New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

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SUMMARY

Many diseases of the anterior surface of the eye can lead to severe disabilities and blindness. A normal anatomy and physiology of the ocular surface is mandatory for good visual acuity. In this thesis, different anterior segment diseases that can affect the normal anatomy have been investigated and new developments in analysis of these diseases. The limbus plays a central role in the maintenance of the ocular surface, it's where the corneal epithelial stem cells are located and renew the corneal epithelium. In Part I we discuss limbal stem cell deficiency, where we looked for new possibilities in limbal stem cell research. One of the causes of limbal stem cell deficiency repeated surgery, which is often the case in melanocytic lesions of the conjunctiva. The topic of Part II is conjunctival melanoma and benign melanocytic lesions such as PAM and conjunctival nevi. We focused on detection and differentiation methods for conjunctival melanoma, especially cytology, which is a detection method that is minimal invasive and therefore less damaging to the ocular surface. Herpes simplex keratitis and infectious corneal ulcer (Part III) are both also capable of severely damaging the ocular surface. We investigated the influence of genetic differences of lactoferrin and IL-10 in patients with infectious corneal disease and between patients and controls.

Limbal stem cell deficiency (Part I)

Accurate follow up of limbal transplants in animals is difficult, since some rejections are not clinically visible. With E-GFP we were able to create an animal limbal transplant model in which we could accurately follow transplant survival in vivo (Chapter 2). The in vivo follow up of the transplants creates large amounts of data on various time points of the same animal, and both transplant survival and transplant behavior can be assessed. Another advantage of the E-GFP model is the reduction in the number of animals used in an experiment because of the excellent in vivo follow up.

We showed that local clodonate liposome injections were able to improve transplant survival. However, without immunosuppressive treatment both allogeneic and syngeneic transplants survived around 14 days; this demonstrated that the immunogenic properties of the E-GFP are a drawback of the E-GFP limbal transplant model. In the future, other treatment options like cyclosporine or tacrolimus can also be investigated.

The E-GFP makes this new model suitable for new fluorescent imaging techniques. The confocal microscope technique is able to give more data than fluorescence microscopy. The laser of a confocal laser scanning microscope is able to scan the deeper layers of the transplant, and a computer program is able to reconstruct a three-dimensional image from these deeper layer images. The 3D models created, can give new insight in vascular growth patterns, as shown in Chapter 2. We found a large amount of E-GFP positive blood vessels underneath the transplant, which must have been created by the transplant itself. Perhaps confocal microscopy can also be used in the clinical setting of limbal stem cell deficiencies, and in the analysis of patients with a limbal transplant.

Nowadays, with the ability to culture limbal stem cells in vitro, the original limbal transplant that consists of partly corneal epithelium and partly conjunctival cells will be mostly replaced by this new but expensive technique. However, such new culture techniques can be easily incorporated in our model, as the E-GFP makes it especially suitable to investigate

transplant behavior over time. In addition, immunofluorescent staining techniques in combination with E-GFP fluorescence can create new insides in transplant acceptance and expansion. Furthermore, this model can also be used to investigate the newly found stem cell crypts.

Conjunctival melanoma (Part II)

Conjunctival melanoma and PAM are known for their recurrences, patients are therefore regularly examined and repeatedly biopsied. These repeated invasive procedures can damage the eye and especially the limbus since most are located near the limbal region. We therefore examined in Chapter 4, 5, and 6 less invasive diagnostic techniques for conjunctival melanoma.

Our own nation wide study, described in Chapter 3 of this thesis shows the Dutch survival rates, and the risk factors for mortality, local recurrence and distant metastasis, which are similar to previously published data. The main risk factors for mortality were tumor location and tumor thickness, i.e. a nonepibulbar location significantly decreased survival chances, as did thicker tumors (>2mm). The nonepibulbar location was also a risk factor for local recurrence, which could be due to the more difficult surgical approach of tumors in this location. Chapter 3 also indicates that local recurrence rates could be decreased, by applying additional brachytherapy with Iridium or Strontium after surgical tumor removal. Many primary treatment options have been applied in the last decades, but reliable randomized studies are lacking, mostly because of the rarity of the tumor. Large international collaborations are therefore needed to investigate different treatment options and subsequent outcomes. For the clinician it is important to examine the caruncula, fornix, and palpebral conjunctiva intensively in patients with pigmented conjunctival lesions. At the slightest suspicion of a conjunctival melanoma further investigations like cytology and histological biopsies are warranted.

Cytology

Early stage conjunctival melanoma can be difficult to differentiate from a conjunctival nevi, as is the development of a conjunctival melanoma in a PAM lesion. Although a histological biopsy is the gold standard for diagnosing conjunctival melanoma, in Chapter 4 we show that cytology can be an alternative. Tumor cells with severe atypia arise to the epithelial surface in conjunctival melanoma, where they are available for sampling. The sensitivity, specificity, and negative predictive value (85%, 78%, and 93% respectively) are acceptable, however, the positive predictive value is low (59%), creating a high amount of false-positive outcomes. Further research has to prove whether repeated smears will increase the predictive values. An advantage of cytology is the minimally invasive technique which can be repeated almost endlessly in short periods of time. A biopsy cannot be repeated endlessly, it causes more discomfort for the patient, destruction of conjunctival tissue. and requires more time and resources than cytology. Cytology is therefore probably more cost effective. Exfoliative cytology sometimes has the disadvantage of producing small amounts of cells, thereby interfering with the diagnostic process. The Biopore membrane is a device that is able to sample large amount of cells from the ocular surface, however, the large size of the instrument

makes sampling of the fornix and caruncula difficult (Chapter 5). Both the exfoliative and Biopore cytology provide additional data for the ophthalmologist. With the additional data, a better decision on treatment options can be made. Still only a minority of the larger oncological centers have cytology available for ocular surface tumors. We recommend that all major ophthalmic centers should have impression cytology and/or exfoliative cytology, as has been stated by Singh. However, an experienced cyto-pathologist has to be available as well. In the future new techniques like confocale microscopy might also help the clinician to differentiate PAM, conjunctival nevi, and conjunctival melanoma. Reliable data on sensitivity and specificity of the confocale microscope are lacking.

Differentiation

Histological differentiation of a conjunctival melanoma from benign melanocytic lesions can sometimes be difficult, especially lesions from adolescent patients. A reliable marker for conjunctival melanoma could be helpful; in Chapter 6 we investigated S100A1 as a possible candidate to differentiate conjunctival nevi from conjunctival melanoma. Further studies need to examine whether S100A1 is also able to differentiate between a conjunctival Spitz nevus and a conjunctival melanoma, which would be interesting since histologically spitz nevi resemble conjunctival melanoma very closely. Furthermore, S100A1 and S100B could be potential candidates for serum markers for early detection of conjunctival metastasis.

Cell lines

All our previous studies have in some way been limited by the low incidence of conjunctival melanomas. Cell lines of a conjunctival melanoma can help to improve and expand our knowledge of this tumor. In Chapter 7 we describe the fourth conjunctival melanoma cell line ever. It is a stable cell line with a relatively high turnover and distorted karyogram which could be due to the origin of the cell line from a recurrence of a conjunctival melanoma after excision and local brachytherapy.
When all four known conjunctival melanoma cell lines in the world are combined together new research areas can be explored, like genomics or proteomics. Also animal models for conjunctival melanoma can be easily developed when cell lines are available.

Corneal infections (Part III)

HSV

The recurrent nature of HSV keratitis, especially the immune stromal keratitis and the stromal necrotic form, is causing major destruction of the cornea, and therefore responsible for large part of blindness and low vision in the Western World, despite it's low prevalence (0.15%). Many parts of herpetic keratitis are not fully understood, including the low prevelance, while most individuals appear to have HSV shedding in their tears. Furthermore, there is no clear explanation for the wide range in recurrence frequencies in HSV keratitis patients. Since most people shed HSV in their tears, an efficient anti-HSV mechanism in ei-

ther the tear fluid or ocular surface must exist. We have investigated lactoferrin as one of the possible candidates that could influence HSV occurrence and outcome. A relation has been shown between lactoferrin and HSV keratitis in animal models, we therefore expected a lower lactoferrin concentration in HSV patient, however we (Chapter 8) were not able to demonstrate that in our study population group, nor were we able to find a relation between lactoferrin concentration and any clinical parameter. However lactoferrin gene polymorphisms were associated with the occurrence of HSV keratitis (Chapter 8), the Asp561 allele seems to have a protective role. The Glu561Asp polymorphism was not associated with the clinical outcome of the HSV keratitis. The different structure of the Asp561 lactoferrin variant could be the cause of the difference in susceptibility. In the future in vivo and in vitro studies with recombinant lactoferrin Asp561 and Glu561 could support this theory.
Since HSV keratitis infection and recurrence is a complex process, involving many cytokines and chemokines, other proteins than lactoferrin could be involved in the susceptibility to HSV keratitis. Interleukin(IL)-10, IL-12, and INF-γ are examples of proteins that influence HSV infections, polymorphisms in these genes can be candidates for future research. Not only host factors will influence HSV infections, different HSV strains can be responsible for the diversity seen in clinical outcome. Currently, several IL10 polymorphisms are being studied in the HSV-patient group.

Corneal ulcer

In contrast to HSV infections, causes of infectious corneal ulcers are better understood. Trauma by foreign bodies or contact lenses cause epithelial defects that act as a porte d'entrée for micro-organisms. However, many individuals experience corneal epithelial defects but only a minority will develop an infectious corneal ulcer. Bacterial load, virulence of invading organism, and the immune system all play a role in the development of an corneal infection. In patients with contact lenses and poor hygiene the bacterial load is high and are therefore more prone to develop an infection, moreover the contact lens boxes often contain very virulent micro-organisms.

Lactoferrin gene polymorphisms

Lactoferrin as a member of the innate immune system can influence corneal infections through its antibacterial or immunomodulating effects. Lactoferrin polymorphisms that were investigated in Chapter 8 were also investigated in Chapter 9 in patients with an infectious corneal ulcer. No differences were found in lactoferrin polymorphisms between infectious corneal ulcer patients and healthy controls, while in HSV-patient a difference was found for polymorphism Glu561Asp. In contrast to HSV keratitis, corneal ulcers probably need an epithelial defect before infection occurs, therefore its less likely that lactoferrin could have and influence on the chance of infection. Although lactoferrin polymorphisms could have an influence on bacterial load in the tear film. We did find a trend towards slower epithelial healing in patients with the Glu561 allele. Indicating, that the lactoferrin polymorphisms Glu561Asp probably has an influence on the functionality of the lactoferrin protein, as we also indicated in Chapter 8 with HSV keratitis patients. In the future, differences in anti-inflammatory, antibacterial, epithelial healing effect of the various lactoferrin forms can be

tested with recombinant lactoferrins in in vitro and in vivo tests.

IL-10 promotor gene polymorphisms

Both bacteria and immune system are responsible for the corneal damage in bacterial corneal ulcers. IL-10 is a potent suppressor of the immune system, and polymorphisms in the promotor region of the IL-10 gene cause different expression levels of the IL-10 in vitro. We therefore investigated whether IL-10 polymorphisms -C819T, -G1082A, -A2763C, and -A2849G have an influence on infectious corneal ulcers (Chapter 10). We showed that the -819C allele and 2849AA genotype had a protective effect on the development of corneal ulcers. The 2849AA genotype is associated with lower IL-10 levels, which could have made the local immune system of the eye more ready to prevent corneal infections. However, we also showed in chapter 10 that once an infection is established it is more favourable to have a genotype that is associated with higher IL-10 levels. Hence, patients with the 2763A allele or the IL10.1 haplotype, which are both associated with lower IL-10 levels have a worse clinical outcome, i.e. larger corneal ulcers, longer duration of epithelial defect, and longer duration of treatment. The reverse was seen for the IL10.5 haplotype, a high IL-10 producer Whether these polymorphisms also have an influence on the local IL-10 production in the eye is not known. But higher IL-10 levels could cause less inflammation and therefore less destruction to the cornea. In the future it needs to be investigated whether IL-10 polymorphisms have an influence on local IL-10 production in the eye, and also whether IL-10 as adjuvant therapy can improve the clinical outcome.

Besides the influence of lactoferrin and IL-10 gene polymorphisms on infectious corneal ulcer, other gene polymorphisms could also influence the occurrence and development of a infectious corneal ulcer. Possibly in the future, a risk profile for infectious corneal ulcers can be developed on basis of several gene polymorphisms.