Angiogenesis and screening in uveal melanoma

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GENERAL INTRODUCTION TO UVEAL MELANOMA

Epidemiology

Uveal melanoma is the most common primary intraocular tumour in adults. It has an annual incidence of 6-8 per million subjects per year in Caucasian populations. Its incidence is 15 to 50 times lower in Africans and Orientals.[1-2] The incidence increases between ages 30 and 70 and most often this tumour is observed in the sixth decade of life.[3] In Leiden, between 2000 and 2004, 529 uveal melanomas were identified on a general population of 16.268.145 people; this would indicate an annual incidence of 6.5 per million. Assuming that 80% of uveal melanomas are identified in Leiden (and very large tumours that certainly have to be enucleated not), this would bring us to an incidence of 8.1 per million. The survival is poor, even when no clinically apparent metastases are found at the time of enucleation. Many patients have micrometastases at the time of treatment of the primary tumour but the presence of such micrometastases cannot be determined with current imaging techniques or blood tests.[4-5] In Leiden, the 5-year and 10-year tumour-related survival rates are 59% and 72% respectively (Chapter 2). This tumour is rare in comparison with other types of cancer, and the interest in this tumour has been limited worldwide. The World Cancer Report, edited by the International Agency for Research on Cancer of the WHO, does not even mention eye cancer. This is surprising when we realize the unique position and research importance of this tumour: the lack of lymphatic vessels and the purely haematogenous way of spreading, the lack of mutations in growth-inhibition pathways,[6] the lack of genetic inheritance, the role of ocular immune privilege, and the homogeneity of the tumour. All of these findings turn this type of cancer into an ideal model to learn more about cancer and its basic mechanisms.[7] Furthermore, as there is hardly any way to treat metastases, further research is essential to improve patient survival.

Melanocytes in the human eye

Embryologically, the uveal tract, which consists of the choroid, ciliary body and iris, is derived from neuro-ectoderm, in which vascular channels have developed. As uveal melanomas are very rare in the iris, and seem to have a different behaviour there, this thesis is limited to melanomas in the ciliary body and choroids. Melanocytes are derived from the neural crest cells and determine the pigmentation of the choroid. In the foetus, melanosomes, pigmented granules marking melanocytes, can be recognised as early as the seventh month, especially in the region of the outer choroid and supra choroid.

In the mature eye, melanocytes occur in the choroidal stroma and provide its brown colour. Melanocytes contain fine pigment granules (melanosomes), 0.3-0.4 μm wide, oval

in shape, yellowish to dark brown in colour, and always smaller than those of the retinal pigment epithelium (which are up to 1 µm in diameter and 2-3 µm in length). Choroidal melanocytes form an almost continuous layer in the outer choroid, spreading in the plane of the choroidal space and forming a thin three-dimensional network. Cell numbers vary regionally with age, race and general pigmentation: they are most numerous around the optic disc, less so in the periphery and in the inner choroid. They outline vessels, including the veins and ampullae of the venae vorticosae.[8]

Although uveal melanocytes are of the same embryologic origin as cutaneous melanocytes, uveal melanomas seem to have a totally different incidence, prevalence, and metastatic behaviour than skin melanoma. They are also very different compared to conjunctival melanoma or iris tumours.

Aetiology of uveal melanoma

Characteristics influencing the occurrence of uveal melanoma are race, sun exposure and hormones.
Uveal melanomas increase with fair iris colour (elevated risk for blue or grey irises)[9-10] and red or blond hair. There is an elevated risk for Northern European ancestry as compared to Mediterranean heritage; this suggests some genetic influence.[11] In America, we see a higher risk for persons born in the southern United States as compared with those born in the North.[12]

No definite relation could be demonstrated between uveal melanoma development and UV exposal. In fact, only a small percentage of UV-rays actually reaches the choroid.[13] Few data are published on the relation between summer sun exposure,[14] sunburn of the eye or snow blindness and an increased risk for melanoma.[15] Some studies predict an increased risk for outdoor work[16-17] while other studies predict a lower risk.[11] The fact that the incidence for uveal melanoma has remained stable during the past 25 years, in contrast to conjunctival and skin melanoma, is an argument against a major impact of sun exposure.[18-19]

No hormonal influence on this tumour has been demonstrated.[19] One study observed a decreased risk of uveal melanoma for women who had ever been pregnant [relative risk (RR) = 0.60, 95% confidence interval (CI) = 0.37 -0.95]; with an increase of this protective effect with more live births.[20]

Localisation of the tumour

Most uveal melanomas occur in the choroid (80%), fewer in the ciliary body (15%) and the iris (5%). Nevertheless, uveal melanomas in the ciliary body have a worse prognosis than iris or choroidal uveal melanoma.[21-22] In this thesis, iris melanomas were excluded.

Prognostic factors in uveal melanoma

Many clinical and histo-pathological factors have a proven prognostic value in uveal melanoma. The major factors are: tumour diameter, tumour thickness, cell type (epithelioid cells carry a bad prognosis), number of mitoses, microvascular density, presence of PAS-positive patterns and monosomy of chromosome 3.[23-24] Other potential prognostic factors are the presence of a retinal detachment,[25] glaucoma, nucleolar diameter,[26] HLA expression,[27-28] the presence of macrophages,[29] necrosis, vascular invasion, and extra-scleral extension.[30-31] Many of the above mentioned parameters are interrelated.

Treatment of uveal melanoma

In the past, instant enucleation was the treatment of choice for uveal melanoma. In the last decades eye-sparing treatments were developed, like brachytherapy with ruthenium (introduced in Leiden in 1981), radioactive iodine or strontium, local resection, proton beam irradiation and thermotherapy. Enucleation is still the treatment of choice for large tumours, and for a small number of tumours insufficiently responding to brachytherapy, or recurring after the initial treatment. Enucleation is also considered when complications as neovascular glaucoma or total loss of sight occur.[32]

Brachytherapy is nowadays carried out both successfully and safely on a routine basis. The COMS study has demonstrated that survival rate after irradiation is not inferior to immediate enucleation[33]. Brachytherapy is applied using radioactive palladium 104, cobalt 60, strontium 90, ruthenium 106 or iodine 125 plaques.[34-37]
Another form of irradiation is proton beam irradiation.[31, 38] This technique is a potentially eye-saving treatment of very large tumours or tumours near the optic disc.
Thermotherapy is applied alone or in combination with brachytherapy. It reduces the amount of irradiation needed to treat the tumour and induces a quick flattening of the tumour. It is applied transpupillary using an 810 nm diode laser.[39-41]

Recently, stereo-tactic irradiation has been introduced for treatment of large tumours of the ciliary body or as an alternative to proton beam irradiation. No long-term results with this technique are available today.[42-43]

All treatments result in a good control of the primary eye tumour, with low recurrence rates. The main problems and complications are loss of sight, cataract, vitreous haemorrhage, and the formation of irradiation neuropathy or retinopathy, sometimes resulting in neovascular glaucoma.[44-46]

Treatment of uveal melanoma metastases

Uveal melanoma metastases occur in liver (56-90%), lung (31%), bone (7-23%) and skin (17-36.5%).[47-50] At the time of diagnosis of metastases, 60% of patients have liver metastases and post mortem examination revealed a 90-100% incidence.[49] A few studies have reported cardiac metastases,[50-51] breast metastases,[52] brain,[53] contralateral choroid[54-55] and regional lymph nodes.[56]

No effective treatment for uveal melanoma metastases has been found yet, although isolated liver perfusion and resection of isolated liver metastases show some promise for selected cases. Surgery of the liver is only indicated if the metastases are limited in quantity without a diffuse pattern, sparing at least one lobe of the liver. In these cases, a complete resection is possible in 27.5% of the patients.[57]

General chemotherapy has been applied in a limited number of patients, usually with DTIC (dacarbazine), a chemotherapy agent widely used for cutaneous melanoma. The rate of responses is very low, in the order of 5%, and median survival is not longer than 4 to 9 months.[58-65] Nevertheless, the site of metastases influences the success rate of this intravenous treatment: patients without liver metastases seem to have a far higher response (up to 43%).[66]

Chemo-immunotherapy includes therapy based on interferon, interleukine 2, or BCG. Some ocular melanoma patients were included in cutaneous melanoma chemotherapy protocols[67-70] with limited success. This therapy seems to prolong life, about five to seven months, but reports are sporadic and include very few patients.

Some treatments are focused on the liver, as this is the main locus of metastasis in this tumour. Different strategies are used: chemo-embolisation, intra-arterial liver chemotherapy and liver perfusion. Chemo-embolisation is the administration of a chemotherapeutic agent together with polyvinyl particles. This gives a longer exposition of the tumour to the agent. Mavligit et al.[71] demonstrated significant regression of metastatic masses and longer survival in some patients.

Intra-arterial liver chemotherapy (delivered through the arteria hepatica) also leads to longer hepatic exposition to the chemotherapeutic agent. Mostly used agents with this technique are Fudr, BCNU (carboplatin-based chemotherapy), doxorubicin, cisplatin and fotomustin.[72-74]

Isolated liver perfusion is a local treatment of the liver. The vascular system of the liver is isolated from the rest of the body and the liver is perfused extra-corporally. Hyperthermia between 39.5 and 40 °C in combination with different agents (interferon-γ, TNF, and melphalan) has also been used. This treatment is limited to patients with a relatively good general health and with few large liver metastases.[75-78]

Immunotherapy is still experimental and based on stimulating the immune system, often the cytotoxic T cells. Vaccination based on different melanoma tumour antigens (e.g. MAGE, Melan-A, gp100, tyrosinases, NA17) has been studied in a limited number of patients,[79-81] and a specific T-cell response could be found after vaccination.

AIMS OF THIS THESIS

To improve patient survival in uveal melanoma, it is necessary to investigate the aetiology, basic mechanisms (genetics, immunology, and angiogenesis) of tumour growth and metastases, and to develop tests for early recognition of metastases and open new avenues for treatment of metastases.

In this thesis, we focussed on two important subjects of study in uveal melanoma: 1) angiogenesis and angiogenic growth factors, and 2) to find early markers in serum and aqueous fluid of uveal melanoma eyes which may allow for screening of micrometastases or detecting local recurrence.

Angiogenesis in uveal melanoma

Introduction on Angiogenesis

The importance of angiogenesis (the formation of new vessels out of existing vessels) for tumours has been recognized for centuries. Already in 1619, Sir W. Harvey (1578-1657) treated a tumour by depriving it of its blood supply[1]. Since the 1970s, J. Folkman and coworkers really started to work on angiogenesis, making Folkman the founder of the field of angiogenesis research. He has made several discoveries on the mechanisms of angiogenesis, which have opened a field of investigation now pursued worldwide. Dr Folkman's hypothesis[82] that solid tumours are angiogenesis-dependent, initiated studies of angiogenesis in tumour biology and in disciplines as diverse as developmental biology, ophthalmology and dermatology. His laboratory reported the first purified angiogenic molecule, the first angiogenesis inhibitor and proposed the concept of angiogenic disease. All of these discoveries have been translated into clinical trials involving over 30 angiogenesis inhibitors.

Angiogenesis in uveal melanoma

If a tumour starts to expand, the need for oxygen and nutrients increases. To fulfil this need, a first pathway can be the incorporation of existing vessels into the tumour. Secondly, the development of newly formed vessels by sprouting from pre-existing vessels can be induced (angiogenesis). It is likely that incorporation of choroidal vessels occurs in the early growth of uveal melanoma. It is unknown however, but most likely, that uveal melanomas are dependent on angiogenesis for growth beyond a certain size.

The mechanism and induction of angiogenesis

Hypoxia is an important regulator of angiogenesis. Vascular Endothelial Growth Factor (VEGF-A) is strongly upregulated by hypoxia, EGFR stimulation and c-erb stimulation.[84] Hypoxia upregulates the transcription factor hypoxia-inducible factor (HIF)-1 alpha, which binds to the VEGF-A promoter, thus inducing transcription of VEGF-A.[85]

Similar to VEGF-A, the VEGF-R1 promoter contains a HIF-1-binding sequence, providing a direct mechanism for the upregulation of VEGF-R1. In contrast, VEGF-R2 does not show such a binding sequence, suggesting that the hypoxia-induced upregulation of VEGF-R2 proceeds via an indirect yet unknown mechanism such as induction via VEGF-A itself or posttranscriptional activation. In response to hypoxia, the increased levels of VEGF-A increased VEGF-R2 expression-induced angiogenesis.[86]

Oxygen can be considered a repellent of angiogenesis, whereas the lack of oxygen is a strong attractant for vessels sprouts. In cells beyond the oxygen diffusion limit around a vessel, hypoxia activates hypoxia-inducible transcription factors (HIF) that turn on the expression of angiogenic genes, such as VEGF, which induces vessels to branch towards the hypoxic tissue.[87] Little is known about the role of hypoxia in uveal melanoma. Folberg et al.[88] demonstrated that uveal melanoma rarely demonstrate necrosis, although it does occur occasionally. We found expression of HIF-1α, an ischemia induced protein, in 90% of uveal melanomas, strongly supporting a role of hypoxic signalling in these tumours (unpublished results).

Cytokines involved in angiogenesis of uveal melanoma

A large number of angiogenic cytokines have been described. As in other tumour types, one can speculate that a complex interaction of these cytokines is responsible for the induction and maintenance of angiogenesis in uveal melanomas.

VEGF family

Vascular Endothelial Growth Factor is one of the major known angiogenic factors. Today four VEGF factors are known: VEGF-A, -B, -C, -D and one viral VEGF homologue known as VEGF-E. VEGF-A has been found in five different isoforms with different protein weights (121, 145, 165, 189 and 206 AA). These cytokines bind with varying affinity to three receptors: VEGF-R1 (Flt1), -R2 (KDR), and VEGF-R3 (Flt-4) and one soluble receptor sVEGF-R1.

VEGF-A

VEGF-A is the principal actor influencing angiogenesis and vasculogenesis, mainly through VEGF-R2. It is produced by cells in close contact to endothelial cells (EC), suggesting paracrine regulation of blood vessel formation; VEGF-A is also secreted by the EC themselves. It exerts a direct effect on EC via interaction with the cellular receptors VEGF-R1 and VEGF-R2 and induces EC to proliferate, to migrate, to assemble into tubes, and to survive.[85] VEGF-A has a close relationship with hypoxia and ischemia. It increases the expression of the NO synthase enzyme in endothelial cells, inducing angiogenesis.[89-92] VEGF-A could have an as yet unidentified effect on the melanoma cells themselves.[85]

There is still discussion whether VEGF-A has a major role in angiogenesis of uveal melanoma. Findings in favour of a role of VEGF-A in uveal melanoma are mRNA and protein expression by uveal melanoma cells, as well as the increased presence of VEGF-A in aqueous and vitreous of uveal melanoma eyes.[93] In addition, Niederkorn et al.[94] showed an effect for the VEGF-A-reducing therapy anecortave acetate in an anterior chamber tumour in mice.

Immuno-histochemical staining techniques could only show a modest expression of VEGF-A in uveal melanoma itself, although in situ hybridisation showed a clear mRNA expression[95] (Chapter 3). VEGF-A influences angiogenesis mainly through VEGF-R2 and to a lesser extent through VEGF-R1. Expression of both receptors was found in the vasculature of uveal melanoma (Missotten, data not published), and was associated with areas of increased microvascular density.

However, VEGF-A is also reported to have a major influence in developing and sustaining retinal neovascularisation as well as neovascularisation of the iris. As such, an anti-VEGF-A therapy could be a useful tool in the management of some secondary complications of uveal melanoma treatment, especially iris neovascularisation and neovascular glaucoma. Specifically in eyes treated with proton beam irradiation, Boyd[93] and our group both found a very high expression of VEGF-A (Chapter 3).

Experimental therapy of retinal neovascularisation with VEGF and VEGF-receptor inhibition has been successful, both in mice and man.[96-97] Clinical trials with anti-VEGF drugs are being or have been conducted for diabetic retinopathy and choroidal neovascular membranes and have recently been granted FDA approval for the treatment of age related maculopathy.

VEGF B

The function of VEGF-B is comparable with VEGF-A, although it is a less potent angiogenic factor. VEGF-B is not induced by hypoxia, in contrast to VEGF-A. A high expression of VEGF-B has been found both in uveal melanoma cell lines and primary tumour tissue (Chapter 4).

VEGF-C

VEGF-C was first known as the lymphangiogenesis-inducing factor. Nevertheless, it has also a major role in angiogenesis induction[98] and stimulates migration and proliferation of endothelial cells.[99] VEGF-R3, the main receptor for VEGF-C, is reported to be upregulated in angiogenic blood vessels near to tumour cells that expressed VEGF-C, which suggests that VEGF-C secreted by the tumour cells acts as a paracrine angiogenic growth factor, not only for lymphatic but also for blood vessels.[100] VEGF-R3 has been demonstrated to be upregulated in human cutaneous melanoma even on angiogenic vessels, where in normal skin tissue only lymph vessels stain for VEGF-R3.[101] In uveal melanoma, a high expression of VEGF-C was found on tumour tissue[102] (Missotten, data not published), in combination with a very high expression of VEGF-R3 on tumour vessels[103] (Missotten, data not published). The high expression of VEGF-C and VEGF-R3 in uveal melanoma suggests a major role for this cytokine in uveal melanoma. In addition, Seftor and coworkers[104] demonstrated that more invasive tumours expressed more VEGF-C. Clarijs suggested a role for VEGF-C in recruiting macrophages that would induce fluid-conducting spaces in the stromal tissue of the tumour.[103]

VEGF D

The role of VEGF-D in angiogenesis is less clear. It seems to have a similar function as VEGF-C, and is involved in lymphangiogenesis.[99] A low expression of VEGF-D was demonstrated in uveal melanoma (Chapter 4).

Other major angiogenic molecules

P1GF

Placental Growth Factor (PlGF)[105] attaches to VEGF-R1 and sVEGF-R. PlGF works by amplifying the angiogenic activity of VEGF-A.

basic Fibroblast Growth Factor bFGF

IJland et al.[106] demonstrated a high expression of basic Fibroblast Growth Factor in uveal melanoma cell lines. Boyd and co-workers confirmed this in uveal melanoma eyes.[93] The results make further research of the role of this angiogenic factor in uveal melanoma necessary.

Hepatocyte Growth Factor/Scatter Factor (HGF/SF)

Although no HGF/SF expression was found in uveal melanoma, its receptor c-met was found.[32] HGF/SF has a role as promoter of angiogenesis.[108] HGF/SF also gives a mitogenic response and would be a key step in local invasion and targeted dissemination to the liver. C-met expression was high in uveal melanoma with co-expression of vimentin and keratin. Uveal melanoma cell cultures that co-express vimentin and keratin were found to be sixfold more invasive through collagenous extracellular matrices in vitro, compared with uveal melanoma expressing vimentin only.[107] The role of HGF/SF in angiogenesis remains to be demonstrated, however.

PEDF

PEDF is a VEGF antagonist. Hypoxia downregulates the expression of PEDF, and it is postulated that proliferative retinopathies would be associated with decreased expression of PEDF causing a disequilibrium between promotion and inhibition of angiogenesis and allowing pro-angiogenic factors as VEGF to induce neovascularization.[109] Until now, expression of this factor has not been investigated in uveal melanoma.

Microvascular density in uveal melanoma

Since the introduction by Folkman in 1970 the most widely used method to assess angiogenesis in human neoplasm is the quantification of the microvascular density (MVD) of primary tumours by using specific markers for endothelial cells, such as factor VIII-related antigen, CD31 (platelet/endothelial cell adhesion molecule/PECAM), and CD34. In several tumour types it has been shown that MVD is not only significantly associated with prognosis, but also with anti-cancer therapy success.[110] In uveal melanoma, different results have been reported, depending on the antibodies used and the area analysed (Table 1). Association with a worse prognosis was demonstrated using CD34[111-112] and Factor VIII.[113] Schaling et al.[114] could not demonstrate a relation between MVD and prognosis; this may be due to a limited number of samples used in his study.

Author	Antibody *(n)*	Results
Foss et al. 1996[113]	Factor VIII *(123)*	MVD FVIII is an important prognostic factor in hot spots (0.25 mm^2). In a multivariate model only MVD and tumour size were entered.
Schaling et al. 1996[114]	Factor VIII *(40)*	MVD FVIII = 1-12.5 vessels / 0.216 mm^2 in randomly chosen areas. This MVD FVIII was not correlated with prognosis.
Lane et al. 1997[115]	UEA-I *(63)*	Total vessel counts did not correlate with prognostic factors. No prognostic significance for MVD_{UEA-I}.
Makitie et al. 1999[111]	CD34, SMA, Factor VIII *(134)*	MVD_{CD34} = 5-121 vessels / 0.313 mm^2 in hot spots. MVD_{CD34} and FVIII are independent predictors of prognosis. MVD_{SMA} is not related to prognosis.
Chen et al. 2002[112]	CD34 *(200)*	MVD_{CD34} is a prognostic factor, when counted in hot spots. Co-localisation with Melan-A and S100 protein suggest not an exclusive measure of tumour vascularity. CD34 also labels melanocytes.

UEA-I = Ulex Europaeus agglutinin I. SMA = alpha-Smooth Muscle Actin.

Table 1. Microvascular density in uveal melanoma

Lymphangiogenesis

Clarijs and co-workers investigated lymph vessels in uveal melanoma. No indications were found for lymphangiogenesis in this tumour, but this could not be ruled out completely using CD31, PAL-E and VEGF-R3 staining. Absence of lymph vessels was also confirmed by the lack of Prox-1 expression (Figure 1).

Although it seems unlikely that lymph vessels are present in the eye, we should acknowledge that lymph vessels are still very hard to demonstrate. Some articles state that in solid tumours, tumour lymphangiogenesis is a major component of the metastatic process.[116] In contrast, other articles report that functional lymphatics are absent within tumours but in contrast enlarged at the periphery.[117] An explanation would be that neoplastic cells that grow in a confined space generate mechanical stress, which may compress the newly formed lymphatic channels inside the tumour, whereas at the periphery, excess VEGF-C could cause lymph vessels to enlarge.[118]

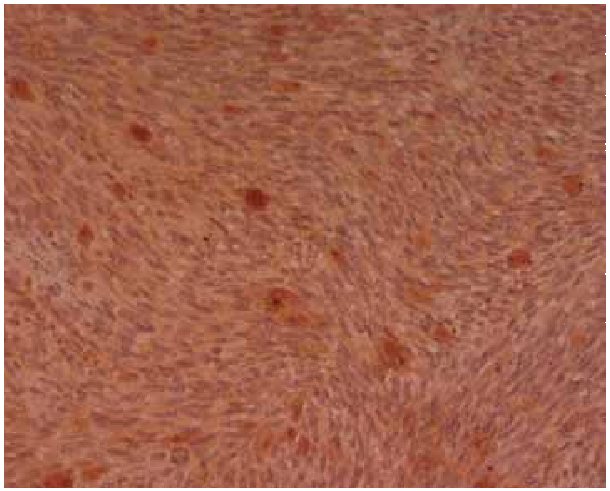

Figure 1. Lack of expression of Prox-1 in uveal melanoma

The lack of lymph vessels is also supported by clinical experience: whereas conjunctival melanomas and sometimes iris melanomas with deep penetration in the ciliary body do metastasize to local lymph nodes, uveal melanomas do not. In cases of human cancer, the occurrence of lymphangiogenesis during tumour development and progression has not been demonstrated clearly. However data support a role for VEGF-C in promoting dissemination of metastases to the lymph nodes.[119]

Detection and screening for uveal melanoma

Five years survival in uveal melanomas is only 57-60% for large tumours (>2 mm in apical height and >10 mm in basal diameter).[120-121] In the last decades, new treatment modalities have been used to improve survival including systemic chemotherapy[122] or immunotherapy. As the liver is frequently involved in uveal melanoma metastasis, and hepatic metastasis reduces the life expectance significantly,[123] therapy has also been focused on treatment of isolated liver metastasis. Positive effects are reported recently with isolated liver perfusion chemotherapy.[123-125] As such, a screening for early liver metastases becomes more relevant.
It is suggested that at the moment of enucleation, all or most uveal melanomas have already spread haematogenously.[126] Nevertheless, only half of these patients also develop macroscopic metastases in later life. It is assumed that at different locations in the body, the tumour stays 'dormant', probably in liver and/or bone marrow.[127-129] The present challenge is to identify and treat metastases early.

Presently, screening is advised to be (semi-) annually with chest x-ray, liver ultrasound and/or liver function tests, especially in high-risk melanomas [124,130-131] (Table 2).

COMS Study [75]	Leiden Screening Protocol
Inclusion criteria: Greater than 10 mm in apical height or at least 16 mm in diameter, and those measuring between 8-10 mm in apical height that were too close to the optic nerve for optimal radiotherapy.	**Inclusion criteria:** Larger than 6 mm in apical height or at least 12 mm in diameter. All tumors within one disk diameter of the optic nerve. Healthy constitution of the patient; > 70 years.
Initial metastases screening: – Systematic evaluation (clinical and laboratory examination) – Chest x-ray – Liver function tests	**Initial metastases screening:** – Systematic evaluation (clinical and laboratory examination) – Chest x-ray – Liver function tests (LDH and γGT) and S100B – Liver ultrasound
Follow up – Annual follow-up – Systemic evaluation – Chest x-ray – Liver function tests	**Follow up** – Semi-annual follow-up – Liver ultrasound – Liver function tests (LDH and γGT) and S100B
Additional tests if suspicion of metastases: – Liver ultrasound – FNAB – Liver CT	**Additional tests if suspicion of metastases:** – Liver CT – FNAB

Table2. Comparison of screening of large uveal melanomas at Leiden University, and as proposed by the COMS study. 5

Functional tests and serum markers for uveal melanoma and melanoma metastases

Liver function tests

So far, lactate dehydrogenase (LDH),[124] Alkaline Phosphatase (AP) and γGT (gamma glutamyl transpeptidase) have been found to be relevant in uveal melanoma metastases screening.[134] Kaiserman reported early detection of metastases using LDH, ALAT and γGT in 33 patients. Others have reported a general low sensitivity for liver function tests (LFTs).[130,135] One of the problems is that LFTs sometimes seem to rise, but within normal limits. This means that not absolute values, but relative changes should be monitored. Patients with extensive metastatic uveal melanoma with normal LFTs have also been reported.[136]

In general, higher LDH, AP and γGT are correlated with poor survival. The transaminases and bilirubin serum levels seem to be of limited use. Kaiserman showed that in 20% of cases LFTs could predict metastases earlier than liver ultrasound (US).[134]

S100

In cutaneous melanoma S100B has proved to be a valid marker for micro- and/or macro metastasis[37] and of highly prognostic importance.[138] It has a sensitivity rate of 80% in advanced metastatic cutaneous melanoma patients.[139]

In uveal melanomas, there is a high expression of S100B protein as investigated by immunohistochemistry.[140-141] However, serum S100B is not a prognostic marker in primary uveal melanomas at the moment of enucleation of large tumours (Chapter 6).[142] Nevertheless, S100B correlates better with metastases than liver function tests (LFT) or other serum markers (Missotten et al., submitted).

MIA

The melanoma inhibitory activity (MIA) protein has been found to be highly prognostic in cutaneous melanoma. Schaller demonstrated that MIA is not of prognostic value in uveal melanoma serum, but is highly upregulated once metastases are present.[143] Others confirmed this upregulation, but MIA levels could not predict metastasis earlier than the standard liver function tests (Missotten et al., submitted).

Carcino-embryonal antigen (CEA)

In 1976, Shields et al. demonstrated an increase of CEA in metastasized uveal melanoma. As CEA might be increased in many tumours, it has been rarely used in uveal melanoma screening.[144-145]

5-S-cysteinyldopa and 6-hydroxy-5-methoxyindole-2-carboxylic acid (melanin metabolites)

5-S-CD, an intermediate in melanin synthesis has been found to be of prognostic value in skin melanomas, both in urine and in serum. In uveal melanoma there is only one report on serum, aqueous and vitreous humour of 16 patients.[146] 5-S-CD was increased in metastatic patients. 5-S-CD is detected by high performance liquid chromatography (HPLC). HPLC is a rather laborious and certainly not a standard test in clinical chemistry laboratories. S100B, MIA and LDH have proved to predict prognosis better than 5-S-CD in cutaneous melanoma.[147-148] No research has been done yet on 6-H-5-MI-2-C in uveal melanoma. In cutaneous melanoma, 6-H-5-MI-2-C did not prove to be a better prognostic marker than S100B or 5-S-CD.[149]

Tumour specific T cells

All tumours are characterised by specific tumour-associated antigens and this holds also true for uveal melanoma. Specific melanoma-associated antigens are e.g. MART-1, Tyrosine (TRP-1), gp100, MAGE1, HMP/NG2, ß-catenin, BAGE, RAGE, GAGE, PRAME, ATP6S1, MUM-1, NA17-A.[150] Specific T cells against these tumour antigens may indicate a generalized tumour spread. In a non-metastatic patient, however, the tumour load is too low for antigens to be detected by ELISPOT (enzyme-linked immunospot). Estimations predict that no more than 40 specific T cells would be present among every 1 000 000 peripheral blood lymphocytes. Therefore these specific T cells are of limited use in evaluating tumour spread.[151] An additional problem is that the melanoma antigens are HLA-specific; this means that the same antigens cannot be used in the whole population (e.g. 40% of Caucasian population is HLA.A2 positive). Nevertheless, these techniques have proven to be very useful in monitoring tumour vaccination therapy.

Circulating Tumour Cells

The detection of tumour cells by PCR screening for upregulated tumour-associated antigens (e.g. tyrosinase) in patient serum, has been tested in many tumours.[152] In uveal melanoma, it was possible to detect as few as ten circulating melanocytes in five ml of blood with tyrosinase PCR.[153] It seemed of no clinical use, as all tested metastatic sera were negative;[154] these tests have low reliability and insufficient detection levels. Up to now they failed to predict the likelihood of developing metastatic disease in cutaneous melanoma.[155-156] Keilholtz et al found three uveal melanoma patients positive for tyrosinase mRNA in 21 patients, and two of these high risk patients (> 15 mm basal diameter) developed liver metastases within three months.[157] Future techniques in detecting circulating tumour cells may improve the current results with immunobead-based detection of cells.[158]

Aims of this thesis

The aims of this thesis are to develop a better understanding of the role of angiogenesis and angiogenic growth factors in uveal melanoma in relation to prognosis (Part I), and to develop new tests for screening and early detection of uveal melanoma in the eye and systemic circulation (Part II).

In a general study into prognostic factors, the influence of clinical and histopathologic parameters on the prognosis in uveal melanoma was examined in 600 eyes enucleated between 1973 and 2003 (**Chapter 2**). The influence of these factors was examined before and after the introduction of brachytherapy in our hospital (1981). We also analysed the number of secondary enucleations and their indications. This enabled us to propose a model to predict more accurately the survival of these patients. In **Chapter 3**, an important factor in tumour angiogenesis, Vascular Endothelial Growth factor, is examined in uveal melanoma eyes. Its presence in tumour tissue and its distribution in the tumour eye is examined, both in eyes after primary enucleation or secondarily after irradiation. All members of the VEGF-family were also determined in uveal melanoma cell line supernatants and primary melanoma tissue (**Chapter 4**). These factors were also demonstrated in intraocular tumours in a mouse model, following injection of human melanoma cell lines in murine eyes.

An important group of proteins for presentation of tumour-specific antigens and an alternative target for therapy are the heat shock protein family (stress-protein family). The expression of these proteins, both in primary enucleated and in eyes with prior radiotherapy, are determined in **Chapter 5**.

Part II starts in Chapter 6 that describes the value of one of these serum tests, S100B, at the time that metastases have formed. S100B is an important serum factor in the diagnosis of metastasised cutaneous melanoma.

In **Chapter 7**, the introduction of new techniques for the examination of the cellular proteome is discussed. In a small series of aqueous fluid samples of uveal melanoma patients, we analysed whether a distinction could be made between normal eyes and uveal melanoma eyes on the basis of their proteome.

Finally, conclusions drawn from above mentioned studies are summarized and put into perspective (**Chapter 8**).

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