Ocular inflammation in age-related eye diseases

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SUMMARY AND GENERAL DISCUSSION

INFLAMMATION IN AGE-RELATED EYE DISEASES

The subject of this thesis concerns the role of inflammation in the pathogenesis of age-related eye diseases, most notably uveal melanoma and glaucoma. Aging is inevitable and refers to a multidimensional process of physical, psychological, and social changes. With an ever-increasing growth of the proportion of population aged 60 years or older worldwide, we are confronted with an ever increasing number of elderly people suffering from age-related diseases.[1] As we age, the cells in our body endure progressive amounts of oxidative and metabolic stress, which eventually leads to increasing amounts of tissue stress and damage.[2-4] Low-grade inflammatory responses (para-inflammation) are then required at these local sites of injury in order to restore tissue functionality and homeostasis. Due to the plasticity of our immune system, we are able to respond to tissue injury/stress in a short period of time without causing immunological imbalance. This is the case under normal aging conditions. However, changes secondary to aging, such as metabolic and genetic abnormalities, altered vascular perfusion or degenerative conditions, may result in dysfunction of the immune system's regulatory activity and lead to a chronic and dysregulated inflammatory state.[5] Although specific and different genetic and environmental factors are involved, I argue that inflammation is an important unifying component that contributes to the pathogenesis of age-related eye diseases, such as uveal melanoma and glaucoma.

INFLAMMATION IN UVEAL MELANOMA

Primary uveal melanoma

It has been shown that macrophages and lymphocytes are present in uveal melanoma and that they are related to an unfavorable prognosis and decreased survival.[6-9] Increased inflammation is not present in all uveal melanoma, but specifically in those with a high risk for metastases formation. High risk tumors carry specific tumor characteristics such as the presence of epithelioid cells and high vascular density, associated with the presence of monosomy 3 and the loss of BAP1 expression. [10-13] In a murine intraocular melanoma model, intratumoral accumulation of M2 type macrophages was shown particularly to foster tumor growth in elderly mice, indicating that macrophages in aged mice have a strong tumor-promoting role.[14] Tumor-associated macrophages are directly involved in the

formation of new vessels making it possible for tumors to survive by supplying nutrients and creating a route for cancer cells to disseminate hematogenously.[15-17] In humans (Chapter 2), pro-angiogenic and anti-inflammatory M2-type macrophages are characterized by a higher expression of CD163, and our lab found that the majority of macrophages present in human uveal melanoma carry this phenotype.[18] Interestingly, tumors with monosomy 3 exhibit increased number of M2-type macrophages and more robust angiogenesis than tumors with disomy 3.[18] In addition, infiltratration by lymphocytes is also seen in uveal melanoma; in Chapter 2 we examined the subtypes of intratumoral T cells which are present in these tumors and we concluded that the infiltrate is pluriform. Tumors can evade effective antitumor immune responses by the recruitment of Tregs into the tumor. Infiltration of Tregs under physiologic conditions is necessary in order to dampen local inflammatory responses by regulating and suppressing other T cells, macrophages and APCs.[19] Mougiakakos et al.[20] have shown that intratumoral FoxP3$^+$ Tregs are an independent prognostic marker for poor clinical prognosis in COX-2-positive uveal melanoma. Other tumor-specific T cell subsets were not analyzed. Our data indicates that the number of CD163$^+$ M2-type macrophages correlates with CD3$^+$ FoxP3$^+$ regulatory T cells while CD8$^+$ T cells were the most abundant T lymphocytes. Moreover, both the presence of intratumoral macrophages as well as of lymphocytes was associated with monosomy of chromosome 3. More recently, our lab showed that this inflammation is related to the loss of BAP1 expression, a gene present on chromosome 3.[11] We believe that the intrinsic malignant properties of the tumor may lead to mixed inflammatory responses that support a pro-tumorigenic microenvironment. The recruitment of immunosuppressive immune cells into the tumor, such as M2-type macrophages and FoxP3$^+$ T regs, may explain the paradoxical finding of increased numbers of cytotoxic CD8$^+$ T cells and ineffective antitumor activity.

Irradiated uveal melanoma

Until the 1970s, the traditional treatment of uveal melanoma was enucleation, which was subsequently followed by a shift toward more eye-salvaging approaches.[21] While eye retention following local irradiation is achieved in more than 80% of cases after 5 years[22-24], secondary enucleation may be required when tumor recurrence or when radiation-related ocular side effects occur. At present, not much has been documented regarding inflammation after local irradiation of uveal melanoma. Because local immune responses may play a role in removing tumor debris or in either stimulating or suppressing antitumor immune responses, we analyzed the presence of inflammatory cells in uveal melanoma

containing-eyes, which had either been enucleated after prior irradiation or had undergone primary enucleation (Chapter 3). We observed that prior irradiation has no effect on the number and type of tumor-infiltrating macrophages, which showed a similar variability to uveal melanoma in primarily-enucleated eyes. This corresponds to a previous case-control analysis of matched pairs of irradiated and non-irradiated uveal melanoma.[25] Surprisingly, previously-irradiated uveal melanoma contained more lymphocytes than non-irradiated tumors. As the numbers of infiltrating cells were higher in epitheloid tumors, one possible explanation is that primary tumor characteristics such as the genetic status and not the treatment itself determine cellular infiltration. Due to limited non-necrotic tumor material in secondarily enucleated eyes, we were unfortunately unable to determine the chromosomal status of these tumors.

Experimental and future considerations

The inflammatory infiltrate in uveal melanoma is a complex phenomenon which raises many questions. One of the major pitfalls of working with human uveal melanoma-containing eyes is the fact that we were unable to assess the functional activity of these tumor-infiltrating leucocytes, as functional immunologic studies were not possible on our tumor sections. However, it has in the meantime been possible (G. Gezgin, unpublished results) to grow T cells from uveal melanoma, which were able to respond with IFN-γ production when exposed to autologous tumor cells. However, we do not know the specificity of these tumor-infiltrating T cells. Furthermore, the outgrowth of the tumors shows that the T cells were not efficient in removing the tumor in vivo. Although we have shown that the number of infiltrating macrophages is correlated to the number of (regulatory) T cells, it remains unclear whether these cells actually interact and what the outcome of these interactions would be on macrophage and T cell function. Another limitation of our study is its cross-sectional design because it provides only a "snapshot" of the moment of enucleation of the tumor-containing eye and does not reflect potential changes in infiltrating immune cells over time. Gezgin[11] and Robertson[26] recently showed that in uveal melanoma, the presence and type of leukocyte infiltrate depends on the developmental stage of the tumor: tumors with an extra 8q exhibited an influx of macrophages, while the loss of one chromosome 3/BAP1 expression was associated with a T cell infiltrate as well. With regard to irradiated uveal melanoma, it remains unclear whether the inflammatory infiltrate is a consequence of the characteristics of the primary tumor before irradiation or due to irradiation. Future studies involving information on biopsies obtained prior to irradiation

could give us more insight into the intrinsic properties of the tumor, and analysis of these tumors after secondary enucleation may help us to understand the changes in tumor behavior in locally uncontrolled uveal melanomas.

INFLAMMATION IN GLAUCOMA

Glaucoma is a global unmet medical challenge because of its prevalence in especially the elderly population, its debilitating consequences, and lack of effective treatment. Currently, the best and most advanced treatments only work to delay loss of retinal function. Although elevation of IOP is considered a major risk factor associated with optic nerve damage, glaucoma is no longer viewed simply as a neurodegenerative condition caused by elevated IOP. A conservative estimation is that 20-30% of patients with glaucoma exhibit a normal IOP range, and progressive visual field deterioration is observed in patients with perfectly controlled IOP levels.[27] To date, the precise pathogenesis of glaucoma is not fully understood. Recently, it has been suggested that inflammatory responses directed towards retinal and optic nerve proteins may be related to the development of glaucomatous optic neuropathy.[28]

The role of the immune system in glaucoma has been described as either neuroprotective or neurodestructive. For example, T cell-mediated immune responses may initially be beneficial to limit neurodegeneration.[29-31] The recruitment of macrophages and T cells allows early communication of the immune system with cellular debris, destruction of damaged cells, and removal of pathogenic agents. Subsequently, these immune cells mediate the protection of neurons against degenerative conditions in the aging eye, which is referred to as "protective immunity".[32] An initial immune response may be beneficial and necessary to promote tissue repair and limit neurodegeneration. However, failure to control aberrant, stress-induced immune responses due to accumulating risk factors (e.g. IOP, ischemia) along with aging-related oxidative stress likely switches the immune response from a neuroprotective response into a neuroinflammatory degenerative process that facilitates the progression of glaucoma. In Chapter 4, we extensively review the role of host immune responses in relation to RGC and axon survival following glaucomatous tissue stress.

Inflammation in experimental glaucoma models

The possibility that inflammation plays an important role in the damaging effects of high IOP or any type of neural insult (e.g. ischemia, traumatic optic nerve injury) has garnered much interest, however, definitive evidence to support a role for an autoimmune pathogenesis in glaucoma is lacking. We determined (Chapter 5 and 6) whether elevated IOP triggers secondary events, which could lead to prolonged or progressive neuronal damage even when the IOP has subsequently returned to a normal range. An elevated IOP is known to induce expression of Hsps in the retina.[33] Hsps are highly conserved proteins that can potently stimulate immune responses and are implicated in the autoimmune responses of rheumatoid arthritis, atherosclerosis, and type I diabetes.[34-37] Autoantibodies against Hsps were detected in the serum of some glaucoma patients[38], which further supports the involvement of autoimmune responses to Hsps in glaucoma. Here, we report (Chapter 5) that a 3-week transient elevation of IOP induces a high expression of Hsp27 in RGCs, which is consistent with what has been reported in human patients and experimental models of glaucoma.[28,33,39,40] Elevation of IOP induces activation of $CD11b^+$ microglia/ macrophages and infiltration of T cells into the retina. More importantly, this leads to $CD4^+$ T cell responses specific to Hsp27 that were both required and sufficient for propagating a prolonged and progressive phase of glaucomatous neurodegeneration after the IOP had already returned to normal ranges. When ocular hypertension was induced in immunodeficient mice, the progressive loss of RGCs and axons did not occur. However, when we transferred diseased T cells isolated from glaucomatous wild type mice to immunodeficient mice with induced high IOP, we observed exacerbated RGC and axon neurodegeneration. Remarkably, acute retinal or optic nerve damage caused by ischemic (acutely elevated IOP) or traumatic injury also induced T-cell mediated autoimmune responses, leading to chronic RGC and axon loss that continued to occur long after the period of initial injury. Suppression of these $CD4^+$ T cell-mediated immune responses by injecting neutralizing and blocking antibodies into the vitreous cavity of the eye led to a drastic decrease in RGC death against acute ischemic injury. We are the first to show that blocking local $CD4^+$ T cell-activity is not only neuroprotective, but also preserves the retinal function by protecting other retinal cell types against the secondary effects of ischemia (Chapter 6).

To determine whether Hsp-specific T cell responses also play an important role in the pathogenesis of glaucomatous neurodegeneration in humans, we examined Hsp-responsive T cells and autoantibodies in POAG patients. We noticed a significant increase

in the numbers of Hsp27- but also of Hsp60-reactive T cells and autoantibodies in the serum of POAG patients compared to age-matched healthy volunteers.

The neuroprotective effect of a germfree environment in experimental glaucoma models

So far, the role of environmental factors and their contribution to the development of glaucoma are not yet known. As the eye is an immunologically-privileged site, a critical gap in our knowledge are the factors which lead to the induction of destructive inflammatory cells. Because HSPs are highly conserved in sequences from bacteria to humans[41], we hypothesized that these damaging inflammatory reactions in the human eye may develop following induction of HSP-specific T cells by previous exposure to bacteria. These T cells would subsequently recognize damaged RGCs and neuronal cells, leading to the autoimmune attack of RGCs, resulting in optic neuropathy. In our hypothesis, the crucial environmental factor contributing to glaucoma development is the patient's exposure to specific bacterial strains. In Chapter 4, we compared the development and progression of glaucoma between DBA/2J mice housed under pathogen-free and completely germfree conditions. DBA/2J mice are genetically predisposed to develop spontaneous glaucoma in an age-related manner. Interestingly, a germfree environment compromised the development of glaucomatous neurodegeneration in DBA/2J mice despite persistent ocular hypertension in old germfree DBA/2J mice. Similar results were observed when we experimentally induced glaucoma in mice using the microbead model. It seems that mice that have never been exposed to any bacterial stimuli harbor more tolerant and "untrained" microglia/macrophages compared to conventionally-raised mice, as measured by their activation status and functional activity (unpublished data).

Experimental and future considerations

We believe that our study provides evidence that involvement of T cells requires local inflammation in the retina and a disruption in the blood-ocular barrier that occurs after injury. We propose that activated microglia/macrophages function as APCs that capture retinal antigens from damaged or injured RGCs and stimulate an autoimmune attack against the retina and optic nerve through activation and recruitment of $CD4^+$ T cells in the absence of sustained elevated IOP, ischemic or traumatic injury. These microglia/macrophages can also secrete inflammatory cytokines, such as TNF-α and IL-1β, both of which may further impair the integrity of the blood-retina barrier, allowing T cells to infiltrate into the retina.[42-44] Identification of a key role of autoreactive $CD4^+$ T cells in

glaucomatous and acute (ischemic, traumatic) injury-induced neurodegeneration may hold larger implications and contribute to our understanding of the molecular mechanisms underlying chronic neurodegeneration in the CNS in general. Our findings may provide a possible connection between acute CNS injury, such as ischemic stroke and traumatic brain injury, to Alzheimer's disease later in life.[45-47] Furthermore, it may also explain why current glaucoma therapies solely directed at lowering IOP are not always effective in the long-term. Our findings give way to a novel theory in this field: that we are able to prevent and treat neurodegenerative eye conditions through immunomodulation. The eye is more easily accessible compared to the CNS, and by treating it locally, we can reduce systemic side effects and have fewer problems with insufficient penetration of drugs in targeted sites.

Finally, we need to remember that glaucoma pathogenesis is multifactorial which involves a complex interplay of elevated IOP-induced events and genetic/epigenetic/age-related susceptibility factors that contribute to neurodegeneration. Our comprehensive study contributes only one piece to the larger meshwork of complex pathogenic mechanisms underlying glaucoma.

CONCLUDING REMARKS

Although uveal melanoma and glaucoma differ in their pathogenesis, it is believed that these diseases share two unifying elements: 1) both are diseases of the elderly; and 2) both involve inflammation. Due to the extreme plasticity of our immune system, we can respond to tissue injury/stress in a short period of time without causing immunological responses with damaging effects, especially during aging. The immune system is however not a mere bystander in the process of neurodegeneration, protecting our body from harmful injury or infections, but can also be involved in detrimental and chronic immune responses leading to neurodegenerative diseases, such as glaucoma. On the other hand, due to the immune-privileged property of the eye, tumors like uveal melanoma are permitted to grow quietly and unhampered by escaping immune detection and attack. Therefore, understanding the mechanisms underlying the mosaic of mechanisms which link immune privilege, protective immunity, and autoimmune diseases is crucial to understand the pathogenesis of age-related diseases. Further studies can shed light on elusive aspects of the role of inflammation in age-related eye diseases.

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