Ocular responses to foreign corneal and tumor issue

Essen, T.H. van

CHAPTER 8

Summary and General Discussion

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Ocular Immunology

The immunological characteristics of the eye are intriguing and characterized by immune-privilege and immune deviation. Because of its avascularity, the cornea has already a higher threshold before immune responses take place. Directly behind the cornea is the anterior chamber where immune responses are influenced by an active system of immunosuppression, known as ACAID (Anterior Chamber Associated Immune Deviation).[1] This immunoregulatory system raises an additional threshold for any immune response. In this complex system, we aim to place the artificial cornea made out of fish collagen, which should ideally not elicit an immune response.

Fish Scale-derived Collagen Matrix

The Fish Scale-derived Collagen Matrix (FSCM) represents a new approach to replace human donor corneas. We need an alternative to human donor corneas as there is a worldwide donor shortage, even in the developed world. This is tragic, as cornea transplantation is the most successful form of organ transplantation, largely attributable to the immune privilege that the cornea exhibits.[2, 3]

The make-up of the FSCM is similar to the biosynthetic artificial cornea, used already in a phase one clinical study, with respect to being an acellular collagen scaffold.[4] The FSCM differs by being of natural (fish) origin and not being artificially constructed: its natural origin should ideally lead to a cheaper fabrication process. The challenge is to keep the modification process after harvesting the scales as simple and straightforward as possible. This could give some difficulties as the FSCM differs from batch to batch and also individually, as every scale has different dimensions and is not uniform with regard to thickness. However, technical modifications may help to overcome this.

The most well-known alternative to normal human donor cornea transplantation is the Boston Keratoprosthesis (Kpro),[5] which is used in cases where regular cornea transplantation would not suffice. The Boston Kpro consists of a central optical cylinder, a titanium back plate and a locking ring, with a donor cornea in the middle. This Boston Kpro is a good solution for cases where cornea transplantations have failed, however, it does not reduce the shortage of human donor corneas, as it still needs a donor cornea sandwiched between the optical cylinder and the back plate. Alternatives to the donor cornea for the Boston Kpro are being investigated.[6] The FSCM has the features needed to serve as an alternative for human donor corneas. This collagen scaffold has the same arrangement of collagen fibers, although a bit denser, and facilitates the regrowth of human corneal epithelial cells and adhesion of keratocytes (corneal fibroblasts) in vitro. The regrowth of corneal epithelial cells is comparable for human epithelial cell lines or primary cultured human corneal epithelial cells. The anterior surface of the initial FSCM is not smooth; it exhibits a micropattern that differs from location to location. Yet the growth of human corneal epithelial cells is not hampered by these different surfaces. The keratocytes adhere to the FSCM, which we proved by the positive staining for integrin-α6 and –β1. No infiltration of keratocytes was observed during the relatively short follow-up periods

(maximal 2.5 months). The results were similar to those with the biosynthetic artificial cornea used in a phase 1 clinical trial, where the first signs of keratocyte immigration were seen after 6 months at the skirts, anchoring it in place, and where signs of repopulation were seen in 7 out of 10 patients at 12 months.[4]

The FSMC is highly permeable for oxygen and glucose, at an even higher permeability rate than the human cornea.[7, 8] This is important in order to maintain a vital epithelial layer. The technical aspects allow suturing of the FSCM into place into the recipient corneal bed. As with all ocular tissues, subtle handling is advised, as the FSCM can tear upon too brisk manipulation. This could prove itself a cumbersome threshold to overcome, as tearing during surgery is a serious problem, needing the FSCM to be replaced. The FSCM is a bit tougher than the human cornea, which provides advantages and disadvantages. One of the advantages is that the curvature of the FSCM will be maintained, however, its disadvantage is that a reasonable adequate curvature with regard to refraction should already be achieved during the production process. The smoothness of the FSCM's rim depends on the cutting procedure; in case of irregularities at the edge, a softer FSCM is more forgiving then a brisk version.

The FSCM facilitates a similar direct light transmission in the visible spectrum as the human cornea (90% versus 91%). This high rate of light transmission is not directly correlated with a good visual acuity, as for this the curvature and, to a lesser amount, the light scatter of the scaffold are important as well. The required curvature is individually based and furthermore largely influenced by sutures and in a lesser way by corneal swelling, which is also the case with human donor corneal transplants. The light scattering of the first versions of the FSCM with the pattern still on top was equal to early to moderate cataract with log(s) = 1.62. This does not necessarily influence visual acuity,[9, 10] but could produce some haze and lead to decreased contrast vision. Unpublished data of experiments using the FSCM with the micro-pattern removed, revealed improved light scatter values. As far as we know, no forward light scatter values for the biosynthetic artificial cornea have been published. The optical properties of Boston Kpro have been extensively investigated, but we could not find a comparison with the human cornea with regard to forward light scatter.[11]

Most importantly, our animal studies in which we implanted the FSCM in non-immunoprivileged locations show that the FSCM has an excellent biocompatibility, with similar immune responses as sham-surgery and no sensitization of the immune system upon subcutaneous implantation. When there is no specific immune response triggered when the FSCM is implanted subcutaneously, it is reasonable to expect that such an immune response is also absent in an avascular tissue such as the cornea.

Upon intra-stromal introduction into the cornea of rabbits, the FSCM maintained its transparency, and elicited neither corneal melting, neovascularization nor any immune response, during the six weeks we left it in place. This confirms that the FSCM transmits enough oxygen and nutrients to keep the overlaying epithelium vital and that the FSCM is biocompatible with the cornea. Studies on the biosynthetic artificial cornea showed already that repopulation by epithelial cells is feasible.[4]

There are a few features of the FSCM that require some adjustment and further research. We know that human corneal epithelial cells can repopulate the FSCM surface in vitro, but we need to confirm this in vivo. Are we going to use the FSCM for anterior lamellar keratoplasty only, thus leaving the recipient's corneal endothelium intact, or are we aiming for penetrating keratoplasty as well? Before we can make such decisions, we need to know first whether the FSCM and the overlaying epithelium remain stable without an endothelial layer on its posterior surface. Secondly, we need to know how strong the interaction is between the corneal fibroblast and the FSCM. Will the connection between the FSCM and the rest of the cornea be strong enough to compete with the insertion of a human donor cornea?

Long term animal-studies are needed to determine the long term compatibility of the FSCM, especially with regard to the stability of the scaffold and its interaction with the cornea fibroblasts

Currently, the research focusses on making the FSCM less brisk and to produce a smoother surface and on its use as an emergency patch at the time of corneal perforation. A clinical study to determine its applicability is ongoing.

Uveal Melanoma

While the ocular immune privilege allows acceptance of foreign tissue in the cornea, it also allows growth of intraocular tumors. We looked into the immunology of Uveal Melanoma (UM) because we want to control or cure this malignancy. The tumor is located in the ciliary body and choroid, which are richly vascularized, and lie outside the blood-retina-barrier. These tissues contain the rich networks of innate immune cells (bone marrow-derived resident macrophages and dendritic cells). These cells, together with the parenchymal cells, secrete immune mediators, that support immune-privilege.[12] Several studies show that the immune system is active at the tumor site, yet it is apparently not doing its job effectively in halting and destroying the tumor. We showed that higher HLA protein expression on the tumor surface is associated with shorter patient survival.[13] [14] It could be that certain HLA-alleles are predisposing for this, as we know for example that several autoimmune diseases have HLA-associations such as with HLA-B27, and there are specific regulators that influence the level of class I expression. However, our research revealed no association for a specific HLA-genotype predisposing to an increased HLA protein expression in uveal melanoma.

We know that in most tumors, HLA expression is downregulated or skewed towards the non-classical HLA types.[15] In contrast, in uveal melanoma, classical HLA expression is upregulated. What drives this increased HLA class I expression? Is this directly influenced by tumor genetics or indirectly by the immune infiltrate? Based on the analysis of data from 28 enucleated eyes with uveal melanoma, we found that there was no dosage effect of chromosome 6p, on which the genes encoding the HLA antigens are located. We confirmed the known association between a higher expression and monosomy of chromosome 3. The protein levels of the HLA Class I and II molecules were positively associated with their mRNA gene expression, which is to be expected. The genes encoding the

peptide-loading molecules had similarly raised levels. These peptide-loading molecules are needed and responsible for putting the peptides in the groove of the HLA molecules before they are presented on the cell's outer surface. The HLA molecules are not functional without them. Altogether, this indicates that expression of the HLA antigens is not impaired.

We confirmed that an increased amount of tumor-infiltrating immune cells was associated with increased levels of HLA on the tumor cell surface. This was as expected, based on previous research.[16] . What is new in uveal melanoma is that we show that when the human immune cells are absent, the amount of HLA allele expression subsequently indeed decreases. We demonstrated this by analyzing xenografts of the human tumor in immunodeficient female SCID mice. This finding proves our earlier statement that the increased HLA levels are not directly influenced by the genetics of the tumor, but that the HLA expression level is under the influence of the immunological triggers in its direct environment. We can therefore assume that in uveal melanoma the HLA-system responds normally to the input of the immune system and is not under direct influence of the tumor. Knowing this, the next question to be answered will be whether the tumor will be attacked and removed by the lymphocytes when implanted in mice with a patent immune system, or whether the uveal melanoma will still be able to create an environment which renders the mouse's immune system ineffective?

Which factors of the immune response in uveal melanoma are responsible for triggering the increased HLA expression? It could very well be that the increased HLA expression in uveal melanoma is under influence of interferon-gamma (IFNγ), as the tumor-infiltrating lymphocytes and macrophages produce IFNγ which activates HLA transcriptional regulators. [17, 18] IFNγ is predominantly secreted by CD4+ T helper 1 cells (Th1), CD8+ cytotoxic T cells, and NK cells, and to a lower degree by the professional antigen-presenting cells (APCs) including macrophages, and lastly also by B-cells.

We previously showed that IFNγ stimulation resulted in an increased expression of HLA Class I and Class II molecules in uveal melanoma cell lines.[19, 20] Those studies were done on a limited number of available cell lines, yet demonstrated that *in vitro* the HLA regulation is not aberrant. The UM cell lines during co-culture with IFNγ-secreting T cells, synthesize chemokines such as CXCL8-11, CCL2 and CCL5, VEGF and ICAM1 that create a tumor-promoting environment by attracting monocytes, including M2 type macrophages. M2 macrophages are known to promote angiogenesis.[21]

Normally, the presence of classical HLA-alleles together with infiltration of a tumor by immune cells should lead to the destruction of the tumor. In uveal melanoma, it does not. This could be due to an increase in PD-L1 levels in response to IFNγ exposure, which makes UM cells resistant to lysis by cytotoxic T-cells.[22] Overall, it is clear that the tumor's immune response is skewed towards angiogenesis and growth factors,[23] instead of tumor cell lysis.

A recent study treating metastatic uveal melanoma patients with an adoptive transfer of autologous tumor-infiltrating lymphocytes showed objective tumor regression in 35% of these patients. The

tumor infiltrating lymphocytes were selected based on tumor reactivity and subsequently expanded in cell culture, prior to their transfer.[24] This study demonstrates that tumor infiltrating lymphocytes do exist in metastases of uveal melanoma and can be triggered to act against their autologous tumor during in vitro culture, which leads to our hypothesis that the HLA alleles in uveal melanoma mostly, but not only, present peptides that are not recognized as pathogenic

Recently, Gezgin et al. showed that tumors with many infiltrating lymphocytes have a loss of BAP1 expression. In this thesis, we show that monosomy of chromosome 3 and the gene-expression profile class 2, are associated with loss of BAP1: [25] both prognostic factors are associated with a lower BAP1 gene expression and a negative BAP1 immunostaining. We also confirm that on its own, BAP1 gene-expression and BAP1 immunostaining are predictive of death due to metastasis. This was recently again confirmed by the TCGA study (2017). Gezgin's subsequent work shows that it is possible that BAP1 is an inflammatory regulator in uveal melanoma. It may be that BAP1 by itself promoted the secretion of regulators of a pro-angiogenesis environment, or that it promotes the influx of cytokine-producing T cells or macrophages.

Further research should be directed at finding peptides that are being presented as stimulators of anti-uveal melanoma T cells. Another option is to aimed at expanding possible therapies based at peptides that can be recognized such as PRAME, which has been demonstrated to be expressed in uveal melanoma.[26, 27] Another research focus could be aimed at counteracting the skewing of the immune response towards angiogenesis and growth factors.

Conclusion

We used the corneal immunosuppressive environment to test the possibility of inserting a Fish Scale-Derived Collagen Matrix in a corneal pocket, and the excellent results and lack of primary and secondary immune responses led to a clinical trial, which is currently underway.

The immunosuppressive environment of the eye allows the growth of malignant melanocytes which leads to the formation of uveal melanoma. The association between increased numbers of macrophages and lymphocytes in uveal melanoma and an increased development of uveal melanoma metastases suggest an influence of pro-angiogenic macrophages, The relation between loss of BAP1 expression and a very bad prognosis and the influx of leukocytes into the primary tumor, suggests that BAP1 functions as a regulator of inflammation. Further research will focus on the role of BAP1 in the regulation of inflammation in the uveal melanoma microenvironment.

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