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

Keijser, S.

New Developments in Analysis of Ocular Surface Diseases

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New Developments in Analysis of Ocular Surface Diseases

Nieuwe ontwikkelingen in
analyse van ziekten van het oogoppervlak

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“Want al het lijden verandert na de eindstreep in een herinnering aan genot en hoe groter het lijden is geweest hoe meer genot”

uit “De Renner”
Tim Krabbé, 1978

Voor mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

General introduction

A first step in the process of vision is the proper focusing of the visual image on the retina. This focusing is brought about through the refraction of light by the cornea and the lens. The refractive power of the cornea is constant and makes up about 2/3 of the total refractive power of the eye. The lens has an adaptive refractive power from a few to maximally 15 to 20 diopters allowing us to see nearby and far away objects. The high refractive power and low scattering properties of the cornea are largely due to the specific lamellar organization of its stroma. This lamellar organization is carefully controlled by the endothelium on the back of the cornea and by the corneal epithelium in front of it. From this it is evident that a healthy clear corneal epithelium is crucial for vision in general and for good visual acuity in particular. The corneal epithelial cells provide a smooth refractive surface and in addition form a first defense barrier for injuries from the environment. Moreover, the microvilli at the outermost epithelial surface provide a base for the tear film. The tears contribute to a balanced healthy environment of the ocular surface, and contains a range of important proteins. Anti-inflammatory proteins present in the tears like Interleukin-10 (IL-10) and lactoferrin inhibit excessive immune reactions thereby preventing corneal inflammation and subsequent scarring of the epithelium and stroma. Lactoferrin and lysozyme are anti-microbial tear proteins that contribute to the reduction and/or eventually prevention of ocular surface infections.

The corneal epithelial cells are continuously replenished by stem cells at the limbus. Without functioning stem cells at the limbus the conjunctival epithelium would invade the corneal surface causing a severe decrease in transparency and visual acuity. The local limbal environment is important for optimal function of the limbal stem cells. Their anatomical location in palisades protects the stem cells. Additional protection is provided by surrounding pigmentation and dendritic cells. Blood vessels in the limbal region provide metabolic factors, and direct access of the immunological active cells to the cornea. When the anatomy is disturbed, the function of the limbal stem cells can be compromised. Acquired anatomical changes to the limbal region have a variety of causes ranging from tumors, to excessive inflammation, and surgery. Pigmented conjunctival lesions like primary acquired melanosis, nevi, and conjunctival melanoma frequently arise at the limbus, and surgical removal or biopsies can influence the limbal environment. Cytological smears are less disturbing for the ocular surface than biopsies for histological examination. Part II of this thesis investigates conjunctival pigmented lesions, with emphasis on impression and exfoliative cytology.

Limbal stem cell deficiency is the topic of Part I of this thesis, describing a new animal limbal transplant model. In the final part (Part III), corneal infections like herpes simplex keratitis and corneal ulcers are discussed. We have investigated whether single nucleotide polymorphisms in the lactoferrin and IL10 gene are involved in the susceptibility of these infections.

PART I

LIMBAL STEM CELL DEFICIENCY

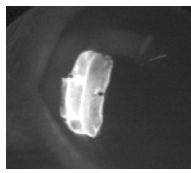
INTRODUCTION

Introduction

The cornea is the highly specialized part in front of the eye and its clarity and refractive ability are crucial for vision. A damaged corneal surface will reduce visual acuity and can ultimately lead to blindness. Most commonly the corneal epithelium is involved, but sometimes the corneal epithelial stem cells are also involved. These stem cells, located in the limbal region, play an important role in the continuous replacement of the basal corneal epithelial cells and thus in the maintenance of the corneal surface. Corneal stem cell dysfunction can lead to blindness, because the normal clear corneal epithelium is replaced by an opaque and vascularised conjunctival epithelium. Currently, there is no drug treatment for corneal stem cell deficiency and treatment has therefore been limited to transplantation of healthy limbal tissue. In patients with a healthy contralateral eye, an autologous transplant is usually successful; when both eyes are involved, allograft transplantation must be performed.[1,2] However, allograft transplants are less successful, and need intensive systemic and local immuno-suppressive treatment to prevent rejection. Cyclosporin and corticosteroids are being used to prevent transplant rejection,[3,4] but these drugs have serious side effects such as infections and tumor development in the long term.[5] Since total limbal deficiency is a rare disease and human tissue for research is limited, animal models are being used to study limbal transplant rejection to find less harmful treatments. Using current animal models it is difficult to assess transplant rejection; even the model described by Mills et al.[6] cannot predict transplant survival accurately. In this model the limbal transplants were followed both clinically and with PCR techniques. Sometimes no clinical rejection was seen but, unexpectedly, PCR analysis failed to prove transplant survival. However, in these cases the reliability of the PCR-test can be questioned, since at most only 200 corneal epithelial cells were available for analysis. The objective of this part of the thesis was to establish a new limbal transplant animal model in which transplant survival can be followed accurately in vivo.

Corneal epithelial stem cells

Anatomically the cornea consists of five layers: epithelium, Bowman's membrane, stroma, Descement's membrane, and endothelium; 90% of the corneal thickness is made up by the stroma. The cornea is an unique tissue, and its transparency is due to its regular lamellar stromal structure together with its avascularity and its relative dehydrated state. The corneal epithelium and endothelium help the cornea to maintain these characteristics. The central and peripheral corneal epithelium consists of 5 to 6 layers of epithelial cells, while at the limbal region the epithelium is 3-4 cell layers thicker. The limbus, a transitional region, marked on one side by the transparent cornea and on the other side by the whitish opaque and vascularised sclera and conjunctiva, is a circular zone characterized by the palisades of Vogt.[7,8] The limbus is the place where the corneal epithelial stem cells have their residence:[9,10,11,12] and the stem cells are thought to be located in the palisades of Vogt. However, recent research suggests that the corneal stem cells are located in 5 to 7 crypts in the limbal region. These so called limbal epithelial crypts are groups of cells that extend from the interpalisade rete ridge to the conjunctival stroma.[13] Stem cells are unique cells that are slow-cycling during homeostasis, poorly differentiated, have a high capacity for error-free self renewal, and a long life span.[14,10] The limbal basal cells are known to have some of these character-



istics[9,10,15,16,17] Corneal epithelial cell migration and differentiation starts at the limbus and occurs in a centripetal and vertical direction.[18] It starts with an asymmetrical cell division of the limbal stem cell. One daughter cell remains undifferentiated and will replenish the stem cell pool (self renewal), the other daughter cell is a transient amplifying cell (TAC) that will further divide and differentiate. The TACs have limited self renewal capacity and will give rise to post-mitotic cells (PMC) that eventually become terminally-differentiated corneal epithelial cells (TDC) (Figure 1).[19] For the protection and nourishment of the corneal epithelial stem cells the limbus is pigmented, forms palisades of Vogt, and is highly vascularised and innervated.[8] The limbal region is also the place where the largest numbers of Langerhans cells (dendritic cells) are found.[20]

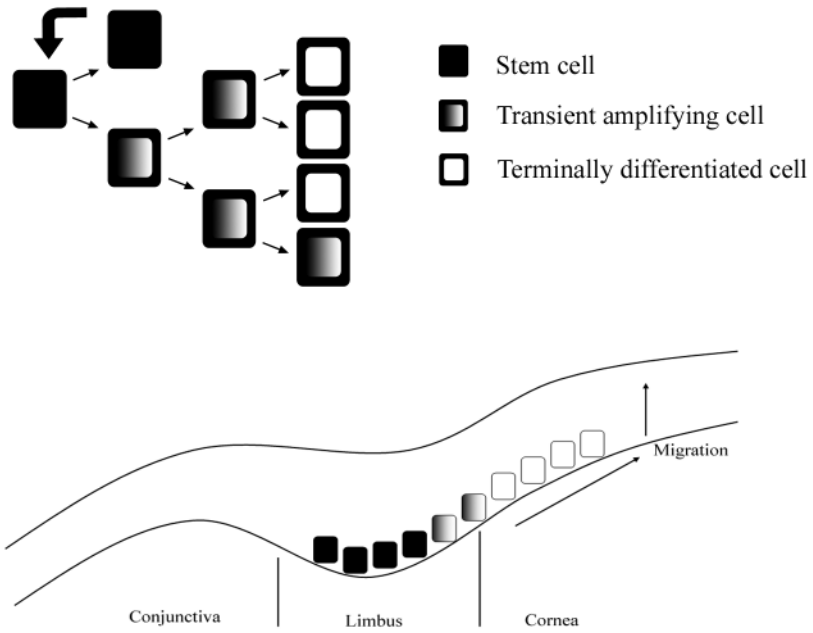


Figure 1. Limbal stem cell concept.

Proof of corneal epithelial stem cells

As has been mentioned above, the limbal epithelial basal cells have the characterstics of a stem cell. Keratin 3 and 12 are keratins that are specific for the corneal epithelium and are present at the suprabasal layers of the limbal epithelium, but not at the basal layers, indicating that the basal limbal region contains the least differentiated epithelial cells.[9,15] Furthermore, the limbal basal epithelial cells have a higher proliferative capacity in culture than the central or peripheral corneal epithelial cells.[16,17] Long time-intervals for thymidine incorporation further proved the long life cycle of the limbal basal cells.[10] Further support for the limbal stem cell theory comes from experimental studies and clinical observations of abnormal corneal wound healing when the limbal epithelium is partly or completely re-

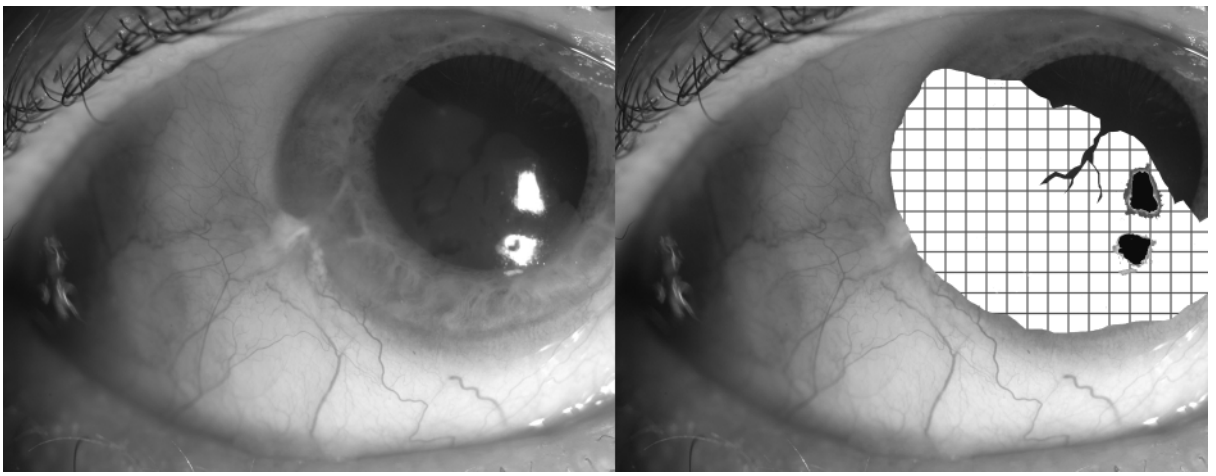


Figure 2. Partial limbal stem cell deficiency. The area with squares shows the partial limbal stem cell deficiency, where the cornea is partly opacified. The black areas inside the squares are light reflections.

moved.[21,22,23,24] The abnormal epithelium is characterized by conjunctivalization, vascularization, and chronic inflammation, all characteristics of limbal stem cell deficiency. In addition, pigmented epithelial migration lines from the limbus to the central cornea can be seen in patients with heavily pigmented eyes and eccentric corneal epithelial defects.[25] Many attemps have been made to find limbal stem cell markers. The basal limbal epithelial cells are marked by high expression of p63, alpha9-integrin, alpha-enolase, ACG2 (ATP-binding cassette subfamily G member 2) and the absence of CD71, Connexin 43, gap junctions[11,26,27,28], but none of these markers are absolutely specific.[29]

Limbal stem cell deficiency

When the limbal stem cells are damaged, the corneal epithelium is slowly replaced by vascularised conjunctiva at the site of the damage (Figure 2). Since the conjunctival epithelium does not have the clarity of the corneal epithelium, the visual acuity will be decreased depending on the number and sites of limbal stem cells involved. Besides the opacification and vascularisation of the cornea, other characteristics for limbal disease are: loss of palisades of Vogt, epithelial instability resulting in ulceration or recurrent erosions, chronic inflammation, tear film dysfunction, and increased incidence of infectious keratitis.[30] Most patients therefore complain about reduced vision, irritation, pain, and light sensitivity, depending on the extent of limbal dysfunction.

There are a variety of causes of limbal stem cell dysfunction, which can be subdivided into genetic and acquired (Table 1). The acquired limbal stem cell disorders form the majority of cases seen clinically. To prove limbal stem cell deficiency, impression cytology can help to diagnose abnormal epithelium by detecting goblet cells or keratinisation.[41,42] Limbal stem cell deficiency may be localized (partial), or diffuse (complete, 360º). In localized limbal stem cell deficiency, some sectors of the limbal and corneal epithelium are normal while the conjunctivalisation is restricted to the regions devoid of healthy epithelium. The stem cell dysfunction can be either unilateral or bilateral, which is of major consequence for the treat-

ment given, as will be discussed in the next paragraph.

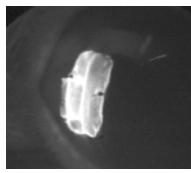


Table 1. Causes of limbal stem cell dysfunction

Genetic causes	
	Aniridea[31]
	Epithelial and stromal dystrophies[32]
	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)[33]
	Lacrimo-auriculo-dento-digital (LADD) sydrome[34]
Acquired causes	
	Chemical and thermal burns[35]
	Contact lens wear[36]
	Chronic inflammation[37]
	Multiple surgeries at the limbus[30]
	Cicatricial pemphigoid[30]
	Stevens Johnson Syndrome[30]
	Pterygium[38]
	Drug toxicity[39]
	Ocular surface squamous neoplasia[40]
	Idiopathic[30]

Treatment of limbal stem cell dysfunction

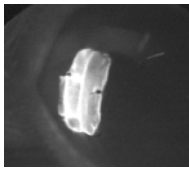
Currently there is no drug therapy for limbal stem cell failure, and the treatment is therefore mostly limited to transplantation. Before the introduction of the limbal stem cell concept, corneal transplants were performed to improve visual acuity.[43] However, this is a solution for the short term since the actual problem, which is situated at the limbus, is not resolved. A variety of transplant techniques have been developed, aimed at solving the stem cell deficiency itself (Table 2). Conjunctival autografts were first used to treat limbal stem cell deficiency;[44] the conjunctival epithelium of these grafts was thought to be able to change its characteristics to become similar to the corneal epithelium. Later, this was called conjunctival transdifferentation.[45] In time, the conjunctival transplants were modified to also include parts of the limbus.[46,36] Using a rabbit model, Tsai et al. proved that transplants which included the limbus were more successful than conjunctival grafts.[47] Therefore most current transplantations involve the limbal region, with exception of transplantation of mucous membrane or amniotic membranes which will be mentioned later. The transplants that contain mostly conjunctival epithelium and limbal tissue are called conjunctival-limbal grafts, for kerato-limbal grafts the biggest part is corneal and limbal tissue. The conjunctival-limbal grafts are mostly used when there is a living donor, i.e. autologous transplants and living-related donors (a relative of the patient). The kerato-limbal transplant probably contains more limbal stem cell, however, it is more damaging to the donor eye and is therefore more often used when there is cadaveric tissue.[54]

Table 2. Different transplantations performed for limbal stem cell deficiency

Transplantation method	
Autografts	Conjunctival graft[44]
	Conjunctival-limbal graft[46]
	Oral mucosa[66]
Allografts	Living-related conjunctival graft[48]
	Living-related conjunctival-limbal graft[49,50]
	Cadaveric conjunctival-limbal graft[51]
	Cadaveric keratoepithelialplasty[52]
	Cadaveric kerato-limbal graft[53]

Limbal autografts have the advantage of not being rejected and are successful; however, a contralateral healthy eye must be available, since a donor eye with subclinical limbal dysfunction gives limited results.[36] Another major drawback is the possibility to induce limbal deficiency in the contralateral donor eye.[55,56] Recent developments made it possible to culture limbal tissue ex vivo from a 1-2 mm^2 biopsy,[57,58,59] thereby reducing the risk of limbal deficiency of the contralateral donor eye and increasing the number of patients that are suitable for autografting. However, when patients with severe bilateral disease are not suitable for autografts, limbal allografts are an alternative. The limbal allografts can come from a deceased donor (cadaveric graft). The high concentration of Langerhans cells (dendritic cells), abundancy of HLA-DR antigens, and the good vascularisation makes these transplants at risk for rejection.[20] HLA matching of limbal allografts might help to improve transplant survival.[60] Similarly, living-related transplants are also used to decrease the risk of transplant rejection.[49,50] Although there are reports of a good long term clinical outcome with limbal allografts, long term persistence of donor corneal epithelial cells is still a topic of discussion.[61,62,63,64,65,60] Since some reports failed to show surviving donor epithelial cells, it was suggested that the transplant improved the local environment to such an extent that residual limbal stem cells were able to survive and be functional, possibly by providing growth factors or other mediators for the host stem cells. On the other hand, donor cells were recently found till 3.5 years after limbal allograft transplantation.[64]

Alternatives for limbal allografts are either transplantation of amnion or autologous oral mucosa. Autologous oral mucosal epithelium has been introduced by Nishida et al,[66] who were able to improve the corneal surface and the visual acuity in four patients. Because the mucous transplant is autologous there is no rejection, while another advantage is the abundance of donor material in the oral cavity. Transplanted amnion, the most inner layer of the placental membranes, will also not reject since amnion lacks HLA-A, B or DR antigens.[67,68] Amnion transplants have been used in ophthalmology for corneal surface disease since 1940.[69] In 1995 amnion transplants were re-introduced in humans to treat ocular surface disease including limbal stem cell deficiency.[70] The amnion helps to improve epithelialization by providing a basement membrane and several growth factors;[71] it also inhibits fibrosis, angiogenesis, and inflammation.[72,73,74] Amnion is used in partial limbal stem cell deficient



eyes (less 50 %); when more than 50 % of the limbus is involved it can be combined with a limbal graft, however, the beneficial effect of the amnion is not clear.[75,76,54]
Since most patients with limbal stem cell deficiency also have stromal involvement, penetrating keratoplasty is sometimes combined with a limbal transplant. There are indications that simultaneous transplantation of a limbal graft and a full thickness corneal button decreases graft survival.[3] Therefore it is recommended that a limbal transplant must be performed first, and that the corneal transplant follows after three months, when the corneal epithelium is stable and the eye not inflamed.[77,78]
Despite the variety of surgical techniques to reconstruct the ocular surface in limbal stem cell deficient eyes, it remains a disease that is difficult to treat. The success rate of a limbal transplants declines from 75-80% in the first year to 47-50% at three-year follow-up.[75,72,73,79] Good prospective, case-controlled clinical trails for the various surgical techniques are still lacking.

Transplant rejection

That limbal allografts are prone to rejection has been mentioned above. Especially the vascularization and high amount of dendritic cells at the limbal region are factors that increase the chance for rejection. Transplant rejection is a T-cell mediated response to allo-antigens. In animal models high amounts of both CD4 and CD8 positive T cells and macrophages can be found.[6,80,81] Treatment options to prevent allograft rejection may differ from a combination of topical prednisolon with systemic cyclosporine A, or systemic tacrolimus (prograft) and mycofenolaatmofetil (cellcept). How long the therapy must last is not clear, but probably as long as there is a living allogeneic transplant. In patients who have a successful clinical outcome after limbal allograft transplantation, it is difficult to assess clinically whether the corneal epithelium is all donor-derived or if the host cells have repopulated the surface. Djalilian et al.[64] showed long term survival of donor cells and they support the use of long term immuno-suppression. However, others failed to establish donor cell survival and therefore question the use of systemic long term immuno-suppression.[65] Also tacrolimus, a macrolid antibiotic with immuno-suppressive characteristics and mycophenolate mofetil (CellCept) have been used to prevent rejection of limbal allografts.[3,82,60] It must be noted that long term systemic immuno-suppressive treatment is known to be related to tumor development and susceptibility to infections.[5] Since both can be life-threatening, questions must be raised whether the increase in visual acuity by a limbal allograft is worth the increased risk to develop cancers and infections. Therefore new less harmful therapies must be investigated.
In animal models, local clodronate liposome treatment has been used to prevent corneal graft rejection. Clodronate liposomes are liposomes which contain clodronate, which is a modified form of Ostac®. Both liposomes and clodronate itself are non-toxic. When macrophages consume and breakdown the liposomes, clodronate will be released intracellularly. Clodronate will therefore accumulate inside the macrophage, and when a certain threshold is reached, the cell is irreversibly damaged and will undergo apoptosis. In a corneal transplantation model clodronate liposomes have been used to locally deplete macrophages and thereby increase corneal transplant survival.[83,84] This therapy has not yet

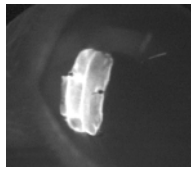
been registered for humans.

Animal models

A variety of animal models have been developed, using mice, rats, and rabbits.[6,85,86,87,88,89,80,47,81] Most animal models have disadvantages, which is probably inherent to the use of an animal model. Rabbit models are not suitable to investigate the immunological background of limbal transplantations since the animals are not inbred and there is a limited availability of antibodies. However, when compared to mice and rats, the rabbit eye probably more closely resembles the human eye. The mouse model is probably the best model to investigate the immunological process, because of the wide variety of mice strains and a huge amount of antibodies available. The size of the mouse eye and cornea are a limitation and limbal transplantations are therefore difficult to standardize. The only operation that probably can be reliably performed is the kerato-epithelial transplant. The rat model is a compromise between the mouse and rabbit models. The rat eye is large enough to perform limbal transplants, there are inbred strains, but only a limited availability of antibodies. Mills [6] was the first to use the rat for limbal transplantation; in this model the limbal transplants were followed up both by PCR, and clinically. They showed that donor corneal epithelial cells did not survive much longer than seven days despite immuno-suppressive treatment. However, there was a discrepancy with the clinical follow-up where the treated allografts survived much longer. This could be similar to limbal transplants in humans, since clinically they perform well, while there is difficulty in showing donor epithelial cells some time after transplantation.[61,63] The animal model by Mills[6] is a reliable model, but we started our experiments with the hope that transplant follow-up could be improved, as is further discussed below and in Chapter 2.

Enhanced Green Fluorescent Protein

Green fluorescent protein was first discovered in the jellyfish *Aequorea* by Shimomura et al.[90] The major advantage of GFP is its intrinsic fluorescent capacity, for most other fluorescent proteins a co-factor is needed. Enhanced green fluorescent protein (E-GFP) is an enhanced version of GFP. When the protein is excitated with blue light of around 488 nm it will emit green light from around 508 nm. It is a protein with a chromophore inside, which is responsible for the fluorescent characteristics. A fluorescent microscope is needed to illuminate the chromophore with pure blue light, the green light that is produced can than be recorded by the microscope. E-GFP has been widely used for research purposes, it can be used to track proteins in living cells.[91] Also in animal models E-GFP is used to track tumor growth, tumor metastasis, tumor angiogenesis, and gene expression[92,93,94,95,96]; it is considered to be the least immunogenic fluorescent protein. GFP has also been used to study corneal epithelial migration.[18,97] In Chapter 2 we investigated whether E-GFP could be used to monitor limbal transplant behaviour, and whether it could used to reliably detect transplant rejection.



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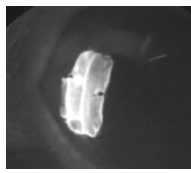

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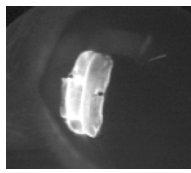

CHAPTER 2

A NEW MODEL FOR LIMBAL TRANSPLANTATION USING E-GFP FOR FOLLOW-UP OF TRANSPLANT SURVIVAL.

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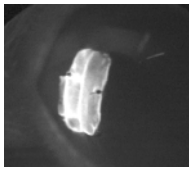
Abstract

Introduction: Limbal transplants in humans show a high rate of rejection even under local and systemic immunotherapy. In order to test immuno-modulatory treatments a new limbal transplant model in the rat was developed using enhanced green-fluorescent protein (E-GFP) as marker for follow-up.

Methods: Sixty E-GFP-positive limbal transplants from Sprague-Dawley TgN(act-EGFP)Osb4 rats were transplanted onto 18 wild type inbred Sprague-Dawley (isografts) rats, six wild type litter mate Sprague-Dawley (sibling) rats, 18 Fischer 344 (allografts) rats, and 18 Fischer 344 rats depleted from monocytes and macrophages by subconjunctival treatment with clodronate liposomes. All rats were monitored three times a week with fluorescence microscopy, until fluorescence had disappeared. At postoperative days 6, 9, 12, and 15, three rats of all groups were sacrificed for immunohistochemical analysis of infiltrating cells.

Results: Using a modified digital fluorescence microscope, we were able to monitor transplant behavior over time without disturbance of the ocular surface. The average days of rejection were 14 days in the isograft group, the sibling group, and the untreated allograft group. However, the average day of rejection in the allogeneic macrophage-depleted group was 27 days. Marked infiltration of macrophages and lymphocytes was seen in the untreated isografts and allografts. In the clodronate liposome-treated allografts infiltration was minor. A successful new limbal transplant model is described.

Conclusion: The transplant can be accurately followed-up in vivo by E-GFP labeling of the donor tissue without disturbing the corneal surface. Although E-GFP itself proved to be immunogeneic local clodronate liposome injections significantly increased graft survival. So the model seems to be useful for testing immuno-suppressive or modulatory agents in limbal transplantation studies.



Introduction

The stem cells of the corneal epithelium are located in the limbus. [1] When the limbus is damaged, the outgrowth of corneal epithelial cells is disturbed and there is a good chance that conjunctival epithelium will cover the corneal surface, leading to loss of corneal transparency and to visual impairment. In order to provide the damaged limbus with new corneal stem cells, a limbal transplantation can be performed to restore transparency and vision and reduce discomfort. In case of unilateral damage an autologous transplant can be performed, [2,3] for bilateral damage an allotransplant is indicated. [4]

The behavior of limbal grafts is not well understood. Little is known about the outgrowth of corneal epithelium from the grafted limbus. Transplant survival is a controversial subject, as some claim a limited survival [5,6,7] while others claim a prolonged survival of limbal grafts in humans. [8,9,10,11]

Animal models are being used to increase our knowledge about limbal transplants, using rabbits, rats, or mice. [12,13,14,15,16,17,18] Some authors follow the transplant clinically, while some use laboratory techniques to determine transplant survival. However, many of these techniques have a low sensitivity or specificity, and therefore have difficulty in showing the difference between transplant survival or rejection. Usually epithelial cells must be removed from the corneal surface to assess outgrowth or limbal transplant survival.[12] These techniques cause some damage to the corneal epithelium, and can therefore influence the transplant's behavior. We have developed a new rat limbal transplant model, in which we used donor tissue from enhanced green-fluorescent protein (E-GFP)- transgenic rats. E-GFP is a protein that emits green light when excited with blue light. Because of this characteristic it was possible to monitor in vivo the graft acceptance or rejection in detail, without the need to interfere with the corneal surface.

Materials and Methods

Experimental animals

Female Sprague-Dawley TgN(act-EGFP)Osb4 ("green" rat) rats, and female Sprague-Dawley TgN-Osb4 (no E-GFP) were bred within the animal facility of our institution. Breeding pairs of this E-GFP-transgenic SD strain was a generous gift from Dr. Masaru Okabe, Genome Information Research Center, Osaka University, Japan. In these transgenic rats, E-GFP is connected to a chicken beta-actin promoter, and a CMV enhancer. [19] Therefore all cells, which produce beta-actin, will also express E-GFP. [19,20,21] Inbred female Fischer 344 (F344) and inbred female Sprague-Dawley (SD) rats were obtained from Charles River (Maastricht, The Netherlands). All animals were allowed unlimited access to water and rat chow. The ARVO statement for the use of animals in ophthalmic and vision research was followed.

Limbal transplantation

Donor rats were anesthetized with Hypnorm 0.4 ml/kg (Janssen Pharmaceutica, Beerse, Belgium) and midazolam 0.4 mg/kg (Roche Nederland, Mijdrecht, The Netherlands). In addition, local anesthesia was obtained with one drop of oxybuprocaine 0.4 % (Théa

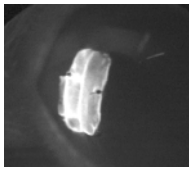
Pharma, Ukkel, Belgium) and a subconjunctival injection (20 µl) of marcaine 5mg/ml (Astra Zeneca, Zoetermeer, The Netherlands). The limbal transplants were excised with the use of a surgical microscope according to the protocol used by R.A. Mills. [12] In brief: lamellar limbal tissue (1-2 mm wide and 3-4 mm long) was excised from the limbus, using disposable slit and crescent knives. The limbal tissue consisted of a corneal part and a smaller conjunctival part to ensure that the limbus was included. Both parts included epithelium and stroma. After excision, the tissue was kept in sterile RPMI 1640 cell culture medium (GIBCO, Invitrogen Corporation, Paisley, Scotland, UK). After use, the donor was killed by exposure to an overdose of CO_2. The recipient rats received the same anesthesia as the donor rats. A transplant bed was cut out, slightly smaller than that of the donor limbal tissue. The limbal tissue was sutured in place with four to six 10.0 nylon sutures (Alcon Laboratories, Texas, USA). During surgery, the eye was kept hydrated with Willospon (Will-Pharma, Wavre, Belgium), which was regularly irrigated with sterile balanced salt solution. After surgery, chloramphenicol ointment 1% (Yamanouchi Pharma, Leiderdorp, The Netherlands) was applied.

Experimental groups

Isografts (syngeneic) (n=6) were performed with a SD "green" female rat donor and a SD wild type female recipient (Charles River). Allografts (allogeneic) (n=6) were performed with a SD "green" female rat donor and F344 female recipient (Charles River). A second group of allograft recipients (n=6) were treated with 100 µl subconjunctival injections of clodronate liposomes [22] at postoperative days (POD) 0, 2, 4, and 7. Clodronate (Cl2MDP) was a gift of Roche Diagnostics GmbH (Mannheim, Germany), and it was encapsulated in liposomes as described before. [23] We also operated an additional group of isografts (n=6) where the donor and recipient were siblings. In this sibling group, the E-GFP positive donor and E-GFP negative recipient were littermates from the same Spraque-Dawley nest. Six isografts, six sibling-isografts, six allografts, and six clodronate liposome-treated allografts were followed three times weekly with a fluorescent microscope, till no more fluorescence was seen. Hereafter the animals were killed and the eye with the transplant was enucleated for immunohistochemistry. Twelve isografts, 12 allografts, and 12 clodronate liposome-treated allografts were also followed three times a week with the fluorescent microscope, but at POD 6, 9, 12, and 15, three rats per group were sacrificed and the graft-containing eye was enucleated for immunohistochemistry. Before the enucleated eyes were processed for immunohistochemistry, transplants were analysed and recorded with a confocal microscope (LSM510, Carl Zeiss AG, Oberkochen, Germany) and three-dimensional pictures of the transplants were reconstructed.

Follow up of the E-GFP limbal transplant

E-GFP is a protein with fluorescent properties. When the molecule is illuminated with 490 nm light (blue) it will emit 509 nm light (green). We followed the limbal transplant with an orthoplan fluorescence microscope (Leica Microsystems, Wetzlar, Germany), using a blue excitation filter. The images were recorded with a modified Canon A60 digital camera.



Three times a week, the animals were anesthetized with inhalation anesthesia (Isoflurane), and photographed. When fluorescent emission of E-GFP was no longer observed, the transplants were assumed to be fully rejected. Animals were than killed with an overdose of carbon dioxide (CO_2).

Immunohistochemistry

Enucleated eyes were embedded in paraffin using the following protocol. Enucleated eyes were first immersion fixed in buffered paraformaldehyde 4 % (pH 7.0) for 24 hours and subsequently treated with ethanol 70 % for 2 hours, ethanol 99 % for 1 hour, absolute ethanol for 30 min, xylene at room temperature for 1 hour, xylene at 37 °C for 10 minutes, then 1 hour in paraffin at 56 °C, where after the eyes were embedded in paraffin. The paraffin blocks were cut into 4 μm sections and placed on glass slides.

The sections were photographed with a fluorescence microscope for detection of E-GFP. Hereafter the sections were stained with the following antibodies: anti-CD4 for CD4 positive T-lymphocytes, anti-CD8 for CD8 positive T-lymphocytes, anti-CD68 ED1 for macrophages and anti-laminin for blood vessels. The following staining protocol was used: slides with sections were deparaffinized in xylene (4 times, 5 minutes each) and ethanol 99 % (2 times,5 minutes each). The endogenous peroxidase activity was blocked by incubating the slides with methanol/H_2O_2 0.3 % for 20 minutes. After washing the slides with PBS, antigen retrieval was performed by either boiling in citrate buffer (Dako, Glostrup, Denmark) for 10 minutes (CD4 and CD8), or 30 min in 37 ˚C trypsin (laminin), or boiling in TRIS-HCL for 10 minutes (ED-1). Slides were washed in PBS and were incubated overnight with the first antibody at 4 ˚C. Mouse anti-CD4 mAb clone W3/25 (dilution 1:3200)(ab6413, Abcam, Cambridge, UK), mouse anti-CD8a mAb (dilution 1:10000), mouse anti-CD68 mAb clone ED-1 (dilution 1:2000)(BM4000, Acris antibodies, Hiddenhausen, Germany), and rabbit anti-laminin polyclonal, (dilution1:50)(L9393, Sigma-Aldrich, Steinheim, Germany) were used. As negative control, the primary antibody was replaced by PBS. Slides were then incubated with biotinylated anti-mouse anti-rabbit Ig (Dako) for 30 minutes. After washing, the slides were labeled with Streptavidin-HRP (Dako) for 30 minutes; hereafter the labeling was visualized by a 10-minute incubation in DAB (3,3 diaminobenzidine). Slides were counterstained with Mayer's haematoxylin and finally embedded in Kaiser's glycerine. Slides were scored by counting the number of positive cells or vessels in the transplant, and measuring the transplant area. Cellular infiltration is expressed as number of cells per mm^2 and vessel ingrowth as number of vessels per mm^2. E-GFP positive vessels were expressed as percentage of the total number of vessels.

Statistics

For statistical analysis the mean day of rejection was used ± SEM (standard error of the mean). Data was analyzed in SPSS 11.0 (SPSS Inc, Chicago, USA). Kaplan-Meier survival curves with log rank test were used to calculate differences in limbal transplant survival. A p-value of 0.05 or less was considered significant.

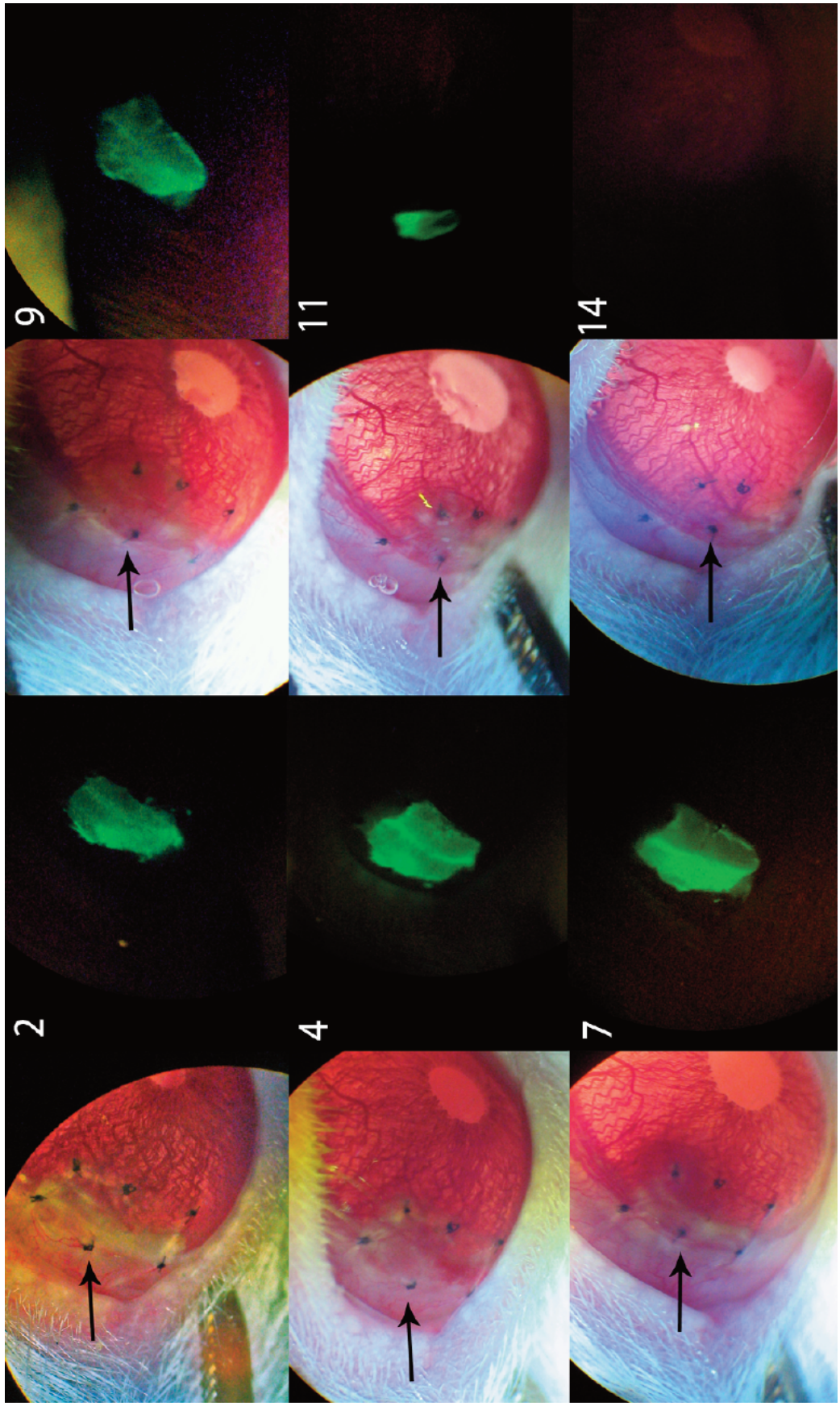
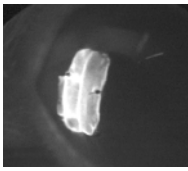


Figure 1. Images of an untreated E-GFP positive isograft at post-operative days 2, 4, 7, 9, 11, and 14. The left-hand pictures are in a normal color setting, the right-hand pictures are the corresponding fluorescent pictures. On day 14, fluorescence is no longer present. The arrows point at the transplant with the six (black) sutures. Note the decreased visibility of the iris vessels below the transplant at days 7 and 9 because of the increase in inflammation.



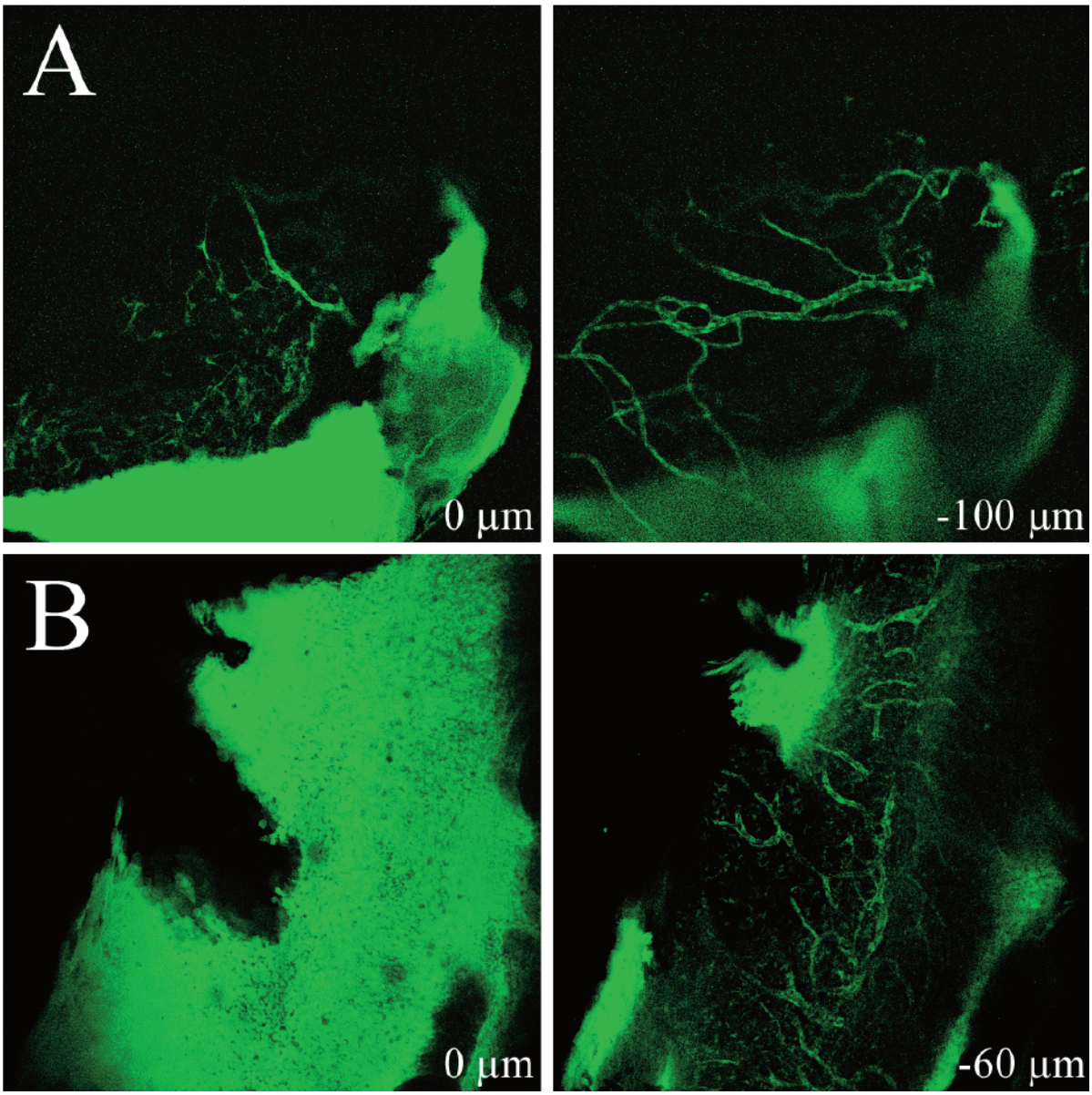


Figure 2. Images from two E-GFP positive transplants taken by confocal microscopy. The right-hand pictures are obtained from a deeper layer than the surface pictures shown on the left side, at 100 µm, and 60 µm depth, respectively. Note the vessels that can be detected underneath the epithelial transplant surface. Figure 2A is taken at day 12 postoperative, and Figure 2B at day 9 postoperative.

Results

Model

The present study revealed that by using fluorescence microscopy and digital camera recording it was easy to follow the E-GFP-labeled grafts by taking pictures at different time intervals after transplantation. Using this system, it is possible to monitor the same graft over time in the same animal (Fig.1). The 3-D confocal reconstructions of the enucleated eyes give a good in-depth insight of the distribution of E-GFP in the transplant and surrounding recipient tissue (3D movies at the Experimental Eye Research website). Fig. 2 partly illustrates this for two transplants.

Graft survival

The limbal isografts had an average transplant survival of 14 ± 0.3 days. Average transplant survival of the allografts (E-GFP-positive SD donor rats, Fisher 344 rats as recipients) was 14 ± 0.9 days (Fig. 3). As these similar results for isografts and allografts were unexpected, we questioned whether rejection really occurred in allogeneic grafts, or that the grafts disappeared because of for instance competition with normal recipient epithelium. We therefore used a treatment which has proven to prolong corneal graft survival by reducing the immunological rejection.[22,24] Recipient rats received subconjunctival injections with clodronate liposomes at POD 0, 2, 4, and 7. The thus treated allogeneic group survived for 27 ± 3.4 days (Fig. 3). This increased survival of clodronate liposome treated allografts is highly significant compared to the untreated allografts and isografts ($p = 0.0018$, Log rank test).

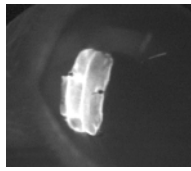
Immunohistochemistry

Immunohistochemical studies were performed to determine whether infiltration of the transplant with immunological cells or blood vessels of the recipient occurred. The histological results are given in Fig. 4. In the isografts, the highest density (cells/mm^2) of macrophages (ED-1), CD4-positive lymphocytes, and CD8-positive lymphocytes were seen on POD 12, while in the allografts, this was on POD 9 (Fig. 5). In the clodronate liposome-treated allogeneic group no significant increase in ED-1, CD4, or CD8 positive cells was seen. There was no difference in total amount of infiltrate between isografts and allografts, however, there was a delay in onset of infiltrate in the isograft group when compared to the allograft group.

Underneath the epithelium of the transplant laminin-positive blood vessels, staining both recipient and donor blood vessels, were seen on POD 6, 9, 12, and 15 in all three groups, and no clear trend was seen over time. However, when looking at the percentage of E-GFP-positive vessels a peak was seen on POD 9 with a decrease to zero on POD 15 in all three groups (Fig. 6).

E-GFP or minor transplantation antigen mismatches?

We conclude from the findings described in the previous paragraph, that in both the syngeneic group and the allogeneic group, immunological rejection forms the basis of the dis-



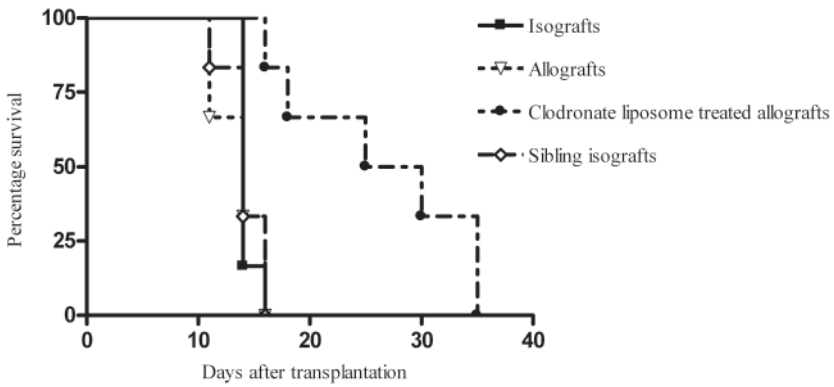


Figure 3. Limbal transplant survival curves for isografts, sibling isografts, allografts, and clodronate liposome treated allografts. Limbal transplants survived significantly (p=0.0018) longer in the allograft group treated subconjunctivally with clodronate liposomes till post operative day 7.

appearance of the grafts. This suggests that E-GFP itself may be a cause of rejection in an otherwise syngeneic combination. However, another option might be that since we had used two different SD strains as "syngeneic" donor and recipient, we might be dealing with minor transplantation antigen mismatches. We therefore did an additional experiment with E-GFP positive and negative littermates as donor-recipient combinations to test this possibility. The outcome was an average transplant survival of 14 ± 0.7 days. We did not perform immunohistochemistry on this material. Statistical testing revealed that the average time of rejection between the isografts, sibling isografts, and the untreated allografts was not significantly different ($p = 0.94$, Log rank test).

Discussion

We have developed an E-GFP labeled limbal transplant model in which the transplant can be followed in vivo without disturbing the corneal environment of the recipient, which was the main goal of this study. The decrease in E-GFP fluorescence seen in both isograft and allograft groups is most likely due to rejection. This conclusion is based on the following findings. First, the decrease in fluorescence can be delayed with clodronate liposome injections. Secondly, the decline in E-GFP fluorescence (Figs. 1 and 3) is preceded by T-lymphocyte and macrophage infiltration (Figs. 4 and 5), which corresponds with the onset of clinical rejection seen 3-5 days prior to full loss of E-GFP fluorescence. Surprisingly, the non-clodronate liposome-treated isografts and allografts were rejected at the same time. This could be due to either a minor antigen mismatch between the donor group and recipient isograft group, or E-GFP is the cause of the rejection. E-GFP is known to cause an immune response

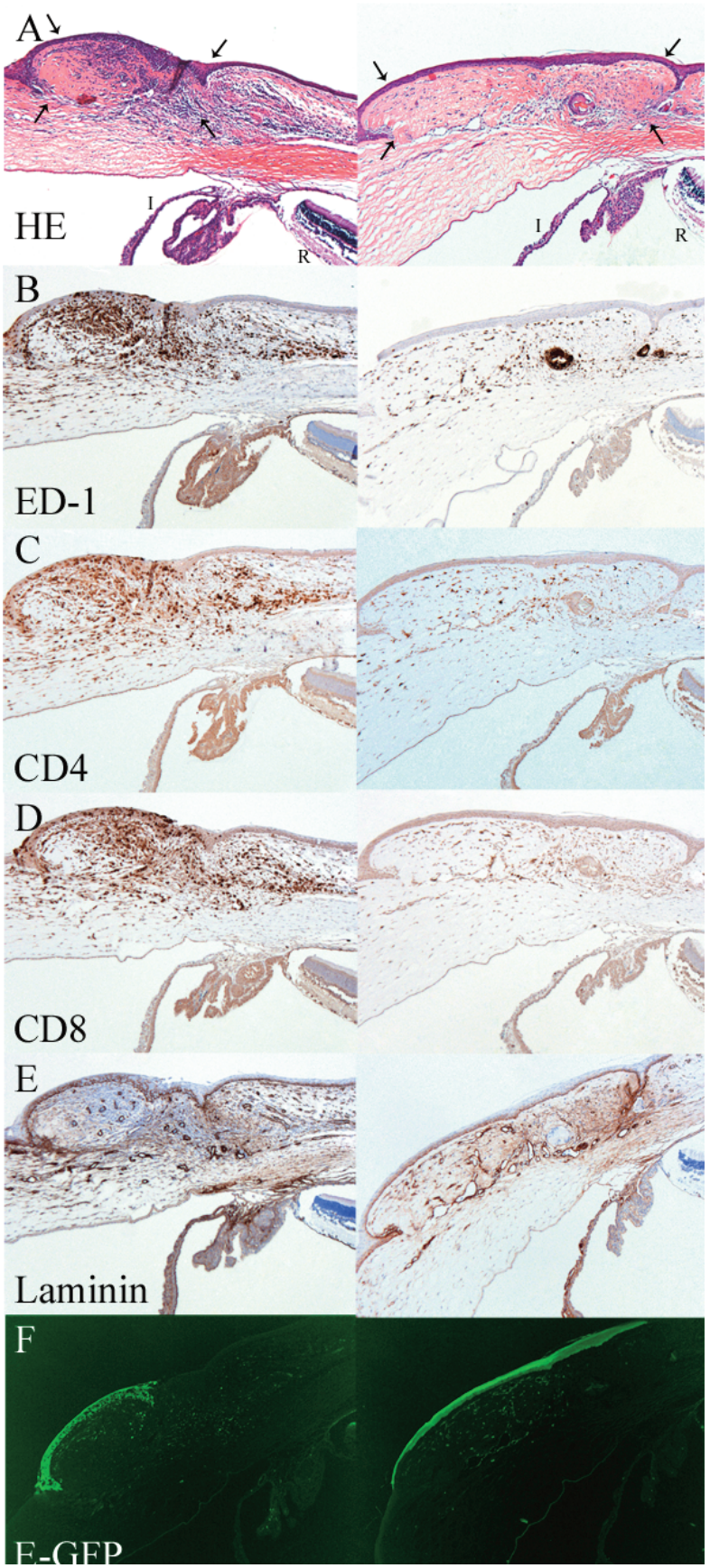
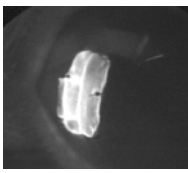


Figure 4. The-left hand pictures are of an untreated allograft, the right-hand pictures are from a clodronate liposome treated allograft. The left and right-hand figures represent consecutive sections. Sections A are stained with HE and show the outline of the transplants (arrows) and the position of the iris (I) and retina (R). Sections B-E show the immunohistochemical stainings for macrophages with anti-ED-1, for T-lymphocytes with anti-CD4 and anti-CD8 and for blood vessels with anti-laminin respectively. Sections F show the presence of E-GFP. Note the abundance of macrophages and CD4 and CD8 positive lymphocytes in the untreated allografts as compared to the clodronate liposome treated allografts. The difference in vessel density is not obvious.



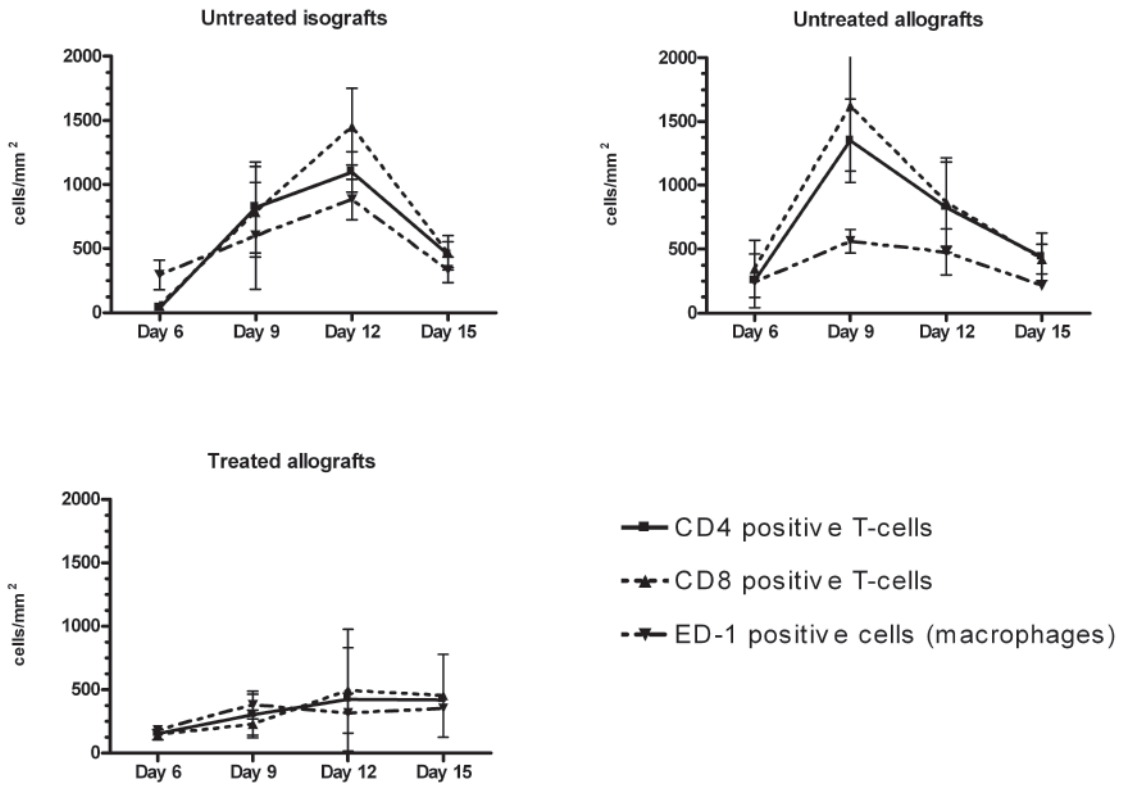


Figure 5. Density of CD4-positive T-lymphocytes, CD8-positive T-lymphocytes, and macrophages in untreated limbal isografts, allografts and clodronate liposome treated allografts. The error bars represent the 95% confidence interval.

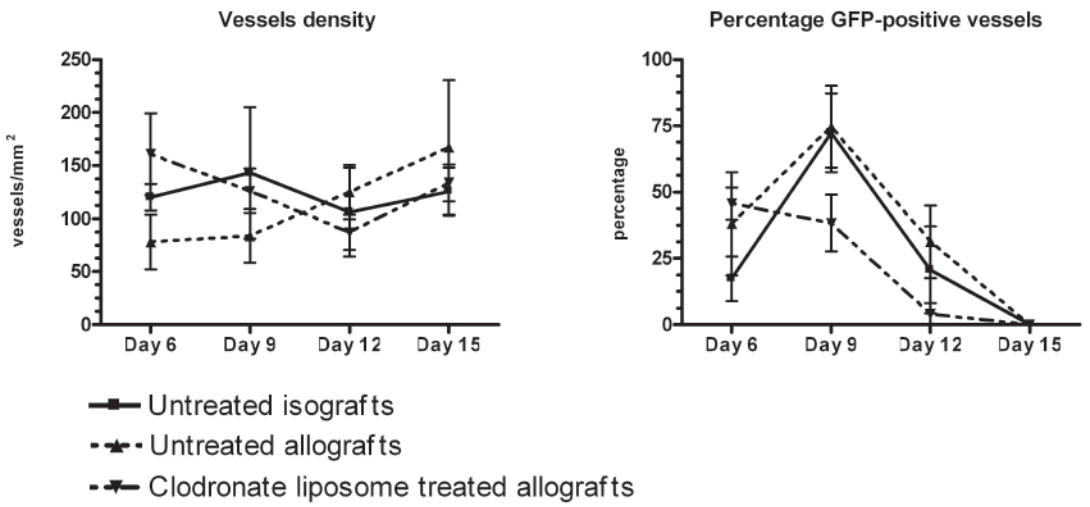
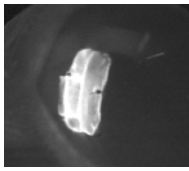


Figure 6. Density of vessels and percentage of E-GFP positive vessels in the limbal isograft, allograft and clodronate liposome treated allograft groups. The error bars represent the 95% confidence interval



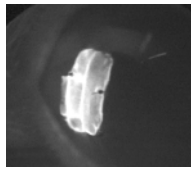
in animals. [25,26,27,28] Therefore we also performed limbal transplants from E-GFP-positive donors to E-GFP-negative siblings of the same family, thereby minimizing the genetic background mismatch. However, these transplants were also rejected around day 14, and therefore it was likely that the E-GFP was involved in the rejection of the isografts. Although the E-GFP survival was the same between the isograft and allograft group, immunohistochemistry showed that the T-lymphocyte and macrophage infiltration developed slightly later in the isograft group than in the allograft group (Fig. 5). This may indicate that although E-GFP can be regarded as a minor antigen, it is less immunogeneic than a full major histocompatibility complex (MHC) mismatch.

When treating the allografts with clodronate liposomes for a relative short period (7 days) an increase in transplant survival from 14 to 27 days occurred, and no increase in T-lymphocyte and macrophage infiltration was seen (Figs. 4 and 5). In corneal transplants treated with clodronate liposomes, survival is much longer, i.e. >100 days.[22] This difference could be due to the vascular environment in which the limbal transplant is sutured, since in prevascularized corneas, corneal transplants survived only 47 days with clodronate liposomes. [24] Moreover, the transplant is situated in a healthy eye, and could therefore be slowly replaced by host epithelium. In humans it is also seen that donor tissue is not detectable some time after transplantation.[29,30,7]

We also looked at vessel growth into the transplant. Clinically it looks as if the vessels penetrate the transplant from the surrounding conjunctiva of the recipient. However, on POD 9, fluorescence microscopy revealed (Fig. 2) that most vessels underneath the transplanted epithelium were E-GFP positive, showing clear signs of neoangiogenesis. The total number of vessels (both recipient and donor) remained stable during the investigated time points, however, in all three groups the density of E-GFP vessels seems to increase up till day 9 while E-GFP vessels were no longer seen at day 15 (Fig. 6). For the non macrophage-depleted groups this could be explained by the rejection that occurred. Why the macrophage-depleted isografts also showed no E-GFP vessels at day 15 while there still was E-GFP positive epithelium is not clear. It could be due to the inhibiting effect of macrophage depletion on hemangiogenesis.[31] It must be noted that for each time point and transplant group only 3 eyes were used for analysis, this low number could have influenced the data.

The present model is very similar to the model used by R.A. Mills [12] however, in that model corneal epithelial cells had to be removed to prove transplant survival, possibly causing disturbance of the corneal surface. Corneal epithelial sampling is limited by sampling errors and the low amount of cells harvested. The E-GFP model has no such disadvantages, and can accurately follow transplant survival. In Mills' model allograft survival did not increase by systemic immuno-suppression with cyclosporine. In our model, it was possible to increase graft survival by short time local immuno-suppression with clodronate liposomes, although only for a period up to 35 days. No limbal deficient rats were used in this study, which is a limitation, but in order to study transplant rejection limbal deficiency is not mandatory. The E-GFP-transplant did not increase in size during the follow-up period in this study probably due to the fact that the transplant is completely surrounded by healthy conjunctival and corneal epithelium blocking a stimulus for the transplant to increase in size. In the future, we will try to create a limbus-deficient rat model and will transplant several transplants onto

one eye to follow-up transplant behavior. The fact that E-GFP in itself acts as a transplantation antigen must be taken into account.



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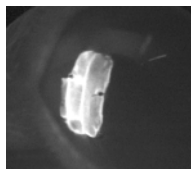


PART II

PIGMENTED CONJUNCTIVAL LESIONS

INTRODUCTION

Introduction

Conjunctival melanoma is the most potentially fatal pigmented lesion of the conjunctiva; it is a malignant lesion with a 30 % mortality in 10 years.[1] It is difficult but crucial to distinguish the malignant conjunctival melanoma from other benign melanocytic conjunctival lesions such as conjunctival nevi and conjunctival melanosis. Although it is a very rare disease, 0.2-0.8 cases per 1.000.000 inhabitants per year,[1,2,3] every ophthalmologist must be aware of this tumour. In the Netherlands, an average of about eight conjunctival melanomas can be diagnosed each year. The clinical presentation of conjunctival melanocytic lesions can help to distinguish between a nevus or primary acquired melanosis and a conjunctival melanoma, although it remains difficult to differentiate between them. Cytological and histological examinations are essential investigations to differentiate between these entities.

Anatomy of the conjunctiva

The most outer parts of the eye are the cornea and the conjunctiva. The cornea is a transparent avascular tissue, while the conjunctiva is opaque and vascularized. The bulbar conjunctiva is a free movable protective mucous membrane that covers the sclera and is continuous with the inner layer of the eyelids (palpebral conjunctiva). The conjunctival fornix is the junction of the bulbar and palpebral conjunctiva, thereby sealing off the orbital cavity from the outside environment (Figure 1). The circular zone where the conjunctiva

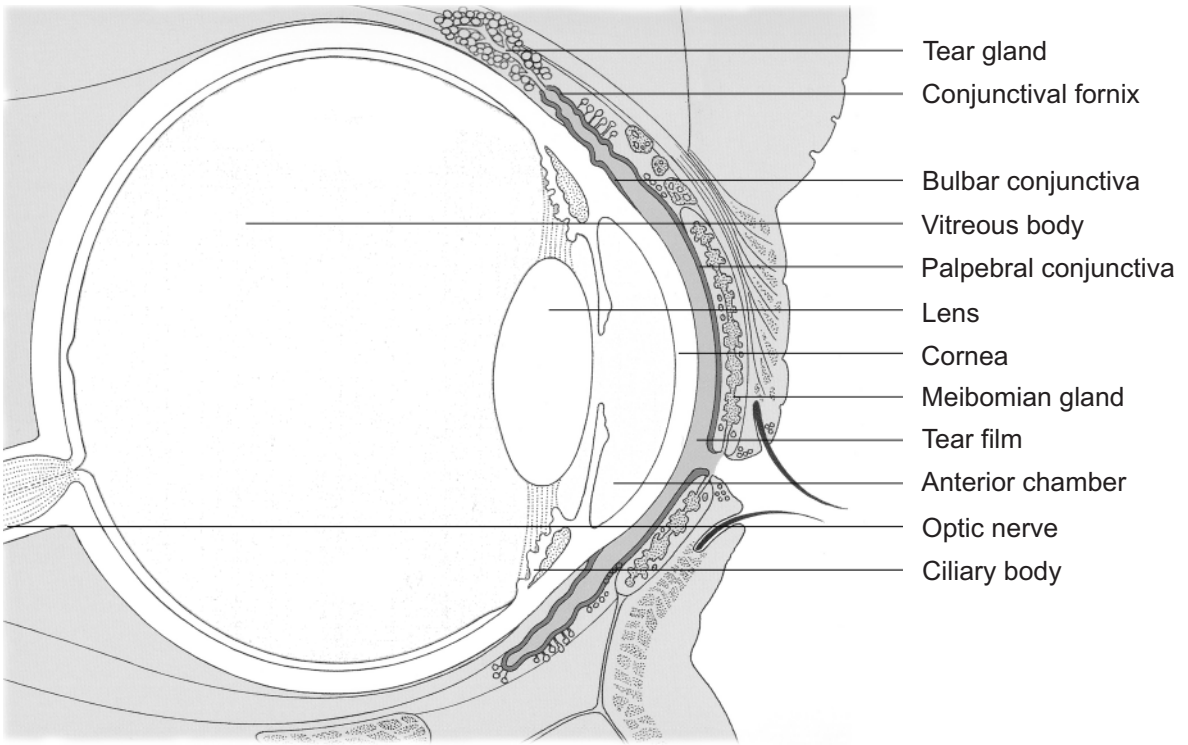
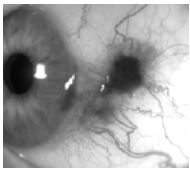


Figure 1. The anatomical structures of the human eye.

ends and cornea begins is the limbal region, where the corneal epithelial stem cells have their residence (see Part I). The conjunctiva consists of two or more layers of stratified columnar epithelial cells. Scattered among the basal cells on the basement membrane melanocytes are present, which stretch out their spidery offshoots between the epithelial cells. Also between the epithelial cells, goblet cells are present which produce mucous. Accessory tear glands are present in the subepithelial stroma of the conjunctiva and they continuously produce basal tears. In the tarsal plates of both the upper and lower eyelid meibomian glands produce lipids that prevent the evaporation of tears from the ocular surface. Lymphatic channels are present throughout the conjunctival stroma; the medial part of the conjunctival lymphatic vessels drain to the submandibular lymph nodes while the lateral part of the conjunctiva drains to the preauricular lymph nodes.[4]

Two special anatomical structures must also be mentioned, the plica semilunaris and the caruncle. The plica semilunaris is a conjunctival fold in the medial portion of the bulbar conjunctiva. The plica is thought to represent a remnant of the nictitating membrane that is found in many vertebrate species.[5] The caruncle is situated at the medial canthus, between the upper and lower tear ducts. It contains both characteristics of conjunctival and cutaneous tissue i.e. sebaceous glands and hair follicles.

During embryonic development, melanocytes migrate from the neural crest[6] to the basal layers of the conjunctival epithelium or to the area just underneath the basement membrane. Racial melanosis, primary acquired melanosis, conjunctival nevus, and conjunctival melanoma all find their origin in these melanocytes.



Nevi

Conjunctival nevi can either be congenital or acquired. The acquired nevus is the most common conjunctival nevus and can be subdivided into junctional nevi, compound nevi, subepithelial nevi, and Spitz nevi. Where the congenital nevus presents itself during the first six months of life, the acquired nevi usually develop during the first or second decade. A nevus starts to develop as a benign proliferation of nevus cells (plump melanocytes without the spidery extensions) at the junction of epithelium and subepithelium (junctional nevus), and usually forms nests.[7] The junctional nevus is a clinically flat lesion since the nevus cells are confined to the epithelium, and are only seen in children.[7]

The nevus cells of a junctional nevus can descend to the subepithelial layers (superficial substantia propria) in a short period of time. The nevus cells frequently drag epithelial surface cells and goblet cells down to the subepithelial layer, where they can develop into inclusion cysts, which may enlarge gradually. The nevus is now a compound nevus, nevus cells are present at the epithelium and in the substantia propia.[7] Clinically, the lesion thickens and inclusions cysts can be seen with a slit lamp (Figure 3).

Over time the epithelial component of the junctional nevus may disappear, leaving only the subepithelial part of the nevus separated from the epithelium by a small band of fibrotic stroma.[7] Hence, the nevus is now called a subepithelial nevus, and is usually clinically elevated and may contain epithelial cysts.

Conjunctival Spitz nevi are rapidly growing nevi, appear solely during childhood, and are very rare.[8] The rapid growth makes these lesions very suspect for conjunctival melanoma.

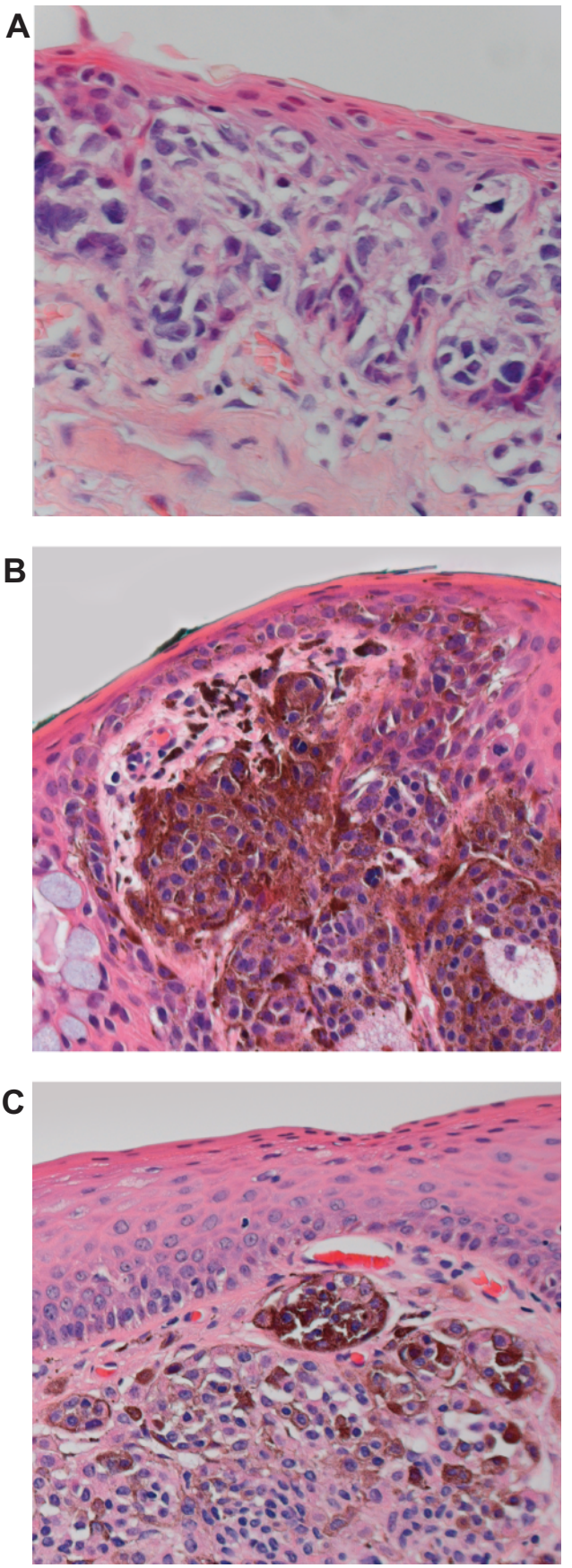


Figure 2. Histological pictures of the three types of conjunctival nevi; **A**, junctional nevus; **B,** compont nevus; and **C**, subepithelial nevus.

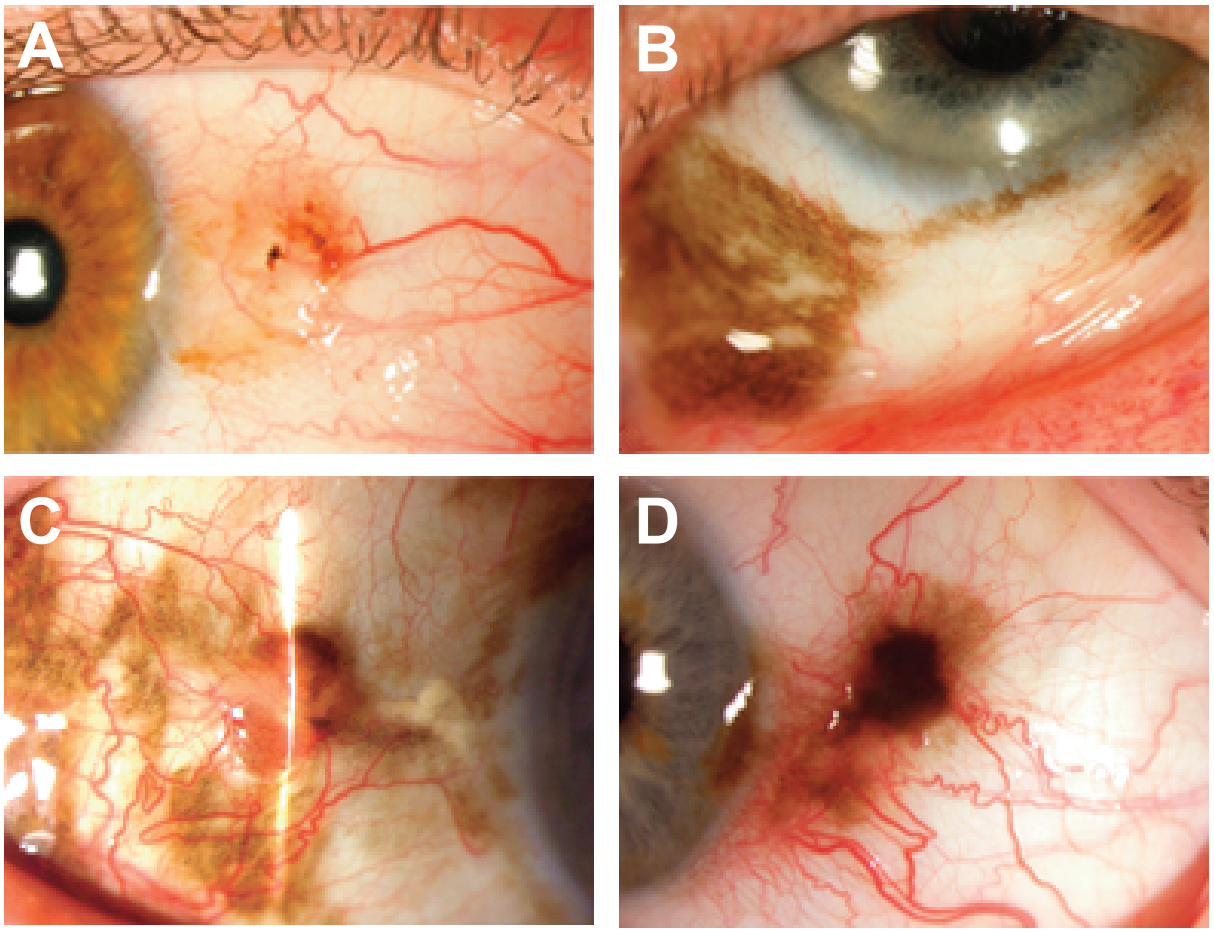


Figure 3. Clinical examples of a conjunctival nevus (fig. 3A), PAM (fig. 3B), PAM with a conjunctival melanoma (fig. 3C), and conjunctival melanoma (fig. 3D).

Also histologically differentiation of a conjunctival Spitz nevus from a conjunctival melanoma is difficult, and even mitotic figures can be found.[7] However, the conjunctival Spitz nevus is strictly benign and used to be called "benign juvenile melanoma", which is a confusing term since a melanoma cannot be benign.
The conjunctival nevus is a benign tumour that very rarely develops into a malignant conjunctival melanoma, i.e. less than one percent.[9,10] Epithelial cysts are an important clinical feature that usually indicates the benign character of a melanocytic lesion. Other important features are the free mobility of the lesion over the sclera and the localisation; nevi are usually situated at the interpalpebral bulbar conjunctiva near the limbus. Lesions in the fornix or the palpebral conjunctiva are suspicious of primary acquired melanosis (PAM) or conjunctival melanoma.
The management of an acquired conjunctival nevus consists of photographic documentation and regular follow up. Growth of a nevus can be a sign for malignant transformation, however, during puberty the lesions may acquire more pigment and can grow under the influence of hormones. The increase in pigmentation, enlargement of the intralesional cysts or inflammation of a nevus may simulate growth, increasing the suspicion of a melanoma, but is mostly restricted to childhood and adolescence. When additional information is required, cytological or histological biopsies can be performed to assess the risk of malignant transformation.

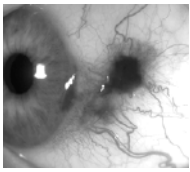
Melanosis

Conjunctival melanosis can be divided in two forms: racial melanosis and primary acquired melanosis (PAM) (Figure 3). Racial melanosis is very common in dark-skinned individuals, and generally affects the limbus of both eyes.[7] It is a benign condition that rarely develops into a conjunctival melanoma; histologically there is an increase in melanin production in spidery melanocytes without atypial characteristic. These rarely transforms into melanocytes with atypia of the nucleus. Atypia of the melanocytes is characterized by abnormalities in nuclear size, nuclear shape, nuclear-cytoplasmatic ratio, chromatin, nuclear membrane, nucleoli,or melanin.
PAM is an acquired melanosis, usually unilaterally in fair-skinned middle-aged individuals. Other terms for PAM used to be benign acquired melanosis, precancerous melanosis, idiopathic acquired melanosis, or intraepithelial atypical melanocytic hyperplasia. To promote a uniform nomenclature the World Health Organisation (WHO) introduced the term primary acquired melanosis. Clinically the pigmentation in PAM appears brown, diffuse, patchy, flat, and noncystic. PAM can wax and wane over time, i.e. extend and diminish in size within the epithelium, without any tumefaction.[11] They are mostly located at the limbal region, but may extend to the cornea and surrounding bulbar conjunctiva, fornix, and palpebral conjunctiva. Histologically, PAM is an intraepithelial proliferation of plump melanocytes (naevus cells) with or without atypia of the nucleus. The proliferation can be present in little nests in the basal layer of the conjunctival epithelium, or in a more linear increase in number of naevoid melanocytes. The atypia can be classified as mild, moderate or severe. This is important to estimate since PAM with atypia has a 50% chance to develop into a conjunctival melanoma.[11,12] Melanoma development in PAM without atypia or with mild atypia is very

rare.[12] PAM lesions that become elevated, or elevated areas within a larger patch of flat pigmentation, or increased vascularization are suspect of the development of a conjunctival melanoma, and must be treated accordingly. Frequently the tumour is unpigmented or of another hue than the original PAM. Unpigmented PAM exists, but is fortunately rare (PAM sine pigmento)

The management of PAM without atypia or with mild atypia consists of regular clinical and photographic follow up and, when indicated, cytological examination or biopsy to assess the atypia. However PAM can be a very extensive disease, and can require many biopsies over time, that can cause damage to the conjunctiva and sometimes limbus. Since cytology is a non-invasive method to asses the atypia, it is an alternative that has to be considered (Chapter 4 and 5). PAM with moderate to severe atypia can be either treated with mitomycin C, cryotherapy, or excision, depending on the localisation and extend of the lesion. PAM is notorious for its multiple recurrences despite adequate treatment; regular follow up is therefore a necessity.

Ocular melanocytosis can be confused with melanosis of the conjunctiva. However, ocular melanocytosis is part of the oculodermal melanocytosis ("nevus of Ota"), a congenital pigmentary skin disorder which may include the periocular skin, (epi-)sclera, uvea, orbit, meninges and soft palate. The pigmentation in PAM is located in the conjunctiva and directly subepithelial and is acquired. The pigmentation in ocular melanocytosis is located in the epi-sclera, and usually the conjunctiva is remarkable unpigmented; it is congenital, but may increase in pigmentation and extension with increasing age. Patients with ocular melanocytosis have an increased risk of uveal melanoma but not of conjunctival melanoma.[13]



Conjunctival melanoma

As has been mentioned in the introduction, conjunctival melanoma is a rare disease, which has the potential to metastasise and to cause metastatic death. Conjunctival melanoma usually arises from PAM, but it can also arise from conjunctival nevi or develop de novo.[1,14,15,16,17,18,2,3] Conjunctival melanoma with surrounding PAM with atypia is seen in approximately 70% of the conjunctival melanomas, indicating that, indeed, PAM with atypia is the most common precursor lesion of conjunctival melanoma.[19] It typically arises around the age of 60,[20,2] and can be located anywhere on the conjunctiva, but mostly in the bulbar conjunctiva near the limbus (Figure 3). It is a disease that primarily affects the Caucasian population, it is very uncommon in the African or Asian population, with a relative risk ratio of at least 8:1.[21,22,23,24] Clinically, conjunctival melanoma is characterized by horizontal and vertical growth with prominent feeder vessels. The pigmentation can vary from unpigmented to brown or black. Exfoliation and impression cytology shows severe atypical melanocytes.

Aetiology of conjunctival melanoma

The aetiology of the conjunctival melanoma is not clear. Ultraviolet radiation has been mentioned to be involved in the development of conjunctival melanomas, since patients with xeroderma pigmentosum develop PAM with atypia and conjunctival melanoma more frequently.[25,26,27] Patients with xeroderma pigmentosum are prone to develop a variety of epithelial and melanocytic ultraviolet radiation-inducible tumours. In addition, many of the

conjunctival melanomas are found in the sun-exposed areas of the conjunctiva, although melanomas are also found in the fornix and the palpebral conjunctiva. In skin melanoma, the importance of ultraviolet radiation has been established; however, it is better to see conjunctival melanoma and skin melanoma as different entities, since the conjunctiva is a mucosa with considerably different anatomy and function. Although there was a major increase in skin melanoma in Denmark presumably due to increase in ultraviolet radiation, no increase in conjunctiva melanoma was seen.[28] Others claim an increase in conjunctival melanoma similar to the increase in skin melanoma.[29,30] So, there is no solid evidence that ultraviolet radiation is involved in the aetiology of conjunctival melanoma.

Differential diagnosis of conjunctival melanoma

A conjunctival melanoma must be differentiated from other conjunctival melanocytic lesions such as a conjunctival nevi, racial melanosis, and PAM, but also from other melanocytic lesions that can be situated in deeper layers. Pigmented episcleral spots or Axenfeld's nerve loops are uveal melanocytes that have migrated to the episclera via either a perforating ciliary vessel or an intrascleral nerve loop. The spots may be painful and are usually located 3-4 mm behind the limbus. Ocular melanocytosis is also a melanocytic lesion and has been discussed previously. Tumour outgrowth of a ciliary body melanoma through the sclera can mimic a conjunctival melanoma,[31] but ultrasound biomicroscopy and ophthalmic examination can be helpful to differentiate between the two. Although it is very rare, a conjunctival metastasis of a skin melanoma must be included in the differential diagnosis.[32] Non-melanocytic lesions that can be mistaken for a conjunctival melanoma are pigmented squamous cell carcinoma, pterygium, pingueculum, Moll gland cystadenoma, apocrine adenocarcinoma, mascara, silver depositions, haemorrhage, and gunpowder.[1,33] For the clinical differentiation between melanin pigmentation or pigmentations of other origins, a UV-lamp can be used. For the differentiation of brown(ish) pigment in tissues, several histological stains are available. The lack of pigmentation in an amelanotic conjunctival melanoma makes the clinical diagnosis even harder.

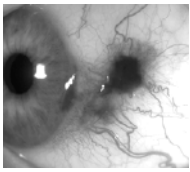
Cytology

The Leiden University Medical Center has more than 25 years of experience with ocular cytology, especially with melanocytic lesions. It is one of our essential examinations for pigmented conjunctival lesions, however, there is not much literature on that subject. A grading system for melanocytic atypia in cytological samples is used in our clinic (Chapter 4). The primary objective for cytology in pigmented conjunctival lesions is the detection of conjunctival melanoma and the follow up of PAM and nevoid lesions. Exfoliative cytology and impression cytology are two different techniques to harvest cells for cytological examination. Exfoliative cytology can be performed through conjunctival scrapings[34,35] or by use of cotton wool swabs.[36] Impression cytology is either done with cellulose acetate filters[37] or with the Biopore membrane.[38] Advantages and disadvantages of both exfoliative and Biopore techniques are further considered in Chapter 5. Cytology can be helpful in differentiating PAM and nevoid lesions from non-melanocytic lesions and it can help to estimate the risk for melanoma development. For PAM lesions it is particularly helpful to know whether and

when a lesion without atypia turns to a lesion with atypia, or when the atypia further increases from mild to moderate or severe. Especially severe atypia is highly associated with the presence of a conjunctival melanoma (Chapter 4). However, cytology only assesses the superficial layers and the atypia of the sampled cells, and cannot differentiate between PAM with moderate/severe atypia and frank invasive melanoma.
An alternative for the cytological examination is a biopsy. A biopsy can reliably diagnose lesions as nevus, PAM, or melanoma, it can also further classify the grade of atypia within the melanocytic proliferation. However, a biopsy is an invasive method that cannot be performed endlessly on an eye with recurrent PAM lesions, while cytology is non-invasive, patient friendly and can be repeated many times. Both histological and cytological methods are essential tools for ocular oncology centres.

Histology

Histological material from melanocytic conjunctival lesions can be obtained by incisional or excisional biopsies. Incisional biopsies are primarily performed on large lesions that are difficult to remove in toto. However, an excisional biopsy is preferred since the lesion can be removed together with the acquired margins, thereby minimalizing the chance of recurrence of the lesion.[16] According to Shields, suspect bulbar lesions that occupy less than 4 clock hours or are less than 15 mm in diameter are suitable for excision.[33] Extreme care in handling the excised lesions will guarantee better material for the (ophthalmic) pathologist to estimate useful details such as margins of excision.



Conjunctival melanoma treatment

The treatment of conjunctival melanoma has changed over time, from radical orbital exenteration to local irradiation or excision. Nowadays, the primary treatment for a conjunctival melanoma is local excision with a 3-5 mm free conjunctival margin,[39] thereby avoiding manipulation of the tumour, and with adjuvant therapy as brachytherapy or cryocoagulation.[39,40,41,42,43] Because conjunctival melanoma cells easily seed when manipulated, local recurrences may be reduced by applying absolute alcohol or Dakin's solution to devitalize any residual atypical melanocytes.[44,45] Excision of larger conjunctival melanomas can leave conjunctival defects that are too large for primary closure, mucosal grafts from the mouth or contralateral eye or amnion membranes can be used. When the tumour is not suitable for local excision, i.e. unfavourable location, very large tumours, or multifocal tumours, proton beam irradiation may be considered.[47] For very large conjunctival melanomas especially with extension into the orbit, orbital exenteration is often the only solution.[48] In large series, the number of exenterations is still uncomfortably high in these advanced cases.[20] Exenteration should only be used as a palliative treatment for no beneficial effect has been found of exenteration on survival.[46]

Recurrences of conjunctival melanoma

Despite complete microsurgical excision, local recurrences of conjunctival melanoma are

often seen, which can be either new primary tumour formation from remaining or recurrent PAM, from remaining melanoma due to incomplete removal or local spread due to surgery, or an in-transit metastasis in a lymphatic vessel. Five years after treatment, 26% - 46% of the patients have a local recurrence, increasing to 51% at 10 years.[49,50,20]

Metastasis of conjunctival melanoma

Because of the existence of lymphatic vessels in the conjunctiva, conjunctival melanomas can spread to the regional lymph nodes (preauricular, submandibular, and cervical lymph nodes). In 65% of the patients with a distant metastasis also had regional lymph node metastases.[51] Haematogenous metastasis can be located in the lung, liver, brain, bone marrow, spleen, and gastrointestinal tract.[17,52] Because the lymph nodes can be the first step towards further spread of the conjunctival melanoma[17,53,52] and has a better prognosis than distant metastasis,[54] use of the sentinel node biopsy has recently been suggested. Good survival chances have been achieved with radical excision of the metastatic lymph nodes,[53] whether removal of the sentinel node also leads to longer survival is not known.

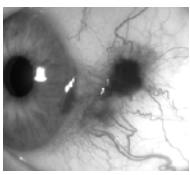
Risk factors

Patients with a conjunctival melanoma have a survival chance of 74% - 93% at 5 years, decreasing to 41% - 87% at 10 years.[55,56,14,16,17,3,49] There are several risk factors that decrease the chance of survival. First of all, tumour thickness is an important risk factor:[57,43,49,58] patients with a tumour thickness of 0.8 mm or more have a lower chance of survival.[49] A worse prognosis is also seen when the conjunctival melanoma is located in the fornix, palpebral conjunctiva, plica, or the caruncle.[57,43,49,58] Other risk factors can be multifocal tumours, orbital invasion, and recurrent disease. Histopathological risk factors are a high mitotic rate, mixed cell tumours, and a pagetoid growth pattern.[49] Whether the origin of the conjunctival melanoma (PAM, nevus, or de novo) is a risk factor for tumour related death is a topic of discussion and may vary with the number of cases of the study.[57,43,16,49,59,53,41]
The local recurrence rate of conjunctival melanomas is influenced by incomplete removal of the lesion (i.e. positive margins), tumour thickness, and type of treatment.[60,43,16] Epithelioid cell type and mixed cell type conjunctival melanomas also have a higher rate of recurrence.[43]
In Chapter 3 the risk factors for recurrences, metastasis and tumour related mortality for the Dutch population are further discussed.

Markers

Histological markers for conjunctival melanomas could be helpful to differentiate a conjunctival nevus or PAM from a conjunctival melanoma. HMB 45, Melan A, and S100 have been used to identify conjunctival melanomas; however these markers only prove the melanocytic origin of the tumour and cannot differentiate between benign and malignant melanocytic lesions. [57,60,61,55,62,63,64]
S100 is a calcium-binding protein and involved in establishing the malignant and metastatic phenotype of various tumours.[65,66,67] The S100 protein family consists of over 20 members. The expression of S100A1, S100A2, S100A3, S100A4, S100A6, and S100B has been stud-

ied previously in cutaneous melanoma.[68,69,70,71,72] Serum S100B has proved its value as a marker for metastasis in skin melanoma, and is of prognostic importance.[70,73] Furthermore, in histological sections S100A6 seems of some use in the distinction between a Spitz-nevus and a cutaneous melanoma.[72] For conjunctival melanoma the research for markers has been limited by the rarity of the tumor, hence material is scarce. In Chapter 6 we will discuss the search for histological markers for conjunctival melanoma.



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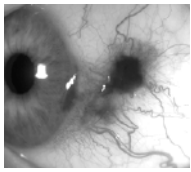

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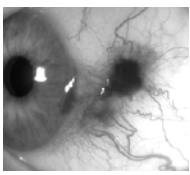

CHAPTER 3

CONJUNCTIVAL MELANOMA IN THE NETHERLANDS: A NATIONWIDE STUDY

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Abstract

Purpose. To evaluate risk factors for local recurrence, regional and distant metastases, and mortality associated with conjunctival melanomas.

Patients. 194 patients with histologically confirmed conjunctival melanoma, diagnosed between 1950 and 2002 in the Netherlands. Data were collected from all university centers and many non-tertiary hospitals, using the National Pathology and the Leiden Oncologic Registration Systems. Based on number of incidences, this study included 70% of the conjunctival melanomas reported.

Methods. Clinical and histopathological data for conjunctival tumors were reviewed and compared with data reported in the literature. Risk factors for local, regional, and distant metastases and survival were analysed using Kaplan-Meier and Cox regression analysis.

Results. Of 194 patients with conjunctival melanoma, 112 had a local recurrence (median 1, range 1–9) during follow-up (median 6.8 y; range 0.1-51.5 y). Location was the most important risk factor for development of local recurrence and significantly more were seen for non-epibulbar (Log Rank P=0.044) tumors. Significantly fewer local recurrence were found for tumors initially treated with excision and adjuvant brachytherapy rather than with excision only (Log rank P=0.008) or with excision and cryotherapy (Log rank P<0.038). Forty-one (21%) patients developed regional lymph node metastases, mostly to the parotid or preauricular lymph nodes (n=26; 13%). Risk factors for regional metastases were tumor thickness (Log rank P<0.001), and tumor diameter (Log rank P=0.010). Forty-nine (25%) patients later (mean 4.37 y) developed distant metastases mainly in lung, liver, skin, and brain. Tumor-related survival was 86.3% (95% Confidence interval [CI], 81.0-91.6) at 5 years, 72% (95% CI, 79.7-64.4) at 10 years, and 67% (95% CI, 58.9-76.1) at 15 years. Main mortality risk factors were non-epibulbar location (Log rank P<0.0001) and tumor thickness (Log rank P=0.0004).

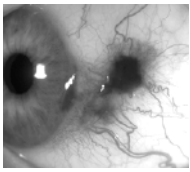
Conclusions. Non-epibulbar tumors more often recur locally and are associated with a shorter survival independent of other risk factors. Tumor thickness is also an important predictor of regional and distant metastases, as well as survival. A prospective study is needed to compare the effect of excision in combination with radiotherapy and excision with cryotherapy on the number of local recurrences, exenteration rate, and survival.

Introduction

Conjunctival melanoma is a rare tumor [1-3] with an incidence of 0.02-0.08 per 100 000 in a Caucasian population,[4-6] accounting for some 1-3% of all ocular malignancies in adults. This malignancy normally occurs around the age of 60 and only rarely before the age of 40 (10%). Conjunctival melanomas may perhaps be associated with sun exposure, like skin melanoma,[8] although they can occur at non sun-exposed sites.[6] In contrast to uveal melanoma,[9] the incidence of conjunctival melanoma is increasing.[6-7]

Local recurrence is reported to be 30–50% at 5 years, 38–51% at 10 years, and 65% after 15 years, depending on treatment.[10-21] Excision without additional treatment is associated with more local recurrences.[22] Main risk factors for patient mortality are thickness and size (basal diameter) as well as a non-epibulbar location (Picture 1) for the primary tumor or one of its recurrences. A contributing factor for prognosis is the origin of the tumor; i.e., 55% are primary acquired melanosis (PAM) and in 25% melanoma de novo, which gives the worse prognosis.[23] Conjunctival nevi rarely develop into a melanoma.[24] The mortality rate is 12-20% at 5 years and up to 30% at 10 years.[10-21] Conjunctival melanomas are known to spread via the lymphatic system, although distant metastases are also found without regional lymph node metastasis.[25]

In this report, data for 194 patients with conjunctival melanoma collected from all university hospitals and many non-tertiary centers in the Netherlands were analyzed to investigate differences in treatment and surgical approach as well as the main risk factors for recurrence, metastasis, and survival.



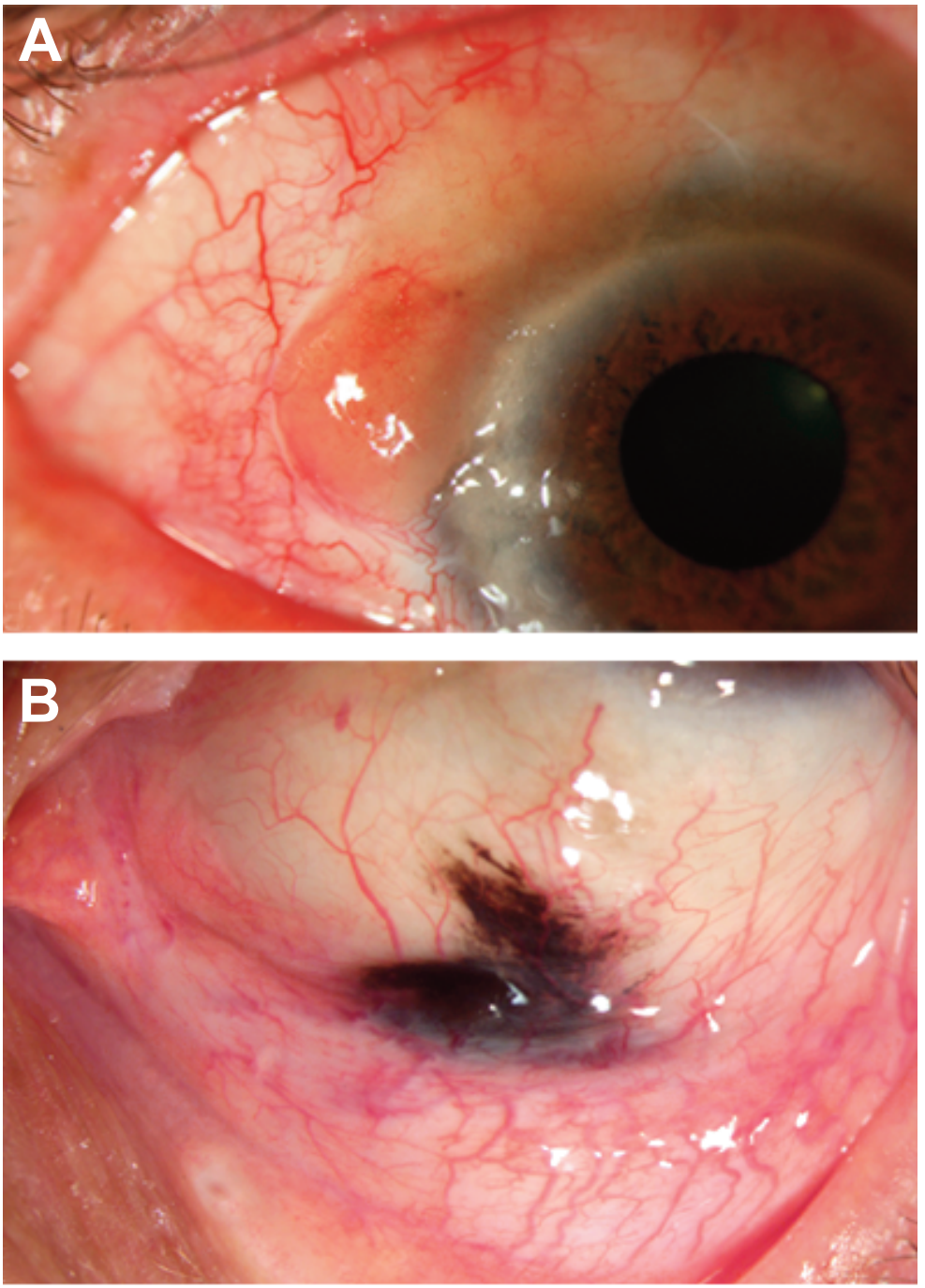
Patients and methods

The Netherlands National Ocular and Orbital Tumor Committee, using the Netherlands Pathology Diagnosis System, helped identify 194 patients diagnosed with malignant conjunctival melanoma between 1950 and 2002. Complete clinical and histopathological data were gathered from patient records and follow-up data were obtained from death certificates, general practitioners, local ophthalmologists, and the Oncologic Registration System of Leiden University Medical Center (LUMC). The mean follow-up was 9.2 years (SD, 8.1 y; median 6.8 y). Only data for patients with histologically proven conjunctival melanoma were analyzed. Patients with a primary eyelid (cutaneous) melanoma were excluded, as were patients with PAM without an invasive component. The study was approved by the Institutional Review Board and adhered to the tenets of the Declaration of Helsinki.

Assessment of tumor characteristics

Of 194 conjunctival melanomas, 178 (92%) primary tumor specimens were reviewed for histopathologic confirmation of the diagnosis at the LUMC while 16 other specimens were assessed by an experienced ophthalmic pathologist elsewhere.

Tumor origin, established on the basis of medical and histopahtological records and photographs, and was classified as ‘nevus’, ‘nevus in combination with primary acquired melanosis (PAM)’, ‘PAM’, and ‘de novo’ melanoma. The clinical variables included age at diagnosis, sex, unilocular (caruncle, fornix, tarsus, limbus, or eyeball) or multilocular (dif-



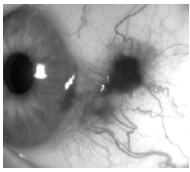
Picture 1. An epibulbar location of a conjunctival melanoma is shown in picture 1A, a non-epibulbar location (inferior conjunctival fornix) is shown in picture 1B.

was 57.4 years (median: 58 y). Regions involved were the limbus (*n*=110, 57%), bulbar conjunctiva (limbus included; *n*=133, 69%), caruncule (*n*=5, 2.6%), tarsus (*n*=11, 5.6%), and fornix (*n*=2, 1%); more than one location was involved in 43 (22%) patients. Origin of the melanomas was PAM (*n*=111, 57.2%), melanoma de novo (*n*=50, 25.7%), nevus in combination with PAM (*n*=9, 5%), and nevus only (*n*=3, 2%). Origin was inconclusive in 21 (11%) patients. Tumor thickness was 2.07 mm (SD 1.9 mm; *n*=153) and the basal diameter was 7.21 mm (SD 4.7 mm; *n*=152).

Primary treatment

Treatment at the moment of histopathological diagnosis (Table 2) was excision alone (*n*=127, 65.5%), excision with topical chemotherapy (Mitomycin C; *n*= 4, 2%), excision and adjuvant cryotherapy (*n*=17, 8.7%), irradiation (*n*=11, 5.6%), and excision with brachytherapy (^{90}Sr/^{90}Y irradiation; 6 x 1000cGy; *n*=20, 10.3%). Exenteration was the primary therapy for advanced conjunctival melanoma in 14 (7.2%) patients and was used in combination with orbital irradiation in 1 patient (0.5%).

Today the standard treatment for primary conjunctival melanoma is excision with adjuvant therapy. However, during the period covered by this study, many different treatment strategies were used (Figure 1). Although in some cases only a few patients represent a certain treatment, we tried to compare different treatments and their outcomes.



Local recurrence analysis and secondary treatments

Within the total follow-up period, 112 patients had a local recurrence (62.6% of the 179 non-exenterated patients). Mean time between the first treatment for histopathologically proven melanoma and first local recurrence was 2.4 years (SD 3.4; median 1.3 y; max 22.6 y). The mean number of local recurrences was 1.6 (range 1–9), resulting in a local recurrence rate of 60.7% (95% CI, 52.9-68.5) after 5 years, 66.8% (95% CI, 59.0-74.6) after 10 years, and 72.2% (95% CI, 63.4-81.0) after 15 years (Figure 2A).

Treatment of the first local recurrence was excision in 63 (56%) patients (4 in combination with topical chemotherapy), 22 of whom ultimately required orbital exenteration for more recurrences or advanced conjunctival melanoma. Twenty-three (20.5%) patients received excision with cryotherapy, with 8 patients ultimately requiring exenteration. Excision with brachytherapy (^{92}Ir irradiation, *n*=3, ^{90}Sr/^{90}Y application, *n*=8) was performed in 11 cases, with two patients being exenterated. One patient was treated for recurrence with irradiation only. In total, 44 (39.3%) patients were exenterated for advanced disease. The probability of recurrence of primary tumors using excision with brachytherapy was lower than when using other treatment modalities (Figure 2B). There were significantly fewer recurrences using excision and brachytherapy of primary tumors (*n*=19) than when using excision with adjuvant cryotherapy (*n*=17; *P*=0.038, log rank test not adjusted for multiple comparisons). Excision with cryotherapy, in comparison to excision only, did not result in less recurrences (*P*=0.759, log rank test, not adjusted for multiple comparisons).

In contrast to other tumor locations, epibulbar involvement showed significantly fewer local recurrences (P=0.044, log rank) (Figure 2C). Analysis showed that local recurrence was not associated with age at time of diagnosis (categories: <50, 50-68, >68y; years; *P*=0.774, log

fuse) location of the primary tumor, date for primary treatment, date for diagnosis of local recurrence, and the presence of regional or hematogenous metastases. Local recurrences at the same or different location in the ocular region, but not PAM without melanoma, were considered 'local recurrence'.[20] The proximity of the melanoma to the limbus was classified as limbal or non-limbal. Basal tumor diameter (mm) was taken from patient records, pathology reports, or photographs, if available (79%), as described by Tuomaala et al[6]. In 42 cases, information about tumor diameter could not be found. Melanoma thickness (mm)was measured by one ophthalmic pathologist on slides of the tumor, according to Breslow.[26] In 41 cases, thickness could not be measured because tumors had been sliced in a tangential plane. Treatment of initial tumors and recurrences were categorized as 'excision only', 'excision and chemotherapy' (topical Mitomycin C), 'excision and cryotherapy', 'external irradiation only', 'brachytherapy after excision', 'orbital exenteration', and 'irradiation and exenteration'. Cryotherapy and ^{90}Sr brachytherapy were available as adjuvant therapy from 1970 onward and were frequently used after 1980 (Figure 1).

Assessment of outcome

Dates of diagnosis and local recurrence were those for histopathologic confirmation of the primary tumor or local recurrence. The interval between diagnosis by a physician and histopathologic confirmation was always less than 2 months. The date for metastasis was taken as the date when dissemination was confirmed by biopsy, imaging, or clinical examination. Regional metastasis was histopathologically confirmed in all 41 cases. Of 49 cases with distant metastases, the metastases were confirmed by histopathology in 24 (49%) patients and by imaging (mostly Computed Tomography) in 25 (51%) patients. Autopsy was performed in three patients. In 12 cases, the location of metastases was not specified. Survival time was defined as the time from histopathological confirmation of the primary tumor to death due to conjunctival melanoma (event), death due to other causes, or time of last presentation.

Statistical analysis

Risk factors for local or regional recurrence and for tumor-related survival were assessed by Kaplan-Meier estimates and with Cox proportional hazards. Hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated. Values of $P<0.05$ were considered significant. Statistical analysis was performed using SPSS 11.0 (SPSS Inc, Chicago, IL) and Stata/SE 8.0 software package (StatCorp, College Station, TX). Kaplan-Meier analysis for local recurrence was analyzed for time to the first local recurrence. The Log Rank test was used to compare variables in Kaplan-)Meier analysis. In Cox regression analysis, the variables "origin" and "therapy" were considered nominal categorical variables.

Results

Patient and tumor characteristics

Our study population consisted of 107 (55%) female and 87 (45%) male patients (Table 1), including 99 (51%) left and 95 (49%) right eyes. Mean age at histopathological diagnosis

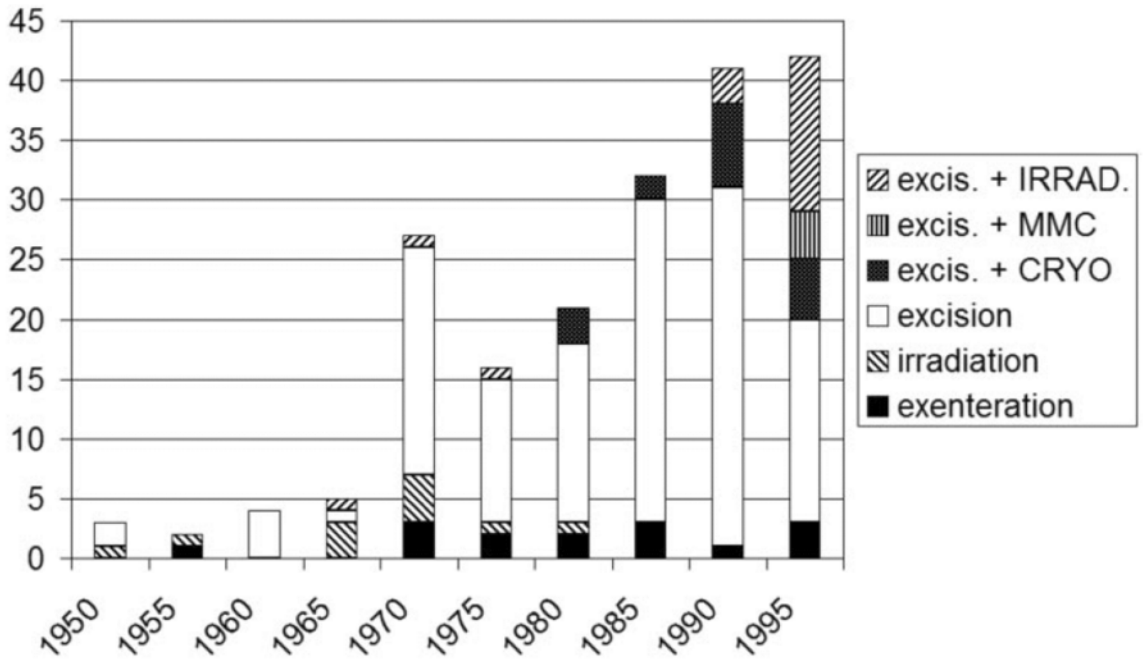
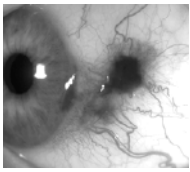


Figure 1. Treatment modality in function of time. The study was made by contributions from all university centers and many general ophthalmologists.



rank test for trend), tumor origin (categories: PAM or non-PAM; *P*=0.61, Log Rank), sex (*P*=0.77, Log Rank), unilocular/multilocular location (*P*=0.82, Log Rank), tumor thickness (<1 mm, 1-2 mm, >2 mm; *P*=0.063, Log Rank test for trend), or basal tumor diameter (0-6 mm, 6-10 mm, >10 mm; *P*=0.571). Cox regression analysis showed that epibulbar location and treatment modality of the primary tumor were the best predictors of local recurrence (see Table 3).

Regional metastases analysis and treatment

Regional metastases developed in 41of 194 patients (*n*=9 regional, *n*=32 regional and distant). Of these 41 patients, 32 were first diagnosed as having only regional dissemination. Mean time between primary tumor diagnosis and regional metastasis was 4.37 years (SD 4.2; median 3.7 y). Mean survival time after metastasis was 8.1 years for regional dissemination only (6 patients still living, 3 died of non-related causes; SD 8.7; median 2.7 y) and 1.72 years for both regional and distant metastases (SD 1.5; median 1.3 y). Regional metastases were found in parotid or preauricular (*n*=26), cervical (*n*=13), sub-mandibular (*n*=9), and axillar (*n*=2) lymph nodes. Both patients with positive axillar lymph nodes had also parotid positive lymph nodes.

Univariate Cox regression analysis (Table 4) indicated that tumor thickness (HR 2.8, *P*<0.001), and basal diameter (HR 1.6, *P*=0.010) were the best predictors of regional metastases. In a multivariate Cox model, only tumor thickness reached statistical significance.

Distant recurrence analysis

Distant metastases developed in 49 patients, of whom 17 cases showed no regional ('skipping') metastases. In 37 patients, the distant metastasis was localized. Metastasis occurred in the lungs (*n*=18 cases), liver (*n*=15), skin (*n*=13), brain (*n*=11), spinal cord (*n*=5), ileum (*n*=3), mesentery (*n*=3), skeletal bones (*n*=3), thyroid gland (*n*= 2), and jaw bone (*n*=2).Mean survival was 1.72 years (SD 1.5; median 1.3 y) for both regional and distant and 0.42 years (SD 0.34; median 0.44 y) for hematogenous metastases. Analysis (Table 5) showed that non-epibulbar lesions were significantly more likely to give rise to a distant metastasis (HR 4.0, *P*<0.001). Univariate Cox analysis showed that increasing tumor diameter was associated with an increasing risk of distant recurrences (HR 2.1, *P*<0.001).

Survival analysis

During the total follow-up period, 47 (24.3%) patients died of metastasis of conjunctival melanoma, 44 (22.7%) of non-melanoma-related causes, and 103 (53%) were still living. Fifty-eight patients (29.9%) developed metastasis. The melanoma-specific survival rate was 86.3% (95% CI 81.0-91.6) at 5 years, 71.2% (95% CI 79.7-64.4) at 10 years, and 67.3% (95% CI 58.9-76.1) at 15 years (Figure 3A).

Survival analysis did not show a significant difference in survival between therapies (overall log rank *P*=0.363). Only patients who underwent a primary exenteration had a significantly lower survival compared to other treatment modalities (*P*=0.036, Log Rank test not adjusted for multiple comparisons). This may be explained by the fact that this group contained significantly larger tumors and at an unfavorable location.

Neither tumor origin (*P*=0.42) nor tumor basal diameter (*P*=0.066, log rank test for trend) showed a significant difference in survival rate. However, survival was significantly better for epibulbar tumors (*P*<0.0001), unilocular location (*P*=0.02), and thin tumors (*P*=0.0004, overall Log Rank for trend). Age at diagnosis (<50 y, 50-68 y, >68 y) was significantly correlated with survival (*P*=0.023, Log Rank test for trend), but older patients had significantly larger (*P*<0.023) and thicker (*P*<0.021) tumors. Cox regression analysis showed the same risk factors as for the development of distant metastases (Table 6), most importantly a non-epibulbar location (HR 3.6, *P*<0.001).

Discussion

Our study suggests that the incidence of conjunctival melanoma in the Netherlands is similar to the previously reported incidence of 0.05/100 000 inhabitants.[4-7] Since 1970, conjunctival melanoma has been diagnosed in 172 patients. With a mean population of 14 575 000 inhabitants in the Netherlands (Central Bureau of Statistics, Voorburg/Heerlen), over the last 30 years an estimated 240 patients would have been diagnosed with this type of melanoma. Thus we probably included more than 70% of possible patients with conjunctival melanoma in the Netherlands after 1970.

Treatment of the local primary tumor has changed in the last decades from irradiation or excision as sole therapy to excision in combination with cryotherapy or brachytherapy. This has enabled us to compare different treatments. For local tumor control, excision in combination with brachytherapy appears to results in fewer local recurrences than when using

Table 1. Patient and tumor characteristics of 194 conjunctival melanoma patients.

Variables	*No. of cases*		*(%)*
Sex			
Male	87		(45)
Female	107		(55)
Location			
Fornix	2		(1)
Caruncular	5		(2.5)
Tarsal	11		(5.5)
Limbal	110		(57)
Epibulbar	133		(69)
Diffuse	43		(22)
Origin of melanoma			
Naevus	3		(2)
Naevus and PAM	9		(4)
PAM	111		(57)
de novo	50		(26)
Inconclusive	21		(11)
Maesurements	*No. of cases*	*Mean*	*SD*
Basal diameter (mm)	153	7.21	4.7
Thickness (mm)	152	2.07	1.9

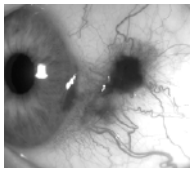


Table 2. Treatment of the primary tumor at moment of its histological diagnosis, and the number of patients with a local recurrence.

Treatment modality	*No. of cases*	*No. of patients with a local Recurrence*	(%)
Excision	127	85	(67)
Excision and Chemotherapy (MMC)	4	4	(100)
Excision and Cryotherapy	17	11	(65)
External Irradiation	11	7	(64)
Excision and Brachytherapy	19	5	(26)
Excision and Brachytherapy and Cryo	1	0	(0)
Exenteration and Irradiation	1	0	(0)
Exenteration	14	0	(0)

MMC = Mitomycin C; intraoperative.

other treatments. Other studies also found a trend toward an increased local recurrence rate after excision without additional treatment compared to excision and ruthenium plaque[27-30] or additional external radiotherapy.[18] Reduction in local recurrences might also reduce the need for secondary exenteration.

The other main risk factor for local recurrences was non-epibulbar location. None of the other variables, such as age at diagnosis, unilocular or multilocular location, origin, thickness, or diameter, were significantly correlated with the local recurrence rate. We confirm the findings of Tuomaala et al.[6] that location is the most important risk factor for local recurrence. The 5-year local recurrence rate was 60.7% (95% CI 53.0-68.5). Others reports have shown lower 5-year rates of between 26% and 52%.[6,11,15,19-20] This difference may be explained by the percentage of tumors treated with adjuvant therapy. However, 10 years after diagnosis, local recurrence seemed to reach comparable levels.[19-20] Therefore, the long-term effect of adjuvant therapy on local recurrences still has to be evaluated. One of our patients had nine local recurrences despite receiving adjuvant brachytherapy and cryotherapy.

Regional metastases occurred in 41 (21%) patients, mostly in the preauricular or parotid (*n*=26) region and cervical (*n*=13) regions. Four patients with only regional metastasis survived for more than 15 years after local treatment, as also reported for parotid gland melanomas.[37-38] This long survival, in the absence of distant metastases, supports an active search for positive lymph nodes in the pre-auricular, cervical, and sub-mandibular regions. This result may also advocate consideration of sentinel lymph node biopsy[39] in trying to treat regional metastases at a sub-clinical stage. Lymphoscintigraphy and sentinel lymph node biopsy is a safe procedure, although its sensitivity is still not known[39-41] and care should be taken to avoid facial paresis. The indication for sentinel lymph node biopsy is still uncertain, but the Cox analysis for regional metastases (Table 4) suggests that tumors thicker than 2 mm with a diameter greater than 10 mm warrant this approach. In our study, primary epibulbar tumors tended to give rise to fewer regional metastases (Table 4), in agreement with Tuomaala et al.[42] who reported significantly fewer regional lymph nodes from limbal tumors.

Forty-seven (24%) patients developed a distant metastasis, mainly in lung, liver, skin, and brain, 40 of these patients had distant metastasis in these four locations. Esmaeli et al.[25] also reported liver and lung as main sites of metastasis. Seventeen patients presented with distant metastasis, without prior or concurrent regional nodal involvement, indicating that not all melanoma patients will benefit from sentinel lymph node biopsy. In contrast to the study of Esmaeli et al, we did not find that the primary tumors that 'skipped' the lymph nodes had a higher rate of local recurrence.

The survival rate was 86.3% at 5 years, 71.2% at 10 years, and 67.2% at 15 years (Figure 3A), similar to other studies (5-year survival 87.6-74%).[4, 21, 25] The total exenteration rate was 30% over a mean follow-up period of 9.2 years, comparable to the 37% reported by Paridaens et al.[33], and the estimated 15-year 32% exenteration rate of reported by Shields and Shields[34]. Main risk factors for distant metastases, as well as for mortality, were non-epibulbar location as well as thick (>2 mm), and multilocular tumors.

Although we found a significant difference in local recurrence analysis, there was no significant difference in survival with different treatment strategies. Only primary exenteration

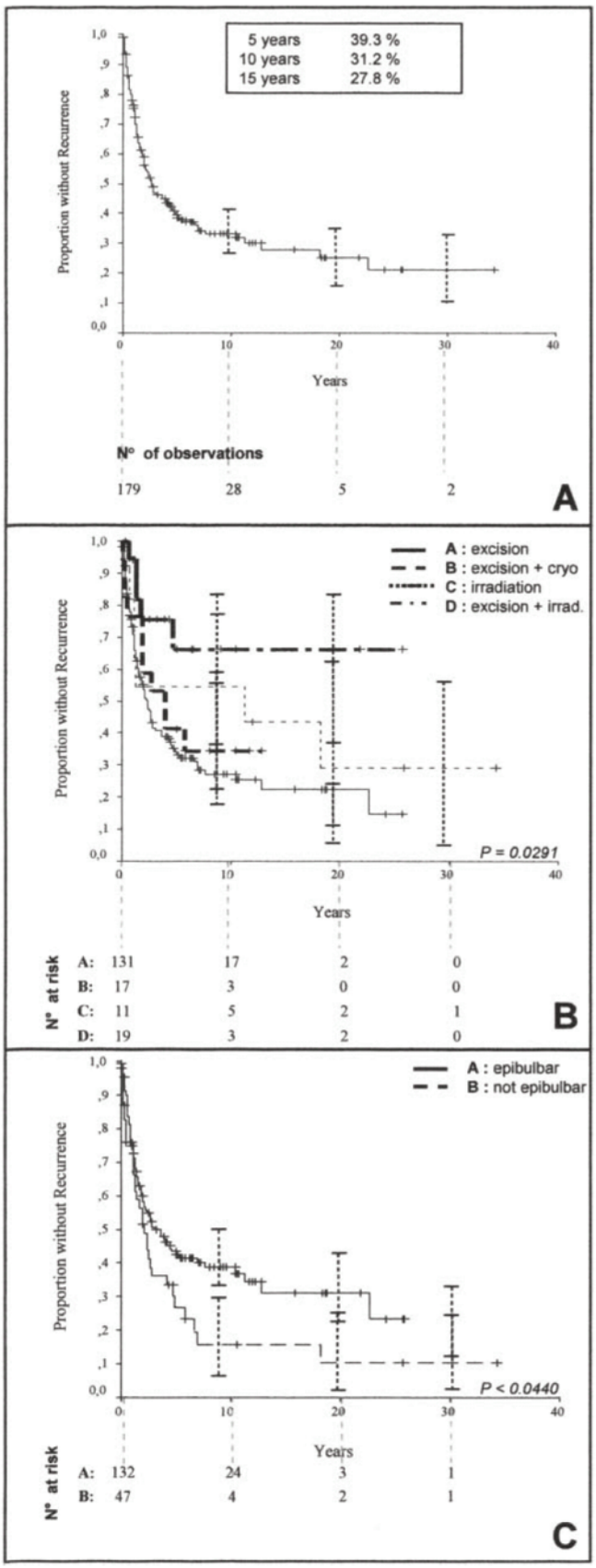


Figure 2. Kaplan Meier recurrence analysis in 179 patients (primary exenterated patients are not included in recurrence analysis). **A**. Overall recurrence rate after the primary treated tumor (time analysed from primary diagnosis to first local recurrence) **B**. Recurrence rate in function of treatment modality of the primary lesion (overall log rank p=0.043). Excision in combination with irradiation had significantly less recurrences (log rank p=0.038). There was no significant difference between excision in combination with cryotherapy when compared with excision only (log rank p=0.759). **C**. Recurrence rate for patients with only a epibulbar component (132 patients) or not (47 patients) (log rank p<0.044).

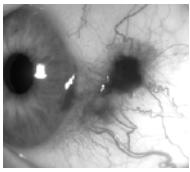


Table 3. Cox regression analysis of the appearance of first local recurrence. Both univariate analysis and a multivariate model are presented. Analysis for 179 patients (patients with primary exenterated tumors are not included in the analysis).

Variable	*Coefficient (SE)*	*Wald ²*	*P*	*Hazard Ratio (95% CI)*
Univariate analysis				
Age at Diagnosis[$]	0.005 (0.005)	0.897	0.344	1.005 (0.994-1.016)
Non-epibulbar location	0.412 (0.206)	3.996	0.046	1.510 (1.008-2.263)
Uni/Multilocular	0.087 (0.170)	0.263	0.608	1.091 (0.782-1.522)
Origin * (referenced to melanoma) [α]		1.768	0.622	
Naevus	-0.515 (0.732)	0.495	0.482	0.598 (0.142-2.509)
Naevus + PAM	-0.624 (0.532)	1.374	0.241	0.536 (0.189-1.520)
PAM	-0.120 (0.219)	0.303	0.582	0.887 (0.578-1.361
Thickness[‡]	0.240 (0.130)	3.433	0.064	1.271 (0.986-1.639)
Basal Diameter[Ÿ]	0.065 (0.115)	0.320	0.571	1.067 (0.852-1.338
Therapy[†] (referenced to excision + brachytherapy) [α]		8.065	0.045	
Excision	1.251 (0.460)	7.404	0.007	3.494 (1.419-8.606)
Excision + cryotherapy	1.129 (0.540)	4.373	0.037	3.093 (1.073-8.911)
Irradiation	0.863 (0.588)	2.151	0.142	2.370 (0.748-7.506)
Multivariate Analysis				
Model (-2log likelihood 1006.905)				
Non-epibulbar location	0.533 (0.211)	6.376	0.012	1.704 (1.127-2.577)
Therapy[†] (referenced to excision + brachytherapy) [α]		9.667	0.022	
Excision	1.331 (0.461)	8.315	0.004	3.783 (1.531-9.347)
Excision + cryotherapy	1.236 (0.542)	5.196	0.023	3.442 (1.189-9.963)
Irradiation	0.768 (0.590)	1.699	0.192	2.156 (0.679-6.848)

$Age at diagnosis : grouped as 0-50 years; 51-67 years; 68-99 years.
*Origin : grouped as naevus, naevus and PAM, PAM or melanoma.
[‡]Thickness (Breslow): ≤ 1mm; 1 > 2 mm; ≥ 2mm.
[Ÿ]Basal diameter : 0-6 mm; 6-10 mm; > 10mm.
†Therapy : excision; excision and cryotherapy; excision and brachytherapy; irradiation.
[α] Origin and Therapy were considered as categorical variates for Cox regression analysis.

for advanced disease was associated with a significantly lower survival, which can be explained by the fact that these tumors were more extensive and had a more frequently unfavorable location. We think that more knowledge of this rare tumor may help us to detect suspect lesions earlier and thus ensure timely referral of patients to ophthalmic oncology centers.

Conclusion

Predictors of poor survival were a non-epibulbar location, tumors thicker than 2 mm, and multifocal location. Survival may be improved by early diagnosis of regional metastases. The long survival rate of some patients with regional metastases also suggests that sentinel lymph node biopsy may increase survival in high-risk patients, e.g., especially in patients with multiple local recurrences, a Breslow tumor thickness of more than 2 mm, and a tumor

Table 4. Cox regression analysis for the appearance of regional metastases in 194 patients. Tumor thickness showed to be the most important risk factor

Variable	*Coefficient (SE)*	*Wald ²*	*P*	*Hazard Ratio (95% CI)*
Univariate analysis				
Age at Diagnosis[$]	0.011 (0.010)	1.234	0.267	1.011 (0.992-1.031)
Non-epibulbar location	0.596 (0.321)	3.456	0.063	1.815 (0.968-3.404)
Uni/Multilocular	0.231 (0.228)	1.027	0.311	1.259 (0.806-1.967)
Origin * (referenced to melanoma) [α]		1.260	0.739	
Naevus	-12.241 (414.426)	0.001	0.976	0.000 (n.a.)
Naevus + PAM	0.312 (0.652)	0.229	0.632	1.366 (0.381-4.903)
PAM	-0.281 (0.370)	0.575	0.448	0.755 (0.366-1.560)
Thickness[‡]	1.019 (0.238)	18.363	<0.001	2.770 (1.738-4.415)
Basal Diameter[Ÿ]	0.489 (0.189)	6.660	0.010	1.631 (1.125-2.364)
Therapy[†] (referenced to excision + brachytherapy) [α]		1.483	0.686	
Excision	0.566 (0.732)	0.597	0.440	1.761 (0.419-7.396)
Excision + cryotherapy	-0.092 (1.001)	0.008	0.927	0.912 (0.128-6.485)
Irradiation	0.741 (0.915)	0.656	0.418	2.098 (0.349-12.602)
Multivariate Analysis				
Model 1 (-2log likelihood 264.401)				
Thickness [‡]	0.874 (0.254)	11.851	0.001	2.397 (1.457-3.942)
Basal Diameter[Ÿ]	0.192 (0.213)	0.811	0.368	1.211 (0.798-1.839)

$,*,‡,Ÿ,†,α : as in Table 3.

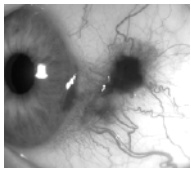


Table 5. Cox regression analysis for the appearance of hematogenous metastases in 194 conjunctival melanoma patients. Epibulbar localisation and tumor thickness are the most important risk factor in our study.

Variable	*Coefficient (SE)*	*Wald ²*	*P*	*Hazard Ratio (95% CI)*
Univariate analysis				
Age at Diagnosis[$]	0.425 (0.180)	5.556	0.018	1.530 (1.074-2.179)
Non-epibulbar location	1.396 (0.289)	23.269	<0.001	4.039 (2.290-7.122)
Uni/Multilocular	0.034 (0.057)	0.343	0.558	1.034 (0.924-1.157)
Origin * (referenced to melanoma) [α]		1.234	0.745	
Naevus	-12.305 (358.641)	0.001	0.973	0.000 (0.000-8.544)
Naevus + PAM	-0.007 (0.637)	0.000	0.991	0.993 (0.285-3.461)
PAM	-0.347 (0.331)	1.100	0.294	0.707 (0.369-1.352)
Thickness[‡]	0.743 (0.196)	14.306	<0.001	2.102 (1.430-3.089)
Basal Diameter[Ÿ]	0.322 (0.172)	3.501	0.061	1.379 (0.985-1.932)
Therapy[†] (referenced to excision + brachytherapy) [α]		3.121	0.373	
Excision	0.711 (0.727)	0.955	0.328	2.036 (0.489-8.471)
Excision + cryotherapy	-0.818 (1.226)	0.466	0.504	0.441 (0.040-4.876)
Irradiation	0.582 (0.918)	0.401	0.526	1.789 (0.296-10.813)
Multivariate Analysis				
Model 1 (-2log likelihood 264.401)				
Non-epibulbar location	1.269 (0.331)	14.694	<0.001	3.557 (1.859-6.805)
Thickness [‡]	0.533 (0.197)	7.345	0.007	1.704 (1.159-2.505)

$,*,‡,Ÿ,†,α : as in Table 3.

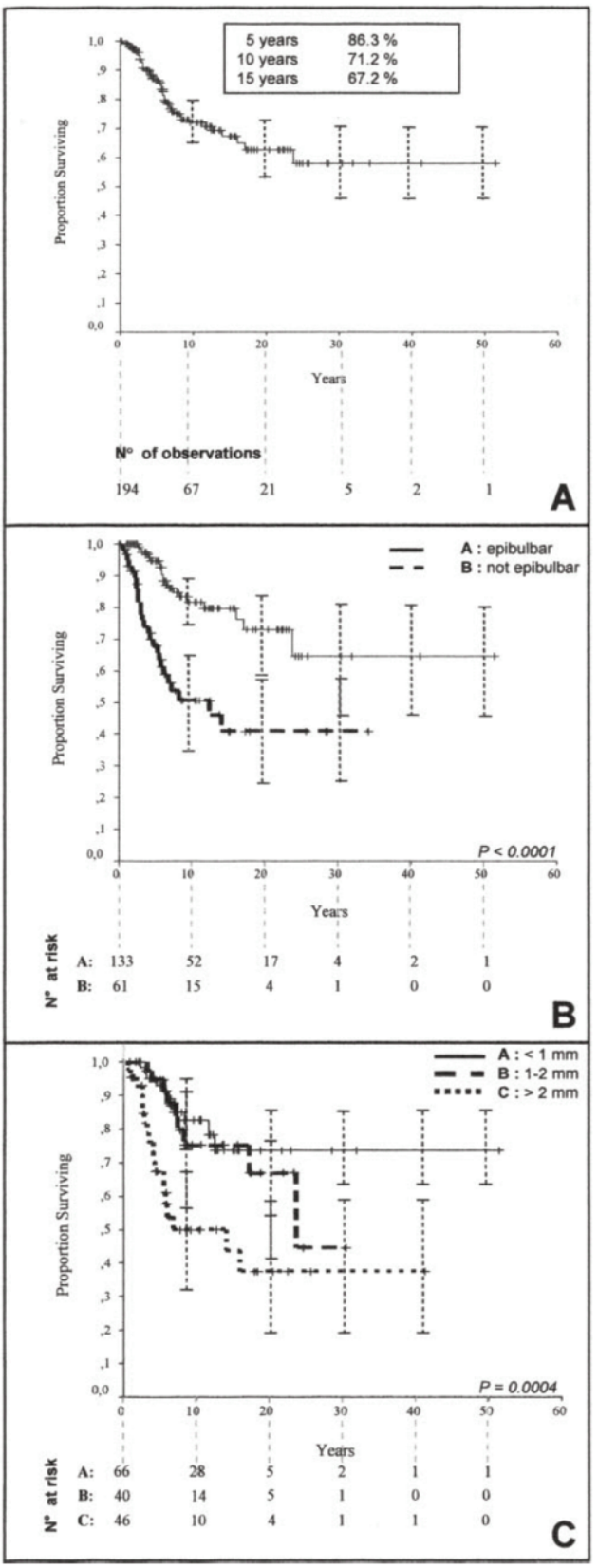
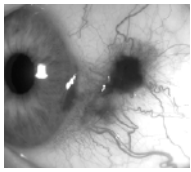


Figure 3. Kaplan Meier survival analysis in 194 patients. **A**. Overall survival in years after primary treated tumor (histopathologically diagnosed conjunctival melanoma). **B**. Survival for epibulbar (61 patients) and non-epibulbar (133 patients) conjunctival melanoma patients. Log rank p<0.0001. **C**. Survival analysis for different tumor thicknesses of the primary lesion (overall Log rank test for trend p=0.0004).

Table 6. Cox regression analysis for survival in 194 conjunctival melanoma patients.

Variable	*Coefficient (SE)*	*Wald ²*	*P*	*Hazard Ratio (95% CI)*
Univariate analysis				
Age at Diagnosis[$]	0.018 (0.010)	3.425	0.064	1.018 (0.999-1.037)
Non-epibulbar location	1.286 (0.294)	19.113	<0.001	3.617 (2.033-6.438)
Uni/Multilocular	0.439 (0.196)	5.210	0.025	1.551 (1.056-2.278)
Origin * (referenced to melanoma) [α]		1.417	0.702	
Naevus	-12.293 (354.707)	0.001	0.973	0.000 (0.000-8.948)
Naevus + PAM	0.192 (0.644)	0.089	0.765	1.212 (0.343-4.279)
PAM	-0.333 (0.341)	0.954	0.329	0.716 (0.367-1.399)
Thickness[‡]	0.678 (0.200)	11.465	0.001	1.969 (1.330-2.916)
Basal Diameter[Ÿ]	0.320 (0.176)	3.299	0.069	1.378 (0.975-1.946)
Therapy[†] (referenced to excision + brachytherapy) [α]		2.788	0.426	
Excision	0.649 (0.728)	0.795	0.372	1.914 (0.460-7.972)
Excision + cryotherapy	-0.819 (1.226)	0.446	0.504	0.441 (0.040-4.873)
Irradiation	0.544 (0.918)	0.351	0.553	1.723 (0.285-10.417)
Multivariate Analysis				
Model 1 (-2log likelihood 264.401)				
Non-epibulbar location	1.148 (0.337)	11.595	0.001	3.152 (1.628-6.103)
Thickness [‡]	0.491 (0.202)	5.914	0.015	1.634 (1.100-2.426)

$,*,‡,Ÿ,†,α : as in Table 3.



diameter greater than 10 mm. Adjuvant brachytherapy may reduce the number of local recurrences. A prospective study is needed to compare the effect of excision in combination with radiotherapy and excision with cryotherapy on the number of local recurrences, exenteration rate, and survival.

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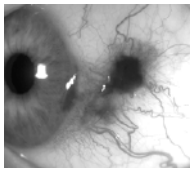

CHAPTER 4

PREDICTIVE VALUE OF EXFOLIATIVE CYTOLOGY IN PIGMENTED CONJUNCTIVAL LESIONS

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Abstract

Purpose. Pigmented lesions of the conjunctiva are often difficult to classify clinically. Exfoliative cytology may be helpful, but reliable data regarding the sensitivity and specificity of this test are currently lacking. We determined the value of exfoliative cytology with regard to pigmented conjunctival lesions.

Methods. 294 smears from 182 patients were screened for malignancy within 6 months of exfoliative cytology. Smears were classified according to the following categories: grade 0, insufficient material for diagnosis; grade 1, normal conjunctival cells; grade 2, melanocytes with mild atypia; grade 3, melanocytes with moderate atypia; and grade 4, melanocytes with severe atypia.

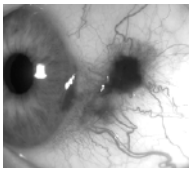
Results. The sensitivity, specificity, positive predictive value, and negative predictive value of exfoliative cytology were 85 %, 78 %, 59 %, and 93 %, respectively.

Conclusion. Exfoliative cytology is a fast, easy, and non-invasive technique that may be used in the evaluation of patients with a pigmented conjunctival lesion.

Introduction

It is often difficult to correctly diagnose pigmented lesions of the conjunctiva on clinical grounds alone.[1] Yet it is important to differentiate between a benign conjunctival nevus, a potentially premalignant primary acquired melanosis (PAM), a malignant conjunctival melanoma, and pigmented squamous cell carcinoma, because the latter two are potentially lethal.

Conjunctival biopsies are usually taken to verify the clinical diagnosis of PAM or melanoma. However, a non-invasive and infrequently used alternative to conjunctival biopsy is the conjunctival smear. Whereas repeated biopsies can cause complications and discomfort to the patient, conjunctival smears can be obtained with minimal damage and discomfort by means of cotton swabs wiped across the conjunctiva. Lopez Cardozo, who first described exfoliative cytology, used a cotton wool tip to collect cells from pigmented conjunctival lesions.[2] In earlier studies, conjunctival scraping was used instead of cotton wool swabs, which is slightly more invasive.[3,4] Egbert et al. first described impression cytology, by which samples were obtained by touching the eye with a cellulose acetate filter or by Biopore membrane impression.[5,6] In our hospital, exfoliative cytology of conjunctival smears has been used for more than 25 years to assess pigmented conjunctival lesions. However, the exact sensitivity and specificity of exfoliative cytology have not yet been determined. Previous studies have reported predictive values from 73% to 100% for exfoliative or impression cytology on pigmented conjunctival lesions, but all examined only 30 patients or less.[7] In this study, we determined the predictive value, sensitivity, and specificity of exfoliative cytology in a large group of patients with a pigmented conjunctival lesion.



Patients and methods

We reviewed the records of all the patients who were diagnosed with a melanocytic conjunctival lesion (nevus, PAM, conjunctival melanoma), and for whom exfoliative cytology had been performed between 1975 and 2003 at the Leiden University Medical Centre. Exfoliative cytology was performed when the clinical diagnosis was uncertain and when a lesion was suspect for conjunctival melanoma or PAM with atypia. In this time period, 328 conjunctival smears were taken from 199 patients with a melanocytic conjunctival lesion. Of these samples, 34 were scored according to a grading system different from the currently used system and the slides were not available for re-evaluation. Slides of 294 smears from 182 patients were available for this study. For 138 patients, a single smear was performed. For 44 patients, multiple smears were taken (in total 156 smears), 101 of these smears were taken because of a primary tumour lesion or a recurrence; 46 smears were taken from the same lesion, because the lesions showed clinical changes. Nine exfoliations were performed after excision of the primary lesion to check complete removal of the lesion.

For all slides, the grade, number of cells, and possible problems with exfoliative cytology were recorded. It was also registered whether histological samples were taken from the same patient within six months after the cytological smear, such matched samples were available in 157 of the 294 cases. Conjunctival melanoma in situ was defined as a complete replacement of the epithelium with atypical melanocytes, without any signs of invasion into the deeper layers of the conjunctiva. PAM with atypia was defined as atypical melanocytes in

the basal layers of the epithelium, or nest formation of atypical melanocytes in the epithelium, or as individual atypical melanocytes in different layers of the epithelium (pagetoid form). No histological material was obtained when the lesion was considered benign; when in doubt the patients were followed regularly to confirm the benign character of the lesion. All cytological samples were reviewed by one cyto-pathologist (MV), all histological slides were reviewed by one ophthalmic pathologist (DW).

All smears were taken as described previously.[2] In brief, all suspected pigmented lesions were gently rubbed with a cotton wool tip, and the cotton wool tip was subsequently dabbed onto several glass slides. Glass slides were air dried, fixed with methanol, and subsequently stained with Giemsa. The exfoliative samples can contain conjunctival cells, goblet cells, melanocytic cells, inflammatory cells, and blood cells. The presence of melanocytic cells is important for the classification of pigmented conjunctival lesions. All samples were investigated for abnormalities in nuclear size, nuclear shape, nuclear-cytoplasmatic ratio, chromatin, nuclear membrane, nucleoli, and melanin. The samples were classified into five different categories (Figure 1): grade 0, insufficient material for diagnosis, grade 1, normal

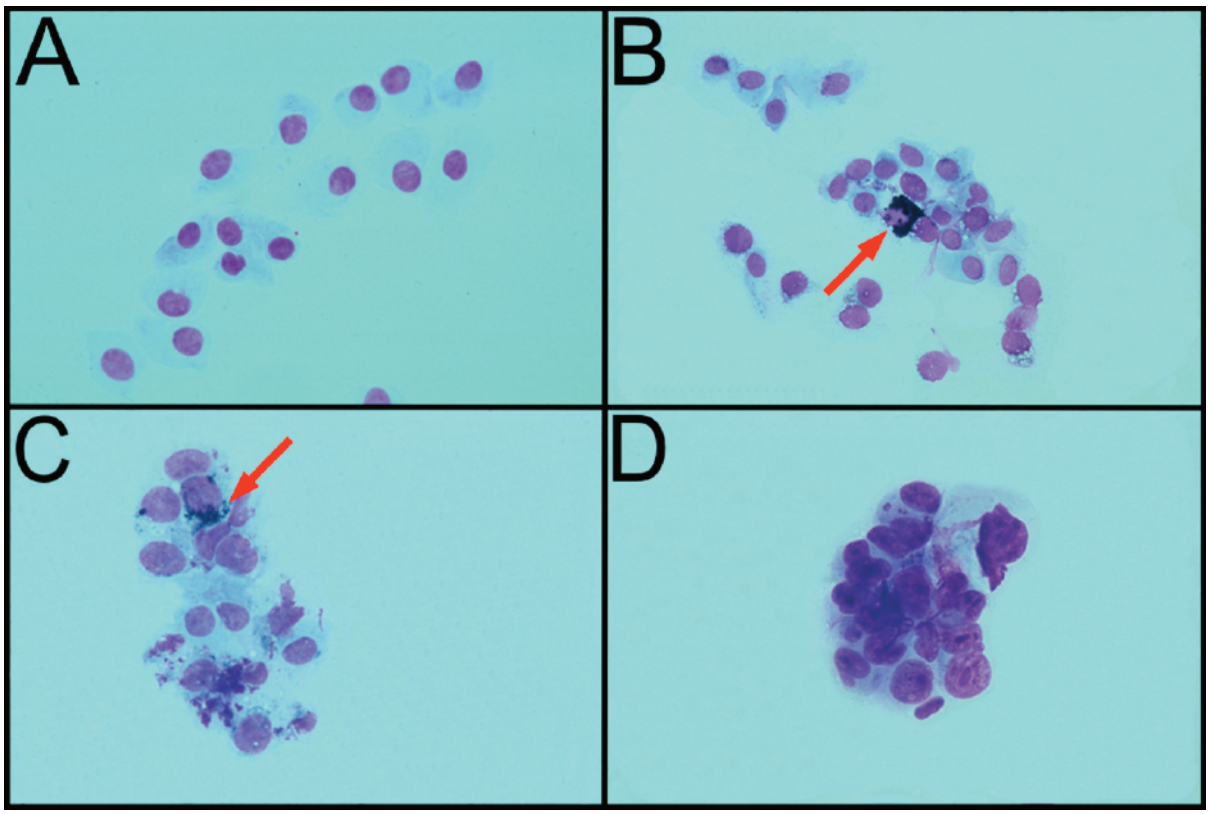


Figure 1. Different grades of atypia in exfoliative cytology samples.
Figure 1A: grade 1, normal conjunctival cells. Figure 1B: grade 2, melanocytes with mild atypia, characterized by slight increased nucleus-cytoplasmatic ratio, and few irregular nuclear membranes. Figure 1C: grade 3, melanocytes with moderate atypia, characterized by large nuclei, irregular nuclear membranes, anisokaryosis, and prominent nucleoli. Figure 1D: grade 4, melanocytes with severe atypia, characterized by very large nuclei and therefore severe increased nucleus-cytoplasmatic ratio, anisokaryosis, very irregular nuclear membranes, and large prominent macro-nucleoli. Red arrow indicates melanin pigment. Figures A-D are Giemsa stained and photographed with 400X magnification.

conjunctival cells with or without melanin pigment, or reactive conjunctival cells typical of inflammation; grade 2, melanocytic cells with mild atypia; grade 3, melanocytic cells with moderate atypia; and grade 4, melanocytic cells with severe atypia.

Statistics

Sensitivity (se), specificity (sp), positive predictive value (ppv), and negative predictive value (npv) were calculated with the following formulas:
se = TP/(TP +FN),sp = TN/(FP + TN), ppv = TP/(TP + FP), npv = TN/(FN +TN), where TP are the true positive results, TN are the true negative results, FP the false positive results, and FN the false negative results.
The 95% confidence intervals (CI) were calculated with the following formulas:
$SE(P) = \sqrt{(P(1-P)/n)}$, 95% CI = mean ± 2SE, where SE is the standard error, P is the percentage found, and *n* the total number of cases.

Results

Samples were obtained from 83 men (45 %) and 99 women (54 %). The average age of the patients was 44 (± SD 22) years.
In 23 (8 %) smears (grade 0) the severity of atypia could not be estimated, primarily due to a low cell count and poor quality of the collected material. These cases were excluded from further analysis. Of the remaining 271 smears, 80 were classified as grade 1, 87 as grade 2, 26 as grade 3, and 78 as grade 4. Of the 157 histological samples, 35 were diagnosed as nevus, 16 as nevus with atypia, 6 as PAM without atypia, 18 as PAM with atypia, 17 as melanoma in situ, 63 as invasive melanoma, and two as a degenerative lesion. Table 1 shows the histological diagnosis versus the exfoliative grading.

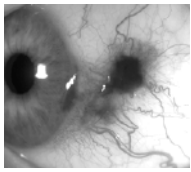


Table 1. Histological diagnosis and the corresponding exfoliative cytology category among 294 smears.

Category of atypia *	Nevus	PAM without atypia	PAM with atypia	Melanoma insitu	Invasive melanoma	Other†	No histology	Total
Grade 0	4	0	0	2	6	0	11	23
Grade 1	17	3	1	1	4	0	54	80
Grade 2	16	1	1	1	5	1	62	87
Grade 3	4	0	8	0	5	0	9	26
Grade 4	10	2	8	13	43	1	1	78
Total	51	6	18	17	63	2	137	294

* Grading system for atypia, grade 0: insufficient material, grade 1: normal cells, grade 2: melanocytes with mild atypia, grade 3 melanocytes with moderate atypia, and grade 4 melanocytes with severe atypia.
† Degenerative lesions

Histologically confirmed conjunctival melanoma (invasive or in situ) was detected in 72 % (95% CI, 62-82) of the smears with grade 4 atypia, whereas melanoma was detected in 6 % (95% CI, 1-12), 7 % (95% CI, 2-12), and 19 % (95% CI, 4-35) of the cases with grade 1, 2, or 3 atypia, respectively (Table 2).
The calculated sensitivity, specificity, positive predictive value, and negative predictive value of atypia grades 1, 2, 3, and 4 to detect a conjunctival melanoma (invasive or in situ) are listed in Table 3. When smears with grade 3 and 4 atypia are grouped together this would result in a sensitivity of 85 % (95% CI, 77-93), specificity of 78 % (95% CI, 73-84), positive predictive value of 59 % (95% CI, 49-68), and a negative predictive value of 93 % (95% CI, 90-97) (Table 3).

Table 2. Cytology grading and melanoma incidence.

Category of atypia	*Conjunctival melanoma †*			
	No	*Yes*	*Total*	
Grade 0	15 65% (45-85)	8 35% (15-55)	23	
Grade 1	75 94% (88-99)	5 6% (1-12)	80	Low chance of melanoma
Grade 2	81 93% (88-99)	6 7% (2-12)	87	
Grade 3	21 81% (65-96)	5 19% (4-35)	26	High chance of melanoma
Grade 4	22 28% (18-38)	56 72% (62-82)	78	
Total	214	80	294	

* Grading system for atypia, grade 0: insufficient material, grade 1: normal cells, grade 2: melanocytes with mild atypia, grade 3 melanocytes with moderate atypia, and grade 4 melanocytes with severe atypia.
† Occurrence of histological-confirmed conjunctival melanoma (invasive or in situ) within six months of exfoliative cytology. Data are numbers, percentages within that grade of atypia, and between brackets 95% confidence intervals.

Table 3. Predictive values for the different grades of atypia to detect a conjunctival melanoma.

	Sensitivity	*Specificity*	*Positive predictive value*	*Negative predictive value*
	% (95% CI)	*% (95% CI)*	*% (95% CI)*	*% (95% CI)*
Grade 1	7 (1-14)	62 (56-69)	6 (1-12)	65 (58-72)
Grade 2	8 (2-15)	59 (52-66)	7 (2-12)	64 (57-71)
Grade 3	7 (1-14)	89 (85-94)	19 (4-35)	73 (67-78)
Grade 4	78 (68-88)	89 (85-93	72 (62-82)	92 (88-96)
Grade 3 and 4 together	85 (77-93)	78 (73-84)	59 (49-68)	93 (90-97)

The sensitivity, specificity, positive predictive value, and negative predictive value are expressed as percentages with a 95% confidence interval.

Table 4. Number of cells in 248 exfoliative smears.

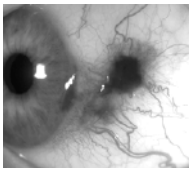
Number of cells	Number of smears (percentage)
Low	57 (23)
Moderate	71 (28)
High	110 (44)
Very High	10 (4)
Total	248 (100)

In 248 of the 294 smears the number of cells could be estimated: low, moderate, high, and very high numbers of cells were present in 57, 71, 110, and 10 smears, respectively (Table 4).

In 8 (35 %) of the 23 smears in which the lesion could not be graded by exfoliative cytology (grade 0), a conjunctival melanoma (invasive or in situ) was histologically confirmed within six months after cytological examination. In all of these cases, atypical cells were seen but the cell count was too low or the cell quality was too poor to grade the lesion.

Discussion

Atypical melanocytic cells that ascend to the epithelial surface of the conjunctiva are indicative of melanoma and PAM with atypia. Benign lesions, such as conjunctival nevus or PAM without atypia, are not associated with superficial atypical melanocytes, although superficial atypical cells can be found in growing nevi in childhood and adolescence [3]. Thus benign lesions in adult patients usually have a normal cytology. PAM without atypia does not progress to melanoma [8]. Our results for the exfoliative cytology of pigmented conjunctival lesions (Table 2) indicate that lesions with melanocytes showing a low grade of atypia (grade 2) at their epithelial surface are rarely associated with a histologically confirmed conjunctival melanoma within 6 months. The likelihood of the presence of a conjunctival melanoma was higher when samples showed higher grades of melanocyte atypia (grades 3 and 4), increasing to 72 % (95% CI, 62-82) for grade 4 smears.



Therefore pigmented lesions with a low grade of atypia (grade 2) do not require aggressive treatment since the risk of melanoma is equal to that of controls (= normal cytological results), within six months of exfoliative examination. Patients should be followed each 6 to 12 months to detect whether the lesion is growing and smears should be taken repeatedly. For grade 4 smears the sensitivity, specificity , positive predictive value, and negative predictive value for diagnosing a conjunctival melanoma are reasonably well (78 %, 89 %, 72 %, and 92 %, respectively), however, when both grades 3 and 4 atypia are considered as a positive marker for conjunctival melanoma the sensitivity increases from 78 % to 85 % (Table 3). Because conjunctival melanoma is a potentially lethal disease (30 % 10-year mortality) [9,10,11,7,12,13,14,15,16], the sensitivity should be as high as possible in order to detect the highest number of conjunctival melanomas. Thus both grade 3 and 4 atypia (moderate and severe atypia) should be considered as positive clinical markers for both in situ and invasive conjunctival melanoma, since cytology cannot differentiate between in situ and invasive

malignancies. If grade 3 or 4 atypia is found, patients should undergo excisional biopsy to confirm the findings. The sensitivity found in this study is comparable with previous studies [7].

Because the chance of conjunctival melanoma still exists in low-grade lesions, a biopsy is always needed when there is strong suspicion on clinical grounds that a pigmented lesion is a conjunctival melanoma. In these cases, atypical melanocytic cells have probably not yet reached the surface of the epithelium, and for this reason cytology reveals only normal conjunctival cells or only a very few atypical cells. It should be noted that four of the five melanomas found in patients with grade 1 cytology did have strongly reactive conjunctival epithelial cells but no melanocytic cells in their smears. Reactive cells can be found in inflamed conjunctivas, such as infected conjunctivas or after topical treatment with mitomycin C. Repeated smears for exfoliative cytology can probably decrease the numbers of missed melanomas in low-grade lesions.

To calculate the predictive value of exfoliative smears, we also used smears without a histological sample, this could be seen as a drawback of the study and a possible bias. However when only smears with a corresponding histological sample would be included, another bias exists since only lesions that are more suspect of conjunctival melanoma are excised. We wanted to present a more realistic picture, so all exfoliative samples were included in this study. For the samples without a histological sample, the clinical follow-up was used to confirm that these lesions were indeed benign.

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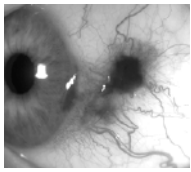

CHAPTER 5

IMPRESSION CYTOLOGY OF MELANOCYTIC CONJUNCTIVAL TUMOURS USING THE BIOPORE MEMBRANE

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Abstract

Background: To compare a new Biopore membrane impression cytology method with the routinely used exfoliative cytology in patients with a melanocytic lesion of the conjunctiva.

Methods: Sixty-eight consecutive patients with a conjunctival melanocytic lesion underwent Biopore membrane impression cytology as well as exfoliative cytology. A histological sample was also available in 26 cases. All Biopore samples were stained immediately with RAL 555. Both Biopore and exfoliative cytology samples were assessed by two cyto-pathologists and graded into four different categories of atypia.

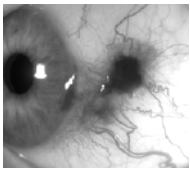
Results: Twenty-three out of 26 Biopores and 20 out of 24 for the exfoliative smears correlated with the corresponding histological sample. Biopore cytology resulted in higher numbers of cells with a greater density compared to exfoliative cytology.

Conclusions: Biopore cytology can be used for cytological sampling of conjunctival melanocytic lesions. Because of the larger amount and higher density of cells obtained with the Biopore membrane, interpretation by a pathologist is easier and faster. Sampling of the fornix, caruncle, and sampling of ocular material in children is difficult with the Biopore method, and exfoliative cytology seems to be the favourable test in those situations.

Introduction

Conjunctival melanoma is a rare malignant tumour, accounting for 2-3% of all ocular tumours.[1,2] The incidence of conjunctival melanoma in Caucasians is 0.02-0.08 per 100.000 inhabitants.[3-6] Conjunctival primary acquired melanosis (PAM) is the most frequently reported precursor of conjunctival melanoma and in general affects the limbal and bulbar conjunctiva, although some conjunctival melanomas evolve from pre-existing nevi or develop de novo.[3-9] Clinically, the differentiation between PAM and a nevus with or without progression to melanoma is often difficult,[10] and a biopsy for histologic examination can be obtained. Cytology could be an alternative to diagnostic biopsies, and is a minimally invasive diagnostic tool, which can also specify the risk of the lesion developing into a conjunctival melanoma without the need for biopsy, as especially the severe atypia is correlated with the presence of a conjunctival melanoma.[11] Cytology can therefore help the ophthalmologist in the diagnosis and subsequent treatment of conjunctival pigmented lesions and follow-up after observation or mitomycin-C treatment.

Exfoliative cytology and impression cytology are two different techniques to acquire cells for cytological analysis. In exfoliative cytology, cells are collected with a cotton-wool swab and mounted on glass slides.[12] Impression cytology is either done with cellulose acetate filters or by use of a Biopore membrane,[13,14] the cellulose acetate filters have already been tested on conjunctival melanocytic lesions.[15] The Biopore membrane has already been used in patients with superficial viral infections, and in case of ocular surface squamous neoplasia.[14,16,17] Biopore impression cytology is a newer technique, that provides a relatively large surface, and can therefore strip off a high amount of cells, still in their original configuration. In this study we investigated whether the Biopore can be used to interpret a melanocytic lesion, and compared the advantages and disadvantages with exfoliative cytology.



Patients and methods

Patients

Sixty-eight patients with a pigmented conjunctival lesion underwent both Biopore and exfoliative cytological sampling between April 2003 and November 2004 (Table I).

There were 33 men with a mean age of 42 years (SD: 22.9, range 8 to 87), and 35 women, with a mean age of 49 years (SD 26.4, range 8 to 92). All patients came from the outpatient clinic of the Department of Ophthalmology at Leiden University Medical Centre, Leiden, The Netherlands. Of 26 of the 68 patients a histological sample was available. The study was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Technique

The eye with the melanocytic lesion was first sampled with the Biopore impression cytology method. The Biopore (Millicell-CM 0.4 μm PICM 012550, Millipore Corp, Bedford, MA, USA) is an 8 mm round membrane disc, which is placed in a plastic ring. Before sampling, three plastic legs are removed from the plastic ring. To obtain a firmer grip on the Biopore

Table 1. Patient characteristics.

Characteristics	*Number*	*(percentage)*
Gender		
Male	33	(49)
Female	35	(51)
Clinical diagnosis		
Nevus	31	(46)
PAM	28	(41)
Melanoma	9	(13)
Location		
Caruncle	10	(15)
Fornix	3	(4)
Bulbus	55	(81)
(Limbal *	36	(65))

* Number of bulbar lesions that were located at the limbus.
PAM = Primary acquired melanosis

membrane, the device is placed in a slightly larger plastic tube. The eye is anaesthetized with 1-2 drops of 0.4% oxybuprocaine (mono free, Théa Pharma, Ukkel, Belgium), and the eyelids are opened for a few seconds to dry the conjunctiva to improve the adherence of cells onto the Biopore membrane. The Biopore membrane is pressed gently onto the conjunctiva, after 3-5 seconds the Biopore is removed and immediately fixed and stained with RAL 555 (555-FIX-RAL, 555-Eosin-RAL, 555-Blue-RAL, Reactifs RAL Bordeaux technopols, Martillac, France). The Biopore membrane is submerged in each of the three RAL 555 solutions (methanol, eosin, methylene blue) for approximately 10 seconds. After staining, the membrane is cut out with a 15-degree knife and fixed with mounting medium on a glass slide for microscopic evaluation.
After Biopore sampling, the same lesions are swabbed with a cotton-wool tip for exfoliative cytology. The cells on the cotton-wool tip are than transferred to several glass slides, procedure is repeated three times to acquire more cells for analysis. The glass slides of the exfoliative cytology are processed under standard protocol used in our hospital.

Cytological interpretation

Exfoliative cytology and Biopore membranes were all interpreted by two cyto-pathologists (MVC and SV). When there was disagreement between the two observers a third independent observed made the final decision. All Biopore samples were numbered, and bias through prior knowledge of the exfoliative cytology was therefore excluded. Both exfoliative smears as well as Biopore samples were graded by a standard grading system used in our hospital. In brief, the samples were screened for: nuclear size, nuclear to cytoplasmatic ratio, irregular nucleus, irregular nuclear chromatin pattern, and prominent nucleoli, and subsequently graded into four different stages, 0: insufficient material for diagnosis, 1: normal epithelial conjunctival cells with or without melanin pigment, reactive conjunctival cells as seen in in-

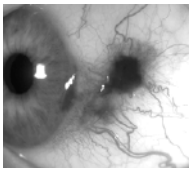
flammation, 2: melanocytes with mild atypia, 3: melanocytes with moderate atypia, 4: melanocytes with severe atypia.[11] The amount of cells collected (low, moderate, high, very high) was noted for all samples.

Statistics

Data was analysed in SPSS 11.0 (SPSS Inc, Chicago, USA). Differences in amount of cells harvested were calculated with a paired samples t-test. The Fisher's Exact Test was used to calculate the significance of the differences in percentage of conjunctival melanomas detected by both methods. It was also used to calculate the differences in correlation between both methods and the histological diagnosis.

Results

Biopore provided a cytological diagnosis in 67 out of the 68 samples (99 %), where exfoliative cytology was able to give a diagnosis in 65 out of the 68 samples (96%). In all four cases (one Biopore, three exfoliative smears) this was due to a very low cell count, and were therefore graded in category 0. There was concordance between the two observers in 58 out of 68 Biopores (85%) and 47 out of 68 exfoliative smears (69%). In 9 of the 10 Biopore disagreements and 19 of the 21 exfoliative disagreements there was only one grade of difference in atypia. Of 64 patients both a Biopore and an exfoliative sample of the same lesion were graded. In 42 of these 64 patients (66 %), Biopore and exfoliation were graded in the same category; 25 (33 %) of the Biopores were graded in a lower category, and one (2 %) Biopore was graded in a higher category than the corresponding exfoliative sample (Table II). Figure I shows exfoliative cytology, Biopore cytology, and histology for four different cases.



A corresponding histological sample was available for 26 Biopores and 24 exfoliative smears. The histological diagnosis was confirmed by the Biopore in 23 of 26 cases (88 %), and in 20 of 24 (83 %) exfoliative smears (p=0.697, Fisher's Exact Test) (Table III). We

Table 2. Cross table for Biopore and exfoliative cytology grading. Numbers within the dotted-lined squares represent the cases where Biopore and exfoliative cytology were graded similarly.

Biopore grading	Exfoliative grading					
	0	1	2	3	4	Total
0	-	1	-	-	-	1
1	1	20	13	-	4	38
2	-	1	11	3	1	16
3	1	-	-	5	-	6
4	1	-	-	-	6	7
Total	3	22	24	8	11	68

Table 3. Correlation between histological diagnosis and cytological diagnosis of the same conjunctival melanocytic lesions.

	Correct correlation	
Histological diagnosis	Exfoliative smears % (numbers)	Biopore % (numbers)
Naevus	75 (12)	92 (13)
PAM without atypia	100 (2)	100 (2)
PAM with atypia	-	100 (1)
Melanoma	89 (9)	78 (9)
Pigmented piqueculum	100 (1)	100 (1)
Total	83 (24)	88 (26)

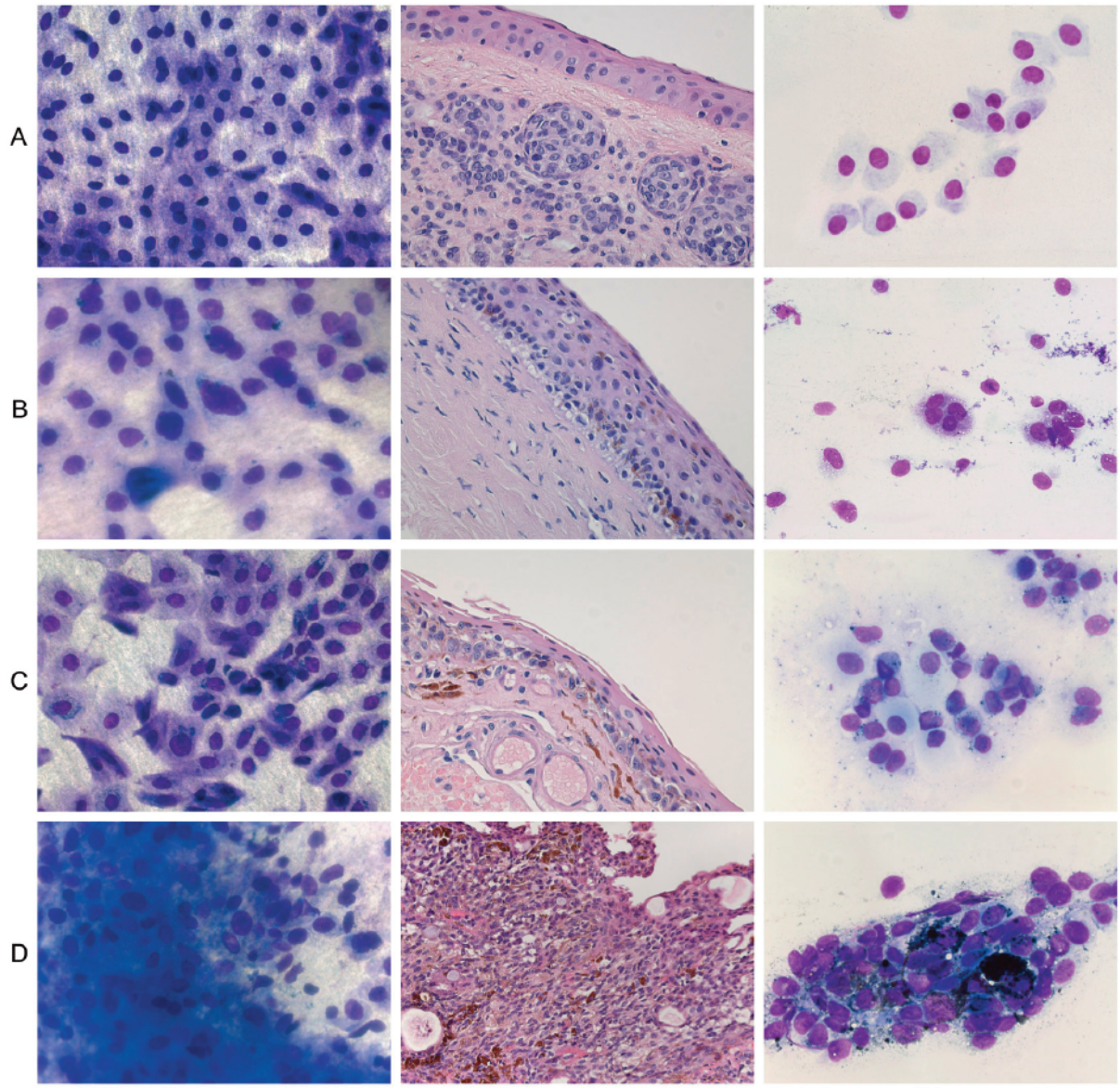
Table 4. Amount of cells

	Exfoliative smears numbers (%)	Biopore numbers (%)	p<0.0001 paired T-test
Low amount of cells	9 (14)	14 (21)	
Medium amount of cells	16 (24)	27 (40)	
High amount of cells	15 (23)	26 (38)	
Very high amount of cells	28 (39)	1 (1)	
Total	68 (100)	68 (100)	

previously noted that atypia grade 3 and 4 should be considered as a positive clinical marker for conjunctival melanoma.[11] Of all histological samples, nine were conjunctival melanomas. In these, seven out of nine (78 %) Biopores and eight out of nine (89 %) exfoliative samples had an atypia grade 3 or 4 (p=1.0, Fisher's Exact Test).
Biopore sampled significantly more cells from the conjunctival surface than exfoliative cytology (p<0.001, paired sample T-test) (Table IV, Figure I). Fewer cells were collected with the Biopore when the lesion was situated in the caruncle (p=0.03, T-test), primarily because the relatively larger Biopore was not able to reach the lesion properly.

Discussion

A variety of pigmented lesions can exist in the conjunctiva, and can be clinically and histologically divided in nevi, melanosis, and malignant melanoma. All these lesions can be further histologically subdivided. In only a part of the melanocytic lesions, melanocytes will arise to the epithelial surface, such as in juvenile intraepithelial nevi, compound nevi, adult onset PAM with moderate and severe atypia, and conjunctival melanoma.[18] Since some melanocytic lesions will be covered with one or more layers of normal epithelium, cytology can only give a realistic picture of a lesion when it is able to sample deeper than the most superficial layer of epithelial cells. Exfoliative cytology is able to sample more than one



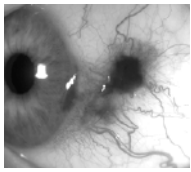


Figure 1. Biopore (left hand column), histology (middle column), and exfoliative cytology (right hand column) samples are shown for four different cases. Figure IA shows a subepithelial conjunctival nevus in a 51 year old female. Exfoliative and Biopore cytology sampled normal cells since the lesion is located underneath the epithelium. Figure IB represents PAM at the limbal region in a 23 year old female. Histology and both cytology methods showing mild atypia. Figure IC represents a PAM at the limbal region in a 57 year old male. Histology and both cytology methods showed PAM with moderate atypia. Note the fine pigmentation around the nucleus in the Biopore and exfoliative samples. Figure ID represents a conjunctival melanoma at the bulbar conjunctiva in a 83 year old female. Histology and cytology showing severe atypia.

epithelial layer since the lesion is rubbed three times on the same spot. Biopore, however, will sample only the first layer of cells on the conjunctiva, unless the Biopore is repeated several times to acquire cells of deeper layers. Similarly, impression cytology with cellulose acetate filters is able to sample deeper layers of the conjunctiva when performed repeatedly.[19] This could probably explain why 33 % of the Biopores was graded in a lower category than the corresponding exfoliative smear, since in most cases only one Biopore was sampled. Further studies need to prove whether the Biopore is able to sample deeper layers when performed repeatedly.

The most important task for cytology is to detect conjunctival melanomas. Exfoliative cytology was able to detect 89 % of the melanomas and Biopore was able to detect 78 %. One of the missed conjunctival melanomas with the Biopore technique was situated in the caruncle, which is a difficult location to sample with the relatively large and flat Biopore. The second conjunctival melanoma was situated under the conjunctival epithelium (local in-transit-metastasis), and could therefore not be reached by both Biopore and exfoliative method. However, when all histological samples were taken into account, Biopore correctly predicted the outcome in 88 % of the lesions, and exfoliative cytology in 83 %.

Other authors also found similar correlations between cytology and histology.[15,16,20] Besides the difficulty of the Biopore to sample the caruncle and fornices, we experienced that the relatively large Biopore is also difficult for sampling in young children.

Advantage of the Biopore is the high yield of cells that are collected on a relatively small surface. The high density of cells makes interpretation also faster when compared to exfoliative smears. With Biopore, the pathologist only has to screen approximately 50 mm2 as compared to exfoliative smears where a total surface of 900 mm2 has to be screened microscopically. The Biopore also had less disagreements in atypia classification between the two observers than the exfoliative samples. Since most of the high risk samples (grade 3 and 4) remained in this category, the disagreements between the two observers was not of major influence for the clinician.

Recently, Singh et al. recommended the introduction of impression cytology for routine clinical practice in major ophthalmic centres.[19] We agree that impression cytology (Biopore or cellulose acetate filters) and/or exfoliative cytology should be available to ophthalmologists in major centres, since these minimal invasive techniques can help the ophthalmologist in the diagnosis of a variety of ocular surface diseases.

Conclusion

Biopore can be used in cytology of melanocytic lesions and is easier and faster to interpret than exfoliative cytology. When a cytological test is indicated, the Biopore can be used complementary to exfoliative smears on bulbar lesions, while exfoliative cytology alone is preferable on lesions situated in the caruncle and fornix, and in young children.

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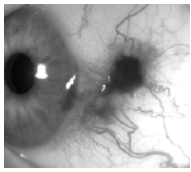

CHAPTER 6

IMMUNOPHENOTYPIC MARKERS TO DIFFERENTIATE BETWEEN BENIGN AND MALIGNANT MELANOCYTIC LESIONS

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Abstract

Background/aims. We investigated the expression of S100A1, S100A6, S100B, MelanA, and CEA in conjunctival nevi, primary acquired melanosis (PAM), conjunctival melanoma, and uveal melanoma, in order to assess their potential usefulness in the pathologic differential diagnosis of these entities.

Methods. Paraffin-embedded sections of 18 conjunctival nevi, 14 PAM, 16 conjunctival melanomas, and 20 uveal melanomas were immunostained for S100A1, S100A6, S100B, MelanA, and CEA, and expression was scored semi quantitatively.

Results. Expression of S100A1 differed significantly between conjunctival nevi and conjunctival melanoma, with percentages of positive cells of 30.6 % and 71.4 %, respectively. Conjunctival melanomas had high average scores for S100A1 and S100B (71.4 %, 62.9 %, respectively), while uveal melanomas also had high S100A1 but low S100B scores (88.5 %, 18.5 %, respectively). MelanA was highly variable; nevi and uveal melanoma had higher average scores than conjunctival melanoma. CEA was hardly detectable in all four groups.

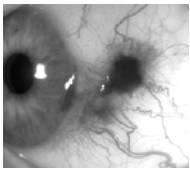
Conclusion. S100A1 seems to be a possible candidate to differentiate conjunctival nevi from conjunctival melanoma. S100B seems to differentiate between uveal melanoma and conjunctival melanoma. However the study size was small and therefore the data has to be confirmed by others.

Introduction

The most common melanocytic lesions of the conjunctiva include conjunctival nevus, primary acquired melanosis (PAM), and conjunctival melanoma. Clinically, their distinction may be difficult,[1] and in case of doubt, histological investigation is required. PAM is subdivided histologically into a typical and an atypical form, and especially the latter, as well as conjunctival melanoma, has a high tendency for local recurrence after treatment.[2,3] The mortality rate of patients with a conjunctival melanoma is about 30 % in 10 years.[2,3,4,5,6,7,8,9,10] Immunophenotypic markers might be of help to distinguish between benign and malignant melanocytic lesions of the conjunctiva. Previous studies addressed S100, MelanA, and HMB45 expression in conjunctival melanoma,[11,12,13,14,15,16,17] and a few studies compared expression patterns on conjunctival nevus or PAM with conjunctival melanoma.[14,15,16,17] Although most markers have proven to be of value in the establishment of the melanocytic origin of various lesions, they are of much less help in the distinction between a melanocytic nevus and a melanoma.[14,15,16]

Calcium-binding proteins like S100 have been implicated in establishing the malignant and metastatic phenotype of various tumours.[18,19,20] The S100 protein family consists of over 20 members. The expression of S100A1, S100A2, S100A3, S100A4, S100A6, and S100B has been studied previously in cutaneous melanoma.[21,22,23,24,25] S100A6 seems of some use in the distinction between a Spitz-nevus and a cutaneous melanoma.[25] The S100B level in serum is of considerable interest as a prognostic marker in cutaneous melanoma, and has been used to monitor patients with metastatic cutaneous melanoma.[23]

In this study we stained histological samples of conjunctival nevus, PAM, conjunctival melanoma, and uveal melanoma for S100A1, S100A6, S100B, MelanA, and CEA.



Materials and methods

Patients and Tissues

Eighteen conjunctival nevi, 14 PAM (one with no atypia, four with mild atypia, six with moderate atypia, and three with severe atypia), 16 conjunctival melanomas, and 20 uveal melanomas (six epithelioid, eight spindle, and six mixed cell type) from different patients were collected from the pathology archives of the Leiden University Medical Centre, Leiden, The Netherlands. Patient records were used to obtain information on local or distant recurrence, and to investigate whether patients with PAM or nevus subsequently developed conjunctival melanoma.

Immunohistochemistry

Specimens were fixed in 4 % neutral buffered formaldehyde and embedded in paraffin. Immunohistochemical reactions were performed using the streptavidin-biotin method. Sections were cut at 4 μm and mounted on glass slides, and deparaffinized in xylene (four times, five minutes each) and ethanol 99 % (two times, five minutes each). The endogenous peroxidase activity was blocked by incubating the slides with methanol/H_2O_2 0.3 % for 20 minutes. After the slides were washed, antigen retrieval was performed by boiling in citrate buffer (Dako, Glostrup, Denmark) for 10 minutes. Slides were washed again in phosphate-buffered

saline (PBS), and they were incubated overnight with the first antibody at 4 °C. Rabbit anti-S100A1 polyclonal, (dilution1:100) (A5109, Dako), mouse anti-S100A6 mAb clone CACY-100 (dilution 1:4000) (S-5049, Sigma-Aldrich, Steinheim, Germany), mouse anti-S100B mAb clone DAK-S100B/2 (dilution 1:100) (M7221, Dako), mouse anti-MelanA mAb clone A103 (dilution 1:100) (M7196, Dako), and mouse anti-CEA mAb clone 11-7 (dilution 1:50) (M7072, Dako) were used. As negative control, the primary antibody was replaced by PBS. Slides were then incubated with biotinylated anti-mouse anti-rabbit Ig (Dako) for 30 minutes. After washing, the slides were labeled with Streptavidin-HRP (Dako) for 30 minutes; hereafter the labeling was made visible with a 30-minute incubation in AEC (3-amino-9-ethylcarbazole) or DAB (3,3 diaminobenzidine). Slides were counterstained with Mayer's haematoxylin and finally embedded in Kaiser's glycerine. For the CEA-antibody, sections of colon carcinoma were used as a positive control; for S100A1, S100A6, S100B, and MelanA, sections of cutaneous melanoma were used.

Scoring

The immunolabeled slides were interpreted by determining the percentage positively staining cells, scored on a scale of 0 to 100 %, in steps of 10 %. Slides were independently scored by at least two people; in all cases agreement was reached.

Statistics

For statistical analysis, the mean ± SEM (standard error of the mean) was used. Data were analysed in SPSS 11.0 (SPSS Inc, Chicago, USA). A one-way ANOVA test was used to calculate significance between the groups. A two-tailed nonparametric Mann-Whitney U test was used to compare two variables.

Results

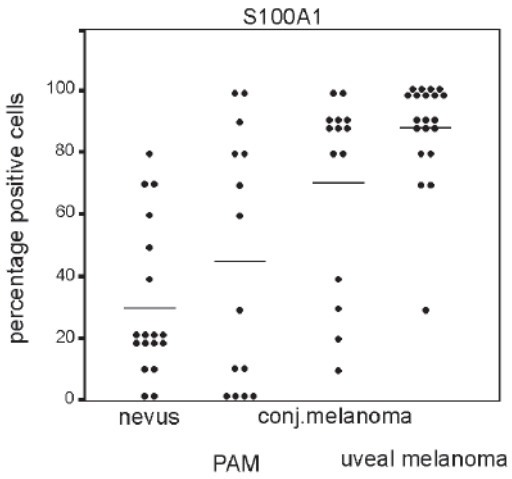
Of a total of about 400 slides, six were lost due to technical problems or lack of residual tissue in the blocks. As a consequence, only 13 of 14 PAM specimens could be stained with S100A6 and MelanA, 14 of 16 conjunctival melanoma were stained with S100A1 and S100B. Figure 2 shows the staining results for S100A1, S100A6, S100B, and CEA staining in representative sections of conjunctival nevi, PAM, conjunctival melanoma, and uveal melanoma.

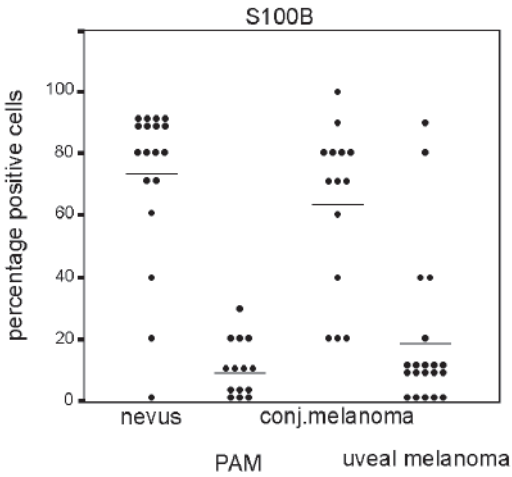
S100A1

S100A1 positively stained more than 10 % of the lesional cells of 16 of the 18 conjunctival nevi (89 %), of 10 of 14 PAM (71 %), of all conjunctival melanomas (n=14, 100%), and all uveal melanoma (n=20, 100%) (Figure 2). Significantly more cells were positive in conjunctival melanoma compared to conjunctival nevi (71.4 %, and 30.6 %, respectively) (p=0.001 Mann-Whitney) (Figure 1), while the PAM reacted intermediately (45.0 % positive cells).

S100A6

In all *uveal* melanomas more than 10 % of the cells stained positively; the average percentage of positive cells was very high (72.1 %). All *conjunctival* tissues showed severe non-specific staining, and could therefore not be scored: most epithelial cells stained positive,





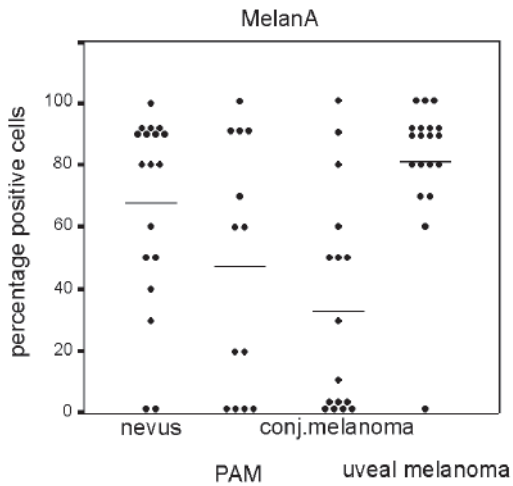
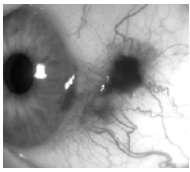


Figure 1. Percentage positive cells after staining for antibodies against S100A1, S100B, and MelanA in conjunctival nevus, PAM, conjunctival melanoma, and uveal melanoma specimens. Each dot represents a single case, the line represents the average score for each group of melanocytic lesions. (S100A1: nevus versus conj. melanoma p=0.001, Mann Whitney U test) (S100B: PAM versus nevus p<0.0001, PAM versus conj. melanoma p<0.0001, conj. melanoma versus uveal melanoma p<0.0001, Mann Whitney U test)



which has been described previously.[21,22]

S100B

More than 10 % of the cells scored positively with S100B of 17 of the 18 conjunctival nevi (94 %), of 8 of 14 PAM (57 %), of all conjunctival melanoma (n=14, 100%), and of 15 of 20 uveal melanoma (75 %). Comparing the average scores for S100B, nevus and conjunctival melanoma scored significantly higher than PAM ($P<0.001$) (Figure 1). The average percentage positive cells in conjunctival melanoma was significantly higher than in the uveal melanoma (62.9 %, 18.5 %, respectively) ($P<0.0001$, Mann-Whitney)

MelanA

MelanA stained more than 10% of the cells in 16 of the 18 nevi (89 %), 9 of 13 PAM (69 %), 9 of 16 conjunctival melanoma (56 %), and 19 of 20 uveal melanoma (95 %). Most nevi and uveal melanoma had a good expression of MelanA, while in PAM and conjunctival melanoma expression was very variable (Figure 1).

CEA

CEA was completely negative in most lesions, but in one nevus, two conjunctival melanomas, and 15 uveal melanomas, groups of non-melanocytic cells were positive (Figure 2). The total number of CEA-positive cells was very low in all categories.

The atypia stage of the PAM lesions did not correlate with the expression levels of the tested antibodies. No significant difference in expression was seen between uveal or conjunctival melanomas in patients with or without metastasis, although for S100A1 and S100A6 there was a trend towards higher expression in uveal melanoma patients with metastatic disease.

Discussion

In this study, we set out to determine whether expression patterns of various markers may differentiate between benign and malignant conjunctival pigmented lesions, as previous studies did not yield a positive result.[14,15,16,17] In our study, S100A1 staining differed between a nevus and a conjunctival melanoma. The nevi had low S100A1 expression (mean 30.6 %), while in most conjunctival melanomas expression was strong (mean 71.4 %) (Figure 1 and 2). It must be noted that because of the rarity of histological sections of conjunctival nevi and conjunctival melanomas, the sample size is small. Since no other studies have been published about S100A1 staining of melanocytic conjunctival lesions, confirmation by others will be awaited. S100B staining did not differ between conjunctival nevi and conjunctival melanoma, however, a lower staining was seen in the PAM lesions (Figure 1). Others also found no difference in S100 expression between conjunctival nevi and conjunctival melanoma.[16,17,26] Steuhl et al. did also find slightly lower expression of S100 in epithelial melanosis than in conjunctival nevi or conjunctival melanoma.[16] However Hitzer et al., and Sharara et al. did not find any difference between PAM and conjunctival nevi, and conjunctival melanoma.[17,26] The former studies used the "general" S100 antibody which probably represents the S100B used in our study.

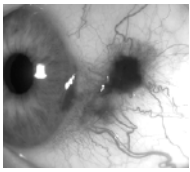
To our surprise MelanA was not present in 7 of the 16 conjunctival melanomas, while the expression in PAM and conjunctival nevi was higher. Others found higher expression of MelanA in conjunctival melanoma,[12,13] although Heegaard et al. found weak staining of the

Table 1. Expression of S100A1, S100A6, and S100B of conjunctival melanoma, uveal melanoma, and cutaneous melanoma

	S100A1	S100A6	S100B
Conjunctival melanoma †	+++	*	++
Uveal melanoma †	+++	+++	+
Cutaneous melanoma ‡	0	++	++

* S100A6 stained very non-specific on the conjunctival melanomas and could therefore not be scored.
† Data from this study.
‡ Data from literature.[21,22,25]

MelanA in most of the conjunctival melanomas.[12] Our findings indicate that MelanA is not a good marker for conjunctival melanoma.
Whether conjunctival melanoma is biologically more closely related to the skin melanoma or the uveal melanoma is still a topic of discussion. We included uveal melanoma samples as a control and to compare expression of the conjunctival lesions with this tumour. The S100B was as abundantly expressed in conjunctival melanomas as the S100A1, while in uveal melanomas the expression of S100A1 was also high, but S100B was low. Others also found higher expression of S100B in conjunctival melanoma than in uveal melanoma.[12,13] Iwamoto claims that conjunctival melanoma is most similar to the epithelioid phenotype of the cutaneous melanoma.[13] Table 1 compares the S100A1, S100A6, and S100B data from this study with data on cutaneous melanoma in the literature. All lesions have similarities for one or two antibodies, but one cannot conclude that conjunctival melanoma resembles cutaneous melanoma more closely than uveal melanoma. Perhaps it is better to see them as three separate identities, although clinically the conjunctival melanoma has more similarities with cutaneous melanoma, since both have the tendency to metastasise to the regional lymph node first. The tested antibodies may not only be used for differentiation between lesions but may also be potential serum metastases markers. Especially molecules with a high expression in the malignant lesion may be good candidates. Serum levels of S100B have proved to be reliable serum markers to follow metastasis in cutaneous melanoma.[23] For both uveal and conjunctival melanoma good serum markers may also be of help in detecting and following primary and metastastic disease. For uveal melanoma, S100A1 and S100A6 could be candidate serum markers, since they are abundantly expressed on uveal melanoma, for conjunctival melanoma S100A1 and S100B could be good candidates to test in the serum. Van Ginkel already implicated that S100A6 could be of influence on malignant transformation of the uveal melanoma. [27] Serum S100B was not able to detect metastatic disease in patients with uveal melanoma,[28] probably since S100B is not well expressed in uveal melanoma, but serum S100A1b was able to detect metastatic disease in uveal melanoma patients (G.S. Missotten et al., submitted). As far as we know, no serum markers have yet been analysed in conjunctival melanoma. In 1976 Michelson et al. reported a slightly elevated blood CEA levels in 45 % of the uveal melanoma patients, although the increase of CEA levels was only marginal.[29] We also investigated the CEA expression of conjunctival



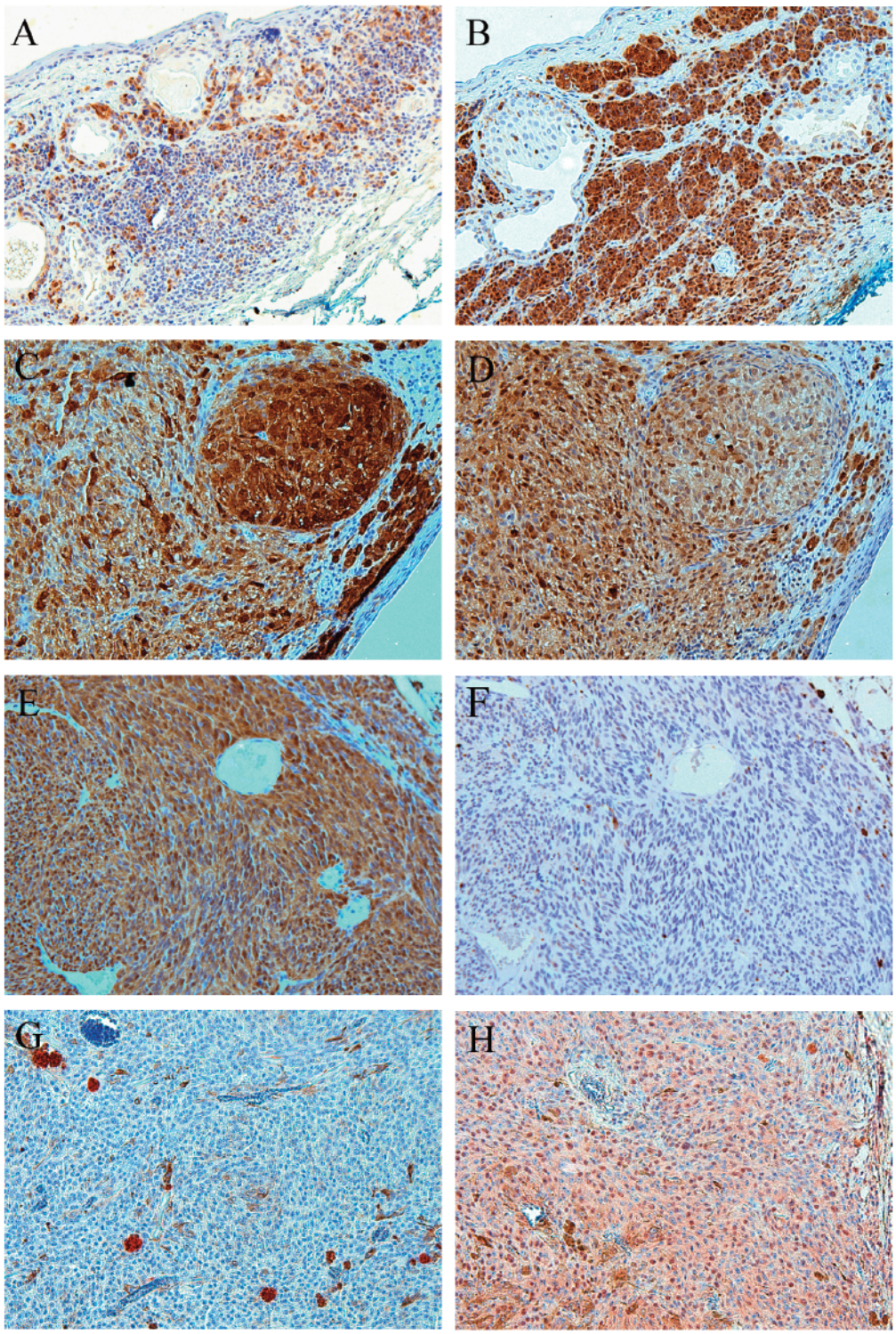
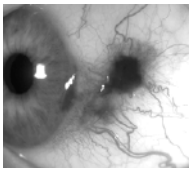


Figure 2.
Figure 2A and 2B represent the S100A1 and S100B expression on conjunctival nevi, respectively. Figure 2C and 2D show the expression of S100A1 and S100B on conjunctival melanoma, respectively. Figure 2E, 2F, 2G, and 2H represent the S100A1, S100B, CEA, and S100A6 expression on uveal melanoma, respectively. Mark the difference in S100A1 expression between conjunctival nevi and conjunctival melanoma, also the S100B expression between uveal and conjunctival melanoma is remarkably different. All figures are with a magnification of 200X.

and uveal lesions, but could not find any tumour labelling, although in some tumours we did find staining of a few non-melanocytic cells (Figure 2), which were microscopically identified as melanophages. As CEA is not expressed, using it as a serum marker seems inefficient.

In conclusion, we think that of those we studied S100A1 is the best marker to differentiate between a nevus and conjunctival melanoma, although it does not provide an absolute cut-off value. Because of the small sample size, further studies will be needed to confirm these findings.



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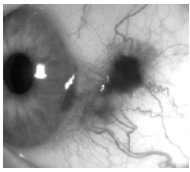

CHAPTER 7

A NEW CELL LINE FROM A RECURRENT CONJUNCTIVAL MELANOMA.

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The incidence of conjunctival melanoma is about 0.2-0.8/1.000.000 cases each year.[1;2] As a consequence our knowledge about this tumor is limited but cell lines derived from these tumours will give more information about them. So far only three conjunctival melanoma cell lines have been described.[3;4] The first cell line, IPC 292, was described in1993 by Aubert et al.[4] Recently Nareyeck et al. developed two additional conjunctival melanoma cell lines (CRMM1 and CRMM2).[3] We wish to report a further, fourth, conjunctival melanoma cell line CM2005.1.

The conjunctival melanoma cell line CM2005.1 was established from tumor material derived from a 84 year old male. The primary tumor was located at the medial side of the inner upper eyelid adjacent to the cornea. The tumour was treated initially by local excision together with adjuvant Iridium 192 brachytherapy. After three years the tumour reappeared on the inner side of the lower eyelid. The tumour extended into the nasal cavity and disseminated further. The patient died one year later from the melanoma which had originated in the conjunctiva. Both the primary tumor and the local recurrence were histologically proven conjunctival melanomas.

From the local recurrence two small tumor specimens were available for cell culture. The tumor material was cut into small pieces with scalpels and transferred to several culture plates. Culture plates contained 10 mL/dish RPMI 1640 (Invitrogen-Gibco, Groningen, The Netherlands) supplemented with 10% FCS (Hyclone, Logan, UT), 100 IU/mL penicillin (Invitrogen-Gibco), and 100 µg/mL streptomycin (Invitrogen-Gibco). Cultures were incubated at 37°C in a humidified atmosphere and a CO_2 content of 5% in air and the culture medium was refreshed every 72-96 hours. After four months a stable cell line had been developed, which we were grown for over 22 passages. Cell doubling time was measured three times by culturing 100.000 cell/plate. The cell count was measured again at days 1, 2, 3, 4, and 5, which resulted in a cell doubling time around 35 hours (Figure 1).

To establish the melanocytic origin of the cultured cells, cytospins were undertaken and immuno-histochemically stained for S100, MelanA, NKI-C3, and HMB 45.

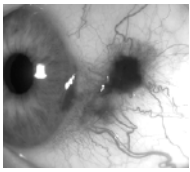
Immunohistochemical reactions were performed using the streptavidin-biotin method. Of the cultured cells 95-100 % were positive for all four primary antibodies thereby proving their melanocytic origin. MelanA, HMB 45, NKI-C3 stained the cultured cells intensively, the S100 labeling was slightly less intense.

To asses the karyotype of cell line CM2005.1, cytogenetic analysis was performed. Chromosome preparations were made according to standard procedures and stained to obtain R or Q banding. Cytogenetic abnormalities were described in accordance with the ISCN.[5]

The following karyogram was found in the majority of cells: 83~96, XX, +X, -Y, -Y, -1, -1, -3, -3, -4, -5, -5, -6, +7, +7, +del (7) (q22), -8, -9, -9, -10, -10, del (11) (q23)x2, -12, +13, +13, +14, -16, -17, -17, -18, -19, +20, -21, -21, -21, -22, +12~16mar (see Figure 2).

Cell line CM2005.1 was derived from a recurrent conjunctival melanoma that showed recurrence after excision and brachytherapy, which could explain the distorted karyogram and the relative high cell doubling time of this cell line. However, the cytogenetic analysis was performed four months after start of the culture, which could have influenced the karyogram and may therefore not represent the original tumor. Nareyeck et al. found cell doubling times around 60h for their conjunctival melanoma cell lines CRMM-1 and CRMM-2.[3]

This fourth conjunctival melanoma cell line is now available for research, and can hopefully attribute to the expansion of our knowledge of conjunctival melanomas.



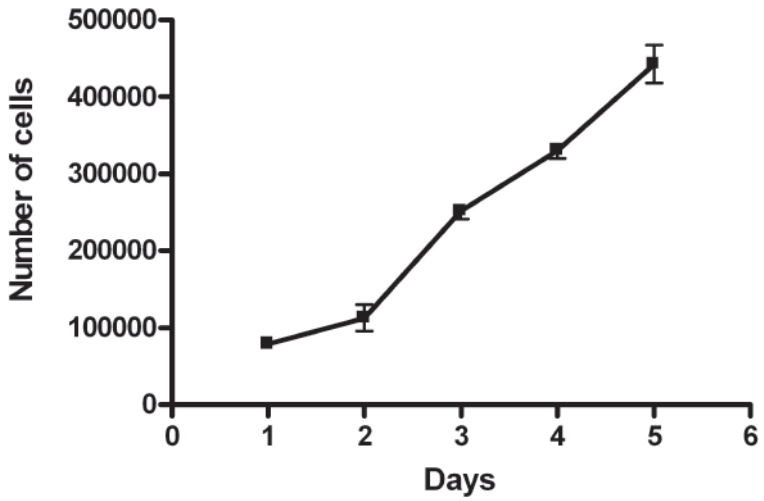


Figure 1. Growth curve of conjunctival melanoma cell line CM2005.1. Showing the number of cells at various time point during culture. Cell doubling time was around 35 hours.

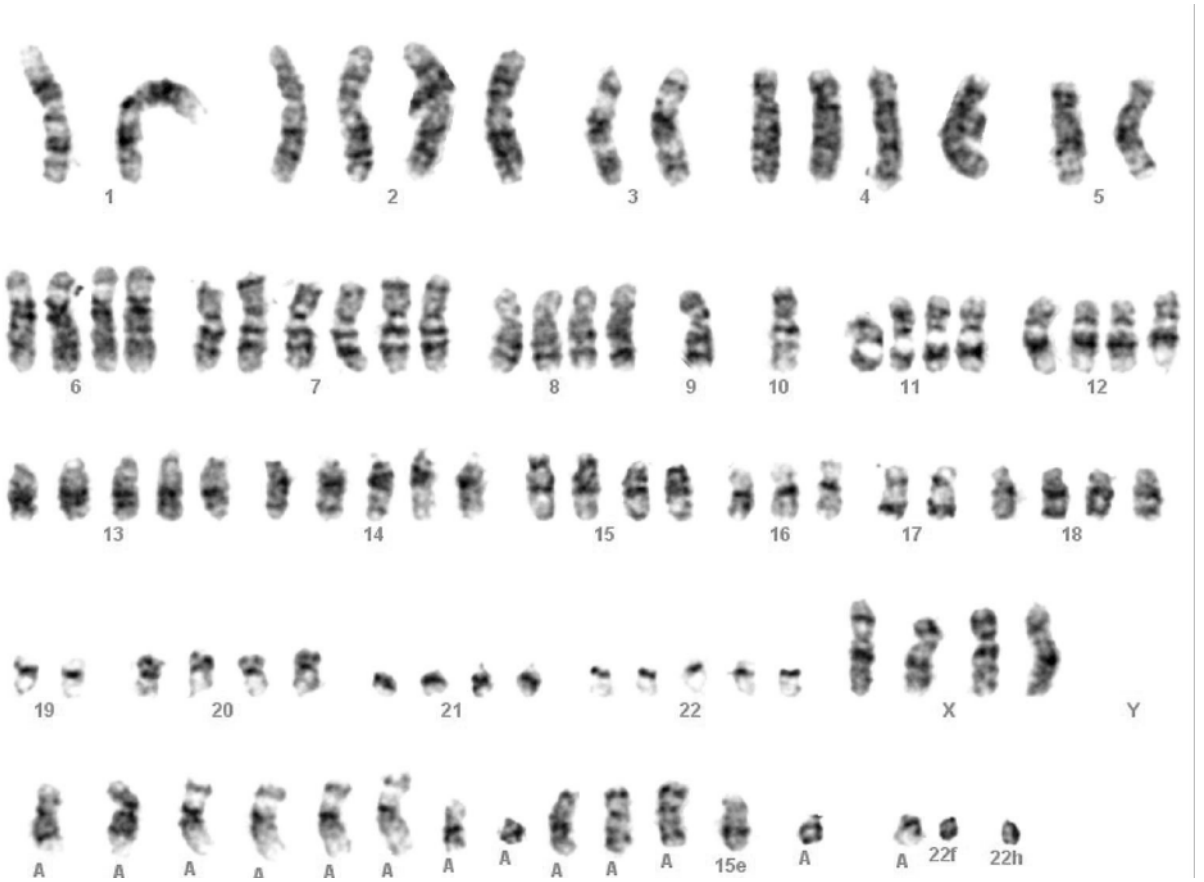


Figure 2. Karyogram of cell line CM2005.1. A very complex karyogram with gains, deletions and rearrangements of almost all chromosomes was observed in the majority of cultured cells.

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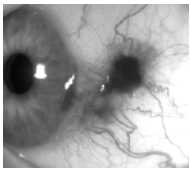


PART III

CORNEAL INFECTIONS

INTRODUCTION

Introduction

The cornea is a highly specialized part of the eye, and its transparency is a requirement for good visual acuity. Corneal infections can damage the integrity of the ocular surface thereby disturbing the transparency of the cornea, leaving scars and decreasing visual acuity. Antimicrobial mechanisms of the eye try to prevent ocular surface infections, whereas anti-inflammatory mechanisms try to limit damage by the immune system. The occurrence of bacterial corneal infections is increasing with the increasing use of contact lenses. Especially bacterial corneal ulcers are a challenge to the ophthalmologist and immediate and strong antibacterial treatment is needed to prevent further visual loss and ocular complications. Another frequent ocular surface infection is the herpetic keratitis, which forms a serious ophthalmologic problem. Because of its latency and recurrences, the herpes simplex virus (HSV) can cause severe damage to the cornea. For a long time, HSV infection was one of the leading causes for corneal transplantation, which decreased after the introduction of strong antiviral medications.[1]

Corneal ulcerations

Infectious corneal ulcers have the potential to perforation the cornea and are therefore a serious threat for the eye.[2,3] In the Western World the incidence of infectious corneal ulcers is about 11 cases per 100.000 inhabitants;[4] however, in other parts of the world like for instance Southern India the incidence can be up to 113 cases per 100.000 inhabitants.[5] In Western Europe, the major causes of infectious corneal ulcers are contact-lens wear, trauma, and ocular surface diseases such as chronic blepharitis, dry eye syndrome, and eyelid pathology. Ocular trauma is the cause of corneal ulceration in 20% of the cases, in 30% the corneal ulcer is contact-lens related.[6] Extended-wear contact lenses are associated with a higher chance to de-

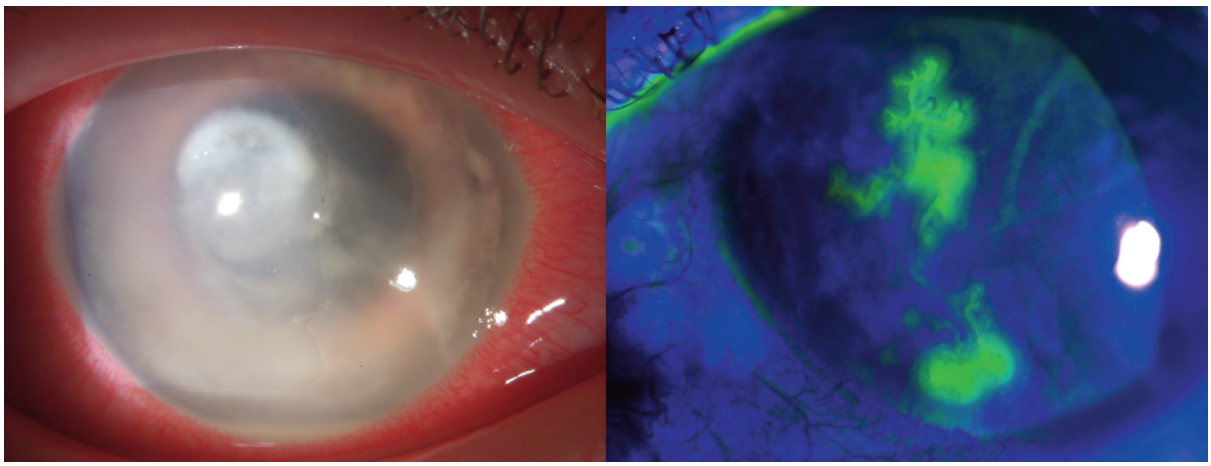
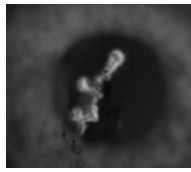


Figure 1. On the left a bacterial corneal ulcer with severe anterior chamber reaction. On the right a herpes simplex keratitis staining with fluorescein with characteristic dendritic shape.



velop corneal ulcers than daily-wear contact lenses (20.9% versus 4.1% corneal ulcers per 10.000 contact lens wearers).[7] Recently, a decrease in the incidence of contact-lens related corneal ulcers was seen, despite an increase of the contact-lens wearing population and is probably due to the shift from extended-wear contact lenses to daily-wear contact lenses.[8] Few reports exist about the incidence of corneal ulcers in patients with daily disposable contact lenses, but it seems that the chance is very low.[8] Other risk factors for corneal ulcers in individuals with contact lenses are overnight wear and improper lens care.[8,7,9]

Culturing the pathogens that cause the corneal ulcer is important for diagnosis and subsequent treatment. However, in 30% to 50% of the cases the bacterial cultures taken in academic centres are negative,[10,11] probably because antibiotic therapy had already been started by the general practitioner. Most commonly found bacteria are *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Pseudomonas aeruginosa*.[6,10] Acanthamoeba can also be found; this is an opportunistic parasite that has the ability to transform from trophozoites to cysts and vice versa. The cysts can survive under extreme conditions such as high temperature, high osmolarity, and nutrition-poor environments; the cysts are also resistant to many antimicrobial agents.

Treatment with antibiotics must be started as soon as possible, since the internal eye is an ideal environment for bacterial growth and since *Pseudomonas aeruginosa* and *Staphylococcus aureus* are able to perforate the cornea within 24 hours.[12] In the absence of the outcome of positive cultures initial therapy must be with broad spectrum antibiotics. A combination of polymyxin B, neomycin, and gramicidin,[10] or cefazolin and gentamicin[13] is effective. In cases of positive cultures the antibiotics must be aimed at the pathogen found. In case of acanthamoeba keratitis, treatment consists of chlorhexidine, polyhexamethylene biguanide, neomycine, and propamidine isethionate (Brolene).[14]

Risk factors that are associated with penetrating keratoplasty, evisceration or enucleation are: older age, delay in referral to ophthalmologist, larger size of ulcer, central location of the ulcer, topical steroid treatment, prior ocular surgery, and poor vision at presentation.[15,16]

Herpes simplex keratitis

Herpes simplex virus (HSV) type 1 mostly causes facial infection, while HSV type 2 is primarily situated in the genital area. Although the site of the infection is different, the clinical signs and symptoms overlap. The HSV viruses are known for their latency and frequent recurrences. In primary infections, the HSV virus infects epithelial cells and spreads further through the tissue; some HSV particles enter the axons of sensory neurons. Via retrograde transport, HSV migrates to the neuronal cell body, where it can remain latent for a long time. The ability of the HSV virus to evade the immune system is the cause of its latency. By infecting the nervous system (ganglia), the HSV has gained access to a tissue that is relatively inaccessible for the immune system because of the low expression of Major Histocompatibility Complex (MHC) on neuronal cell surfaces. During the latency period in the ganglia, the viral metabolism is shut down and viral protein expression on the cell surface is down-regulated. The HSV-1 virus usually acquires latency in the trigeminal ganglion and can be found there in almost 100% of individuals above the age of 60.[17] Serum antibodies can be found in 45% to 88% of a population; a positive serology is influenced by sex, age

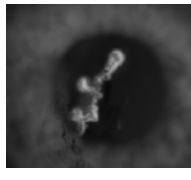
and social economic status.[18] The best known recurrent HSV-1 infection is herpes labialis; about 17-30 % of the western population suffer from this dissease.[19,20,21] Ocular HSV-1 infection has a lower prevalence of about 0.15%,[22,23] but can have a greater social-economic impact, since recurrent infections can cause blindness.[22] Reactivation of ocular HSV from its latent state in the trigeminal ganglion can be caused by a variety of stimuli such as local trauma or tissue damage, stress, ultraviolet light exposure, illness, or hyperthermia.[24,25,26,27] Transmission of HSV virus is usually caused by close contact with mucosal tissue; even corneal transplants can be a source of ocular HSV infection transmission,[28,29,30,31] and it has been suggested that the cornea is an extraneural site for HSV latency.[32,33] Primary ocular infections result in conjunctivitis, blepharitis with vesicles, and epithelial keratitis. Herpetic epithelial keratitis can recur and occurs as a dendritic or geographical shape. A more severe recurrent disease is herpetic stromal keratitis, which is mainly an immunological reaction. Besides infecting the ocular surface HSV is capable of causing trabeculitis, uveitis, and acute retinal necrosis.

The current treatment for herpetic epithelial keratitis is local treatment with aciclovir, a purine-nucleoside analogue that is able to disrupt viral DNA replication. In patients with herpetic stromal keratitis topical prednisolon is used to downregulate the immune system. For severe recurrent disease prophylactic treatment with oral valaciclovir (Zelitrex) is used to reduce the frequency of recurrences. Since the availability of aciclovir, the number of corneal transplants for HSV infections has decreased.[1]

Protection of the eye

The eye is protected against infections by a combination of anatomical, mechanical, antimicrobial, and immunological factors. The intact conjunctival and corneal epithelium forms a barrier against pathogens; once the integrity is broken by trauma or contact-lens wear, pathogens have a chance to infect the deeper layers. Mechanical factors include eyelid blinking and the continuous flow of tears, thereby diluting and removing pathogens such as bacteria, viruses, and parasites from the anterior eye surface. Tears transport metabolic products like oxygen and carbon dioxide, and allow passage of leucocytes after injury. Besides the mechanical function, tears have antimicrobial capabilities. Lysozyme and lactoferrin are tear proteins that are part of the innate immune system and form the first line of defence. Both proteins are known to inhibit growth of bacteria. The adaptive immune system is formed by plasma cells secreting immunoglobulin A or G in the lacrimal gland and the T-cell system. The IgA and IgG secreting plasma cells are probably former B-cells that underwent antigen sensitisation in the gut-associated lymphoid tissue and bronchus-associated lymphoid tissue.[34,35] The conjunctiva but not the cornea is provided with blood vessels and lymphatic vessels, through which T cells can be transported. Blood vessels will invade the cornea in cases of more serious corneal infections, bringing the immune system closer to the site of infection. However, the immune response can seriously damage the cornea, and can even be more destructive than the infection itself.

Under normal circumstances the conjunctiva is densely populated with bacteria; it has been suggested that the normal conjunctival flora has an inhibitory effect on more pathogenic bacteria.[36,37] However, some of these bacteria can be cultured from corneal ulcers or from



infections after surgery.[38,10]

Ocular immune privilege

To protect the micro-anatomical structures of the eye against a potential devastating immune response, the eye is an immune-privileged site. The cornea lacks blood and lymph vessels, which are usually used by the immune system to gain access to an infectious site. Intraocular immune privilege exists in the anterior chamber and is known as the Anterior Chamber Associated Immune Deviation (ACAID), that is able to suppress delayed type hypersensitivity and complement-fixing antibody reactions against anterior chamber antigens. Soluble factors in the anterior chamber (TGF-beta2, alpha-MSH, MIF, IL-10) can suppress T cells or NK cell activities.[39] Furthermore, eye tissues express Fas-ligand (CD95L) that is able to induce apoptosis in activated T-cells.[40,41]

Innate immune system

The innate immune system consists of soluble factors and leucocytes excluding the T and B cells, and forms the non-specific first line of defence. Invasion of tissue by either bacteria or viruses will activate the complement system, macrophages, neutrophils, and NK cells, which are all components of the innate immune system since they use "broad-spectrum" mechanisms and not antigen-specific mechanisms as the T and B cells to cope with infection. Macrophages play a role in the phagocytosis of bacteria or virus particles, thereby processing the pathogen and activating the immune system. In bacterial infections most bacteria are cleared by phagocytes like macrophages and neutrophils. Natural killer (NK) cells are primarily involved in intracellular infections like HSV,[42] as can be illustrated by the occurrence of more severe HSV infections in patients with genetic defects in NK cell function.[43] NK cells recognise infected cells through opsonization of target cells with virus-specific antibodies in an MHC-independent way. Furthermore, HSV induces MHC class I down-regulation in the infected cell thereby making the cells more prone to NK-cell mediated killing.[44] The soluble factors of the innate immune system include lactoferrin, lysozyme, the complement system, interferons (IFNs), and other cytokines or chemokines. Lactoferrin has both antiviral and antibacterial properties and is discussed later in this chapter. Lysozyme is effective against bacteria by perforating the bacterial surface. The complement system can cause direct killing of bacteria, opsonization of bacteria or infected cells, and can act as a chemotactic factor. IFNs (alpha and beta) are cytokines that are able to increase the resistance of cells to viral infections and are produced by infected cells.

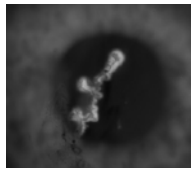
Adaptive immune system

The adaptive immune system uses antigen-specific recognition to clear bacterial or viral infections, and can be divided in a humoral response with antibodies (B cells) or a cellular response by T cells. In viral infections, antibodies against the virus can bind to the infected cell and cause cell death through either formation of a membrane-attack complex with complement or through NK-cell killing by recognition of the antibody by the NK cell. The latter is also called antibody-dependent cell-mediated cytotoxicity (ADCC), which is a critical mech-

anism in the antiviral defence against the HSV virus.[45] In bacterial infections, antibodies can interfere with the motility of bacteria and can block bacterial toxins. The most important effect of antibodies against bacteria is its ability to increase the effectiveness of complement targeting, and to act as an opsonin to make phagocytosis more effective.
The T-cell system consist of CD4- and CD8-positive T cells, which recognise viral antigen in association with major histocompatibility complex (MHC) molecules II and I, respectively. In infected cells, viral particles are degraded and processed on MHC class I on the cells surface for recognition by CD8 positive T cells. MHC class II cells are usually antigen-presenting cells such as macrophages and dendritic cells which present phagocytosed particles in an MHC class II-restricted manner for communication with CD4-positive T cells. CD4-positive T cells act as helper cells that help to induce CD8-positive T cell clones and recruit macrophages. The CD8-positive T cells are cytotoxic T cells, that can cause MHC class I-restricted apoptosis of infected cells. When a CD8-positive T cell recognizes an antigen on the surface of a cell in combination with MHC class I than the target cell will be programmed for apoptosis. For viral infections, the adaptive immune system is crucial for clearance of the virus. For HSV infections both the CD4-positive and the CD8-positive cells are crucial in the clearance of the virus,[46,47] which is further supported by the occurrence of more severe herpes infections in patients with AIDS.[48]

Lactoferrin

Lactoferrin is an important protein in the non-specific defence,[49,50] and can be found in tear fluid, saliva, milk, airway surface liquid, and in intestinal and vaginal secretions.[49,50,51] The main lacrimal gland produces the lactoferrin that can be found in tears. Granules of neutrophils also release lactoferrin during an inflammatory response.[52] Human lactoferrin contains 692 amino acids, and is folded into two symmetrical lobes (C and N terminal lobe).[53] It is active against many pathogens like gram-negative and positive bacteria, viruses, and fungi.[54,55] Besides the antimicrobial effects, lactoferrin also has immuno-modulatory and anti-inflammatory characteristics.[56] The bacteriostatic effect of lactoferrin is partly explained by its iron-binding capacity,[57,58,59] decreasing the local iron concentration, which is essential for bacterial growth. Lactoferrin also has non-iron-dependent antibacterial, antifungal, and antiviral capabilities.[60,49,50,61] It has been shown that the N-terminal cationic domains of lactoferrin have anti-bacterial activity through depolarization of bacterial membranes and by increasing bacterial membrane permeability.[62] Residues 1-47 of the N-terminus are responsible for the antibacterial effect.[63,64]
The antiviral effect of lactoferrin is primarily due to the interference of the lactoferrin protein with the binding of the virus to the cell surface.[65] The lactoferrin protein has a high affinity to heparan sulfate, which is a key glycosaminoglycan for the herpes virus to enter the cell.[66,67,68] The affinity of lactoferrin to bind to heparan sulfate is influenced by size and charge of the lactoferrin protein. Furthermore, in vitro, lactoferrin is able to inhibit infections with human herpes viruses, such as human cytomegalovirus and HSV-1.[69,70] Lactoferrin also suppresses HSV infection on the mouse cornea when applied prior to virus inoculation.[69] Lactoferrin has the largest effect in the initial stages of virus infection, and seems to prevent the herpes simplex virus to enter the cell. The N-terminal lobe of the lactoferirn protein

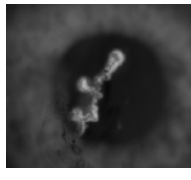


plays a pivotal role in the inhibition of viral infections.[71] High concentrations of lactoferrin, around 1 mg/ml, are needed to efficiently suppress viral infection, which is the case in human tears (about 2 mg/ml).[72]
Lactoferrin polymorphisms encoding amino acids at positions 29 and 561 of the N-terminal alpha-helical region have already been reported.[73,74] In Chapter 8 and 9 we report about a single nucleotide polymorphism encoding amino acid at position 11 of the lactoferrin gene.
The polymorphic forms of the human lactoferrin are defined as Ala11Thr, Lys29Arg, and Asp561Glu, in which Ala = alanine, Thr = threonine, Lys = lysine, Arg = arginine, Asp = aspartic acid, and Glu = glutamic acid. Moreover, it has been reported that lactoferrin Lys29Arg polymorphisms exerts different antibacterial and transcriptional activation activities.[74,75] Chapter 8 and 9 of this thesis investigates the potential role of the three lactoferrin single nucleotide polymorphisms in the development of corneal ulcers or HSV keratitis.

IL-10

IL-10 is a multi-functional cytokine with strong anti-inflammatory and immunosuppressive properties.[76] Cells that are capable of producing IL-10 include macrophages, Th2 cells, neutrophil, and resident corneal cells.[77,78,79]
Especially in HSV infections, the effect of IL-10 has been wel established. In animal models, IL-10 knock-out or IL-10 depletion by antibodies is associated with an increase in HSV corneal disease severity.[79] Similarly, topical IL-10 treatment significantly decreases corneal pathology induced by primary infection with HSV.[80,81,82] Besides the primary infections, IL-10 also seems to play a protective role in recurrent HSV infections in animal models.[83] IL-10 exerts its protective effect by downregulating pro-inflammatory cytokines and thereby decreasing the number of destructive lymphocytes during a stromal HSV infection, i.e. CD4-positive T cells and neutrophils.[83,84,80,81,79]
In infectious corneal ulcers IL-10 seems to have a protective effect for both gram positive and negative bacterial corneal ulcers.[85,86] It can especially prevent corneal perforation in animal models, indicating that the immune system itself also contributes to the corneal damage.
Many single nucleotide polymorphisms (SNP) exist in the promoter region of the IL-10 gene and some are associated with different IL-10 expression levels,[87,88,89] and these IL-10 promotor SNPs are involved in numerous types of infections.[90-98] Regarding HSV infections, people homozygous for the ATA IL-10 haplotype seem to be more resistant to HSV based on serum antibodies.[94] In Chapter 10, we have investigated four key SNPs of the IL-10 promotor gene region, -C819T, -G1082A, -A2763C, and -A2849G, in patients with infectious corneal ulcers.
Combinations of different single nucleotide polymorphisms can form haplotypes. Of the four above mentions SNPs five frequently occuring haplotypes can be inferred; haplotype IL10.1 CGAA, haplotype IL10.2 CACG, haplotype IL10.3 CGAG, haplotype IL10.4 TACG, and haplotype IL10.5 CGCG. These IL-10 haplotypes are associated with IL-10 production in vitro. Haplotypes IL10.1 and IL10.3 are associated with lower IL-10 production, where IL10.2 and IL10.5 are associated with higher IL-10 production. Haplotype IL10.4 is both associated with high and low IL-10 production.[99] Whether the IL-10 haplotypes also influ-

ence the continuous production of IL-10 in resident corneal cells[79] and thereby corneal disease is not known. These IL-10 haplotypes are also investigated in the infectious corneal ulcers patients group in Chapter 10.



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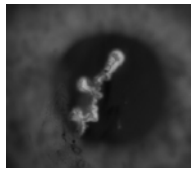

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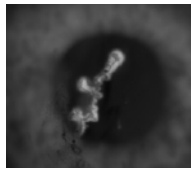

CHAPTER 8

LACTOFERRIN GLU561ASP POLYMORPHISM IS ASSOCIATED WITH SUSCEPTIBILITY TO HERPES SIMPLEX KERATITIS

Experimental Eye Research 2007, accepted.

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Abstract

Lactoferrin plays an important role in the defense against infections, including herpes simplex virus (HSV) keratitis. We studied the impact of three single nucleotide polymorphisms in the human lactoferrin gene on the susceptibility to HSV infections to the eye and the severity of such infections.

Lactoferrin gene polymorphisms were determined by PCR combined with restriction fragment length analysis in 105 HSV keratitis patients and 145 control subjects. Bilateral tear samples were harvested from 50 patients and 40 healthy controls and tear lactoferrin concentrations were determined by ELISA. Patients' records were used to acquire information about the severity of the HSV keratitis.

The frequencies of the Glu561Asp polymorphism, but not those of the Ala11Thr and Lys29Arg polymorphisms, differed significantly between patients and control subjects with an under-representation of the Asp561 allele in the patient group. Furthermore, the values for best corrected visual acuity, frequency of recurrences since onset, and average duration of clinical episodes did not differ among patients with the various lactoferrin genotypes. In addition, tear lactoferrin concentrations were the same in patients with HSV keratitis and healthy controls and also did not differ among patients with the various lactoferrin genotypes.

Lactoferrin Glu561Asp polymorphism is associated with the susceptibility to HSV keratitis with a protective role for lactoferrin variants comprising Asp561. However, no beneficial effects of this lactoferrin variant on the clinical outcome of ocular HSV keratitis were noted.

Introduction

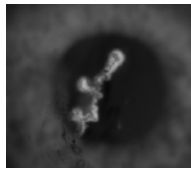
Serological studies indicated that up to 90% of the adults in the Western world have been exposed to herpes simplex virus-1 (HSV) [1]. The percentage of HSV-positive individuals rises with age and virtually all individuals over 60 years harbour HSV in their trigeminal ganglia [2]. However, only 17-33% of the population in the Western world suffer from HSV labialis [3,4,5], and only 0.15% from ocular HSV infection [6,7]. Moreover, it has recently been reported that 46 out of 50 adults (92%) actively shed HSV into the tear fluid, while only 2 out of these 50 subjects had a history of ocular HSV infections [8]. Apparently, the antiviral systems of the outer eye are highly effective in preventing the development of a clinical corneal HSV infection.

Lactoferrin, which is an important component of the non-specific defense against (HSV) infections and excessive inflammation [9,10], is present at high concentrations (about 2 mg/ml)[11] in mucosal secretions, such as tears and milk [9,10,12]. It is a multifunctional protein [9,13] with proven efficacy against HSV [14,15]. In tear fluid, lactoferrin aids in the control of HSV infections by preventing HSV particles to bind to and enter epithelial cells [15,16,17]. Interestingly, lactoferrin also suppresses HSV infection of the mouse cornea when applied prior to the inoculum [14]. Obviously, lactoferrin is an important element of the antiviral systems of the eye. Polymorphisms in the lactoferrin gene leading to amino acid substitutions at position 11 and 29 of the protein have been associated with aggressive periodontitis [18,19], transcriptional activation [19], antibacterial activity [20], and amino acid substitution at position 561 of lactoferrin is associated with corneal amyloidosis [21], and even a polymorphism located in the exon 15 not responsible for an amino acid substitution in the protein was associated with the susceptibility to diarrhea [22]. Based on these considerations we investigated whether lactoferrin gene polymorphisms causing an alanine/threonine amino acid substitution at position 11 (Ala11Thr), lysine/arganine substitution at position 29 (Lys29Arg), or glutamic acid/aspartic acid substitution at position 561 (Glu561Asp) of the lactoferrin protein are associated with the susceptibility to and/or severity of HSV keratitis.

Patients and methods

Patients

Patients with HSV keratitis seen between 2004 and 2006 in our hospital were invited to participate in the study. Inclusion criteria were: positive diagnosis of HSV keratitis by corneal specialist, age between 18 and 90 years, and able to give written informed consent. Exclusion criteria included: an active eye infection and severe dry eyes. Of the total of 115 patients that responded to our invitation to participate in the study 105 were included. The study population consisted of 68 males and 37 females, with an average age of 56 (±16) years. Six cases were later excluded because there was not a clear diagnosis of HSV keratitis and four because of severe dry eyes. Patients' records were used to obtain information about visual acuity at the moment of this study, number of recurrences, average duration of clinical episodes, diabetes mellitus, and contact lens wear. If the patient had been referred to our tertiary center, data on historical parameters were obtained from the patient. All patients received information, both orally and in writing, and signed an informed consent paper. From



68 patients sufficient tears from the affected eye could be collected, but from 18 of these patients no tear sample of the contra lateral eye was available. Tears were harvested with Sugi Steril sponges (Kettenbach Medical, Eschenburg, Germany), which were kept in the inferior conjunctival fornix for a few minutes [23]. A blood sample (<10-ml) was available from all patients and 145 control subjects (55 ± 13 years old) without a history of severe systemic or eye infections. The characteristics of these control subjects have been described previously [24]. Forty healthy subjects underwent a BUT and Schirmer test before a tear sample was collected. This study was approved by the local medical ethics committee (p03-078), and followed the tenets of the Declaration of Helsinki. Power analysis revealed that 80 patients and 80 healthy controls were required for this study assuming a relative risk of 2.0, and an allele frequency of 0.15, and a power of 80%.

Lactoferrin gene polymorphisms

DNA was extracted from the blood samples with the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) according to manufacturer's instructions. The DNA samples were amplified with a primer set for exon 1 and another set for exon 15 of the lactoferrin gene. Sequence for primers for exon 1 are: forward 5'-CTGTGTCTGGCTGGCCGTAGG-3' and reverse, 5'-AATGGCCTGGATACACTGGAT-3', for exon 15: forward 5'-ATTCCATTGCATGGACACAG-3', and reverse 5'-CCCACACAGCTAAGAAAGCA-3'.

PCR reactions were performed in a final reaction volume of 50.16 µl comprised of 37 µl of H_2O, 3 µl of 25 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 1 µl containing 100 ng of DNA, 5 µl of 10x concentrated PCR buffer (Roche), 1 µl containing 10 pmol of each primer (Isogen Life Science, Maarssen, The Netherlands) , 1 µl of DMSO, 2 µl of 25 mM of dNTPs (Invitrogen, Carlsbad, CA, USA), and 0.16 µl of 5 U/ml Taq-polymerase (Promega®). The PCR reaction consisted of one denaturation step of 5 min at 95 °C and subsequently the PCR was done for 35 cycles: 30 sec at 95°C followed by 30 sec at 55°C and 30 sec at 72°C with a final 10 min extension at 72°C. PCR products were detected after separation on a 2 % agarose gel (Invitrogen).

To analyze the Ala11Thr, Lys29Arg, and the Glu561Asp polymorphisms, we used the following restriction enzymes: Hha1 (Gibco BRL, Paisley, Scotland), MBOII (Gibco BRL), and Hga1 (Biolabs, Hitchin, England), respectively. In short, the PCR product (8 µl) was mixed with 1.5 µl of 2 U/ml of restriction enzyme and 1 µl of 10x buffer and then incubated for 3 hours at 37°C. To visualize the restriction fragments, the mixtures containing Hha1 or MBOII were run on spreadexgel (EL300, 50-200 bp, Elchrom, Scientific, Cham, Switzerland) and those containing HgaI on a 2% agarose gel.

ELISA for human lactoferrin

Tear lactoferrin concentrations were quantified by a human lactoferrin-specific ELISA (gift of Dr. H. van Veen, Pharming, Leiden, The Netherlands) as described by Van Berkel et al.[25] using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Tear samples were prediluted in PBS-1% BSA before application into the 96-well plates.

Table 1. Frequencies of genotypes/alleles encoding the amino acids at position 11, 29, and 561 in patients with herpes simplex keratitis and control subjects.

Polymorphisms		Patients		Controls	
		Genotype	Allele	Genotype	Allele
Ala11Thr	Ala/Ala	58	75	55	74
	Ala/Thr	34		37	
	Thr/Thr	8	25	8	26
Lys29Arg	Lys/Lys	54	68	49	66
	Lys/Arg	28		34	
	Arg/Arg	18	32	17	34
Glu561Asp*	Asp/Asp	7	30	16	40
	Asp/Glu	44		49	
	Glu/Glu	49	70	34	60

Data of 105 patients and 145 control subjects are expressed as percentages. All data are in Hardy Weinberg equilibrium.
* p = 0.05, Pearson's Chi Square test, p=0.016 linear by linear association.

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Pearson's Chi-Square test with 2 degrees of freedom was used to compare genotype frequencies between cases and controls. Logistic regression was used to estimate the odds-ratios of various lactoferrin genotypes between the patients and the control subjects with 95% confidence intervals. An ANOVA-test was used to determine the correlation between the clinical parameters and the different lactoferrin genotypes, and to compare the tear lactoferrin concentrations of individuals with different lactoferrin genotypes. For comparison of tear lactoferrin concentrations between the affected eye and the healthy eye in the patients group a paired samples t-test was used. P-values of 0.05 or less were considered significant.

Results

To find out whether polymorphisms in the lactoferrin gene are associated with susceptibility to develop HSV keratitis, we compared the frequencies of the three lactoferrin gene polymorphisms in patients with HSV keratitis and in control subjects. The results revealed significantly different frequencies of the Glu561Asp polymorphism between patients and control subjects (p=0.05 by Pearson's Chi Square). A multiplicative model yields an odds ratio of 1.6 (95% CI of 1.1 to 2.5) for heterozygote individuals and an odd-ratio of 2.7 (95% CI of 1.2 to 6.1) for individuals homozygote for Asp561. No differences between patient and controls were found for the Ala11Thr and Lys29Arg polymorphisms (Table 1), both polymorphisms were in linkage disequilibrium. Next, we investigated whether the various genotypes were involved in the severity of HSV infections. The results showed no differences in best corrected visual acuity, frequency of recurrence of infection, or average duration of clinical episodes among patients with the various genotypes.

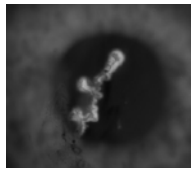


Table 2. Tear lactoferrin levels in individuals with different lactoferrin genotypes.

Polymorphisms		n	Lactoferrin concentration
Ala11Thr	Ala/Ala	39	1.6(0.1)
	Ala/Thr	21	2.0(0.4)
	Thr/Thr	5	2.2(0.6)
Lys29Arg	Lys/Lys	35	1.7(0.1)
	Lys/Arg	17	2.0(0.4)
	Arg/Arg	10	1.9(0.3)
Glu561Asp*	Asp/Asp	2	1.9(0.8)
	Asp/Glu	30	1.9(0.3)
	Glu/Glu	24	1.7(0.2)

Results are shown as mean values and SEM

Furthermore, no significant differences in tear lactoferrin concentrations among the various lactoferrin genotypes were observed (Table 2). In addition, no differences in tear lactoferrin concentrations between the affected eye and contra lateral eye of the patients (1.7 ± 0.1 mg/ml and 1.8 ± 0.2 mg/ml, respectively; n=50) were found, and these values were similar to the tear lactoferrin concentrations of the right and left eye of healthy controls (1.9 ± 0.2 mg/ml and 2.1 ± 0.2 mg/ml, respectively; n=40). No differences in clinical parameters were observed between patients from whom a tear sample was available (n=68) and patients from whom a tear sample was lacking (n=37).

Discussion

We conclude from this study that the lactoferrin Glu561Asp polymorphism is associated with the susceptibility to HSV keratitis, but not with the severity of the disease or frequency of its recurrences. This is based on the following findings. First, the frequencies in the Glu561 and 561Asp alleles, but not those of the Ala11Thr and Lys29Arg lactoferrin polymorphisms, differed significantly between patients and control subjects. These data are in accordance with an earlier report that genetic variation plays an important role in the development of ocular herpetic disease [26]. Secondly, no correlation was found between the various lactoferrin genotypes and the severity of the disease, i.e. best corrected visual acuity, average duration of clinical episodes, and frequency of recurrent infections.

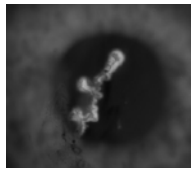
Although our data is derived from a relatively small number of patients, the present number of patients in the present study was higher than that calculated by the power analysis performed at the start of the study. To confirm the association between Glu561Asp polymorphism and HSV keratitis susceptibility our study needs to be replicated in a different, larger cohort. Nevertheless, our data indicates that individuals with lactoferrin comprising Asp561 are less susceptible to HSV keratitis than those with the lactoferrin variant with Glu561. Of note, the lack of positive effect of Glu561 on the frequency of recurrence in HSV patients can be explained by the different ports d'entrée of the virus. In a primary infection the HSV

is thought to infect the corneal epithelium from the outside, i.e. through the lactoferrin containing tear film, while in recurrent disease the HSV is reactivated from its latent state in the trigeminal ganglia and can infect the cornea through the trigeminal nerve fibers in the stroma of the cornea, without any involvement of tear lactoferrin.

The mechanisms by which the Glu561Asp polymorphism can influence the primary ocular HSV infection are not known. The amino acid at position 561 in lactoferrin locates in a loop region at the bottom of the C-lob. The oxygen of the Glu561 side chain may form a weak hydrogen band with the nitrogen of the Trp563 side chain, while the Asp561 lacks this possible hydrogen bond. This may enhance the flexibility of the Asp561 lactoferrin variant compared to the Glu561 variant leading to the exposure of a hydrophobic domain to the environment [21]. Whether this exposed hydrophobic domain contributes to the antiviral activities of lactoferrin needs to be investigated. For this purpose recombinant lactoferrins varying at position 561 will be prepared and their antiviral activities analyzed using established assays. The lack of association between Ala11Thr and Lys29Arg polymorphisms and susceptibility to HSV keratitis together with the association between Glu561Asp polymorphisms and disease susceptibility indicates that the hydrophobic region comprising amino acid 561 affects the antibacterial and antiviral activities differently.

Furthermore, our observation that the tear lactoferrin concentrations did not differ among the various genotypes encoding amino acids at the three positions in the lactoferrin protein excludes the possibility that the protective role of the Asp561 allele is mediated by higher tear lactoferrin levels in these patients. Finally, the tear lactoferrin concentration did not significantly differ between patients and control subjects, which is an interesting finding because in vitro studies have found an influence of the lactoferrin concentration on HSV infections [14,15].

To elucidate genetic determinants of susceptibility to eye infections and/or the severity of these diseases, we first analyzed the association of lactoferrin gene polymorphisms with the susceptibility and/or severity of HSV keratitis. We realize that HSV keratitis is a complex process involving virulence factors of the virus, such as glycoproteins B,C, D, H and L, as well as host molecules, including heparan sulfates and the entry receptors herpes virus entry mediator (HVEM) and nectin-1 and-2 [27,28] cytokines like IL-10 [29], IL-12 [30], and INF-γ [31,32], and glucocorticoids [33] , and antimicrobial proteins/peptides, such as human alpha- and beta-defensins,[34] cathelicidins[35] and lactoferrin/lactofericin[28]. Obviously, polymorphisms in the genes encoding these host factors (or their receptors and/or signalling molecules) may also contribute to the susceptibility and/or the severity of HSV keratitis.



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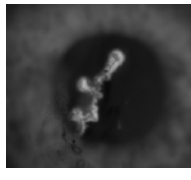

CHAPTER 9

LACTOFERRIN GENE POLYMORPHISM GLU561ASP COULD BE ASSOCIATED WITH EPITHELIAL HEALING OF INFECTIOUS CORNEAL ULCERS

Submitted

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Abstract

Background/aims. Lactoferrin is found in high concentrations in human tears, and plays an important role in host defense against infections and in down-regulation of ocular inflammation. Since polymorphic variants of lactoferrin may differ in their antibacterial activities, we investigated whether lactoferrin gene polymorphism is associated with susceptibility to and outcome of infectious corneal ulcers.

Methods. Information about the various clinical aspects of the corneal ulcers was extracted from patient's records. Lactoferrin gene polymorphisms Ala11Thr, Lys29Arg, and Glu561Asp were determined by restriction fragment length analysis on PCR-amplified genomic DNA in 70 patients with an infectious corneal ulcer. Lactoferrin concentration in tears of 26 patients and 40 healthy controls was quantified by ELISA.

Results. The frequencies of genotypes did not differ between patients and controls. Lactoferrin gene polymorphisms Glu561Asp showed a trend towards delayed epithelial healing for patients homozygous for 561Glu. Polymorphisms Ala11Thr, Lys29Arg did not reveal any differences for clinical outcome in covariate analysis. Lactoferrin concentration in tears of the affected and contralateral eye of the patients did not differ, and were the same as the lactoferrin levels of healthy controls.

Conclusion. Lactoferrin gene polymorphisms Ala11Thr, Lys29Arg, and Glu561Asp are not associated with the susceptibility to infectious corneal ulcers, but patients with glutamic acid at position 561 of the lactoferrin protein showed a trend for delayed healing of the corneal ulcer. Thus, differences in the functionality of the lactoferrin variants may contribute to the clinical outcome of infectious corneal ulcers.

Introduction

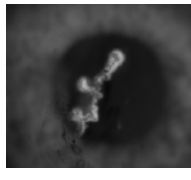
Infectious corneal ulceration is a rare but serious ophthalmologic disease that can cause corneal perforation[1,2] and blindness. In the Western World, the incidence of corneal ulceration is about 11 cases per 100.000 inhabitants;[3] and it has been estimated that worldwide each year 1.5 million people become unilaterally blind due to corneal ulcers.[4] The main risk factors for corneal ulceration are contact lens wear and trauma;[5] 30 % of all corneal ulcerations in the Western World are contact lens related.[6] Regarding the total number of individuals with extended-wear contact lenses, only a small minority develops an infectious corneal ulceration,[7] *Pseudomonas aeruginosa* being the most frequently cultured organism from contact lens associated ulcers.[8]

In general, the risk to develop an infectious corneal ulcer and/or its clinical outcome depends on the type of infectious etiologic agent, extent of precipitating trauma, the intrinsic antimicrobial capacity of the eye, and the inflammatory response of the host.[9] Among the factors determining the host response to eye infections, lactoferrin is a multifunctional protein with antimicrobial and anti-inflammatory activities, [10,11] and it is found in a high concentration in tears (approximately 2 mg/ml) (Keijser et al., submitted).[12] Recently, we investigated lactoferrin gene polymorphisms in patients with herpes simplex keratitis and found an association between polymorphism Glu561Asp and the susceptibility to herpes simplex keratitis. (Keijser et al. submitted). Two other lactoferrin polymorphisms causing amino acid substitutions at position 11 (Ala11Thr) and 29 (Lys29Arg) of the lactoferrin protein are associated with infections of the oral cavity.[13,14] In addition, the lactoferrin polymorphism Lys29Arg displayed differences in antibacterial and transcriptional activation activities.[15] Based on these findings, we investigated whether the lactoferrin genotypes are associated with the susceptibility and clinical outcome of infectious corneal ulcer.

Patients and methods

Patients

Seventy-six patients ages ≥18 years with an infectious corneal ulcer treated between 2004 and 2006 at the university eye clinics of LUMC and UMCU were invited to participate in the study. The study was approved by the local medical ethics committee (No p03-078), and followed the tenets of the Declaration of Helsinki. All patients provided a written informed consent. Patients with herpes simplex keratitis hepatitis C, or severe dry eyes as defined by a tear break up time (BUT) of less than 5 seconds and/or Schirmer test under 5 mm/5 min were excluded. Seventeen patients (36 male, 34 female, mean age 51 (range 18-91)) were included in the study. Twenty-nine patients (42 %) wore contact lenses at onset of the corneal infection, seven patients had diabetes (10%). Six patients were excluded from the study: three because they suffered from severe dry eyes, two with corneal decompensation, and one with a corneal ulceration after radiation of the eye for melanoma of the iris. Patient's records were used to extract demographic information, contact lens wear, best corrected visual acuity at onset, best corrected visual acuity after treatment, size of the ulcer, identification of the pathogen, duration of corneal defect, duration of treatment, and type of treatment. Visual acuity was measured with Snellen charts. All patients signed a written in-



formed consent before a tear sample and 10 ml of blood were collected. From 26 patients sufficient tears from the affected eye could be collected, of four of these patients no contralateral tear sample was available. Tears were acquired with Sugi Steril sponges (Kettenbach Medical, Eschenburg, Germany) which were kept in the inferior conjunctival fornix for a few minutes. Forty healthy subjects underwent a BUT and Schirmer test before a tear sample was collected. A blood sample was available from 145 control subjects without a history of severe systemic or eye infection; the characteristics of these patients have been reported earlier.[16]

PCR-restriction fragment length analysis of lactoferrin gene polymorphisms

We used PCR-restriction fragment length polymorphism analysis to determine the genotypes encoding the amino acids at positions 11, 29, and 561 as described earlier (Keijser et al., submitted). DNA was extracted from the blood samples using the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) and then amplified using a primer set for exon 1 and another set for exon 15 of the lactoferrin gene. Sequence for primers for exon 1 are: forward 5'-CTGTGTCTGGCTGGCCGTAGG-3' and reverse, 5'-AATGGCCTGGATACACTGGAT-3', for exon 15: forward 5'-ATTCCATTGCATGGACACAG-3', and reverse 5'-CCCACACAGCTAAGAAAGCA-3'.

PCR reactions were performed in ~50 µl comprising of 37 µl of H_2O, 3 µl of 25 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 1 µl containing 100 ng of DNA, 5 µl of 10x concentrated PCR buffer (Roche), 1 µl containing 10 pmol of each primer (Isogen Life Science, Maarssen, The Netherlands) , 1 µl of DMSO, 2 µl of 25 mM of dNTPs (Invitrogen, Carlsbad, CA, USA), and 0.16 µl of 5 U/ml Taq-polymerase (Promega®). The PCR reaction consisted of one denaturation step of 5 min at 95 °C and subsequently 35 cycles of 30 sec at 95°C followed by 30 sec at 55°C and 30 sec at 72°C with a final 10 min extension at 72°C. PCR products were detected after separation on a 2 % agarose gel (Invitrogen).

The following restriction enzymes: Hha1 (Gibco BRL, Paisley, Scotland), MBOII (Gibco BRL), and Hga1 (Biolabs, Hitchin, England) were used to detect the genotypes encoding the amino acids at position 11, 29, and 561, respectively. The PCR product (8 µl) was mixed with 1.5 µl of 2 U/ml of restriction enzyme and 1 µl of 10x buffer and then incubated for 3 hours at 37°C. To visualize the restriction fragments, the mixtures containing Hha1 or MBOII were run on spreadexgel (EL300, 50-200 bp, Elchrom, Scientific, Cham, Switzerland) and those containing HgaI on a 2% agarose gel.

ELISA for human lactoferrin

Tear lactoferrin concentrations were quantified by a human lactoferrin-specific ELISA as described earlier (Van Berkel et al.[17], and Keijser et al., submitted).

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Chi-

Square test was used to calculate the significance of the differences in frequencies of various lactoferrin genotypes between the patient group and control subjects. An oneway ANOVA and Cox regression mono- and covariate analysis were used to compare the lactoferrin genotypes with the clinical parameters. The ANOVA-test was also used to compare tear lactoferrin concentrations between the different genotypes. A P-value of 0.05 or less was considered significant.

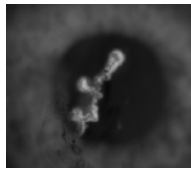
Results

The PCR analysis revealed no differences in the frequencies of the various genotypes of lactoferrin between the patients and the controls (Table 1). Next, we investigated whether the various genotypes may be associated with the clinical outcome of the infectious corneal ulcers. Patients homozygous for alanine at position 11 of the lactoferrin protein are suffering from a worse clinical outcome in monovariate cox regression analysis. The best corrected visual acuity at onset (p=0.028) and the duration of the corneal defect (p=0.017) differed significantly. However, when corneal ulcer size was introduced as a covariate in Cox regression analysis none of the clinical parameter reached significance (Figure 1). Furthermore, monovariate Cox regression analysis showed longer duration of epithelial defect in patients heterozygous for Glu561Asp (p=0.046). When corrected for corneal ulcer size a trend was still visible (p=0.075). In addition, patients with a 561Asp allele showed a faster epithelial healing than patients without the 561Asp allele (p=0.043), when corrected for corneal ulcer size a trend was still visible (p=0.06) (Figure 2). No differences were seen for clinical outcome in polymorphism Lys29Arg.

Table 1 Distribution of the various genotypes in exon 1 and exon 15 of the lactoferrin gene in patients with corneal ulcerations and in controls subjects. There are no significant differences.

		Patients		Control subjects	
Gene polymorphisms causing a substitution at position		Genotype frequencies	Allele frequencies	Genotype frequencies	Allele frequencies
11	Ala/Ala	57	75	55	74
	Ala/Thr	36		37	
	Thr/Thr	7	25	8	26
29	Lys/Lys	53	71	49	66
	Lys/Arg	37		34	
	Arg/Arg	10	29	17	34
561	Asp/Asp	10	40	16	40
	Asp/Glu	59		49	
	Glu/Glu	31	60	35	60

Data are from 70 patients and 145 controls. All genotypes are in Hardy-Weinberg equilibrium.



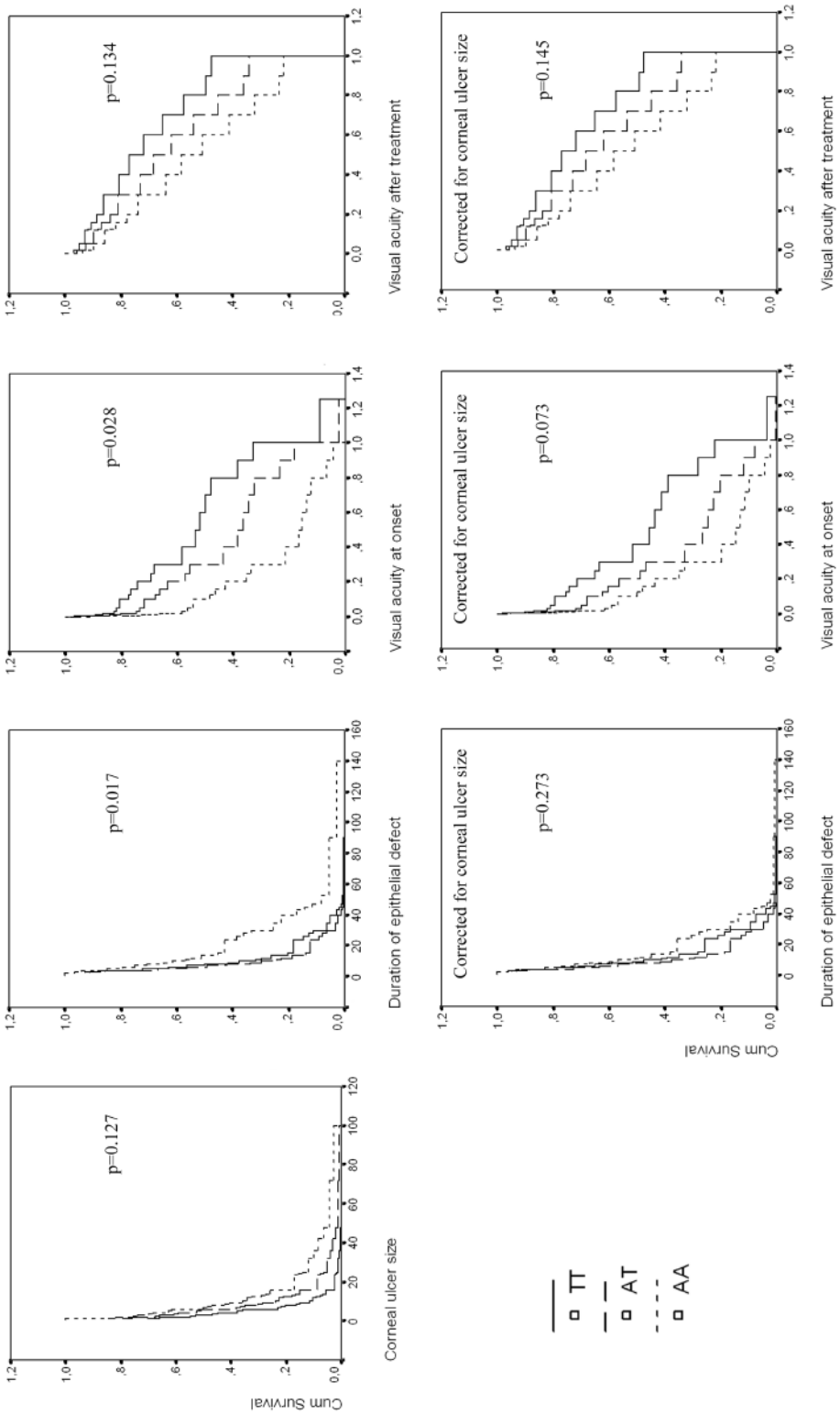


Figure 1. Cox regression analysis of lactoferrin polymorphism ala11thr and clinical outcome of infectious corneal ulcers. Note the reduced significance when corneal ulcer size was introduced as a covariate.

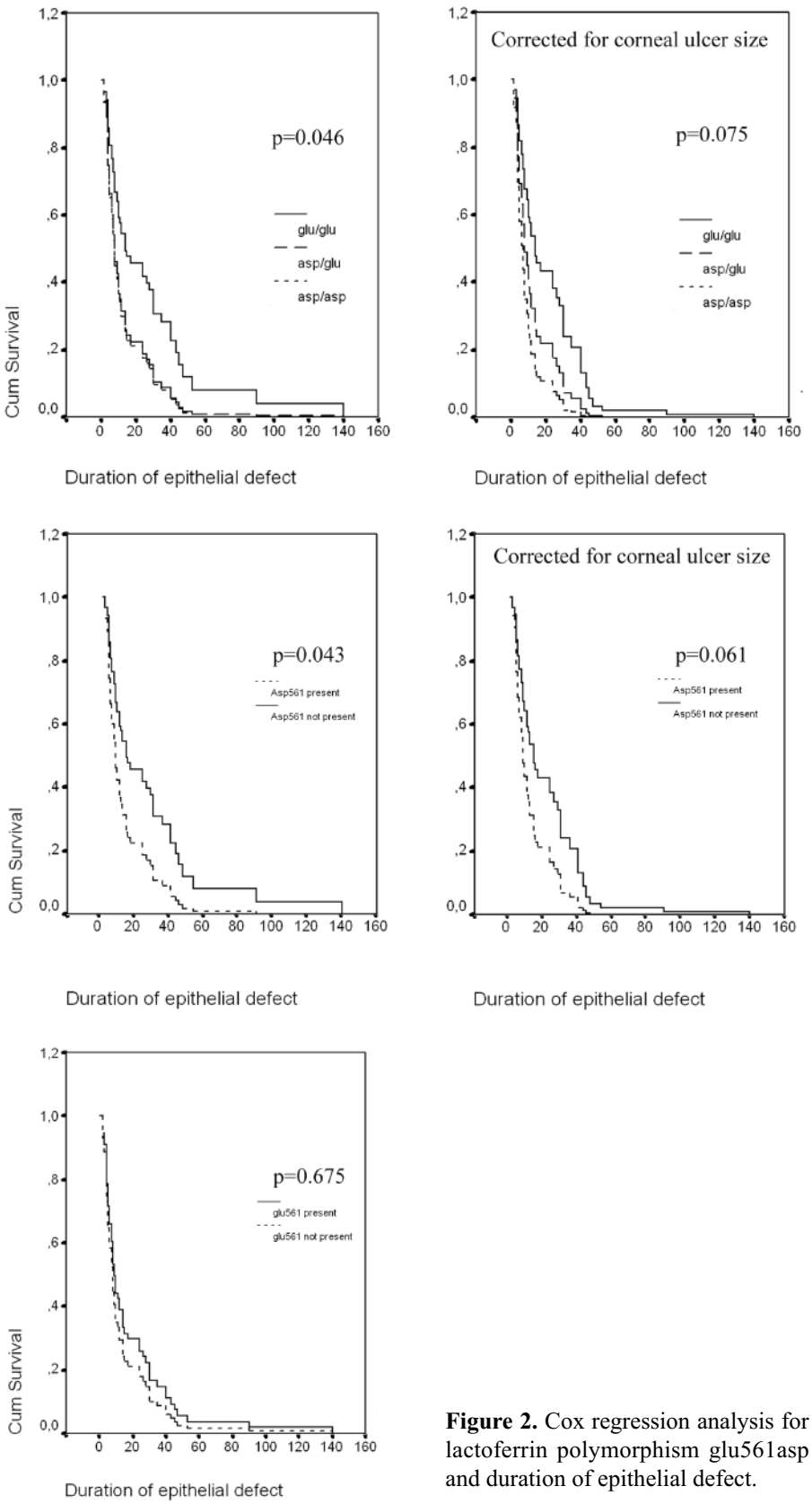


Figure 2. Cox regression analysis for lactoferrin polymorphism glu561asp and duration of epithelial defect.

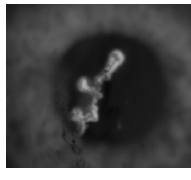
No differences in tear lactoferrin concentrations between the affected eye and the contralateral eye of patients (2.2 ± 0.3 mg/ml and 1.9 ± 0.2 mg/ml, respectively) were observed, and these values were about equal to lactoferrin concentrations in tears of healthy controls (2.1 ± 0.1 mg/ml).
Comparison between patients with and those without contact lenses revealed no differences in clinical outcomes, except the best corrected visual acuity after treatment in patients without contact lenses was significantly (p=0.018) lower than of patients with contact lenses. Patient with contact lenses were evenly distributed among the various lactoferrin genotypes.

Discussion

The main conclusion from the present study is that the lactoferrin gene polymorphism causing an glutamic acid/aspartic acid substitution at position 561 of the protein could be associated with the duration of epithelial defect of infectious corneal ulcers, but not with the susceptibility to this disease. Patients without the 561Asp allele seem to have a slower corneal epithelial healing than patients with 561Asp allele. This finding together with a previous report of an association with susceptibility to Herpes Simplex Keratitis and the Glu561Asp lactoferrin polymorphism (Keijser et al, submitted), indicates that the Glu561Asp polymorphism could influence the functionality of the lactoferrin protein. The Asp561 lactoferrin variant displays more flexibility in the C-lobe than Glu561 leading to the exposure of a hydrophobic domain (Araki-sasaki 2005), which could interfere with the interaction with other proteins. Further studies are required to elucidate the possible role of this amino acid substitution on the biological activities of lactoferrin.
Moreover, no differences in the tear lactoferrin levels among the various lactoferrin gene polymorphisms have been found (Keijser et al, submitted). In agreement with tear lactoferrin levels in Chinese patients with an infectious condition of the eye and healthy controls,[18] the tear lactoferrin levels in the affected and the contralateral eye of the patients were the same and did not differ from the levels in healthy controls, excluding the possibility that differences in tear lactoferrin levels affected the susceptibility to and clinical outcomes of infectious corneal ulcers in our study.
The genotype frequencies for the polymorphisms resulting in an amino acid substitution at positions 11, 29, and 561 of the lactoferrin protein did not differ between patients with infectious corneal ulcers and healthy controls. This observation may seem surprising, but it should be realized that events independent from the susceptibility to infections, such as microtrauma to the corneal epithelium, precede an infectious corneal ulcer. Although no association was found for infectious corneal ulcer patients and susceptibility between lactoferrin gene polymorphisms, others showed that lactoferrin gene polymorphisms causing a lysine/arginine substitution at position 29 and an alanine/threonine substitution at position 11 of the protein are associated with susceptibility to aggressive (bacterial) periodontitis.[19,20]
Obviously, the present lactoferrin gene polymorphisms do not explain susceptibility to infectious corneal ulcers. It is likely that that further polymorphisms within the lactoferrin gene[21] as well as the genes for other host-response factors, such as lysozyme[22] and cytokines like interleukin (IL)-10,[23] IL-12,[24,25] interferon-gamma,[26] and corticosteroids contribute to susceptibility to infectious corneal ulcers. Furthermore, other - yet unidentified - genes,

which control the presence and numbers of bacteria in corneal epithelium, may render some patients less susceptible than others. In addition, numerous factors related to the causative agent(s) could also be involved in the susceptibility. The kind of bacteria found in the cultures is related to the clinical outcome in patients with infectious corneal ulcers,[27] with commonly found bacteria such as *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. One possible confounding factor in our present data could be contact lens wear as it has been reported that this is a risk factor for the development of infectious corneal ulcers.[28] However, we found no difference in contact lens wear among the patient groups with the different lactoferrin gene polymorphisms. Although the number of patients included in our study may seem small, it is in full agreement with the results of our power calculations. Therefore, we believe that our data allow us to draw reliable conclusions.

The results presented here and those reported by others[29,30,31] and us (Keijser et al., submitted) revealing an association between lactoferrin gene polymorphisms and susceptibility to and clinical outcome of mucosal infections underline the notion that genes play a role in the predisposition to and progression of infections of the eye. Once the contribution of the genetic variations in the different genes to susceptibility/progression of infections of the eye have been documented, a better risk profile for patients can be made, which may in the future influence the treatment of infections to the eye.



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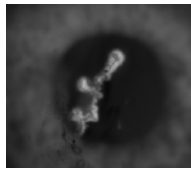

CHAPTER 10

IL-10 PROMOTOR POLYMORPHISMS ASSOCIATED WITH SUSCEPTIBILITY TO AND SEVERITY OF INFECTIOUS CORNEAL ULCERS

Submitted

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Abstract

Purpose. In animal models for bacterial corneal ulcers, high IL-10 levels were associated with a better clinical outcome. We investigated whether IL-10 promotor polymorphisms, known to influence IL-10 expression in vitro, were associated with susceptibility to and/or clinical outcome of infectious corneal ulcers.

Methods. IL-10 promotor polymorphisms C-819T, G-1082A, A-2763C, and A-2849G were determined in 70 patients with infectious corneal ulcers and 115 healthy controls by restriction fragment length PCR analysis. For 51 patients and all healthy controls IL-10 haplotypes could be inferred using the program SNPHAP.

Results. A significant under representation of the -819C allele and A-2849A genotype were observed in the patient group compared to healthy controls, while the -2763A allele was associated with a poor clinical outcome. The IL-10.1 haplotype was associated with a poor clinical outcome, whereas haplotype IL-10.5 showed a trend towards a favorable outcome.

Conclusions. IL-10 promotor polymorphisms that are associated with low IL-10 levels seem to protect against an infectious corneal ulcer. Once a corneal ulcer has developed, IL-10 polymorphisms/haplotypes associated with a high IL-10 expression display a favorable outcome of infectious corneal ulcers.

Introduction

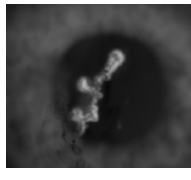
Infectious corneal ulcer can be an aggressive disease with serious complications, such as perforation of the cornea and blindness.1 Known risk factors for infectious corneal ulcers include trauma, contact-lens wear, and dry eyes.2,3 We observed in a previous study that lactoferrin gene polymorphisms may be associated with the susceptibility to and severity of infectious corneal ulcers (Keijser, submitted). However, polymorphisms in other genes may also play a role.

A major part of the damage of the cornea in infectious corneal ulcers arises from actions of the immune system itself. Anti-inflammatory mediators, such as interleukine-10 (IL-10), play a key role in preventing such excessive inflammation. The actions of this cytokine, which is produced by mononuclear phagocytes, Th2 cells and corneal epithelial cells,4 are mediated by the regulation of 1) the expression of other (proinflammatory) cytokines by a variety of cell types in the eye, 2) angiogenesis5 via various mediators, including vascular endothelial growth factor, and 3) antimicrobial defenses against e.g. Pseudomonas aeruginosa.6,7 In infectious corneal ulcers in mice, IL-10 affects the severity of the disease with high IL-10 levels being associated with a favorable outcome.8,9 In humans, different expression levels of IL-10 are related to single nucleotide polymorphisms (SNP) in the promotor region of the IL-10 gene and their haplotypes.10,11,12 These IL-10 promotor polymorphisms are involved in a variety of infections,13,14,15,16,17,18,19,20 including eye infections.21,22 Based on these considerations, we investigated whether IL-10 promotor polymorphisms are associated with the susceptibility to and/or severity of infectious corneal ulcers in man.

Patients and methods

Patients

Blood was obtained from 70 patients with an infectious corneal ulcer from the ophthalmology departments of the Leiden University Medical Center and the University Medical Center Utrecht; there were 36 male and 34 female patients, with an average age of 51 ± 20 years. Patient's records were used to extract demographic information, contact lens wear, best corrected visual acuity at onset, best-corrected visual acuity after treatment, size of the ulcer, identification of the pathogen, duration of the corneal defect, duration of antibiotic treatment, and type of treatment. Visual acuity was measured with Snellen charts. A infectious corneal ulcers was defined as an epithelial corneal defect, stromal infiltrate, purulent discharges, and with or without stromal loss. Since most ulcers resemble a circle, the size of the corneal ulcer was calculated from the diameter of the ulcer. Patients with a history of viral keratitis or a proven acanthamoeba keratitis were excluded from the study. Blood samples were available from 115 healthy unrelated control subjects (42 male, 73 female; average age 42 ± 13 years) from the same geographical region as the patients. This study was approved by the local medical ethics committee (No p03-078) and followed the tenets of the Declaration of Helsinki. All patients signed a written informed consent. From 26 patients and forty controls a tear sample was available.



Determination of IL-10 promotor polymorphisms

DNA was extracted from blood samples with the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) according to the manufacturer's instructions. IL-10 promotor polymorphisms at position C-819T, G-1082A, A-2763C, and A-2849G from the transcriptional start site were analyzed by PCR combined with restriction fragment length analysis, as previously described.[23] In brief, for C-819T and G-1082A the forward primer was: 5-CCA-AGA-CAA-CAC-TAC-TAA-GGC-TTC-TTG-AGG-A-3, and the reverse primer was: 5-AGG-TAG-TGC- TCA-CCA-TGA-CC-3. Restriction enzymes BseRI (New England Biolabs, Ipswich, MA, USA) and MslI (New England Biolabs) were used for restriction fragment length analysis of SNP C-819T and G-1082A, respectively. For SNPs A-2763C and A-2849G the forward primer: 5-TAA-AGA-AGT-CAG-ATC-CGG-GC-3, and the reverse 5-CGC-TGG-CAC-CAC-GCC-CGG-C-3 were used. Digestion was performed with restriction enzymes AlwI (Invitrogen Corporation, Carlsbad, CA, USA) for SNP A-2849G) and DdeI (Invitrogen) for SNP A-2763C.

Quality control

PCRs were run twice in order to obtain reliable data. In addition, the results were read by two independent observers. If the results of the two runs/observers differed (<1% of the cases) a third PCR was performed. All SNPs in the IL-10 promotor in the healthy control group were in Hardy-Weinberg equilibrium.

Table 1. IL-10 promotor allele and genotype frequencies among patients with an infectious corneal ulcer and healthy controls

IL-10 gene polymorphisms causing a substitution at position		Patients		Control subjects	
		Genotype frequencies	Allele frequencies	Genotype frequencies	Allele frequencies
-819	CC	47	69	63	79
	CT	44		32	
	TT	9	31*	5	21
-1082	AA	27	49	24	48
	AG	44		47	
	GG	29	51	29	52
-2763	AA	15	39	17	39
	AC	48		45	
	CC	37	61	38	61
-2849	AA	4 #	31	11	29
	AG	54		36	
	GG	41	69	53	71

A total of 70 patients and 115 healthy volunteers were involved in this study.
* p= 0.035; Chi Square test for allele frequencies
# p= 0.029; Chi-Square test for genotype frequencies

Table 2. IL-10 haplotype frequencies among patients with an infectious corneal ulcer and healthy controls

Haplotype	*SNPs*	*Patients (%)*	Healthy controls (%)
IL-10.1	CGAA	24	27
IL-10.2	CACG	18	26
IL-10.3	CGAG	8	10
IL-10.4	TACG	25	20
IL-10.5	CGCG	10	13

Haplotypes are formed by four distal SNPs in the IL-10 promotor that dictate IL-10 production. Data are from 51 patients and 115 healthy controls.

IL-10 haplotypes

From 51 of the 70 patients, haplotypes were inferred by using SNPHAP version 1.2.1 (http://www-gene.crimr.cam.ac.uk/clayton/software/). Individual haplotypes with a probability under 95% were discarded from further analysis. With SNPs C-819T, G-1082A, A-2763C, and A-2849G, the following most common haplotypes could be inferred: haplotype IL-10.1 (CGAA), haplotype IL-10.2 (CACG), haplotype IL-10.3 (CGAG), haplotype IL-10.4 (TACG), haplotype IL-10.5 (CGCG).

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Pearson Chi-Square tests were used to calculate the significance of the differences in frequencies of various SNPs in the IL-10 promotor and haplotypes between the patients and control subjects. An ANOVA-test and Cox regression analysis were used to determine the correlation between the clinical parameters among the different SNPs in the IL-10 promotor and haplotypes. P-values of 0.05 or less were considered significant. Results are expressed as odds ratios (OR) and 95% confidence intervals (CI).

Results

The allele and genotype frequencies of the four SNPs in the IL-10 promotor (C-819T, G-1082A, A-2763C, and (No p03-078), A-2849G) in patients and controls are reported in Table 1. The -819T allele was found significantly (p=0.035) more frequently in the patient group than in the control group (OR=1.68; CI=1.00-2.80). In addition, the frequency of the A-2849A genotype was significantly lower (p=0.029) in the patient group than in the control group. We calculated an OR of 0.26 (CI=0.07-0.97; p=0.045) for the A-2849A genotype compared to the A-2849G genotype and an OR of 0.52 (CI=0.14-1.99; p=0.34) compared to the G-2849G genotype.

Comparison of the various clinical characteristics defining the disease severity with the four IL-10 promotor polymorphisms revealed significantly larger corneal ulcers (p=0.014),

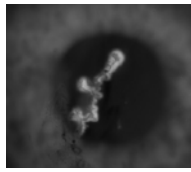


Figure 1. Association between IL-10 promotor polymorphisms -A2763C and parameters for clinical outcome. Cox regression analysis was used to calculate associations between IL-10 promotor polymorphisms -A2763C and parameters for clinical outcome.

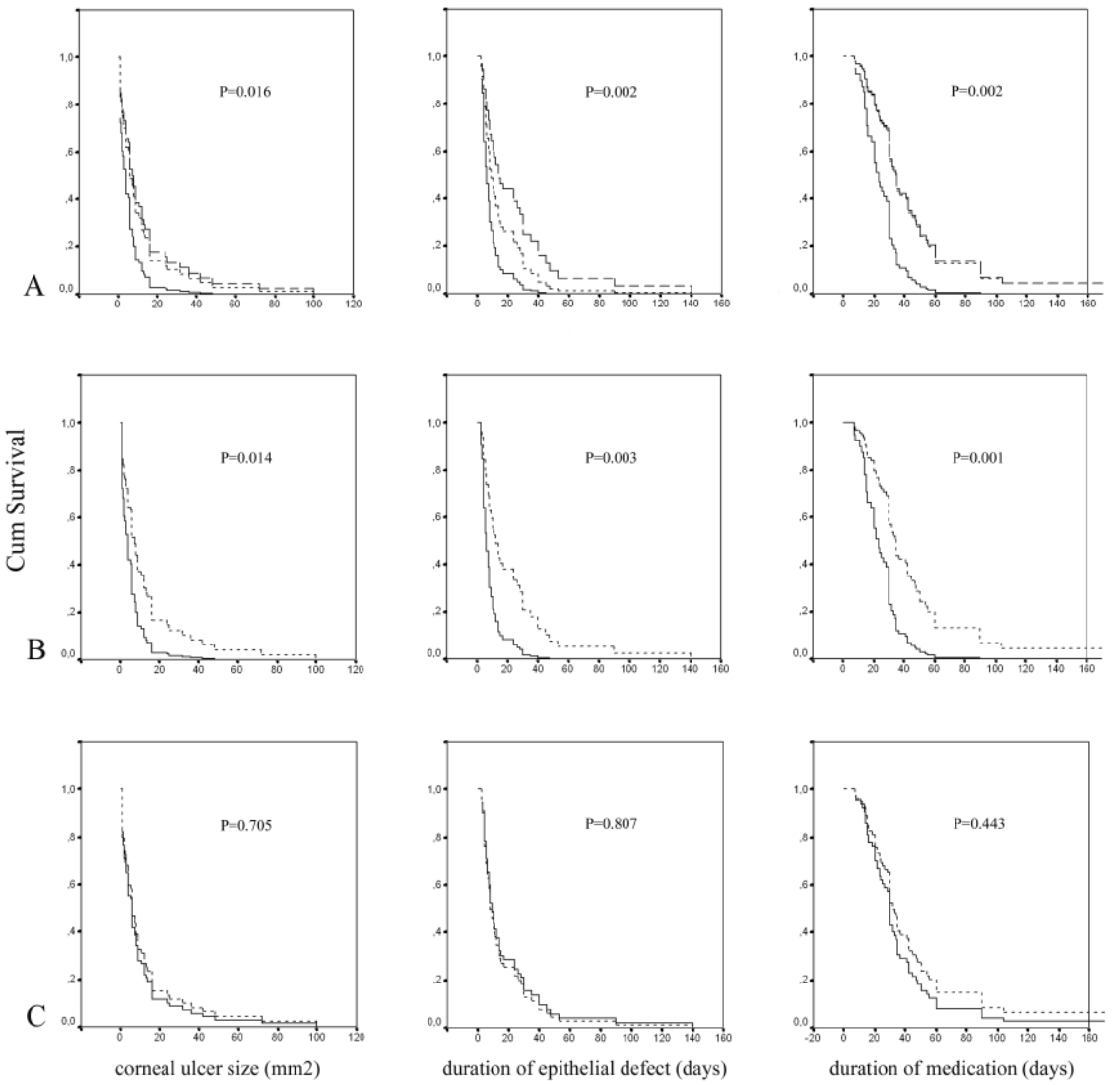
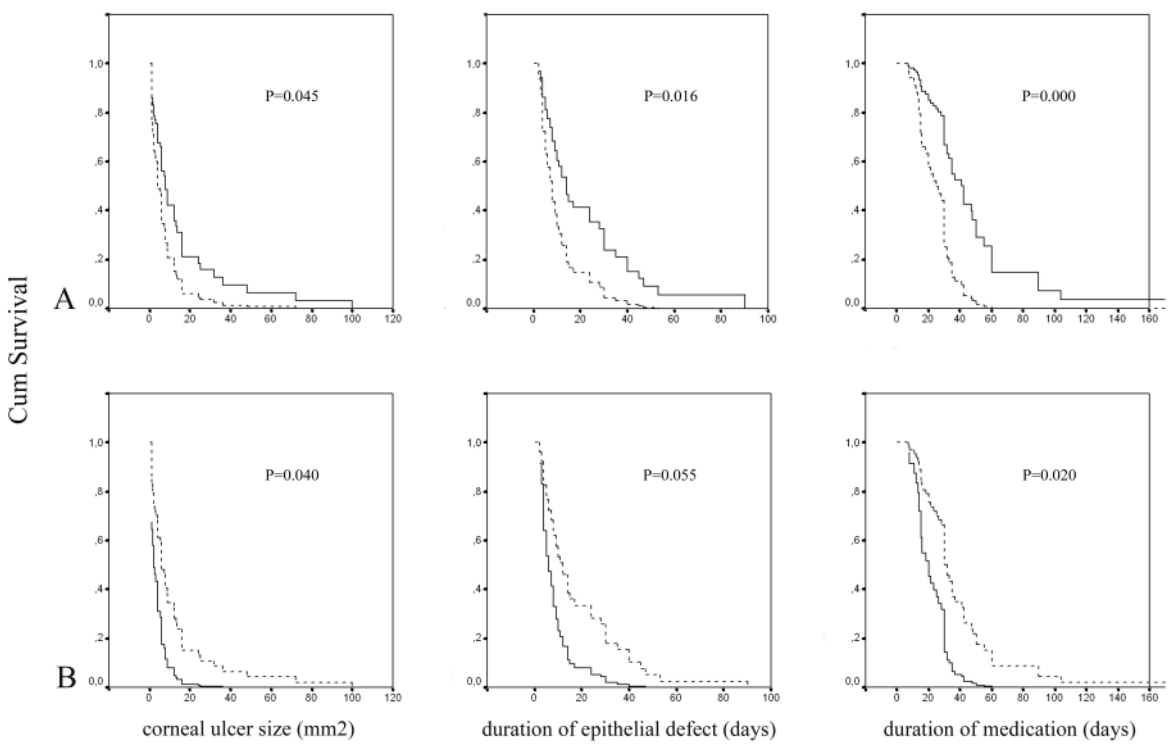


Figure 2. Associations between IL-10 haplotypes 1 and 5 and parameters for clinical severity. Cox regression analysis for associations between IL-10 haplotypes 1 and 5 and parameters for clinical severity. Note the opposite effects of the IL-10.5 and IL-10.1 haplotypes.



2A Haplotype IL10.1
IL10.1 present
IL10.1 not present

2B Haplotype IL10.5
IL10.5 present
IL10.5 not present

longer duration of the epithelial defects (p=0.003), and longer duration of treatment (p=0.001) in patients carrying the -2763A (Figure 1). Corneal ulcer size in patients carrying this allele and those carrying -2763C remained significantly different when duration of medication (p=0.024) and contact lens wear (p=0.011) were introduced as covariates in a Cox regression analysis. However, when duration of an epithelial defect was introduced as a covariate, corneal ulcer size was not significantly different (p=0.162) between patients with the -2763A and those with the -2763C allele. Furthermore, the differences in duration of the epithelial defect between patients with the -2763A allele and those with the -2763C allele remained significant when corneal ulcer size (p=0.028), duration of medication (p=0.032), and contact lens wear (p=0.005) were introduced as covariates. None of the other SNPs in the IL-10 promotor were associated with the severity of infectious corneal ulcers.

Next, we compared the various IL-10 haplotype frequencies between patients and controls and again looked for associations with disease severity. No differences were seen in haplotype frequencies between patients and controls (Table 2). With respect to the disease severity, we found that the IL-10.1 haplotype (CGAA) was associated with larger corneal ulcers (p=0.045), longer duration of epithelial defects (p=0.016), and longer duration of treatment ($p<0.001$) when compared to patients not carrying this haplotype (Figure 2). When duration of an epithelial defect was introduced as a covariate in the Cox regression analysis, no significant difference in corneal ulcer size was seen between patients carrying the IL-10.1 haplotype and those without. Duration of the epithelial defect and medication remained significantly different between these two patient subgroups when the other clinical parameters were introduced as covariates. Furthermore, the presence of the IL-10.5 haplotype was associated with smaller corneal ulcers (p=0.044), shorter duration of epithelial defect (p=0.05) and shorter duration of medication (p=0.02) in a Cox regression analysis. When the clinical parameters were introduced as covariates, only duration of antibiotic medication remained significant (p=0.045) between these two patient subgroups.

Comparison between patients with and those without contact lenses revealed a lower best-corrected visual acuity after treatment in patients without contact lenses (p=0.018) than in patients with contact lenses. All other disease severity parameters were not significantly different between patients with and those without contact lenses. Furthermore, patients with contact lenses were evenly distributed among the various IL-10 promotor genotypes and haplotypes.

IL-10 concentrations were not detectable in patients or control tear samples, both because of low volume and probably very low concentration.

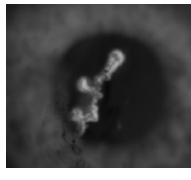
Discussion

The results of this study show that the IL-10 promotor polymorphisms C-819T and A-2849G are associated with susceptibility to infectious corneal ulcers. This conclusion is based on the differences in -819T allele and A-2849A genotype frequency between patients and healthy controls. Carriers of the -819C allele and the A-2849A genotype seem to be better protected against the development of infectious corneal ulcers. It should be realized that the A-2849A genotype is related to haplotype IL-10.1, which is associated with a low IL-10 production,12,24 whereas the -819T allele is related to haplotype IL-10.4, which is not

clearly associated with high levels of IL-10 production. These data suggest that IL-10 is important in the development of corneal ulcers but probably plays different roles in the early and late stage of corneal ulcers and/or in the defense against the different infectious agents. In this connection, it has been reported that low IL-10 levels may cause an impaired elimination of Staphylococcus aureus25 leading to destructive effects on the corneal epithelium, and Pseudomonas aeruginosa are more rapidly eliminated by high IL-10 levels.6 In agreement, others also have found different distributions of IL-10 promotor polymorphisms in infectious diseases,21,15,16,17,20 most of which are related to G-1082A. Furthermore, C-819T is associated with susceptibility to HIV infections19 and with disease severity in patients with chronic hepatitis B infections18 and those suffering from graft versus host disease.26 No relation was found between the IL-10 promotor polymorphisms C-819T and A-2849G and the disease severity in patients with infectious corneal ulcers.

Secondly, IL-10 promotor polymorphisms A-2763C are associated with disease severity; those carrying the allele -2763A suffered from a poor clinical outcome. In agreement, patients carrying haplotype IL-10.1, which comprises the -2763A allele and is associated with low levels of IL-10 in vitro,27,10 displayed a poor clinical outcome. Our data are in accordance with previous studies in mice in which an association between low IL-10 levels and an unfavorable outcome of bacterial corneal ulcers8,9,28 was seen. In addition, the IL-10 promotor polymorphisms A-2763C and IL-10.1 haplotype are associated with the duration of epithelial defect and medication. Interestingly, a trend was seen for a favourable clinical outcome in patients with IL-10.5 (Figure 2), which is related to higher transcriptional activity of the IL-10 gene.10 Although our study size is small, our observations are in alignment with earlier reports that the destruction seen in infectious corneal ulcers is partly caused by the immune system itself. High IL-10 levels could prevent excessive inflammation in response to an infection and therefore result in a less severe clinical appearance. However, replication of our findings in another study involving larger sample populations with similar clinical outcomes will shed further light on the generality of these findings. Nevertheless, it can be suggested that IL-10 therapy may be of additional value in the treatment of infectious corneal ulcers.

In conclusion, IL-10 promotor polymorphisms associated with low IL-10 levels could possibly be protective against infectious corneal ulcers, while IL-10 promotor polymorphisms associated with high IL-10 levels may regulate excessive inflammation and thereby contribute to a favourable clinical outcome.



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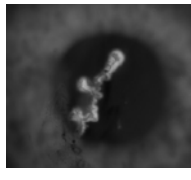

SUMMARY

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.

SAMENVATTING

Veel oogziekten van het voorsegment kunnen leiden tot een visuele handicap of zelfs tot blindheid. Een normale anatomie en fysiologie van het oogoppervlak en het voorsegment is een vereiste voor een goede gezichtsscherpte. In dit proefschrift onderzoeken we verschillende voorsegmentziekten welke de normale anatomie van het oogoppervlak kunnen verstoren. Tevens onderzoeken we nieuwe analysemogelijkheden van deze ziekten. De limbus speelt een centrale rol in het onderhouden van een normaal oogoppervlak, het is de plaats waar de cornea-epitheelstamcellen zich bevinden en het cornea-epitheel zich vernieuwt. Het onderwerp van Deel I van dit proefschrift is limbusstamceldeficiëntie, waar nieuwe mogelijkheden in het limbusonderzoek worden geëxploreerd. Herhaaldelijke chirurgie in de limbusregio is een oorzaak van limbusstamceldeficiëntie, hetgeen vaak wordt gezien bij melanocytaire laesies van de conjunctiva. Conjunctivamelanoom en benigne melanocytaire laesies zoals primary acquired melanosis (PAM) en conjunctivanaevi zijn het onderwerp van Deel II. Daar focussen we op detectie en differentiatie methoden voor conjunctivamelanomen. In het bijzonder richt onze aandacht zich op cytologie, wat een minimaal invasieve methode is en daardoor ook minimale schade aanricht aan het oogoppervlak. Herpes simplex virus (HSV)-keratitis en infectieuze cornea-ulcera kunnen ook allebei het cornea-oppervlak ernstig beschadigen. We onderzochten (Deel III) de invloed van genetische verschillen in het lactoferrine en IL-10-gen bij patiënten met infectieuze corneaziekten en tussen deze patiënten en gezonde individuen.

Limbusstamceldeficiëntie (Deel I)

Het accuraat volgen van limbustransplantaten in diermodellen is moeilijk omdat de afstoting van een transplantaat niet altijd klinisch zichtbaar is. Met Enhanced Green Fluorescent Protein (E-GFP) waren we in staat een diermodel te ontwikkelen waar we de limbustransplantaten accuraat in vivo mee konden volgen (hoofdstuk 2). Het in vivo volgen van de transplantaten creëerde een grote hoeveelheid data van het zelfde proefdier op verschillende momenten in de tijd. Naast transplantaatoverleving kan ook het groeipatroon van het transplantaat worden onderzocht. Een bijkomend voordeel van het E-GFP-model is de reductie van het aantal proefdieren dat nodig is doordat de transplantaten beter gevolgd kunnen worden.

Hoofdstuk 2 beschrijft ook de verlenging van transplantaatoverleving door subconjunctivale injecties met clodonaatliposomen. Zonder immunosuppresieve therapie overleefden allogene en syngene transplantaten niet langer dan 14 dagen. Dit illustreert de nadelige immunogene werking van dit E-GFP diermodel. In de toekomst kunnen andere en nieuwe behandelmogelijkheden in dit limbustransplantatiemodel worden getest. Het E-GFP dat is ingebouwd in het limbustransplantaat maakt het diermodel bruikbaar voor nieuwe afbeeldingsmogelijkheden met behulp van fluorescentie. De confocale fluorescentie- microscoop genereert veel meer data dan de normale fluorescentiemicroscopie. De laser van de confocale fluorescentiemicroscoop maakt het mogelijk om ook diepere weefsellagen te bekijken. Met behulp van een computerprogramma is het mogelijk om een driedimensionale afbeelding te creëren. Deze driedimensionale afbeeldingen geven nieuwe inzichten in de groei van bloedvaten, zoals te zien is in hoofdstuk 2. Wij vonden een aanzienlijk aantal E-GFP positieve bloedvaten aan de onderkant van het transplantaat, die per definitie door het transplantaat zelf moe-

ten zijn gevormd. Confocale microscopie kan mogelijk ook worden gebruikt in de kliniek bij patiënten met limbusstamceldeficiëntie, en mogelijk ook bij patiënten met limbustransplantaten.
Vandaag de dag zijn nieuwe, zeer dure technieken ontwikkeld om patiënten met limbusstamceldeficiëntie te behandelen. Het kweken van patiënt-eigen limbusstamcellen zal het traditionele limbustransplantaat, dat bestaat uit een deel conjunctiva en deel cornea, grotendeels vervangen. Deze nieuwe gekweekte limbusstamcellen kunnen op eenvoudige wijze geïncorporeerd worden in het E-GFP diermodel. Daarnaast kunnen immunofluorescentiekleuringen samen met het E-GFP nieuwe inzichten geven in de acceptatie en groei van het transplantaat. Ook de nieuwe theorie over limbusstamcelcrypten kunnen worden onderzocht met dit nieuwe diermodel.

Gepigmenteerde conjunctiva laesies (Deel II)

Het conjunctivamelanoom en PAM zijn berucht om hun recidieven. Patiënten worden daarom ook regelmatig onderzocht en gebiopteerd. Deze veelvuldige invasieve ingrepen kunnen schade geven aan het oog en aan de limbus in het bijzonder, aangezien veel van deze laesies bij de limbus gelegen zijn. Daarom hebben wij in hoofdstukken 4, 5 en 6 nieuwe minder invasieve methodes om conjunctivale melanomen te diagnosticeren onderzocht. De nationale studie naar conjunctivamelanomen (hoofdstuk 3) beschrijft de overleving, de risicofactoren voor overlijden, de lokale recidieven en de metastasen op afstand; deze zijn gelijkluidend aan eerder gepubliceerde gegevens. . De belangrijkste risicofactor voor overlijden zijn tumorlokalisatie en tumordikte; tumoren met een niet epibulbaire lokalisatie en dikkere tumoren hebben een slechtere overleving. De niet-epibulbaire lokalisatie was ook een risicofactor voor locale recidieven, wat kan worden verklaard door de moeilijkere toegankelijkheid van deze tumoren voor de chirurg. Hoofdstuk 3 laat ook zien dat lokale recidieven mogelijk kunnen worden verminderd als er naast de excisie ook lokaal brachytherapie met Iridium of Strontium wordt toegepast. In de laatste decennia zijn verscheidene primaire behandelingsmogelijkheden voor conjunctivamelanomen toegepast, echter betrouwbare gerandomiseerde studies ontbreken in verband met de lage incidentie van deze tumor. Grootschalige internationale samenwerking is nodig om verschillende behandelingsmogelijkheden en de klinische uitkomsten daarvan te onderzoeken. Voor de clinicus is het belangrijk om bij patiënten met gepigmenteerde conjunctivalaesies de caruncula, fornix, en palpebrale conjunctiva uitvoerig te onderzoeken. Bij de minste verdenking op een conjunctivamelanoom zijn aanvullende onderzoeken zoals cytologie en histologie noodzakelijk.

Cytologie

In een vroeg stadium kan het moeilijk zijn een conjunctivamelanoom te onderscheiden van een conjunctivale naevus, net zoals de ontwikkeling van een conjunctivamelanoom uit een PAM laesie. Alhoewel histologie de gouden standaard is om een conjunctivamelanoom te diagnosticeren, laten wij in hoofdstuk 4 zien dat cytologie een alternatief kan zijn. In een conjunctivamelanoom rijzen atypische cellen naar het epitheliale oppervlak, waar ze toegankelijk zijn voor cytologische afname. De sensitiviteit, specificiteit en negatief voorspellende waarde (respectievelijk 85%, 78% en 93%) zijn acceptabel, echter de positief voorspellende

waarde is laag (59%), waardoor een aanzienlijk aantal valspositieve uitslagen ontstaat. Toekomstig onderzoek moet uitwijzen of herhaaldelijke cytologische uitstrijken de voorspellende waarden doen toenemen. Een groot voordeel van cytologie is het beperkte invasieve karakter van de techniek, waardoor in kortere tijd meerdere cytologische uitstrijken kunnen worden afgenomen. Een biopsie daarentegen kan niet eindeloos worden herhaald, het zorgt voor destructie van conjunctivaweefsel, is minder aangenaam voor de patiënt en kost meer tijd en middelen dan cytologie. Cytologie is daardoor mogelijk meer kosteneffectief.
Een nadeel van exfoliatiecytologie is de mogelijke lage opbrengst van cellen waardoor de beoordeling wordt bemoeilijkt. De Biopore membraan is een apparaat dat wel in staat is grotere hoeveelheden cellen af te nemen van het oogoppervlak (impressiecytologie), echter de grote omvang van het apparaat bemoeilijkt afname in de fornix en bij de caruncula (hoofdstuk 5). Exfoliatiecytologie en Biopore impressiecytologie zorgen voor additionele data voor de oogarts, waarmee waarschijnlijk een betere beslissing genomen kan worden over de behandeling. Slechts een kleine minderheid van de grotere oncologische centra hebben cytologie voor oppervlakkige oogtumoren tot hun beschikking. Wij onderstrepen de uitspraak van Singh dat alle grotere oogheelkundige centra impressiecytologie (Biopore) en/of exfoliatiecytologie tot hun beschikking zouden moeten hebben. Een voorwaarde is tevens de aanwezigheid van een ervaren cyto-patholoog. In de toekomst kunnen nieuwere technieken zoals de confocale microscopie de clinicus mogelijk helpen te differentiëren tussen PAM, conjunctivanaevus, en conjunctivamelanoom. Betrouwbare data over de sensitiviteit en specificiteit ontbreken nog voor de confocale microscopie.

Differentiatie

Differentiatie van een conjunctivamelanoom van een benigne gepigmenteerde laesie door middel van histologie kan soms moeilijk zijn, zeker bij adolescente patiënten. Daarom kan een betrouwbare marker voor het conjunctivamelanoom hulp bieden. In hoofdstuk 6 is S100A1 als mogelijke kandidaat marker naar voren gekomen om een conjunctivamelanoom te onderscheiden van een naevus. Extra onderzoek is nodig om uit te vinden of S100A1 ook in staat is een Spitz naevus te onderscheiden van een conjunctivamelanoom. Dit kan van extra waarde zijn omdat deze twee gepigmenteerde laesies histologisch zeer op elkaar kunnen lijken. Tevens kunnen S100A1 en S100B mogelijke kandidaat-serummarkers zijn voor het vroeg ontdekken van conjunctivametastasen.

Cellijn

Al het voorgaande onderzoek is beperkt geweest door de lage incidentie van het conjunctivamelanoom. Cellijnen van een conjunctivamelanoom kunnen helpen om de kennis over de tumor te verbeteren. In hoofdstuk 7 wordt de vierde conjunctivamelanoom-cellijn ter wereld beschreven. Het is een stabiele cellijn met een relatief hoge celdeling en een sterk verstoord karyogram, mogelijk veroorzaakt door de oorsprong van de cellijn uit een recidief- conjunctivamelanoom na excisie en lokale brachytherapie. Wanneer alle vier de conjunctivamelanoomcellijnen worden gecombineerd, kunnen nieuwe onderzoeksgebieden worden verkend, zoals genomics en proteomics. Ook kunnen diermodellen voor conjunctivamelanomen worden ontwikkeld met behulp van een cellijn.

Cornea infecties (Deel III)

HSV (Herpes Simplex Virus)

Het recidiverende karakter van HSV-keratitis, in het bijzonder dat van de immuunstromale en stromaal necrotische keratitis, zorgt voor een aanzienlijke schade aan het hoornvlies. Zij zijn daardoor ook verantwoordelijk voor een groot deel van de blindheid en slechtziendheid in de westerse wereld, ondanks de lage prevalentie (0.15%). Veel facetten van de herpes keratitis zijn niet goed begrepen, waaronder de lage prevalentie, terwijl de meeste individuen herpes virus in hun traanvocht blijken te hebben. Daarnaast ontbreekt een goede verklaring voor de grote spreiding in recidieffrequenties bij HSV-keratitispatiënten. Aangezien de meeste patiënten HSV in hun tranen hebben, zal een zeer effectief anti-HSV mechanisme in óf de traanfilm óf het oogoppervlak aanwezig moeten zijn om deze aandoening te voorkomen. Lactoferrine hebben wij onderzocht als een van de kandidaten die invloed kan hebben op het ontstaan en de ernst van HSV-keratitis. In diermodellen is een duidelijke relatie gelegd tussen lactoferrine en HSV-keratitis, we hadden daarom een lagere lactoferrineconcentratie verwacht in de HSV- patiëntengroep. We hebben dit echter niet kunnen aantonen (hoofdstuk 8). Ook was er geen relatie met de ernst van de HSV-infectie. Een relatie tussen het ontstaan van HSV- keratitis en lactoferrinegen-polymorfisme op positie 561 is wel aangetoond. Allel Asp561 lijkt een beschermende factor te zijn. Polymorfisme Glu561Asp is niet geassocieerd met de ernst van een HSV-infectie. De verschillende structuur van het Asp561 lactoferrine kan mogelijk de oorzaak zijn voor het verschil in vatbaarheid voor het Herpes Simplex Virus. In de toekomst moeten in vivo- en in vitrostudies met recombinant Asp561 en Glu561 lactoferrine deze theorie bevestigen. Aangezien HSV-keratitis een complex proces is waarbij veel cytokines en chemokines betrokken zijn, is het waarschijnlijk dat andere eiwitten dan lactoferrine ook van invloed zijn op het ontstaan van HSV-keratitis. Interleukine(IL)-10, IL-12 en INF-gamma zijn voorbeelden van eiwitten die invloed kunnen uitoefenen op HSV infecties. Polymorfismes in deze genen kunnen kandidaat zijn voor toekomstig onderzoek. Niet alleen gastheerfactoren beïnvloeden HSV-infecties, verschillende HSV-stammen kunnen ook verantwoordelijk zijn voor verschillen in ernst van de keratitis. Op dit moment worden verschillende IL-10-gen polymorfismes onderzocht in de HSV-patiënten groep.

Cornea ulcus

In tegenstelling tot HSV-infecties worden de oorzaken van infectieuze cornea-ulcera beter begrepen. Trauma's door een corpus alienum of contactlenzen zorgen voor epitheliale defecten in het hoornvlies die als porte d'entrée dienen voor micro-organismen. Veel individuen ondervinden kleine trauma's aan het hoornvlies en een klein deel daarvan ontwikkelt een infectieus cornea ulcus. De hoeveelheid micro-organisme, virulentie van het organisme en het immuunsysteem spelen allemaal een rol in het ontstaan van een cornea-infectie. Vooral bij dragers van contactlenzen met een matige hygiëne is de hoeveelheid bacteriën groot, waardoor zij vatbaarder zijn voor infecties. Bovendien worden vaak virulente micro-organismen gekweekt uit de bewaarbakjes van de contactlenzen.

Lactoferrine gen polymorfisme

Lactoferrine, behorend tot het `innate` immuunsysteem, beïnvloedt cornea-infecties door de antibacteriële en immuunmodulerende eigenschappen. De lactoferrine-polymorfismes die aan de orde zijn gekomen in hoofdstuk 8 hebben we ook onderzocht in hoofdstuk 9 bij patiënten met een infectieus cornea-ulcus. Tussen patiënten met een cornea-ulcus en gezonde proefpersonen zijn geen verschillen in frequentie van lactoferrine-polymorfismes gevonden. Dit in tegenstelling tot de HSV-keratitis-patiëntengroep. Waar bij HSV-keratitis het cornea-epitheel initieel intact is, is bij het cornea-ulcus waarschijnlijk eerst een epitheeldefect nodig voordat de infectie tot stand kan komen. Het is daardoor minder aannemelijk dat lactoferrine invloed heeft op het ontstaan van een infectieus cornea-ulcus, alhoewel lactoferrine wel mogelijk invloed heeft op de hoeveelheid bacteriën op het oogoppervlak. We hebben wel een trend gevonden voor een tragere epitheliale genezing in patiënten met een Glu561 lactoferrine allel. Dit geeft aan dat het Glu561Asp polymorfisme waarschijnlijk een functioneel effect heeft, zoals we ook al hebben gezien in hoofdstuk 8. In de toekomst kunnen verschillen in wondgenezing, anti-inflammatoire en antibacteriële effecten van de verschillende lactoferrines in vitro en in vivo worden onderzocht met behulp van recombinante lactoferrines.

IL-10 promotor gen polymorfismes

Zowel het infecterende micro-organisme als het immuunsysteem zijn verantwoordelijk voor schade aan het hoornvlies in infectieuze cornea-ulcera. IL-10 is een sterke suppressor van het immuunsysteem, en polymorfismes in de promotorregio van het IL-10 gen zorgen voor verschillen in IL-10 expressielevels in vitro. Daarom hebben wij onderzocht of IL-10 polymorfismes -C819T, -G1082A, -A2763C, en -A2849G invloed uitoefenen op infectieuze cornea ulcera (hoofdstuk 10). Het IL-10 -819C allel en -2849AA genotype bleken een beschermend effect te hebben op het ontstaan van een cornea-ulcus. Het -2849AA genotype is geassocieerd met lagere IL-10 waarden, waardoor het locale immuunsysteem mogelijk beter in staat is geweest een cornea ulcus te voorkomen. We hebben in hoofdstuk 10 ook laten zien dat, wanneer er eenmaal een infectie tot stand is gekomen, het gunstiger is om een genotype te hebben dat geassocieerd is met hogere IL-10 waarden. Met andere woorden: patiënten met het 2763A allel of IL-10.1 haplotype – beide geassocieerd met lage IL-10 waarden – hebben een ernstiger klinisch beeld, wat wil zeggen grotere ulcera, langere duur van het epitheliaal defect en langere duur van de behandeling. Het omgekeerde was te zien voor haplotype IL-10.5 dat geassocieerd is met een hoge IL-10 productie. Of deze polymorfismes ook een invloed hebben op de lokale IL-10 productie bij het oog is niet bekend. In theorie kunnen hogere IL-10 waarden zorgen voor een demping van de inflammatie en daardoor voor minder schade aan het hoornvlies. In de toekomst moet worden uitgezocht of deze polymorfismes van invloed zijn op de lokale IL-10 productie en ook of IL-10 als lokaal adjuvante therapie de ernst van de infectie kan beperken. Naast de lactoferrine en IL-10 gen polymorfismes kunnen andere gen polymorfismes ook van invloed zijn op infectieuze cornea-ulcera. Mogelijk kunnen we in de toekomst een risicoprofiel ontwikkelen op basis van verschillende gen-polymorfismes.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 27 april 1977 te Amsterdam. In 1995 haalde hij het eindexamen VWO aan het Fioretti College te Lisse. In hetzelfde jaar werd hij uitgeloot voor de studie geneeskunde en begon met de studie Medische Biologie aan de Universiteit van Amsterdam. In 1996 werd de propedeuse Medische Biologie behaald en na te zijn ingeloot begonnen met de studie Geneeskunde aan de Universiteit van Leiden. Het doctoraal examen werd in 2001 behaald en het arts examen werd in 2003 afgelegd. Tijdens zijn studie is hij een jaar bestuurslid geweest van de studievereniging M.F.L.S., waar hij ook verschillende evenementen heeft helpen organiseren. In 2003 is hij begonnen met promotieonderzoek aan de afdeling Oogheelkunde aan het Leids Universitair Medisch Centrum onder leiding van Prof. dr. R.J.W. de Keizer, Dr. M.J. Jager en Prof. C.C. Sterk. Dit onderzoek naar verscheidene oogziekten in het voorsegment heeft geresulteerd in dit proefschrift. In 2005 is hij aangenomen voor de opleiding tot oogarts aan het Leids Universitair Medisch Centrum (Prof. C.C. Sterk, Prof dr. G.P.M. Luyten).