Knowledge-based treatment in uveal melanoma

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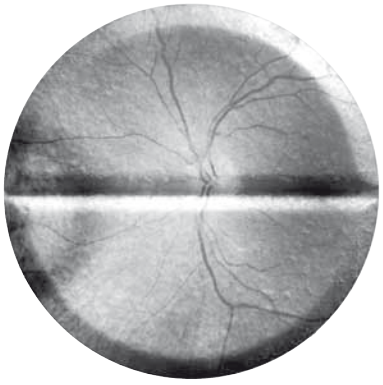
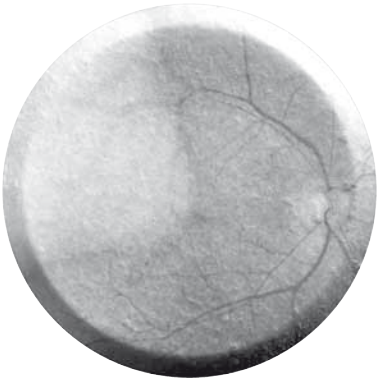
SUMMARY
AND
GENERAL DISCUSSION





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.

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