CHAPTER 2

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

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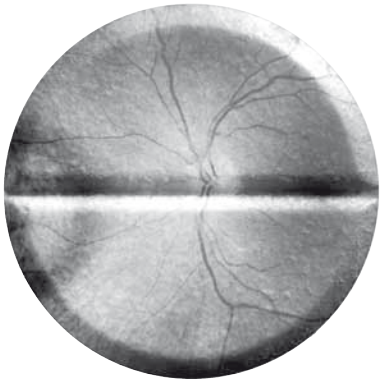

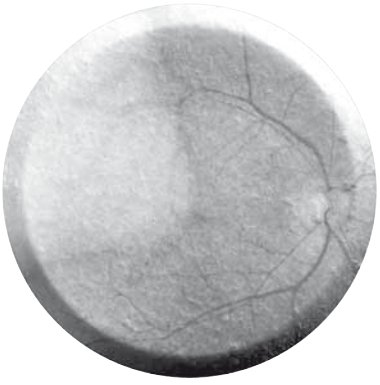

2.1 UVEAL MELANOMA AND ANGIOGENESIS

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

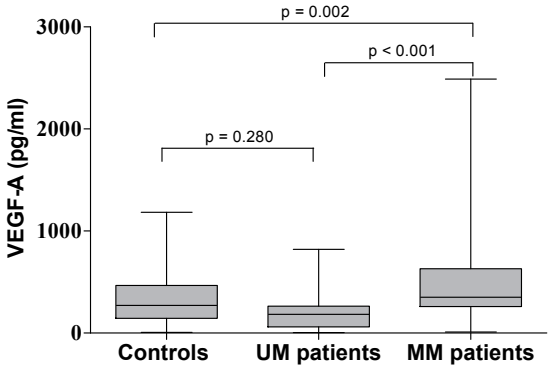

FIGURE 1. Concentration of serum VEGF-A in (metastatic) UM patients and controls.
Concentration of serum VEGF-A in the control group, and in patients with and without metastatic uveal melanoma. P-values (Mann–Whitney test) between the different groups are indicated in the graph. Each box shows the median, 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

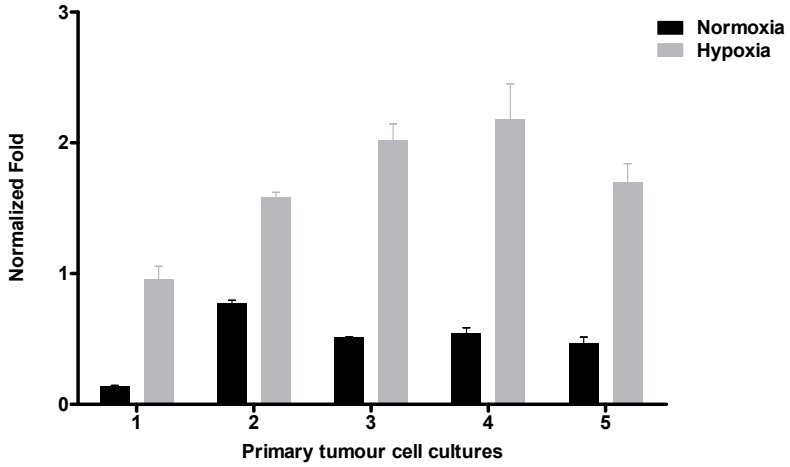

FIGURE 2. VEGF-A mRNA expression in primary UM cell cultures following exposure to hypoxia. The amount of VEGF-A mRNA expression was measured with quantitative real-time RT-PCR in primary UM cell cultures (cultures 1–5) under normoxic (black) and hypoxic (gray) conditions after 24 h. Expression is demonstrated 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

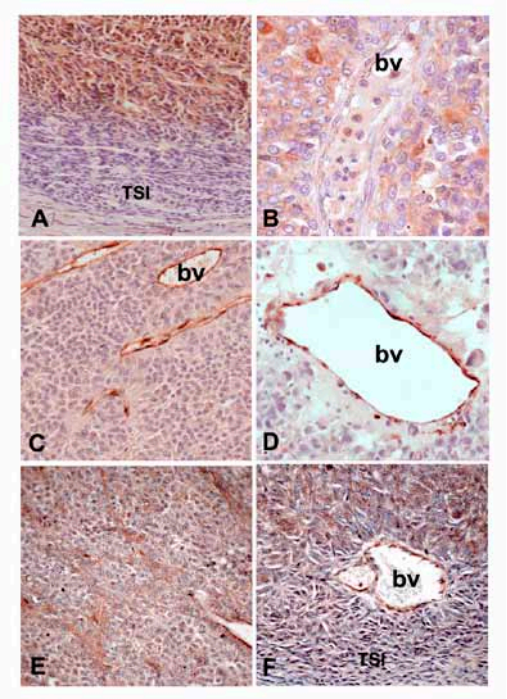

FIGURE 3. Immunoperoxidase labelling of MMP-1, -2 and -9 in uveal melanoma. A and B. Tumor cells immunolabelled for MMP-1, although not at the tumor-sceral interface (TSI). **B.** Tumor cells are also seen within a blood vessel (bv) **C and D.** Blood vessels (bv) show strong MMP-2 immunostaining, with 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).

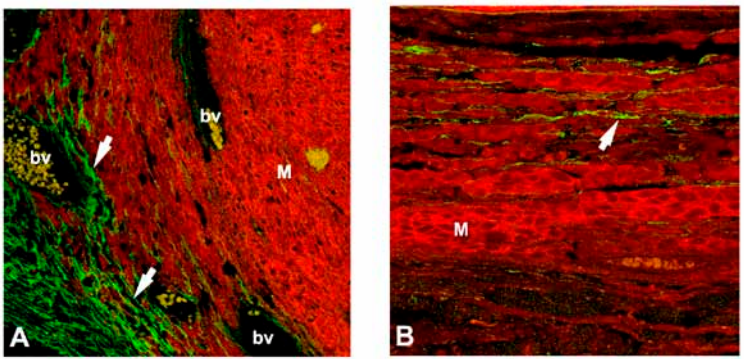

FIGURE 4. Co-immunolabelling for MMP-2 and EMMPRIN (CD147) in uveal melanoma, visualized using confocal microscopy. A and B.
Stromal cells (presumptive fibroblasts; arrowheads) near the tumour edge show strong MMP-2 immunostaining (green). Tumor cells (M) display distinct cell-surface EMMPRIN immunostaining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

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