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CHAPTER 1

General introduction

Parts have been used in
Inflammation in uveal melanoma.
INTRODUCTION

The link between inflammation and cancer

Cancer-related inflammation has been studied extensively. Inflammatory processes are present in many tumors, and influence the behaviour of malignant cells in many ways: a smouldering inflammation can contribute to angiogenesis and metastasis, but also to anti-tumor immune responses. Inflammation can impact reactions to chemotherapeutic agents. Several hallmarks of cancer have been defined, such as evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, tissue invasion and metastasis, and the more recently added inflammatory microenvironment. The seventh identified hallmark of cancer is Cancer-Related Inflammation (CRI), which is characterized by leukocyte infiltration and the presence of soluble mediators, such as cytokines and chemokines.1, 2 One would expect that the infiltration of leukocytes into tumors would lead to tumor cell elimination before the tumor becomes clinically apparent: however, this is often not the case. One of the major obstacles to anti-tumor activity of infiltrating leucocytes may be local immune suppression within the tumor microenvironment.3 This thesis summarizes what we know, and what we need to find out, about the role of inflammation in uveal melanoma. Particular emphasis is placed on the characterization of the unique features of inflammation in the tumor microenvironment, and the relation with other factors that are critical in the progression and metastatic spread of uveal melanoma cells. This general introduction will give an overview of uveal melanoma, its immunology, and the role of intratumoral immune infiltrates and cytokines in this malignancy.

Uveal melanoma

Uveal melanoma is a rare tumor, nevertheless, it is the most common primary intraocular malignancy in adults. It is called melanoma, as it originates from the melanocytes in the eye. Melanocytes can be found all over the body, producing the pigment melanin. Since the same type of cell is involved in cutaneous melanoma, one might think that ocular melanoma is related to melanoma of the skin; however, they are epidemiologically, genetically, and prognostically distinct. For instance, initiating pathways differ completely, as in cutaneous melanoma, BRAF gene mutations are initiators of malignancy, while in uveal melanoma, this role is played by mutations in the GNAQ and GNA11 genes.4-8 Such mutations activate biochemical pathways that induce cell division.

Uvea is the Latin word for ‘grape’, and this is what it looks like when you peel off the cover of the eyeball. It consists of three parts: the choroid, the ciliary body and the iris. All three parts can give rise to melanomas. People with ocular melanoma usually notice a problem such as blurred or distorted vision, and sometimes the melanoma causes a dark spot, cloud, or area of obscurity in the field of vision. Melanomas can be effectively treated
with eye-conserving treatment approaches, such as Ruthenium or Iodine plaque brachytherapy, laser treatment, stereotactic radiotherapy or proton beam radiotherapy. It is necessary to understand the patient's needs, with respect to tumor control, visual conservation, and preservation of the eye, so as to prioritize outcomes accordingly. If none of these forms of conservative therapy is appropriate, then enucleation is advised. Ocular melanoma metastasizes hematogeneously. Metastatic disease develops in up to 50% of patients, and usually involved the liver. Reported median survival time after diagnosis of liver metastasis ranges between 4 and 12 months. Treatment only rarely prolongs survival, because metastases are highly resistant to most chemotherapeutic agents and because they are not usually resectable. In conclusion, uveal melanoma arises in pigment cells, and on the one hand endangers vision, and on the other, is a malignant disease that may endanger life.

**Prognostic factors**

One of the factors that has been demonstrated to be of prognostic significance is a large tumor size (as measured by largest tumor diameter and thickness). A Tumor Node Metastasis (TNM) staging classification of uveal melanoma was originally developed by the American Joint Committee on Cancer (AJCC), and was revised in 2010 (figure 1). By combining size with tumor involvement of the ciliary body, and extrascleral extension (two other parameters associated with a poor prognosis), one can classify patients into prognostic groups.

However, it is difficult to exactly predict clinical outcome in individual cases of uveal melanoma on the basis of intraocular tumor size because of the spectrum of clinical, morphologic, and cytologic changes and a lack of discrete stages. Examination of an enucleated eye can reveal several important tumor features. The tumor is classified into two main cell types: spindle and epithelioid. Spindle cells are highly cohesive fusiform cells with small nuclei. Epithelioid cells are large polyhedral cells with abundant cytoplasm and contain large round nuclei with round nucleoli. Patients with tumors composed

![FIGURE 1. TNM classification for ciliary body and choroidal uveal melanoma based on thickness and diameter.](image)
of pure spindle cells have a more favorable prognosis, and those with a component of epithelioid cells (mixed- or epithelioid-cell types) have a worse prognosis. Chromosomal markers in the tumor (i.e. chromosome 8q gain, chromosome 6p gain and chromosome 3 loss)\textsuperscript{17} and gene-expression profiling\textsuperscript{18} can be used to define groups at high risk for subsequent development of metastatic disease. Other parameters related to poor prognosis include immunologic determinants. Our group previously described the existence of an inflammatory phenotype, which consists of an increased presence of different types of infiltrating immune cells, as well as an increase in the expression of molecules associated with inflammation, such as HLA class I and II antigens\textsuperscript{21} (see below).

**Prognostic impact of intratumoral immune infiltrates**

The presence of infiltrating immune cells in and around tumors and their relation with clinical outcome have led to the hypothesis that the immune microenvironment is an important prognostic factor in cancer.\textsuperscript{19} The presence of large numbers of lymphocytes is associated with a good prognosis in many cancer types, such as cutaneous melanoma, non-Hodgkin’s lymphoma, non-small cell lung cancer, breast, ovarian, head, neck, esophageal, urothelial, and colorectal cancer (reviewed by Mlecnik\textsuperscript{20}). In uveal melanoma, however, an inflammatory phenotype is associated with a poor outcome.\textsuperscript{21} Uveal melanoma may show increased numbers of CD3\textsuperscript{+} T lymphocytes and CD11b\textsuperscript{+} macrophages.\textsuperscript{22} High densities of inflammatory cells occur more frequently in epithelioid-cell-type tumors. Moreover, beyond just the presence, the composition, the nature, and functional orientation of the immune infiltrate must be analyzed in context with other environmental factors, e.g. hypoxia, to comprehend their significance.\textsuperscript{23}

**Immunology**

The main functions of the immune system are to eliminate pathogens and eradicate potentially clonogenic cells, while preventing auto-reactive responses that are harmful to the host. A complex interplay between immune cells exists, and deregulation of this stimulatory and inhibitory balance is directly associated with many human diseases, which include inflammatory and autoimmune disorders, infection and cancer (Text box). The ocular immune system and its role in tumor biology have been intensively studied, leading to the discovery of the ocular immune privilege incorporating the phenomenon of anterior chamber-associated immune deviation (ACAID).\textsuperscript{24} Ocular immune privilege helps to minimize immunopathologic processes, thereby preserving vision. In uveal melanoma, cells contributing to the immune response are thus of interest. The inflammatory response associated with regular wound healing is usually self-limiting, and once tissue regeneration is complete, inflammation subsides. Initial stages of tumor development with continuous proliferation have been referred to as “wounds that do not heal”,\textsuperscript{24} based on the continuous cell renewal and proliferation induced by tumor-associated inflammation.
Early and persistent inflammatory responses help to create an environment suitable for neoplastic progression by providing diverse factors that alter tissue homeostasis. The tumor microenvironment essentially consists of tumor-infiltrating cells, vasculature, extracellular matrix (ECM), and other matrix-associated molecules. Understanding the roles of each type of cell and signaling pathway involved in cancer initiation and progression is critical to the development of treatments. Moreover, tumor cells may modulate the functions of surrounding cells to facilitate their own growth, survival, invasion, and metastasis. This interplay between cancer cells and surrounding components opens new possibilities for novel treatments.

**Recruitment of immune cells**

Conventional macrophages and dendritic cells have functions such as antigen presentation and T cell activation, which may be important in providing anti-tumor immunity (Table 1). They may also produce immunosuppressive cytokines, which may suppress

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### Table 1. Tumor-infiltrating leukocytes (adapted from Grivennikov).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tumor-inhibiting</th>
<th>Tumor-promoting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage, dendritic cell, myeloid-derived suppressor cell</td>
<td>Antigen presentation; production of cytokines</td>
<td>Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors</td>
</tr>
<tr>
<td>Mast cell</td>
<td></td>
<td>Production of cytokines</td>
</tr>
<tr>
<td>B cell</td>
<td>Production of tumor-specific antibodies</td>
<td>Production of cytokines and antibodies; activation of mast cells; immunosuppression</td>
</tr>
<tr>
<td>CD8+ (cytotoxic) T cell</td>
<td>Direct lysis of cancer cells; production of cytotoxic cytokines</td>
<td>Production of cytokines?</td>
</tr>
<tr>
<td>CD4+ T cell Th1/Th2/Th17 cells</td>
<td>Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; activation of CTLs; production of cytokines</td>
<td>Education of macrophages; production of cytokines; B cell activation</td>
</tr>
<tr>
<td>CD4+ Treg cell</td>
<td>Suppression of inflammation (cytokines and other suppressive mechanisms)</td>
<td>Immunosuppression; production of cytokines</td>
</tr>
<tr>
<td>Natural killer cell</td>
<td>Direct cytotoxicity towards cancer cells; production of cytotoxic cytokines</td>
<td></td>
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</tbody>
</table>
tumor immunity. Interaction of local T cells and macrophages may lead to inhibition or stimulation of malignant growth.

**Macrophages**

Macrophages are involved in tumor development as well as wound healing. Macrophages are monocytes that originate in the bone marrow and differentiate on extravasation from the blood circulation. They are recruited to sites of tissue injury, inflammation or cell proliferation by specific chemokines, such as monocyte chemotactic protein (MCP-1). Macrophages are a major source of growth factors and cytokines that profoundly affect other cells in their vicinity. Tumor-associated macrophages (TAMs) mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis. A high TAM content generally correlates with poor prognosis.

TAMs come in two kinds: M1 and M2 macrophages. M1 macrophages are activated by IFNγ and microbial products, and express high levels of proinflammatory cytokines, major histocompatibility complex (MHC) molecules, inducible nitric oxide synthase, and are capable of killing pathogens and priming anti-tumor immune responses. M2 macrophages have low MHC class II and IL-12 expression and show increased expression of anti-inflammatory cytokine IL-10, scavenger receptor A, and arginase. These “alternatively” activated macrophages have only a low tumoricidal activity, mostly promoting tissue remodeling and angiogenesis. Cytokines produced by cells in the tumor microenvironment determine whether TAMs become M1 or M2. Furthermore, M1 and M2 macrophages are plastic and their phenotype is defined by their gene expression profile rather than by deterministic differentiation pathways and lineage choices.

**T cells**

Besides TAMs, the other immune cells that are frequently found in tumors are lymphocytes (T cells). Mature T cells are classified according to their effector functions as: (1) CD8+ cytotoxic T cells (CTLs); (2) CD4+ helper T (Th) cells, which include Th1, Th2, Th17; (3) regulatory T (Treg) cells and (4) natural killer T (NK) cells. Importantly, T cells can exert both tumor-suppressive and -promoting effects, as determined by their effector functions. As reviewed by Grivennikov, increased numbers of T cells, specifically activated CTLs, correlate with better survival in some cancers, including cutaneous melanoma. However, there is also evidence that many of the T cell subsets found in solid tumors, such as uveal melanoma, are involved in tumor promotion, progression and metastasis. In contrast, NK cells seem to be the only ones that do not play a protumorigenic role. Different types of tumor-infiltrating leukocytes (TIL) can affect each other’s function. For example, myeloid-derived suppressor cells (MDSCs) may induce the maturation of CD4+ Tregs, which suppress the immune response. As with TAMs, the tumor-promoting functions of T lymphocytes are mediated by cytokines.
Chapter 1

Soluble mediators: cytokines and chemokines

The cytokine and chemokine expression profiles of the tumor microenvironment may be more relevant than the specific immune cell content. Different cytokines can either promote or inhibit tumor development and progression, regardless of their source.33 TAMs are an important source of cytokines. The perpetuation of inflammation is largely achieved through positive feedback loops that induce chemokine synthesis in malignant and stromal cells, leading to further recruitment of inflammatory cells.2 When we look at the macro-environment of the eye, one can see that the eye has two liquid compartments that can contain soluble inflammatory mediators. Aqueous humor (AqH) is secreted into the posterior chamber by the ciliary body, specifically the non-pigmented epithelium of the ciliary body (pars plicata). It flows through the narrow cleft between the front of the lens and the back of the iris, through the pupil and into the anterior chamber, and drains out of the eye via the trabecular meshwork. From here, it drains out of Schlemm’s canal by one of two ways: directly, via the aqueous vein to the episcleral vein, or indirectly, via collector channels to the episcleral vein by the intrascleral plexus and eventually into the veins of the orbit. The gel in the vitreous chamber provides an unhindered path for light to reach the retina, and the vitreous has the important mechanical role of supporting the ocular shape, promoting the adherence between the retina and the choroid. Most of this humor consists of water (99%) as well as a lower amount of collagen, salt, sugar, and proteins in micro amounts. The soluble factors produced by the uveal melanoma cells and surrounding immune cells may have an effect on these two compartments.

Human leukocyte antigens

A specific immunological characteristic of a uveal melanoma cell is its HLA expression. The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, is controlled by genes located on chromosome 6.34 It encodes cell surface molecules specialized to present antigenic peptides to the T-cell receptor (TCR) on T cells. MHC molecules that present antigen (Ag) are divided into two main classes. The MHC class I molecules are present on the surface of all nucleated cells and platelets. The heavy chain of the class I molecule is encoded by genes at HLA-A, HLA-B, and HLA-C loci. Lymphocytes that express CD8 molecules react with MHC class I molecules. These CD8 lymphocytes often have a cytotoxic function, requiring them to be capable of recognizing any infected cell. Loss of MHC class I antigens may thus be advantageous to tumor cells, as the CD8 cytotoxic lymphocytes may no longer be able to recognize their target. However, an other type of killer cell, the NK cell, is specifically able to lyse cells that have lost their MHC class I antigen expression. Other class I MHC genes encode nonclassical MHC molecules, such as HLA-G (which may play a role in protecting the fetus from the maternal immune response) and HLA-E (which presents peptides to certain receptors on natural killer cells). MHC class II molecules are usually present only on professional
Lymphocytes reactive to class II molecules express CD4, and are often helper T cells. CD8 cells may respond to class I on the tumor cells and CD4 cells to class II antigens: the level of HLA expression by tumor cells may thus affect lymphocyte function.

**Impact of Oxygen availability**

A wide range of environmental factors may influence tumor behaviour, such as oxygen levels. At some point, all solid malignancies outgrow their blood supply, and becoming deprived of oxygen and nutrients. When cancer cells extend beyond the diffusion limits of nearby blood vessels, they metabolically adapt by preferentially undergoing glycolysis (even in the presence of oxygen). This escape mechanism not only provides a survival advantage over non-transformed cells but also ensures that only the most successful cancer cells persist. As a consequence, oxygen-deficient (hypoxic) regions develop within the tumor. Local hypoxia stimulates cells to release pro-inflammatory mediators, recruiting inflammatory and immune cells, stimulating local angiogenesis and providing surviving cancer cells with additional growth factors. Recruitment of leukocytes, including TAM, is largely dependent on mediators such as vascular endothelial growth factor (VEGF). As most growing tumors contain some areas of hypoxia, it is not clear whether hypoxia is the direct driver of tumor angiogenesis or whether hypoxic stimuli generate inflammatory signals that drive angiogenesis. Indeed, exposing uveal melanoma cells in vitro to a hypoxic environment induced increased VEGF production. There is no doubt that hypoxia plays an important role in cancer; however, whether such hypoxia induces macrophage migration and differentiation in uveal melanoma is still not known.

**OUTLINE OF THIS THESIS**

We review existing information on the role and function of the inflammatory microenvironment in uveal melanoma in Chapter 2.

Destabilization of the genome of cancer cells causes a genetically heterogeneous population of tumor cells, which are selected for their ability to proliferate, evade host defenses, and invade other tissues. Different areas of uveal melanoma may be heterogeneous with regard to loss of chromosome 3. A single tumor can contain areas of cells that carry only one copy of chromosome 3, and areas of cells with the normal two copies. We wondered whether there was a difference in prognosis when only part of the tumor displays monosomy 3, compared with tumors in which most tumor cells display this aberration. In Chapter 3, we were able to compare the number of nuclei having monosomy 3 in tumors with variable frequencies of cells with this chromosomal aberration and evaluate the impact of % nuclei with monosomy 3 on survival.
The focus of the studies in the Chapters 4 and 5 is on the phenotypic analysis of infiltrating inflammatory cells, especially macrophages and T lymphocytes, in uveal melanoma. The leukocyte composition and the balance between a pro-tumoral versus anti-tumoral microenvironment might be relevant for clinical outcome.

Whether uveal melanoma-related inflammation is localized in the tumor, or also affects the rest of the eye, is described in Chapter 6 and 7. We investigated whether the inflammatory cells in uveal melanoma have any effect on the rest of the eye, by analyzing the amount of inflammatory cytokines in the aqueous humor (AqH) and vitreous body (CV) from eyes with uveal melanoma.

A decrease in tissue oxygenation is very often associated with the tumor microenvironment. This hypoxia plays a role in cancer, however, an open question is whether macrophage migration and differentiation in uveal melanoma is induced by hypoxia, as described in other tumors. Experiments addressing this topic are described in Chapter 8.
Several specific associations are known between HLA antigens and ocular diseases. A certain local immune response may protect against the development of uveal melanoma by providing a local immunosurveillance system, for example against aberrant melanocytes. Whether the HLA genes of a patient influence local inflammation and/or HLA expression of the tumor is analyzed in Chapter 9.

A strong tumor-associated inflammatory response can be initiated by cancer therapy. Radiation and chemotherapy cause massive necrotic death of cancer cells and surrounding tissues, which in turn triggers an inflammatory reaction analogous to a wound-healing response. The net outcome of therapy-induced inflammation is controversial, as on the one hand it can have tumor-promoting functions that accompany rapid tumor growth, but on the other hand it can enhance the cross-presentation of tumor antigens and subsequent induction of an antitumor immune response. The presence of a leukocytic infiltrate is analyzed in uveal melanoma treated with experimental transscleral thermotherapy (Chapter 10) and with prior irradiation (Chapter 11).

Finally, the conclusions of the different chapters (Figure 2, Table 2) are summarized and discussed.

### Table 2. Overview of eyes used

| (Selection of) 50 primary enucleated uveal melanoma | 3–7, 9, 11 |
| Fresh tissue from eight primary enucleated uveal melanoma | 8 |
| Eleven experimental TSTT treated enucleated uveal melanoma | 10 |
| 47 secondary enucleated uveal melanoma | 11 |
REFERENCES

Introduction


