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Summary and general discussion

Parts have been used in Chapter 1-1-5 Cancer Immunology
Section 1 Basic Principles
Eds. Singh and Damato: Clinical Ophthalmic Oncology, 2nd Edition
The subject of this thesis is the role of immunological factors in the behaviour of uveal melanoma. Inflammatory responses may contribute to architectural changes in this malignancy, as tumor-associated inflammation often goes hand in hand with tumor development. Inflammation in cancer can augment genomic instability, cause local immunosuppression, enhance neoangiogenesis, and thus promote tumor progression and metastatic spread. In this thesis, I state that inflammation is a critical mediator of neoplastic progression in uveal melanoma.

Uveal melanoma contain malignant cells, but also inflammatory cells (tumor-associated leukocytes) and surrounding structures, which may contain blood vessels, fibroblasts, and vascular mimicry channels. We define the tumor microenvironment as the total functional and structural constellation of neoplastic and non-neoplastic cells. We are specifically interested in the functional interactions between cells, in particular through soluble components, e.g. cytokines and chemokines, that may be derived from either neoplastic or non-neoplastic cells. The characteristics of the prognostically-bad inflammatory microenvironment of uveal melanoma, known as the inflammatory phenotype, is reviewed in Chapter 2; increased inflammation is not present in all uveal melanoma, but specifically in those carrying an increased risk of metastasis formation.

Uveal melanoma are heterogeneous. One option is that metastases may result from clonal populations of cells that pre-exist within the parent tumor and are subsequently selected for during growth and the formation of metastases. As the time between enucleation and clinical metastasis can be over ten years, uveal melanoma shed tumor cells hematogeneously, and such cells may remain dormant for many years, evolving into progressive metastases. Monosomy of chromosome 3 in uveal melanoma is strongly associated with the development of such metastases. However, heterogeneity within the tumor may lead to false-negative biopsies: sampling a small portion of a tumor as is done by taking a biopsy may produce a wrong test result. In addition, heterogeneity may limit the accuracy of monosomy 3 analysis: there is disagreement among ocular oncologists as to what percentage of cells in a tumor must exhibit monosomy 3 before the tumor can be said to have monosomy 3. In Chapter 3, we show that a percentage of >5% of uveal melanoma cells with monosomy 3 as determined by FISH on isolated nuclei is already associated with a significantly reduced 5-year survival, indicating that monosomy 3 in as few as >5% of tumor cells is related to death from metastatic disease. These results seem comparable to division by cell type; there is no consensus as to which percentage of tumors needs to be present to call a tumor “mixed cell type”, but it seems that even a few epithelioid cells do markedly worsen prognosis. Applying a cut-off value of 30% seems to lead to the best accuracy, although an assessment of clinical practice patterns showed that FISH-based detection of monosomy 3 in uveal melanoma has not been performed.
in a standardized manner. FISH is a widely available, versatile technology, and when performed optimally has the potential to be a valuable tool for determining the prognosis of uveal melanoma.

To investigate the details of the inflammatory phenotype, we determined the presence of leukocytic subsets in tumor sections of uveal melanoma. Lymphocytes and macrophages were already known to be negative prognostic factors for survival in uveal melanoma. However, no comprehensive phenotypic study of the leukocytic infiltrate had been performed so far. We analyzed the phenotype of inflammatory cells in uveal melanoma slides, especially studying macrophage and lymphocyte subsets (Chapter 4 and 5). Pro-angiogenic and anti-inflammatory M2-type macrophages supposedly contribute to tumor growth and CD163 is a marker of type 2 macrophages. Immunofluorescence-double staining for the macrophage markers CD68 and CD163 was performed and showed that most tumor-associated macrophages in uveal melanoma exhibit the M2-phenotype. Tumors with monosomy 3 exhibited significantly more M2-type macrophages than tumors with disomy of chromosome 3. Furthermore, there is substantial evidence supporting a role for myeloid cells and their precursors in the neovascularization of tumors. CD34 staining, which identifies blood vessels and helps to measure microvascular density, showed a positive association with the density of M2-type macrophages. Tumors with loss of one chromosome 3 are therefore characterized by more pro-angiogenic macrophages and more blood vessels. Studies will be performed to determine the cause of this difference.

Uveal melanoma can also be infiltrated by lymphocytic cells; however, histopathologic studies into cellular infiltration of tumors rarely distinguish between the various subsets of T cells. Our analysis showed that the majority of T cells are CD8+ T-cells, with an almost significant correlation with a bad prognosis. There is a close inter-relationship between all T-cell subsets, as well as with the expression of class I and class II MHC by the tumor cell. The cell ratios, e.g. CD4+/CD8+ or CD8+/Treg ratio, of tumor-infiltrating T cells did not show a relation with patient survival.

Tumors can evade immune responses by the recruitment of Tregs into the tumor microenvironment. Infiltration of Tregs is also assumed to be beneficial for local control of inflammation, probably by “cooling down” the inflammatory process. The presence or absence of Foxp3 has been used to characterize Treg subsets, which appears to be a critical component of Treg function. The clinical relevance of these cells in uveal melanoma was initially shown by Mougiakakos et al., who showed that the intratumoral Treg-frequency was an independent prognostic marker for poor prognosis in COX-2-positive uveal melanoma. Other tumor-specific T-cell subsets were not analyzed. Different types of T cells differentially influence the polarization and function of the APC: depending on the type
of T cell, APC can either suppress or stimulate immunity. Studies that evaluate the type of macrophages in relation to the number and type of T cells in patients are scarce. In our study, the number of CD163+ M2 macrophages correlated with CD3+FoxP3+ regulatory T cells, while CD8+ T cells were the most abundant T lymphocytes.

Macrophages and lymphocytes were mostly absent in tumors with disomy 3, and regarding the presence of a high lymphocytic infiltrate, a trend towards an association with a worse survival was seen. Several genetic studies on the smallest regions of deletion in uveal melanoma have found multiple regions in chromosome 3 on both the p and q arms, but these regions do not generally overlap between different studies,12 and none of them has been linked to inflammation. Therefore, more than one gene in different locations on chromosome 3 may be important for tumor aggressiveness.

We think that as the inflammatory phenotype in uveal melanoma is linked to monosomy 3, this type of inflammation is the one that follows tumor development. Uveal melanoma may trigger an intrinsic inflammatory response that builds up a pro-tumorigenic microenvironment, similar to most solid tumors12A. In addition to autonomous cell proliferation, certain oncogenes, or loss of tumor suppressor genes, may induce a transcriptional program that leads to remodeling of the tumor microenvironment through recruitment of leukocytes, expression of tumor-promoting chemokines and cytokines, and induction of an angiogenic switch, leading to metastasis formation.

Communication between tumor and immune cells

We determined the presence of cytokines in the ocular fluids, i.e. in the aqueous humor (AqH) in the anterior segment and in the vitreous in the posterior part of the eye. Inflammation-related cytokines were increased in the AqH of uveal melanoma-containing eyes (Chapter 6). To our surprise, hardly any associations were seen between the presence of cytokines and clinical, genetic or histopathological tumor characteristics. MCP-3 in the AqH correlated with the presence of infiltrating macrophages. In general, we noticed that the aqueous of a uveal melanoma-containing eye contains many inflammatory cytokines, independent of the presence of an inflammatory phenotype. However, several of the factors present in the AqH are known to inhibit T-cell activity. Thus, this inhibitory ocular microenvironment, composed of soluble immunosuppressive factors within the eye, might inhibit the activity of immune-competent cells, including CTLs.

Similarly, many cytokines and chemokines were found elevated in the vitreous of eyes with uveal melanoma; however, we also made the novel observation that one cytokine (IL-1ra) is actually decreased in tumor-bearing eyes. In Chapter 7, we found a correlation between tumor size and cytokine levels and show that certain elevations in cytokines correlate with immune cell infiltrate in the tumor. These findings are clinically important
Macrophages are recruited as monocytes from the bloodstream to the tumor microenvironment in response to chemokine production by tumor cells or by their environment. Another factor that may play a role is hypoxia, which is known to stimulate homing of myeloid cells to tumors. Low oxygen tension results in the expression of genes associated with angiogenesis, metastasis, and invasion. This transcriptional response pathway is mediated to a large extent by the transcription factor complexes of hypoxia-inducible factors (HIFs). HIF1 activity promotes neovascularization by the induction of a variety of proangiogenic factors including VEGF-A, that stimulate vessel formation within hypoxic areas. In addition, HIF activity also regulates the expression of chemo-attractant factors, including MCP-1, M-CSE, VEGF-A, TNFα, and SDF-1α, each capable of attracting myeloid cells to hypoxic tissues. Clarification of this interaction in uveal melanoma is partly described in Chapter 8. Although in primary uveal melanoma cultures numerous genes respond to hypoxia, hypoxia did not result in an altered monocyte migration in our in vitro assay. In contrast to supernatant of different tumor types, uveal melanoma culture supernatant failed to bring forth the polarization of macrophages into M2-type cells.

A uveal melanoma cell characteristic, loss of classical HLA Class I molecules, is associated with an improved prognosis. Understanding the pathological mechanism of HLA expression and inflammation in uveal melanoma is therefore important. In our study (Chapter 9), it seemed that with the possible exception of an association between HLA-A2 and fewer infiltrating macrophages, and between HLA-DR6 and increased HLA-DR expression, HLA genotype does not determine the amount of HLA expression and macrophage infiltration. Due to multiple testing, these results have to be validated in larger studies.

**Experimental considerations**

The inflammatory infiltrate in uveal melanoma is a complex issue with many unsolved questions. The term “inflammatory phenotype” was proposed to describe a combination of characteristics related to inflammation and is mainly present in tumors with a bad prognosis. The inflammatory phenotype is characterized by high numbers of macrophages and a high level of HLA class I and II expression, and is further associated with the presence of epithelioid cells. Tumor-infiltrating macrophages in uveal melanoma are believed to be mainly M2 macrophages, but a thorough knowledge of the different type of macrophages with different activity levels is still lacking. To this end, the type and numbers of macrophages in the tumor have to be more carefully analyzed by the simultaneous use of several markers that allow discrimination of the different macrophages subsets in one run. Therefore, new and better subset-specific markers need to be identified.
Although there are not hard guidelines, a statistic caveat is the small effective sample size of patients in our studies who died of uveal melanoma, as only they matter in survival analysis. The low number of cases may affect the reliability of the multivariate models. Another issue is the large number of comparisons done for cytokine analysis in relation to the p values reported. In case of multiple comparisons, only attention should be given to p values which are much smaller than the nominal value of alpha 0.05. In interpreting our findings, it is important to notice that most p-values were borderline.

Overall investigation of the primary tumor microenvironment allowed us to uncover additional intra-tumoral immune profiles showing a strong presence of CD8+ CTLs with a weaker presence of CD4+ cells. What makes the same T cell subset anti-tumorigenic in one cancer and pro-tumorigenic in another cancer remains largely unknown. Although this study shows that the number of infiltrating macrophages is related to the number of (regulatory) T cells, it is not clear whether these cells interact and what the outcome of interactions is with respect to macrophage function. In order to do that, the functional properties of all infiltrating T-cell subsets should be evaluated. In addition to tumor infiltration by macrophages and T-cells, studies have shown that myeloid-derived suppressor cells (MDSCs) also infiltrate tumors, inhibiting T-cell and dendritic cell functions and facilitating tumor growth, angiogenesis, and metastasis.

Considerations regarding therapy
Is targeting the tumor microenvironment the way to treat uveal melanoma? The opportunity to change the microenvironment to anti-tumor, encouraging a tumor-specific adaptive immune response, and inhibit metastatic spread, provides exciting new targets for anticancer immune-therapies. The treatment of uveal melanoma may take advantage of therapies that interfere with M2 macrophages, if combined with standard or immunotherapeutic regimens. Therapeutic modalities may attack at several levels, i.e. at the attraction, the differentiation or the activation level of macrophages. Treatment to alter or decrease the number of macrophages has the potential to alter the tumor microenvironment, thus reducing angiogenesis and tumor progression, which may suppress tumor growth and metastasis. Encouragingly, many drugs that target cancer-related inflammation are currently in clinical trials for other diseases.

Obstacles to such studies include the rarity of UM, and clinical trials have only attracted limited numbers of patients with different characteristics (often unresectable or metastatic UM patients) in phase I or II trials. Immune-based therapies have a long history in the treatment of cutaneous melanoma, and sometimes also uveal melanoma patients are included. Some immunological approaches in (ongoing) trials in uveal melanoma patients include anti-PD-L1, fully human monoclonal anti-CTLA4 antibody (Iplimumab), IFN alpha-2b, MHC II uveal melanoma vaccines, and a DC vaccine therapy.
program, a first trial of adjuvant therapy in non-metastasized patients. Since most studies were initiated to block the tumor cells, not much is documented about the immunological effects let alone with regard to macrophage differentiation or specific T-cell responses.

To analyze the immunological effects of therapy, we attempted to screen treated tumors for inflammatory cells. In one study, experimental trans-scleral thermotherapy (TSTT) was applied on eleven uveal melanomas prior to enucleation, and showed that it had an effect on immunological parameters with local loss of expression of HSPs, S100, and higher densities of M2 macrophages around the treated areas (Chapter 10). In the other study (Chapter 11), we analyzed 46 uveal melanoma-containing eyes that had to be enucleated due to tumor recurrence, non-responsiveness to prior irradiation, or complications. Surprisingly, previously-irradiated uveal melanoma contained more lymphocytes than non-irradiated tumors. Both sets had approximately the same macrophage content, similar to a previous study on matched pairs of primarily and secondarily enucleated eyes. We did not match the control group for each case, which might have affected the conclusions. As the numbers of infiltrating cells were higher in epithelioid tumors, it may be that the primary tumor characteristics and not the treatment determine cell infiltration. Those two studies are rather explorative by providing a snapshot of the moment of enucleation, and therefore speculative regarding “influx”, as this cannot be directly observed nor substantiated from matched controls.

Contrary to primary uveal melanoma, the biological prognostic characteristics of its metastases are not well established. The presence of mononuclear inflammatory infiltrate in uveal melanoma metastasis has been reported. One important consideration that should be made when designing clinical trials to test the effectiveness of various therapies of metastatic uveal melanoma, is the fact that in metastatic uveal melanoma, monosomy 3 is associated with highly aggressive, rapidly progressive disease, while a prominent mononuclear inflammatory infiltrate in the metastases is associated with a better prognosis. This is in contrast to what we find in primary tumors (this thesis). This recent study of exclusive metastasis material (with a small number of eleven patients included) indicates that host factors are relevant in the development and progression of metastatic disease and implies that immunologic therapies to treat metastatic uveal melanoma may have the potential to impact patient survival. In addition, Kivela’s group found that hepatic metastases had significantly more intermediate and dendritic types of CD68-immunopositive macrophages than round ones. The difference in the type of inflammatory cellular infiltrate between primary and metastatic tumors might have to be taken into account. In addition to a combination of anti-inflammatory approaches that target the tumor microenvironment with more knowledge-based selective tumoricidal drugs, future therapies should also take notice of the intra-tumoral genetic variation (e.g.
monosomy 3) that affects inflammation and immunity. A still open question is whether leukocyte infiltration predicts the response to therapy, as suggested by a previous study.26 As reviewed,27 pre-therapeutic immune response can determine the efficacy of conventional chemotherapies, and therefore anticancer immune responses may be indispensable for the achievement of optimal therapeutic results.

**Concluding remarks**

Leukocytic infiltration is a common feature of human cancers, including those that develop in immunoprivileged sites, such as the eye. The infiltration of myeloid and T-cells into tumors is part of the host response against cancer. Our data show that in uveal melanoma, high densities of immune cells are associated to tumor progression, as they are associated with the loss of one chromosome 3. In particular, macrophages may have a trophic function instead of being hostile to the tumor. However, this nature of the tumor microenvironment offers therapeutic opportunities. Ultimately, by combining selective impairment of the neoplastic cell populations residing in the eye with anti-infiltrate treatments, it may be possible to bring uveal melanoma growth, invasion, and metastasis to a halt. Despite the exciting advances in the field of uveal melanoma inflammation research, many questions remain. The signaling pathways involved in cancer-related inflammation remain unclear. What is the relationship between loss of one chromosome 3 and the induction of uveal melanoma-related inflammation? Is it mainly the increased tumor growth which leads to large tumors and thus intratumoral hypoxia, which influences the cellular components and mediators of the uveal melanoma inflammatory response? How can uveal melanoma-related inflammatory pathways be targeted for drug development? Defining the roles of inflammatory mediators and the underlying signaling pathways will be critical to increase the understanding of uveal melanoma progression.
REFERENCES


