclide therapy with 3 radiolabeled somatostatin analogs.* J.Nucl.Med. 2005. 46 Suppl 1:62S-66S.
23. Elbadri, A. A., Shaw, C., Johnston, C. F., Archer, D. B., and Buchanan, K. D. *The distribution of neuropeptides in the ocular tissues of several mammals: a comparative study.* Comp Biochem.Physiol C. 1991. 100:625-627.
24. Helboe, L. and Moller, M. *Immunohistochemical localization of somatostatin receptor subtypes sst1 and sst2 in the rat retina.* Invest Ophthalmol.Vis.Sci. 1999. 40:2376-2382.
25. Troger, J., Sellemond, S., Kieselbach, G., Kralinger, M., Schmid, E., Teuchner, B., Nguyen, Q. A., Schretter-Irschick, E., and Gottinger, W. *Inhibitory effect of certain neuropeptides on the proliferation of human retinal pigment epithelial cells.* Br.J.Ophthalmol. 2003. 87:1403-1408.
26. Ardjomand, N., Ardjomand, N., Schaffler, G., Radner, H., and El Shabrawi, Y. *Expression of somatostatin receptors in uveal melanomas.* Invest Ophthalmol.Vis.Sci. 2003. 44:980-987.
27. Chen, P. W., Murray, T. G., Salgaller, M. L., and Ksander, B. R. *Expression of MAGE genes in ocular melanoma cell lines.* J.Immunother.(1997.) 1997. 20:265-275.
28. Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer 1995. 62:155-161.
29. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
30. Luyten, G. P., Naus, N. C., Mooy, C. M., Hagemeijer, A., Kan-Mitchell, J., Van Drunen, E., Vuzevski, V., De Jong, P. T., and Luider, T. M. *Establishment and characterization of primary and metastatic uveal melanoma cell lines.* Int.J.Cancer 1996. 66:380-387.
31. Verbik, D. J., Murray, T. G., Tran, J. M., and Ksander, B. R. *Melanomas that develop within the eye inhibit lymphocyte proliferation.* Int.J.Cancer 1997. 73:470-478.
32. Melis, M., Forrer, F., Capello, A., Bijster, M., Bernard, B. F., Reubi, J. C., Krenning, E. P., and de Jong, M. *Up-regulation of somatostatin receptor density on rat CA20948 tumors escaped from low dose [(177) Lu-DOTA(0),Tyr(3)]octreotate therapy.* Q.J.Nucl.Med.Mol.Imaging 2007. 51:324-333.
33. Naus, N. C., Verhoeven, A. C., Van Drunen, E., Slater, R., Mooy, C. M., Paridaens, D. A., Luyten, G. P., and de Klein, A. *Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization.* Clin.Cancer Res. 2002. 8:534-539.
34. Hopman, A. H., Ramaekers, F. C., Raap, A. K., Beck, J. L., Devilee, P., van der, P. M., and Vooijs, G. P. *In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors.* Histochemistry 1988. 89:307-316.
35. van Dekken, H., Pizzolo, J. G., Reuter, V. E., and Melamed, M. R. *Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes.* Cytogenet.Cell Genet. 1990. 54:103-107.
36. Reubi, J. C., Schaer, J. C., and Waser, B. *Cholecystokinin(CCK)-A and CCK-B/gastrin receptors in human tumors.* Cancer Res. 1997. 57:1377-1386.
37. Bodei, L., Hofland, L. J., Ferone, D., Mooy, C. M., Kros, J. M., Paridaens, D. A., Baarsma, S. G., Ferdeghini, M., Van Hagen, M. P., Krenning, E. P., and Kwekkeboom, D. J. *In vivo and in vitro detection of dopamine d2 receptors in uveal melanomas.* Cancer Biother.Radiopharm. 2003. 18:895-902.

38. Ferrara, N., Gerber, H. P., and LeCouter, J. *The biology of VEGF and its receptors.* Nat.Med. 2003. 9:669-676.
39. Bustin, S. A. *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays.* J.Mol.Endocrinol. 2000. 25:169-193.
40. Zuidervaart, W., van der Velden, P. A., Hurks, M. H., van Nieuwpoort, F. A., Out-Luiting, C. J., Singh, A. D., Frants, R. R., Jager, M. J., and Gruis, N. A. *Gene expression profiling identifies tumour markers potentially playing a role in uveal melanoma development.* Br.J.Cancer 2003. 89:1914-1919.
41. Rubin, H. *The role of selection in progressive neoplastic transformation.* Adv.Cancer Res. 2001. 83:159-207.
42. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
43. Eskelin, S., Pyrhonen, S., Summanen, P., Prause, J. U., and Kivela, T. *Screening for metastatic malignant melanoma of the uvea revisited.* Cancer 1999. 85:1151-1159.
44. *Assessment of metastatic disease status at death in 435 patients with large choroidal melanoma in the Collaborative Ocular Melanoma Study (COMS): COMS report no. 15.* Arch.Ophthalmol. 2001. 119:670-676.
45. Lorigan, J. G., Wallace, S., and Mavligit, G. M. *The prevalence and location of metastases from ocular melanoma: imaging study in 110 patients.* AJR Am.J.Roentgenol. 1991. 157:1279-1281.
46. Aoyama, T., Mastrangelo, M. J., Berd, D., Nathan, F. E., Shields, C. L., Shields, J. A., Rosato, E. L., Rosato, F. E., and Sato, T. *Protracted survival after resection of metastatic uveal melanoma.* Cancer 2000. 89:1561-1568.
47. Eskelin, S., Pyrhonen, S., Summanen, P., Hahka-Kemppinen, M., and Kivela, T. *Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment.* Ophthalmology 2000. 107:1443-1449.
48. Moody, T. W., Chan, D., Fahrenkrug, J., and Jensen, R. T. *Neuropeptides as autocrine growth factors in cancer cells.* Curr.Pharm.Des 2003. 9:495-509.
49. Zia, H., Hida, T., Jakowlew, S., Birrer, M., Gozes, Y., Reubi, J. C., Fridkin, M., Gozes, I., and Moody, T. W. *Breast cancer growth is inhibited by vasoactive intestinal peptide (VIP) hybrid, a synthetic VIP receptor antagonist.* Cancer Res. 1996. 56:3486-3489.
50. Jiang, S., Kopras, E., McMichael, M., Bell, R. H., Jr., and Ulrich, C. D. *Vasoactive intestinal peptide (VIP) stimulates in vitro growth of VIP-1 receptor-bearing human pancreatic adenocarcinoma-derived cells.* Cancer Res. 1997. 57:1475-1480.
51. Lilling, G., Wollman, Y., Goldstein, M. N., Rubinraut, S., Fridkin, M., Brenneman, D. E., and Gozes, I. *Inhibition of human neuroblastoma growth by a specific VIP antagonist.* J.Mol.Neurosci. 1994. 5:231-239.
52. Reubi, J. C. *Peptide receptors as molecular targets for cancer diagnosis and therapy.* Endocr.Rev. 2003. 24:389-427.

CHAPTER 8

UVEAL MELANOMA ASSOCIATED PEPTIDES IDENTIFIED WITH A PHAGE DISPLAY LIBRARY AND IN VITRO PANNING

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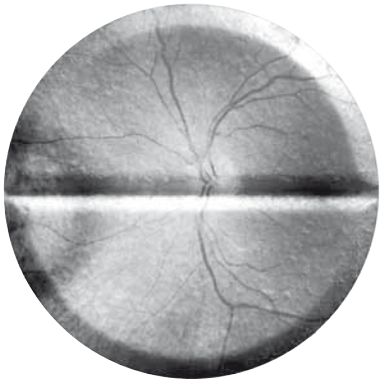
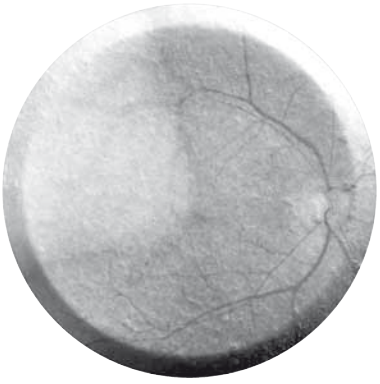
Article submitted





ABSTRACT

Introduction. Uveal melanoma (UM) is the most common primary intraocular tumor, in which still 50% of patients may die due to metastatic disease. Specific targeting of UM for imaging and delivery of drugs is urgently needed for improved identification and subsequent killing of tumor cells. Ligand-specific antigens that are preferentially expressed by primary or metastatic UM cells are ideal targets for tumor-targeting applications.

Material and Methods. UM cell lines were screened in vitro for tumor-specific peptides using a phage-displayed peptide library. Binding and internalization of peptides to cell lines was visualized using fluorescein and rhodamine-labeled synthetic peptides and confocal microscopy.

Results. Phage display experiments resulted in the identification of three UM-associated peptides (UMAPs). After labeling with fluorescein or rhodamine, internalization of these peptides in several UM cell lines was visualized by confocal fluorescent microscopy. One peptide, UMAP1, was identified multiple times in phages that had bound to metastatic cell lines, while UMAP2 showed homology to the insulin-like growth factor 1 receptor (IGF-1R).

Discussion. Three UMAPs were identified by screening phage peptide libraries and shown to successfully internalize in targeted UM cells. UMAP1 may have potential for future usage for UM-targeted treatment. UMAP2 is especially interesting because of its homology to the IGF-1R, which is a bad prognostic marker in UM.

INTRODUCTION

Isolation of tumor-specific ligands has been extensively researched in this last decade, as specific tumor agents can be used for in vivo imaging and thus for early detection of tumors and micrometastases. In case of uveal melanoma (UM), the most common intraocular tumor in adults, these micrometastases are believed to play an important role: in spite of an effective treatment of primary UMs by enucleation or radiotherapy, with a local tumor control rate of 97% of all treated cases [1-3], still half of all patients may die due to UM-related metastatic disease [4,5]. The occurrence of metastases has even been described 35-40 years after initial diagnosis and treatment of the primary UM tumor [5,6]. It has been proposed that dissemination occurs several years before identification of the primary tumor and its treatment [7]. Non-invasive detection of dormant micrometastases using specific peptides coupled to a fluorescent probe or a radionuclide and subsequent treatment with specific tumor ligands that are conjugated to an anticancer drug may improve survival. Specific peptide-based agents that achieve apoptosis, or block angiogenesis and proliferation of UM metastatic tissue, would be of great value in sparing healthy tissue.

For this purpose, various melanoma-derived autoantigens and cell surface receptors have been explored in uveal melanoma. Cutaneous melanoma markers such as MAGE are not widely distributed in ocular melanoma tissue [8]. For histopathologic diagnosis of uveal melanoma, antibodies such as S-100 (specific for a protein derived from bovine brain cross-reacting with melanoma and melanocytes), HBM45 (specific for gp100) and A103, (recognizes the Melan-A/Mart-1 protein) are used [9-11].

Unfortunately, most of these receptors are not UM specific and are also expressed by normal cells (melanocytes). Therefore, tartgeting these receptors may cause significant side-effects.

Phage display is a powerful technique for the isolation of peptides that bind to a particular target with high affinity and specificity. In contrast to larger molecules, such as proteins and antibodies, small peptides can efficiently penetrate tissues and are relatively easy to synthesise. Several studies describe the successful detection of organ and tumor-specific peptides using phage peptide libraries, for example in bladder cancer, brain and kidney tissue [12,13]. Using phage display, Howell et al. [14] have reported the identification of heptapeptides that specifically bind to human tumor melanin, as demonstrated in human metastatic melanoma tumor-bearing nude mice. These heptapeptides may be used as a tool in targeted therapy for (metastatic) melanoma [14]. In addition, phages alone are also known to be highly immunogenic. Treatment of B16-F10 melanoma in mice with tumor specific (selected with phage display) phages induced a massive infiltration of neutrophils, which delayed tumor growth and increased survival [15].

Using in vitro biopanning on (metastatic and primary) UM cells, we report the identification of UM associated peptides (UMAPs) selected from phage display libraries. Additionally, we demonstrate the potent binding and internalisation of fluorescein and rhodamine-conjugated synthetic peptides in UM cells.

MATERIAL AND METHODS

Cell Lines

Four UM cell lines were used as targets in biopanning experiments. Mel270 and 92.1 are primary tumor-derived cell lines, while OMM1 and OMM2.5 are cell lines derived from metastases. Mel270 and OMM2.5 were provided 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 [16-20]. All cell lines were cultured in complete RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin/streptomycin (GIBCO, Life Technologies, Paisley, UK) at 37°C and 5% CO_2 (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). The cells were passaged once or twice a week using trypsin (0.05%).

Mel1, a normal melanocyte culture established in our own laboratory, was used for pre-adsorption (diminishing tumor aspecificity in subsequent incubation rounds) and as a control for internalisation experiments. In addition, HUVECs (human umbilical vein endothelial cells) and SAOS-2 (primary osteosarcoma cells) served as controls in internalisation assays.

Biopanning

M13 phage display libraries containing random hepta- and dodecapeptides fused to the N-terminus of minor coat protein pIII (Ph.D.-C7C and Ph.D.12 Phage Display Peptide Library, New England Biolabs, Ipswich, MA, USA) were used for in vitro experiments.

Biopanning was performed for three to four rounds, starting with an equal mixture of 10^{11} pfu of each library, with and without pre-adsorption on normal melanocytes (Mel1). In case of pre-adsorption, the mixed Ph.D.-C7C and Ph.D.12 libraries were first incubated with each of the four UM cell lines. The selected phages were amplified and subjected to pre-adsorption on a Mel1 cell monolayer, grown in a T25 tissue culture flask (Corning, NY), incubated with phages ($2x10^{-11}$ pfu) for 1 h at 37°C for melanocyte-specific phage depletion. Subsequently, the medium, including phages, was transferred to 90-100% confluent UM monolayers in T75 flasks and incubated again for 1 h at 37°C. After incubation, cells were washed five times with 10 ml of phosphate-buffered saline (PBS) and treated with trypsin (0.05%) for 10 min. at 37°C to inactivate non-internalized phages. Subsequently, 10 ml of RPMI was added and the cells were harvested in a 15 ml tube and spun down. Cell pellets were washed another

3-5 times with PBS. Internalized phages were recovered by lysing the cells in 1 ml of cell culture lysis buffer (Promega, Benelux) for 15 min. Phages were precipitated by addition of 250 µl of a 20% PEG8000/ 2.5 M NaCl solution and overnight incubation at 4°C. Pellets were resuspended in 100 µl TBS. The internalized phages were amplified and subjected to another round (round 3) of biopanning on their respective cell lines. Round 3 phages were subjected to an additional round of pre-adsorption and biopanning (round 4) on their respective cell lines. Phages from round 3 and 4 were sequenced.

Phage titering, amplification, and sequencing

Phage titers were determined after each round of selection and amplification by mixing appropriate dilutions of the phage suspension with 100 µl of an overnight culture of E.coli XL-1 Blue F^+ cells. After five minutes of incubation at room temperature, three ml of topagar was added and the mixture was plated onto LB agar plates containing kanamycin. Plaques were counted after overnight incubation at 37°C. Five to ten plaques were picked after each round, amplified and phage DNA was isolated as described by the manufacturer (New England Biolabs, Ipswich, MA, USA). Sequencing of phage DNA was performed by automated dideoxy sequencing with chain terminator dyes (LGTC, Leiden).

Phages were amplified essentially as described in the manual (New England Biolabs, Ipswich, MA, USA).except that XL-1 cells were used.

Synthetic peptides

UMAP 1 was synthesized using F-moc chemistry on a peptide synthesizer (LIC, Leiden). The lysine-residue was labeled with fluorescein using FITC (Sigma-Aldrich). The peptide was purified by HPLC, and analyzed by MALDI-TOF mass spectrometry. Rhodamine-labeled UMAP2 and 3, and fluorescein-labeled UMAP3 were purchased from the peptide synthesis facility at the LUMC (Leiden, The Netherlands).

Internalisation assay and Confocal imaging

For internalisation assays, cells were cultured on eight-chamber slides (Nalgen Nunc Int.) to ~70% confluency. Prior to addition of peptides, medium was refreshed (200 µl RPMI 1640) and 50 µl of a 0.2mM solution of peptide in 50% ethanol was added. After 30 min incubation, cells were washed 2 times with 200 µl of medium to remove unbound peptides. Images were recorded on a Leica SP1 confocal microscope equipped with an Argon laser (488 excitation and emission 500-540 nm).

RESULTS

Selection of UM cell-binding peptides

Two primary and two metastatic UM cell lines were used to select melanoma-specific phages from 7- and 12-mer random peptide phage libraries. To assess whether cell-binding phages were enriched during the panning experiments, phages eluted after each round of selection were amplified and their DNA sequenced.

After three rounds of biopanning, four peptide sequences dominated in the selected phage pool in specific cell lines as shown in Table 1. One peptide that was repeatedly amplified (9 of 10 plaques) from phages targeting the metastatic UM cell lines OMM 1 and OMM2.5, consisted of the following sequence: DLNYFTLSSKRE and was named uveal melanoma associated peptide 1 (UMAP1). This peptide was identified during biopanning without pre-adsorption onto normal melanocytes, and may thus carry melanocyte-lineage and not melanoma-specific characteristics. UMAP1 has also been reported in a liver cancer phage display experiment as Titanium Oxide binding peptide (www.freshpatents.com).

Two other potentially-interesting peptides, found after pre-adsorption by normal melanocytes, were UMAP2 (YITPYAHLRGGN) from metastatic cell line OMM2.5, and UMAP3 (ELQVMPIHIAAS) which were selected from both primary UM cell lines 92.1 and Mel270.

The sequence of UMAP2 was identified to be a homologue of the rat insulin-like growth factor receptor [21]. UMAP3 could not be linked to any known receptor or protein.

TABLE 1. Identified peptide sequences after biopanning in UM primary and metastatic cell lines.

Peptide	Sequence *	Label	Cell line of origin	Remarks
UMAP 1	DLNYFTLSSKRE *** (9/10)	FITC	OMM2.5, OMM1	Without counterselection on melanocytes
UMAP 2	YITPYAHLRGGN**(9/9)	Rhodamine	OMM2.5	Present in round 3 and 4
UMAP 3	ELQVMPIHIAAS (4/17)	FITC and Rhodamine	Mel270, 92.1	Present in round 3 and 4
	CLSYLPHKC (3/5), CFSSHPHVC*1/5) CLSYLPHKC (2/5),	n/a	OMM1, Mel270	Round 3, lost in round 4 (no clear consensus sequence in round 4)
	HYSRYNPGPHPL (3/17)	n/a	92.1	Round 3 only
	SPITISSWFPMP (3/17)	n/a		3 & 4 suspicious
	SVSVGMKPSPRP (2/17)	n./a		3 & 4 aspecific

Umap= uveal melanoma associated peptide , n/a = not applicable.
* (x/y) x plaques of total y plaques sequenced in rounds 3 and 4 have this sequence for the indicated cell lines.
homologous to rat IGF-1 receptor. * homologous to Titanium Oxide binding peptide SSWSSPITTAAV.
minor sequences NFMESLPRLGMH (92.1), CNHLNHHLC (92.1), ITSTSPMFATPP (omm1), NADNQMTWRHVL (OMM1),

TABLE 2. Internalisation capacities of synthetic labelled peptides.

	UMAP 1	UMAP 2	UMAP 3R	UMAP 3F
Mel270	+	+	+/-	+/-
OMM2.5	+	++	+	+/-
92.1	+	+	+	+/-
OMM1	+	-	+/-	-

- no internalisation,+/- moderate internalisation, + internalisation, ++ excessive internalisation
UMAP= uveal melanoma associated peptide
R= rhodamine
F= FITC

Two peptides from the PhD-C7C library with the related sequences CLSYLPHKC and CFSSHPHVC, named UMAP4 and 5, were isolated from Mel270 and OMM1 cells during round 3 only. In general, peptides from the PhD-C7C library were highly underrepresented. A peptide with the sequence HYSRYNPGPHPL dominated in the 92.1 pool during round 3, but was not retrieved in round 4. On a few occasions, peptides with the sequences SPITISSWFPMP and SVSVGMKPSPRP were selected. These peptides were considered aspecific because of their homology to a titanium-oxide binding peptide (SSWSSPITTAAV) or to the well-known target unrelated peptide (SVSVGMKPSPRP) [22]

A total of three peptides were synthesized and either labelled with fluorescein (UMAP 1 and 3) or rhodamine (UMAP 2 and 3).

Internalization of selected peptides by UM and other cells

The biopanning method is expected to favor the selection of internalizing phages. We therefore investigated whether the synthesized peptides demonstrated internalization in UM cells in vitro. UMAP1 showed a fast and efficient penetration in all UM cell lines both in the cytoplasm as well as in the nucleus, with high concentrations in the nucleolus (Table 2, Figure 1). As expected, also normal melanocytes were penetrated by UMAP1, since no negative selection against these cells had taken place. UMAP1 was not internalized by SAOS-2 and HUVEC cells.

All UM cell lines showed a similar mottled distribution of UMAP 2 and 3, which were primarily concentrated in the cytoplasm. UMAP 2 was especially internalized in the OMM2.5 cell line, from which the peptide was initially selected (Figure 2), and showed less penetration in the other UM cells. UMAP 3 only moderately penetrated cell lines Mel270, 92.1 and OMM2.5, and was not internalized by SAOS-2 and HUVEC cells. Moreover, labelling did not influence staining patterns since also fluorescein-labelled UMAP 3 showed a moderate mottled internalisation (Figure 3).

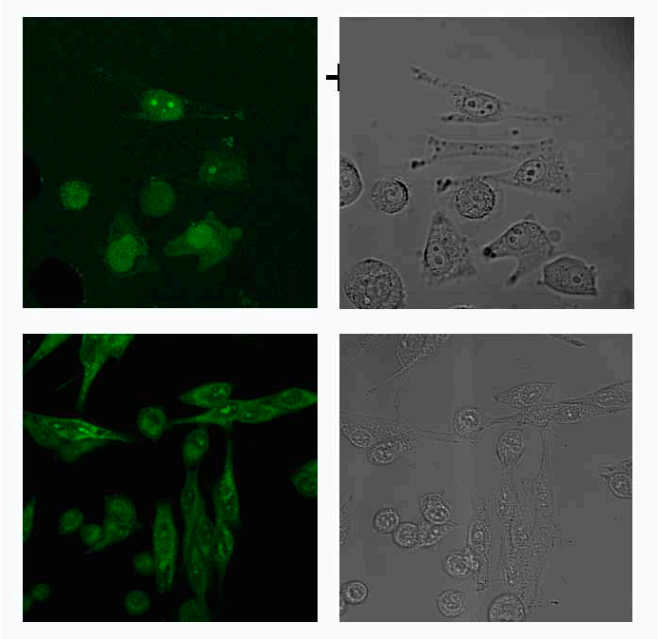


FIGURE 1. Internalization of UMAP 1.
UMAP1 showed penetration of all UM cell lines both in the cytoplasm as well as in the nucleus, with high concentrations in the nucleolus (OMM2.5 (top) and Mel 270 (bottom)). UMAP1 was not internalized in control cell lines SAOS-2 and HUVEC cells (not shown).

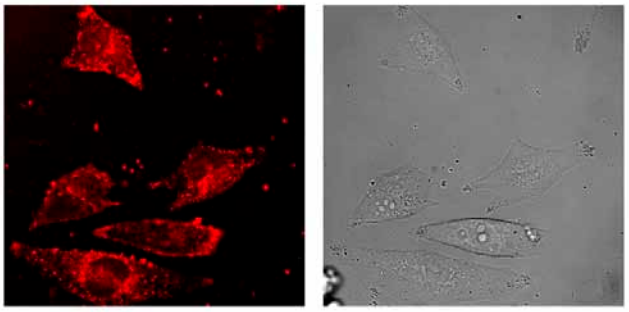


FIGURE 2. Internalization of UMAP 2.
UMAP 2 shows a mottled distribution primarily concentrated in the cytoplasm. UMAP 2 was especially internalized by OMM2.5 from which the peptide was initially selected.

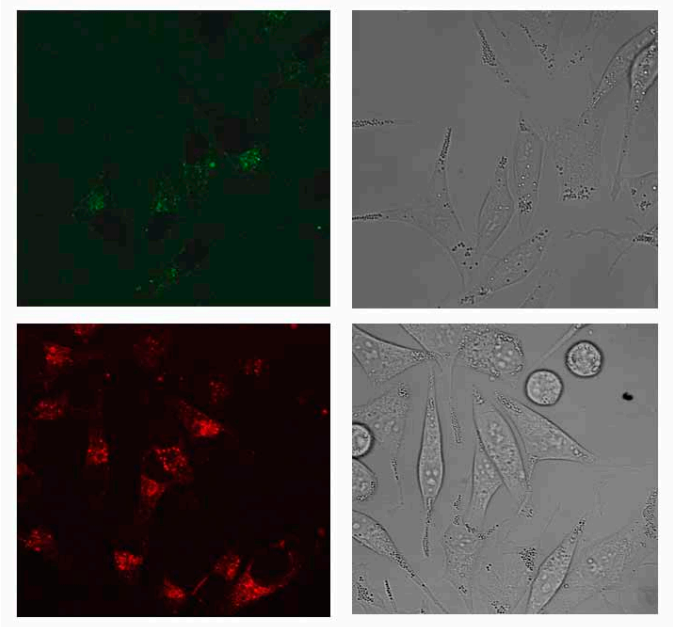


FIGURE 3. Internalization of UMAP 3 in UM cell line 92.1
Shows a mottled moderate staining with both FITC (top)and rhodamine label (bottom).

DISCUSSION

In cancer research, phage display libraries have been used widely for the identification of tumor-targeting peptides and antibodies. [23] Although UM is the most common intraocular tumor in adults, it remains a rare disease and very few reports exist of phage display experiments involving UM. We set out to identify specific UM peptides with good binding and internalization capacities and that can be used for in vivo imaging of (micro) metastases and as a tool for targeted treatment of UM primary and metastatic disease.

Four peptides were identified and subsequently amplified after several in vitro panning rounds. UMAP1 proved to be the most potent peptide, demonstrating excellent internalisation ability in primary and metastatic UM cell lines. This peptide was also internalized by normal melanocytes (due to the lack of counter-selection during biopanning). This is an unfavourable event in regard to the effect of targeted therapies on healthy tissue. However, the current most widely used treatment for UM related metastatic disease, chemotherapy, demonstrates no specificity at all [24]. In addition, UMAP1 was not internalized by SAOS-2 and HUVEC cells, demonstrating partial selectivity.

The low specificity of UMAP1 is also evident from its presence in some patents as a titanium oxide and antibody-binding peptide (www.frespatents.com). UMAP1 has also previously been selected by phage display on human embryonic stem cells [25] and heptocarcinoma cells [26]

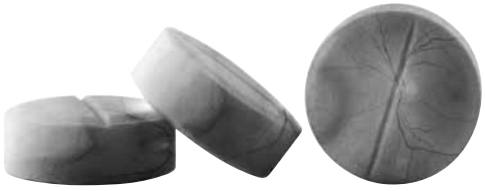
UMAP2 showed a mild preference for metastatic cell line OMM2.5, from which the peptide was initially selected. Although internalisation was mottled, large amounts of peptide were visible in the cytoplasm of almost all cells. The sequence of UMAP2 has been identified as a homologue of the rat insulin-like growth factor receptor [21]. Interestingly, Economou et al described the expression of insulin-like growth factor-1 (IGF-1) in uveal melanoma to be associated with a bad prognosis [27,28]. IGF-1 binds to the IGF-1 receptor leading to phosphorylation of IGF-1R, which then activates key signal molecules involved in tumor transformation, maintenance of malignant phenotype, promotion of cell growth, and prevention of apoptosis [29]. IGF-1 is mainly produced by the liver, of which the metastatic cell line OMM2.5 originated (liver metastases). Since UM almost exclusively disseminate to the liver, the expression of IGFR may therefore explain the preferential liver-homing of UM cells [5]. One would expect to detect a peptide which shows homology with IGF rather than its receptor and to have human similarity. However, one should realize that the Ph.D.-12 phage library does not cover the entire landscape of all possible dodecapeptides (which is ~4x10e15) so exact homologues to human peptides will be extremely rare in the starting quantity of 1x10e11 pfu. Besides, the degree of amino acid homology between rat and human IGF receptor is 85% at the C-terminal and 98% at the tyrosine kinase domain [30]. In addition, heterodimeric hybridization of receptors have been described regarding IGFR and Insulin Receptor (IR) [31,32] which are of the same tyrosine kinase family and have a high degree of sequence similarity [33]. This phenomenon may also explain the binding and internalization of UMAP3 with UM cells.

In summary, at least three UMAPs could be identified using phage peptide libraries and in vitro panning. In addition, synthetic constructed peptides that corresponded to these UMAPs were shown to successfully internalize targeted UM cells. Whether these UMAPs can be used clinically for in vivo imaging (radiolabelled) or targeted therapy remains to be investigated.

REFERENCE LIST

1. Jampol, L. M., Moy, C. S., Murray, T. G., Reynolds, S. M., Albert, D. M., Schachat, A. P., Diddie, K. R., Engstrom, R. E., Jr., Finger, P. T., Hovland, K. R., Joffe, L., Olsen, K. R., and Wells, C. G. *The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma: IV. Local treatment failure and enucleation in the first 5 years after brachytherapy. COMS report no. 19.* Ophthalmology 2002. 109:2197-2206.
2. Shields, C. L., Shields, J. A., Karlsson, U., Menduke, H., and Brady, L. W. *Enucleation after plaque radiotherapy for posterior uveal melanoma. Histopathologic findings.* Ophthalmology 1990. 97:1665-1670.
3. Shields, C. L., Cater, J., Shields, J. A., Chao, A., Krema, H., Materin, M., and Brady, L. W. *Combined plaque radiotherapy and transpupillary thermotherapy for choroidal melanoma: tumor control and treatment complications in 270 consecutive patients.* Arch.Ophthalmol. 2002. 120:933-940.
4. Augsburger, J. J., Correa, Z. M., Freire, J., and Brady, L. W. *Long-term survival in choroidal and ciliary body melanoma after enucleation versus plaque radiation therapy.* Ophthalmology 1998. 105:1670-1678.
5. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
6. Coupland, S. E., Sidiki, S., Clark, B. J., McClaren, K., Kyle, P., and Lee, W. R. *Metastatic choroidal melanoma to the contralateral orbit 40 years after enucleation.* Arch.Ophthalmol. 1996. 114:751-756.
7. Eskelin, S., Pyrhonen, S., Summanen, P., Hahka-Kemppinen, M., and Kivela, T. *Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment.* Ophthalmology 2000. 107:1443-1449.
8. Luyten, G. P., van der Spek, C. W., Brand, I., Sintnicolaas, K., de Waard-Siebinga, I., Jager, M. J., de Jong, P. T., Schrier, P. I., and Luider, T. M. *Expression of MAGE, gp100 and tyrosinase genes in uveal melanoma cell lines.* Melanoma Res. 1998. 8:11-16.
9. Stefansson, K., Wollmann, R., and Jerkovic, M. *S-100 protein in soft-tissue tumors derived from Schwann cells and melanocytes.* Am.J.Pathol. 1982. 106:261-268.
10. Adema, G. J., de Boer, A. J., van 't, H. R., Denijn, M., Ruiter, D. J., Vogel, A. M., and Figdor, C. G. *Melanocyte lineage-specific antigens recognized by monoclonal antibodies NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA.* Am.J.Pathol. 1993. 143:1579-1585.
11. Heegaard, S., Jensen, O. A., and Prause, J. U. *Immunohistochemical diagnosis of malignant melanoma of the conjunctiva and uvea: comparison of the novel antibody against melan-A with S100 protein and HMB-45.* Melanoma Res. 2000. 10:350-354.
12. Pasqualini, R. and Ruoslahti, E. *Organ targeting in vivo using phage display peptide libraries.* Nature 1996. 380:364-366.
13. Lee, S. M., Lee, E. J., Hong, H. Y., Kwon, M. K., Kwon, T. H., Choi, J. Y., Park, R. W., Kwon, T. G., Yoo, E. S., Yoon, G. S., Kim, I. S., Ruoslahti, E., and Lee, B. H. *Targeting bladder tumor cells in vivo and in the urine with a peptide identified by phage display.* Mol.Cancer Res. 2007. 5:11-19.
14. Howell, R. C., Revskaya, E., Pazo, V., Nosanchuk, J. D., Casadevall, A., and Dadachova, E. *Phage display library derived peptides that bind to human tumor melanin as potential vehicles for targeted radionuclide therapy of metastatic melanoma.* Bioconjug.Chem. 2007. 18:1739-1748.
15. Eriksson, F., Culp, W. D., Massey, R., Egevad, L., Garland, D., Persson, M. A., and Pisa, P. *Tumor specific phage particles promote tumor regression in a mouse melanoma model.* Cancer Immunol.Immunother. 2007. 56:677-687.

16. Chen, P. W., Murray, T. G., Salgaller, M. L., and Ksander, B. R. *Expression of MAGE genes in ocular melanoma cell lines.* J.Immunother. 1997. 20:265-275.
17. De Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer 1995. 62:155-161.
18. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
19. Luyten, G. P., Naus, N. C., Mooy, C. M., Hagemeijer, A., Kan-Mitchell, J., van, D. E., Vuzevski, V., de Jong, P. T., and Luider, T. M. *Establishment and characterization of primary and metastatic uveal melanoma cell lines.* Int.J.Cancer 1996. 66:380-387.
20. Verbik, D. J., Murray, T. G., Tran, J. M., and Ksander, B. R. *Melanomas that develop within the eye inhibit lymphocyte proliferation.* Int.J.Cancer 1997. 73:470-478.
21. Higgins, L. M., Lambkin, I., Donnelly, G., Byrne, D., Wilson, C., Dee, J., Smith, M., and O'Mahony, D. J. *In vivo phage display to identify M cell-targeting ligands.* Pharm.Res. 2004. 21:695-705.
22. Menendez, A. and Scott, J. K. *The nature of target-unrelated peptides recovered in the screening of phage-displayed random peptide libraries with antibodies.* Anal.Biochem. 2005. 336:145-157.
23. Deutscher, S. L. *Phage display in molecular imaging and diagnosis of cancer.* Chem.Rev. 2010. 110:3196-3211.
24. Augsburger, J. J., Correa, Z. M., and Shaikh, A. H. *Effectiveness of treatments for metastatic uveal melanoma.* Am.J.Ophthalmol. 2009. 148:119-127.
25. Derda, R., Musah, S., Orner, B. P., Klim, J. R., Li, L., and Kiessling, L. L. *High-throughput discovery of synthetic surfaces that support proliferation of pluripotent cells.* J.Am.Chem.Soc. 2010. 132:1289-1295.
26. Li, J., Wang L., Han H., Du B., Qian M. *Selection and preliminary identification of hepatocarcinoma-binding and internalizing peptide* . Current Immunology. 2007. 01 (chinese journal)
27. Economou, M. A., All-Ericsson, C., Bykov, V., Girnita, L., Bartolazzi, A., Larsson, O., and Seregard, S. *Receptors for the liver synthesized growth factors IGF-1 and HGF/SF in uveal melanoma: intercorrelation and prognostic implications.* Invest Ophthalmol.Vis.Sci. 2005. 46:4372-4375.
28. All-Ericsson, C., Girnita, L., Seregard, S., Bartolazzi, A., Jager, M. J., and Larsson, O. *Insulin-like growth factor-1 receptor in uveal melanoma: a predictor for metastatic disease and a potential therapeutic target.* Invest Ophthalmol.Vis.Sci. 2002. 43:1-8.
29. Baserga, R. *The insulin-like growth factor I receptor: a key to tumor growth?* Cancer Res. 1995. 55:249-252.
30. Pedrini, M. T., Giorgino, F., and Smith, R. J. *cDNA cloning of the rat IGF I receptors: structural analysis of rat and human IGF I and insulin receptors reveals differences in alternative splicing and receptor-specific domain conservation.* Biochem.Biophys.Res.Commun. 1994. 202:1038-1046.
31. Moxham, C. P., Duronio, V., and Jacobs, S. Insulin-like growth factor I receptor beta-subunit heterogeneity. Evidence for hybrid tetramers composed of insulin-like growth factor I and insulin receptor heterodimers. J.Biol.Chem. 1989. 264:13238-13244.
32. Entingh-Pearsall, A. and Kahn, C. R. Differential roles of the insulin and insulin-like growth factor-I (IGF-I) receptors in response to insulin and IGF-I. J.Biol.Chem. 2004. 279:38016-38024.
33. Nakae, J., Kido, Y., and Accili, D. Distinct and overlapping functions of insulin and IGF-I receptors. Endocr.Rev. 2001. 22:818-835.



PART IV

ONCOGENIC PATHWAYS INVOLVED IN UVEAL MELANOMA AND TREATMENT OPTIONS

CHAPTER 9

EPISODIC SRC ACTIVATION IN UVEAL MELANOMA REVEALED BY KINASE ACTIVITY PROFILING

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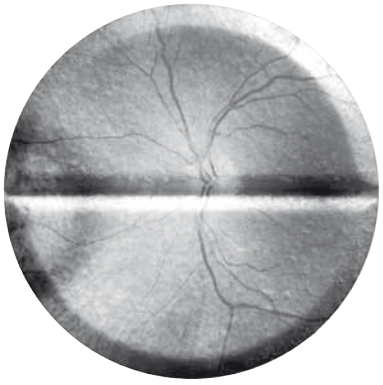

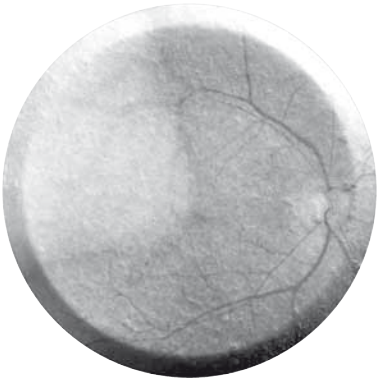

ABSTRACT

The RAS/RAF/MEK/ERK pathway is involved in the balance between melanocyte proliferation and differentiation. The same pathway is constitutively activated in cutaneous and uveal melanoma and related to tumor growth and survival. Whereas mutant BRAF and NRAS are responsible for activation of the RAS/RAF/MEK/ERK pathway in most cutaneous melanoma, mutations in these genes are usually absent in uveal melanoma. We set out to explore this pathway and used mitogen-activated protein kinase profiling and tyrosine kinase arrays and identified Src as a kinase that is associated with ERK1/2 activation in uveal melanoma. However, low Src levels and reduced ERK1/2 activation in metastatasis cell lines suggest that proliferation in metastases can become independent of Src and RAS/RAF/MEK/ERK signaling. Inhibition of Src led to growth reduction of primary uveal melanoma cultures and cell lines and thereby identified Src kinase as a potential target in primary uveal melanoma treatment. Metastasis cell lines displayed a more resistant phenotype and indicate that in metastases a different approach may be required.

INTRODUCTION

Uveal melanoma (UM) is a rare neoplasm, which arises from melanocytes in the eyes. It usually affects people in their sixties with an incidence rate of approximately 6-8 new cases per million per year among Caucasians [1,2]. Little is known about the molecular pathogenesis of UM as compared to cutaneous melanoma (CM). CM and UM share the same embryonic origin and similar histological features, but mutations that regulate proliferation and cause loss of cell cycle control in CM can hardly be found in UM. Whereas p16-regulated cell cycle control is targeted by deletion of chromosome 9p or mutation of CDKN2A in CM, most of UM cell lines posses a wildtype p16-encoding gene that is, however, not expressed due to epigenetic modification of the CDKN2A gene [3]. The same may be true for activation of the RAS-RAF-MEK-ERK, or the classical mitogen-activated protein kinase (MAPK) pathway. MAPK activation is crucial in the development of melanocytic neoplasia and constitutive activation of this pathway has been associated with many different types of cancer [4,5]. In CM, activation of the MAPK pathway has been shown to occur by a variety of mechanisms, including autocrine growth factor stimulation and mutation of the NRAS (20% of cases) and BRAF (60% of cases) genes [6-8]. BRAF mutations have only rarely been reported in UM and activating mutations in NRAS, which are found in 25% of all cancers, have never been reported [9-14]. However, we and others have found that UM are heterogeneous and that, with more sensitive techniques, the percentage of mutant BRAF-positive UM may be higher [15,16]. The lack of mutations in the majority of cells is in contrast with immunohistochemistry and Western blot analysis that have shown activation of ERK1/2 in most UM [13,17,18]. Still, pharmacological inhibition of MAPK/ERK kinases 1 and 2 (MEK1/2) and genetic targeting of BRAF with siRNA resulted in a reduced proliferation of UM cell lines [19,20]. This indicates that although mutations are absent, the RAS-RAF-MEK-ERK pathway is essential for UM growth and suggests that an upstream factor is involved in autonomous UM proliferation. Recently, c-Kit was shown to be upregulated in uveal melanoma and involved in an autocrine loop that also involved the RAS-RAF-MEK-ERK pathway [20]. An incomplete response to c-Kit inhibition indicates that additional factors are involved [21]. Also the GNAQ gene was shown to be mutated in almost half of the uveal melanoma [22]. GNAQ is part of the G-protein heterotrimer and represents the GTP-binding part that couples GPCR signaling to MAPK activation which marks it as a potential therapeutic target. However, targeting downstream signaling molecules may be just as effective as they may be shared with other mutant pathways. Tyrosine kinase activity profiling in uveal melanoma was used to explore the involved kinases. Based on a UM cell line and two related metastasis cell lines which revealed reduced ERK1/2 activation in metastases, we were able to identify Src as a crucial upstream tyrosine kinase for ERK1/2 activation in primary UM. Unfortunately, metastasis cell lines appeared less dependent on Src and may indicate that metastasis may require an alternative approach for intervention.

MATERIAL AND METHODS

Cell lines and tumor material

Eleven cell lines derived from primary UM (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290) and UM metastases (OMM-1, -2.3, and -2.5) were analyzed for kinase activity [23-26]. UM cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 3mM L-glutamine (Gibco), 2% penicillin/streptomycin and 10% FBS (Hyclone, Logan, UT). Primary UM were cultured in Amniochrome Pro Medium (Lonza Group Ltd, Basel, Switzerland). All cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Cell lysates were obtained by lysing cells in M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL), supplemented with 1% Halt Protease Inhibitor Cocktail, EDTA-free (Pierce) and 1% Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were measured by using the BCA Protein Assay kit (Pierce). Cell lysates were also acquired from three fresh primary UM samples obtained by enucleation and from three liver metastases of three different patients, in which the diagnosis was confirmed.

Phospho-MAPK Array

The Human Phospho-MAPK Array (R&D Systems, Abingdon, UK) was used to simultaneously detect the relative levels of nine MAPkinases and nine other serine/threonine kinases in cell lines, primary UM and liver metastasis. In this array, capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Experiments were carried out according to the manufacturer's guidelines. In short, cell lysates were diluted and incubated with the array. After binding of both phosphorylated and unphosphorylated kinases, unbound material was washed away. A cocktail of phospho-site specific biotinylated antibodies was used to detect phosphorylated proteins via Streptavidin-HRP and chemiluminescence. The X-ray films of the blots were scanned and analyzed using the G-boxHR (Syngene, Frederick, MD). Control spots with mouse, goat and rabbit antibodies were used for background correction.

PamGene Tyrosine Kinase Array

Experiments were performed by using a 4-array semi-automated system (PamStation 4, PamGene, `s-Hertogenbosch, The Netherlands) designed for processing PamChip-4 arrays. The PamChip Tyrosine Kinase Array (PamGene) contains 144 phospho-peptides, immobilized on a porous microarray surface via the peptide N terminus, representing tyrosine kinase substrates. Each array was blocked with 0.2% Bovine Serum Albumin, fraction V (Calbiochem Immunochemicals, Merck KGaA, Darmstadt, Germany) by pumping it through the porous microarray for 30 cycles of 30 seconds. Thereafter, each array was washed three times for 8 seconds with 1x ABL Protein Tyrosine Kinase Reaction Buffer solution (New England Biolabs, Ipswich, MA). Next, incubation was performed at 30°C with the reaction mix, containing 5µg cell lysate, 4µl 100x BSA (New England Biolabs, Ipswich, MA),

0.4µl 10mM ATP (Sigma-Aldrich, Zwijndrecht, the Netherlands), 0.5µl 1mg/ml Monoclonal anti-phosphotyrosine FITC conjugate (clone Py20, Exalpha Biologicals, Maynard, MA), adjusted to 40 µl with distilled H_2O. The sample was pulsed back and forth through the porous material for 45 cycles, which is coupled to the base of a well to maximize reaction kinetics and to reduce analysis time. Every 5 pump cycles, a 16-bit TIFF image was taken with a built-in CCD camera.

Blocking experiments were carried out with Src family-selective tyrosine kinase inhibitors, PP1, PP2 (Biomol international, LP, of Plymouth Meeting, PA) and PP3 (the inactive analogue, Calbiochem), at an end concentration of 10µM in line with a large body of literature. Each particular inhibitor was mixed with lysates of cell lines and tissue together with the reaction mix just before incubation on the array.

Acquired data from the PamStation 4 was captured with the supplied software package BioNavigator (Version 0.3.1; PamGene). For the purpose of finding differentially phosphorylated substrates, the data was imported in the LIMMA package and we applied the empirical Bayes method [27]. Background subtracted data was normalized for differences between experiments and substrates and P-values of 0.05 or less were corrected for multiple testing using Benjamini and Hochberg correction. Substrates with a corrected P-value of 0.05 or less were assumed significant.

Western blot analysis

Cell lysates (10 µg) were separated on 12.5% SDS-PAGE gels and proteins were transferred to Hybond-polyvinyldifluoride membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% skim milk in PBS-Tween 0.1% solution and probed at room temperature for 1 hour with antibodies specific to each antigen: Phospho-Src (Tyr527; dilution 1:1000), Phospho-Src Family (Tyr416; dilution 1:1000) and Src (36D10; dilution 1:1000) antibody (all from Cell Signaling Technology, Hertfordshire, UK). An antibody against Actin (Abcam, Cambridge, UK) was used as a loading control. Membranes were subsequently incubated at room temperature with horseradish peroxidase-conjugated IgG anti-mouse or anti-rabbit secondary antibodies for 1 hour. Supersignal West Femto ECL (Pierce) was used to visualize protein bands on the membrane.

siRNA treatment

Sub-confluent cell cultures were grown without antibiotics 24 hours prior to transfection in RPMI 1640 medium. A mixture of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and two different siRNA constructs (40 nM) were incubated in standard medium with reduced serum (1%) all in line with the advice of the manufacturer. The siRNA constructs (Stealth) were predesigned and validated (~70% knock down) by the manufacturer (Invitrogen). After 24 and 48 hours the cells were harvested and RNA and protein lysates were prepared.

WST-1 assay

Cell proliferation in response to PP1 (10 and 50μM) was measured by mitochondrial function using the WST-1 proliferation reagent (Sigma-Aldrich) as previously described [28]. This assay measures tetrazolium reductase activity in the mitochondria which serves as a measure of cell viability. In short, 96-well plates were filled with 1250 uveal melanoma cells per well. One (tumor 1-5) or six days (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3, and -2.5) after treatment, WST-1 reagent was added and absorbance was measured at 450nm on a multiwell spectrophotometer. The median and standard error of 8 wells were taken at each time and dosage point.

QPCR

The cell lines (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3, and -2.5) were analyzed for Src gene expression. Primers for Src and the reference gene, β-actin, were developed with Beacon Designer software (Premier Biosoft, Palo Alto, CA). Primer sequences for Src: 5'-GCTGCGGCTGGAGGTCAAG-3' (forward) and 5'-AGACATCGTGCCAGGCTTCAG-3' (reverse). Primer sequence for β actin; 5'-CGGGACCTGACTGACTACCTC-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse). The PCR reaction settings were 95°C for 5 min, then 40 cycles at 96°C for 15 s and 60°C for 45 s. DNA melting point of the amplicons were acquired by measuring the fluorescence of SYBR Green during a linear temperature transition from 70°C to 97°C at 0.2°C each 10 seconds with accompanying software (Bio-Rad, Hercules, CA).

RESULTS

ERK1/2 activation in uveal melanoma

An antibody array was applied to investigate the mapk pathway in ten um cell lines, three primary UM and three UM metastasis. We observed uniform HSP27 phosphorylation with the exception of three UM cell lines (OCM1, -3, -8). UM displaying activated ERK1/2 as well as phosphorylated HSP27 were most common whereas signals for phosphorylated ERK1/2 were low in metastasis tissue (MET1-3) and metastatic UM cell lines (OMM1, OMM2.3 and OMM2.5) (Fig. 1). Remarkably, two of the metastatic cell lines (OMM2.3, OMM2.5) are derived from the same patient as cell line Mel270 but contained far less activated ERK1/2.

Differential kinase activity in uveal melanoma

Reduction of ERK1/2 activation in metastatic cell lines compared to the primary UM cell lines provides a model to identify the underlying mechanism of ERK1/2 activation in the absence of BRAF and NRAS mutations.

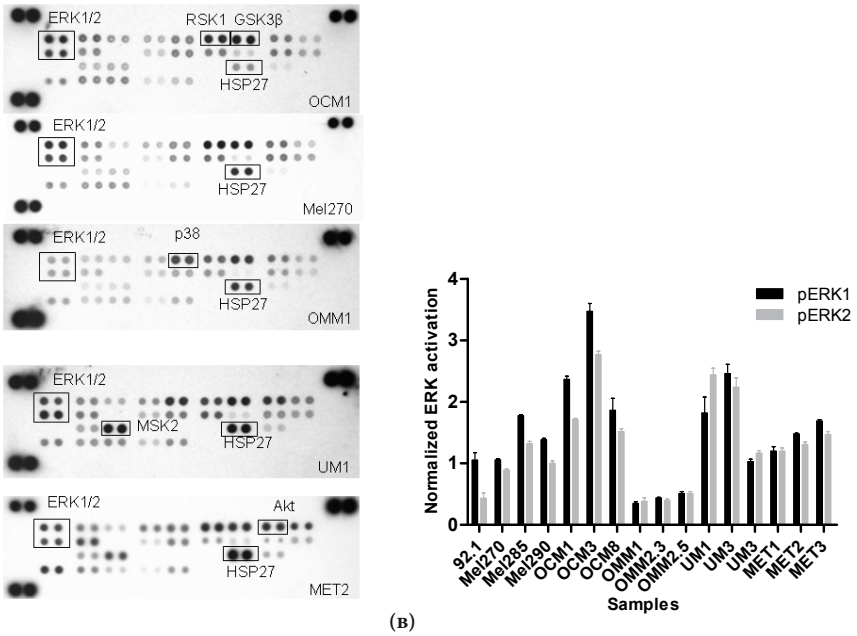

FIGURE 1. MAPK activation in primary UM and UM metastases was studied with a MAPK antibody array. We observed uniform HSP27 phosphorylation in both cell lines and tissue samples except for OCM1, -3 and -8 (A). Activated ERK1/2 was normalized with HSP27 and shown to be low in UM metastases (MET1-3) while metastatic cell lines just passed the background (OMM1, -2.3, -2.5) (B).

To investigate whether a kinase is differentially activated between primary UM cell lines and metastatic UM cell lines we used peptide-based tyrosine kinase arrays [29]. The UM cell lines displayed a high kinase activity while the metastatic UM cell lines displayed a low kinase activity although the same amount of lysate was incubated (Fig. 2A). After normalization, we could analyze the kinase data and identify nine substrates that were significantly differentially phosphorylated between primary and metastatic UM cell lines (Fig. 2B, Tab. 1). Primary UM and metastatic tissue also showed differential phosphorylation of these nine peptides though not as clearly as observed in the cell lines (Fig. 2C).

Candidate kinase: Src

We identified nine peptides derived from eight proteins that were differentially phosphorylated between primary and metastatic cell line lysates. based on a literature search we identified candidate tyrosine kinases for eight out of nine peptides (Table 1) [30-33]. Among the candidates, Src and Src family members were most prominent. In order to validate the candidacy of Src we performed *in vitro* inhibition experiments with the Src-kinase specific inhibitors PP1 and the PP1 analogue, PP2. We added PP1 and PP2 (10μM) to lysates of primary UM tissue and of a primary UM cell line and we measured the inhibitory effect of these Src inhibitors using the kinase array (Fig. 3A). Seven out of nine substrates that identified

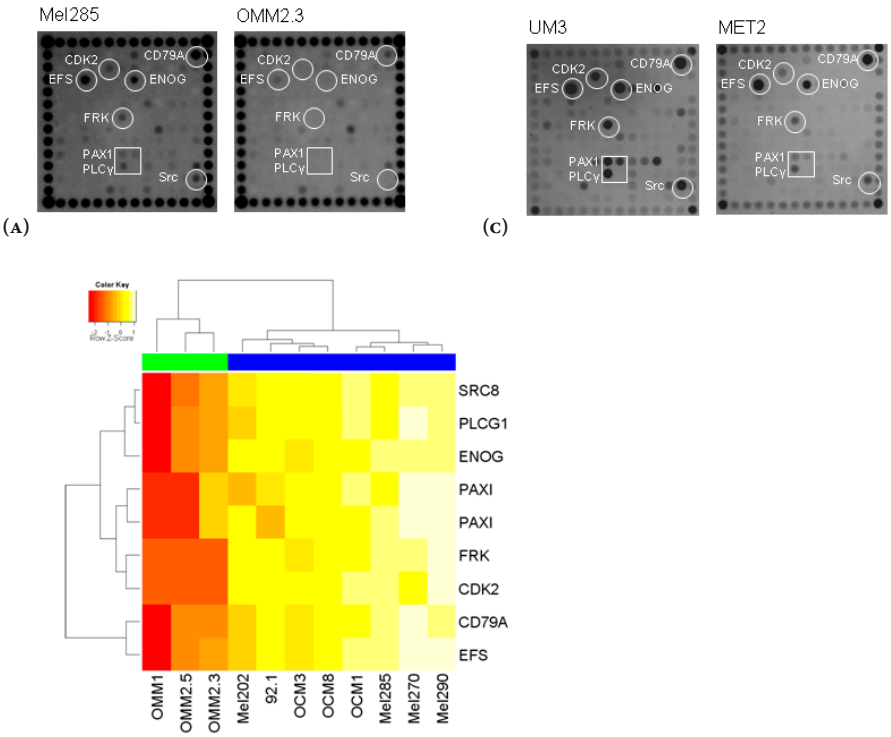

FIGURE 2. Tyrosine kinase activity was measured with an array of peptide substrates.
Two representative examples of a UM cell line and a metastatic cell line (A). Analysis with eBayes identified nine substrates, representing eight proteins, to be significantly (p=0.01) differentially phosphorylated between UM and metastatic cell lines (B). UM (UM1-3) tyrosine kinase activity is high compared to liver metastasis (MET1-3) (two representative arrays are shown) (C).

TABLE 1. Tyrosine kinase substrates on the kinase array that were differentially phosphorylated between primary UM cell lines and metastatic cell lines.

Substrate	UniProt ID	Position	Log Fold Change	Adj. P value	Kinase
CDK2	P24941	T14/Y15	5.3	0.00005	Lyn
FRK	P42685	Y387	6.2	0.0002	unknown
SRC8	Q14247	Y499	4.6	0.006	Src
ENOG	P09104	Y43	4.7	0.01	Src
EFS	O43281	Y253	3.1	0.01	Src
PLCG1	P19174	Y771	4.2	0.01	Syk, Sky, GFRs
CD79A	P11912	Y182/Y188	3.1	0.01	Lyn
PAXI	P49023	Y118	4.0	0.02	FAK, Src, Brk
PAXI	P49023	Y31	4.3	0.03	FAK, Src, Brk

Tyrosine kinase substrate specificities are included.

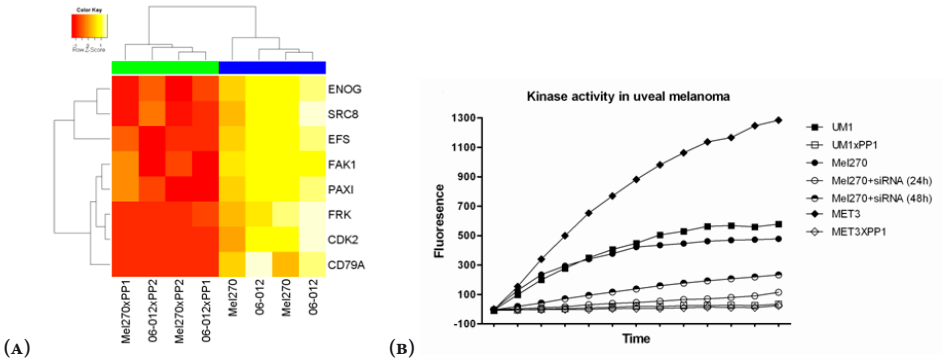

FIGURE 3. UM1 (06-12) and Mel270 treatment with Src inhibitors (PP1/PP2) identified eight substrates with a significant reduction in phosphorylation (A).
Inhibition of EFS peptide phosphorylation by genetic (Src-siRNA) and pharmacological means (PP1) in cell line Mel270 and PP1 treatment of cell lysates of UM (UM1) and metastasis tissue (MET3) (B).

Src in the first screen displayed a significantly reduced phosphorylation when PP1 or PP2 were added to lysates of UM1 and Mel270 (Fig. 3A). The PLCG1 peptide and one of the PAX1 (Y31) substrates did not reach significance but were still phosphorylated at a reduced level after PP1 and PP2 treatment. The peptide representing FAK1 Y576/Y577 is a genuine substrate for Src that was not detected in the UM cell line comparison but phosphorylation was significantly downregulated by PP1 and PP2 treatment. In the control experiment in which we added the inactive analogue of PP1 (PP3) to cell lysates, we did not observe a loss of kinase activity (not shown).

Kinase activity of metastasis tissue and UM tissue differed marginally (Fig. 2C) and incubation with PP1 (10μM) resulted in a decimation of kinase activity similar to the inhibition that we observed in UM tissue (Fig. 3B). In order to validate Src activity, Mel270 was transfected with two siRNA constructs that target Src and reduced kinase activity (Fig. 3B).

Regulation of ERK1/2 and growth

To investigate whether Src contributes to ERK1/2 activation in Mel270 we analyzed the two Src siRNA transfected cell cultures with the MAPK antibody array. Twenty four and 48 hours after transfection with Src siRNA we observed a reduced ERK1 phosphorylation whereas ERK2 phosphorylation was minimally affected (Fig. 4).

Whether Src inhibition and consequently a lowered ERK activation in UM cell lines is associated with a reduced growth was investigated with the WST-1 viability assay (Fig 5). All UM cell lines showed a PP1-dose and time (1 to 6 days) dependent reduction in cell viability but the magnitude of the response differed widely. In general, the metastatic UM cell lines were less affected by PP1. We also determined the growth inhibition rate of PP1 in cultures of five primary UM cell cultures and observed an increased sensitivity to PP1 treatment

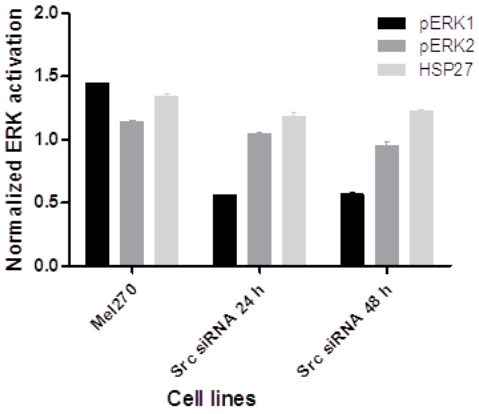

FIGURE 4. ERK1/2 activation in Mel270 24 and 48 hours after transfection with Src siRNA. Phosphorylated HSP27 is included as reference signal.

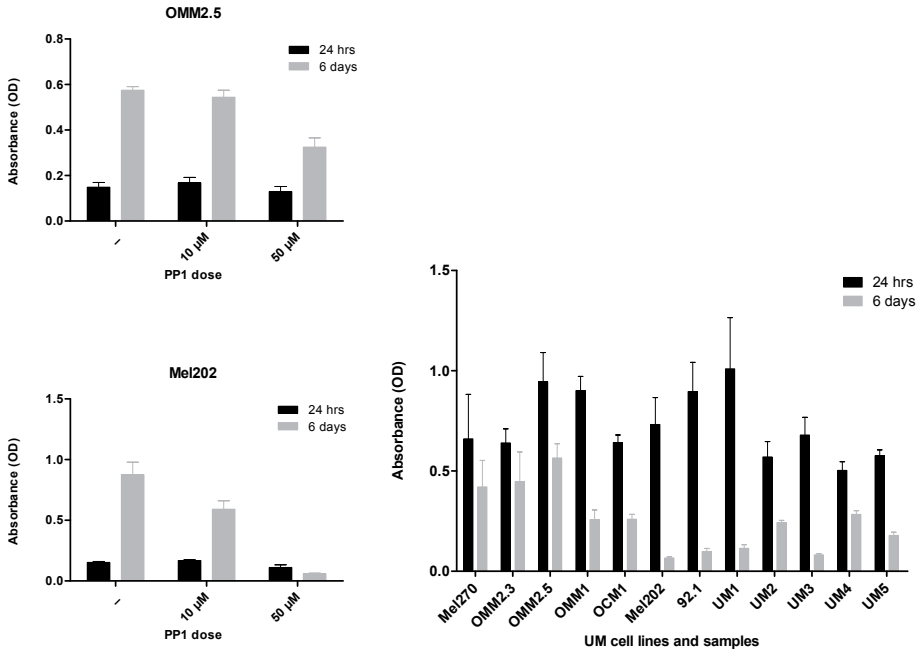

FIGURE. 5. UM cell lines and primary cultures were cultured with PP1 (10μM and 50μM). After 24 hours and 3 days (UM cultures) and 6 days (cell lines) the viability was tested with the WST-1 assay. Two representative cell culture experiments for which all time points and conditions are shown (A). Growth inhibition by PP1 (50 μM) after 24 hours and 3/6 days was normalized to the control culture of each individual cell line (B).

compared to the cell lines. We had to take samples at day 3 of PP1 treatment because hereafter massive cell death occurred (Fig 5B).

Src protein is reduced in metastasis cell line

Src is regulated by phosphorylation of tyrosine residues at position 416 (Y416) and 527 (Y527). Expression of phosphorylated Src Y416 which is associated with an active conformation, was low in the metastatic UM cell lines (Fig. 6A). Surprisingly, phosphorylation of Y527 that is associated with an inactive conformation, was also low and subsequent analysis indicated that Src expression is low in the metastatic UM cell lines. Therefore the difference between kinase activity in metastatic cell lines (OMM1, OMM2.3, OMM2.5) and UM cell lines (OCM1, OCM3, OCM8, Mel202, Mel270, Mel285, Mel290 and 92.1) seems to be the result of a difference in Src expression.

To investigate the origin of lowered Src expression we performed gene expression analysis (Fig. 6B). Src gene expression varied widely in the cell lines and the metastatic cell lines but a correlation between protein and gene expression was not observed in UM cell lines.

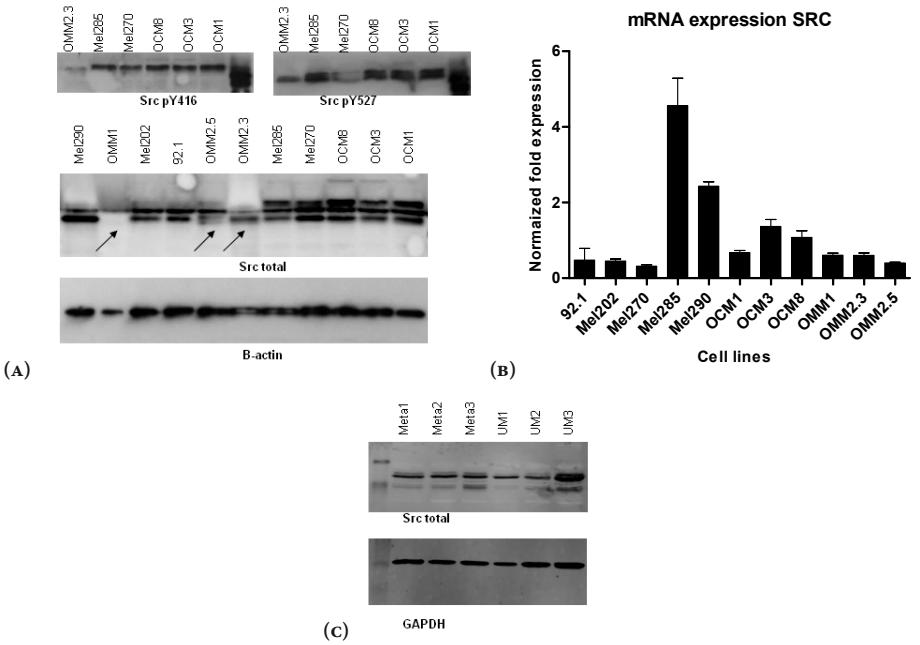

FIGURE. 6. Western analysis of Src in the UM cell lines for activating phosphorylation (Y416) inactivating phosphorylation (Y527) and total Src expression (A).
Src gene expression measured by QPCR varied widely but did not correlate with variation in protein expression (B). UM and metastasis tissue all displayed medium Src kinase expression except for UM3 that presents a high level of expression (C).

Western analysis of Src expression in UM and metastasis tissue revealed in only one out of three primary UM a very high Src expression whereas all three metastasis tissue displayed medium expression of Src protein (Fig. 6C).

DISCUSSION

Constitutive activation of ERK1/2 has often been reported for UM [13,17,18]. Using a more quantitative approach we distinguished a decrease of active ERK1/2 in metastatic cell lines and fresh liver metastasis, suggesting loss of ERK1/2 activation during UM progression. The latter is unexpected since ERK1/2 activation is generally associated with mitogen signaling and is known to determine malignant potential *in vitro*. However, in endometrial and breast cancer, ERK1/2 activation has been associated with a good prognosis [34 35]. A possible explanation is provided by the observation that ERK1/2 is involved in oncogene and stress induced senescence [36,37]. This mechanism is thought to be an important defense for cells that are at risk of neoplastic transformation and needs to be circumvented by tumor cells in order to proliferate. Loss of activated ERK1/2 may relieve the associated inhibitory mechanisms in a direct manner but also requires for alternative mitogenic signals to take over in UM metastasis.

The metastatic and UM cell lines provide a unique model to identify the mechanisms that regulate ERK1/2 activation in uveal melanoma. Previous work already showed that ERK1/2 phosphorylation in UM depends on the MAPK pathway although mutations in the usual suspects (e.g. BRAF and NRAS) are lacking [19]. We investigated the possibility of a tyrosine kinase with differential activities in UM and UM metastasis to be responsible for ERK1/2 activation, using an array of kinase activity assays. Src was revealed as differentially activated tyrosine kinase and this was supported by incubation with Src specific kinase inhibitors PP1 and PP2. Moreover, by treating the cell lysates instead of the cell cultures, we minimized the secondary effects of the inhibitors. However, PP1 and PP2 affect most of the Src-family of tyrosine kinases and the observed reduction of kinase activity therefore does not specifically mark Src. Multi-target inhibitors are a problem in molecular analysis but may be beneficial in the clinical application since in CM a switch from Src to Yes signaling has been reported in brain metastases [38]. In order to specifically inhibit Src we targeted the Src gene expression with a siRNA approach. We detected a reduced kinase activity in Mel270 upon transfection and this was correlated with a reduced ERK1 activation. ERK2 activation appeared unaffected which could be due to the limited efficacy of siRNA treatment or it could indicate the activity of another, yet unidentified, kinase. Low Src protein expression in conjunction with loss of ERK1/2 activation in metastatic UM cell lines however supports the hypothesis that in UM, Src kinase is involved in ERK1/2 activation. Gene expression analysis revealed no significant differences between metastatic and UM cell lines and thereby indicated that

post-transcriptional mechanisms are most likely involved in Src down regulation. Src is both a kinase as well as a client protein for the chaperone HSP90 that is expressed in UM [39]. Whether HSP90 is reduced in metastases and whether treatment with HSP90 inhibitors depends on Src signaling is part of future investigation [40,41]. Inhibition of Src kinase activity resulted in a strong growth reduction in all UM cultures while in UM cell lines the response varied more widely. The genetic background of the cell lines might play a role in the observed variation. However, all UM cell lines displayed Src kinase activity and PP1 sensitivity irrespective of c-kit upregulation (Mel270) or BRAF V600E (OCM1) and GNAQ Q209L (Mel202) mutation status [20,22]. Tissue of UM and UM liver metastasis displayed more or less comparable Src levels. Incubation of the lysates with Src inhibitors resulted in comparable reduction of kinase activity in UM and metastasis tissue. The possibility that there exist Src negative clones in liver metastasis can however not be ruled out on the basis of these data. Clinical trials targeting Src kinase activity in uveal melanoma should therefore anticipate on this potential risk.

In conclusion: we have identified differential ERK1/2 activation in UM and metastatic UM cell lines. Using tyrosine kinase activity profiling we identified Src as a determinant of ERK1/2 activation and showed that Src expression and kinase activity together with ERK1/2 activation is reduced in UM metastases cell lines.

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REFERENCE LIST

1. Egan, K. M., Seddon, J. M., Glynn, R. J., Gragoudas, E. S., and Albert, D. M. *Epidemiologic aspects of uveal melanoma.* Surv Ophthalmol 1988. 32:239-51.
2. Singh, A. D. and Topham, A. *Incidence of uveal melanoma in the United States: 1973-1997.* Ophthalmology 2003. 110:956-961.
3. van der Velden, P. A., Metzelaar-Blok, J. A., Bergman, W., Monique, H., Hurks, H., Frants, R. R., Gruis, N. A., and Jager, M. J. *Promoter Hypermethylation: A Common Cause of Reduced p16(INK4a) Expression in Uveal Melanoma.* Cancer Res 2001. 61:5303-5306.
4. Goding, C. R. *Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage.* Genes Dev. 2000. 14:1712-1728.
5. Reddy, K. B., Nabha, S. M., and Atanaskova, N. *Role of MAP kinase in tumor progression and invasion.* Cancer Metastasis Rev. 2003. 22:395-403.
6. Satyamoorthy, K., Li, G., Gerrero, M. R., Brose, M. S., Volpe, P., Weber, B. L., Van Belle, P., Elder, D. E., and Herlyn, M. *Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation.* Cancer Res. 2003. 63:756-759.
7. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. *Mutations of the BRAF gene in human cancer.* Nature 2002. 417:949-954.
8. van Elsas, A., Zerp, S., van der, F. S., Kruse-Wolters, M., Vacca, A., Ruiter, D. J., and Schrier, P. *Analysis of N-ras mutations in human cutaneous melanoma: tumor heterogeneity detected by polymerase chain reaction/single-stranded conformation polymorphism analysis.* Recent Results Cancer Res. 1995. 139:57-67.:57-67.
9. Edmunds, S. C., Cree, I. A., Di Nicolantonio, F., Hungerford, J. L., Hurren, J. S., and Kelsell, D. P. *Absence of BRAF gene mutations in uveal melanomas in contrast to cutaneous melanomas.* Br.J.Cancer. 2003. 88:1403-1405.
10. Cohen, Y., Goldenberg-Cohen, N., Parrella, P., Chowers, I., Merbs, S. L., Pe'er, J., and Sidransky, D. *Lack of BRAF mutation in primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:2876-2878.
11. Cruz, F., III, Rubin, B. P., Wilson, D., Town, A., Schroeder, A., Haley, A., Bainbridge, T., Heinrich, M. C., and Corless, C. L. *Absence of BRAF and NRAS mutations in uveal melanoma.* Cancer Res. 2003. 63:5761-5766.
12. Mooy, C. M., Van der Helm, M. J., Van der Kwast, T. H., de Jong, P. T., Ruiter, D. J., and Zwarthoff, E. C. *No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited.* Br.J.Cancer 1991. 64:411-413.
13. Rimoldi, D., Salvi, S., Lienard, D., Lejeune, F. J., Speiser, D., Zografos, L., and Cerottini, J. C. *Lack of BRAF mutations in uveal melanoma.* Cancer Res. 2003. 63:5712-5715.
14. Soparker, C. N., O'Brien, J. M., and Albert, D. M. *Investigation of the role of the ras protooncogene point mutation in human uveal melanomas.* Invest Ophthalmol.Vis.Sci. 1993. 34:2203-2209.
15. Janssen, C. S., Sibbett, R., Henriquez, F. L., McKay, I. C., Kemp, E. G., and Roberts, F. *The T1799A point mutation is present in posterior uveal melanoma.* Br.J.Cancer. 2008. 99:1673-1677.

16. Maat, W., Kilic, E., Luyten, G. P., de Klein, A., Jager, M. J., Gruis, N. A., and van der Velden, P. A. *Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2008. 49:23-27.
17. Weber, A., Hengge, U. R., Urbanik, D., Markwart, A., Mirmohammadsaegh, A., Reichel, M. B., Wittekind, C., Wiedemann, P., and Tannapfel, A. *Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea.* Lab Invest 2003. 83:1771-1776.
18. Zuidervaart, W., van Nieuwpoort, F., Stark, M., Dijkman, R., Packer, L., Borgstein, A. M., Pavey, S., van, d., V, Out, C., Jager, M. J., Hayward, N. K., and Gruis, N. A. *Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS.* Br.J.Cancer 2005. 92:2032-2038.
19. Calipel, A., Mouriaux, F., Glotin, A. L., Malecaze, F., Faussat, A. M., and Mascarelli, F. *Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells.* J.Biol.Chem. 2006. 281:9238-9250.
20. Lefevre, G., Glotin, A. L., Calipel, A., Mouriaux, F., Tran, T., Kherrouche, Z., Maurage, C. A., Auclair, C., and Mascarelli, F. *Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis.* J.Biol.Chem. 2004. 279:31769-31779.
21. Hofmann, U. B., Kauczok-Vetter, C. S., Houben, R., and Becker, J. C. *Overexpression of the KIT/SCF in Uveal Melanoma Does Not Translate into Clinical Efficacy of Imatinib Mesylate.* Clin.Cancer Res. 2009. 15:324-329.
22. Van Raamsdonk, C. D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J. M., Simpson, E. M., Barsh, G. S., and Bastian, B. C. *Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi.* Nature. 2008.
23. Chen, P. W., Murray, T. G., Salgaller, M. L., and Ksander, B. R. *Expression of MAGE genes in ocular melanoma cell lines.* J.Immunother. 1997. 20:265-275.
24. Waard-Siebinga, I., Blom, D. J., Griffioen, M., Schrier, P. I., Hoogendoorn, E., Beverstock, G., Danen, E. H., and Jager, M. J. *Establishment and characterization of an uveal-melanoma cell line.* Int.J.Cancer. 1995. 62:155-161.
25. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. *Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes.* Invest Ophthalmol.Vis.Sci. 1989. 30:829-834.
26. Luyten, G. P., Naus, N. C., Mooy, C. M., Hagemeijer, A., Kan-Mitchell, J., Van Drunen, E., Vuzevski, V., de Jong, P. T., and Luider, T. M. *Establishment and characterization of primary and metastatic uveal melanoma cell lines.* Int.J.Cancer. 1996. 66:380-387.
27. Smyth, G. K. *Linear models and empirical bayes methods for assessing differential expression in microarray experiments.* Stat.Appl.Genet.Mol.Biol. 2004. 3:Article3. Epub;%2004 Feb 12.:Article3.
28. Narayanan, R., Kenney, M. C., Kamjoo, S., Trinh, T. H., Seigel, G. M., Resende, G. P., and Kuppermann, B. D. *Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells.* Invest Ophthalmol.Vis.Sci. 2005. 46:304-309.
29. Lemeer, S., Jopling, C., Naji, F., Ruijtenbeek, R., Slijper, M., Heck, A. J., and den Hertog, J. *Protein-tyrosine kinase activity profiling in knock down zebrafish embryos.* PLoS.ONE. 2007. 2:e581.
30. Thomas, S. M. and Brugge, J. S. *Cellular functions regulated by Src family kinases.* Annu.Rev.Cell Dev. Biol. 1997. 13:513-609.
31. Cooper, J. A., Reiss, N. A., Schwartz, R. J., and Hunter, T. *Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus.* Nature. 1983. 302:218-223.

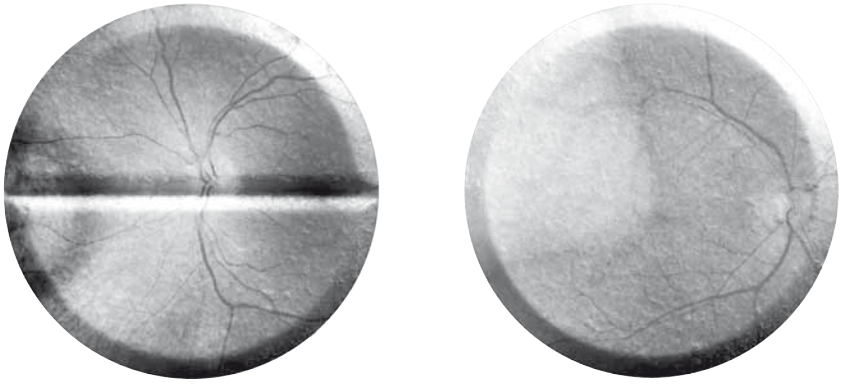
32. Diella, F., Cameron, S., Gemund, C., Linding, R., Via, A., Kuster, B., Sicheritz-Ponten, T., Blom, N., and Gibson, T. J. *Phospho.ELM: a database of experimentally verified phosphorylation sites in eukaryotic proteins.* BMC.Bioinformatics. 2004. 5:79.:79.
33. Koike, A., Kobayashi, Y., and Takagi, T. *Kinase pathway database: an integrated protein-kinase and NLP-based protein-interaction resource.* Genome Res. 2003. 13:1231-1243.
34. Milde-Langosch, K., Bamberger, A. M., Rieck, G., Grund, D., Hemminger, G., Muller, V., and Loning, T. *Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer.* Br.J.Cancer. 2005. %20;92:2206-2215.
35. Mizumoto, Y., Kyo, S., Mori, N., Sakaguchi, J., Ohno, S., Maida, Y., Hashimoto, M., Takakura, M., and Inoue, M. *Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis.* Cancer Sci. 2007. 98:652-658.
36. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. *The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2.* Embo J 1998. 17:5001-14.
37. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.* Cell 1997. 88:593-602.
38. Summy, J. M. and Gallick, G. E. *Src family kinases in tumor progression and metastasis.* Cancer Metastasis Rev. 2003. 22:337-358.
39. Missotten, G. S., Journee-de Korver, J. G., Wolff-Rouendaal, D., Keunen, J. E., Schlingemann, R. O., and Jager, M. J. *Heat shock protein expression in the eye and in uveal melanoma.* Invest Ophthalmol. Vis.Sci. 2003. 44:3059-3065.
40. Faingold, D., Marshall, J. C., Antecka, E., Di Cesare, S., Odashiro, A. N., Bakalian, S., Fernandes, B. F., and Burnier, M. N., Jr. *Immune expression and inhibition of heat shock protein 90 in uveal melanoma.* Clin.Cancer Res. 2008. 14:847-855.
41. Babchia, N., Calipel, A., Mouriaux, F., Faussat, A. M., and Mascarelli, F. *HSP90 inhibitors 17-AAG and 17-DMAG target wild-type B-Raf signaling for the proliferation of human uveal melanoma cell lines.* Invest Ophthalmol.Vis.Sci. 2008. ..

CHAPTER 10

SRC KINASE IN UVEAL MELANOMA IS ASSOCIATED WITH MONOSOMY 3 AND CAN BE INHIBITED BY DASATINIB

M. el Filali, M. Versluis, I. Bronkhorst, A. Baghat, G.P.M. Luyten, M. J. Jager, P.A. van der Velden

Article submitted



ABSTRACT

Purpose: Uveal melanoma (UM) leads to metastasis in up to 50% of the patients. Patients at risk are readily identified using an array of prognostic markers but an effective treatment is lacking. We recently identified Src as an important tyrosine kinase that conveys MAPK activation in UM. GNAQ/GNA11 mutations are the common denominators of oncogene signalling in UM and Src is a likely downstream kinase. We describe the preclinical analysis of Dasatinib, a known inhibitor of Src kinase, in UM.

Experimental design: Eight tumours were removed, cultured and exposed to Dasatinib treatment. Proliferation and MAPK signalling were evaluated. Additionally, 36 UM samples were analyzed for Src/ERK signalling, GNAQ/GNA11 mutation status, chromosome 3 and known histological prognostic parameters.

Results: Growth arrest was observed in 5 of 8 UM cultures and molecular analysis indicated that Dasatinib inhibited MAPK via Src. Treatment efficacy associated with MAPK and Src kinase activity as UM cells with the highest Src activity and MAPK activation displayed the strongest growth inhibition. Furthermore, treatment responses tended to be better in UM cultures with monosomy 3. In the cohort of primary UM, Src expression was highly correlated with MAPK activation and monosomy 3.

Conclusions: We identified the Src family kinase inhibitor, Dasatinib, as a treatment option for UM. Dasatinib inhibits UM proliferation and may also inhibit UM progression as an association between Src and monosomy 3 was revealed. Consequently, monosomy 3 analysis in tumour tissue may suffice both the prognosis and choice of treatment.

GENERAL INTRODUCTION

Uveal melanoma (UM) is a rare tumour affecting 7/1.0 million of the Western population per year [1]. Still, it is the most common tumour in the eye in adults; 30 % of the tumours is asymptomatic and only discovered at routine examination [2]. This malignancy may ultimately lead to metastatic disease in up to 50% of the patients [3].

UM commonly spreads hematogeneously because there are hardly any lymph vessels present in the eye, and most often metastasis of UM are found in the liver. Once liver metastases have been detected, life expectancy is a mere 2-6 months on average since hardly any effective treatment is available for these patients [4]. This highlights the necessity for effective adjuvant and clinical treatment options which can be applied in high risk and metastasis patients.

Identifying high risk patients is possible with an array of diagnostic and prognostic markers. Well-known prognostic factors for UM are an advanced age, tumour thickness/diameter, and localization in the ciliary body [5]. Besides these histopathological parameters, there are also genetic aberrations that are associated with a poor prognosis. Most significantly, loss of one copy of chromosome 3 is observed in almost half of the tumours and is associated with a poor prognosis [6]. The risk associated with monosomy 3 is further modulated by other chromosomal aberrations such as deletion of chromosome 1p and gain of chromosomes 6p and 8q [7]. How these aberrations, besides being a prognostic factor, contribute to the metastasis of UM is still unknown but the recent detection of truncating mutations in the BAP1 gene may elucidate a possible mechanism [8].

Recently, two recurrent mutations were identified in the GNAQ and the GNA11 genes. For both the GNAQ and the GNA11 gene, a mutation in glutamine-encoding codon 209 has been described (Q209), as well as a mutation in R183 (less frequent). Mutations in the GNAQ and GNA11 gene were shown to be common in UM with frequencies of respectively 45% and 32% [9,10]. GNAQ and GNA11 encode for Gαq-type subunits of the heterotrimeric G-protein and the mutations result in constitutive G-protein activation which mediates intracellular signals and activates the Mitogen-activated protein kinase (MAPK) pathway, promoting proliferation and cell survival [11]. Cellular components that couple GNAQ/GNA11 to MAPK signalling are most likely the effector IP3 and Src tyrosine kinase [12].

Recently, we showed that in UM, Src expression and Src kinase activity is correlated with MAPK activation [13]. Whether Src is the intermediate kinase between oncogenic GNAQ/GNA11 signalling and MAPK activation in UM remains to be shown, but it potentially provides an opportunity for pharmaceutical intervention. Treatment of UM cell lines with specific MAPK and Src inhibitors has already been shown to reduce proliferation [13], demonstrating the importance of the MAPK pathway and Src kinase for UM growth.

Dasatinib is an inhibitor of the Src-family kinases as well as other kinases such as BCR-ABL, c-Kit, PDGFR-alpha and beta, and ephrin-receptor kinase [14,15]. It has been approved for the treatment of patients with BCR-ABL-positive chronic myeloid leukaemia (CML),

Philadelphia Chromosome positive (Ph+) acute lymphoblastic leukaemia (ALL) and results in remission and improved survival in these malignancies. For several other diseases including solid tumours, Dasatinib is still under investigation [16]. The single daily oral dose of 100 mg, and manageable side effects, make Dasatinib an interesting candidate for treatment of patients with metastatic disease and/or patients with high risk UM.

We investigated in vitro the efficacy of Dasatinib and analyzed the underlying molecular mechanisms in order to identify potential biomarkers.

MATERIALS AND METHODS

Patient tumour material and primary 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 tumor samples. Fresh tumor tissue (n=36), obtained immediately after enucleation of the eye, was frozen in liquid nitrogen-cooled isopentane and used to isolate DNA, protein lysates or to develop a cell culture (n=8). Histologic sections were prepared from tissues fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin. Hematoxylin-eosin-stained 4-µm sections were examined for cell type, largest basal diameter, prominence, ciliary body involvement and scleral invasion.

Primary tumour cell cultures

Primary UM cell cultures (n=8) were cultured in Amniochrome® Pro Medium (Lonza Group Ltd, Basel, Switzerland) and incubated in an atmosphere with 5% CO_2 at a temperature of 370C. (HERAcell 240 CO_2 Incubator, Thermo Fisher Scientific Inc, USA). The cells were passaged once or twice a week using trypsin (0.05%).

PCR

GNAQ/GNA11 genes were amplified with a Polymerase Chain Reaction (PCR). A PCR was conducted using chromosomal DNA, isolated from frozen tumours using the QIAamp DNA minikit from Qiagen (Germany). The following protocol was used for amplification of exon 5 of the GNA11 and the GNAQ genes:

94°C, 1min; (96°C, 15sec; 63°C, 15sec; 72°C, 1min) 7X; (96°C, 15sec; 61°C, 15sec; 71°C, 1min) 8X; (96°C, 15sec; 60°C, 15sec; 72°C, 1min), 36X; 72°C, 1min, end.

The following primers were used CGCTGTGTCCTTTCAGGATGGTG, GNA11 Forward and GCCCACCTCGTTGTCCGACT, GNA11 Reverse.

The forward primer for GNAQ: CCCTAAGTTTGTAAGTAGTGCTATATTTATGTTG,

while the reverse primer was ATGATAATCCATTGCCTGTCTAAAGAACAC. After amplification, DNA clean-up was performed using the Nucleospin Extract II columns of Macherey-Nagel (Germany) following the manufacturers protocol. For sequencing analysis, samples were prepared by adding 10 pmol of reverse primer to the amplified DNA.

Sequencing and Karyotyping

Sequencing for GNAQ c.626A>C (~30%) and GNAQ c.626A>T (~70%) and GNA11 c.626A>T (100%) mutations was performed at the Leiden Genomic Technology Center (LGTC) department of the LUMC. Screening for monosomy 3 with karyotyping in all uveal melanoma samples and primary cultures was performed at the Department of Clinical Genetics of the LUMC.

Dasatinib treatment

Several Dasatinib (Toronto Research Chemicals Cat# D193600) concentrations were used to investigate treatment options in vitro. Dasatinib (stock solution concentration of 20 mM in DMSO) was dissolved in Amniochrome® Pro Medium (Lonza Group Ltd, Basel, Switzerland) to obtain concentrations ranging from 2 nM to 2 μM and used in experiments as described. As control, we used standard Amniochrome® Pro Medium.

Cell proliferation

The effect of Dasatinib on UM cell viability was measured by mitochondrial function using the water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Indianapolis, IN, USA). In short, 96-well plates were filled with 1200 uveal melanoma cells per well, with either regular medium (control) or Dasatinib solutions (200nM to 2μM), and placed in an incubation chamber (20% O_2, 5% CO_2, 37°C). After the indicated incubation time (1, 2, or 3 days) 10ul of WST-1 reagent was added to each well and absorbance was measured at 450nm (n=8) on a multiwell spectrophotometer (Perkin Elmer, Wellesley, MA).

PamGene tyrosine-kinase array

To ascertain tyrosine-kinase activity in lysates of UM tissue and primary cultures, the PamChip-4 of PamGene B.V. ('s Hertogenbosch, The Netherlands) was used, as described previously [13]. In short, this array consists of 144 peptides spotted on a porous carrier. The 144 peptides are known tyrosine-kinase substrates and phosphorylation of these peptides by cell lysates can be monitored because of a generic antibody that recognizes phosphorylated tyrosine. Each chip consists of four arrays, so four lysates can be measured at the same time. In this experiment, we incubated a control lysate with and without addition of Dasatinib at an end concentration of 200nM on an array following the manufacturer's protocol.

Western blot analysis

Cell lysates were obtained by lysing cells of UM cryosamples and primary UM cultures (with and without Dasatinib treatment) in M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL, USA), supplemented with 1% Halt Protease Inhibitor Cocktail, EDTA-free (Pierce) and 1% Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were measured by using the BCA Protein Assay kit (Pierce).

Cell lysates, containing the same amount of protein, were mixed with equal volumes of 6× sample loading buffer, boiled for 10 min, cooled on ice, and then analyzed by 10% SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a PVDF membrane (Millipore). Subsequently, the membrane was blocked with Li-Cor blocking buffer for 1 h at room temperature and incubated with primary antibodies (rabbit anti-Src, rabbit anti-Erk1/2, mouse anti-pErk1/2, rabbit anti-GAPDH (loading control) (Cell Signalling, Boston, USA) followed by an infrared labeled secondary antibody (goat anti-rabbit800 and goat anti-mouse680 (LI-COR, Lincoln, USA). Labeled PVDF membranes were scanned with the Li-Cor scanner and quantification of proteins was performed using the Li-Cor software.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc. Chicago, Illinois, USA). One-way ANOVA was used to analyze significant difference in cell proliferation of treated and untreated cells. The significance of differences in Src and ERK1/2 expression between samples with different histologic and molecular parameters was determined using unpaired T-tests. Additionally, all parameters were analyzed for survival and prediction of metastatic disease using the Kaplan-Meier analysis, log rank testing, cox regression and multivariate analysis. P-values for categorical parameters (associations) were obtained by Independent-Samples T Test (t-test for Equality of Means) and for the numerical data by Pearson correlation (2-tailed). $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of UM growth by Dasatinib

The application of the experimental Src inhibitors PP1 and PP2 has already shown that Src inhibition substantially reduces cell proliferation in UM cell lines [13]. The aim of the study was to analyze the efficacy of the commercially-available Src kinase-inhibitor Dasatinib in primary UM cell cultures. UM cell viability was significantly reduced by Dasatinib in a dose-dependent manner in five out of eight UM cell cultures (Fig. 1). Three cultures appeared Dasatinib resistant and displayed no growth arrest.

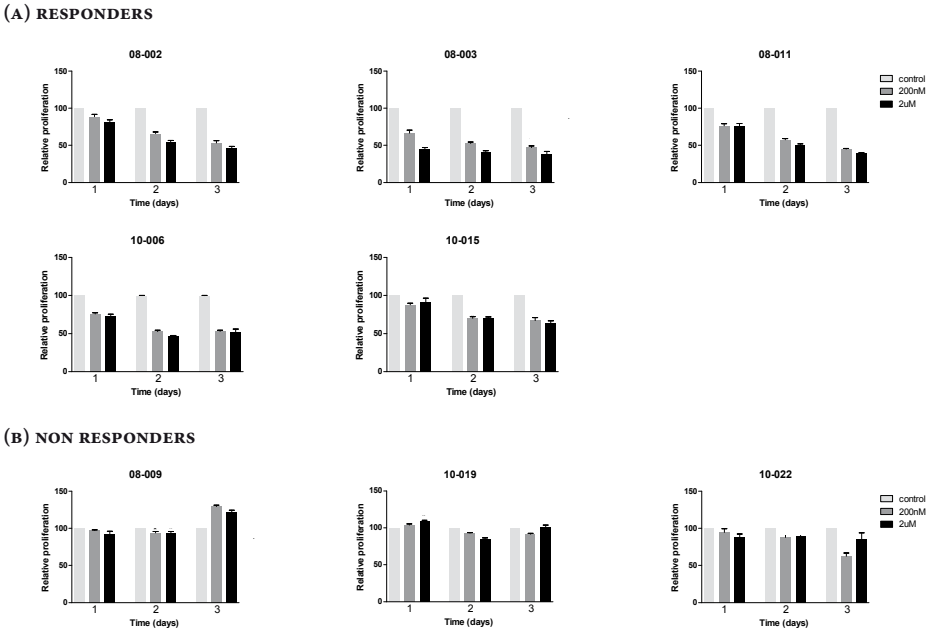


FIGURE 1. Cell viability of primary UM cell cultures treated with Dasatinib.
Cell viability was measured at 3 consecutive days. Each time point embodied a control culture and two Dasatinib treatments (200 nM and 2 μM). The untreated control culture is set at 100% and in five out of eight cultures viability was reduced upon Dasatinib treatment.

MAPK involvement

In order to investigate the molecular mechanisms by which Dasatinib induces growth arrest, we compared MAPK activation (pERK) and Src kinase activity in responders and non-responders. Primary cell cultures were exposed to two concentrations of Dasatinib. Basal expression of both Src and ERK varied widely in UM tissue cultures but revealed no correlation with treatment efficacy. Neither Src nor ERK expression was reduced after Dasatinib treatment (Fig. 2a). However, Dasatinib treatment significantly reduced activated ERK (pERK) in UM cell cultures (Fig. 2a and b). The degree of pERK down-regulation varied widely and tended to be highest in the responders as compared to the non-responders. Furthermore, the degree of ERK activation, prior to treatment, also seemed to be higher in the responding cultures (not shown).

Src and pERK expression in primary UM samples

In order to validate the correlation between Src and MAPK activation, Src and pERK expression in 36 UM was analyzed (Table 1). Expression levels of Src protein and ERK/pERK were quantified with Western analysis. All samples expressed Src (range of 1.17-55.83 integrated

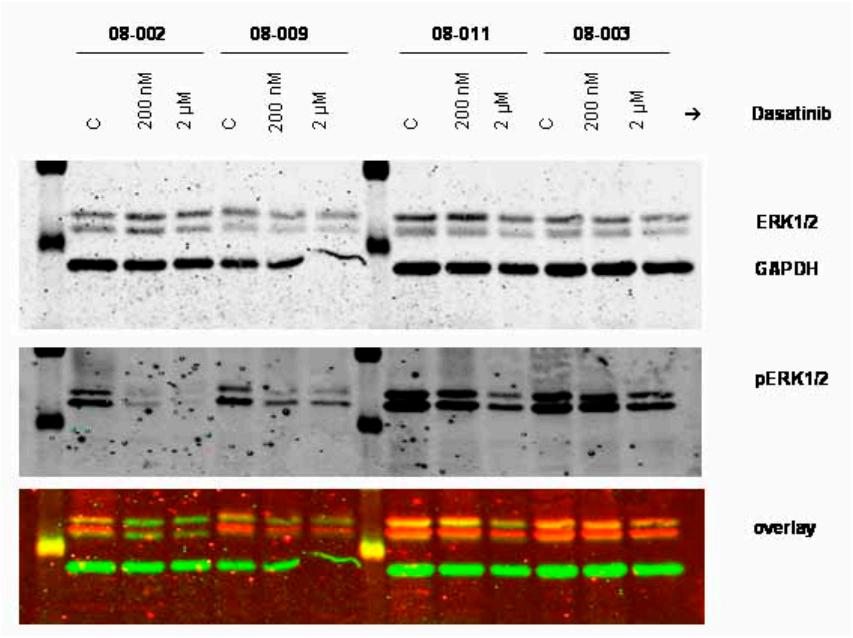


FIGURE 2A. ERK/pERK analysis in UM cell cultures treated with Dasatinib.
ERK/pERK analysis of 4 representative UM cell cultures treated for 48 hours with two concentrations of Dasatinib. Simultaneous hybridization with total ERK (top) and pERK (middle) specific antibodies allows quantification of pERK relative to ERK presence (bottom). Red in the overlay represents high pERK while yellow represents reduced pERK and absence of pERK is represented by a green signal.

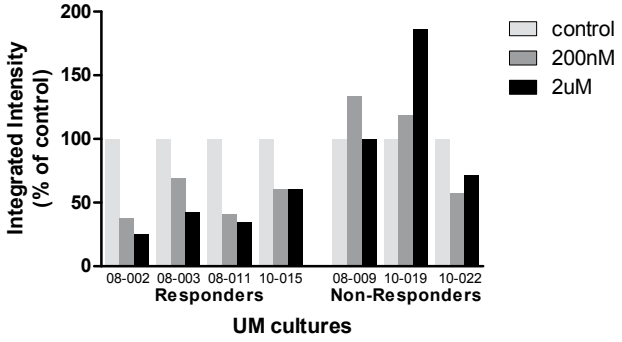


FIGURE 2B. ERK/pERK quantification in UM cell cultures treated with Dasatinib.
Activated ERK (pERK) in UM cell cultures after Dasatinib treatment in the responders and the non-responders. Relative ERK activation (pERK/ERK) is normalized to untreated control cultures.

TABLE 1. Distribution of Src expression and clinical and pathological features of 36 cases of uveal melanoma. Src expression was determined using Western blot analysis.

	Total		Associations		
Clinicopathologic factors	**N**	**%**	**Src expression**	**Standard Deviation**	**P- value**
Gender					.10
Male	18		19,64	10,20	
Female	18		30,97	14,46	
Eye					.98
Right	24		25,26	13,59	
Left	12		25,39	14,24	
Histopathologic cell type					.33
Spindle	8		21,14	7,99	
Mixed/ Epithelioid	28		26,49	14,72	
CB involvement					**.03**
Not present	21		21,17	10,74	
Present	15		31,10	15,38	
Scleral invasion					
None	8		25,92	15,85	
Intrascleral	24		25,73	12,74	
Extrascleral	4		21,49	17,55	
Chromosome 3 status					**.001**
Disomy 3	11		14,21	9,64	
Monosomy 3	25		30,18	12,25	
Metastasis					.10
No	23		22,49	12,31	
Yes	13		30,28	14,84	
Death					.20
Yes, due to other cause	3		29,37	17,72	
Yes, due to metastasis	11		29,32	16,02	
No	22		22,74	11,77	
GNA11					.99
No	17		25,32	17,00	
Yes	19		25,29	10,16	
GNAQ					.89
No	17		24,48	10,46	
Yes	16		23,82	16,34	
GNA11/GNAQ status					.75
GNA11	19		25,29	10,16	
GNAQ	16		23,82	16,34	
			Correlations		
			Coefficient		P-value

Age at diagnosis (year)	36	0.41	.01
Tumor diameter (mm)	36	0.19	.26
Tumor thickness (mm)	36	-0.04	.82
Erk expression (SD)	**36**	**0.40**	**0.02**
pErk expression (SD)	**36**	**0.71**	**<.001**

Data indicate the mean ± SD for the categorial parameters and correlation coefficients for the numerical variables.

P-values for categorical parameters (associations) were obtained by Independent-Samples T Test (t-test for Equality of Means) and for the numerical data by Spearman correlation (2-tailed).

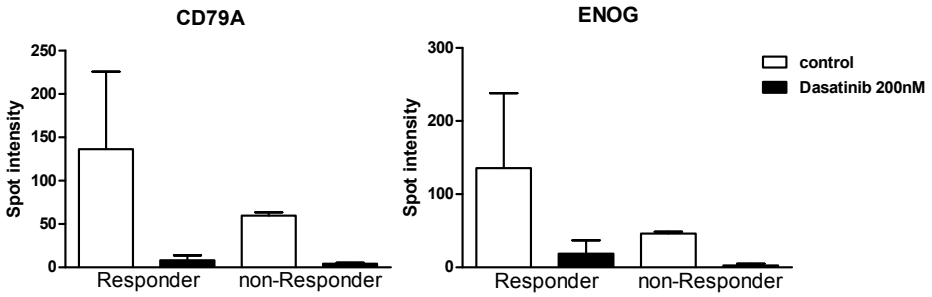


FIGURE 3A. Src kinase activity in UM cultures.

Phosphorylation of ENOG and CD79A peptides on the kinase array represent Src kinase activity in UM cell lysates and this activity is lost upon incubation with Dasatinib. Kinase activity of treated and untreated lysates are plotted for Dasatinib responders (n=3) and non-responders (n=3).

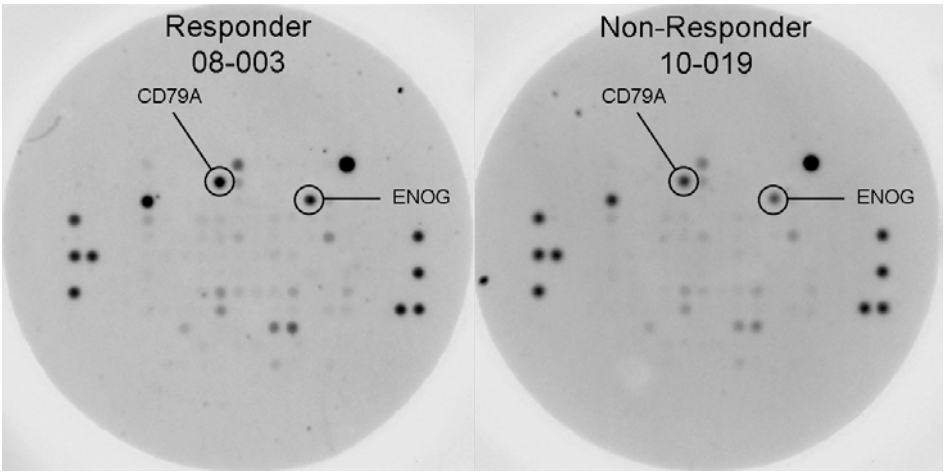


FIGURE 3B. Src kinase activity of Dasatinib responder and non-responder.

Representative kinase arrays of UM that were used for in vitro analysis. The cells of the tumor on the left responded with growth arrest whereas the cell culture of the tumor on the right did not respond to Dasatinib treatment.

intensity), ERK (range of 0.1-22.0 integrated intensity) and pERK (range of 0.84-13.29 integrated intensity) at variable levels (Fig 4a). Src expression was not correlated with ERK expression but the level of pERK was highly correlated with the level of Src ($p<.001$) and Src level predicts pERK to large degree (correlation coefficient = 0.71) (Table 1). Though ERK expression was also found to be correlated with pERK ($p= 0.017$) only part of the activation is predicted by ERK expression (correlation coefficient = 0.40).

Tyrosine-kinase activity

With kinase activity arrays Src activity in response to Dasatinib treatment was analyzed. Lysates of Dasatinib-treated UM cells all displayed reduced Src kinase activity, still a correlation with treatment efficacy was observed. UM cultures that responded to Dasatinib treatment with growth arrest presented a higher basal Src activity compared to the non-responding tumor cultures and hence a stronger reduction of kinase activity was observed (Fig 3a). Peptides on the kinase array, such as ENOG and CD79A, that are known substrates for Src kinase were highly phosphorylated by lysates of the Dasatinib sensitive UM cultures (Fig 3b).

Molecular mechanisms

Known molecular mechanisms in UM were analysed and compared to treatment efficacy. GNAQ and GNA11 mutations of Q209 represent early events in UM development that could be related to Src activity in UM. However, mutations in GNAQ/GNA11 are very common and hence only 2 wiltype tumor cultures were analyzed. The GNAQ/GNA11 wildtype UM were found to be present in both the responder and non-responder group. In addition, the GNAQ and GNA11 status of primary UM was assessed (n=36) and no correlation was detected between GNAQ/GNA11 mutation status and Src/pERK expression. Out of the 36 analyzed tumors, 19 displayed the GNA11 mutation and 16 contained the GNAQ mutation while for one sample, the sequence analysis failed.

Monosomy 3 is a late event and strongly correlated with metastasis development. Eight primary UM cultures were analyzed for chromosome 3 and all three non-responders displayed a normal chromosome 3 karyotype whereas three out of five responders displayed monosomy 3. These data suggest that Dasatinib efficacy and ERK activation through Src signaling may be correlated with tumor progression, and this was further analyzed using primary UM tissue.

Using 35 UM samples, we observed that the level of Src expression was significantly correlated with monosomy 3 ($p = 0.0014$): the mean Src expression in UM samples with monosomy 3 was much higher (mean of 29.87; SEM 2.452) than in UM samples with disomy 3 (mean of 14.91; SEM 3.249). Analysis for pERK demonstrated a similar trend, as expression of pERK was elevated in the UM samples with monosomy 3 (mean of 7.13; SEM 0.60) in comparison to UM samples containing 2 copies of chromosome 3 (mean of 5.05; SEM 0.92). This

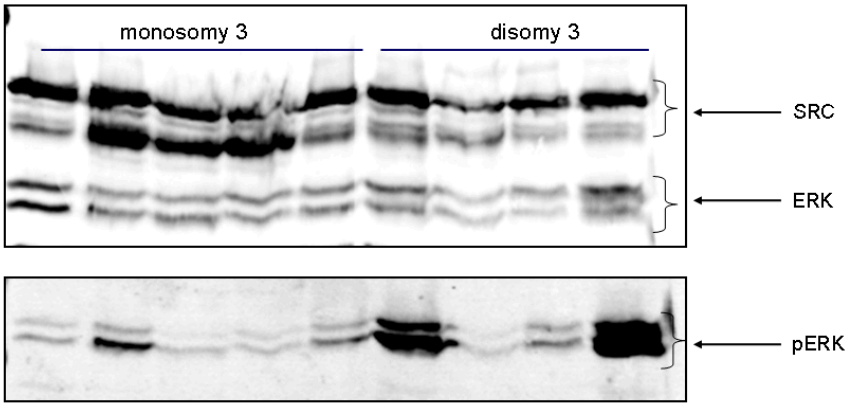


FIGURE 4A. Expression of Src, ERK and pERK in primary UM samples.
Representative example of the Western analysis that was conducted on 36 UM samples in order to analyze ERK activation and Src expression.

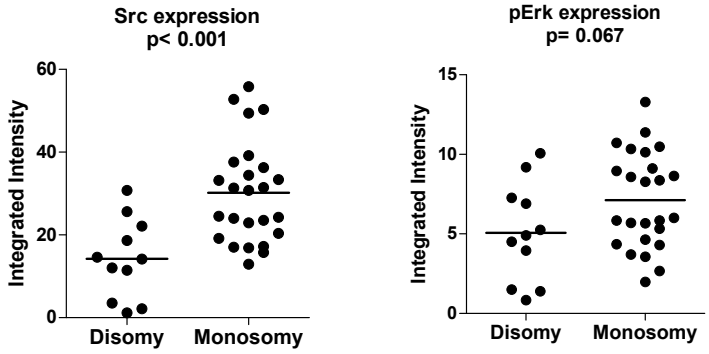


FIGURE 4B. Src/pERK expression and chromosome 3 status in UM tissue.
Protein expression levels of Src and pERK were measured and correlated with chromosome 3 status of 35 UM using unpaired T-test.

difference did however not reach statistical significance (p =0.068) (Fig. 4B). The analysis of the total amount of ERK1/2 expression and monosomy 3 revealed neither an association nor a trend (mean expression disomy 5.6, monosomy 4.6, p=0.750).

According to the TNM classification (7th edition) five tumors were stage I, four were stage IIA, ten were stage IIB, 14 were stage IIIA, one was stage IIIB, and one was stage IV. Besides ciliary body involvement (p=0.03), neither TNM classification, nor other clinical and histopathological parameters demonstrated an association with Src or pERK expression (Table 1).

Prognosis

Patient data and survival were updated till September 2010 and the mean follow-up at the time of analysis was 43 months (range, 3-121). During this period, 14 patients died, 11 due to metastatic disease. Two patients with metastasis were still alive at the end of follow-up and no patients were lost to follow up. According to Kaplan-Meier analysis and log rank testing, ciliary body involvement (hazard ratio 3.31, $p<0.05$), TNM classification (stage IV; hazard ratio 28.2, $p<0.05$) and monosomy 3 (hazard ratio 7.17, $p<0.05$), were significantly associated with a decreased survival. Src and pERK expression was not correlated to prognosis.

DISCUSSION

Src has previously been identified in UM cell lines as an important kinase involved in UM growth via the activation of MAPK signaling. Based on this, preclinical analysis with Dasatinib was performed, a Src family-kinase inhibitor that is used in the treatment of leukemia.

Using 8 primary UM cell cultures, we tested whether UM cell growth could be inhibited using Dasatinib. In about 60% of the UM cultures, cell proliferation was reduced after treatment with Dasatinib whereas in the remaining cultures, proliferation appeared unaffected. To investigate the underlying mechanism, MAPK activation in response to Dasatinib treatment was analyzed. In cell cultures that responded to Dasatinib with growth arrest, ERK activation (pERK) appeared to be higher prior to treatment and was also more strongly reduced upon Dasatinib treatment, compared to the non-sensitive cell cultures. To investigate the molecular mechanism of Dasatinib induced growth inhibition, the response was compared with the molecular characteristics of the UM. Tumors with GNAQ and GNA11 wildtype genotypes were detected in both the responders and the non-responders and could not distinguish the treatment groups. Monosomy 3 was however only detected in the responders while the non-responders all contained two copies of chromosome 3. This may indicate that a modifier of Src signaling is located on chromosome 3.

We also determined Src and MAPK activation status in 36 UM samples obtained after enucleation. A very strong correlation was detected between Src expression and ERK activation (pERK), supporting the notion that Src is associated with MAPK-driven UM proliferation and survival. Furthermore, we evaluated the possibility that Src upregulation and MAPK activation is due to somatic mutations in the heterotrimeric G protein alpha-subunit (GNAQ and GNA11) gene [10-13]. GNAQ/GNA11 mutations are the first oncogenic mutations in UM and were shown to occur in about 78% of tested primary UM samples [9,10]. In our UM cohort we detected an even higher abundance of GNAQ and GNA11 mutations (>90%) and hence we could not detect an association with Src mutation status and MAPK activation because a wild type group was lacking. Mutation of these genes in UM development appears to be an

early event and underlines the importance of secondary lesions as possible determinants of metastatic disease and elevated Src/MAPK activation.

Subsequent comparison of chromosome 3 status with Src and MAPK activation status in the primary tumors, and, in support of our in vitro analysis, revealed a significant association between Src expression and monosomy 3 (p= 0.0014). Src protein expression was approximately two-fold higher in monosomy 3 UM samples compared to disomy 3 UM samples. Monosomy 3 has been established as one of the most important molecular markers in UM patient survival and actually represents a late event in UM development [6,17,18]. This association supports in vitro data that suggest the presence of a Src modifier on chromosome 3. Recently, somatic mutations were identified in the gene encoding BRCA1-associated protein 1 (BAP1) on chromosome 3p21.1 which was significantly associated with metastatic occurrence [8]. BAP1 has been shown to possess tumor suppressor activity and mutations have been reported in some forms of cancer [19,20]. BAP1 mutation is a late event which may determine malignant conversion and should be analyzed for its role in Src/MAPK activation.

Previously, we reported a decreased Src activation in metastatic cell lines compared to primary UM cell lines [13]. However, in the same study residual Src activity was shown in UM metastasis tissue. Further analysis regarding the discrepancy between metastatic tissue and metastatic UM cell lines with regard to Src activity should be performed as loss of Src activity could be a potential cause of Dasatinib treatment resistance.

Uveal melanoma metastasizes almost exclusively to the liver and most current treatment options focus on this site [3]. Currently, conventional metastasis treatment including (systemic or intra-hepatic) chemotherapy or partial hepatectomy, provides no significant prolongation of survival compared to no treatment [4]. We propose the use of chromosome 3 status, ERK activation and Src kinase expression level of the primary UM and related metastases as a tool to select patients eligible for treatment with Dasatinib. In the future, prophylactic treatment of high risk patients could be an additional possibility hence biomarker analysis should be performed on both patients treated locally and patients treated with enucleation. Assessment of monosomy 3 by fine-needle-aspiration biopsy has been shown to be feasible in patients undergoing brachytherapy [21]. However, biopsies would only be clinically relevant if successful treatment options for metastases patients would become available.

In summary, we have shown that in selected UM, Dasatinib inhibits cell growth in vitro. The UM cultures that respond to Dasatinib treatment, differ in MAPK and Src kinase activity compared to non-responding primary UM cultures. Moreover, with monosomy 3 we may have revealed the underlying mechanism and hence we propose Dasatinib as a possible therapy for UM and we suggest monosomy 3, Src expression and MAPK activition as potential biomarkers for early adjuvant treatment.

Acknowledgements

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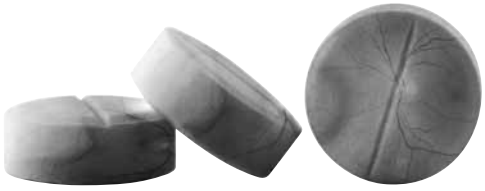
REFERENCE LIST

1. Singh AD, Bergman L, Seregard S. Uveal melanoma: epidemiologic aspects. Ophthalmol.Clin.North Am. 2005;18:75-84, viii.
2. Damato B. Detection of uveal melanoma by optometrists in the United Kingdom. Ophthalmic Physiol Opt. 2001;21:268-71.
3. Kujala E, Makitie T, Kivela T. Very long-term prognosis of patients with malignant uveal melanoma. Invest Ophthalmol.Vis.Sci. 2003;44:4651-9.
4. Augsburger JJ, Correa ZM, Shaikh AH. Effectiveness of treatments for metastatic uveal melanoma. Am.J.Ophthalmol. 2009;148:119-27.
5. Mooy CM, de Jong PT. Prognostic parameters in uveal melanoma: a review. Surv.Ophthalmol. 1996;41:215-28.
6. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. Lancet 1996;347:1222-5.
7. Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. Cancer Genet.Cytogenet. 1990;45:249-53.
8. Harbour JW, Onken MD, Roberson ED, Duan S, Cao L, Worley LA et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. Science. 2010;330:1410-3.
9. Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature. 2008.
10. Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T et al. Mutations in GNA11 in uveal melanoma. N.Engl.J.Med. 2010;363:2191-9.
11. Hubbard KB, Hepler JR. Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cell Signal. 2006;18:135-50.
12. Cramer H, Schmenger K, Heinrich K, Horstmeyer A, Boning H, Breit A et al. Coupling of endothelin receptors to the ERK/MAP kinase pathway. Roles of palmitoylation and G(alpha)q. Eur.J.Biochem. 2001;268:5449-59.
13. Maat W, el Filali M, Dirks-Mulder A, Luyten GP, Gruis NA, Desjardins L et al. Episodic Src activation in uveal melanoma revealed by kinase activity profiling. Br.J.Cancer. 2009;101:312-9.
14. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399-401.
15. Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z et al. Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. J.Biol.Chem. 2004;279:31769-79.
16. Lindauer M, Hochhaus A. Dasatinib. Recent Results Cancer Res. 2010;184:83-102.
17. Sisley K, Rennie IG, Parsons MA, Jacques R, Hammond DW, Bell SM et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. Genes Chromosomes.Cancer 1997;19:22-8.
18. Damato B, Duke C, Coupland SE, Hiscott P, Smith PA, Campbell I et al. Cytogenetics of uveal melanoma: a 7-year clinical experience. Ophthalmology 2007;114:1925-31.
19. Jensen DE, Proctor M, Marquis ST, Gardner HP, Ha SI, Chodosh LA et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene 1998;16:1097-112.
20. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318:1108-13.

21. Shields CL, Ganguly A, Materin MA, Teixeira L, Mashayekhi A, Swanson LA et al. Chromosome 3 analysis of uveal melanoma using fine-needle aspiration biopsy at the time of plaque radiotherapy in 140 consecutive cases. Trans.Am.Ophthalmol.Soc. 2007;105:43-52.



PART V

SUMMARY AND GENERAL DISCUSSION

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ACKNOWLEDGEMENTS

CURRICULUM VITAE

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SUMMARY
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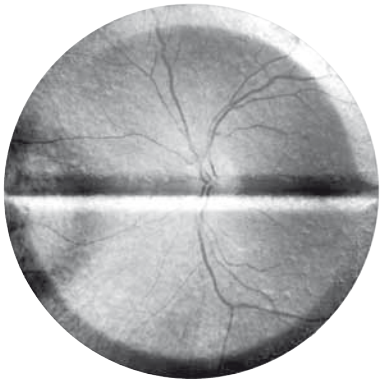
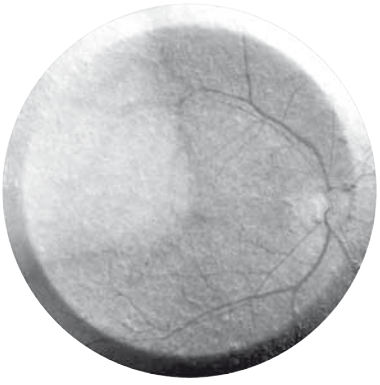




SUMMARY AND GENERAL DISCUSSION

Medical care of uveal melanoma has many aspects, as it involves treatment of the primary tumor, dealing with the complications of ocular radiation therapy, and the prevention and treatment of metastatic disease. In this thesis, I set out to analyze the efficacy and safety of some already available treatment modalities and subsequently explored new therapeutic options to reduce complications of current treatments and prolong survival.

It would be ideal if pre-clinical analysis in a laboratory setting (in vitro and in vivo) could be used to predict which treatment will have the best outcome. Knowledge of molecular mechanisms and pathways may allow efficient patient selection and help to prevent undesirable side effects.

In this discussion, I will first describe anti-angiogenic therapy and subsequently consider targeted therapy, which will be divided in cellular targeting and molecular targeting of tumor cells.

VEGF-regulated tumor angiogenesis in uveal melanoma

In general oncology, anti-angiogenic therapy has been explored extensively under the assumption that a tumor cannot grow beyond the limits of oxygen and nutrient diffusion (about 1-2 mm), unless (tumor)angiogenesis occurs [1]. Prevention of the formation of new vessels is expected to stop tumor growth. The clinical course of UM is often slow, implicating dormancy, which has been associated with an avascular phase. Change of this angiogenic phenotype, which is known as the 'angiogenic switch' and due to an alteration in the balance of inhibitory and stimulatory factors, may be associated with the induction of vessel growth and tumor proliferation [2,3]. VEGF is an important pro-angiogenic factor which is produced by tumor as well as adjacent cells and regulates vasopermeability and proliferation and migration of endothelial cells [4]. VEGF is especially upregulated in ischemic tumor areas due to Hypoxia-Inducible Factor (HIF)-1α stabilization [5]. We therefore analyzed the regulation of expression of VEGF in combination with the HIF-1α pathway in uveal melanoma cells in vitro (*Chapter 4*). Hypoxic conditions strongly induce VEGF mRNA expression in uveal melanoma cell lines and primary uveal melanoma cultures, although hypoxia did not increase cell proliferation. On the contrary, UM cell proliferation was significantly reduced under hypoxic conditions in comparison to UM cells in a normoxic environment. This indicates that VEGF cannot independently increase uveal melanoma growth through a possible feedback loop. However, it is likely that VEGF induction may play a role in the development of new vessels.

In several tumors including colon carcinoma, soft tissue sarcomas and gastric cancer, serum VEGF levels have been found to be an indicator of metastases [6-8]. We therefore extended our analysis by determination of VEGF expression in primary UM in order to determine a possible role for VEGF expression in malignant dissemination. The mRNA VEGF expression

of primary tumor tissue varied widely and did not predict survival nor was it correlated with the presence of bad prognostic factors. The importance of VEGF production was shown by testing serum from UM patients: we detected a significantly higher level of VEGF in sera of patients suffering from UM-related metastatic disease compared to UM patients without metastases and 'healthy people'. This has been confirmed in subsequent clinical studies [9] and in an experimental UM metastatic mouse model [10]. The high amounts of VEGF in the sera of patients with UM metastases suggest that VEGF plays a role in the growth of metastases, and the use of VEGF-inhibiting agents as treatment should therefore be considered.

Pharmacologic effects of anti-angiogenic treatment in uveal melanoma

Anti-angiogenic treatment which mainly targets VEGF has been used in the treatment of colorectal, lung and renal cancer [11-13], rendering a possibility for uveal melanoma treatment (*Chapter 2 and 3*). We therefore analyzed the anti-melanoma effect of bevacizumab (Avastin), the first approved monoclonal antibody against VEGF that has been used for treatment of metastases in colorectal cancer. When we injected bevacizumab intraocularly to treat a murine melanoma present in the anterior chamber, we, unexpectedly, found an acceleration of intraocular tumor growth (*Chapter 5*). In addition, following bevacizumab treatment, these anterior chamber tumors demonstrated more intraocular and intratumoral hemorrhages; however, no difference in vessel amount could be ascertained. In vitro treatment with bevacizumab stimulated expression of VEGF mRNA expression in murine melanoma as well as in human uveal melanoma cells. Combined, our in vivo/vitro results suggest that high VEGF levels caused tumor growth acceleration and hemorrhages by increasing vascular permeability [14]. These 'adverse' effects of bevacizumab have been described before: in mice bearing intracerebral glioma, anti-VEGF treatment with pegaptanib (Macugen) increased GLUT-1 expression (a glucose transporter upregulated by HIF-1α similar to VEGF) [5]. This 'pseudohypoxia' has been shown to increase tumorigenesis in other types of cancer cells: anti-VEGFR treatment of mice bearing pancreatic neuroendocrine tumors resulted in an initial tumor stasis followed by tumor recurrence. The relapsing tumor expressed high levels of mRNAs for pro-angiogenic factors while demonstrating several hypoxic regions [15], and treated mice developed more invasive tumors and metastatic lesions [16]. Bevacizumab has now been in clinical use since 2004 and treatment often prolongs overall survival of cancer patients by a few months, without really curing metastatic disease [17]. However, the FDA has recently prohibited the use of bevacizumab (monotherapy) for metastatic breast cancer, since several studies have revealed that VEGF inhibitors eventually may promote tumorigenesis and metastatic dissemination in this malignancy [16,18].

Ophthalmologists have been using bevacizumab for several indications, though not for the treatment of ocular tumors. Anti-VEGF treatment has been used in ocular diseases which are characterised by vessel leakage and neovascularisation, such as age-related macula degenera-

tion and diabetic retinopathy. Regarding uveal melanoma, bevacizumab is frequently utilized for the treatment of radiation retinopathy. 'Off-label' use of intravitreal bevacizumab to treat macular edema and neovascularization in radiation retinopathy often demonstrates a decrease of macular edema and an improvement of visual acuity [19-21]. Still, the use of VEGF inhibitors in uveal melanoma-bearing eyes should be considered carefully, since the possibility that there are living uveal melanoma cells in eyes treated with radiotherapy cannot be excluded [22,23]: a possible effect on micrometastases which could be present in the eye or systemically has never been investigated. An alternative for treatment of radiation retinopathy consists of intraocular steroid injections. Triamcinolone acetonide (TA) is a glucocorticoid that has already been shown to exhibit a temporary positive effect in patients with radiation maculopathy [24]. TA [25-27] has been shown to have an anti-angiogenic effect, though the mechanism through which this effect comes about is not clear. We analyzed the effect of TA on UM cell growth and VEGF expression in vitro (*Chapter 6*). Our results show no apparent stimulating or inhibiting effect of TA on uveal melanoma cell proliferation or on VEGF mRNA and protein expression. One of the disadvantages of TA, however, is that it causes ocular hypertension in about 30% of the cases [28] and may lead to glaucomatous damage. A steroid-derived substance, anecortave acetate (AA), which is an angiostatic cortisone, has been developed to be devoid of corticosteroid side effects such as ocular hypertension. Hence, AA may be a good alternative to TA. A first study of AA administered in a juxtascleral depot to treat subfoveal choroidal neovascularization in age-related macular degeneration showed good results in the prevention of further vessel development [29]. In our experiments, AA did not induce cell proliferation or affect VEGF, pigment epithelium derived factor (PEDF; pro-angiogenic factor) or trombospondin-1 (TSP-1, anti-angiogenic factor) expression in vitro, similar to TA. In addition, AA has been found to significantly reduce tumor growth in intraocular tumor-bearing mice [30]. Tumor inhibition was presumably due to the angiostatic properties of AA because the compound did not affect tumor cell proliferation in vitro as was confirmed in *Chapter 6*. It is therefore regrettable that AA is not a marketed drug.

Anti-VEGF treatment is valuable in many ocular diseases. As tumor angiogenesis plays a role in uveal melanoma growth, it is probably also important in metastases, and systemic administration has been shown to reduce metastatic out growth in experimental models [31]. Studies are needed to determine the use of anti-VEGF drugs for single or combination therapy for prevention or treatment of metastases in UM patients.

Tumor-specific targeting

One of the main challenges in cancer treatment is selective and potent delivery of drugs to tumor cells. This motivated us to search for ligands that are selective for uveal melanoma. These ligands may be used as a diagnostic tool and/or used for targeted therapy of primary and (micro) metastatic uveal melanoma. Our first approach was to isolate uveal melanoma selective peptides, based on embryonic origin. During embryogenesis, neural crest cells

migrate to the diencephalon and to the uvea, where they give rise to pigmented melanocytes. Neural crest cells are able to produce neurohormones like somatostatin (SST). SST inhibits the release of growth hormone and thyroid-stimulating hormone by binding to specific G protein-coupled receptors [32].

SST analogues, like octreotide and octreotate, can be radiolabelled and are currently being used in the diagnosis and therapy of patients suffering from SSTR-expressing tumors [33-36]. In *Chapter 9*, expression of SSTR2 on uveal melanoma cell lines was analysed by autoradiography using radio labelled-ocreotate. Cell lines created from primary UM cells showed very low or no receptor-specific binding. By contrast, cell lines obtained from metastatic melanoma cells showed high binding. In addition, a two- to four-times higher mRNA expression level of SSTR2 was demonstrated in metastatic cells compared to primary UM cells. These result implicate a possible association between SSTR2 expression and UM malignancy. Unfortunately, analysis of primary UM tissue revealed an overall low expression level of SSTR2, and no association with known histologic and genetic prognostic markers or with survival. We therefore conclude that SSTR is not a valuable tool to further investigate for use in uveal melanoma.

As an alternative approach to select uveal melanoma selective peptides, we subsequently used phage peptide libraries. Phage display is a powerful technique for the isolation of peptides that bind to a particular target with high affinity and specificity. In contrast to larger molecules, such as proteins and antibodies, small peptides can efficiently penetrate tissues and are relatively easy to synthesize. Several studies describe the successful detection of organ and tumor-specific peptides using phage peptide libraries [37,38]. Using phage display, Howell et al. identified heptapeptides that specifically bound to human tumor melanin, as demonstrated in nude mice that carried human metastatic melanoma tissue. These heptapeptides could be used as a tool in targeted therapy for (metastatic) melanoma [39]. In *Chapter 8* we describe the identification of uveal melanoma associated peptides (UMAPs). In the future, these UMAPs may be used clinically for in vivo imaging of micro metastases. It has been proposed that patients who develop clinical metastases from uveal melanoma often harbour micro metastasis for years [40]. UMAPs radiolabelled with radionuclides could be used in scintigraphy to detect such micro metastases. In addition, the same peptides labeled with therapeutic β-emitting radionuclides, may be used in targeted therapy and prevent or decelerate the occurrence of 'full-blown' metastatic disease.

In our study, UMAP1 proved to show excellent internalisation ability in primary and metastatic UM cell lines. This peptide was also internalized by normal melanocytes due to the lack of a negative selection with normal melanocytes. Other control cells such as SAOSH and HUVEC cells did not internalise UMAP1, demonstrating a partial selectivity.

UMAP2 showed a strong preference for metastatic cell line OMM2.5 and the sequence of UMAP2 has been identified as a homologue of the rat insulin-like growth factor receptor

[41]. Interestingly, Economou et al demonstrated the expression of insulin growth factor-1 receptor (IGF-1R) in uveal melanoma to be associated with a bad prognosis [42-44]. Besides representing an opportunity for targeting UM cells, UMAP2 may be used to study the mechanisms that are involved in proliferation and progression; for instance IGF-1R signaling couples to MAPK signaling that is responsible for proliferation and survival signaling. The lack of tumor-specificity of the UMAPs that we identified made us focus on other targeted therapies.

Oncogenic pathways involved in uveal melanoma and treatment options

The RAS-RAF-MEK-ERK, or classical mitogen-activated protein kinase (MAPK) pathway, is essential in the development of melanocytic neoplasia and constitutive activation of this pathway has been associated with many different types of cancer [45,46]. Knowledge of these pathways and the molecular mechanisms that underlay aberrant signaling is required to predict treatment efficacy and/or failure. Recently, mutations have been discovered in the GNAQ and GNA11 gene, which encode Gαq-type subunits of the heterotrimeric G-protein. These mutations occur in 77 % of uveal melanomas [47,48], and result in constitutive G-protein activation which mediates intracellular signals and activates the MAPK pathway [49]. This discovery has created a great interest in these pathways, as interference may be used therapeutically.

A molecule that links GNAQ/GNA11 to MAPK signaling as well as IGF-1R signaling is Src tyrosine kinase [50]. We identified Src as a crucial upstream tyrosine kinase, based on analysis of several UM cell lines which revealed ERK1/2 activation in primary UM cell lines (*Chapter 9*).

In addition, metastatic tissue displayed considerable Src kinase activity, most likely resembling the in vivo condition (*Chapter 9*). Src kinase was efficiently inhibited by incubation with Dasatinib. In *Chapter 10*, we further analyze the Src and MAPK activation in primary uveal melanoma. Using primary UM cell lines, we observed that Src is associated with the MAPK pathway resulting in UM proliferation and survival, and this was confirmed by a significant correlation between Src/ERK expression levels and ERK activation (pERK). In addition, we evaluated the possibility of Src upregulation and MAPK activation due to GNAQ/GNA11 mutations. In our primary UM samples we detected a high occurrence of GNAQ and GNA11 mutations (>90%) but no association with Src and MAPK activation. The mutation of these genes in UM development appears to be an early event and indicates the importance of secondary lesions as possible triggers of elevated Src and MAPK activation. We further analyzed whether there was an association with the presence of monosomy 3, which is one of the most important prognostic markers in UM patient survival and may actually represent a late event in UM development [51-53]. In agreement, we did find a significant association between Src expression and monosomy 3. Based on the apparently pivotal role of Src in MAPK-driven proliferation, inhibition of Src may provide a new treatment option. We demonstrate inhibi-

tion of cell growth in 60% of UM cell cultures treated with dasatinib; a commercially available tyrosine kinase inhibitor of the src-family kinase. Further analysis revealed that basale Src kinase activity and ex vivo reduction after incubation with Dasatinib was much higher in the sensitive cultures in contrast to the non-responding cells. In addition, all three non-responders displayed a normal chromosome 3 karyogram whereas three out of five responders displayed monosomy 3. Based on this pre-clinical evaluation, optimal treatment selection should include evaluation of primary and metastastic tumor tissue for basal Src kinase activity of the cells and testing ex vivo cultured cells for reduction of expression of kinase activity after incubation with specific drugs, using for example kinase arrays. In addition, determination of chromosome 3 status could be of value, but this remains to be ascertained by others as well. Tyrosine kinase inhibitors, such as Dasatinib, are interesting due to their ability to target multiple kinases and therefore have a wide application in several different malignancies. Unfortunately, this promiscuous nature may also cause "off-target" effects demonstrated in several clinical trials [54,55]. Tumor cell targeting with specific ligands and of specific cellular components, such as Src, which play an important role in uveal melanoma tumorigenesis may provide new treatment options and reduce adverse side effects.

Conclusion and future prospects

UM is a disease with many faces: ophthalmologists treat the primary tumor, but the patient faces the problem of developing metastases, which are often deadly after a short period. Collaboration with geneticists, biochemists, and oncologists is of the greatest importance to develop an effective approach to prevent or treat metastases. Recent insight, as described in this thesis, indicates the need for knowledge-based treatment of UM. The 'pseudohypoxic' and tumor promoting effects of bevacizumab as described in this thesis is especially relevant. Bevacizumab is frequently used off-label to treat macular edema in UM patients suffering of radiation retinopathy, without the knowledge of possible effect on still viable UM cells. The FDA has recently prohibited the use of bevacizumab (monotherapy) for metastatic breast cancer, and future (clinical) experiments should be performed to establish the clinical safety in case of UM.

We further observed that specific peptides, such as UMAP1, which can successfully be internalized by targeted UM cells, have demonstrated potential for UM-targeted treatment. These peptides have to be investigated in vivo, to ascertain whether they are a viable clinical tool. Labeling the peptides with radionuclides, and demonstrating specificity for UM cells are some of the challenges which have to be overcome. Another aspect in the patient-specific treatment of UM is the in vitro analysis of primary UM samples to predict treatment responses. In case of Dasatinib, we describe treatment responses associated with monosomy 3 and 'kinase' activity in different primary UM samples. In vitro and ex-vivo pre-clinical analysis in association with genetic testing for specific gene mutations will be of future relevance.

REFERENCE LIST

1. Holmgren, L., O'Reilly, M. S., and Folkman, J. *Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression.* Nat.Med. 1995. 1:149-153.
2. Machein, M. R. and Plate, K. H. *Role of VEGF in developmental angiogenesis and in tumor angiogenesis in the brain.* Cancer Treat.Res. 2004. 117:191-218.
3. Boyd, S. R., Tan, D., Bunce, C., Gittos, A., Neale, M. H., Hungerford, J. L., Charnock-Jones, S., and Cree, I. A. *Vascular endothelial growth factor is elevated in ocular fluids of eyes harbouring uveal melanoma: identification of a potential therapeutic window.* Br.J.Ophthalmol. 2002. 86:448-452.
4. Ferrara, N., Gerber, H. P., and LeCouter, J. *The biology of VEGF and its receptors.* Nat.Med. 2003. 9:669-676.
5. Verhoeff, J. J., Stalpers, L. J., Claes, A., Hovinga, K. E., Musters, G. D., Peter, V. W., Richel, D. J., Leenders, W. P., and van Furth, W. R. *Tumour control by whole brain irradiation of anti-VEGF-treated mice bearing intracerebral glioma.* Eur.J.Cancer 2009. 45:3074-3080.
6. Karayiannakis, A. J., Syrigos, K. N., Polychronidis, A., Zbar, A., Kouraklis, G., Simopoulos, C., and Karatzas, G. *Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery.* Ann.Surg. 2002. 236:37-42.
7. Celen, O., Kahraman, I., Yildirim, E., and Berberoglu, U. *Correlation of vascular endothelial growth factor (VEGF) and CEA with clinicopathological variables in colorectal cancer patients.* Neoplasma 2004. 51:293-299.
8. Hayes, A. J., Mostyn-Jones, A., Koban, M. U., A'Hern, R., Burton, P., and Thomas, J. M. *Serum vascular endothelial growth factor as a tumour marker in soft tissue sarcoma.* Br.J.Surg. 2004. 91:242-247.
9. Barak, V., Pe, E. J., Kalickman, I., and Frenkel, S. *VEGF as a Biomarker for Metastatic Uveal Melanoma in Humans.* Curr.Eye Res. 2011.
10. Crosby, M. B., Yang, H., Gao, W., Zhang, L., and Grossniklaus, H. E. *Serum vascular endothelial growth factor (VEGF) levels correlate with number and location of micrometastases in a murine model of uveal melanoma.* Br.J.Ophthalmol. 2011. 95:112-117.
11. Kabbinavar, F., Hurwitz, H. I., Fehrenbacher, L., Meropol, N. J., Novotny, W. F., Lieberman, G., Griffing, S., and Bergsland, E. *Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer.* J.Clin.Oncol. 2003. 21:60-65.
12. Yang, J. C., Haworth, L., Sherry, R. M., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Steinberg, S. M., Chen, H. X., and Rosenberg, S. A. *A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer.* N.Engl.J.Med. 2003. 349:427-434.
13. Cobleigh, M. A., Langmuir, V. K., Sledge, G. W., Miller, K. D., Haney, L., Novotny, W. F., Reimann, J. D., and Vassel, A. *A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer.* Semin.Oncol. 2003. 30:117-124.
14. Criscuolo, G. R., Merrill, M. J., and Oldfield, E. H. *Further characterization of malignant glioma-derived vascular permeability factor.* J.Neurosurg. 1988. 69:254-262.
15. Casanovas, O., Hicklin, D. J., Bergers, G., and Hanahan, D. *Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors.* Cancer Cell 2005. 8:299-309.
16. Paez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Vinals, F., Inoue, M., Bergers, G., Hanahan, D., and Casanovas, O. *Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis.* Cancer Cell 2009. 15:220-231.
17. Kerbel, R. S. *Tumor angiogenesis.* N.Engl.J.Med. 2008. 358:2039-2049.

18. Ebos, J. M., Lee, C. R., Bogdanovic, E., Alami, J., Van Slyke, P., Francia, G., Xu, P., Mutsaers, A. J., Dumont, D. J., and Kerbel, R. S. *Vascular endothelial growth factor-mediated decrease in plasma soluble vascular endothelial growth factor receptor-2 levels as a surrogate biomarker for tumor growth.* Cancer Res. 2008. 68:521-529.
19. Mason, J. O., III, Albert, M. A., Jr., Persaud, T. O., and Vail, R. S. *Intravitreal bevacizumab treatment for radiation macular edema after plaque radiotherapy for choroidal melanoma.* Retina 2007. 27:903-907.
20. Finger, P. T. *Radiation retinopathy is treatable with anti-vascular endothelial growth factor bevacizumab (Avastin).* Int.J.Radiat.Oncol.Biol.Phys. 2008. 70:974-977.
21. Gupta, A. and Muecke, J. S. *Treatment of radiation maculopathy with intravitreal injection of bevacizumab (Avastin).* Retina 2008. 28:964-968.
22. Pe'er, J., Stefani, F. H., Seregard, S., Kivela, T., Lommatzsch, P., Prause, J. U., Sobottka, B., Damato, B., and Chowers, I. *Cell proliferation activity in posterior uveal melanoma after Ru-106 brachytherapy: an EORTC ocular oncology group study.* Br.J.Ophthalmol. 2001. 85:1208-1212.
23. Harbour, J. W., Char, D. H., Kroll, S., Quivey, J. M., and Castro, J. *Metastatic risk for distinct patterns of postirradiation local recurrence of posterior uveal melanoma.* Ophthalmology 1997. 104:1785-1792.
24. Shields, C. L., Demirci, H., Dai, V., Marr, B. P., Mashayekhi, A., Materin, M. A., Manquez, M. E., and Shields, J. A. *Intravitreal triamcinolone acetonide for radiation maculopathy after plaque radiotherapy for choroidal melanoma.* Retina 2005. 25:868-874.
25. Murata, M., Shimizu, S., Horiuchi, S., and Taira, M. *Inhibitory effect of triamcinolone acetonide on corneal neovascularization.* Graefes Arch.Clin.Exp.Ophthalmol. 2006. 244:205-209.
26. Hashimoto, I., Nakanishi, H., Shono, Y., Toda, M., Tsuda, H., and Arase, S. *Angiostatic effects of corticosteroid on wound healing of the rabbit ear.* J.Med.Invest 2002. 49:61-66.
27. Antoszyk, A. N., Gottlieb, J. L., Machemer, R., and Hatchell, D. L. *The effects of intravitreal triamcinolone acetonide on experimental pre-retinal neovascularization.* Graefes Arch.Clin.Exp.Ophthalmol. 1993. 231:34-40.
28. Jaissle, G. B., Szurman, P., and Bartz-Schmidt, K. U. *[Ocular side effects and complications of intravitreal triamcinolone acetonide injection].* Ophthalmologe 2004. 101:121-128.
29. Schmidt-Erfurth, U., Michels, S., Michels, R., and Aue, A. *Anecortave acetate for the treatment of subfoveal choroidal neovascularization secondary to age-related macular degeneration.* Eur.J.Ophthalmol. 2005. 15:482-485.
30. Clark, A. F., Mellon, J., Li, X. Y., Ma, D., Leher, H., Apte, R., Alizadeh, H., Hegde, S., McLenaghan, A., Mayhew, E., D'Orazio, T. J., and Niederkorn, J. Y. *Inhibition of intraocular tumor growth by topical application of the angiostatic steroid anecortave acetate.* Invest Ophthalmol.Vis.Sci. 1999. 40:2158-2162.
31. Yang, H., Jager, M. J., and Grossniklaus, H. E. *Bevacizumab suppression of establishment of micrometastases in experimental ocular melanoma.* Invest Ophthalmol.Vis.Sci. 2010. 51:2835-2842.
32. Johnson, L. R. *Regulation of gastrointestinal mucosal growth.* Physiol Rev. 1988. 68:456-502.
33. de, J. M., Breeman, W. A., Valkema, R., Bernard, B. F., and Krenning, E. P. *Combination radionuclide therapy using 177Lu- and 90Y-labeled somatostatin analogs.* J.Nucl.Med. 2005. 46 Suppl 1:13S-17S.
34. Krenning, E. P., de, J. M., Kooij, P. P., Breeman, W. A., Bakker, W. H., de Herder, W. W., Van Eijck, C. H., Kwekkeboom, D. J., Jamar, F., Pauwels, S., and Valkema, R. *Radiolabelled somatostatin analogue(s) for peptide receptor scintigraphy and radionuclide therapy.* Ann.Oncol. 1999. 10 Suppl 2:S23-S29.
35. Krenning, E. P., Valkema, R., Kwekkeboom, D. J., de Herder, W. W., Van Eijck, C. H., de, J. M., Pauwels, S., and Reubi, J. C. *Molecular imaging as in vivo molecular pathology for gastroenteropancreatic neuroendocrine tumors: implications for follow-up after therapy.* J.Nucl.Med. 2005. 46 Suppl 1:76S-82S.
36. Kwekkeboom, D. J., Mueller-Brand, J., Paganelli, G., Anthony, L. B., Pauwels, S., Kvols, L. K., O'dorisio, T. M., Valkema, R., Bodei, L., Chinol, M., Maecke, H. R., and Krenning, E. P. *Overview of results of*

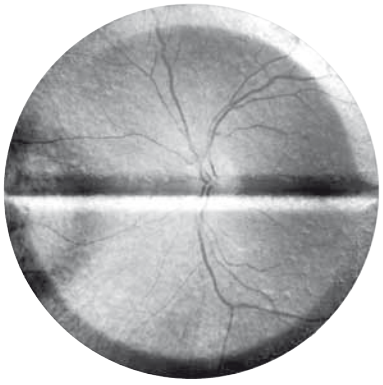
peptide receptor radionuclide therapy with 3 radiolabeled somatostatin analogs. J.Nucl.Med. 2005. 46 Suppl 1:62S-66S.

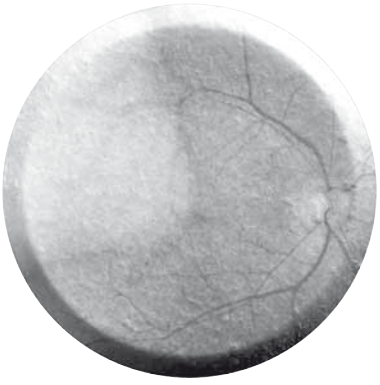
37. Pasqualini, R. and Ruoslahti, E. *Organ targeting in vivo using phage display peptide libraries.* Nature 1996. 380:364-366.
38. Lee, S. M., Lee, E. J., Hong, H. Y., Kwon, M. K., Kwon, T. H., Choi, J. Y., Park, R. W., Kwon, T. G., Yoo, E. S., Yoon, G. S., Kim, I. S., Ruoslahti, E., and Lee, B. H. *Targeting bladder tumor cells in vivo and in the urine with a peptide identified by phage display.* Mol.Cancer Res. 2007. 5:11-19.
39. Howell, R. C., Revskaya, E., Pazo, V., Nosanchuk, J. D., Casadevall, A., and Dadachova, E. *Phage display library derived peptides that bind to human tumor melanin as potential vehicles for targeted radionuclide therapy of metastatic melanoma.* Bioconjug.Chem. 2007. 18:1739-1748.
40. Eskelin, S., Pyrhonen, S., Summanen, P., Hahka-Kemppinen, M., and Kivela, T. *Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment.* Ophthalmology 2000. 107:1443-1449.
41. Higgins, L. M., Lambkin, I., Donnelly, G., Byrne, D., Wilson, C., Dee, J., Smith, M., and O'Mahony, D. J. *In vivo phage display to identify M cell-targeting ligands.* Pharm.Res. 2004. 21:695-705.
42. Economou, M. A., All-Ericsson, C., Bykov, V., Girnita, L., Bartolazzi, A., Larsson, O., and Seregard, S. *Receptors for the liver synthesized growth factors IGF-1 and HGF/SF in uveal melanoma: intercorrelation and prognostic implications.* Invest Ophthalmol.Vis.Sci. 2005. 46:4372-4375.
43. All-Ericsson, C., Girnita, L., Seregard, S., Bartolazzi, A., Jager, M. J., and Larsson, O. *Insulin-like growth factor-1 receptor in uveal melanoma: a predictor for metastatic disease and a potential therapeutic target.* Invest Ophthalmol.Vis.Sci. 2002. 43:1-8.
44. Kujala, E., Makitie, T., and Kivela, T. *Very long-term prognosis of patients with malignant uveal melanoma.* Invest Ophthalmol.Vis.Sci. 2003. 44:4651-4659.
45. Goding, C. R. *Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage.* Genes Dev. 2000. 14:1712-1728.
46. Reddy, K. B., Nabha, S. M., and Atanaskova, N. *Role of MAP kinase in tumor progression and invasion.* Cancer Metastasis Rev. 2003. 22:395-403.
47. Van Raamsdonk, C. D., Griewank, K. G., Crosby, M. B., Garrido, M. C., Vemula, S., Wiesner, T., Obenauf, A. C., Wackernagel, W., Green, G., Bouvier, N., Sozen, M. M., Baimukanova, G., Roy, R., Heguy, A., Dolgalev, I., Khanin, R., Busam, K., Speicher, M. R., O'Brien, J., and Bastian, B. C. *Mutations in GNA11 in uveal melanoma.* N.Engl.J.Med. 2010. 363:2191-2199.
48. Van Raamsdonk, C. D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J. M., Simpson, E. M., Barsh, G. S., and Bastian, B. C. *Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi.* Nature 2009. 457:599-602.
49. Hubbard, K. B. and Hepler, J. R. *Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins.* Cell Signal. 2006. 18:135-150.
50. Cramer, H., Schmenger, K., Heinrich, K., Horstmeyer, A., Boning, H., Breit, A., Piiper, A., Lundstrom, K., Muller-Esterl, W., and Schroeder, C. *Coupling of endothelin receptors to the ERK/MAP kinase pathway. Roles of palmitoylation and G(alpha)q.* Eur.J.Biochem. 2001. 268:5449-5459.
51. Prescher, G., Bornfeld, N., Hirche, H., Horsthemke, B., Jockel, K. H., and Becher, R. *Prognostic implications of monosomy 3 in uveal melanoma.* Lancet 1996. 347:1222-1225.
52. Sisley, K., Rennie, I. G., Parsons, M. A., Jacques, R., Hammond, D. W., Bell, S. M., Potter, A. M., and Rees, R. C. *Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis.* Genes Chromosomes.Cancer 1997. 19:22-28.
53. Damato, B., Duke, C., Coupland, S. E., Hiscott, P., Smith, P. A., Campbell, I., Douglas, A., and Howard, P. *Cytogenetics of uveal melanoma: a 7-year clinical experience.* Ophthalmology 2007. 114:1925-1931.

54. Shepard, D. R. and Garcia, J. A. *Toxicity associated with the long-term use of targeted therapies in patients with advanced renal cell carcinoma.* Expert.Rev.Anticancer Ther. 2009. 9:795-805.

55. Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B. J., Anderson, D. J., Alvarado, R., Ludlam, M. J., Stokoe, D., Gloor, S. L., Vigers, G., Morales, T., Aliagas, I., Liu, B., Sideris, S., Hoeflich, K. P., Jaiswal, B. S., Seshagiri, S., Koeppen, H., Belvin, M., Friedman, L. S., and Malek, S. *RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth.* Nature. 2010.

SAMENVATTING





SAMENVATTING

De medische zorg van oogmelanomen kent vele aspecten; behandeling van de primaire tumor, het omgaan met de complicaties van radiotherapie, en de preventie en behandeling van metastasen. In dit proefschrift is gezocht naar de werking en veiligheid van een aantal reeds beschikbare behandelmethoden. Ook zijn nieuwe therapeutische mogelijkheden onderzocht om complicaties van de huidige behandelingen te verminderen en de overleving van patiënten te verbeteren. Dit proefschrift beschrijft het mechanisme van tumor vaatnieuwvorming in oogmelanomen en de effecten van de behandeling die dit tegen moet gaan. Verder wordt er ingegaan op de mogelijkheden om oogmelanomen op cel en moleculair niveau te behandelen.

Behandeling van tumor vaatnieuwvorming in oogmelanomen

Het remmen van tumor vaatnieuwvorming is uitgebreid onderzocht als mogelijke therapie voor kanker met de vooronderstelling dat tumorgroei beperkt wordt door diffusie van zuurstof en voedingsstoffen. Het remmen van vaatnieuwvorming zal naar verwachting de groei van tumoren stoppen. Het klinische verloop van oogmelanomen is vaak traag en wordt in verband gebracht met een vaatarme groeifase. VEGF is een belangrijke vaatgroei factor, die wordt geproduceerd door de tumor en aangrenzende cellen. VEGF is vooral aanwezig in zuurstof arme gebieden en daarom onderzochten we de regulatie van VEGF expressie in oogmelanoom cellen (*Hoofdstuk 4*). Onder zuurstofarme omstandigheden neemt VEGF expressie toe en de tumorgroei neemt af.

In een aantal tumoren waaronder darmkanker en maagkanker, zijn VEGF niveaus in het bloed een goede indicator van de aanwezigheid van uitzaaiingen. En ook in het oogmelanoom is VEGF in het bloed van patiënten die lijden aan uitzaaiingen aanzienlijk verhoogd in vergelijking met patiënten zonder uitzaaiingen en 'gezonde mensen'. De grote hoeveelheden VEGF in het bloed van patiënten met uitzaaiingen suggereert dat VEGF een rol speelt in de groei van metastasen, en het gebruik van VEGF-remmende middelen als behandeling moet daarom worden overwogen.

Hoofdstuk 3 beschrijft het effect van een VEGF neutraliserend antilichaam (Avastin; bevacizumab) op oogmelanomen. In een diermodel vonden we, geheel onverwacht, een versnelling van de intraoculaire tumorgroei na behandeling met bevacizumab, en dit ging gepaard met bloedingen van de tumor (*Hoofdstuk 5*). Mogelijk dat de paradoxale VEGF verhoging die we in oogmelanoom cellen zien na bevacizumab behandeling, tumor vaatdoorlaatbaarheid en daarmee tumorgroei, verhoogt. Deze 'ongewenste' effecten van bevacizumab zijn al eerder beschreven en de FDA heeft onlangs het gebruik van bevacizumab (monotherapie) voor gemetastaseerde borstkanker verboden, omdat diverse studies hebben aangetoond dat VEGF-remmers uiteindelijk tumorgroei en uitzaaiingen kunnen bevorderen .

Onderwijl gebruiken oogartsen bevacizumab voor verschillende indicaties, maar niet voor de behandeling van oculaire tumoren. Anti-VEGF behandeling wordt gebruikt bij oogaandoeningen die worden gekenmerkt door vaatlekkage en vaatnieuwvorming zoals leeftijds- gerelateerde macula degeneratie en diabetische retinopathie. Bij oogmelanomen kan bevacizumab gebruikt worden voor de behandeling van bestralingsretinopathie. Toch moet het gebruik van VEGF-remmers in ogen behandeld voor een oogmelanoom zorgvuldig worden afgewogen, omdat de mogelijkheid dat er nog levende oogmelanoomcellen in het oog aanwezig zijn, niet kan worden uitgesloten. Een alternatief voor de behandeling van bestralingsretinopathie bestaat uit intraoculaire corticosteroïdinjecties. Triamcinolonacetonide (TA) is een glucocorticoïd dat een tijdelijk positief effect vertoont bij patiënten met bestralingsmaculopathie. Onze resultaten tonen aan dat er geen stimulerend of remmend effect van TA op oogmelanoom celproliferatie of op VEGF expressie is (*Hoofdstuk 6*). Één van de nadelen van TA, echter, is dat het oogdrukstijging veroorzaakt in ongeveer 30% van de gevallen en kan leiden tot glaucomateuze schade. Een afgeleide stof, anecortave acetaat (AA), is ontwikkeld om de bijwerkingen zoals oculaire hypertensie te verminderen. In onze experimenten geeft AA vergelijkbare resultaten als met TA (*Hoofdstuk 6*).

Anti-VEGF behandeling is waardevol in vele oogziekten. Als tumor vaatnieuwvorming een rol speelt in oogmelanoom groei, is het waarschijnlijk ook belangrijk in metastasen, en systemische toediening heeft aangetoond dat de groei van uitzaaiingen in experimentele modellen verminderd. Studies zijn nodig om een mogelijke rol voor het gebruik van anti-VEGF geneesmiddelen bij de preventie of behandeling van uitzaaiingen in de oogmelanoompatienten te bepalen.

Tumor specifieke peptiden

Één van de belangrijkste uitdagingen in de behandeling van kanker is selectieve en effectieve aflevering van geneesmiddelen aan tumorcellen. Oogmelanoom specifieke peptiden kunnen worden gebruikt als een diagnostisch hulpmiddel of voor een gerichte behandeling. In tegenstelling tot grotere moleculen, zoals eiwitten en antistoffen, kunnen kleine peptiden efficiënt weefsels penetreren en zijn relatief makkelijk te synthetiseren.

Oogmelanoom-selectieve peptiden kunnen op basis van embryonale oorsprong verondersteld worden. SSTR2 wordt geexpresseerd door cellen uit de neurale lijst waartoe oogmelanocyten ook behoren en in *Hoofdstuk 7*, is de expressie van SSTR2 op oogmelanoom cellen geanalyseerd. Oogmelanoom cellen vertoonden een zeer lage SSTR2 expressie en daardoor, nauwelijks receptor-specifieke binding door het bijbehorende hormoon, terwijl cellen van oogmelanoom uitgezaaiingen een hoge expressie en hormoonbinding vertoonden. Dit suggereert een mogelijk verband tussen SSTR2 expressie en oogmelanoom progressie. Uit analyse van klinische en moleculaire kenmerken bleek echter geen relatie met uitzaaiingen.

Een andere benadering om tumorspecifieke peptiden te identificeren is door random peptiden te screenen voor oogmelanoom penetrerend vermogen. In *Hoofdstuk 8* wordt de identificatie van oogmelanoom geassocieerde peptiden (UMAPs) beschreven. In de toekomst kunnen deze UMAPs klinisch gebruikt worden om micro-uitzaaiingen op te sporen. Daarnaast kunnen dezelfde peptiden worden gebruikt in gerichte therapie om metastasen te voorkomen danwel te vertragen. Of deze peptiden klinisch gebruikt kunnen gaan worden is afhankelijk van de specificiteit waarmee oogmelanomen gedetecteerd en behandeld kunnen worden. Voorlopige analyse op cellen laat een beperkte mate van specificiteit zien maar alleen analyse in een diermodel kan hier uitsluitsel over geven.

UMAP2 vertoonde een sterke voorkeur voor de cellen van het gemetastaseerde oogmelanoom. De sequentie van UMAP2 werd geïdentificeerd als een homoloog van de rat ' insulin-like growth factor receptor' (IGF-1R) waarvan al aangetoond is dat de expressie in oogmelanomen geassocieerd is met uitzaaiingen. IGF-1R is bovendien betrokken bij tumorgroei; IGFR signalering verloopt via de mitogeen-activated protein kinase (MAPK) route. UMAP2 is daarmee ook uitermate geschikt om de mechanismen die betrokken zijn bij celgroei en tumorprogressie te bestuderen.

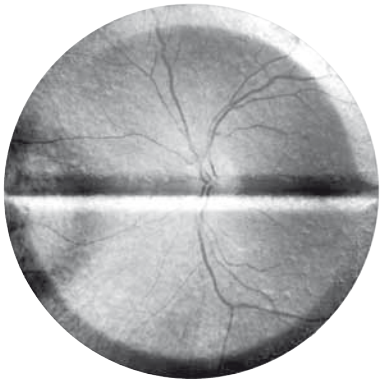
Tumor signalen en behandelingopties

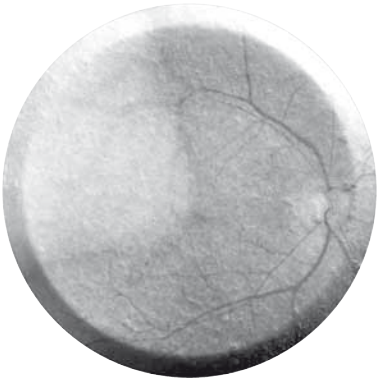
Activering van de RAS-RAF-MEK-ERK, of klassieke mitogeen-activated protein kinase (MAPK) route, is in verband gebracht met melanomen en vele andere soorten kanker. Kennis van deze route en de moleculaire mechanismen die ten grondslag liggen aan activering van deze route is nodig om de werkzaamheid van behandelingen te voorspellen. Voor oogmelanomen worden mutaties in het GNAQ en GNA11-gen verantwoordelijk gehouden voor MAPK activering en deze mutaties komen voor in een overgrote meerderheid. Src kinase is een mogelijk tussenstation voor mutaties en MAPK activering. In *Hoofdstuk 9* wordt de rol van Src bij oogmelanoom beschreven en hoe dit samenhangt met tumorgroei. Op basis van de belangrijke rol van Src in de MAPK proliferatie, is remming van Src overwogen als behandeloptie. Het commercieel beschikbare Dasatinib, een tyrosine kinase remmer van de Src-kinase familie, kan de oogmelanoom celgroei inhiberen in 60% van de geteste celculturen (*Hoofdstuk 10*). Verdere analyse wees uit dat basale Src kinase-activiteit en verlies van chromosoom 3 voorspellend zijn voor het behandelingseffect. Tyrosine kinase-remmers, zoals Dasatinib, zijn interessant vanwege hun vermogen om op meerdere kinasen gericht te zijn en hebben dus een brede toepassing in verschillende maligniteiten. Helaas kan dit ook leiden tot "off-target"-effecten aangetoond in verschillende klinische studies. Tumorcel 'targeting' met specifieke liganden tegen specifieke cellulaire componenten, zoals Src, die een belangrijke rol speelt bij oogmelanomen, bieden nieuwe behandelingsmogelijkheden en de afname van negatieve bijwerkingen.

ACKNOWLEDGEMENTS

CURRICULUM VITAE

LIST OF PUBLICATIONS





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منذ كنت صغيرة، و أنتم تبذلون كل جهدكم من أجل دراستي،
أعطيتموني الحب و الثقة بالنفس، علّمتموني المعنى الحقيقي للحياة،
منذ أن أصبحت امّاً و أنا ادرك يوما بعد يوم عظمة تضحيتم من أجل تربيتي أنا و إخوتي،
أسأل أالله أن يرشدني لكي أتّبع كامل خطاكم يا خير من نصح و أرشد
سأبقى أدين لكم بكل نجاح طالما حييت
لكم كلّ حبي ..

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CURRICULUM VITAE

The author of this thesis was born on January 19th, 1982, in Leidschendam, The Netherlands. She attended high school at the Erasmus College in Zoetermeer and started her medical training at Leiden University in 2000. In 2005, the author performed a short research project at the departments of Ophthalmology, Nuclear Medicine and Human Genetics of the Erasmus MC under supervision of Prof. Dr. Gré P.M. Luyten. This resulted in a successful publication and stimulated her enthusiasm for further research in ophthalmology. After receiving her M.D. degree in 2006, she started with her Ph.D at the department of Ophthalmology at the Leiden University Medical Center under supervision of Dr. Martine J. Jager and later on also of Dr. Pieter van der Velden. The author presented her work in Europe, the Middle-east and the United States at several ophthalmological conferences. She received an award from Santen pharmaceuticals at the annual meeting of the Association for Research in Vision and Ophthalmology 2008 for her poster presentation.
In March 2010, the author commenced her residency in Ophthalmology under the supervision of Prof. Dr. Gré P.M. Luyten.

LIST OF PUBLICATIONS

1. M. el Filali, S.F.T.M. de Bruijn. 'Neuro-Imaging, uw diagnose? Tijdschr Neurol Neurochir 2007;108:300-1.

2. M. el Filali, E. Kilic, M. Melis, A. de Klein, M. de Jong, G.P.M Luyten. Expression of the SST receptor 2 in uveal melanoma is not a prognostic marker. Graefes Arch Clin Exp Ophthalmol. 2008 Nov;246(11):1585-92.

3. M. el Filali, I. Homminga, W. Maat, P.A. van der Velden, M.J. Jager. Triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth. Mol Vis. 2008 Sep 24;14:1752-9.

4. W. Maat, M. Filali, A. Dirks-Mulder, G.P.M. Luyten, N.A. Gruis, L. Desjardins, P. Boender, M.J. Jager, P.A. van der Velden. Episodic Src activation in uveal melanoma revealed by kinase activity profiling. Br J Cancer. 2009 Jul 21;101(2):312-9

5. M. el Filali, G.S. Missotten, W. Maat, L.V. Ly, G.P.M. Luyten, P.A. van der Velden, M.J. Jager. Regulation of VEGF-A in uveal melanoma. Invest Ophthalmol Vis Sci. 2010 May;51(5):2329-37.

6. M.J. Jager, L.V. Ly, M. el Filali, M.C. Madigan. Macrophages in uveal melanoma and in experimental ocular tumor models: Friends or foes? Prog Retin Eye Res. 2010 Dec; 30: 129-146

7. M. el Filali, P.A. van der Velden, M. J. Jager. Anti-angiogenic therapy in uveal melanoma. Dev Ophthalmol. 2012;49:117-36.

8. M. el Filali, L.V. Ly, G.P.M. Luyten, M. Versluis, H. E. Grossniklaus, P.A. van der Velden, M.J. Jager. Bevacizumab and intraocular tumors: an intriguing paradox. Molecular Vision 2012 in press

9. M. el Filali, M. Versluis, I. Bronkhorst, A. Baghat, G.P.M. Luyten, M. J. Jager, P.A. van der Velden. Src kinase is associated with MAPK activation and monosomy 3 in uveal melanoma and can be inhibited by Dasatinib. Article submitted

10. M. el Filali , R. Olsthoorn, M.J. Jager, G.P.M. Luyten. Uveal melanoma associated peptides identified with a phage display peptide library and in vitro panning. Article submitted