Herpetic Keratitis in Humans: Interaction between Virus and Host

Rui Duan

The research described in this thesis was conducted at the Department of Virology of the Erasmus Medical Center in Rotterdam, The Netherlands. These studies were funded by grants from the Stichting Wetenschappelijk Onderzoek Oogziekenhuis Prof. Dr. H.J. Flieringa, Algemene Nederlandse Vereniging ter Voorkoming van Blindheid, Hoornvlies Stichting Nederland, Stichting OOG and Stichting voor Ooglijders and the International Consortium on Anti-Virals.

Printing of this thesis was financially supported by:
ViroVentures companies (consisting of ViroClinics BV, CoroNovative BV & ViroNovative BV), U-CyTech biosciences, Becton Dickinson Benelux N.V.

Design of the cover: Tommy Wang

ISBN: 978-90-5335-189-5
Printed by: Offsetdrukkerij Ridderprint B.V., Ridderkerk, The Netherlands

Herpetic Keratitis in Humans: Interaction between Virus and Host

Herpetische Keratitis in de mens: interactie tussen virus en gastheer

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the rector magnificus

Prof.dr. S.W.J. Lamberts

and in accordance with the decision of the Doctorate Board

The public defense shall be held on
Tuesday 30th June 2009 13:30 o'clock

By

Rui Duan

Born in Beijing, China

Doctoral Committee

Promotor:
Prof.dr. A.D.M.E. Osterhaus

Copromotors:
dr. G.M.G.M Verjans
dr. L . Remeijer

Other members:
Prof.dr. C.A.B. Boucher
Prof.dr. G. van Rij
Prof.dr. H.G.M Niesters

Table of Contents

Chapter 1

General Introduction

Human Herpesviruses

Morphology and classification

Our first knowledge of human herpes can be traced back to the ancient Greeks, who coined the phrase: 'herpes'. Hippocrates used this term to describe lesions that appeared to creep or crawl along the skin. The virus that causes this condition, herpes simplex virus (HSV), has been described in more detail over the past 3 decades. The spectrum of herpetic disease continues to expand. Nowadays, the structure of this virus is well documented (Figure 1).

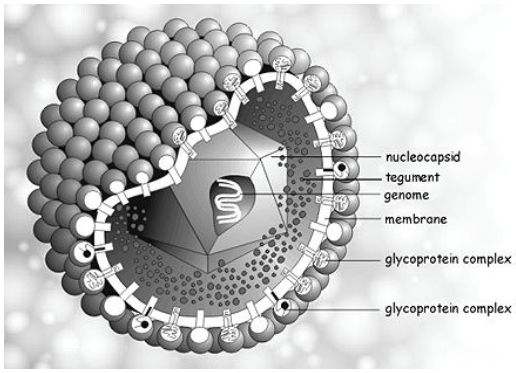


Figure 1. Diagram of the herpesvirus virion structure.
(Adapted from website: http://web.njit.edu/~pkb3/)

Herpesviruses are linear double-stranded DNA viruses, consisting of an envelope, a tegument, a nucleocapsid, and a core. The size of herpesvirus virions varies from 125-260 nm, and the shape varies from spherical to pleomorphic. Virus-encoded glycoproteins, exhibited as spikes are embedded in the envelope, which wraps the capsid. Herpesviruses encode a large group of enzymes involved in nucleic acid metabolism, DNA synthesis, and assembling capsid into the cell nucleus. Virions are processed in the cytoplasm. Production of infectious virus accompanies the destruction of the infected cells. All herpesviruses are able to remain latent in the host.

More than 100 different herpesviruses are known to infect vertebrates. Only 8 of them can infect humans: human herpesvirus 1 to 8 (HHV1-HHV8). They are classified into three subfamilies based on their biological properties and DNA sequence homology: alpha-, beta-, gamma-herpesvirinae (Table 1).

Table1. Subfamilies of human herpesviruses

Type	Synonym	Subfamily	Primary Target Cell	Site of Latency
HHV-1	Herpes simplex virus-1 (HSV-1)	α	Mucoepithelial	Neurons
HHV-2	Herpes simplex virus-2 (HSV-2)	α	Mucoepithelial	Neurons
HHV-3	Varicella zoster virus (VZV)	α	Mucoepithelial	Neurons
HHV-4	Epstein-Barr virus (EBV)	γ	B-cells and epithelial cells	B-cells
HHV-5	Cytomegalovirus (CMV)	β	Monocyte, lymphocyte, and epithelial cells	Monocytes, lymphocytes
HHV-6	Roseolovirus	β	T-cells and epithelial cells	T-cells
HHV-7	Roseolovirus	β	T-cells and epithelial cells	T-cells
HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	γ	Lymphocyte and other cells	B-cell

Alphaherpesvirinae

Members of this subfamily have a variable host range and a relative short reproductive cycle. They spread rapidly in culture, destruct infected cells, and establish latent infections in neural tissue. Human alphaherpesvirinae include herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella zoster virus (VZV).

Betaherpesvirinae

Members of this subfamily have a more restricted host range, a long reproductive cycle and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalia). Betaherpesviruses establish latent infections in secretory glands, lymphoreticular cells and kidneys. Human betaherpesvirinae include cytomegalovirus (HHV-5), human herpesvirus type 6 (HHV-6), and HHV-7.

Gammaherpesvirinae

The host range of this subfamily is the most restricted of all the herpesviruses. Gammaherpesvirinae predominantly infect B and T cells. They establish latent infections in lymphoid tissues. Human gammaherpesvirinae include: Epstein-Barr virus (EBV, HHV-4), and HHV-8 [1].

Genomic Organization of HSV

Herpes simplex virus is the prototype of human herpesviruses, and includes two serotypes: HSV-1 and HSV-2. HSV is the first herpesvirus discovered in humans and is the most extensively investigated among all herpesviruses. The natural host of HSV are humans and the virus can be cultured easily *in vitro* to high cell-free virus stocks. The ability to cause various infections in different hosts, including rodents, to remain latent in the hosts, and to reactivate to cause clinical lesions are characteristics of herpes simplex virus[1].

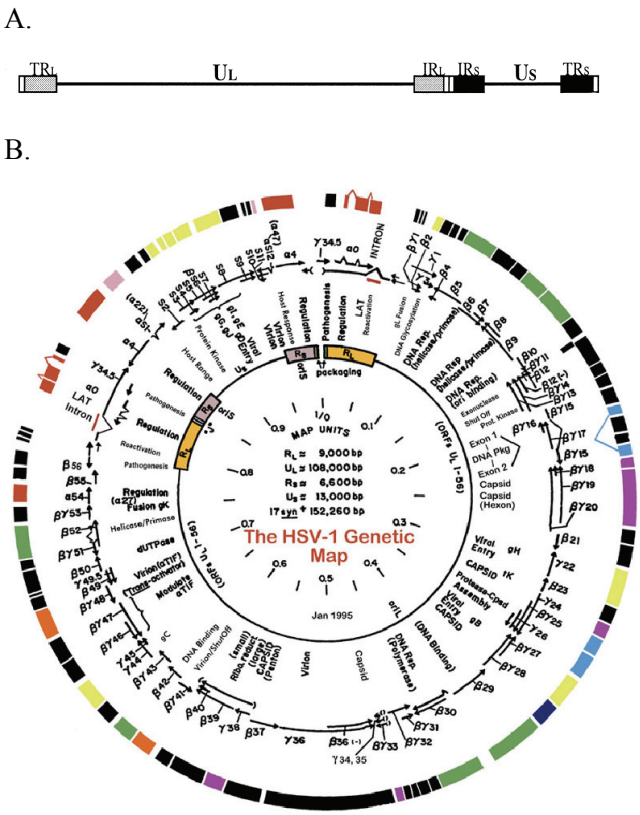


Figure 2. (A). Diagram of HSV-1 genome with its unique long (UL) and unique short (US) regions, which are flanked by inverted repeats (IR and TR) (Adapted from: Maertzdorf J et.1993). (B). Genetic map of HSV-1, indicating the location of genes and the encoding protein
(Adapted from website: http://www.dbc.uci.edu/~faculty/wagner/hsv3f.html).

The genome of HSV is about 150 kb long, with a GC content of 68% and 69% for HSV-1 and HSV-2 respectively. The linear double stranded DNA consists of two covalently linked components, which are designated as unique long (UL) and unique short (US) sequences (Figure 2A). The US and UL sequences are flanked by inverted repeats, that allow the inversion of the two components to create four isomers. The HSV-1 genome encodes at least 84 polypeptides, several of which have multiple functions. Thus, the HSV genome encodes more than 100 different functions [2] (Figure 2B). The tegument proteins and glycoproteins are considered the major targets of the host defense [3, 4].

Epidemiological unrelated HSV strains can be distinguished at the DNA level either by nucleotide mutations or by the difference of variability in the number of repeated sequences located within specific regions of the viral genome: US1, US4,

US7, US8 and US12 [5]. Variations in the number of these reiterated sequences located within the introns of US1 and US12 have been used to distinguish between different HSV-1 strains in epidemiological studies [5, 6]. Hayward *et al*. were the first to suggest that restriction endonuclease pattern could be used to differentiate between HSV strains [7]. This method, referred to as restriction fragment length polymorphism (RFLP) analysis, has been used to demonstrate that individuals can be infected with different strains of HSV at the same anatomical site [8]. A limitation of the RFLP method, however, is that it is laborious and needs infectious virus. The workload and the requirement of infectious virus, have limited its application in clinical studies. To overcome these shortcomings, Maertzdorf J *et al*. have developed a PCR-based technique to differentiate HSV-1 strains. This PCR-based assay employs amplification of the hypervariable regions containing introns of US1 and US12 genes to get variable amplicon lengths from different HSV-1 strains [9]. Recently, Norberg *et al.* have designed a PCR-RFLP assay to classify clinical HSV-1 isolates into three distinct genotypes A, B and C. The classification was based on different restriction enzyme cleavage patterns in distinct regions of the HSV-1 viral US4, US7, and US8 genes [10].

Replication and latency of HSV

Attachment and penetration

The initial step of HSV infection is the attachment of the virus to the cell membrane. The cell surface receptors include HVEM (herpes- virus entry mediator); a member of the TNF receptor family), nectin-1 and -2 (members of the immuno- globulin superfamily), and heparan sulphate [11, 12]. The HSV ligands for heparan sulphate are glycoproteins B (gB), gC, and gD. Glycoprotein D is the ligand of HVEM, nectin-1 and nectin-2 [3]. Recently Satoh T *et al.* demonstrated that the paired immunoglobulin-like type 2 receptor α (PILRα) is an additional receptor that associates with HSV-1 gB during the attachment [13].

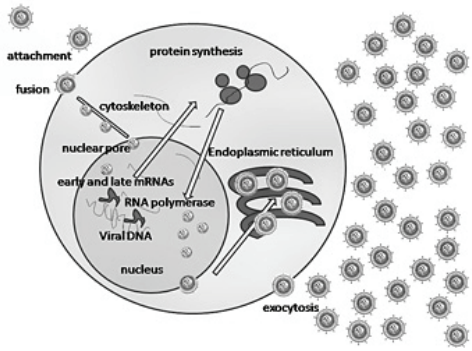


Figure 3. A simplified diagram of HSV replication (Adapted from website: http://www.bioedonline.org/hot-topics/stds.cfm).

Virus penetration involves direct fusion of the viral envelope with the host cell membrane. Viral gB and the heterodimer gH/gL are essential in the fusion step [3]. Upon penetration the viral capsid-tegument structure is released into the cytoplasm where the capsid-tegument structure subdivides into their respective parts. The tegument proteins

execute crucial functions for the initiation of viral replication, while the capsids are transported to the host nuclei at which viral DNA is released.

Gene expression

HSV transcription and protein synthesis is temporally regulated. HSV genes are divided into 3 groups based on their time and requirements for expression: α, β and γ proteins (Figure 4). In the immediate-early phase (alpha-phase, 2-4 hours post-infection), five alpha genes are expressed. They encode for infected cell proteins (ICPs): ICP0, ICP4, ICP22, ICP27 and ICP47. Alpha genes are important in priming the cell for subsequent viral gene expression and to mobilize the cellular transcription machinery. Beta genes are expressed in the early phase (beta-phase, 5-7 hours post-infection) and precede the onset of viral DNA synthesis. They are either directly or indirectly involved in genome replication. During the late phase (gamma-phase, timing depends on viral DNA synthesis) viral structural proteins are expressed in high abundance. Gamma proteins are involved in assembling the capsid in the nucleus and modifying the membrane for virion formation [4].

Assembling and processing

Viral capsids assemble in the nucleus and bud into the cytoplasm from the nuclear membrane by a process of envelopment and de-envelopment. Virions acquire their final envelope by budding through the membrane of the trans-Golgi network [14, 15]. Enveloped infectious virions can either remain cell-associated and spread directly from cell-to-cell, or are released from the infected cells as cell-free virus [15] (Figure 4).

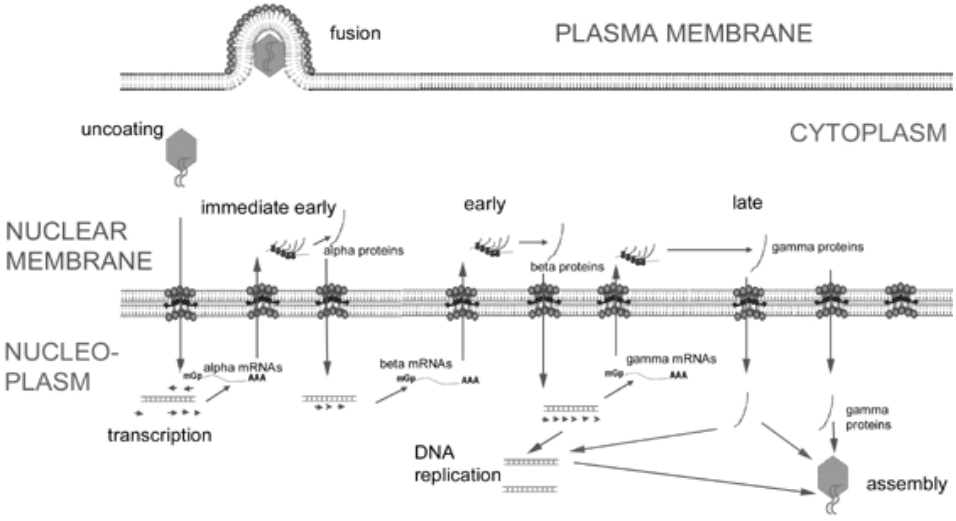


Figure 4. Expression of immediate early, early and late genes during lytic HSV infection (Adapted from website: http://pathmicro.med.sc.edu/mhunt/dna1.htm).

Latency

Latency is a state of infection in which the viral genome is present in a non-replicating state in an infected cell from which the virus can reactivate intermittently. The ability of HSV to establish latency in the ganglia of the peripheral nervous system and periodically reactivate is critical to its survival and spread in human populations [16]. The confirmed sites of HSV-1 latency are sensory neurons within the sensory ganglia innervating the site of primary infection [17]. Following replication in mucosal cells, HSV enters sensory neurons by fusion at the axonal termini. The capsid, which contains viral DNA, is carried by retrograde axonal transport to the nucleus in the cell body of neurons. Non-neuronal sites of latency may exist, and some groups advocate the cornea as extra-neuronal site of HSV-1 latency [18, 19]. During the latency phase, the lytic gene expression is repressed and the latency-associated transcript (LAT) is overtly expressed.

There is growing evidence that the HSV-1 latency in sensory ganglia is controlled by the adaptive immune system. The function of $CD8^+$ T cells in HSV-1 has been demonstrated both in mice and humans. HSV-specific $CD8^+$ T cells inhibit HSV-1 reactivation by means of IFN-γ or cytolytic effector molecules such as perforin and granzyme B [20-23]. The establishment and maintenance of HSV latency is an interaction between the virus, neurons and a local virus-specific T-cells response.

Reactivation

Reactivation of latent HSV-1 can be induced by local stimuli, such as trauma, tissue damage and UV light exposure, or by systemic stimuli, like stress, hyperthermia, and hormonal imbalance [24]. During these events virus particles are carried by axonal transport back to the peripheral tissue; usually to the cells at or near the site of primary infection. Some productive phase transcripts and proteins can be detected in sensory neurons, but infectious virus only appears at the periphery. Reactivation may vary in severity from asymptomatic to severe recurrent disease lesions, depending on the virus load and host immune status. The frequency of reactivation has been reported to correlate with the severity of the primary infection and the number of neurons harboring latent virus [25]. In experimental models virus reactivation can be achieved by experimental methods, like nerve dissection, corneal scarification, UV-irradiation, cellotape stripping [26], hyperthermic stress [27] or *in vitro*, by culture of latently infected ganglia.

Disease manifestations and epidemiology of HSV infections

Irrespective of the HSV serotype, HSV primarily affects skin and mucous tissues by the orofacial route. Primary and recurrent infections of HSV and VZV differ in their clinical presentation. Primary VZV infection causes disseminated disease (varicella or chickenpox)

and the recurrent VZV infection is largely confounded to one or a few more dermatomes (shingles or herpes zoster). In contrast, HSV causes more localized infections principally at the same anatomic location during both primary and recurrent disease. Clinical lesions appear 2-20 days after primary infection and last for about 1-3 weeks. Whereas only 20% of the primary infection cases develop clinical disease, virus shedding and subsequent transmission can occur in both clinical and subclinical cases [28]. Severity of HSV infection depends on a variety of viral and host factors such as virus strain, virus dose, route of entry and release, replication rate, and the age and immune status of the host [29, 30]. HSV-1 accounts for the majority of non-genital HSV-induced infections in humans, with 60% to 80% of the world population reportedly HSV-1 seropositive [28]. Although most genital HSV infections are historically caused by HSV-2, today an increasing proportion is attributed by HSV-1 [31, 32].

Epidemiology of ocular HSV infections

Ocular manifestations are only observed in about 1% of those exposed to HSV-1 infection. Only about 5% of ocular infections are primary infections [33]. The incidence of herpetic eye diseases is reported to be about 8 new cases per 100,000 person-years. Overall, the prevalence of ocular infections has been estimated to be 149 cases per 100,000 person years [34]. The majority of primary clinical diseases appear as blepharitis, conjunctivitis and epithelial keratitis (54-63%). While among the recurrent diseases, which are the main problems to cause irreversible vision loss, infectious epithelial keratitis (IEK) and herpetic stromal keratitis (HSK) are the main clinical manifestations [35, 36]. Recurrence rates of ocular HSV infections have been recorded in one-third up to 63% of patients and are more frequent in children and young adults [37, 38]. Nowadays, the introduction of the antiviral drug acyclovir (ACV), and the combined use of antiviral and immunosuppressive treatment have dramatically improved the visual prognosis of ocular HSV-1 infections [39-41].

Immune response to HSV

Upon HSV infection, the host immune system exhibits two types of responses to clear the virus. A non-specific immune response, referred to as innate immunity, starts immediately after viral infection. Macrophages, nature killer (NK) cells, and polymorphonuclear cells (PMNs) infiltrate the site of infection to provide the first line of defense. This innate immune response will last several days. However, complete viral clearance and control of latency largely depends on the development of an adaptive immune response involving both T- and B-cells. It takes several days to initiate an adaptive immune response and once installed this will last for the whole life of the host [42].

Innate immune response

For the naive host, innate immunity is very important to combat infections. Macrophages and PMNs are the main effector cell types involved and eliminate the virus directly by phagocytosis or indirectly by the secretion of immune-stimulatory cytokines and chemokines like tumor necrosis factor (TNF), interleukin 1 (IL-1), and IL-6 and IL-8, respectively [42]. Nature killer cells exert their anti-viral functions by secreting IFN-γ to inhibit the viral replication [43, 44]. They also recognize viral antigens expressed by HSV-infected cells independent of molecules of the major histocompatibility complex (MHC). Opsonization of virus-infected cells by virus-specific antibody or complement, will lead to recognition and cell killing by macrophages, PMNs and NK cells [45]. The down-regulation of MHC class I, due to HSV-encoded immunemodulatory molecules like ICP47, render infected cells more susceptible to NK cell killing [46]. Cytokines and chemokines secreted by cells of both the innate and adaptive immune system, and the infected cells themselves, are of importance in the defense against viral infections. Cytokines are mainly in charge of inhibiting virus replication, activating lymphocytes, and the induction of MHC class II, and co-stimulatory molecules [42]. Chemokines have a more pro-inflammatory effect and have a pivotal role attracting inflammatory cells to the site of infection [47, 48]. Overall, innate immunity prevents the dissemination of the virus in the early stage of infection, and plays a pivotal role in the initiation of the subsequent adaptive immune response.

Adaptive immune response

Both the humoral and cellular arms of the adaptive immune system play an important role in the control of HSV infections. In humoral immunity, antibodies neutralize free viral particles or opsonise virus-infected cells by complement-mediated cytotoxicity or antibody dependent cytotoxicity (ADCC) to reduce the viral load and the number of virus-infected cells [49]. Herpesvirus glycoproteins and outer capsid proteins represent the targets for neutralizing antibodies. Binding of the neutralizing antibodies prevents viral attachment to host cells. Severe cases of herpesvirus infections observed in patients with antibody deficiency syndromes implicate a pivotal role of antibodies against herpesvirus infections

Generally, T-cells exert their main function to recognize and eliminate virus-infected cells. T-cells can be subdivided into two subsets, $CD4^+$ and $CD8^+$ T-cells. Both T-cell subsets play a central role in antiviral immunity either directly by their cytotoxic properties or indirectly by their secretion of cytokines upon activation, and in the case of $CD4^+$ T-cells, by the stimulation of a virus-specific humoral immune response [50]. Patients with impaired T-cell immunity, like AIDS patients and those under immunesuppresive therapy, usually develop severe herpetic diseases. This finding indicates that cellular immunity plays

an important role in controlling HSV infections [51]. In this system, both $CD4^+$ and $CD8^+$ T-cells are of significance [52, 53].

Pathology of corneal HSV infections

Corneal morphology

The cornea is the most important light-refracting structure of the eye. It produces the initial images and cast them onto the lens behind it. The human cornea is composed of five layers: epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium (Figure 5).

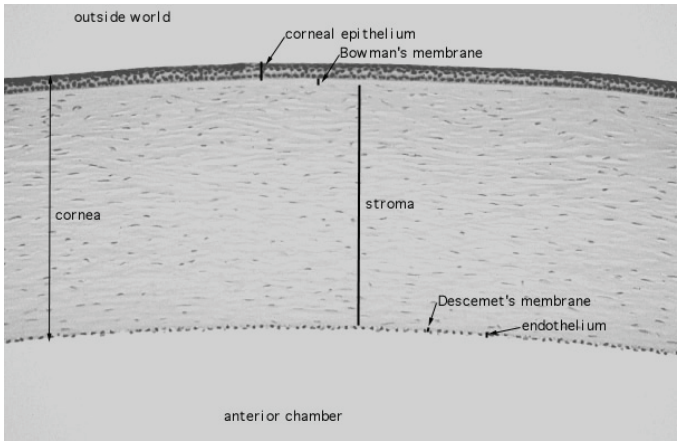


Figure 5. Structure of normal human cornea. (Adapted from http://www.bu.edu/histology/p/08002ooa.htm).

The cornea epithelium is a non-keratinising squamous epithelium, with approximately five to six layers of fast-growing and easily regenerating cells reaching to the thickness of about 50µm. The Bowman's layer is composed of fine collagen fibrils and is about 10µm thick. This acellular layer is limited anteriorly by the basement membrane of the corneal epithelium. It is assumed to provide a barrier to protect the corneal stroma from trauma and tumour cells. The corneal stroma is a 0.5mm thick, transparent middle layer, consisting of regularly arranged collagen fibbers, extracellular matrix, and sparsely populated keratocytes. The extracellular matrix, composed mainly of sulfated glycosaminoglycans, plays an important role in maintaining the regular array of collagen fibrils. Keratocytes are the predominant cells of the stroma. In response to injury, keratocytes migrate into the wounded area and transform into myofibroblasts involved in repairing damaged corneal tissue, which will lead to the scar formation. The Descemet's membrane, which closely resembles the lens capsule, is produced by the endothelium and is about 10µm thick. The endothelium lies on the posterior surface of the cornea and forms the anterior boundary of

the anterior aqueous chamber as a single layer of flattened hexagonally arranged cells. Soluble nutrients in the aqueous humour reach the corneal stroma through a passive movement of water through Descemet's membrane and the endothelium. The cells within these two layers prevent to excessive hydration of the extracellular matrix of the corneal stroma by active dehydration of the cornea.

Ocular HSV-1 infection disease

Among the eight human herpesviruses, HSV-1 and VZV are the most common causes of ocular herpesvirus infections [54]. Both viruses can infect all parts of the human eye [36]. Ocular disease caused by HSV-1 ranges in severity from a self-limiting blepharitis, conjunctivitis, or epithelial keratitis, to potential blinding ocular disease like HSK and acute retinal necrosis.

Corneal HSV-1 infections

The majority of the corneal HSV-1 infections are superficial. In genuine IEK, the virus replicates in the epithelial layer of the cornea and causes dendritic ulcers with a clearly defined and characteristic fractal-like shape. The disease subsides normally without scarring. The deeper layers of the cornea are involved in about 20% of recurrent herpetic keratitis (HK) cases. If the infection involves the deeper layers, it can damage the integrity of Bowman's layer and reach down into the stromal layer to induce HSK, which may lead to scarring of the cornea, loss of vision, and sometimes even corneal blindness [55] (Figure 6). HSK is the most common cause of infectious corneal blindness in developed countries worldwide.

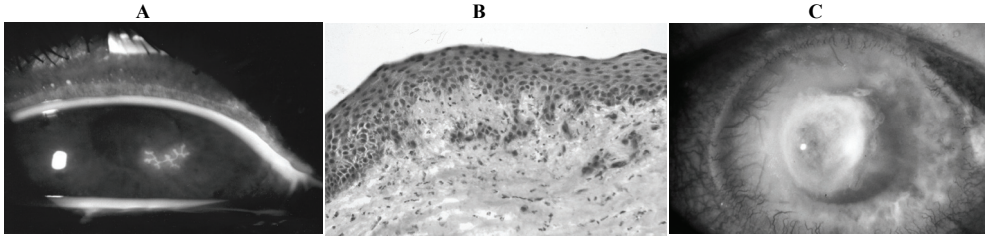


Figure 6. A. Clinical picture of herpetic keratitis. (A), Classical fractal-like epithelial lesions of the cornea of a patient with infectious epithelial keratitis. (B) and (C),in situ and ex vivo pathology of the cornea of the same patient with herpetic stromal keratitis. (B), hematoxylin and eosin staining of the excised cornea showing a dense inflammatory cell infiltrate with hyperplasia of the epithelium. (C), macroscopic picture of the affected cornea showing corneal scarification and edema (Adapted from the thesis of Remeijer L in 2002, Human Herpes Simples Virus Keratitis: The pathogenesis revisited).

Pathology of herpetic stromal keratitis

HSV-1 is a leading cause of corneal disease and loss of vision in humans, largely because of its recurrent nature [40]. Permanent loss of vision is commonly associated with HSK. HSK is characterized as a chronic immunopathogenic disease. The pathology of HSK involves the complex interplay between the triggering virus, corneal resident cells and infiltrating inflammatory cells including T-cells and PMNs. Murine HSK models have provided invaluable insights in the pathogenic immunologic mechanisms involved in the initiation and perpetuation of HSK. The development of HSK in experimentally infected mice can be divided into three stages: the preclinical phase, which starts immediately after the HSV-1 infection until approximately 7 days post infection (dpi); the clinical phase that starts at approximately 7dpi and progresses through 21 dpi and finally the resolution phase, which starts at approximately 21 dpi until at least 40 dpi. In the clinical phase, corneal opacity and neo-vascularisation reach their peak intensity. These symptoms will persist until the end of the resolution phase [56]. Figure 7 summarizes the events involved in murine HSK.

PMNs infiltrate the HSV-1 infected cornea at two different time points during HSK disease. Within 18 hours after virus inoculation, PMNs infiltrate the cornea. Their accumulation is transient and is involved in clearance of infectious virus [57, 58]. After disappearance of the first wave of PMNs, $CD4^+$ T-cells enter the cornea stroma to initiate the clinical phase of HSK. $CD4^+$ T-cells are essential for the development of HSK, orchestrating the extravasation and activation of the second wave of PMNs, which are the main effector cells involved in corneal destruction [59, 60]. Langerhans' cells and macrophages also migrate to the site of infection in early stage of HSK [61, 62].

The immunopathogenic processes in HSK result from the local coordinated host response to the triggering virus involving the following immune cells: dendritic cells, macrophages,

T-cells, PMNs and corneal resident cells. These interactions are coordinated by cytokines and chemokines. Cytokines with known roles in HSK include INF-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-17. TNF-α and IL-1 are pluripotent cytokines that influence several aspects of HSK, including the infiltration of PMNs, MHC class II positive dendritic cells, and T-cells following HSV-1 infection [63, 64]. The infiltration of inflammatory cells is controlled by chemokines that are produced in response to IL-1 and TNF-α by cornea resident cells, like corneal epithelial cells, keratocytes, macrophages, and dendritic cells. Recent studies from our group have established that IL-17 is over-expressed in human HSK corneas, and that the IL-17 receptor is constitutively expressed by corneal fibroblasts. IL-17 and TNF-α synergistically induce the secretion of IL-6, and the PMN-attracting chemokine IL-8 by cultured human corneal fibroblasts [65].

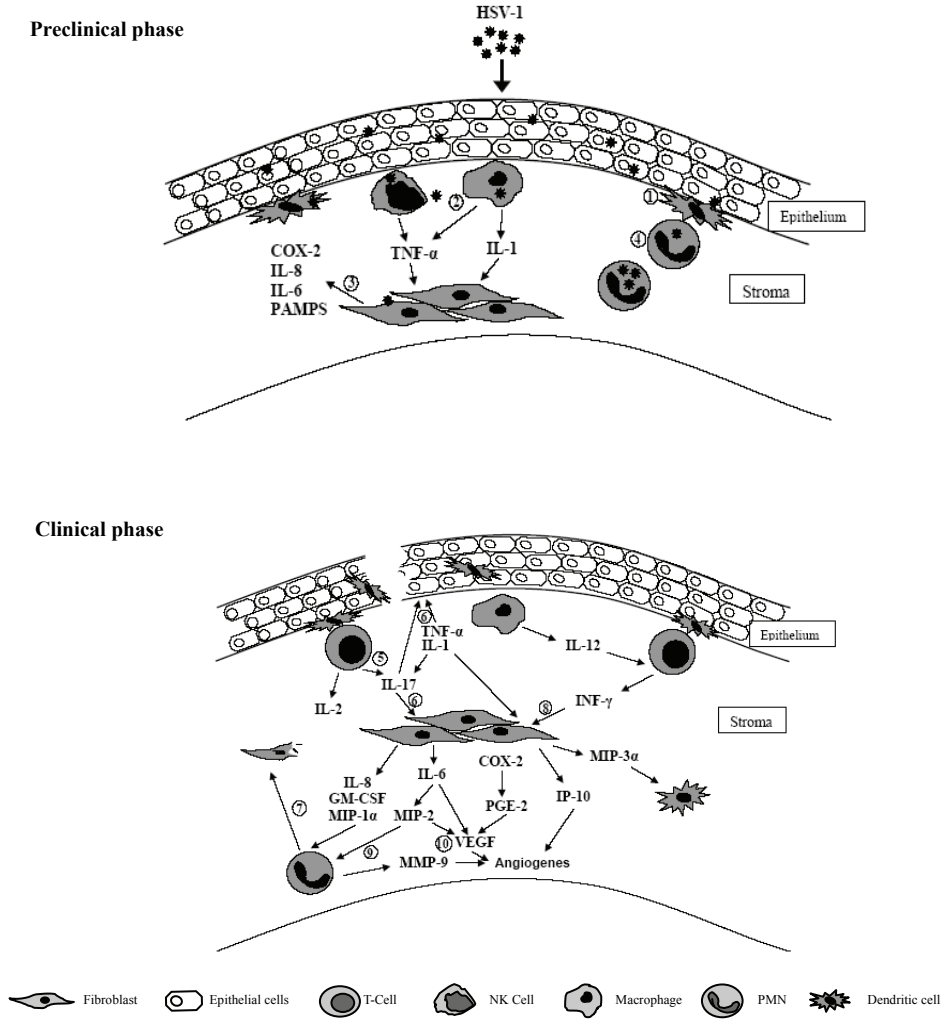


Figure 7. Early events in HSK pathogenesis. HSV-1 infection results in the production of a plethora of proinflammtory cytokines and chemokines early after infection. These cytokines and chemokines create an inflammatory milieu that makes the corneal environment favourable for a prompt inflammatory cell influx. In the preclinical phase: (1) Dendritic cells activated by HSV-1 viral particles. (2) NK cells and macrophages are activated. They kill the HSV-1 virus directly and start to secrete TNF-α and IL-1. (3) Upon the stimulation of IL-1 and TNF-α, corneal fibroblasts start to secrete IL-8, IL-6 and PAMPS. COX-2 is also produced by infected fibroblasts. (4) Upon the attraction of IL-8 and PAMPS, PMNs infiltrate into the cornea to clear up the HSV-1 virus. In the clinical phase: (5) Infiltrated T-cells are activated by HSV-1 or self antigens that are presented by dendritic cells. (6) T-cell express IL-1, combined with other cytokines, secreted by macrophages, stimulate the corneal epithelial cells and fibroblasts to secrete GM-CSF, IL-8, MIP-1α and IL-6. (7) PMNs are activated by GM-CSF and start to kill the infected and none-infected fibroblasts. (8) Macrophages secrete IL-12 and stimulate T-cells to produce INF-γ. INF-γ together with TNF-α induce fibroblasts to produce antiangiogenic factor IP-10. (9) Besides recruitment of T-cells IL-6 also induce MIP-2 production which related to the PMN recruitment. (10) COX-2 induced PGE-2, IL-6, and MIP-2 will induce the corneal angiogenesis via VEGF upregulation.
Abbreviations: IL, interleukin; GM-CSF, Granulocyte macrophage colony-stimulating factor; IFN-γ, interferon gamma; COX-2, cycloxygenase 2; PGE2, prostaglandin E2; MIP, macrophage inflammatory protein; IP-10, interferon inducible protein 10; PMN, Polymorphonuclear neutrophil, NK cells, natural killer cells.

Treatment of corneal HSV infections

Because most cases of IEK resolve spontaneously within 3 weeks, treatment is largely focused to minimize stromal damage and scarring. Gentle epithelial debridement and topical antiviral treatment, like the use of trifluorothymidine (TFT), ACV or gancyclovir (GCV) are recommended to remove the virus and inhibit HSV-1 replication in the corneal epithelium, respectively. Response to topical therapy usually occurs within 2-5 days, with complete resolution in 2 weeks [66]. Failure of epithelial healing after 2-3 weeks suggests antiviral-induced corneal toxicity or drug-resistant corneal HSV-1. Patients with HSK and endotheliitis should receive a combined corticosteroid and antiviral therapy to control the local inflammatory response and to prevent or limit viral replication in the immune-suppressed corneal tissue, respectively. Whereas corticosteroid therapy should be tapered to the lowest dosage necessary to control inflammation, antiviral therapy should be continued until resolution of the lesion [67]. Because corneal cytotoxity is a common adverse effect of topical antiviral treatment, use of systemic ACV, or newer related anti-HSV drugs like valcyclovir and famcyclovir, is increasingly preferred in HK patients. Moreover, systemic ACV prophylaxis decreases the HK recurrences rate of about 45%, particular in patients with a clinical history of HSK, and achieves therapeutic concentrations in the aqueous humor to control HSV keratouveitis [68].

Patients with chronic or recurrent HSK commonly develop visually significant corneal opacities or corneal ulcers. For these patients corneal transplantation, also referred to as penetrating keratoplasty (PKP), is the only therapeutic option for visual rehabilitation [36]. The prognosis for a successful graft in patients with a history of herpetic keratitis is lower compared to non-HK patients. This is largely attributable to surgical trauma- and corticosteroid-induced HSV reactivation of the endogenous latent virus depositing infectious virus in the graft leading to graft failure [69]. Alternatively, post-PKP HK may be caused by an exogenous HSV-1 strain present within the transplanted graft: graft-to-host transmission [70-73]. Tapering immune suppression and long-term prophylactic antiviral medication reduces the rate of recurrent HK and improves graft survival in HK patients [74]. Because the visus prognosis of patients with post-PKP HK is poor, the early identification of PKP patients at risk is of major importance. The presence of HSV and particularly the HSV-1 genome load within excised corneas may be of diagnostic value to identify those at high risk to develop post-PKP HK, a common cause of corneal graft failure.

Current anti-HSV drugs

Three classes of anti-viral drugs are effectively used to treat HSV infections. Members of the first class are nucleoside analogues like ACV, GCV and penciclovir. Their discovery and implementation represented a milestone in the management of HSV infections. Compared to the first generation of nucleoside analogue anti-herpesvirus drugs like TFT, these new nucleoside analogues have shown a remark- able efficacy and safety with topical and systemic admini- stration. Consequently, nucleo- side analogues have become the standard anti-HSV drugs. These drugs are pro-drugs that require metabolic activation by three phosphorylation steps to achieve antiviral effect. The first phosphorylation step is mediated by the viral enzyme thymidine kinase (TK). The next two phosphorylation steps are dependent of cellular kinases. The active component acts as guanosine analogue competing with the natural nucleoside, resulting in a competitive inhibition of the viral DNA polymerase. Valaciclovir, famciclovir and valganciclovir are equivalent anti-HSV drugs developed for oral administration [76-79]. The main pharmacological differences between these drugs lie in the bioavailability and the intracellular half-life.

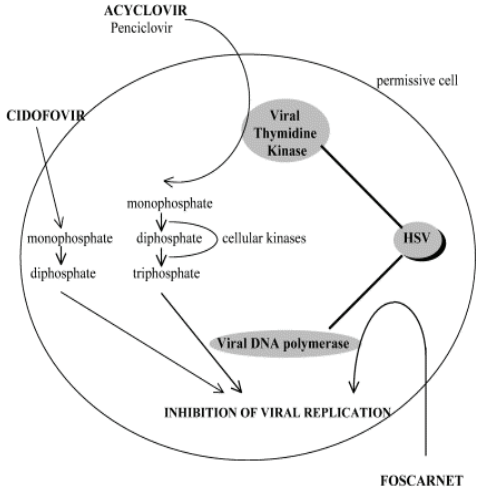


Figure 8. Effector mechanisms of anti-viral drugs interacting with lytic HSV replication (Adapted from reference [75]).

The second class of anti-herpesvirus drugs include DNA nucleoside analogues and pyrophosphate analogues, which become incorporated into viral DNA during replication. These drugs directly inhibit the HSV DNA polymerase and are HSV TK-independent [80]. Incorporation of the drug will disrupt further chain elongation. They include the DNA nucleoside analogues: idoxuridine, vidarabine, and the pyrophosphate analogue foscarnet (FOS). FOS is recommended for severe HSV infections refractory to ACV therapy, but nephrotoxicity is a common side affect opposing the general use of FOS in anti-HSV therapy.

The third class of anti-herpesvirus drugs are acyclic nucleoside phosphonates including cidofovir. Like FOS, these drugs directly interfere with the DNA polymerase and have the disadvantage of being nephrotoxic.

The major weakness of all current anti-HSV drugs is that they only inhibit productive

infection, but are not effective against latent virus. Figure 7 shows the mechanisms of action of the anti-HSV drugs. For reasons of toxicity of the class 2 and 3 anti-HSV drugs, both the nucleoside analogues and TFT are preferred as first-line therapy for the treatment of ocular HSV-1 infections.

Prevalence and mechanisms of HSV resistance to ACV

Early upon the introduction of ACV in the mid 80s, ACV resistant (ACV^R) HSV-1 have been identified [81, 82]. The identification of these viruses in clinical isolates obtained in the pre-ACV era and from patients not treated with ACV indicates that ACV^R HSV-1 variants arise spontaneously without ACV pressure from the natural variability of the HSV population [83]. The frequency of HSV-1 ACV^R variants in ACV sensitive (ACV^S) clinical isolates and laboratory strains range between 10^{-4} and 10^{-5} and remained unchanged despite the widespread use of ACV [84]. Among immune-compromised patients with HSV-1 disease the prevalence of ACV^R HSV-1 isolates is higher (4.3-14%) [85, 86]compared to immune-competent patients (0.1-0.6%) [85, 87-91]. This difference is most likely due to longer mucosal persistence of ACV^R HSV variants caused by impaired local immune responses. About 50% of the ACV^R HSV-1 are cross-resistant to GCV and only rarely to FOS. In contrast to herpes genitalis and herpes labialis, large surveys on the incidence of ACV resistance in HK patients are lacking [75, 77, 92-94]. Currently, only few anecdotal case reports have been published on corneal ACV^R HSV-1 in HK patients [77, 95-98].

In about 95% of the cases, ACV^R is associated with alterations within the HSV-1 TK gene. Less frequent are mutations in DNA polymerase, which may lead to cross-resistance to FOS [99, 100]. HSV-1 TK is a 376 amino acid (aa) long protein encoded by a open reading frame of 1128bp. It contains an ATP binding site (aa 51-63), a nucleoside-binding site (aa 168-176) [101], and 5 evolutionary conserved regions located at aa 55-66, 79-91, 162-178, 212-226, and 281-292 [75]. TK mutations conferring ACV^R are frequently found in these regions and the 7-Gs homopolymer repeat located at nucleotides 430-436 [96, 102]. About 50% of the ACV^R-associated aa polymorphisms are due to nucleotide insertions or deletions, leading to a frameshift reading that potentially results in a truncated TK protein. The remaining polymorphisms of the ACV^R isolates are single aa changes in the TK protein at sites essential to the enzymatic function of TK. More than 90% of ACV^R HSV-1 are TK deficient (TK^D), which lack TK activity or have reduced TK activity, and the remaining viruses have an altered TK substrate specificity (TK^A).

Whereas TK is not essential for HSV-1 replication [103], TK is considered to be involved in HSV-1 pathogenicity and reactivation [104, 105]. Some groups have claimed that ACV^R strains are not capable of reactivation [105, 106], whereas other groups have reported the opposite [84, 107]. Because these studies have been performed in experimental rodent models the relevance of their findings in a clinical setting remains largely unknown.

The widespread use of ACV to treat successive episodes of disease in patients with recrudescent HSV-1 disease may induce and subsequently select for ACV^R HSV-1 strains thereby altering the effectiveness of ACV therapy in time. Detailed analyses on the ACV sensitivity and genetic characteristics of sequential HSV-1 isolates recovered from the same anatomic site of patients with recurrent HSV-1 disease are a prerequisite to understand the incidence and clinical significance of ACV^R HSV-1 in patients with recrudescent recurrent HSV-1 disease.

Scope of the thesis

The aim of the research described in this thesis is to increase our understanding of the virus and host factors involved in the pathology of HSV-1-induced keratitis in humans.

Chapter 2 expands on previously reported role of IL-17 on the activation of cornea resident cells leading to the secretion of pro-inflammatory cytokines and chemokines. Since granulocyte macrophage colony stimulating factor (GM-CSF) is considered to play a key role in chronic inflammatory diseases by governing the survival and function of infiltrating PMNs, the present study addresses the putative role of GM-CSF in the pathogenesis of human HSK.

Chapter 3 expands on studies in the HSK mouse suggesting that different clinical patterns of herpetic ocular disease may be attributed -at least in part- to the differing biological behaviour of specific HSV-1 strains. The prevalence and clinical consequences of the US4 and US7 HSV-1 genotypes is addressed in 178 unrelated HK patients.

Chapter 4 expands on studies reporting that cornea transplantation in patients with a history of HK confers a high rate of post-PKP complications, including recurrent HK, epithelial defects, and eventually graft rejection. The prevalence and clinical consequences of HSV-1, HSV-2 and VZV in human corneal tissues (n= 450) obtained after PKP is determined.

Chapters 5 and 6 relate to ACV resistance of corneal HSV-1 isolates obtained from HK patients. Chapter 5 reports on the prevalence and molecular characteristics of corneal acyclovir-resistant HSV-1 isolates among 173 immune-competent HK patients. Chapter 6 describes the detailed analyses of ACV susceptibility and genetic characterization of sequential corneal HSV-1 isolates of 15 patients with recrudescent HK.

References

1. Pellett PE RB. Herpesviridae: A Brief Introduction. In: Knipe DM HP, Griffin DE, et al., ed. Fields virology. 5th ed. Vol. 2: Philadelphia: Lippincott Williams & Wilkins, 2007:2007:2479- 2497.
2. Whitley RJ, Roizman B. Herpes simplex virus infections. Lancet 2001;357:1513-8
3. Reske A, Pollara G, Krummenacher C, Chain BM and Katz DR. Understanding HSV-1 entry glycoproteins. Rev Med Virol 2007;17:205-15
4. Roizman B KD, Whitley RJ. Herpes simplex viruses. In: Knipe DM HP, Griffin DE, et al., ed. Fields virology. 5th ed. Vol. 2: Philadelphia: Lippincott Williams & Wilkins, 2007:2007:2501- 2603
5. Umene K, Yoshida M. Reiterated sequences of herpes simplex virus type 1 (HSV-1) genome can serve as physical markers for the differentiation of HSV-1 strains. Arch Virol 1989;106:281-99
6. Umene K, Sakaoka H. Homogeneity and diversity of genome polymorphism in a set of herpes simplex virus type 1 strains classified as the same genotypic group. Arch Virol 1991;119:53-65
7. Hayward GS, Frenkel N and Roizman B. Anatomy of herpes simplex virus DNA: strain differences and heterogeneity in the locations of restriction endonuclease cleavage sites. Proc Natl Acad Sci U S A 1975;72:1768-72
8. Buchman TG, Roizman B and Nahmias AJ. Demonstration of exogenous genital reinfection with herpes simplex virus type 2 by restriction endonuclease fingerprinting of viral DNA. J Infect Dis 1979;140:295-304
9. Maertzdorf J, Remeijer L, Van Der Lelij A, et al. Amplification of reiterated sequences of herpes simplex virus type 1 (HSV-1) genome to discriminate between clinical HSV-1 isolates. J Clin Microbiol 1999;37:3518-23
10. Norberg P, Bergstrom T and Liljeqvist JA. Genotyping of clinical herpes simplex virus type 1 isolates by use of restriction enzymes. J Clin Microbiol 2006;44:4511-4
11. Spear PG. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol 2004;6:401-10
12. Spear PG, Eisenberg RJ and Cohen GH. Three classes of cell surface receptors for alphaherpesvirus entry. Virology 2000;275:1-8
13. Satoh T, Arii J, Suenaga T, et al. PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. Cell 2008;132:935-44
14. Granzow H, Klupp BG, Fuchs W, Veits J, Osterrieder N and Mettenleiter TC. Egress of alphaherpesviruses: comparative ultrastructural study. J Virol 2001;75:3675-84
15. Harley CA, Dasgupta A and Wilson DW. Characterization of herpes simplex virus-containing organelles by subcellular fractionation: role for organelle acidification in assembly of infectious particles. J Virol 2001;75:1236-51
16. Efstathiou S, Field HJ, Griffiths PD, et al. Herpes simplex virus latency and nucleoside analogues. Antiviral Res 1999;41:85-100
17. Miller CS, Danaher RJ and Jacob RJ. Molecular aspects of herpes simplex virus I latency, reactivation, and recurrence. Crit Rev Oral Biol Med 1998;9:541-62
18. Gordon YJ, Romanowski E, Araullo-Cruz T and McKnight JL. HSV-1 corneal latency. Invest Ophthalmol Vis Sci 1991;32:663-5
19. Polcicova K, Biswas PS, Banerjee K, Wisner TW, Rouse BT and Johnson DC. Herpes keratitis in the absence of anterograde transport of virus from sensory ganglia to the cornea. Proc Natl Acad Sci U S A 2005;102:11462-7
20. Khanna KM, Bonneau RH, Kinchington PR and Hendricks RL. Herpes simplex virus-specific memory CD8+ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity 2003;18:593-603
21. Liu T, Khanna KM, Carriere BN and Hendricks RL. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. J Virol 2001;75:11178-84
22. Liu T, Khanna KM, Chen X, Fink DJ and Hendricks RL. CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. J Exp Med 2000;191:1459-66
23. Verjans GM, Hintzen RQ, van Dun JM, et al. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. Proc Natl Acad Sci U S A 2007;104:3496-501
24. Sawtell NM. The probability of in vivo reactivation of herpes simplex virus type 1 increases with the number of latently infected neurons in the ganglia. J Virol 1998;72:6888-92
25. Cohrs RJ, Gilden DH. Human herpesvirus latency. Brain Pathol 2001;11:465-74
26. Blyth WA, Harbour DA and Hill TJ. Effect of acyclovir on recurrence of herpes simplex skin lesions in mice. J Gen Virol 1980;48:417-9
27. Sawtell NM, Thompson RL. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. J Virol 1992;66:2150-6
28. Fatahzadeh M, Schwartz RA. Human herpes simplex virus infections: epidemiology, pathogenesis,

symptomatology, diagnosis, and management. J Am Acad Dermatol 2007;57:737-63; quiz 764-6
29. Mettenleiter TC. Initiation and spread of alpha-herpesvirus infections. Trends Microbiol 1994;2:2-4
30. Rajcani J. Molecular mechanisms of virus spread and virion components as tools of virulence. A review. Acta Microbiol Immunol Hung 2003;50:407-31
31. Gupta R, Warren T and Wald A. Genital herpes. Lancet 2007;370:2127-37
32. Ribes JA, Steele AD, Seabolt JP and Baker DJ. Six-year study of the incidence of herpes in genital and nongenital cultures in a central Kentucky medical center patient population. J Clin Microbiol 2001;39:3321-5
33. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. Cornea 2001;20:1-13
34. Liesegang TJ, Melton LJ, 3rd, Daly PJ and Ilstrup DM. Epidemiology of ocular herpes simplex. Incidence in Rochester, Minn, 1950 through 1982. Arch Ophthalmol 1989;107:1155-9
35. Darougar S, Wishart MS and Viswalingam ND. Epidemiological and clinical features of primary herpes simplex virus ocular infection. Br J Ophthalmol 1985;69:2-6
36. Remeijer L, Osterhaus A and Verjans G. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul Immunol Inflamm 2004;12:255-85
37. Shuster JJ, Kaufman HE and Nesburn AB. Statistical analysis of the rate of recurrence of herpesvirus ocular epithelial disease. Am J Ophthalmol 1981;91:328-31
38. Wishart MS, Darougar S and Viswalingam ND. Recurrent herpes simplex virus ocular infection: epidemiological and clinical features. Br J Ophthalmol 1987;71:669-72
39. Carroll JM, Martola EL, Laibson PR and Dohlman CH. The recurrence of herpetic keratitis following idoxuridine therapy. Am J Ophthalmol 1967;63:103-7
40. Group. HEDS. Acyclovir for the prevention of recurrent herpes simplex virus eye disease. Herpetic Eye Disease Study Group. N Engl J Med 1998;339:300-6
41. Williams HP, Falcon MG and Jones BR. Corticosteroids in the management of herpetic eye disease. Trans Ophthalmol Soc U K 1977;97:341-4
42. Ahmed R BC. Immunity to viruses. In: WE P, ed. Fundamental Immunology 4th ed. Vol. Phliladelphia: Lippincott-Raven 1999:1999: 1295-1334
43. Biron CA, Nguyen KB, Pien GC, Cousens LP and Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189-220
44. Habu S, Akamatsu K, Tamaoki N and Okumura K. In vivo significance of NK cell on resistance against virus (HSV-1) infections in mice. J Immunol 1984;133:2743-7
45. Fitzgerald-Bocarsly P, Howell DM, Pettera L, Tehrani S and Lopez C. Immediate-early gene expression is sufficient for induction of natural killer cell-mediated lysis of herpes simplex virus type 1-infected fibroblasts. J Virol 1991;65:3151-60
46. Lanier LL. Natural killer cell receptors and MHC class I interactions. Curr Opin Immunol 1997;9:126-31
47. Schall TJ, Bacon KB. Chemokines, leukocyte trafficking, and inflammation. Curr Opin Immunol 1994;6:865-73
48. Ward SG, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. Biochem J 1998;333 (Pt 3):457-70
49. Kohl S. Role of antibody-dependent cellular cytotoxicity in defense against herpes simplex virus infections. Rev Infect Dis 1991;13:108-14
50. Schmid DS, Mawle AC. T cell responses to herpes simplex viruses in humans. Rev Infect Dis 1991;13 Suppl 11:S946-9
51. Schmid DS, Rouse BT. The role of T cell immunity in control of herpes simplex virus. Curr Top Microbiol Immunol 1992;179:57-74
52. Rinaldo CR, Jr., Torpey DJ, 3rd. Cell-mediated immunity and immunosuppression in herpes simplex virus infection. Immunodeficiency 1993;5:33-90
53. Hukkanen V, Broberg E, Salmi A and Eralinna JP. Cytokines in experimental herpes simplex virus infection. Int Rev Immunol 2002;21:355-71
54. Ragozzino MW, Melton LJ, 3rd, Kurland LT, Chu CP and Perry HO. Population-based study of herpes zoster and its sequelae. Medicine (Baltimore) 1982;61:310-6
55. Kaye S, Choudhary A. Herpes simplex keratitis. Prog Retin Eye Res 2006;25:355-80
56. Lepisto AJ, Frank GM and Hendricks RL. How herpes simplex virus type 1 rescinds corneal privilege. Chem Immunol Allergy 2007;92:203-12
57. Thomas J, Kanangat S and Rouse BT. Herpes simplex virus replication-induced expression of chemokines and proinflammatory cytokines in the eye: implications in herpetic stromal keratitis. J Interferon Cytokine Res 1998;18:681-90
58. Tumpey TM, Chen SH, Oakes JE and Lausch RN. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. J Virol 1996;70:898-904
59. Niemialtowski MG, Rouse BT. Predominance of Th1 cells in ocular tissues during herpetic stromal

keratitis. J Immunol 1992;149:3035-9
60. Streilein JW, Dana MR and Ksander BR. Immunity causing blindness: five different paths to herpes stromal keratitis. Immunol Today 1997;18:443-9
61. Bauer D, Mrzyk S, van Rooijen N, Steuhl KP and Heiligenhaus A. Macrophage-depletion influences the course of murine HSV-1 keratitis. Curr Eye Res 2000;20:45-53
62. Jager MJ, Bradley D, Atherton S and Streilein JW. Presence of Langerhans cells in the central cornea linked to the development of ocular herpes in mice. Exp Eye Res 1992;54:835-41
63. Biswas PS, Rouse BT. Early events in HSV keratitis--setting the stage for a blinding disease. Microbes Infect 2005;7:799-810
64. Keadle TL, Usui N, Laycock KA, Miller JK, Pepose JS and Stuart PM. IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis. Invest Ophthalmol Vis Sci 2000;41:96-102
65. Maertzdorf J, Osterhaus AD and Verjans GM. IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts. J Immunol 2002;169:5897-903
66. Wilhelmus KR. Therapeutic interventions for herpes simplex virus epithelial keratitis. Cochrane Database Syst Rev 2008:CD002898
67. Wilhelmus KR, Gee L, Hauck WW, et al. Herpetic Eye Disease Study. A controlled trial of topical corticosteroids for herpes simplex stromal keratitis. Ophthalmology 1994;101:1883-95; discussion 1895-6
68. Barron BA, Gee L, Hauck WW, et al. Herpetic Eye Disease Study. A controlled trial of oral acyclovir for herpes simplex stromal keratitis. Ophthalmology 1994;101:1871-82
69. Panda A, Kumar TS. Prognosis of keratoplasty in viral keratitis. Ann Ophthalmol 1991;23:410-3
70. Biswas S, Suresh P, Bonshek RE, Corbitt G, Tullo AB and Ridgway AE. Graft failure in human donor corneas due to transmission of herpes simplex virus. Br J Ophthalmol 2000;84:701-5
71. Openshaw H, McNeill JI, Lin XH, Niland J and Cantin EM. Herpes simplex virus DNA in normal corneas: persistence without viral shedding from ganglia. J Med Virol 1995;46:75-80
72. Remeijer L, Maertzdorf J, Doornenbal P, Verjans GM and Osterhaus AD. Herpes simplex virus 1 transmission through corneal transplantation. Lancet 2001;357:442
73. Thuret G, Acquart S, Gain P, et al. Ultrastructural demonstration of replicative herpes simplex virus type 1 transmission through corneal graft. Transplantation 2004;77:325-6
74. Panda A, Vanathi M, Kumar A, Dash Y and Priya S. Corneal graft rejection. Surv Ophthalmol 2007;52:375-96
75. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. J Clin Virol 2003;26:29-37
76. Bacon TH, Howard BA, Spender LC and Boyd MR. Activity of penciclovir in antiviral assays against herpes simplex virus. J Antimicrob Chemother 1996;37:303-13
77. Bacon TH, Levin MJ, Leary JJ, Sarisky RT and Sutton D. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. Clin Microbiol Rev 2003;16:114-28
78. Faulds D, Heel RC. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs 1990;39:597-638
79. Reardon JE, Spector T. Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. J Biol Chem 1989;264:7405-11
80. Chrisp P, Clissold SP. Foscarnet. A review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs 1991;41:104-29
81. Crumpacker CS, Schnipper LE, Marlowe SI, Kowalsky PN, Hershey BJ and Levin MJ. Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N Engl J Med 1982;306:343-6
82. Elion GB, Furman PA, Fyfe JA, de Miranda P, Beauchamp L and Schaeffer HJ. The selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. Reproduced from Proc. Natl. Acad. Sci. USA 74, 5716-5720 (1977). Rev Med Virol 1999;9:147-52; discussion 152-3
83. Parris DS, Harrington JE. Herpes simplex virus variants restraint to high concentrations of acyclovir exist in clinical isolates. Antimicrob Agents Chemother 1982;22:71-7
84. Sarisky RT, Nguyen TT, Duffy KE, Wittrock RJ and Leary JJ. Difference in incidence of spontaneous mutations between Herpes simplex virus types 1 and 2. Antimicrob Agents Chemother 2000;44:1524-9
85. Christophers J, Clayton J, Craske J, et al. Survey of resistance of herpes simplex virus to acyclovir in northwest England. Antimicrob Agents Chemother 1998;42:868-72
86. Englund JA, Zimmerman ME, Swierkosz EM, Goodman JL, Scholl DR and Balfour HH, Jr. Herpes simplex virus resistant to acyclovir. A study in a tertiary care center. Ann Intern Med 1990;112:416-22
87. Boon RJ, Bacon TH, Robey HL, et al. Antiviral susceptibilities of herpes simplex virus from

immunocompetent subjects with recurrent herpes labialis: a UK-based survey. J Antimicrob Chemother 2000;46:1051
88. Fife KH, Crumpacker CS, Mertz GJ, Hill EL and Boone GS. Recurrence and resistance patterns of herpes simplex virus following cessation of > or = 6 years of chronic suppression with acyclovir. Acyclovir Study Group. J Infect Dis 1994;169:1338-41
89. Mertz GJ, Jones CC, Mills J, et al. Long-term acyclovir suppression of frequently recurring genital herpes simplex virus infection. A multicenter double-blind trial. Jama 1988;260:201-6
90. Nugier F, Colin JN, Aymard M and Langlois M. Occurrence and characterization of acyclovir-resistant herpes simplex virus isolates: report on a two-year sensitivity screening survey. J Med Virol 1992;36:1-12
91. Whitley RJ, Gnann JW, Jr. Acyclovir: a decade later. N Engl J Med 1992;327:782-9
92. Chibo D, Druce J, Sasadeusz J and Birch C. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Res 2004;61:83-91
93. Danve-Szatanek C, Aymard M, Thouvenot D, et al. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. J Clin Microbiol 2004;42:242-9
94. Stranska R, Schuurman R, Nienhuis E, et al. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. J Clin Virol 2005;32:7-18
95. Bodaghi B, Mougin C, Michelson S, et al. Acyclovir-resistant bilateral keratitis associated with mutations in the HSV-1 thymidine kinase gene. Exp Eye Res 2000;71:353-9
96. Sarisky RT, Cano R, Nguyen TT, et al. Biochemical characterization of a virus isolate, recovered from a patient with herpes keratitis, that was clinically resistant to acyclovir. Clin Infect Dis 2001;33:2034-9
97. Yao YF, Inoue Y, Kase T, Uchihori Y, Mori Y and Ohashi Y. Clinical characteristics of acyclovir-resistant herpetic keratitis and experimental studies of isolates. Graefes Arch Clin Exp Ophthalmol 1996;234 Suppl 1:S126-32
98. Zhang W, Suzuki T, Shiraishi A, Shimamura I, Inoue Y and Ohashi Y. Dendritic keratitis caused by an acyclovir-resistant herpes simplex virus with frameshift mutation. Cornea 2007;26:105-6
99. Hill EL, Hunter GA and Ellis MN. In vitro and in vivo characterization of herpes simplex virus clinical isolates recovered from patients infected with human immunodeficiency virus. Antimicrob Agents Chemother 1991;35:2322-8
100. Pottage JC, Jr., Kessler HA. Herpes simplex virus resistance to acyclovir: clinical relevance. Infect Agents Dis 1995;4:115-24
101. Balasubramaniam NK, Veerisetty V and Gentry GA. Herpesviral deoxythymidine kinases contain a site analogous to the phosphoryl-binding arginine-rich region of porcine adenylate kinase; comparison of secondary structure predictions and conservation. J Gen Virol 1990;71 (Pt 12):2979-87
102. van Doornum GJ, Guldemeester J, Osterhaus AD and Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 2003;41:576-80
103. Jamieson AT, Gentry GA and Subak-Sharpe JH. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J Gen Virol 1974;24:465-80
104. Efstathiou S, Kemp S, Darby G and Minson AC. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J Gen Virol 1989;70 (Pt 4):869-79
105. Jacobson JG, Ruffner KL, Kosz-Vnenchak M, et al. Herpes simplex virus thymidine kinase and specific stages of latency in murine trigeminal ganglia. J Virol 1993;67:6903-8
106. Coen DM, Kosz-Vnenchak M, Jacobson JG, et al. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc Natl Acad Sci U S A 1989;86:4736-40
107. Tenser RB, Gaydos A and Hay KA. Reactivation of thymidine kinase-defective herpes simplex virus is enhanced by nucleoside. J Virol 1996;70:1271-6.

Chapter 2

Granulocyte Macrophage Colony Stimulating Factor Expression in Human Herpetic Stromal Keratitis (HSK): Implications for the Role of Neutrophils in HSK

Rui Duan, Lies Remeijer, Jessica M. van Dun, Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

Investigative Ophthalmology & Visual Science ***2007*** *Jan; 48(1): 277-84.*

Abstract

Purpose: Granulocyte macrophage colony stimulating factor (GM-CSF) is considered to play a key role in chronic inflammatory diseases by governing the survival and function of infiltrating neutrophils. The objective of this study was to address the putative role of GM-CSF in the pathogenesis of human herpetic stromal keratitis (HSK).

Methods: Primary human corneal fibroblast (HCF) cultures, and a telomerase-immortalized human corneal epithelial (HCE) cell line representative for native HCE, were stimulated with the known HSK inducing cytokines interferon γ (IFN-γ), interleukin 1β (IL-1β) and tumor necrosis factor (TNF-α). Alternatively, the T cell cytokine IL-17 was added solely or simultaneously. Human neutrophils were incubated with conditioned medium (CM) of the HCF and HCE stimulated with the aforementioned cytokines, or recombinant GM-CSF, and their viability or activation status determined by flowcytometry. GM-CSF and IL-8 secretion levels in the CM were determined by ELISA. The antibody-dependent cellular cytotoxicity (ADCC) of neutrophils towards herpes simplex virus (HSV) infected HCF was determined by flowcytometry. The expression of GM-CSF was determined in HSK and control corneal buttons by real-time RT-PCR and immunohistology.

Results: Compared to IFN-γ, CM of either cell type stimulated with IL-1β, or in case of HCE stimulated with TNF-α or IL-17, delayed neutrophil apoptosis significantly. Only for HCF, IL-17 exhibited a synergistic effect with TNF-α. The anti-apoptotic activity was - in part - attributable to GM-CSF secreted by the activated HCF and HCE. GM-CSF stimulation of neutrophils induced their activation and secretion of IL-8. GM-CSF did not increase the ADCC reaction significantly of neutrophils towards HSV-infected HCF. Finally, GM-CSF is expressed in corneas of HSK patients but not controls.

Conclusions: The data presented suggest that GM-CSF, expressed by corneal resident cells like HCF and HCE, may play a role in the immunopathogenesis of HSK by prolonging the survival and modulating the effector function of corneal infiltrating neutrophils.

Introduction

Herpes simplex type 1 (HSV-1) infection of the cornea can induce keratitis clinically classified into herpetic epithelial keratitis (HEK) and herpetic stromal keratitis (HSK) [1]. HEK is an acute inflammation and results from viral toxicity of infected corneal epithelial cells. In contrast, HSK is characterized as a chronic immunopathogenic disease in which tissue injury and eventually blindness is due to the complex interplay between cells of the innate and adoptive immune response to antigens expressed in the corneal tissue[1, 2]. Studies performed on the experimental HSK mouse model greatly improved our understanding of the pathogenesis of HSK. Whereas, dendritic cells, macrophages and $CD4^+$ T cells play a pivotal role in the induction of the disease, neutrophils are considered as the main cell type directly involved in the destruction of corneal architecture[2]. The extravasation and function of neutrophils is coordinated by cyto- and chemokines expressed within the cornea[3-5]. The cells secreting these immune modulatory factors remain ill-defined. Until recently, corneal infiltrating inflammatory cells have been advocated as the main source. Evidence is accumulating that tissue resident cells like fibroblasts play an important role as well[6-8]. Activated human corneal epithelial cells (HCE) and fibroblasts (HCF) secrete key cytokines like interleukin 6 (IL-6) and IL-8[9-11]. We have recently extended these studies by demonstrating that human corneal fibroblasts secrete a broad variety of chemokines upon stimulation with proinflammatory cytokines[6]. Moreover, we showed that the T cell cytokine IL-17, expressed within affected corneas of HSK patients, had a modulatory effect on the secretion of these chemokines[6].

Neutrophils normally live for less than 24 hours within the peripheral circulation. They undergo constitutive spontaneous cell death, referred to as apoptosis, as a mechanism to facilitate normal cell turnover and immune system homeostasis. Their rate of apoptosis is delayed upon egress into tissues and subsequent exposure to specific cytokines.[12] Conversely, extended neutrophil survival within tissues can result in persistent inflammation and tissue damage if these cells are stimulated to secrete their cytotoxic molecules like proteases and reactive oxidants[13]. Besides being indispensable for the growth and development of granulocyte-macrophage progenitors, granulocyte macrophage colony stimulating factor (GM-CSF) is a major regulator governing the effector function of both mature macrophages and neutrophils[14]. It delays apoptosis and induces the release of proteolytic enzymes and oxygen free radicals, the latter referred to as the oxidative burst of neutrophils[15, 16]. There is mounting evidence for a pro-inflammatory role of GM-CSF in chronic inflammatory diseases[18]. Rheumatoid arthritis is associated with sustained overproduction of cytokines such as IL-1, tumor necrosis factor (TNF-α), IL-6 and GM-CSF.[19] Neutralization of IL-1, TNF-α and GM-CSF has been shown to ameliorate the disease symptoms in its representative experimental animal models[19-21]. Moreover, mice deficient in GM-CSF were largely disease resistant[22]. Considering the similarities between

the pathogenic mechanisms and cell types involved in experimental arthritis and HSK, we explored the putative role of GM-CSF in human HSK. To address this issue, we determined the intra-corneal expression and induction of GM-CSF secretion by HCF and HCE upon stimulation with the known HSK inducing cytokines IFN-γ, TNF-α and IL-1β. The combinative effect of IL-17 and the role of GM-CSF on human neutrophil function were emphasized. The data show that GM-CSF is expressed in corneas of HSK patients and has a regulatory effect on neutrophils by prolonging neutrophil survival and function.

Materials and Methods

Cytokines and mAb treatment

Human recombinant IL-1β, IL-17, TNF-α, and IFN-γ were obtained from PeproTech (London, UK). Recombinant human GM-CSF (rhGM-CSF) was obtained from R&D Systems (Abingdon, UK). For blocking experiments, a neutralizing mouse monoclonal antibody (mAb) directed to human GM-CSF (clone 3209.1; 5 μg/ml; R&D Systems) and an isotype matched control mAb was used (clone 107.3; BD Biosciences, Erembodegem, Belgium). The optimal concentration of the anti-GM-CSF mAb was predefined using GM-CSF and neutrophils in pilot experiments (data not shown). Secretion levels of GM-CSF (U-CyTech, Utrecht, The Netherlands) and IL-8 (Biosource, Etten-Leur, The Netherlands) in cell-free conditioned media (CM) of cytokine stimulated HCF, HCE and neutrophils were measured according the manufacturers instructions, respectively. The detection limit of both ELISAs was 10 pg/ml.

Human corneal cell cultures

The local ethical committee approved the study and informed consent was obtained from all subjects donating clinical specimen. The study adhered to the tenets of the Declaration of Helsinki. Primary HCF cultures were generated from 4 individual donor corneas, control corneas obtained from the Dutch Cornea Bank (Amsterdam; The Netherlands), that had been rejected for transplantation use due to low endothelial cell counts, and from 2 corneas of HSK patients that underwent therapeutic keratoplasty to restore sight. The corneas were finely minced and digested with collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands) essentially as described elsewhere[6]. Adherent cells were cultured in six-well plates in medium consisting of a 1:1 ratio (v/v) of Dulbecco's Modified Eagle Medium (DMEM) and F-12 Nutrient Mixture (Ham F12) (Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and antibiotics (from here referred to as HCF medium). HCF cultures, with a fibroblast-like morphology, were grown in bulk in 162 cm^2 flasks and cryopreserved in aliquots. HCF cultures were not contaminated with corneal epithelial or endothelial cells

(data not shown). Passage 5 - 7 HCF cultures were used throughout the study. As numerous efforts failed to generate primary human corneal epithelial cell cultures to sufficient cell numbers, a human telomerase-immortalized corneal epithelial cell line (HCE) was used as alternative throughout the study[23]. This cell line, closely resembling native human corneal epithelial cell, was maintained in defined keratinocyte-serum free medium (SFM medium; Invitrogen, Carlbad, CA) [23, 24]. For cytokine stimulation experiments, HCF and HCE were grown in 6 well plates in HCF and SFM medium, respectively. At confluence, approximately 3 x 10^5 cells/well for both cell types, medium of the HCF cultures was replaced with a serum-free medium (referred to as SF-HCF medium) consisting of DMEM and Ham F12 (1:1; v/v) supplemented with Insulin-Transferrin-Selenium-X supplement and 0.5% bovine serum albumin (BSA) (all obtained Invitrogen). HCF was left for 5 days on serum-free medium before stimulation with cytokines. Serum-free medium was used to maintain a more native biosynthetic phenotype and appearance and to reduce background levels of cytokine and chemokine production[6]. Analogously, the SFM of the HCE cultures was replaced before addition of the cytokines. The HCF and HCE cultures were incubated in triplicate for 48 hours at 37^{o}C with stimulatory cytokines added at previously defined optimal concentrations: IL-17 (100 ng/ml), IL-1β (100 ng/ml), TNF-α (50 ng/ml) and IFN-γ (100 U/ml) in a total volume of 1ml[6]. Subsequently, cell-free CM was collected and frozen in aliquots at –70^{o}C. Experiments were repeated at least 3-times.

Assessment of cytokine induced viability and activation of human neutrophils

Human neutrophils were isolated from heparinized venous blood of healthy individuals using Polymorphprep™ (Axis-Shield, Oslo, Norway), and residual erythrocytes lysed with BD Pharm Lyse™ (BD Biosciences), according to the manufacturer instructions. The isolated cell fraction typically contained at least 90 to 95% granulocytes with a viability of >95% as determined by May-Grünwald/Giemsa and trypan blue exclusion staining, respectively. The granulocytes obtained consisted almost exclusively of neutrophils with traces of eosinophils (<1%) as assessed by differential CD16 (Fluorescein-conjugated anti CD16; clone 3G8; BD Biosciences) expression by both granulocyte subsets using flowcytometric analyses.[25] Moreover, the latter technique confirmed the frequency of granulocytes in the isolated cells, as defined by May-Grünwald/Giemsa staining, judged on the differential forward and side scatter pattern of mononuclear cells versus granulocytes (data not shown). The neutrophils were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotics (referred to as R10F; 5x10^6 neutrophils in 500 µl medium) and incubated with diluted SF-HCF or SFM medium, or CM of mock- and cytokine-stimulated HCF and HCE for 18 hours at 37^{o}C in a CO_2-incubator. The total assay volume was 0.5 ml and the dilution, with R10F, of the HCF (1:100) and HCE (1:20) CM was defined in pilot experiments for optimal distinction between neutrophil survival using the CM of the cells stimulated with the different cytokines. For blocking experiments, the

CM were similarly diluted prior to the addition of anti-GM-CSF and the isotype control mAb. The neutrophils were stained with 20 µg/ml 7-amino actinomycin D (7AAD; Sigma-Aldrich) for 20 minutes at 37°C and examined by flowcytometry. Based on the study of Philpott and co-workers 7AAD negative cells were considered as viable non-apoptotic cells (V_{cells}).[26] Samples were acquired on a FACSCalibur device (BD Biosciences). The forward scatter (FSC) and side scatter (SSC) acquisition threshold was set to include all neutrophils, including dead neutrophil events (D_{cells}), but to exclude mononuclear cells. Debris was excluded by gating in FSC-7AAD dot plots during data analyses. Percentages of viable 7AAD-negative events (% V_{cells}) were calculated with the formula 100 × (number of V_{cells})/(number of V_{cells} + number of D_{cells}).

Alternatively, neutrophils were incubated, in a total assay volume of 0.5 ml, with increasing doses of rhGM-CSF, the known neutrophil activating mitogen lipopolysaccha-ride (LPS; 1 µg/ml; Sigma-Aldrich) or solely R10F for 30 minutes or 18 hours at 37°C in a CO_2-incubator to determine their activation status or viability, respectively. Neutrophils stimulated for 30 minutes were stained with fluochrome-conjugated mAbs directed to CD11b (Fluorescein-conjugated; clone M1/70; BD Biosciences) and CD62L (phycoerythrin-conjugated; clone Dreg-56; BD Biosciences), or their respective isotype controls A95-1 (BD Biosciences) and MOPC-21 (BD Biosciences), respectively. Cell surface expression was analyzed by flowcytometry on a FACSCalibur device (BD Biosciences). The CM of stimulated neutrophils, collected after 6 hours that was defined as the optimal time point in pilot experiments (data not shown), was analyzed for IL-8 secretion levels by a commercial ELISA (R&D Systems).

Antibody dependent cellular cytotoxicity assay

The HCF were infected with a recombinant HSV-1 virus (strain v44), expressing VP16 linked to the green fluorescent protein (GFP), at a multiplicity of infection of 0.02 and incubated overnight. This recombinant HSV-1 strain replicates with virtually normal kinetics and yields and incorporate the fusion protein into the virion, resulting in autofluorescent particles[27]. As control, HCF were treated similarly but without addition of virus: referred to as mock-infected HCF. The HSV-1- and mock-HCF (i.e. target cells) were trypsinized, extensively washed, and incubated at 4°C for 1 hour in HCF medium with 1:200 (v/v) diluted heat-inactivated human pooled serum of donors serologically defined as HSV seronegative or -positive. Subsequently, neutrophils (i.e. effector cells) were added at different effector/target-ratios and incubated for 4 hours at 37°C. Alternatively, rhGM-CSF was added to a final concentration of 100 pg/ml. During the last 20 minutes of incubation TO-PRO-3 iodide (TP3; final concentration at 25 nmol/L; Invitrogen) was added to discriminate between viable and nonviable cells[28]. Samples were acquired on a FACSCalibur device (BD Biosciences). The FSC acquisition threshold was set to include nonviable events. Debris was excluded by gating in FSC-TP3 dot plots during data analyses.

A region to exclude GFP-negative events was defined in GFP-TP3 or GFP-fluorescence channel 3 (FL3) dot plots of the data acquired from cultures that contained mock-infected HCF. GFP-positive events derived from HCF cultures, infected with the GFP expressing HSV-1 strain, were displayed in FSC-TP3 or GFP-TP3 dot plots for the definition of viable HSV-1 infected GFP-positive (GFP^+ V_{cells}) events (i.e. GFP^+; TP3-negative events) and nonviable or dead GFP-positive (GFP^+ D_{cells}) events (i.e. GFP^+; TP3-positive events). Percentages of dead GFP-positive cells (% GFP^+ D_{cells}) were calculated with the formula 100 × (number of GFP^+ D_{cells}) / (number of GFP^+ V_{cells} + number of GFP^+ D_{cells}).

RNA isolation and real-time reverse transcriptase polymerase chain reaction analyses

Total cellular RNA was extracted from HSK and control corneas with TRIzol LS reagent (Invitrogen) and subsequently purified using an RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's protocols. The cornea buttons analyzed were obtained from patients with severe HSV-induced HSK after therapeutic penetrating keratoplasty. Donor corneas, rejected for transplantation purposes, were included as controls corneas. For real-time reverse transcriptase - polymerase chain reaction (RT-PCR) analyses, RNA was converted into single stranded copy DNA using random primers and reverse transcriptase (all from Invitrogen) according to the manufacturer's protocols. Relative expression levels of GM-CSF and the house keeping gene β-actin were measured using the 5' fluorogenic nuclease assay in real-time quantitative PCR using TaqMan chemistry on the ABI 7000 Prism real-time PCR instrument (Applied Biosystems, Warrington, UK). The GM-CSF and β-actin primer probe sets were obtained from Applied Biosystems assays-on-demand (ID No. Hs00171266 and Hs99999903, respectively). PCR was conducted using the following cycle parameters: 95°C, 12 minutes for 1 cycle (95°C, 20 s; 60°C, 1 minute), for 45 cycles. Analysis was conducted using the sequence detection software supplied with the ABI 7000. The software calculates the threshold cycle (Ct) for each reaction and this was used to enumerate the amount of starting template in the reaction. The Ct values for each set of duplicate reactions were averaged for all subsequent calculations. A difference in Ct values (ΔCt) was calculated for each gene by taking the mean Ct of gene of interest and subtracting the mean Ct for β-actin for each cDNA sample. Relative mRNA expression levels were calculated using the formula $2^{-\Delta Ct}$.

Immunohistochemistry

Corneal buttons, obtained within 3 hours after surgery, were fixed with formalin, embedded in paraffin and subsequently cut into 4 µm-thick sections. Following antigen retrieval by pronase (1 mg/ml; Sigma-Aldrich) treatment at 37°C for 12 minutes, sections were treated with avidin/biotin-blocking kit (Vector Laboratories, Peterborough, UK) to block non-specific binding sites of biotin/avidin-system reagents. Consecutive slides form

each cornea were stained with the unconjugated moAbs anti-human GM-CSF (clone 3209.1; R&D Systems) or IgG1 isotype control (clone 107.3; BD Biosciences). Subsequently, the streptavidin–biotin immunoperoxidase method was used. The reagents used for the subsequent steps like biotinylated goat anti-polyvalent immunoglobulin, peroxidase-labeled streptavidin and 3-amino-9-ethyl carbazole were obtained from Lab Vision (Fremont, CA).

Statistical analyses

The data are presented as the mean ± standard error mean (SEM). A two-tailed paired *t* test or a one-way ANOVA with Bonferonni's posttest was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). A p value of <0.05 was taken as indicative of statistical significance.

Results

Conditioned medium from cytokine stimulated human corneal fibroblast and epithelial cell lines delay spontaneous neutrophil cell death

Studies in the experimental HSK mouse model have demonstrated that neutrophils are the main cell type associated with collateral tissue damage[1, 2]. Apoptosis is the physiologic cell death of neutrophils. However, neutrophil apoptosis within inflamed tissue can be delayed when neighboring cells like tissue resident cells generate survival factors.[12] We determined whether human corneal fibroblasts and a human corneal epithelial cell line, closely resembling native human corneal epithelial cell[23, 24], secrete neutrophil survival factors in vitro upon stimulation with cytokines known to be involved in the development of HSK: TNF-α, IL-1β and IFN-γ. Compared to mock treated cells, CM of IL-1β and TNF-α treated HCE significantly delayed in neutrophil cell death at the same level. In contrast, only CM of IL-1β treated HCF improved neutrophil survival significantly (Fig. 1). For both cell types IFN-γ stimulation had no effect. Subsequently, we determined the combinatory effect of IL-17, a proinflammatory T cell cytokine potentially involved in the immunopathogenesis of HSK[6]. In contrast to HCF, the CM of IL-17 treated HCE showed a significant delay in neutrophil cell death (Fig. 1). However, in contrast to HCE, incubation of HCF with combinations of the aforementioned cytokines showed that IL-17 had a synergistic effect only on the TNF-α-induced secretion of (a) neutrophil survival factor(s) (Fig. 1).

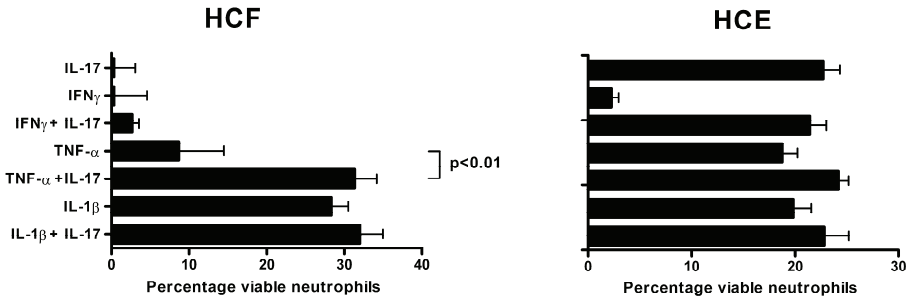


Figure1. Conditioned medium of cytokine stimulated human corneal fibroblasts (HCF) and a human corneal epithelial cell line (HCE) differentially delay neutrophil cell death. Neutrophils were incubated overnight in diluted conditioned medium (CM), generated by stimulating HCF or HCE for 48 hours with the indicated cytokines, and the percentage of viable neutrophils was assessed. The CM were diluted with assay medium: 1:100 (HCF) and 1:20 (HCE). The Results, expressed as the mean ± SEM of three independent experiments, are the net values: cytokine minus medium stimulation. The p values were calculated using one-way ANOVA with Bonferonni post test.

GM-CSF secreted by cytokine treated human corneal fibroblast and epithelial cell lines contributes to neutrophil survival

Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases and appears to be largely mediated by the production of GM-CSF.[16,17] To determine whether GM-CSF was contributing to the observed delay in neutrophil cell death, we determined the GM-CSF secretion levels in CM of the cytokine treated HCF and HCE cell cultures. For both cell types, only IL-1β and TNF-α stimulation induced GM-CSF secretion and IL-17 exerted a synergistic effect with TNF-α. Overall, HCF secreted approximately 3-times more GM-CSF than HCE (Fig. 2).

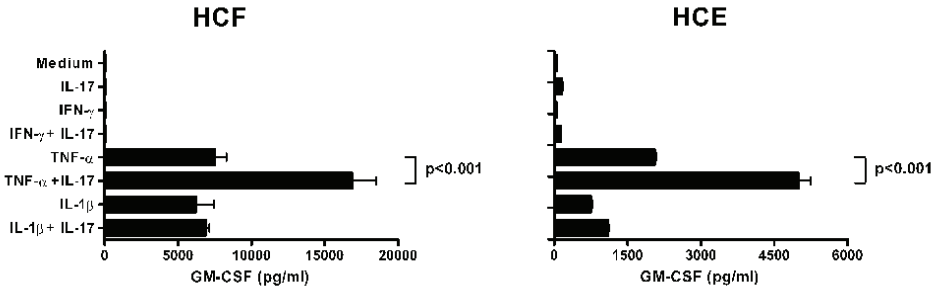


Figure 2. Human corneal fibroblasts (HCF) and a human corneal epithelial cell line (HCE) secrete GM-CSF upon cytokine stimulation. The HCF and HCE were stimulated for 48 h with the indicated cytokines and the concentration of GM-CSF present in the conditioned medium was assessed by ELISA. Results are expressed as the mean ± SEM of a representative experiment performed in triplicate. The p values were calculated using one-way ANOVA with Bonferonni post test.

To assess the potential role of GM-CSF in contributing to neutrophil survival directly, the CM obtained from the HCF and HCE cultures was immunodepleted of GM-CSF using a specific mAb. First, the kinetics of neutrophil survival in relation to increasing amounts of

rhGM-CSF was determined. rhGM-CSF inhibited neutrophil cell death, even at the lowest concentrations, during the first for 6 hours of incubation. Only at 20 hours a clear decline of viable neutrophil numbers, in a dose-response fashion, was observed (Fig. 3A). Secondly, we defined that the anti-GM-CSF mAb, at a concentration of 5µg/ml, inhibited the neutrophil anti-apoptotic effect of 100 pg/ml rhGM-CSF significantly (Fig. 3B).

Finally, the CM of both cell types displaying high neutrophil survival activity were analyzed. Compared to the isotype control or untreated CM, pretreatment of the CM with a neutralizing anti-GM-CSF mAb significantly blocked neutrophil survival (Fig. 3C and 3D). However, for all anti-GM-CSF treated CM analyzed residual neutrophil survival factor activity remained present.

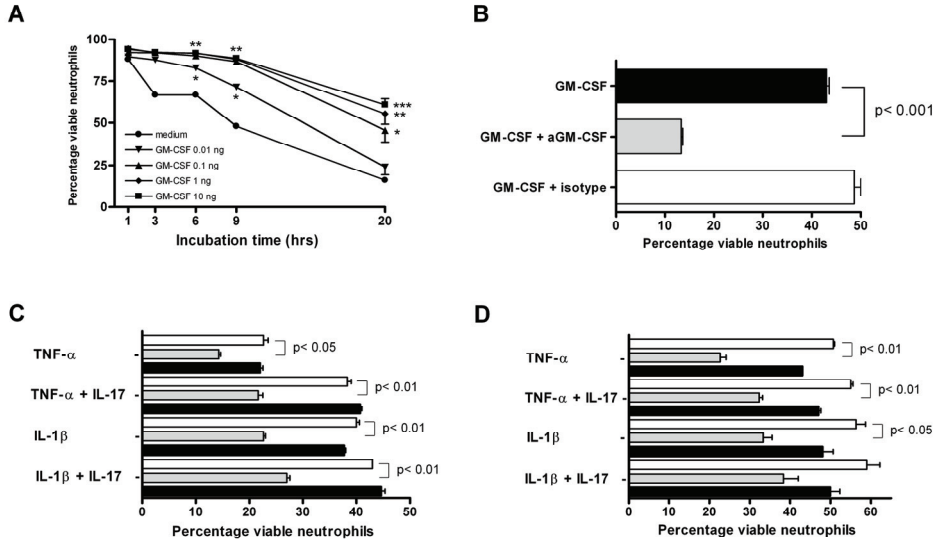


Figure 3. GM-CSF secreted by cytokine stimulated human corneal fibroblasts (HCF) and a human corneal epithelial cell line (HCE) contributes to neutrophil survival. A, Kinetics of neutrophil cell death in relation to increasing concentrations of rhGM-CSF. Neutrophils were incubating with increasing amounts of rhGM-CSF, or as control medium only, for the indicated time points at 37°C. * p< 0.05, **p<0.01 and*** p<0.001 compared to medium, respectively. B, Anti-GM-CSF mAb specifically neutralizes rhGM-CSF-mediated neutrophil survival. rhGM-CSF (100 pg/ml) was preincubated with anti-GM-CSF (GM-CSF + aGM-CSF) or isotype control mAb (GM-CSF + isotype), both at 5 µg/ml, for 30 minutes and subsequently neutrophils added for overnight culture at 37°C. C and D, diluted conditioned media from HCF (C; 1:100 diluted with assay medium) or HCE (D;1:20 diluted) cultures, stimulated with the indicated cytokines, were pretreated with 5µg/ml anti-GM-CSF (gray bars) or an isotype control mAb (black bars), and subsequently neutrophils added and incubated overnight at 37°C. White bars, untreated conditioned media. All results are expressed as the mean ± SEM percentage viable neutrophils of representative experiments performed in triplicate. The p values were calculated using one-way ANOVA with Bonferonni post test.

GM-CSF stimulation modulates the expression of adhesion molecules and secretion of IL-8 by neutrophils

The functional activity of neutrophils is modulated on several levels. Two particularly important aspects are the priming of neutrophils to undergo an oxidative burst, i.e. release of reactive oxygen derivates, and simultaneously the mobilization of granule contents.16

Examples of the latter are CD11b, a component of the integrin Mac-1 (i.e. CD11b/CD18-dimer), and the adhesion molecule L-selectin (CD62L) [16, 29]. Consequently, these adhesion molecules have been advocated as surrogate markers for the neutrophil oxidative burst[29]. Additionally, these molecules are important for neutrophil recruitment and transmigration through the endothelial layer into tissues. Both adhesion molecules are expressed by resting neutrophils. Upon neutrophil activation, CD11b is mobilized from granules to the cell surface and membrane bound CD62L is proteolytically shed[29]. To evaluate the role of GM-CSF on the expression of these neutrophil activation markers, neutrophils were incubated with increasing amounts of GM-CSF and the cell surface expression of CD11b and CD62L was analyzed by flowcytometry. GM-CSF treatment induced, in a dose-response fashion, the up- and downregulation of CD11b and CD62L, respectively (Fig. 4A).

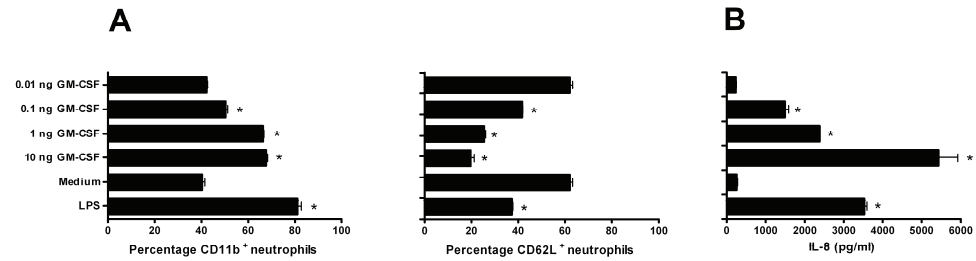


Figure 4. GM-CSF stimulation induces the activation and IL-8 secretion of neutrophils. Freshly isolated neutrophils were incubating with increasing amounts of GM-CSF, LPS (1 µg/ml), or as control medium only, for 30 minutes (A) or 6 hours (B) at 37°C. The expression of CD11b and CD62L on neutrophils was analyzed by flowcytometry. The secretion levels of IL-8 were determined by ELISA in cell-free conditioned medium. Results are expressed as the mean ± SEM of a representative experiment performed in triplicate. Probabilities were calculated with one-way ANOVA with Bonferonni post test. * p<,0.01 compared to medium.

In addition to intra-lesional mononuclear cells like macrophages and T cells, tissue infiltrating neutrophils have been identified as a prominent source of proinflammatory cytokines and chemokines. Here, we demonstrated that GM-CSF treatment of neutrophils induced IL-8 secretion in a dose-response fashion (Fig. 4B). Neutrophils stimulated with LPS, used as positive control, responded accordingly in both assays.

GM-CSF does not significantly increase neutrophil antibody-dependent cellular cytotoxicity of HSV-1 infected human corneal fibroblasts.

Anti-HSV antibodies, both secretory IgA and IgG, are commonly detected in the tear film of patients with HSV keratitis[30, 31]. In contrast to the experimental HSK mouse model, HSV infected corneal fibroblast have been detected in corneas of HSK patients[1, 2]. Lysis of HSV-infected corneal fibroblasts by means of ADCC, considered as the major antiviral mechanism of neutrophils, may play a role in the pathogenesis of HSK. Accordingly, we have addressed this issue by analyzing the killing of HSV-1 infected HCF by neutrophils in

the presence of serum of HSV seropositive versus seronegative donors. Infected HCF were not killed upon incubation with serum only and mock-infected HCF were not killed by the neutrophils upon incubation with either serum samples (data not shown). Neutrophils lysed HSV-1 infected HCF preincubated with serum of HSV seropositive donors to a greater extent, only significantly different at the highest neutrophil (effector; E)/ HCF (target; T) ratio: E/T of 100, than cells incubated with HSV seronegative serum (Fig. 5). Addition of rhGM-CSF to ADCC assays has been shown to amplify the neutrophil cytolytic efficiency[32]. Conversely, rhGM-CSF tended to increase, although not significantly, the neutrophil-mediated killing of HSV-1 infected HCF in the presence of HSV seropositive serum only at the highest E/T ratio (Fig. 5).

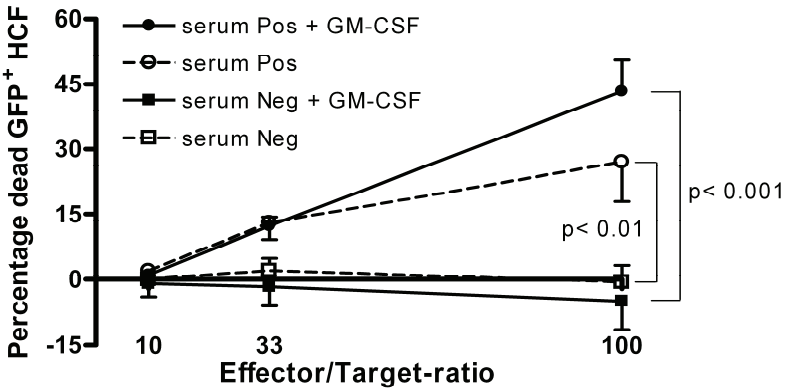


Figure 5. GM-CSF tends to increase the neutrophil antibody-dependent cellular cytotoxicity reaction towards HCFs. HCF infected with a GFP expressing HSV-1, preincubated with pooled serum of seronegative (serum Neg) or seropositive (serum Pos) donors, were used as target cells. Neutrophils were effector cells. The effector-target ratio was as indicated, the incubation time was 4 hours. Concentration of rhGM-CSF was 100 pg/ml. Shown are the combined results of three independent experiments performed in triplicate, expressed as the percentage, mean ± SEM, of dead GFP+ HCFs identified as HSV-1 infected. Probabilities were calculated with a two-tailed paired t-test.

GM-CSF is expressed in corneas of HSK patients

Demonstrating that HCF secrete GM-CSF upon cytokine treatment in vitro, challenges the question whether GM-CSF is expressed in corneas of HSK patients. We first determined the expression of GM-CSF transcripts in HSK corneas and compared it to control corneas. The relative transcript levels of GM-CSF were significantly higher in cornea buttons of HSK patients compared to controls (Fig. 6).

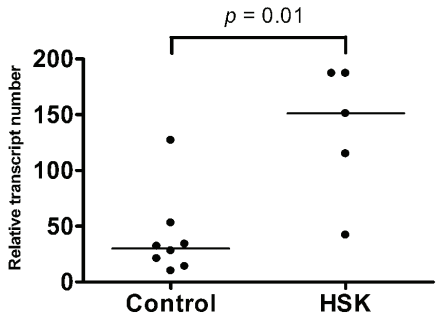


Figure 6. Quantification of GM-CSF mRNA expression by real-timeRT-PCR in human corneal buttons. A comparison between cornealbuttons from eyes of eight control subjects and five patients with HSK is shown. The y-axis represents the relative transcript number of GM-CSF compared with the housekeeping gene β-actin. The median value is indicated with a line. The probability was calculated using a two-tailed paired t-test.

Secondly, to determine the cell type expressing GM-CSF immunohistologic staining was performed on cornea sections of HSK patients. Compared to isotype control, staining of HSK corneas with a GM-CSF-specific mAb revealed noticeable GM-CSF expression in the corneal epithelial cell layer. In contrast, limited GM-CSF expression within the corneal stroma was detected which, based on their cell morphology and localization, could be addressed to infiltrating inflammatory cells. The corneal fibroblasts did not appear to express GM-CSF. No GM-CSF expression was detected in control corneas rejected for transplantation purposes (data not shown). Representative stainings for GM-CSF and an isotype control on corneas of 2 HSK patients are shown in Figure 7.

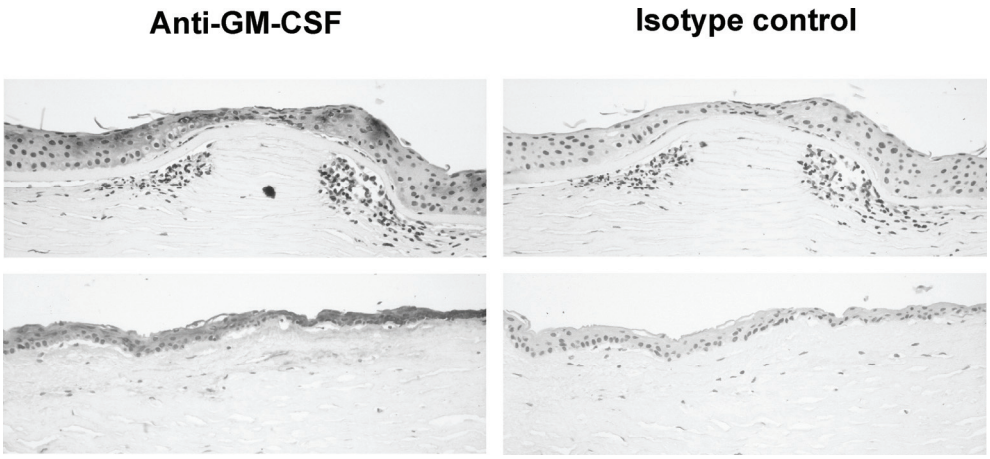


Figure 7. GM-CSF is expressed by cornea-resident cells in corneas of patients with herpetic stromal keratitis. Immunohistologic staining for GM-CSF (left) and isotype control (right) were performed on cornea buttons of two HSK patients. All images represent the central view of the cornea with the epithelial cell layer pointing upwards. Hematoxylin counterstaining; magnification,×200.

Discussion

The deposition of functional neutrophils in inflamed tissue is essential in the host defense to curtail infections, but a subsequent controlled elimination of these cells is of similar importance to prevent the development of a chronic potentially pathogenic inflammation[12, 33]. Current knowledge on the pathogenic processes involved in chronic inflammatory responses challenges the previous unappreciated role of tissue resident cells. Tissue resident cells are able to impose a stromal address code on tissue infiltrating inflammatory cells, which governs their accumulation, survival, activation and differentiation[34]. It is generally accepted that mesenchymal cells, upon activation with proinflammatory cytokines of macrophage/monocyte (IL-1β and TNFα) and T cell (IFN-γ) origin, are involved in the recruitment of inflammatory cells like neutrophils via the expression of adhesion molecules and chemoatractants[34]. However, the factors involved in prolonging survival and modulating the effector function of these pathogenic neutrophils in HSK remain ill-defined. Our laboratory is engaged in an ongoing effort to define the possible role of corneal resident cells, in addition to infiltrating inflammatory cells, in the disease process. This report evaluates the potential role of GM-CSF in relation to the role of neutrophils in the pathology of human HSK.

The results presented here demonstrate that GM-CSF is expressed in corneas of HSK patients, but not in control corneas. The proinflammatory cytokines TNF-α and IL-1β induced GM-CSF secretion by HCF and HCE. These findings are concordant with similar studies on other cell types[17]. However, the data are in contradiction to a previous study of Cubitt and co-workers on corneal epithelial cells and HCF[35]. Whereas both corneal cell types secreted copious amounts of GM-CSF upon IL-1α stimulation, GM-CSF secretion could only be induced in HCE by TNF-α.[35] This puzzling discrepancy between our data and the latter study, both studies used similar amounts of TNF-α and cell types, remains inexplicable. Possibly, differences in culture conditions or donors of which the HCF were obtained are involved. Unfortunately, efforts culturing corneal epithelial cells from the cornea donors included, to compare both cell types in our study, were unsuccessful. The similarities in GM-CSF secretion levels following TNF-α and IL-1α/β stimulation of the immortalized HCE cell line described here (Fig. 2), and the primary HCE cultures applied by Cubitt and co-workers[35], once more underlines the close resembles of this HCE cell line to native HCE[23, 24].

We have recently shown that IL-17, expressed in corneas of HSK patients, modulates the secretion of various chemokines by cytokine stimulated HCF[6]. Here, we demonstrate a new aspect of the T cell specific cytokine IL-17 in terms of its stimulatory action on both HCF and HCE. Whereas IL-17 itself had no effect, IL-17 selectively enhanced TNF-α induced GM-CSF secretion by both cell types, consistent with previous studies on different cell types[36-38]. The expression of GM-CSF is regulated both by transcriptional and

post-transciptional mechanisms[39]. IL-17 shares mainly properties with IL-1β and TNF-α that these three cytokines activate the transcription factor NF-κB in a variety of cell types including mesenchymal cells like fibroblasts[40]. In human colonic myofibroblasts, the synergistic effect of IL-17 to TNF-α in GM-CSF release was mediated both by the additive effect of IL-17 on TNF-α-induced NF-κB-DNA binding activity as well as the effect of IL-17 to stabilize the rapid degradation of the GM-CSF transcript in TNF-α stimulated cells[38]. Interestingly, the IL-1β induced GM-CSF mRNAs were stable and were not affected by IL-17 in colonic myofibroblasts[38]. Given the similarity between cell types, colonic myofibroblasts and HCF, these mechanisms may have contributed to the synergistic effect of IL-17 on TNF-α induced HCF secretion of GM-CSF. In case of HCE, the mechanisms involved are unknown and necessitate additional research.

In contrast to corneal epithelial and infiltrating inflammatory cells, corneal fibroblast do not appear to be the cellular source of GM-CSF in cornea buttons of patients with severe HSK (Fig. 7). However, the *in vitro* data on primary HCF cultures proved otherwise (Fig. 2). Penetrating keratoplasty (PKP) is the last option to treat patients with severe HSK to restore sight. Immunosuppressive treatment of these patients, for often many months, prior to PKP may have differentially inhibited GM-CSF production by the corneal fibroblast compared to epithelial cells *in situ*. For obvious reasons cornea buttons from untreated HSK patients are not available. Studies on the experimental HSK mouse model may provide more insight into this discrepancy.

We demonstrated that the production of GM-CSF is functionally significant. Conditioned medium from both cell types stimulated with TNF-α or IL-1β, with or without addition of IL-17, prolonged the survival of human neutrophils and this effect was blocked significantly by a neutralizing GM-CSF specific mAb. However, given the relatively low GM-CSF levels in the aforementioned CM of cytokine treated HCF and HCE, compared to the combination of TNF-α and IL-17, other neutrophil survival factors than GM-CSF are most likely involved (Fig. 1). Noticeably, the CM of IL-17 stimulated HCE that was devoid of GM-CSF exerted sustained neutrophil survival activity. The potential fibroblast factors involved may be cytokines like G-CSF or IL-6[12]. Previously, others and our group have demonstrated that these proinflammatory cytokines induce IL-6 secretion by HCF and HCE[6,10]. Studies are of interest to unravel the total spectrum of neutrophil survival factors differentially secreted by cytokine stimulated corneal resident cells.

In contrast to the HSK mouse model, HSV-infected corneal fibroblast have been demonstrated in corneas of HSK patients[41,42]. The presence of HSV-specific antibodies within tears of these patients poses the possibility of a neutrophil ADCC response, as part of their pathogenic role in HSK.[30,31] The *in vitro* data presented here underlines this option and are in agreement with other studies using different target cells[43,44]. Neutrophil ADCC depends on the expression of both Fc γ receptors and Mac-1[45]. GM-CSF has been shown to improve mAb-dependent cellular cytotoxicity of neutrophils towards various tumor cell

lines.[32, 45] This effect was largely attributable to the upregulation of Mac-1, mediating adhesion and subsequent the neutrophilic oxidative burst once neutrophil-target cell conjugates have been established by mAb-antigen complexes on the target cell[45]. Conversely, addition of GM-CSF did not significantly enhanced killing of the HSV-infected HCF (Fig. 4). This, despite the ability of GM-CSF to induce the early upregulation of CD11b and shedding of CD62L (Fig. 3A), described as surrogate markers for the neutrophil oxidative burst response, similar as described by others[29]. Possibly cell type differences (e.g. expression of Mac-1 ligands) and/or the use of serum antibodies compared to mAbs may have accounted for this incongruity[32, 45].

The current study is the first to detect GM-CSF expression in corneas of HSK patients. *In vitro* studies showed that cytokine stimulated HCF and HCE secreted GM-CSF capable of inducing the survival, activation and even secretion of the major neutrophil chemoattractant IL-8 (Fig. 3B). Because chronic inflammation is critical to the loss of corneal function in the setting of HSK, our data suggest that GM-CSF may represent a promising therapeutic target to antagonize neutrophil-mediated tissue destruction. Additionally, cytokine induced expression of GM-CSF may initiate the egress and activation of macrophages into HSV-infected corneas, as has been demonstrated in GM-CSF transgenic mice[46]. Studies are in progress to investigate the putative proinflammatory role of GM-CSF in the experimental HSK mouse model.

References

1. Remeijer L, Osterhaus ADME, Verjans GMGM. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul Immunol Inflamm. 2004;12:255-285.
2. Biswas PS, Rouse BT. Early events in HSV keratitis - setting the stage for a blinding disease. Microbes Infect. 2005;7:799-810.
3. Yan XT, Tumpey TM, Kunkel SL, Oakes JE, Lausch RN. Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea. Invest Ophthalmol Vis Sci. 1998;39:1854-1862.
4. Tang Q, Hendricks RL. Interferon gamma regulates platelet endothelial cell adhesion molecule 1 expression and neutrophil infiltration into herpes simplex virus-infected mouse corneas. J Exp Med. 1996;184:1435-1447.
5. Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. J Immunol. 1997;158:1383-1391.
6. Maertzdorf J, Osterhaus ADME, Verjans GMGM. IL-17 Expression in Human Herpetic Stromal Keratitis: Modulatory Effects on Chemokine Production by Corneal Fibroblasts. J. Immunol. 2002;169:5897-5903.
7. Shirane J, Nakayama T, Nagakubo D, et al. Corneal epithelial cells and stromal keratocytes efficently produce CC chemokine-ligand 20 (CCL20) and attract cells expressing its receptor CCR6 in mouse herpetic stromal keratitis. Curr Eye Res. 2004;28:297-306.
8. Biswas PS, Banerjee K, Kim B, Kinchington PR, Rouse BT. Role of inflammatory cytokine-induced cyclooxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis. J Virol. 2005;79:10589-600.
9. Cubitt CL, Tang Q, Monteiro CA, Lausch RN, Oakes JE. IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. Invest Ophthalmol Vis Sci. 1993;34:3199-3206.
10. Cubitt CL, Lausch RN, Oakes JE. Differences in interleukin-6 gene expression between cultured human corneal epithelial cells and keratocytes. Invest Ophthalmol Vis Sci. 1995;36:330-336.
11. Biswas PS, Banerjee K, Kim B, Rouse BT. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. J Immunol. 2004;172:3736-3744.
12. Simon HU. Neutrophil apoptosis pathways and their modifications in inflammation. Immunol Rev. 2003;193:101-110.
13. Theilgaard-Monch K, Porse BT, Borregaard N. Systems biology of neutrophil differentiation and immune response. Curr Opin Immunol. 2006;18:54-60.
14. Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. Crit Rev Immunol. 2005;25:405-428.
15. Coxon A, Tang T, Mayadas TN. Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo. A role for granulocyte/macrophage colony-stimulating factor. J Exp Med. 1999;190:923-934.
16. Kobayashi SD, Voyich JM, Whitney AR, Deleo FR. Spontaneous neutrophil apoptosis and regulation of cell survival by granulocyte macrophage-colony stimulating factor.
J Leukoc Biol. 2005;78:1408-1418.
17. Hamilton JA. GM-CSF in inflammation and autoimmunity. Trends Immunol. 2002; 23:403-8.
18. Andreakos ET, Foxwell BM, Brennan FM, Maini RN, Feldmann M. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. Cytokine Growth Factor Rev. 2002;13:299-313.
19. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med. 2001;344:907-916.
20. Dinarello CA. The many worlds of reducing interleukin-1. Arthritis Rheum. 2005;52:1960-1967.
21. Cook AD, Braine EL, Campbell IK, Rich MJ, Hamilton JA. Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage colony-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease.
Arthritis Res. 2001;3:293-298.
22. Lawlor KE, Wong PK, Campbell IK, Rooijen NV, Wicks IP. Acute CD4+ T lymphocyte-dependent interleukin-1-driven arthritis selectively requires interleukin-2 and interleukin-4, joint macrophages, granulocyte-macrophage colony-stimulating factor, interleukin-6, and leukemia inhibitory factor. Arthritis Rheum. 2005;52:3749-3754.
23. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. Invest Ophthalmol Vis Sci. 2003;44:2496–2506.
24. Li H, Zhang J, Kumar A, Zheng M, Atherton SS, Yu FS. Herpes simplex virus 1 infection induces the

expression of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells. Immunology. 2006;117:167-176.
25. Gopinath R, Nutman TB. Identification of eosinophils in lysed whole blood using side scatter and CD16 negativity. Cytometry. 1997;30:313-316.
26. Philpott NJ, Turner AJ, Scopes J, et al. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood. 1996;87:2244-2251.
27. Barreca C, O'Hare P. Suppression of herpes simplex virus 1 in MDBK cells via the interferon pathway. J Virol. 2004;78:8641-8653.
28. Lee-MacAry AE, Ross EL, Davies D, et al. Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. J Immunol Methods. 2001; 252:83-92.
29. Wittmann S, Rothe G, Schmitz G, Frohlich D. Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response.
Cytometry. 2004;57:53-62.
30. Pramod NP, Dhevahi E, Sudhamathi K, Kannan K, Thyagarajan SP. Tear secretory IgA: evaluation of usefulness as a diagnostic marker in herpetic keratitis. Ocul Immunol Inflamm. 1999;7:61-67.
31. McBride BW, Ward KA. Herpes simplex-specific IgG subclass response in herpetic keratitis. J Med Virol. 1987;21:179-189.
32. Ottonello L, Morone P, Dapino P, Dallegri F. Monoclonal Lym-1 antibody-dependent lysis of B-lymphoblastoid tumor targets by human complement and cytokinine-exposed mononuclear and neutrophilic polymorphonuclear leukocytes. Blood. 1996;87:5171-5178.
33. Nathan C. Points of control in inflammation. Nature. 2002;420:846-852.
34. Parsonage G, Filer AD, Haworth O, et al. A stromal address code defined by fibroblasts. Trends Immunol. 2005;26:150-156.
35. Cubitt CL, Lausch RN, Oakes JE. Differential regulation of granulocyte-macrophage colony-stimulating factor gene expression in human corneal cells by pro-inflammatory cytokines. J Immunol. 1994;153:232-240.
36. Laan M, Prause O, Miyamoto M, et al. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. Eur Respir J. 2003;21:387-393.
37. Numasaki M, Tomioka Y, Takahashi H, Sasaki H. L-17 and IL-17F modulate GM-CSF production by lung microvascular endothelial cells stimulated with IL-1beta and/or TNF-alpha. Immunol Lett. 2004;95:175-184.
38. Andoh A, Yasui H, Inatomi O, et al. Interleukin-17 augments tumor necrosis factor-alpha-induced granulocyte and granulocyte/macrophage colony-stimulating factor release from human colonic myofibroblasts. J Gastroenterol. 2005;40:802-810.
39. Brizzi MF, Rossi PR, Rosso A, Avanzi GC, Pegoraro L. Transcriptional and post-transcriptional regulation of granulocyte-macrophage colony-stimulating factor production in human growth factor dependent M-07e cells. Br J Haematol. 1995;90:258-265.
40. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med. 1996;183:2593-603.
41. Verjans GMGM, Remeijer L, Mooy CM, Osterhaus ADME. Herpes simplex virus-specific T cells infiltrate the cornea of patients with herpetic stromal keratitis: no evidence for autoreactive T cells. Invest Ophthalmol Vis Sci. 2000;41:2607-2612.
42. Kaye SB, Baker K, Bonshek R, et al. Human herpesviruses in the cornea. Br J Ophthalmol. 2000;84:563-571.
43. Siebens H, Tevethia SS, Babior BM. Neutrophil-mediated antibody-dependent killing of herpes-simplex-virus-infected cells. Blood. 1979;54:88-94.
44. Fujimiya Y, Rouse BT, Babiuk LA. Human neutrophil-mediated destruction of antibody sensitized herpes simplex virus type I infected cells. Can J Microbiol. 1978;24:182-186.
45. Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. Blood. 2002;99:4166-4173.
46. Lang RA, Metcalf D, Cuthbertson RA, et al. Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. Cell. 1987;51:675-686.

Chapter 3

Prevalence of Herpes Simplex Virus Type 1 Glycoprotein G (gG) and gI Genotypes in Patients with Herpetic Keratitis

Rui Duan, Jessica M. van Dun, Lies Remeijer, Martin Siemerink, Paul G.H. Mulder, Peter Norberg, Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

British Journal Of Ophthalmology. ***2008*** *Sep;92(9):1195-200.*

Abstract

Aim: Recent phylogenetic analyses on the herpes simplex virus type 1 (HSV-1) genes US4, encoding glycoprotein G (gG) and US7, encoding gI, of clinical HSV-1 isolates have led to the classification of HSV-1 into three genotypes, arbitrarily designated as A, B and C. The prevalence of the HSV-1 gG and gI genotypes and their potential disease association was determined in a large cohort of patients with herpetic keratitis (HK). **Methods:** Primary corneal HSV-1 isolates of 178 HK patients were genotyped by a PCR-based restriction fragment length polymorphism method targeting the viral genes US4 and US7. **Results:** Genotype B was more frequently expressed by the corneal HSV-1 isolates compared to genotypes A and C. Fifty-five of 178 corneal isolates (31%) had different genotypes in both loci. No clinically relevant associations were observed between the HSV-1 genotypes and disease outcome in the HK patients studied. **Conclusions:** The data presented demonstrate a high frequency of recombinant corneal HSV-1 isolates and suggest that clinical outcome of HSV-1-induced keratitis is independent of a gG or gI genotype.

Introduction

Herpes simplex virus type 1 (HSV-1) infection is a leading cause of visual morbidity in developed countries. The prevalence of ocular HSV-1 disease, mainly affecting the anterior segment of the eye, has been estimated at 149 cases per 100,000 US citizens.[1] Corneal HSV-1 infections cause a spectrum of clinical manifestations ranging from blepharitis, acute infectious epithelial keratitis (IEK) to the potentially blinding chronic inflammatory disease herpetic stromal keratitis (HSK). Patients who have had a corneal HSV-1 infection risk recurrent corneal disease throughout life. Particularly prolonged or recurrent episodes of herpetic keratitis (HK) can result in the development of HSK.[2]

The pathogenesis of ocular HSV-1 disease is dependent on a number of factors, including the genetic constitution of the virus and host and the host's immune system. Current knowledge on the pathogenesis of HK is largely based on studies on experimental mouse models, which have provided important insights into the role of host factors involved in both the prevention and immunopathology of HK.[3,4] Differences among inbred mouse strains have a strong influence on the reactivation of HSV-1 and the disease phenotype.[5,6] In humans, specific HLA alleles, polymorphisms in the genes encoding interleukin 10, apolipoprotein E and toll-like receptors, and a gene located within a region on the long arm of chromosome 21 have been described to affect HSV-1 disease.[7-12] By comparison, only a few studies have addressed the role of the viral genotype in the disease process.[13,14] Studies in experimental animal models have shown that different clinical patterns of herpetic ocular disease may be attributed at least in part to the differing biological behavior of specific HSV-1 strains.[15-19]

Recently, Norberg and colleagues have described that clinical HSV-1 isolates can be divided into three distinct genotypes, arbitrarily designated as genotype A, B and C.[20] The classification was based on DNA sequencing of the viral genes, located in the unique short (US) region of the HSV-1 genome, encoding glycoprotein G (gG; gene US4), gI (US7) and gE (US8). The gE and gI proteins have been shown to form a complex involved in cell-to-cell spread and the binding of IgG to its Fc receptor. The gG protein is essential for virus entry through the apical surfaces of polarized cells.[21] Genotype-specific synonymous and non-synonymous nucleotide substitutions have been identified, the latter mutations may modify the biological activity of the encoding proteins.[20,21] The contribution of the viral genotype to the clinical entity or outcome of HSV-1-induced diseases is still unknown.

The objective of the current study was two-fold. First, we determined the prevalence of the US4 and US7 HSV-1 genotypes in a large cohort of 178 unrelated HSV-1 isolates recovered from affected corneas of HK patients. Second, we explored the possible association between the viral genotypes and the clinical outcome of HSV-1-induced keratitis.

Patients and Methods

Patients and clinical specimens

Corneal swabs (n= 178) were obtained for diagnostic purposes at the Rotterdam Eye Hospital (Rotterdam, The Netherlands) between 1981 and 2002 from otherwise healthy patients with suspected herpetic corneal lesions. The classification of HK was defined on clinical criteria.[2] The corneal swabs were inoculated on human embryonic lung fibroblasts. The virus was harvested when approximately 75% of the monolayer showed viral cytopathic effect, typed for HSV-1 or -2 by immunocytology and PCR and subsequently culture supernatant frozen in aliquots as described previously.[21] The clinical items scored retrospectively were previous history of ocular disease, gender, age, number of recurrences, regimen and response to therapy (steroid and/or antiviral), cornea transplantation(s), glaucoma, anatomic location of the lesion and clinical presentation of HK at time of virus culture and at end of follow-up period (6 ± 2.9 years).The study was performed according to the tenets of the Declaration of Helsinki, approved by the local Ethical Committee and written informed consent was obtained from all patients.

HSV-1 genotyping

Total DNA was isolated from the virus culture supernatants using the MagNA Pure LC total nucleic acid isolation kit and the MagNAPure LC isolation station according to the manufacturer's instructions (Roche diagnostics, Almere, The Netherlands). Classification of the corneal HSV-1 isolates into the three distinct genotypes A, B and C was based on a PCR-based restriction fragment length polymorphism (RFLP) method as described in detail previously.[23] In brief, the PCR was carried out in a 50 µl volume containing 2.5 U Taq-polymerase with the corresponding buffer containing 15 mM $MgCl_2$, 0.5 µM of each of the primers, 200 µM dNTP (all from Roche diagnostics) and 10 µl of the purified DNA sample on a Peltier Thermal Cycler 200 DNA Engine (Bio-Rad, Veenendaal, The Netherlands. The gG gene (US4) was amplified using the following primers: forward 5'-GACTCTCCCACCGCCATCAG-3' (HSV-1 genome sequence nucleotide positions 136912-136932; GenBank accesion number NC_001806), reverse 5'-TGTCTTCGGGCGACTGGTCT-3' (nucleotide positions 137162-137181). For the gI gene (US7) the following primers were used: forward 5'-CCTGCTTATTCTCGGGGAGCTTC-3' (nucleotide positions 139934-139957) and reverse 5'-AGCAGTTTCGGGTCGCAGGA-3' (nucleotide positions 140325-140344). Genotype classification was done by restriction enzyme (RE) analysis. Ten microliters of the unpurified PCR product was mixed with *Pfl*M I and *Dde* I (US4 gene) or *Sac* I and *Ple* I (US7 gene), supplemented with the correct buffer, bovine serum albumin and water to a final volume of 20 µl (all from New England Biolabs, Leusden, The Netherlands). After

incubation for 3 hours at 37°C, the samples were run on 3% Metaphor agarose gel (BioWhittaker) along with a 50 bp DNA marker (Invitrogen, Breda, The Netherlands. Differential RE cleavage patterns of the amplicons, based on genotype-specific point mutations within the RE sites, enabled accurate classification of the HSV-1 isolate analyzed.[20, 23]

Statistics

Comparisons of categorical or numeric variables within the group of patients and the US4 and US7 viral genotypes were done by use of the χ^2-test, Kruskal-Wallis test or the one-way analysis of variance with Bonferroni's multiple comparison test. In total 13 clinical variables were explored in this way.

Results

Recently, Norberg and colleagues described a PCR-RFLP assay for rapid and accurate genotyping of clinical HSV-1 isolates.[23] This assay is based on genotype-specific mutations within RE sites of discrete regions within US4 and US7.[20] This assay was applied to determine the genotype prevalence among unrelated corneal HSV-1 isolates from 178 HK patients. The HK patient group consisted of 108 men and 70 women (mean age, 50 ± 22 years). The HK clinical entities included IEK (n= 63), HSK (n= 81), blepharitis (n= 24) and keratouveitis (n= 10). Based on the genotyping target US4, 46 isolates were classified as genotype A, 104 isolates were genotype B and 28 isolates were genotype C. Based on the US7 gene, 52 isolates were classified as genotype A, 86 isolates were genotype B and 40 isolates were genotype C (Table 1). Interestingly, 55 out of 178 isolates (31%) had different genotype identities in the 2 genotyping targets, suggesting that these isolates are intergenic recombinants. The most prevalent combinations between US4 and US7 found were A/B (n= 14), B/A (n= 21) and B/C (n= 14). Contrastingly, the US4/US7 combinations A/C (n= 2), C/A (n= 1) and C/B (n= 3) were less common (Table 1).

Table 1. Results of cornea HSV-1 isolate genotyping based on HSV-1 US4 and US7 polymorphisms

	US4 genotype		
	A (n= 46)	B (n= 104)	C (n= 28)
US7 genotype			
A (n= 52)	30 (16.9)[a]	21 (11.8)	1 (0.6)
B (n= 86)	14 (7.9)	69 (38.8)	3 (1.7)
C (n= 40)	2 (1.1)	14 (7.9)	24 (13.5)

a Data are the number (%) of corneal HSV-1 isolates assigned to the indicated US4 and US7 genotypes A, B or C

Table 2. Comparison of the corneal herpes simplex virus type 1 US4 and US7 genotypes and clinical parameters of the herpetic keratitis patients studied

	US4 genotype[b]				US7 genotype[b]			
Clinical parameters[a]	**A**	**B**	**C**	**p[c]**	**A**	**B**	**C**	***p*[c]**
Affected eye								
Right (n= 94)	18 (19)	57 (61)	19 (20)	**.046**	22 (23)	44 (47)	28 (30)	**.028**
Left (n= 84)	28 (33)	47 (56)	9 (11)		30 (36)	42 (50)	12 (14)	
Gender								
Male (n= 108)	25 (23)	64 (59)	19 (18)	.353	27 (25)	50 (46)	31 (29)	**.035**
Female (n= 70)	22 (31)	39 (56)	9 (16)		25 (36)	36 (51)	9 (13)	
HSV-1 blepharitis								
Yes (n= 24)	10 (42)	14 (58)	0 (0)	**.029**	7 (29)	12 (50)	5 (21)	.976
No (n= 154)	36 (23)	90 (58)	28 (18)		45 (29)	74 (48)	35 (23)	
Corneal insensibility								
Yes (n= 21)	4 (19)	16 (76)	1 (6)	.171	3 (14)	16 (76)	2 (10)	**.024**
No (n= 157)	42 (27)	88 (56)	27 (17)		49 (31)	70 (45)	38 (24)	
AC sensitation								
Yes (n= 32)	8 (25)	23 (72)	1 (3)	.076	10 (31)	19 (59)	3 (9)	.134
No (n= 146)	38 (26)	81 (56)	27 (19)		42 (29)	67 (46)	37 (25)	
Glaucoma								
Yes (n= 39)	10 (6)	23 (13)	6 (3)	.996	11 (6)	19 (11)	9 (5)	.986
No (n= 139)	36 (2)	81 (46)	22 (12)		41 (23)	67 (38)	31 (17)	
HK entity: history								
IEK (n= 63)	16 (36)	36 (35)	11 (37)	.778	16 (31)	32 (38)	15 (36)	.872
HSK (n= 85)	21 (47)	49 (48)	15 (50)		27 (53)	38 (45)	20 (48)	
Conjunctivitis (n= 17)	4 (9)	12 (12)	1 (3)		3 (6)	10 (12)	4 (10)	
Other (n= 13)	4 (9)	6 (6)	3 (10)		5 (10)	5 (6)	3 97)	
HK entity: end follow-up								
IEK (n= 50)	15 (34)	27 (27)	8 (24)	.666	14 (27)	25 (29)	11(26)	.983
HSK (n= 93)	22 (50)	53 (53)	18 (53)		28 (55)	42 (49)	23 (55)	
Conjunctivitis (n= 16)	4 (9)	11 (11)	4 (12)		4 (8)	10 (12)	5 (12)	
Other (n= 19)	3 (7)	9 (9)	4 (12)		5 (10)	8 (9)	3 (7)	
Corneal transplantation								
Yes (n= 49)	12 (7)	33 (19)	4 (2)	.180	17 (10)	25 (14)	7 (4)	.245
No (n= 129)	34 (19)	71 (40)	24 (13)		35 (20)	61 (34)	33 (19)	
Outcome therapy								
Improvement (n= 147)	39 (27)	84 (57)	24 (16)	.262	44 (30)	67 (43)	36 (24)	.373
Stable (n= 5)	0	5 (100)	0		1 (20)	4 (80)	0	
Worse (n= 6)	4 (67)	2 (33)	0		3 (50)	3 (50)	0	

[a] HK, herpetic keratitis; AC sensitation, inflammatory cells present in the aqueous chamber; IEK, infectious epithelial. keratitis and HSK, herpetic keratitis

[b] Data are the number (%) of corneal HSV-1 isolates assigned to the indicated US4 and US7 genotypes A, B or C.

[c] Results of 2-sided χ^2-test. Statistical significant genotype associations are shown in bold.

Next, we linked the genotype data to the patient's clinical records to look for a possible association between the patient's corneal HSV-1 genotype and outcome of disease. Of the 13 clinical variables considered, four variables were identified with at least one or both p-values for either US4 or US7 smaller than 0.05. The outcomes of the analyses are shown

in Table 2 and Figure 1A. The relative frequencies of genotypes A and C were significantly lower and higher in the right and left eye of the patient for both the US4 (p= 0.046) and US7 gene (p= 0.028), respectively. In case of US7, a lower proportion of female compared to male HK patients were infected with HSV-1 genotype C (p= 0.035). None of the HK patients who developed HSV-1-induced blepharitis were infected with an US4-based genotype C virus, while this particular genotype was identified among 28 of 154 (18%) of the patients with blepharitis (p= 0.029). The US7-based genotype B was significantly associated with reduced sensitivity of the diseased cornea (p= 0.024; Table 2). All other clinical parameters of interest, either before or at end of follow-up, including the presence of inflammatory cells in the aqueous chamber (AC), glaucoma, clinical presentation of herpetic keratitis, transplantation of the affected cornea, outcome of therapy, patient's age and number of recurrences did not show a significant association with either genotype (Table 2 and Figure 1A).

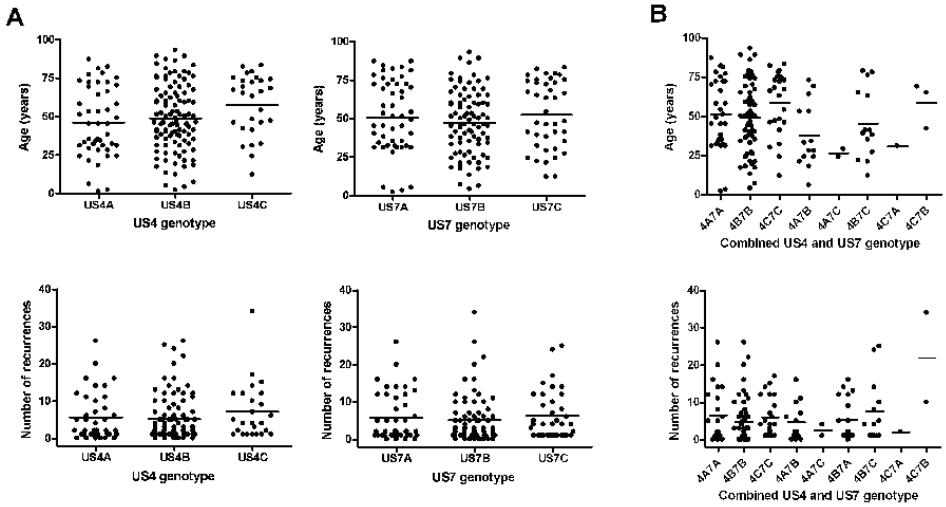


Figure 1. The genotype of corneal HSV-1 isolates is not significantly associated with the age or number of recurrences of herpetic keratitis patients. (A) Corneal HSV-1 isolates of 178 herpetic keratitis patients were genotyped on the HSV-1 US4 and US7 locus and the 3 alleles of each locus, arbitrarily defined as A, B or C, analyzed for the possible association with the age (upper panel) and number of recurrences until end of follow-up (lower panel). (B) The US4 and US7 genotypes were combined and analyzed as in (A). The data were analysed by one-way analysis of variance with Bonferroni's multiple comparison testing. Bars represent the mean values.

About one-third of the corneal HSV-1 isolates had different genotypes in both loci, raising the possibility that a specific US4 and US7 genotype combination may affect the clinical presentation and outcome of disease. The clinical parameters were evaluated for their possible association with either US4 or US7 genotype combination (n= 9) (Table 3 and Figure 1B). Except for the anatomic location of the lesion (left or right eye) and

Table 3. Comparison of the corneal herpes simplex virus type 1 US4 and US7 genotype combinations and clinical parameters of the herpetic keratitis patients studied

	US4 and US7 genotype combinations[b]									
Clinical parameters[a]	**4A7A**	**4B7B**	**4C7C**	**4A7B**	**4A7C**	**4B7A**	**4B7C**	**4C7A**	**4C7B**	**p[c]**
Affected eye										
Right (n= 94)	10 (11)	30 (32)	16 (17)	10(10)	1 (1)	13 (14)	11(12)	1 (1)	2 (2)	**.038**
Left (n= 84)	20 (24)	39 (46)	8 (10)	4 (5)	1 (1)	8 (10)	3 (4)	0	1 (1)	
Gender										
Male (n= 108)	15 (14)	42 (38)	18 (17)	7 (7)	2 (2)	12 (11)	11 (10)	0	1 (1)	.271
Female (n= 70)	15 (21)	27 (39)	6 (9)	7 (10)	0	9 (13)	3 (4)	1 (1)	2 (3)	
HSV-1 blepharitis										
Yes (n= 24)	5 (21)	9 (38)	0	3 (13)	2 (8)	2 (8)	3 (13)	0	0	**.013**
No (n= 154)	25 (16)	60 (39)	24 (16)	11 (7)	0	19 (12)	11 (7)	1 (1)	3 (2)	
Corneal insensibility										
Yes (n= 21)	1 (5)	13 (62)	1 (5)	3 (14)	0	2 (10)	1 (5)	0	0	.330
No (n= 157)	29 (18)	56 (36)	23 (15)	11 (7)	2 (1)	19 (12)	13 (8)	1 (1)	3 (2)	
AC sensitation										
Yes (n= 32)	4 (13)	15 (47)	1 (3)	4 (13)	0	6 (19)	2 (6)	0	0	.403
No (n= 146)	26 (18)	54 (37)	23 (16)	10 (7)	2 (1)	15 (10)	12 (8)	1 (1)	3 (2)	
Glaucoma										
Yes (n= 39)	6 (15)	14 (36)	5 (13)	4 (10)	0	5 (13)	4 (10)	0	1 (3)	.980
No (n= 139)	24 (17)	55 (40)	19 (14)	10 (7)	2 (1)	16 (12)	10 (7)	1 (1)	2 (1)	
HK entity: history										
IEK (n= 63)	9 (14)	25 (40)	9 (14)	6 (10)	1 (2)	6 (10)	5 (8)	1 (2)	1 (2)	.980
HSK (n= 85)	16 (19)	32 (38)	13 (15)	4 (5)	1 (1)	11 (13)	6 (7)	0	2 (2)	
Conjunctivitis (n= 17)	2 (12)	8 (47)	1 (6)	2 (12)	0	1 (6)	3 (18)	0	0	
Other (n= 13)	3 (23)	4 (31)	1 (8)	2 (15)	0	3 (23)	0	0	0	
HK entity: end follow-up										
IEK (n= 50)	8 (16)	19 (38)	7 (14)	6 (12)	1 (2)	5 (10)	3 (6)	1 (2)	0	.991
HSK (n= 93)	16 (17)	34 (37)	15 (16)	5 (5)	1 (1)	12 (13)	7 (8)	0	3 (3)	
Conjunctivitis (n= 16)	3 (19)	7 (44)	1 (6)	1 (6)	0	1 (6)	3 (19)	0	0	
Other (n= 19)	3 (16)	9 (47)	1 (5)	2 (11)	0	3 (16)	1 (5)	0	0	
Corneal transplantation										
Yes (n= 49)	9 (18)	22 (45)	4 (8)	3 (6)	0	8 (16)	3 (6)	0	0	.631
No (n= 129)	21 (16)	47 (36)	20 (16)	11 (9)	2 (2)	13 (10)	11 (9)	1 (1)	3 (2)	
Outcome therapy										
Improvement (n= 147)	26 (18)	54 (37)	21 (14)	11 (7)	2 (1)	17 (12)	13 (9)	1 (1)	2 (1)	.922
Stable (n= 5)	0	4 (80)	0	0	0	1 (20)	0	0	0	
Worse (n= 6)	2 (33)	1 (17)	0	2 (33)	0	1 (17)	0	0	0	

[a] HK, herpetic keratitis; AC sensitation, inflammatory cells present in the aqueous chamber; IEK, infectious epithelial keratitis and HSK, herpetic keratitis

[b] Data are the number (%) of corneal HSV-1 isolates assigned to the indicated US4 and US7 genotype combinations indicated.

[c] Results of 2-sided χ2-test. Statistical significant genotype associations are shown in bold.

blepharitis, no significant association with either US4 or US7 genotype combination was detected (Table 3). In comparison, lesions of patients with corneal HSV-1 isolates expressing the genotype combination US4A/US7A were more often located in the left eye (p= 0.038)

and none of the 24 patients with an US4C/US7C genotype combination had blepharitis (p= 0.013) (Table 3). Patients with an US4B/US7C genotype combination were predominantly males with lesions in their right eye. However, the number of patients expressing this genotype combination is too low to draw conclusions and warrants re-evaluation in a larger group of HK patients.

Discussion

Herpetic keratitis caused by HSV-1 is the most common infective cause of corneal blindness in developed countries, largely because of its recurrent nature.[2] The clinical manifestations of HK vary extensively. The most common presentations are blepharitis and conjunctivitis, with the least common being HSK.[1] Despite the development of new therapeutic regimens targeting both the virus and host immune response involved, HSK remains the most devastating manifestations of herpetic keratitis.[2] Whereas more than 80% of the adult population has acquired HSV-1, only 0.15% will develop herpetic keratitis. Successful treatment of the disease will benefit from the elucidation of factors, of virus or host origin, predisposing to the development and severity of HK in the population. The recent division of HSV-1 into three distinct genotypes has raised the question whether either viral genotype may be associated with clinical disease patterns.[20]

In the present study, we first determined the frequency of the 3 viral genotypes within a large panel of 178 unrelated corneal HSV-1 isolates of HK patients. The genotype of the isolates was defined by a validated PCR-RFLP method targeting the polymorphic locations within the viral genes US4 and US7.[23] The genotype frequencies were not equally distributed. Genotype B was more frequently expressed by the corneal HSV-1 isolates compared to genotypes A and C. The genotype frequencies among HK patients resemble those described recently on 28 Swedish clinical isolates obtained from patients with various diseases including genital and oral HSV-1 infections.[20,23] Although these data suggest that neither genotype predisposes to the development of HK, a larger cohort of patients with other HSV-1 diseases, including patients from other geographical locations in the world, are warranted to validate this assumption.

Whereas the majority of the corneal HSV-1 isolates displayed a similar genotype for both the US4 and US7 gene, the genotypes of about one-third of the isolates were disparate. The data suggest that the latter isolates are intergenic recombinants with recombination points located between the respective genes. A similar high frequency of intergenic recombinants has been reported previously, suggesting the relative instability of the HSV-1 genome between both genes.[20,23] Among the intergenic recombinant corneal HSV-1 isolates, combinations of genotypes A and B were overrepresented whereas genotype A and C combinations were only rarely identified. These differences may in part be due to the genetic distance between genotype A and B compared to C.[20] Alternatively, genotypes A

and B may have been introduced in The Netherlands earlier than genotype C, facilitating genotypes A an B strains to recombine over a longer time frame.

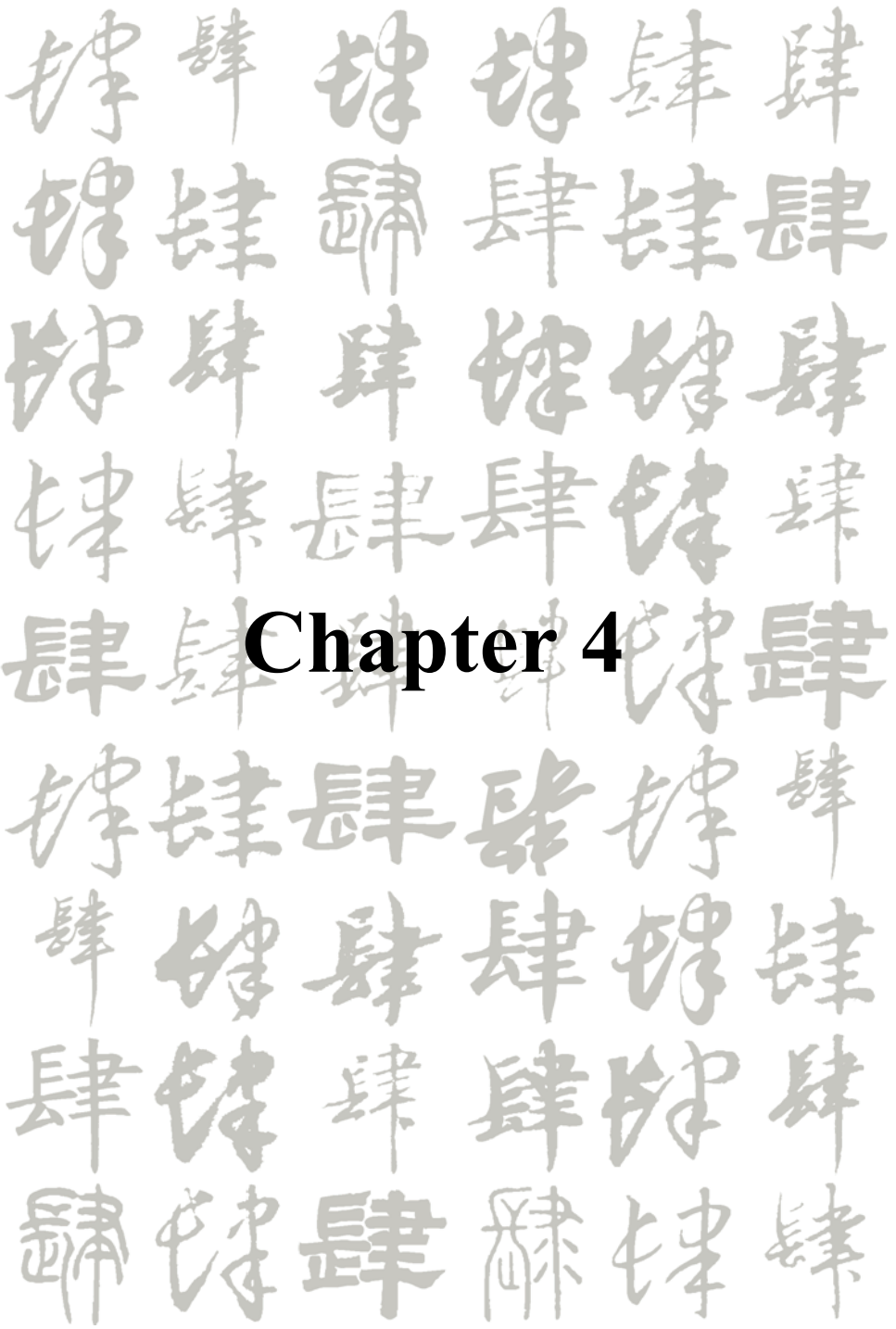
As for other viral infections, the clinical outcome of HK depends on the interaction between virus and host.[2,4] Based on previous studies in experimental HK mouse models, the genetic make-up of the virus may affect the pathology of the disease.[14-17] Consequently, the second objective of the present study was to determine the possible association between the viral genotypes identified and the patients' clinical parameters. Among the set of clinical data analyzed, several clinical parameters appeared to be associated to some extent with a specific genotype. However, the clinical relevance of the associations found is questionable. Particularly, the statistically significant associations between genotype C and US4AUS7A and disease in the right eye and, only in case of US7, its high prevalence in male compared to female HK patients are inexplicable. Additionally, two other disease-related parameters including blepharitis and reduced corneal sensitivity of the diseased eye tended to be more or less associated with specific viral genotypes. This is an explorative study in order to look for clinically relevant associations or clinically relevant combinations of associations. For that reason no correction for multiple testing was applied. A formal multiple testing correction for 13 variables and two tests per variable would have led to such a small alpha value per test that relevant (combinations of) associations would have certainly been overlooked as non-significant. Of course, the price to be paid here is that one should afterwards critically judge the resulting (combinations of) associations for clinical relevance, as they could have quite easily arisen by chance.

Hitherto, the data do not provide conclusive evidence that the US4 and US7 genotypes predispose to the clinical outcome of HK. The data presented leaves us with the question which viral genotypes determine the outcome of disease in HK patients and how to identify these candidate markers. The extensively studied HSK mouse model will be of value to screen potential virulence genes before in depth analyses on HSK patients. The disease closely mimics the immunopathology of human HSK and the application of inbred mouse strains enables to standardize the plethora of host factors involved in the disease process.[1,2] Furthermore, experiments in the HSK mouse model have shown HSV-1 strain differences in reactivation and pathogenesis. For instance, the HSV-1 reference strains McKrae and 17syn^{+} reactivate with similar frequency in mouse and rabbit models of disease, whereas strain KOS does not reactivate in these animals.[24-26] Moreover, corneal infection of A/J mice with the HSV-1 strains KOS and RE induces HSK, which is orchestrated by different effector cells. The disease in KOS and RE infected A/J mice is dependent on CD8 and CD4 T-cells, respectively.[27] Thus, virulence genes encoded by HSV-1 truly affect the severity of corneal disease in mice. Among the 80 HSV-1 genes, only few have been formally tested in mice for their role in HSV keratitis. Polymorphisms within the HSV-1 genes UL9, UL33, UL36, UL41, UL42 and US1 have been show to affect corneal virulence in mice and are of interest to be included in human studies.[14] However, it should be kept in mind that the

genetics of HSV-1 virulence is complicated with the constellation of genes encoded by each strain. Both the interactions between viral proteins, and viral-host protein interactions, determine the outcome of disease. Future studies are mandatory to identify and determine the role of HSV-1 virulence genes involved in HSV-1-induced keratitis. This may eventually provide new diagnostic means to identify and treat patients more effectively that are at risk to develop severe herpetic keratitis.

References

1. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. *Cornea* 2001;**20**:1-13.
2. Remeijer L, Osterhaus ADME, Verjans GMGM. Human herpes simplex virus keratitis: the pathogenesis revisited. *Ocul Immunol Inflamm* 2004;**12**:255-85.
3. Brandt CR. The role of viral and host genes in corneal infection with herpes simplex virus type 1. *Exp Eye Res* 2005;**80**:607-21.
4. Kaye S, Choudhary A. Herpes simplex keratitis. *Prog Retin Eye Res* 2006; **25**:355-80.
5. Harbour DA, Hill TJ, Blyth WA. Acute and recurrent herpes simplex in several strains of mice. *J Gen Virol* 1981;**55**:31–40.
6. Caspary L, Schindling B, Dundarov S, Falke D. Infections of susceptible and resistant mouse strains with HSV types 1 and 2. *Arch Virol* 1980;**65**:219–27.
7. Lekstrom-Himes JA, Hohman P, Warren T, et al. Association of major histocompatibility complex determinants with the development of symptomatic and asymptomatic genital herpes simplex virus type 2 infections. *J Infect Dis* 1999;**179**:1077–85.
8. Hurme M, Haanpaa M, Nurmikko T, *et al*. IL-10 gene polymorphism and herpesvirus infections. *J Med Virol* 2003;**70**:S48–50.
9. Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA. Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* 1997;**349**:241–4.
10. Bochud P-Y, Magaret AS, Koelle DM, Aderem A, Wald A. Polymorphisms in *TLR2* are associated with increased viral shedding and lesional rate in patients with genital herpes simplex virus type 2 infection. *J Infect Dis* 2007;**196**:505–9.
11. Zhang SY, Jouanguy E, Ugolini S, et al. *TLR3* deficiency in patients with herpes simplex encephalitis. *Science* 2007;**317**:1522–7.
12. Hobbs MR, Jones BB, Otterud BE, Leppert M, Kriesel JD. Identification of a herpes simplex labialis susceptibility region on human chromosome 21. *J Infect Dis* 2007;**197**:340–6.
13. Umene K, Inoue T, Inoue Y, Shimomura Y. Genotyping of herpes simplex virus type 1 strains isolated from ocular materials of patients with herpetic keratitis. J *Med Virol* 2003;**71**:75-81.
14. Brandt CR. The role of viral and host genes in corneal infection with herpes simplex virus type 1. *Exp Ye Res* 2005;**80**:607-21.
15. Wander AH, Centifanto YM, Kaufman HE. Strain specificity of clinical isolates of herpes simplex virus. *Arch Ophthalmol* 1980;**98**:1458-61.
16. Brandt CR, Grau DR. Mixed infection with herpes simplex virus type 1 generates recombinants with increased ocular and neurovirulence. *Invest Ophthalmol Vis Sci* 1990; **31**:2214-23.
17. Brandt CR, Kolb AW, Shah DD, *et al*. Multiple determinants contribute to the virulence of HSV ocular and CNS infection and identification of serine 34 of the US1 gene as an ocular disease determinant. *Invest Ophthalmol Vis Sci* 2003; **44**:2657-68.
18. Kim JK, Kim YK, Hong J, et al. Isolation of the enhanced neurovirulent HSV-1 strains from Korean patients. *Virus Genes* 2003; **26**:115–8.
19. Bower JR, Mao H, Durishin C, et al. Intra-strain variants of herpes simplex virus type 1 isolated from a neonate with fatal disseminated infection differ in the ICP34.5 gene, glycoprotein processing, and neuroinvasiveness. *J Virol* 1999;**73**:3843–53.
20. Norberg P, Bergstrom T, Rekabdar E, *et al.* Phylogenetic analysis of clinical herpes simplex virus type 1 isolates identified three genetic groups and recombinant viruses. *J Virol* 2004;**78**:10755-64.
21. Roizman B, Knipe DM, Whitley RJ. Herpes simplex viruses. In: Knipe DM, Howley PM, Griffin DE, et al., eds. Fields virology. 5th ed. Vol 2. Philadelphia: Lippincott Williams & Wilkins, 2007:2501- 2603.
22. Roest RW, Maertzdorf J, Kant M, *et al.* High incidence of genotypic variance between sequential herpes simplex virus type 2 isolates from HIV-1-seropositive patients with recurrent genital herpes. *J Infect Dis* 2006;**194**:1115-8.
23. Norberg P, Bergstrom T, Liljeqvist JA. Genotyping of clinical herpes simplex virus type 1 isolates by use of restriction enzymes. *J Clin Microbiol* 2006;**44**:4511-4.
24. Sawtell NM, Thompson RL. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J Virol* 1992;**66**:2150-6.
25. Hill JM, Garza HH Jr, Helmy MF, *et al*. Nerve growth factor antibody stimulates reactivation of ocular herpes simplex virus type 1 in latently infected rabbits. *J Neurovirol* 1997;**3**:206-11.
26. Stroop WG, Banks MC. Herpes simplex virus type 1 strain KOS-63 does not cause acute or recurrent ocular disease and does not reactivate from ganglionic latency in vivo. *Acta Neuropathol* 1994;**87**:14–22.
27. Hendricks RL, Tumpey TM. Contribution of virus and immune factors to herpes simplex virus type I-induced corneal pathology. *Invest Ophthalmol Vis Sci*. 1990; **31**:1929-39.



Chapter 4

Prevalence and Clinical Consequences of Herpes Simplex Virus Type 1 DNA in Human Cornea Tissues

Lies Remeijer, Rui Duan, Jessica M. van Dun, Mark A. Wefers Bettink,
Albert D.M.E. Osterhaus, Georges M.G.M. Verjans
Journal of Infectious Disease. Accepted

Abstract

Background. To determine the prevalence and clinical consequences of herpes simplex virus type 1 (HSV-1), HSV-2 and varicella zoster virus (VZV) in cornea tissues obtained after penetrating keratoplasty (PKP). **Methods.** Excised corneas of patients with herpetic keratitis (HK patients; n=83) and without a history of HK (non-HK patients; n=367) were analyzed by (real-time) PCR and virus culture for the presence of HSV-1, -2 and VZV. Additionally, post-PKP donor corneascleral rims (n=273) were analyzed. Medical records of the transplanted patients were reviewed to determine the risk factors influencing intra-cornea viral load and graft survival. **Results.** HSV-1 was the most prevalent herpesvirus. Presence and HSV-1 DNA load was higher in corneas of HK patients. HSV-1 DNA load in HK patient corneas correlated with age, recurrence free interval, cornea neovascularization, pre-PKP steroid treatment and severity of disease. Herpesvirus DNA was detected in 2 of 273 corneascleral rims. Graft survival was inversely correlated with cornea HSV-1 DNA load in HK patients. **Conclusions.** The data presented argue for the implementation of real-time HSV-1 PCR on excised corneas of HK patients to improve post-PKP diagnosis and therapy. Screening of donor cornea tissues for herpesviruses is redundant to prevent newly acquired post-PKP HK.

Introduction

Cornea transplantation, also known as penetrating keratoplasty (PKP), is the most common and successful human tissue transplantation procedure. During the past decades the incidence of graft rejection, the leading cause of cornea graft failure, has lowered extensively. This is largely attributable to improvements in the pre- and post-PKP phase including updated procedures in eye banking and operating techniques, and more effective immune-suppressive and antiviral drugs [1].

The two human alpha-herpesviruses (αHHV) varicella zoster virus (VZV) and particularly herpes simplex virus type 1 (HSV-1) are the principal herpesviruses causing herpetic keratitis (HK) [2]. Herpetic keratitis ranges from a superficial infection to the chronic inflammatory disease herpetic stromal keratitis (HSK), the leading cause of cornea blindness in developed countries [5-7]. About 5-10% of all PKPs are performed on HSK patients to restore sight [3,4,8,9]. Cornea transplantation on HK patients confers a high rate of post-PKP complications, including recurrent HK, epithelial defects, secondary infections and eventually graft rejection. The incidence of post-PKP infectious keratitis varies between 1.76 -7.4% [10-13]. In addition to recurrent disease in transplanted HSK patients, HK may develop in cornea grafts of patients transplanted for reasons unrelated to ocular herpesvirus infections [14-16]. This clinical entity, defined as newly acquired herpetic keratitis occurs in about 0.9% of all PKP recipients [14]. Recurrent HK is predominantly initiated by reactivation of the endogenous latent HSV-1 strain [17]. Alternatively, the virus may be acquired from the donor cornea tissue (graft-to-host transmission) [18-21].

Because the visual prognosis of patients with post-PKP HK is poor, the identification of PKP patients at risk is of major importance [10-13]. Detection and quantification of αHHV DNA in recipient and donor corneal tissues obtained at time of PKP may be of diagnostic value to identify those patients at high risk to develop post-PKP HK. To our knowledge, this issue has not been examined extensively [22-25].

The aim of this prospective, cross-sectional study was two-fold. First, we determined the prevalence and quantity of HSV-1, -2 and VZV DNA in excised corneas of a large cohort of PKP patients with HK (HK patients; n=83) and without a clinical history of cornea αHHV infections (non-HK patients; n=367). Second, we determined whether the presence and DNA load of αHHVs detected in the recipient cornea buttons, and the surplus corneascleral rims of donor corneas obtained post-PKP, might influence graft survival.

Materials and Methods

Patients and clinical specimens

Cornea buttons were obtained from 450 consecutive patients, undergoing therapeutic PKP between 1999 and 2004 for visually disabling corneal disease at the Rotterdam Eye Hospital (Rotterdam; Netherlands). The cohort consisted of 83 HK patients and 367 non-HK patients (Table 1). The classification of HK was defined on clinical criteria [5,6]. Recipient corneas, surplus corneascleral rims of the donor corneas obtained after trepanation of the central cornea (n=273), and 84 clear eye bank corneas rejected for transplant purposes due to low endothelial cell counts (<2,300 cells/mm^2) were stored within 4 hours after surgery at –70^{o}C.

The patients' records were reviewed for clinical features before PKP, during and at end of follow-up (>4 years). The clinical entities scored were age, sex, regimen and response to preoperative therapy (steroids and antivirals), recurrence free interval (RFI), cornea vascularization, post-PKP HK recurrences, cornea epithelial defects or secondary infections, and graft status at end of follow-up. A graft failure event was scored when there was partial loss of cornea transparency or complete graft failure. In the HK patient group, 62 and 21 patients underwent a primary PKP or cornea regraft, respectively. Cornea vascularization was scored counting quadrants of deep vascularization. In contrast to non-HK patients, 50 HK patients (60.2%) received pre-PKP topical steroid treatment consisting of fluorometholon-acetate (0.1%; n=12), dexamethasone-di-natrium-phosphate (0.1%; n=29) or prednisolon-acetate (1%; n=9). Long-term pre-PKP systemic acyclovir prophylaxis was applied to 39 HK patients (47%). The RFI, defined as time from last clinical herpetic keratitis episode until PKP, varied from 0.5 to 468 months (mean 39.6 ± 78.4 months). The study was performed according to the tenets of the Declaration of Helsinki, approved by the local Ethical Committee and written informed consent was obtained.

Nucleic acid extraction and cDNA synthesis

The cornea tissues were divided through its centre into two equal parts. Tissues were triturated and DNA isolated using the MagnaPure DNA tissue kit II (Roche Diagnostics) combined with the MagnaPure LC isolation station (Roche Diagnostics). The eluted DNA was resuspended in 50µl elution buffer. Total RNA was isolated from triturated one-forth surplus cornea buttons containing >1 HSV-1 genome equivalent copies (gec) per corneal cell using Trizol (Invitrogen). The RNA was reversed transcribed using random hexamer primers, dNTPs and reverse transcriptase (Supersript II) according to the manufacturer's instructions (Invitrogen). The isolation of DNA and RNA was performed as described previously [26].

Detection of αHHVs by PCR

The αHHV genomes were detected by two different PCR assays: conventional PCR and real-time PCR (qPCR) assays. The conventional qualitative PCR assays included amplification of the isolated DNA with αHHV-specific primers and subsequent Southern blotting as described previously [27]. The qPCR assays were performed as individual assays in which the human single copy housekeeping gene *Homo sapiens* hydroxyl-methyl-bilane synthase (HMBS) was run in parallel with each virus target sequence of interest [28] The sequences and target genes of the primers/probe-pairs used have been published previously: HSV-1 genes US4 (glycoprotein G; gG) [29] latency associated transcript (LAT) [30], UL44 (gC) [31] and UL54 (ICP27) [31], HSV-2 US6 (gD) [29], VZV gene 38 [29], and the HMBS gene [28]. The amplification of the DNA and cDNA samples and detection was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems) as described previously [29]. For standardization of quantitative virus detection assays, commercially available quantified DNA control panels (Advanced Biotechnologies) and high-titer virus preparations derived from culture supernatants were used [26,29]. The sensitivity, as defined by the 95% hit rate on the EM counted virus stocks, of the qPCR assays were approximately 200 αHHV geq/ml.

Virus culture

Human embryonic lung fibroblasts were grown in 24-well plates in medium consisting of Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Invitrogen). For each assay, a one-forth surplus cornea button tissue section of selected cases was triturated in PBS, inoculated on the monolayers and evaluated for cytopathic effect regularly for 2 weeks. When cytopathic effect was observed, the viruses were typed by immunofluorescence [29].

Statistical analyses

Clinical and laboratory data were analyzed using the SPSS statistical software package version 15 (SPSS Inc.). The Spearman's rank correlation test was used to detect associations between viral load and recipients' age, RFI and graft survival. Fisher's exact test was used to compare categorical data. The Mann-Whitney U or Kruskal-Wallis tests were used to compare the means of two or multiple groups, respectively. The graft survival was calculated using the Kaplan Meier method and compared between groups using the log rank test.

Results

Prevalence of HSV-1, HSV-2 and VZV in recipient and donor cornea tissues

To determine the prevalence of αHHVs in human cornea tissues, excised corneas of PKP patients and donor corneascleral rims were prospectively collected between 1999 and 2004. The patients were divided on clinical grounds into two groups: HK patients (n=83) and non-HK patients (n=367) (Table 1).

Table 1. Characteristics of cornea transplant patients and number of excised cornea buttons containing human alpha herpesvirus DNA.

		Gender	Age	No. (%) corneas with viral DNA[a]		
Diagnosis at time of corneal transplantation	N (%)[a]	(F/M)[b]	(years ± SD)	HSV-1	HSV-2	VZV
Patients with history of herpetic keratitis (N=83)						
Infectious epithelial keratitis	1 (1)	0 / 1	36	0 (0)	0 (0)	0 (0)
Immune stromal keratitis	22 (27)	13 / 9	64 ± 16	8 (36)	0 (0)	0 (0)
Necrotizing stromal keratitis	35 (42)	12 / 23	56 ± 16	22 (63)	0 (0)	1 (3)
Herpetic keratouveitis	2 (2)	1 / 1	43 ± 13	1 (50)	0 (0)	0 (0)
Endotheliitis	3 (4)	1 / 2	56 ± 26	0 (0)	0 (0)	0 (0)
Allograft failure with initial HSK diagnosis	20 (24)	6 / 14	65 ± 13	9 (45)	0 (0)	0 (0)
Patients with no history of herpetic keratitis (N=367)						
Allograft failure	53 (14)	24 / 28	63 ± 19	0 (0)	0 (0)	0 (0)
Corneal decompensation	9 (3)	2 / 7	56 ± 17	2 (22)	0 (0)	0 (0)
Bullous keratopathy	23 (6)	16 / 7	66 ± 19	4 (18)	0 (0)	2 (9)
Pseudophake bullous keratopathy	50 (14)	23 / 27	73 ± 9	7 (14)	2 (4)	0 (0)
Fuchs's endothelial dystrophy	97 (26)	62 / 35	72 ± 10	0 (0)	2 (2)	0 (0)
Keratoconus	57 (16)	18 / 39	36 ± 13	2 (4)	1 (2)	1 (2)
Trauma	21 (6)	7 / 14	43 ± 20	0 (0)	1 (5)	0 (0)
Other (e.g scrofulosis, macular corneal dystrophy)	57 (16)	30 / 27	55 ± 22	0 (0)	1 (2)	2 (4)

[a]Number and percentages between brackets.
[b]F, female and M, male.

The prevalence of αHHV DNA in corneas was determined by conventional qualitative PCR (HK patients: n=22 and non-HK patients: n=53) or qPCR (HK patients: n=61 and non-HK patients: n=314). The reason for the separate assays used was that the qPCR assay was set-up and validated for research purposes at our laboratory early 2003. Unfortunately, samples assayed with qualitative PCR could not be re-analyzed by qPCR. The data of both assays are combined when appropriate. HSV-1 DNA was detected more frequently in corneas of HK than non-HK patients, 40 of 83 (48%) and 15 of 367 (4.1%), respectively (p<0.0001; Fisher's exact test) (Table 1). Moreover, the median HSV-1 DNA load (± interquartile range) was significantly higher in corneas of HK compared to non-HK patients, 0.20±5.1 HSV-1 gec/cornea cell (range, 0.0003 to1,548) and 0.002±0.11 (range 0.0004 to 500), respectively (p=0.012) (Fig. 1A). In comparison, HSV-2 and VZV were detected

more frequently in corneas of non-HK patients (Table 1). The median HSV-2 and VZV DNA load in non-HK corneas was 0.0007±0.02 (range 0.0002 to 2.69) and 0.007± 0.02 (range 0.0004 to 0.264), respectively. No cornea contained DNA of multiple αHHVs (data not shown).

Table 2. Detection of HSV-1 transcripts and infectious virus in HSV-1 DNA positive cornea explant buttons

Patient No.	Age (years)	Sex	Clinical Diagnosis[a]	HSV-1 gec/cell[b]	Recurrence free interval[c]	Relative HSV-1 transcript number[d]				HSV-1 culture[e]
						gG	gC	ICP27	LAT	
108	75	M	rePKP for HK	1,548	8	163	377	2,977	2,664	**Yes**
217	84	F	FED	500	NA	62	294	586	1,006	No
33	73	M	NSK	466	6	61	639	8,931	9,151	No
161	52	M	rePKP for HK	26	6	212	177	2,091	2,583	No
249	76	F	rePKP for HK	19	6	3	6	70	139	No
209	72	M	NSK	16	1	1	32	539	832	No

NOTE. [a]Clinical diagnosis at time of surgery. RePKP for herpetic keratitis (HK), regraft for recurrent herpetic keratitis in graft; FED, Fuch's endothelial dystrophy and NSK, necrotizing stromal keratitis.
[b]HSV-1 genome equivalent copies (gec) per corneal cell.
[c]Recurrence free interval, time in months from last clinical herpetic keratitis episode until cornea transplantation. NA, not applicable.
[d]Mean relative transcript levels of the indicated HSV-1 genes normalized for β-actin. gG, glycoprotein G; gC, glycoprotein C; ICP27, infectious cell protein 27 and LAT, latency associated transcript.
eRecovery of infectious virus from the affected corneal tissue.

Several corneas of HK (n= 10) and non-HK patients (n=2), who did not show signs of active disease at time of surgery, had relatively high HSV-1 DNA loads (>1 HSV-1 gec/cornea cell) suggesting virus replication in the excised corneas. This issue was investigated by determining the expression of selected HSV-1 transcripts and to culture the virus from the surplus cornea tissue on indicator cells (Table 2). During a lytic infection the HSV-1 genes are sequentially expressed in infected cells in a temporal cascade: immediate early (α, e.g. ICP27), early (β) and subsequently the late (γ; gG and gC) genes. The LAT gene is transcribed both during lytic infection and latency. The true late (γ2) genes, including gC, are the only HSV-1 genes expressed after the initiation of viral DNA synthesis, prerequisite to generate viral progeny [32]. Detection of HSV-1 transcripts, including the γ2 gene encoding gC, was restricted to corneas containing >15 HSV-1 gec/cornea cell (Table 2). Whereas the RT-PCR data demonstrate transcription of all three HSV-1 transcript categories, infectious HSV-1 could only be isolated from the cornea of a patient regrafted for HSK (patient #108) containing the highest viral load (1,548 HSV-1 gec/cornea cell) (Table 2; data not shown).

Various infectious agents can be transmitted by PKP [33]. Cornea donors are screened serologically for several viruses, including hepatitis B and C virus, and donor corneas are commonly cultured to detect bacterial and fungal infections [34]. Various groups have reported on the presence of HSV-1 in donor cornea tissues and graft-to-host transmission of HSV-1 through PKP [18-21], advocating the implementation of HSV-1 screening of donor corneas before PKP. This issue was addressed by determining the presence of αHHV DNA

in 273 donor corneascleral rims obtained after PKP and 84 clear eye bank corneas rejected for PKP purposes. Whereas none of the eye bank corneas contained αHHV DNA, HSV-1 DNA was detected in only 2 of 273 (0,73 %) corneascleral rims. HSV-2 and VZV DNA could not be detected (Table 3).

Correlation between intra-cornea HSV-1 DNA load and clinical parameters at time of PKP

The HSV-1 DNA qPCR data were linked to the patients' clinical records to determine possible associations between the HSV-1 DNA load in corneas and the clinical parameters at time of PKP.

Table 3. Prevalence of human alpha herpesvirus DNA in recipient and donor cornea tissues.

Origin of cornea tissues	No. (%) corneas with viral DNA[a]		
	HSV-1	HSV-2	VZV
Excised corneas of HK patients (N=83)	40 (48)	0 (0)	1 (1)
Excised corneas of non-HK patients (N=367)	15 (4)	7 (2)	5 (1)
Corneascleral rims (N=273)	2 (1)	0 (0)	0 (0)
Eye bank corneas (N=84)	0 (0)	0 (0)	0 (0)

NOTE. HK and non-HK patients, patients with and without a history of herpetic keratitis, respectively.
[a]Number and percentages between brackets.

Surprisingly, in both HK and non-HK patients, the HSV-1 DNA load correlated significantly with the patients' age (Fig. 1B; r=0.491, *p*=0.009) and (Fig. 1C; r=0.582, *p*=0.029) respectively. In non-HK corneas, this correlation was almost significant when the viral loads of all αHHVs were combined (Fig. 1D; r=0.370, *p*=0.058). Preoperative antiviral and steroid treatment influenced the HSV-1 DNA load in corneas of HK patients. The viral load was significantly higher in patients treated with steroids (Fig. 1E; *p*=0.002), whereas antiviral treatment tended to correlate with higher cornea HSV-1 DNA loads (Fig. 1F; *p*=0.16). The latter observation is most likely due to the use of antiviral treatment in the more severe HK cases at our hospital. Both the type of antivirals and steroids used preoperatively did not correlate with the intra-cornea HSV-1 DNA load (data not shown). The HSV-1 DNA load in HK corneas diminished gradually with increasing grade of cornea vascularization (Fig. 1G; p=0.20). Relatively higher numbers of HK patients with HSV-1 DNA^{pos} corneas had fulminant disease (data not shown), and HK patients regrafted for herpetic keratitis had significantly higher cornea HSV-1 loads compared to immune stromal keratitis patients (Fig. 1H; p=0.041). Interestingly, the RFI was significantly shorter among HK patients with HSV-1 DNA^{pos} corneas compared to those with HSV-1 DNA^{neg} corneas (Fig. 1I; p=0.008). The inverse correlation between RFI and cornea HSV-1 DNA load affirms this observation (Fig. 1J; r=-0.385, p=0.047).

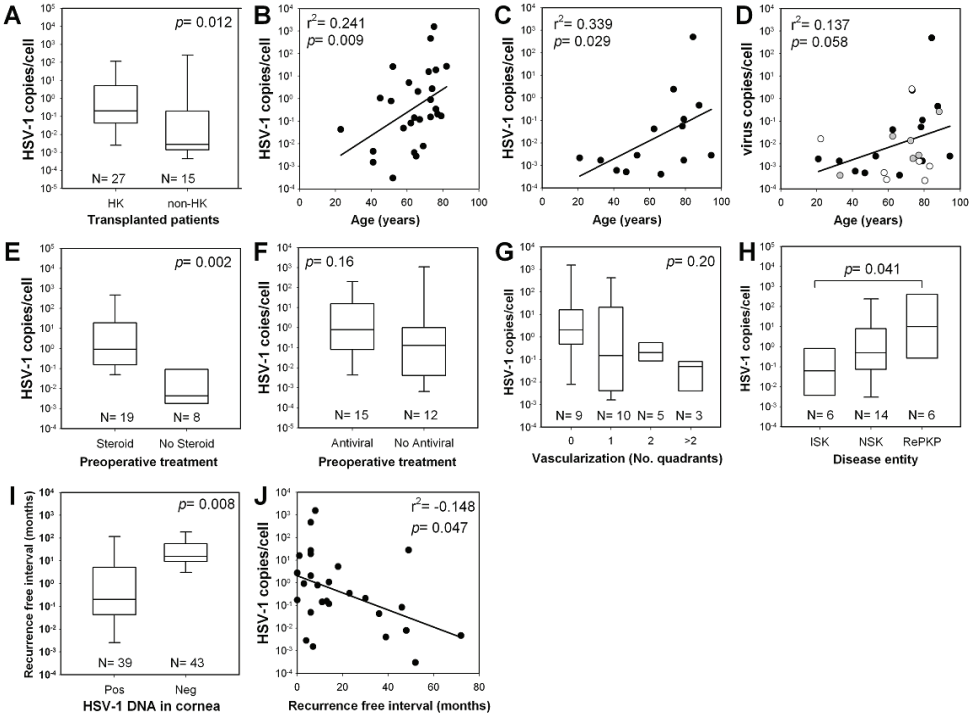


Figure 1. Correlations of clinical entities and HSV-1 DNA load in excised corneas of cornea transplant patients. A, intra-corneal HSV-1 DNA load in transplanted patients with (HK) and without (non-HK) a history of herpetic keratitis. B-D, intra-corneal HSV-1 DNA load in HK patient (B) and non-HK corneas (C), and the combinatory human alpha-herpesvirus DNA load in corneas of non-HK patients (D) in relation to the patients' age. Data presented in subfigure D are the intra-corneal HSV-1 (black symbols), HSV-2 (white symbols) and VZV (grey symbols) DNA load in relation to the non-HK patients' age. E-H, intra-corneal HSV-1 DNA load of HK patients in relation to preoperative treatment with steroids (E) and antivirals (F), corneal vascularization (G) and disease entity (H). The presence of HSV-1 DNA (G) and the HSV-1 DNA load (H) in corneas in relation to the recurrence free interval. The subfigures A and E to I, and B-D and J, represent conventional box-and-whisker plots and scatter plots, respectively. The statistical analyses used were the Mann-Whitney U test (A, E, F and I), Kruskal-Wallis test (G and H) and Spearman's rank correlation test (B-D and J). The numbers in the bar graphs indicated the number of patients within each subgroup. Pos, HSV-1 DNA positive corneas, Neg, HSV-1 DNA negative corneas, ISK, immune stromal keratitis; NSK, necrotizing stromal keratitis and rePKP, corneal regraft.

Correlation between intra-cornea HSV-1 DNA load and graft survival

At the end of follow-up (average 52±30 months), 27 of 83 (32.5%) transplanted HK patients, and 107 of 367 (29.1%) transplanted non-HK patients, had a graft failure. In both patient groups, graft failure was more common in patients with a history of previous graft rejection in the operated eye. Cornea regraft failure occurred at a similar rate between HK and non-HK patients, 10 of 21 (48%) and 31 of 57 (54%) cornea regrafts were rejected, respectively.

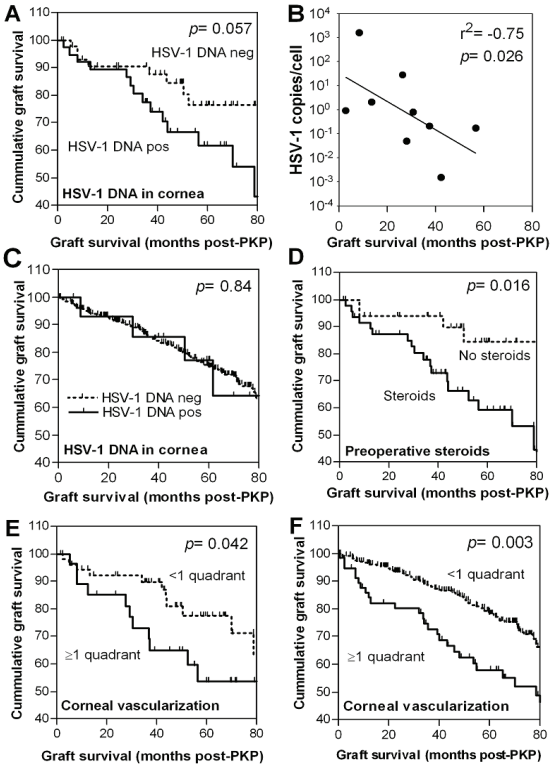


Figure 2. Factors influencing corneal graft survival in cornea transplant patients. Kaplan-Meier survival curves comparing graft rejections in HK patients (**A**) and non-HK patients (**B**) with HSV-1 DNA negative (dashed line) and HSV-1 DNA positive excised corneas (solid dashed line). **C**, scatter plot comparing the intra-corneal HSV-1 DNA load in excised corneas with graft survival in HK patients. **D**, Kaplan-Meier survival curves comparing corneal graft survival in HK patients who received preoperative steroid (solid line) or no steroid treatment (dashed line). **E-F**, Kaplan-Meier survival curve comparing graft rejections in HK patients (**E**) and non-HK patients (**F**) with ≥1 quadrant (solid line) and <1 quadrant (dashed line) deep corneal vascularization at time of transplantation. The statistical analyses used were the Logrank test (A-F) and the Spearman's rank correlation test (B). The bars in the survival curves represent censored events

Next, the PCR data were linked to the patients' clinical records to determine if intra-corneal HSV-1 DNA may have affected graft survival. HK patients with HSV-1 DNApos corneas tended to have a higher risk of graft failure (Fig. 2A; p=0.057). The significant inverse correlation between intra-cornea HSV-1 DNA load and graft survival in HK patients (Fig. 2B; r=-0.75, p=0.026) strengthens this association. In contrast, the presence of HSV-1 DNA in the cornea buttons of non-HK patients did not affect graft survival significantly (Fig. 2C; p=0.84). The hazard ratio (HR) was 0.90 with a 95% confidence interval (95% CI) of 0.35 to 2.35. The numbers of VZV and HSV-2 DNA positive non-HK cornea buttons were too small to be analyzed.

Among the other clinical factors potentially influencing graft survival only cornea vascularization and pre-operative steroid treatment were significantly different. Preoperative steroid treatment of HK patients lowered the graft survival significantly (Fig. 2D; *p*=0.016) (HR=2.99, 95% CI=1.20 to 5.87). In both HK and non-HK patients, graft survival was lower in corneas with ≥1 quadrant deep cornea vascularization (Fig. 2E; *p*=0.042) (HR=2.14, 95% CI=1.04 to 5.98) and (Fig. 2F; *p*=0.003) (HR=2.10, 95% CI=1.54 to 4.34), respectively. Presence of HSV-1 DNA in cornea buttons did not correlate with the number of post-PKP HK recurrences, and cornea epithelial defects or secondary infections

(data not shown).

Next, we addressed the possibility to identify PKP patients at risk acquiring HSV-1 from the cornea graft. As described above, only 2 of 273 corneascleral rims contained HSV-1 DNA and HSV-2 and VZV DNA could not be detected. Both HSV-1 DNA^{pos} grafts were transplanted into HK patients who at end of follow-up, May 2008, did not develop post-PKP HK or graft failure. Furthermore, none of the transplanted non-HK patients with αHHV DNA positive corneas developed HK and 6 non-HK patients developed culture proven newly acquired HK. However, none of these patients had αHHV DNA in their corneas nor did they receive a graft in which αHHV DNA was detectable in the donor's corneascleral rim (data not shown). Because all 6 non-HK patients were HSV-1 seropositive the newly acquired HK is most likely caused by reactivation of the endogenous HSV-1 strain.

Discussion

In the present study, we determined the prevalence and diagnostic value of detecting αHHV-specific DNA in human donor and recipient cornea tissues. Three main findings are reported. First, αHHV DNA was rarely detected in donor cornea tissues and excised corneas of non-HK patients. Second, viral DNA load of HSV-1, the most prevalent αHHV detected in the corneas analyzed, correlated with the patients' age in both PKP patient groups. Third, HSV-1 DNA load in corneas was inversely correlated with graft survival of HK patients.

Laboratory diagnostics on cornea specimens is of additional value to diagnose cornea disease. The introduction of qualitative PCR, and more recently qPCR, has greatly improved the sensitivity to detect the etiologic agent in clinical specimens of suspected infectious keratitis cases [29]. As reported earlier, HSV-1 was the most prevalent αHHV detected in cornea tissues and more frequently identified in corneas of HK patients (48%) compared to non-HK patients (4%) [22-25]. The low number of HSV-2 and VZV DNA positive cornea tissues, as described by other groups [22-25], is most likely due to the different anatomical location of latent HSV-2 compared to HSV-1 and VZV, and the relative low reactivation frequency of VZV compared to HSV in immunocompetent individuals [32,35].

Whereas the data confirm the clinical diagnosis in the majority of the HK patients, detection of αHHV DNA in corneas of non-HK patients is puzzling. The data obtained on non-HK corneas may be due to cross-contamination or less likely misdiagnosis of the cornea disease. The James Hill lab has recently shown that about one-third of the 50 healthy adults analyzed daily, shed HSV-1 at least once in their tears during a 30-day surveillance period [36]. This intermittent and asymptomatic shedding of HSV-1, along with the 2-log lower median HSV-1 DNA load in corneas of non-HK patients compared to HK patients (Fig. 1A), suggests that the presence of αHHV DNA in non-HK corneas is

most likely irrelevant to the disease and represent events of asymptomatic shedding of reactivated virus in the respective patients [34,37].

HSV-1 remnants have been detected in healed corneas of HK patients and some groups have interpreted this as cornea HSV-1 latency [38-40]. However, incomplete clearance of viral DNA following HSV-1-induced intra-corneal immune responses is equally plausible. Indeed, studies in mice have shown that infectious virus and subsequently HSV-1 DNA becomes undetectable when immune cells infiltrate the infected cornea [41]. In our study, and a recent study on a limited number of HK patient corneas [25], the corneal HSV-1 DNA load decreased gradually with increasing RFI (Fig. 1I-J). Furthermore, the viral load correlated with fulminant disease (Fig. 1H) and pre-operative steroid treatment (Fig. 1E), whereas cornea neovascularization was inversely correlated with the cornea HSV-1 DNA load (Fig. 1G). Compared to clinically quiescent HSV-1 cornea lesions, active HK lesions are densely infiltrated with lymphocytes [2,41]. Steroids inhibit inflammation and deep cornea neovascularization facilitates the egress of inflammatory cells into affected tissues [41,42]. Altogether, the data support the notion that local inflammatory responses are involved in clearing the virus from the infected cornea tissue. This, combined with the detection limit of the PCR assays performed on only one-half of the corneas analyzed, may account for the inability to detect HSV-1 DNA in the remaining half of the HK patients analyzed (Table 1).

An interesting yet not reported finding in our study was that the intra-corneal HSV-1 DNA load correlated with the patients' age in both PKP patient groups (Fig. 1), suggesting a general phenomenon was involved. Whereas the microbiological spectrum of infectious keratitis is similar between the elderly (>60 years old) and younger patients, the incidence of HK and associated morbidity is more common in the elderly [43,44]. Similar observations have been made in mice. Unlike young mice, adult mice are more susceptible to severe HSV-1-induced cornea inflammation, which appeared to be largely attributable to age-related alterations of the immune system [45]. Decline in immune function with advancing age is a hallmark of aging. As a result, infectious diseases cause more morbidity and mortality to the elderly [46]. Accordingly, the association between age and HSV-1 DNA load is most likely due to the age-related deprived immune control of latent HSV-1 in the innervating ganglion [26, 47]. Alternatively, corneas of the elderly may be more susceptible to HSV-1 infections.

Although the cornea is an immune privileged site, cornea graft rejection is the major cause of graft failure arguing to identify and treat those PKP patients at high-risk for graft rejection [1-3]. Extended cornea neovascularization, a history of graft rejection and a history of herpetic keratitis in the operated eye are considered as important high-risk factors for graft rejection [2,3]. Analogous to previous reports, corneal neovascularization and cornea regrafting negatively influenced graft survival in both patient groups [1-4]. In contrast, however, the incidence of graft failure was not significantly different between HK

and non-HK patients during the 4-year follow-up period. This discrepancy may in part be attributable to the fact that our hospital, the only hospital in the Netherlands primary focused on ophthalmologic diseases, is the national referral center for patients with complicated cornea disease including severe herpetic keratitis. Alternatively, the favorable results on HK patients may reflect the instigation of effective combined antiviral and local immunosuppressive therapy immediately after transplantation.

An additional important finding of our study was that the presence and particularly the load of HSV-1 DNA in excised corneas negatively influences graft survival in HK patients, but not non-HK patients (Fig. 2). This parameter correlated with graft survival independently with the aforementioned high-risk factors. However, as would be expected, the cornea HSV-1 load correlated with HK severity at time of surgery. Inevitably, interpretation of the clinical data relies on the experience of the ophthalmologist, arguing for the need to include updated laboratory diagnostic tools to optimize the differential diagnosis without ambiguity.

In conclusion, the data demonstrate that the HSV-1 DNA load in corneas of HK patients correlated with age, recurrence free interval, cornea neovascularization, pre-PKP steroid treatment, severity of disease at time of surgery and cornea graft rejection. Implementation of HSV-1 qPCR assays on excised corneas of HK patients will improve post-PKP diagnosis and therapy to prevent severe HSV-1 related complications in cornea grafting, especially in the elderly patients. Contrastingly, HSV-1 qPCR assays on donor cornea tissues have no diagnostic value to predict the development of newly acquired herpetic keratitis in non-HK patients or graft-to-host transmission of HSV-1 in PKP patients in general.

References

1. Panda A, Vanathi M, Kumar A, Dash Y, Priya S. Corneal graft rejection. Surv Ophthalmol **2007**;52:375-96.
2. Remeijer L, Osterhaus AD, Verjans GM. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul Immunol Inflamm **2004**;12:255-85.
3. Koay PY, Lee WH, Figueiredo FC. Opinions on risk factors and management of corneal graft rejection in the United Kingdom. Cornea **2005**;24:292-6.
4. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group. Ophthalmology **1994**;101: 1536-47.
5. The Collaborative Corneal Transplantation Studies Research Group. The Collaborative Corneal Transplantation Studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. Arch Ophthalmol **1992**;110:1392–403.
6. Holland EJ, Schwartz GS. Classification of herpes simplex virus keratitis. Cornea **1999**;18:144-54.
7. Liesegang TJ. Classification of herpes simplex virus keratitis and anterior uveitis. Cornea **1999**;18:127-43.
8. Foster CS, Duncan J. Penetrating keratoplasty for herpes simplex keratitis. Am J Ophthalmol **1981**;92:336-43.
9. Beekhuis WH, Renardel de Lavalette JG, Schaap GJ. Therapeutic keratoplasty for active herpetic corneal disease: viral culture and prognosis. Doc Ophthalmol **1983**;55:31-5.
10. Panda A, Kumar TS. Prognosis of keratoplasty in viral keratitis. Ann Ophthalmol **1991**;23:410–413.
11. Vajpayee RB, Sharma N, Sinha R, et al. Infectious keratitis following keratoplasty. Surv Ophthalmol **2007**;52:1-12..
12. Lomholt JA, Baggesen K, Ehlers N. Recurrence and rejection rates following corneal transplantation for herpes simplex keratitis. Acta Ophthalmol Scand **1995**;73:29-32.
13. Sterk CC, Jager MJ, Swart-vd BM. Recurrent herpetic keratitis in penetrating keratoplasty. Doc Ophthalmol **1995**;90:29-33.
14. Remeijer L, Doornenbal P, Geerards AJ, et al. Newly acquired herpes simplex virus keratitis after penetrating keratoplasty. Ophthalmology **1997**;104:648-52.
15. Borderie VM, Meritet JF, Chaumeil C, et al. Culture-proven herpetic keratitis after penetrating keratoplasty in patients with no previous history of herpes disease. Cornea **2004**;23:118-24.
16. Rezende RA, Uchoa UB, Raber IM, et al. New onset of herpes simplex virus epithelial keratitis after penetrating keratoplasty. Am J Ophthalmol **2004**;137:415-9.
17. Remeijer L, Maertzdorf J, Buitenwerf J, et al. Corneal herpes simplex virus type 1 superinfection in patients with recrudescent herpetic keratitis. Invest Ophthalmol Vis Sci **2002**;43:358-63.
18. Biswas S, Suresh P, Bonshek RE, et al. Graft failure in human donor corneas due to transmission of herpes simplex virus. Br J Ophthalmol **2000**;84:701-5.
19. Remeijer L, Maertzdorf J, Doornenbal P, et al. Herpes simplex virus 1 transmission through corneal transplantation. Lancet **2001**;357:442.
20. Thuret G, Acquart S, Gain P, et al. Ultrastructural demonstration of replicative herpes simplex virus type 1 transmission through corneal graft. Transplantation **2004**;77:325-326.
21. Openshaw H, McNeill JI, Lin XH, et al. Herpes simplex virus DNA in normal corneas: persistence without viral shedding from ganglia. J Med Virol **1995**;46:75-80.
22. van Gelderen BE, Van der LA, Treffers WF, van der GR. Detection of herpes simplex virus type 1, 2 and varicella zoster virus DNA in recipient corneal buttons. Br J Ophthalmol **2000**;84:1238-43.
23. Kaye SB, Baker K, Bonshek R, et al. Human herpesviruses in the cornea. Br J Ophthalmol **2000**;84(6):563-71.
24. Robert PY, Adenis JP, Denis F, et al. Herpes simplex virus DNA in corneal transplants: prospective study of 38 recipients. J Med Virol **2003**;71:69-74.
25. Shimomura Y, Deai T, Fukuda M, et al. Corneal buttons obtained from patients with HSK harbor high copy numbers of the HSV genome. Cornea **2007**;26:190-3.
26. Verjans GM, Hintzen RQ, van Dun JM, et al. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. Proc Natl Acad Sci USA **2007**;104:3496-501.
27. Doornenbal P, Seerp Baarsma G, Quint WG, et al. Diagnostic assays in cytomegalovirus retinitis: detection of herpesvirus by simultaneous application of the polymerase chain reaction and local antibody analysis on ocular fluid. Br J Ophthalmol **1996**;80:235-40.
28. Moberg M, Gustavsson I, Gyllensten U. Real-time PCR-based system for simultaneous quantification of human papillomavirus types associated with high risk of cervical cancer. J Clin Microbiol **2003**;41:3221-8.

29. van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture.J Clin Microbiol **2003**;41:576-80.
30. Cohrs RJ, Randall J, Smith J, et al. Analysis of individual human trigeminal ganglia for latent herpes simplex virus type 1 and varicella-zoster virus nucleic acids using real-time PCR. J Virol **2000**;74:11464-71.
31. Loutsch JM, Sainz B Jr, Marquart ME, et al. Effect of famciclovir on herpes simplex virus type 1 corneal disease and establishment of latency in rabbits. Antimicrob Agents Chemother **2001**;45:2044-53.
32. Roizman B, Knipe DM, Whitley RJ. Herpes simplex viruses. In: Fields Virology, eds Knipe DM and Howley P. Lippincott Williams & Wilkens, Philadelphia: **2007**: 2502-76.
33. O'Day D. Diseases potentially transmitted through corneal transplantation. Ophthalmology **1989**;96:1133–37.
34. Asimakis P, Kirkness CM. Storage of donor corneas, surgery, outcome, and complications of penetrating keratoplasty. Curr Opin Ophthalmol **1996**;7:35-40.
35. Cohen JI, Straus SE, Arvin AM. Varicella zoster virus: replication, pathogenesis and management. In: Fields Virology, eds Knipe DM and Howley P. Lippincott Williams & Wilkens, Philadelphia: **2007**:2773- 2818.
36. Kaufman HE, Azcuy AM, Varnell ED, Sloop GD, Thompson HW, Hill JM. HSV-1 DNA in tears and saliva of normal adults. Invest Ophthalmol Vis Sci **2005**;46:241-7.
37. Toma HS, Murina AT, Areaux RG Jr, et al. Ocular HSV-1 latency, reactivation and recurrent disease. Semin Ophthalmol **2008**;23:249-73.
38. Kaye SB, Lynas C, Patterson A, Risk JM, McCarthy K, Hart CA. Evidence for herpes simplex viral latency in the human cornea. Br J Ophthalmol **1991**;75:195-200.
39. Perng GC, Zwaagstra JC, Ghiasi H, Kaiwar R, Brown DJ, Nesburn AB, Wechsler SL. Similarities in regulation of the HSV-1 LAT promoter in corneal and neuronal cells. Invest Ophthalmol Vis Sci **1994**;35:2981-9.
40. Zheng X. Reactivation and donor-host transmission of herpes simplex virus after corneal transplantation. Cornea **2002**;21:S90-3.
41. Biswas PS, Rouse BT. Early events in HSV keratitis: setting the stage for a blinding disease. Microbes Infect **2005**;7:799-810.
42. Guess S, Stone DU, Chodosh J. Evidence-based treatment of herpes simplex virus keratitis: a systematic review. Ocul Surf **2007**;5:240-50.
43. Butler TK, Spencer NA, Chan CC, Singh Gilhotra J, McClellan K. Infective keratitis in older patients: a 4 year review, 1998-2002. Br J Ophthalmol **2005**;89: 591-6.
44. van der Meulen IJ, van Rooij J, Nieuwendaal CP, Van Cleijnenbreugel H, Geerards AJ, Remeijer L. Age-related risk factors, culture outcomes, and prognosis in patients admitted with infectious keratitis to two Dutch tertiary referral centers. Cornea **2008**;27:539-44.
45. Turner J, Turner OC, Baird N, Orme IM, Wilcox CL, Baldwin SL. Influence of increased age on the development of herpes stromal keratitis. Exp Gerontol **2003**;38:1205-12.
46. Kumar R, Burns EA. Age-related decline in immunity: implications for vaccine responsiveness. Expert Rev Vaccines **2008**;7:467-79.
47. Sheridan BS, Knickelbein JE, Hendricks RL. CD8 T cells and latent herpes simplex virus type 1: keeping the peace in sensory ganglia. Expert Opin Biol Ther **2007**;7:1323-31.

Chapter 5

Acyclovir Resistant Corneal Herpes Simplex Virus Type 1 Isolates in Patients with Herpetic Keratitis

Rui Duan, Rory D. de Vries, Albert D.M.E. Osterhaus, Lies Remeijer, Georges M.G.M. Verjans

Journal of Infectious Disease. 2008 Sep 1;198(5):659-63.

Abstract

The prevalence and molecular characteristics of corneal acyclovir-resistant HSV-1 isolates (ACV^R) was determined in 173 immunecompetent herpetic keratitis (HK) patients. Eleven out of 173 (6.4%) isolates were ACV^R and 9 of these patients were refractory to ACV therapy. Thereof, 5 and 1 ACV^R isolates were cross-resistant to gancyclovir or both gancyclovir and foscarnet, respectively. Ten of the ACV^R isolates had mutations in the thymidine kinase gene presumably conferring the ACV^R phenotype. The data demonstrate a relatively high prevalence of corneal HSV-1 ACV^R isolates in HK patients, emphasizing the need for ACV susceptibility monitoring of HK patients refractory to ACV therapy.

Introduction

Herpes simplex virus type 1 (HSV-1) is a leading cause of corneal disease and blindness in humans, largely because of its recurrent nature [1]. Herpetic keratitis (HK) manifests predominantly as an infectious epithelial keratitis (IEK) or herpetic stromal keratitis (HSK). The drugs of choice for treating HK are nucleoside analogues like acyclovir (ACV) and gancyclovir (GCV), and less frequently the viral DNA synthesis inhibitors foscarnet (FOS) and cidofovir [2]. These treatments, along with corticoid-steroids in case of HSK, have resulted in a significant reduction of HSV-1-induced corneal blindness [1,2]. The antiviral activity and selectivity of these compounds is different. Whereas both ACV and GCV depend on metabolic activation by the HSV-encoded thymidine kinase (TK), FOS inhibits the viral DNA polymerase directly as a substrate analogue of the pyrophosphate formed during DNA synthesis [3]. Among immune-compromised patients with HSV-1 disease the prevalence of ACV resistant (ACV^R) HSV-1 isolates is much higher (4.3-14%) than in immunecompetent patients (0.1-0.6%) [4-6]. This difference is most likely due to longer mucosal persistence of ACV^R HSV variants caused by impaired local immune responses [1]. In about 95% of the cases, ACV resistance is associated with alterations within the HSV-1 TK gene. Less frequent are mutations in DNA polymerase, which may lead to cross-resistance to FOS [7,8]. In contrast to herpes genital and herpes labialis, large surveys on the incidence of ACV resistance in HK patients are lacking [4-8]. Currently, only a few anecdotal case reports have reported on corneal HSV-1 ACV^R in HK patients [4,9-12]. In this study we have determined the prevalence and molecular characteristics of corneal HSV-1 ACV^R in a cohort of 173 HK patients. Additionally, the cross-resistance to GCV and FOS was determined.

Material and Methods

Patients and clinical samples.

Corneal swabs (n= 173) were obtained for diagnostic purposes at the Rotterdam Eye Hospital (Rotterdam, The Netherlands) between 1981 and 2005 from otherwise healthy patients with suspected herpetic corneal lesions. Virus was grown and typed for HSV-1 as described previously [13]. The classification of HK was defined on clinical criteria [1]. The study was performed according to the tenets of the Declaration of Helsinki, approved by the local Ethical Committee and written informed consent was obtained.

In vitro antiviral susceptibility assays.

The susceptibility of corneal HSV-1 isolates to ACV (GlaxoSmithKline), GCV (Roche) and FOS (AstraZeneca) was determined by a real-time PCR (qPCR) assay as described

previously [14]. The plaque reduction assay (PRA) was employed to confirm the qPCR results [14]. Briefly, Vero monolayers in 24-well culture plates were inoculated with 50 plaque-forming units HSV-1. After 1 hr at 37°C, the viral inoculum was removed and the cells incubated in triplicate with different concentrations ACV, GCV or FOS diluted in DMEM supplemented with 1% fetal bovine serum and 0.6% methylcellulose (Acros Organics). At 72 hrs post inoculation (p.i.), plaques were visualized after fixation of the monolayers with 4% paraformaldehyde and staining with 0.2% crystal violet.

The qPCR was performed similar to PRA with the exception that the cells were infected with 100-fold diluted corneal HSV-1 isolate stocks [14]. At 24 hours p.i., supernatant was discarded and cells treated with 300 µl lysis buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.45% Tween 20, 0.45% Nonidet-P40, 200 µg/ml proteinase K) and incubated at 56°C for 90 min. Ten µl of the 10-fold diluted lysate was subjected to qPCR using the Applied Biosystems 7000 Sequence Detection system as described previously [13]. Viral load was determined from a standard curve generated on a stock of HSV-1 strain McIntyre counted by electron microscopy (Advanced Biotechnologies) [13] and the HSV-1 isolates were assayed >2-times. The IC_{50} was defined as the concentration of antiviral drug that reduced the number of plaques or viral copies by 50% compared to the control infected cells without antiviral added. Isolates were considered resistant to ACV and GCV at IC_{50}>2 µM for PRA and IC_{50}>1 µM for qPCR. For FOS, isolates were considered resistant at IC_{50}>200 µM for PRA [14].

DNA sequencing.

DNA was isolated from supernatants of HSV-1-infected Vero cellsusing MagNA Pure LC total nucleic acid kit (Roche). The TK gene was amplified by PCR with 200 pmol of forward (5'-TGGCGTGAAACTCCCGCACCT-3') and reverse (5'-CCCATAAACGCGGCGAATCG-3') primer. Amplification conditions included denaturation for 4 min at 94°C, 30 cycles (1 min at 94°C, 45 s at 50°C and 2 min at 72°C) and a final extension step for 5 min at 72°C. The 1,131 bp amplicon was purified using a MINelute Gel extraction kit (QIAGEN). The entire TK open reading frame was sequenced on the ABIprism 3130 xl genetic analyzer with the BigDye Terminator v.1 cycle sequencing kit (Applied Biosystems) using both external primers as above, as well as sense (5'-CGCCCAGATAACAATGGGC-3') and anti-sense (5'-TCTGTCTTTTTATTGCCGTCAT-3') internal primers.

Western blotting.

The TK protein size was determined by Western blotting using two polyclonal antibodies directed against different regions of HSV-1 TK (Santa Cruz; (sc-28037 and sc-28038). Extracts from HSV-1-infected Vero cells were prepared by lysis and

denaturation for 10 min at 95°C, and subjected to electrophoresed through an acrylamide gel and blotted on a PVDF membrane (Amersham). After overnight blocking with 5% milk powder, the PVDF membrane was incubated with the primary (1:500) and secondary antibody (1:5000; HRP-conjugated donkey-anti-goat; Santa Cruz). Subsequently, chemiluminescent substrate (ECL detection reagent; Amersham) was applied to the membrane for 1 min prior to exposure of the membrane to X-ray film according to the manufacturer's instructions.

Results

Prevalence of corneal HSV-1 ACV^R isolates in HK patients.

The HK patient cohort consisted of 102 men and 71 women (mean age 50 ± 22 years), clinical entities included IEK (n= 79), HSK (n= 85) and keratouveitis (KU; n=9). All patients were treated with ACV during the year preceding sampling. At time of sampling, 25 patients received ACV and 4 patients received trifluorothymidine treatment. qPCR analyses revealed that 11 of 173 isolates (6.4%) were ACV^R, which was confirmed by subsequent PRA assays (Table 1). Among these 11 ACV^R isolates, 5 isolates were resistant to GCV (R3, R4, R6, R8 and R9) and one isolate (R3) was cross-resistant to both GCV and FOS (Table 1). The IC_{50}s determined by PRA and qPCR were reproducible and correlated significantly for both ACV (r= 0,82, p= 0.02) and GCV (r= 0,94, p<0.0001; Spearman's rank correlation test). Four of the 11 patients received topical ACV at time of sampling, which is significantly more than patients with ACV-sensitive (ACV^S; 21 of 162) HSV-1 (p<0.01; Chi-square test). Nine of the patients (R1-9) with ACV^R isolates were clinically refractory to ACV treatment (e.g. slow epithelial healing and development of severe HSK). Patients R1, R2, R5, R7 and R9 were switched to FOS or GCV therapy (R6 and R8). This was initially beneficial to all patients, but during the follow-up period of >3 years corneal blindness developed in the affected eyes of 7 of 11 patients (R2-R5 and R7-9).

Molecular analysis of HSV-1 ACV^R isolates.

Since approximately 95% of the mutations causing ACV^R occur in the TK gene [4,7,8], the entire TK gene of the 11 ACV^R isolates was sequenced. Alignment with ACV^R (n= 74) and ACV^S (n= 40) HSV-1 TK sequences deposited at Genbank revealed numerous amino acid (Aa) substitutions in all ACV^R isolates (Table 1) [7,8; data not shown]. Ten of 11 ACV^R isolates had mutations in the TK gene potentially conferring the ACV^R phenotype. Two isolates had an Aa substitution in the nucleotide-binding site of TK at residue 178 (Leu178Arg; R1 and R7) [7,8]. Isolates R4 and R6 had an in-frame deletion of three nucleotides leading to loss of glutamic acid and isoleucin at positions 36 and 194,

Table 1. Characteristics of patients with herpetic keratitis and corneal herpes simplex virus (HSV)–1 isolates resistant to ACV.

Patient (Age, years; sex)	IC_{50}, $\mu Mol/L$[a]					Mutation in TK[b]	Clinical presentation				
	ACV		GCV		FOS		At culture date	Refractory to ACV therapy	Recurrences no.	At end of follow-up	Corneal blindness
	PRA	qPCR	PRA	qPCR	PRA						
R1 (40; F)	9.5	4.4	0.2	0.4	47	L178R	IEK	Yes	14	NSK	No
R2 (78; F)	2.8	1.5	1.3	0.2	101	T345P	NSK	Yes	4	NSK in graft	Yes
R3 (69; M)	5.8	3.5	5.2	3.9	241	None	KU	Yes	Chronic	NSK in graft	Yes
R4 (72; M)	>50	45.5	>50	>50	40	Del36E	NSK	Yes	4	NSK in two regrafts	Yes
R5 (52; M)	21.5	3.2	1.8	1.1	46	S181N	NSK	Yes			
R6 (67; M)	35.4	13.5	27.4	29.1	81	Del194I	NSK	Yes	1	ISK in graft	No
R7 (29; M)	16.1	4.5	1.6	0.7	78	L178R	NSK				
R8 (51; M)	12.6	7.6	13.3	3.1	54	Frameshift 146	NSK	Yes	18	Two regrafts for NSK	Yes
R9 (69; M)	10.6	4.3	14.3	7.4	54	Frameshift 146	IEK	Yes	12	NSK and uveitis	Yes
R10 (79; M)	2.4	1.4	0.2	0.2	41	I78S	IEK	No	None	Lesion healed in 7 days	No
R11 (33; F)	3.2	1.3	0.2	0.6	105	D258N	ISK	No	None	Lesion healed in 7 days	No

NOTE. ACV, acyclovir; FOS, foscarnet; GCV, gancyclovir; IEK, infectious epithelial keratitis; ISK, infectious stromal keratitis; KU, keratouveitis; NSK, necrotizing stromal keratitis; PRA, plaque-reduction assay; qPCR, real-time polymerase chain reaction; *TK,* HSV gene for thymidine kinase.

a. Cutoff values for both resistance to ACV and resistance to GCV are >1µmol/L in qPCR and >2µmol/L in PRA, and cutoff values for resistance to FOS are >200µmol/L; strains that are cross-resistant to either GCV or FOS are underlined. For qPCR, the mean _ SD values for isolates sensitive to ACV and for the isolates sensitive to GCV were 0.2 ±0.1 µmol/L (range 0.1– 0.6 µmol/L) and 0.2 ±0.3 µmol/L (range 0.1–1.1), respectively; for PRA, the mean ±SD values for isolates sensitive to ACV, isolates sensitive to GCV, and isolates sensitive to FOS were 0.3 ± 0.3 µmol/L (range, 0.1–1.9 µmol/L), 0.3 ± 0.4 µmol/L (range, 0.2–1.8µmol/L) and 50 ±18 (range, 10–91 µmol/L), respectively.

b. The TK gene sequence of each isolate was compared with that of ACV-susceptible HSV-1 reference strain KOS (GenBank accession number GI:125436); amino acid substitutions, deletions (del), or frameshift mutations potentially conferring resistance to ACV are shown. The GenBank accession numbers of isolates from patients R01–R11 are EU541360, EU541361, EU541362, EU541363, EU541364, EU541365, EU541366, EU541367, EU541368, EU541369, and EU541370, respectively.

respectively. Four other Aa substitutions, not documented for ACV^S HSV-1 isolates, were found: isoleucine to serine (residue 78, R10), serine to asparagine (residue 181, R5), aspartic acid to asparagine (residue 258, R11) and threonine to proline (residue 354, R2). The isolates R8 and R9 had an identical single nucleotide insertion in the G repeat region, located between residues 430-437 [7,8], leading to a frameshift and premature stop codon at residue 208 (Table 1). The remaining Aa substitutions have been found in both ACV^S and ACV^R strains and therefore unlikely associated with resistance [4,7,8].

Western blotting confirmed the predicted truncation of the TK protein of isolates R8 and R9 (Figure 1). With the N-terminal antibody the truncated TK protein was detected, whereas no protein was visible with the internal antibody. This antibody binds at residues 215-216 (Santa Cruz), which is C-terminal to the frameshift location. The remaining Aa substitutions identified in the ACV^R isolates were also observed in ACV^S isolates, presumably representing natural polymorphisms [2; data not shown].

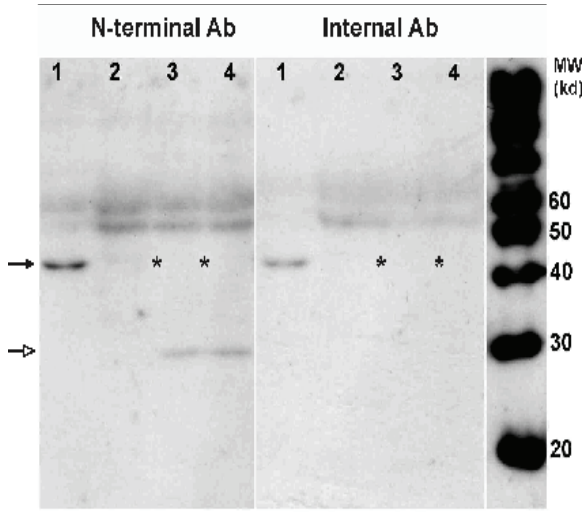


Figure 1. Western blot analysis of altered HSV-1 encoded thymidine kinase (TK) protein in acyclovir (ACV) resistant corneal HSV-1 isolates. Shown are the western blotting results of two ACV resistant corneal HSV-1 isolates R8 (lane 3) and R9 (lane 4), and the ACV sensitive HSV-1 reference strain KOS (lane 1). Lane 2 shows the mock-infected negative control. The anti-HSV-1 TK polyclonal antibodies used were directed to the N-terminal (sc-28037) or the internal TK region (sc-28038) located between residues 50-100 and 215-265, respectively (both antibodies from Santa Cruz). No signal is seen with the internal antibody, indicated with asterisks, as the frameshift mutation causes an altered residue composition after residue 146, which is not recognized by the internal antibody. The location of the wildtype and truncated TK proteins are indicated with closed and open arrows, respectively.

Discussion

Since the discovery of ACV 30 years ago, the remarkable efficacy and safety of this drug represents a milestone in the treatment of HSV infections [3,4]. Until now, resistance to ACV was rarely reported in otherwise healthy patients [4-6]. This study is the first to report an unprecedented relatively high prevalence of corneal HSV-1 ACV^R isolates (6.4%) in immunecompetent HK patients. We hypothesize that the high prevalence could be due to the unique immune privileged site of the cornea, which differs from other HSV-1 infection sites [15]. In the Rotterdam Eye Hospital, where all patients were included, prophylactic ACV treatment has largely increased since 1994. Since all ACV^R isolates identified came from patients who presented after 1994, we hypothesize that the increase of ACV

prescriptions may have influenced the emergence of corneal HSV-1 ACV^R at our hospital.

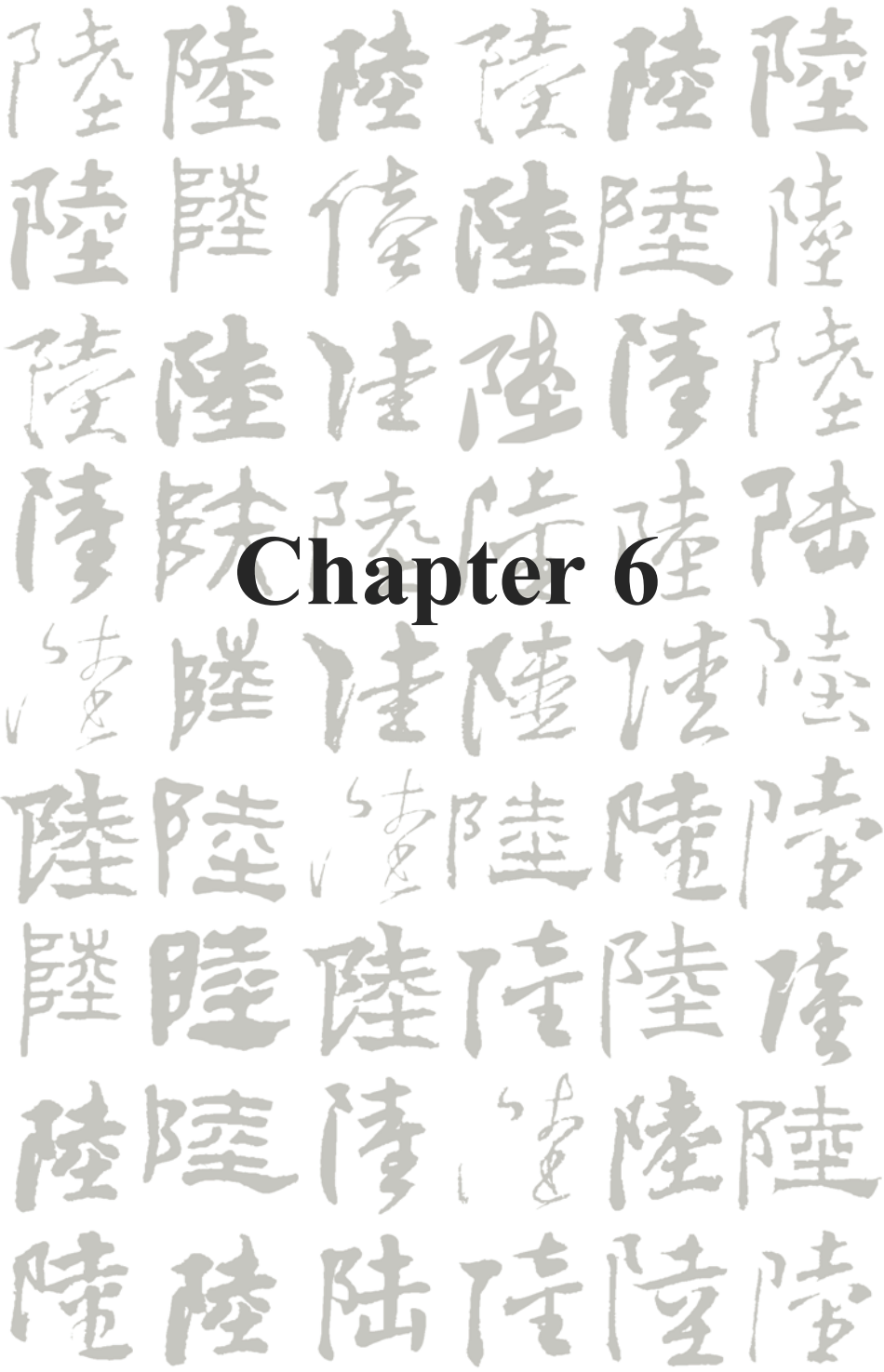
Several mutations in the HSV-1 TK gene were detected that may confer ACV resistance in 10 of 11 corneal ACV^R isolates. These mutations have not described in ACV^S strains and include multiple Aa deletions and substitutions [7,8; data not shown]. The arginine to leucine substitution at residue 178 in isolates R1 and R7 is of particular interest as it is located within the nucleoside-binding site of TK [7,8]. We also report a G insertion at nucleotide position 430 in 2 ACV^R isolates (R8 and R9) with a resulting frameshift leading to a truncated TK protein (Fig. 1). The GCV/FOS-cross-resistant ACV^R isolate R3 had no obvious mutations in the TK gene suggesting that mutations within the DNA polymerase gene are involved [7,8]. Four of 5 ACV/GCV-cross-resistant isolates (R4, R6, R8, R9) had frameshift mutations and deletions in the TK gene. These mutations are probably more detrimental to TK to convert ACV and GCV. Although the molecular and functional data suggest the significance of the mutations identified, no definitive conclusion can be drawn for these newly identified TK alterations and their role in ACV resistance without confirmatory testing by site-directed mutagenesis.

In vitro resistance assays and molecular characterization of HSV-1 isolates are strong diagnostic tools and should be implemented to rationalize a switch in antiviral therapy in patients refractory to treatment [3,4]. In our patient cohort, 9 of 11 patients with HSV-1 ACV^R were clinically resistant to ACV therapy. Seven of these patients were eventually switched to other antivirals. Whereas all patients responded initially, corneal blindness developed eventually in 7 of 11 patients. Because the majority of these patients had HSK, a chronic T-cell mediated inflammatory corneal disease, their ACV^R phenotype may only in part be responsible for the deterioration of disease in the long run [1]. Nonetheless, if the anti-viral resistance data were known beforehand a switch to an alternative antiviral would have been initialized to lower the corneal load, potentially limiting both virus-mediated cytopathology and intra-corneal deposition of viral antigens recognized by the pathogenic intra-corneal T-cell response [1].

In conclusion, the data presented show a relatively high prevalence of ACV resistance in HK patients, compared to other immunecompetent individuals suffering from HSV-1-induced disease [4-6]. The presence of HSV-1 ACV^R strains is commonly associated with clinical treatment resistance including extension, persistence, or recurrent lesions. Given the high prevalence and more serious disease, we suggest that resistance patterns of corneal HSV-1 isolates from HK patients refractory to antiviral therapy should be implemented as a standard diagnostic tool. This immediate *in vitro* testing can rationalize a switch to treatment with GCV, trifluridine and vidarabine or more toxic antivirals like FOS or cidofovir. This will save precious therapeutic time and may ultimately prevent development of severe disease including corneal blindness.

References

1. Remeijer L, Osterhaus ADME, Verjans GMGM. Human herpes simplex virus keratitis: the pathogenesis revisited. *Ocul Immunol Inflamm* **2004**;12:255-85.
2. Herpetic Eye Disease Study Group. Oral acyclovir for herpes simplex virus eye disease: effect on prevention of epithelial keratitis and stromal keratitis. *Arch Ophthalmol* **2000**; 118:1030-6.
3. Naesens L, De Clercq E. Recent developments in herpesvirus therapy. *Herpes* **2001**; 8:12-6.
4. Bacon TH, Levin MJ, Leary JJ, Sarisky RT, Sutton D. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. *Clin Microbiol Rev* **2003**;16:114–128.
5. Stránská R, Schuurman R, Nienhuis E, et al. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. *J Clin Virol* **2005**; 32:7-18.
6. Danve-Szatanek C, Aymard M, Thouvenot D, et al. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. *J Clin Microbiol* **2004**;42:24–9.
7. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. J Clin Virol **2003**;26:29-37.
8. Chibo D, Druce J, Sasadeusz J, Birch C. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. *Antiviral Res* **2004**; 61:83-91.
9. Yao YF, Inoue Y, Kase T, Uchihori Y, Mori Y, Ohashi Y. Clinical characteristics of acyclovir-resistant herpetic keratitis and experimental studies of isolates. *Graefes Arch Clin Exp Ophthalmol* **1996**; 234:S126-32.
10. Bodaghi B, Mougin C, Michelson S, Agut H, Dighiero P, Offret H, Frau E. Acyclovir-resistant bilateral keratitis associated with mutations in the HSV-1 thymidine kinase gene. *Exp Eye Res.* **2000**; 71:353-9.
11. Sarisky RT, Cano R, Nguyen TT, et al. Biochemical characterization of a virus isolate, recovered from a patient with herpes keratitis, that was clinically resistant to acyclovir. *Clin Infect Dis* **2001** ;33:2034-9.
12. Zhang W, Suzuki T, Shiraishi A, Shimamura I, Inoue Y, Ohashi Y. Dendritic keratitis caused by an acyclovir-resistant herpes simplex virus with frameshift mutation. *Cornea* **2007**;26:105-6.
13. van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol* **2003**;41:576-80.
14. Thi TN, Deback C, Malet I, Bonnafous P, Ait-Arkoub Z, Agut H. Rapid determination of antiviral drug susceptibility of herpes simplex virus types 1 and 2 by real-time PCR. *Antiviral Res* **2006**;69:152-7.
15. Lepisto AJ, Frank GM, Hendricks RL. How herpes simplex virus type 1 rescinds corneal privilege. Chem Immunol Allergy 2007;92:203-12.



Chapter 6

Acyclovir Susceptibility and Genetic Characteristics of Sequential Herpes Simplex Virus Type 1 Corneal Isolates from Patients with Recurrent Herpetic Keratitis

Rui Duan, Rory D. de Vries, Jessica M. van Dun, Freek B. van Loenen, Albert D.M.E. Osterhaus, Lies Remeijer, Georges M.G.M. Verjans

Journal of Infectious Disease. Submitted

Abstract

Purpose. The incidence and clinical significance of HSV-1 acyclovir resistance (ACV^R) in patients with recurrent herpetic keratitis (rHK) was determined. **Methods.** Sequential corneal isolates (n=39) from 15 immunecompetent rHK patients were assayed for ACV susceptibility and genotyped by analyzing the hypervariable regions of the HSV-1 genes US1, US4, US7, and US12. The thymidine kinase (TK) gene of each isolate was sequenced and the proportion of ACV^R viruses within isolates was determined. **Results.** Uniform ACV^R or ACV sensitive (ACV^S) sequential isolates were identified in 4 and 2 patients, respectively. Notably, ACV susceptibility of sequential isolates changed from $ACV^S \rightarrow ACV^R$ (n=5 patients) or $ACV^R \rightarrow ACV^S$ (n=3 patients). The ACV^R phenotype of the isolates correlated with the patients' unresponsiveness to ACV therapy. Combined analyses of the TK gene and genotype of sequential isolates showed that ACV^S isolates contained multiple ACV^R variants of the same virus and that an identical ACV^R HSV-1 strain reappeared in the patient's cornea during rHK. **Conclusions.** Corneal HSV-1 isolates are mixtures of ACV^S and ACV^R viruses that shared the same genotype, but have different TK sequences. Recovery of the same ACV^R virus during consecutive HK episodes suggests that ACV^R HSV-1 establishes latency and reactivates intermittently to cause rHK.

Introduction

Herpes simplex virus type 1 (HSV-1) is a highly prevalent human pathogen infecting 60-80% of the human population worldwide [1]. The virus infects mucosal surfaces and induces diseases including herpes labialis and herpetic keratitis (HK). HSV-1 establishes a latent infection of neurons in sensory ganglia innervating the anatomic site of primary infection and reactivates intermittently to cause recurrent disease [1]. Corneal morbidity is mainly due to prolonged or recurrent HK episodes [2,3]. Recurrent herpetic keratitis (rHK) may be caused by reactivation of the latent virus acquired during primary infection or due to a reinfection with a different strain (HSV-1 superinfection) [4].

Therapeutic intervention of HK involves systemic or topical treatment with antivirals, and in case of herpetic stromal keratitis (HSK) a combination of antivirals and corticosteroids [3,5-7]. The antiviral drugs applied topically are trifluorothymidine (TFT) and in particular acyclovir (ACV). To prevent rHK the nucleoside analogues famciclovir and valciclovir are administered systemically [3,5,6]. The introduction of ACV in ophthalmic practice has greatly improved the visual outcome of HK patients [5,6]. ACV is a prodrug that is selectively converted by HSV-1 thymidine kinase (TK) to its active form, which in turn abrogates viral replication [7]. Despite the extensive use of ACV, the incidence of ACV^R HSV-1 has not increased significantly [8-10]. The prevalence of ACV resistant (ACV^R) HSV-1 is rare in immunecompetent individuals (0.1-0.7%), but more common in the immunecompromised (4-14%) [8-10]. The mechanism of ACV^R HSV-1 is largely attributed to mutations within the HSV-1 TK gene resulting in a deficient TK protein or altered substrate susceptibility [11-13].

Recently, we reported on the relatively high prevalence of ACV^R corneal HSV-1 isolates (6.4%) in otherwise healthy HK patients. Recovery of ACV^R isolates correlated with the patients' unresponsiveness to ACV therapy and involved specific TK protein polymorphisms [14]. The widespread use of ACV to treat consecutive episodes of herpetic keratitis may induce and subsequently select for ACV^R HSV-1 in rHK patients, thereby altering the effectiveness of ACV therapy in time.

The aim of the current study was to uncover the incidence and clinical significance of ACV^R HSV-1 in rHK patients. We determined the ACV susceptibility and genetic characteristics of sequential corneal HSV-1 isolates (n=39) recovered from 15 immunecompetent rHK patients.

Material and Methods

Patients and clinical specimens

A large databank of corneal HSV-1 isolates (>800 isolates), collected prospectively since 1980 at the Rotterdam Eye Hospital (Rotterdam, Netherlands), was pre-screened for

sequential corneal isolates of rHK patients of which at least 1 isolate/patient being ACV^R, and as control 2 patients with solely ACV sensitive (ACV^s) sequential isolates [14] (data not shown). Sequential isolates were always recovered from the same eye. In total 39 isolates from 15 immunecompetent rHK patients were selected (Table 1). Five of 15 patients have been described previously. The preceding and current IDs of the 5 isolates reanalyzed are: R2→R4A, R4→R5A, R6→R10A, R7→R11A, and R8→R14A [14]. The clinical items scored were age, gender, previous history of ocular disease, therapy regimen and clinical picture at presentation of each recurrence and at end of follow-up. The HK classification was defined on clinical criteria [15,16]. The study was performed according to the tenets of the Declaration of Helsinki, approved by the local Ethical Committee and written informed consent was obtained.

Acyclovir susceptibility assay

The susceptibility of the HSV-1 isolates to ACV (GlaxoSmithKline) was determined by an HSV-1 specific real-time PCR (qPCR) assay as described previously [14,17]. In brief, Vero cell monolayers cultured in 24-wells plates in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (all products from Invitrogen) were inoculated with a 100-fold diluted corneal HSV-1 isolate and incubated in triplicate with different concentrations of ACV (GlaxoSmithKline) at 37°C. At 24 hours post infection (pi), supernatant was discarded and cells were lysed. Five microliters of the 25-fold diluted lysate was subjected to HSV-1 qPCR using the Applied-Biosystems 7000 Sequence Detection system (Applied-Biosystems) as described previously [14,17]. Viral load was derived from Ct values using a standard curve and isolates were assayed >2-times. The IC_{50} was defined as the ACV concentration that reduced the number of viral copies by 50% compared to the control infected cells without ACV. Isolates were considered resistant to ACV at $IC_{50} \geq 1$ μM [14,17].

Plating efficiency assay

The percentage of ACV^R HSV-1 variants within randomly chosen 7 ACV^S and 5 ACV^R isolates was defined by the plating efficiency assay (PEA) as described previously [18]. In brief, isolates were titrated in five 10-fold dilutions on Vero monolayers cultured in 6-wells plates. The inoculum was removed after 1 hr and monolayers were overlaid with DMEM supplemented with 1% FBS and 0.6% methylcellulose (Acros Organics) including 0 μM or 5 μM ACV. Upon incubation for 3 and 10 days pi, respectively, monolayers were fixed with 4% paraformaldehyde (Sigma-Aldrich), stained with 0.2% crystal violet (Sigma-Aldrich) and plaques were counted. Percentage of ACV^R HSV-1 variants was calculated by dividing the mean number of plaques in the 5 μM ACV wells by the mean number of plaques in the control wells without ACV.

DNA sequencing

DNA was isolated from all HSV-1 isolates, and ACV^R plaques obtained by PEA of 7 ACV^S isolates, using the MagNA Pure LC total nucleic acid isolation kit and MagNAPure LC isolation station (Roche). Amplification and sequencing of the entire HSV-1 TK open reading frame (ORF) was performed as described previously [14]. In brief, the TK gene was amplified with HSV-1 TK-specific primers located at the 5' and 3' end of the TK ORF. PCR products were run on 1% agarose gels, TK-specific 1,131-bp amplicons were purified from gel and TK ORF sequenced by use of an ABIprism 3130 analyzer and BigDye Terminator cycle sequencing kit (Applied-Biosystems) with both the external PCR primers and 2 internal sequencing primers [14].

HSV-1 genotyping

All HSV-1 isolates, and the ACV^R plaques of 7 ACV^S isolates obtained by PEA, were genotyped by two different PCR methods. The first method involved PCR-based restriction fragment length polymorphism (RFLP) analyses of the HSV-1 US4 and US7 genes as described previously [19,20]. In brief, isolated DNA was amplified with US4- and US7-specific primer pairs and amplicons digested with the restriction enzymes (RE) *Pfl*M I and *Dde* I, and *Sac* I and *Ple* I (New England Biolabs), respectively. Amplicon digests were run on 3% Metaphor agarose gels (BioWhittaker) along with a 50bp DNA marker (Invitrogen). Differential RE cleavage patterns of the amplicons, based on genotype-specific point mutations within the respective RE sites, facilitated classification into the three HSV-1 genotypes designated previously as A, B and C [19,20].

The second PCR method is based on the stability and inter-strain variability of DNA repeats located within the introns of the HSV-1 genes US1 and US12 [4,21]. The PCR reaction mixtures contained 1.25 U of cloned *Pfu* DNA polymerase (Stratagene), the corresponding buffer supplemented with 5% dimethylsulfoxide, each of the primers at a concentration of 1 mM, and each deoxynucleoside triphosphate, including equimolar amounts of dGTP and 7-deaza-29-dGTP (Boehringer-Mannheim), at a concentration of 200 mM. The forward and reverse primers of US1 and US12 were, 5'-CCACGAAACACAGGGGACGC-3' and 5'-GGATTCGACCTCAGACTCCA-3', and 5'-ACGCCCCCTTTTATTGATCT-3' and 5'-CCACGAAACACAGGGGACGC-3', respectively. Next, a nested PCR was performed using internal primers containing a 5'-FAM label. Nested US1- and US12-specific forward and reverse primers were 5'-6-FAM-TCCGACGACAGAAACCCACC-3' and 5'-GTCCCGGAGGACCACAGTGG-3', and 5'-TGGTGTCCAGGAAGGTGTCC-3' and 5'-6-FAM-TTTTTGCACGGGTAAGCAC-3', respectively. The nested PCR products were separated by capillary electrophoresis (3130XL genetic analyzer ABIprism; Applied-Biosystems) and analyzed for their exact sizes (GeneMapper software version 3.7)

according to the GeneScan-600 LIZ size standard (Applied-Biosystems).

Results

To gain insight into the incidence and clinical significance of ACV^R HSV-1 in rHK patients, the ACV susceptibility and genetic characteristics of 39 sequential corneal HSV-1 isolates of 15 rHK patients were determined (Table 1). Disease severity varied from mild infectious epithelial keratitis to severe HSK. Median age was 55 years (range 42–78 years). From each patient, two (n=8), three (n=6) and five (patient R13) sequential isolates were obtained. Median time interval between isolates was 28 months (range 1–136 months) (Table 1). All patients had a clinical history of HK and, expect for patient R11, received ACV therapy on multiple occasions before acquisition of the first isolate (i.e. isolate A). Thirteen patients (patients R1-R5, R7-R11, and R13-R15) presented with ACV-treatment refractory disease once or more commonly on repeated occasions (Table 1). A switch to topical treatment with different antiviral drugs was provided to the majority of the ACV refractory patients. At the end of follow-up (>5 years), the clinical picture of 9 patients deteriorated (patients R1-R3, R5, R7, R10, R12, R14, and R15), remained stable in 5 patients (patients R4, R6, R8, R9, and R13), and improved in 1 patient (patient R11) (Table 1).

Acyclovir susceptibility and genetic characteristics of sequential corneal HSV-1 isolates

Twenty-five of 38 (66%) isolates analyzed were classified as ACV^R, which correlated with ACV-treatment refractory disease characterized by relatively slow healing corneal lesions (>3 weeks) or frequent recurrences (>2 events/year) (Table 1). The ACV susceptibility profile varied between patients. Uniform ACV^R or ACV^S sequential isolates were identified in patients R1, R4, R5, R9 and R11, and patients R6 and R12, respectively. Notably, ACV susceptibility changed in time from $ACV^S \rightarrow ACV^R$ (patients R2, R3, R7, R13, and R15) and $ACV^R \rightarrow ACV^S$ (patients R8, R10, and R14) (Table 1).

Because TK deficiency accounts for approximately 95% of ACV^R HSV-1 [11-13], the TK gene of all isolates were sequenced and aligned to HSV-1 TK sequences deposited in Genbank (http://www.ncbi.nlm.nih.gov). The HSV-1 TK gene encodes a 376 amino acids (aa) protein containing 5 regions conserved among herpesviridae (aa 50-66, 79-91, 162-178,

Table 1. Characteristics of recurrent herpetic keratitis patients and acyclovir susceptibility patterns of sequential corneal HSV-1 isolates

Patient	Age[a], Gender	Isolate	Time interval[b]	Clinical picture			Antiviral therapy at culture date (mg/day)	Clinical Resistance[c]	Switch to other antiviral therapy	ACV IC_{50} (μM)[d]
				Before culture	At culture date	At end of follow-up				
R1	46, M	A	181	PKP for KC	IEK in graft	6-times rePKP for NSK	ACV 400x1	Yes	None	1.2
		B	136		IEK in graft		ACV 400x3	Yes	None	1.1
		C	45		NSK in graft		ACV 800x5	Yes	None	1.3
R2	65, M	A	390	Frequent IEK	IEK	ISK and glaucoma	ACV 400x2	No	None	<0.2
		B	17		IEK and glaucoma		ACV 400x2	Yes	TFT	1.9
R3	69, M	A	181	Severe NSK	Bacterial superinfection in HSV ulcer	Failed graft and Recurrent NSK	None	No	None	<0.2
		B	106		Graft rejection with HSV ulcer		ACV 400x2	No	None	<0.2
		C	11		IEK		ACV 400x3	Yes	None	1.2
R4	78, F	A	194	Severe NSK	IEK in graft	NSK in graft	ACV 400x2	Yes	TFT	1.5
		B	3		IEK in graft		ACV 400x3	Yes	TFT	10.6
		C	7		IEK in graft		ValACV 1000x2	Yes	FOS	5.4
R5	72, M	A	486	Sever KU	Ulcer in graft	NSK in graft and epithelial recurrences	None	Yes	None	45.5
		B	38		Ulcer in graft		ACV 800x2	Yes	None	18.3
R6	72, F	A	614	Severe NSK	IEK in graft	NSK in graft	None	No	None	<0.2
		B	66		IEK in graft		None	No	None	<0.2
R7	44, M	A	0	Frequent NSK	IEK	Severe NSK, lost vision after endophthalmitis	None	No	TFT	<0.2
		B	56		IEK		ValACV 500x3	Yes	TFT	ND
		C	3		IEK		ValACV 500x1	Yes	ACV	1.5
R8	42, F	A	117	ISK	IEK	ISK	ACV 400x2	Yes	FOS	9.6
		B	32		IEK		Famvir 500x2	No	FOS	<0.2
R9	69, M	A	430	KU and glaucoma	IEK and glaucoma	KU and glaucoma	ACV 400x3	Yes	None	1.8
		B	22		IEK		Famvir 500x2	Yes	FOS	5.3
		C	56		IEK		Famvir 500x2	Yes	FOS	7.6
R10	67, M	A	651	Severe ISK	IEK in graft	Slow decompensation of graft	None	Yes	GCV	6.9
		B	11		IEK in graft		ACV 400x2	Yes	TFT	<0.2
R11	30, M	A	0	Mild diffuse ISK	IEK	IEK	None	Yes	None	4.5
		B	92		IEK		None	Yes	FOS	2.6
R12	48, F	A	39	Mild diffuse ISK	IEK	NSK and glaucoma	None	No	None	<0.2
		B	24		IEK		None	No	None	<0.2
R13	52, M	A	176	ISK and IEK	IEK	ISK and IEK	ACV 200x1	No	None	<0.2
		B	46		IEK		ACV 400x5	Yes	FOS	8.8
		C	13		IEK		Famvir 250x2	Yes	FOS	8.9
		D	4		IEK		Famvir 500x2	Yes	FOS	2.9
		E	34		IEK		ValACV 500x2	Yes	FOS	8.4
R14	51, M	A	380	Severe NSK	IEK in graft	2-times rePKP for NSK	ACV 400x5	Yes	None	7.6
		B	44		IEK in graft		None	No	GCV	<0.2
R15	55, F	A	213	ISK	IEK	RePKP for neurotrophic ulcus	None	No	None	<0.2
		B	22		IEK		ValACV 500x3	Yes	TFT & GCV	12.5
		C	1		IEK		Famvir 500x2	Yes	TFT &GCV	10.3

NOTE. IEK, infectious epithelial keratitis; NSK, necrotizing stromal keratitis; KU, keratouveitis; ISK, infectious stromal keratitis; rePKP, corneal regraft; PKP, penetrating keratoplasty; KC, keratoconus. ACV, acyclovir; ValACV, valaciclovir; Famvir, famciclovir; TFT, Trifluorothymidine; GCV, gancyclovir and FOS, foscarnet.

a. Patients age (years) at acquisition of isolate A.

b. Time interval (months) between consecutive disease episodes at which the indicated isolates were obtained. Months indicated for samples A refer to time interval between time point of first diagnosis of herpetic keratitis and acquisition of sample A.

c. Clinical resistance was defined as either no healing during ACV treatment within 3 weeks or frequent recurrences of HSV keratitis (>2 events/year).

d. IC_{50} was defined as the concentration of ACV that reduced the viral copies by 50% compared to infected cells in the absence of ACV. Cut-off value for ACV resistance is ≥1μM.

212-226, and 281-292), an ATP- (aa 51–63) and a nucleoside-binding domain (aa 168–176) [11-13]. TK mutations conferring ACV^R are frequently found in these regions, as well as frameshift mutations in the 7G and 6Cs homopolymer repeats located at codons 144-146 and 183-185, respectively [11-13]. The TK sequences obtained revealed numerous nucleotide mutations, of which about half of the mutations resulted in aa substitutions of the TK protein (Table 2). The majority of the aa alterations identified were found in both ACV^S and ACV^R isolates, most likely representing natural aa polymorphisms unrelated to ACV responsiveness [11-13]. In all ACV^R isolates, several ACV^S-unrelated TK aa alterations were detected that potentially conferred their ACV^R phenotype. About half of the ACV^R-associated aa polymorphisms uncovered were due to nucleotide insertions or deletions, leading to frameshift reading that potentially results in a truncated TK protein (Table 2) [11-14]. The remaining polymorphisms were single aa changes in the TK protein at sites like the nucleoside- (patient R11) or ATP-binding site (patient R2), and the C-terminal conserved region (aa 281-292; patient R1 and R5) (Table 2). Except for patients R2 and R3, all primary isolates (isolates A) had unique TK sequences. In 9 of 15 patients, two (patients R1, R3, R4, R6, R9, R11, R12 and R15) or four sequential isolates (patient R13) shared the same TK sequence, which correlated with a similar ACV susceptibility phenotype of the consecutive isolates (Table 2).

To determine whether rHK is due to reactivation of the endogenous strain or HSV-1 superinfection, sequential isolates were genotyped by RFLP analysis (genes US4 and US7) and size fractionation of the DNA repeat containing introns of the HSV-1 genes US1 and US12 (Table 2). The US4 and US7 genotypes of the sequential isolates were identical for each patient, but differed between patients. Whereas the majority of the isolates had an identical genotype at both loci, 12 of 35 (34%) isolates had different US4/US7 genotype identities indicating that these isolates are intergenic recombinants [19,20]. In comparison, the US1 and US12 genotyping assay was more informative, facilitating the differentiation of all isolates. Consistent with our previous study on rHK patients, the sequential isolates from 5 of 15 (33%) patients had different US1/US12 genotypes (patients R1, R5, R6, R13, and R14; Table 2), suggesting corneal HSV-1 superinfection [4].

Frequency and genetic characterization of ACV^R variants in corneal HSV-1 isolates

The intra-individual variation of both the ACV susceptibility and US1/US12 genotype of sequential isolates may be due to corneal HSV-1 superinfection. Alternatively, the isolates may consist of multiple virus strains or represent variants of the same HSV-1 strain with dissimilar susceptibility to ACV. To address these possibilities we characterized the ACV^S isolates by PEA and compared it to paired ACV^R isolate of 5 randomly chosen rHK patients (R2, R8, R10, R13, and R14). In comparison, the sequential ACV^S isolates of patient R12 were analyzed.

Table 2. Genotype and thymidine kinase gene polymorphisms of sequential corneal HSV-1 isolates

Patient	Isolate	IC_{50}[a] (μM)	Nucleotide polymorphisms in TK[b]	Amino acid polymorphisms in TK[c]	Amplicon length[d]		Genotype		GenBank accession No.[e]
					US1	US12	US4	US7	
R1	A	1.0	T16G, **del107-109AAG**, G122A, A266G, A412G, A528G, C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, **T872G**, (T915C, C1053T), & A1126C	C6G, **del36E**, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, **L291R**, & N376H	281	253+361	B	B	FJ655092
	B	1.0	G16T, (G102A), 107-109AAG, A122G, **T259C**, T575C, (T613C, G615T, T672C, C699T, T774C), T799G, A858C, G872T, T1053C, (C1068T), & C1126A	G6C, 36E, H41R, **Y87H**, V192A, L267V, E286D, R291L, & H376N	281	**284**	B	B	FJ655093
	C	1.3	Identical to isolate 1B	Identical to isolate 1B	281	**284**	B	B	FJ655093
R2	A	<0.2	T16G, G122A, A266G, A412G, A528G, C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, (T915C, C1053T), & A1126C	C6G, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, & N376H	341	334	C	B	FJ655094
	B	1.9	**G166A**	**G56S**	341	334	C	B	FJ655095
R3	A	<0.2	T16G, G122A, A266G, A412G, A528G, C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, (T915C, C1053T), & A1126C	C6G, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, & N376H	260	207	B	A	FJ655096
	B	<0.2	Identical to isolate 3A	Identical to isolate 3A	260	207	B	A	FJ655096
	C	1.2	**del460C**	**155Fs→182St**	260	207	B	A	FJ655097
R4	A	1.5	T16G, G122A, A266G, A412G, A528G, C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, (T915C, C1053T), **A1060C**, & A1126C	C6G, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, **T354P**, & N376H	289	243	B	C	EU541361
	B	5.4	**A733C**	**T245P** & **T354P**	289	243	B	C	FJ655098
	C	10.6	Identical to isolate 4B	Identical to isolate 4B	289	243	B	C	FJ655098
R5	A	45.5	T16G, **del107-109AAG**, G122A, A266G, A412G, A528G, C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, (T915C, C1053T), & A1126C	C6G, **del36E**, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, & N376H	348	339	B	A	EU541363
	B	18.3	**T872G**	**L291R**	**369+391**	**361**	B	A	FJ655099
R6	A	0.2	(G102A), A412G, (C513T, T694C), G719A, (A801G, & A1065C)	I138V & G240E	389	357	ND	ND	FJ655100
	B	0.2	(G702A)	Identical to isolate 6A	**411**	**400**	ND	ND	FJ655100
R7	A	<0.2	(G68A), G106A, (T171C), A266G, A412G, (T694C, & A801G)	E36K, Q89R, & I138V	408	372	A	A	FJ655101
	B	ND	A367C & T935C	S123R & L312S	408	372	A	A	FJ655102
	C	1.5	**C367A**	**R123S**	408	372	A	A	FJ655103
R8	A	9.6	(G102A), A412G, **ins437G**, (C513T, T694C, G719A, A801G, C995T, & A1065C)	I138V & **146Fs→228St**	326	318	B	B	FJ655104
	B	<0.2	(G102A), A412G, (C513T, T694C), G719A, (A801G), C995T, & (A1065C)	I138V, G240E, & S332L	326	318	B	B	FJ655105
R9	A	1.8	T16G, G122A, A266G, **A382C**, A412G, (A528G), C575T, (C672T, T694C, G723A), G751T, G799T, (A801G), C802A, C858A, (T915C, C1053T), & A1126C	C6G, R41H, Q89R, **M128L**, I138V, A192V, G251C, V267L, P268T, D286E, & N376H	266	209	B	B	FJ655106
	B	5.3	**T238A**	**M128L**, & **Y80N**	266	209	B	B	FJ655107
	C	7.6	Identical to isolate 9B	Identical to isolate 9B	266	209	ND	ND	FJ655107
R10	A	6.9	(G68A), G106A, (T171C), A266G, A412G, (C513T), **del578-580TCA**, (T694C), G719A, (A801G), G842A, & (A1065C)	S23N, E36K, Q89R, I138V, **del194I**, G240E, & R281Q	215	211	C	C	EU541365
	B	<0.2	578-580TCA	194I	215	211	C	C	FJ655108
R11	A	2.6	(G68A), G106A, (T171C), A266G, A412G, (C513T), **T533G**, (T694C), G719A, (A801G), G842A, & (A1065C)	E36K, Q89R, I138V, **L178R**, G240E, & R281Q	215	209	C	C	EU541366
	B	4.5	Identical to isolate 11A	Identical to isolate 11A	215	209	C	C	EU541366
R12	A	<0.2	A412G, (T694C, & A801G)	I138V	254	242	A	A	FJ655109
	B	<0.2	Identical to isolate 12A	Identical to isolate 12A	254	242	A	A	FJ655109
R13	A	<0.2	T16G, G122A, A266G, A412G, (C468T, C513T, T694C), G719A, (A801G), G842A, & (A1065C)	C6G, R41H, Q89R, I138V, G240E, & R281Q	413	451	C	C	FJ655110
	B	8.8	**del548C**	**185Fs→→376**	**391**	451	C	C	FJ655111
	C	8.9	Identical to isolate 13B	Identical to isolate 13B	**460**	451	C	C	FJ655111
	D	2.9	Identical to isolate 13C	Identical to isolate 13C	**391**	**383**	C	C	FJ655111
	E	8.4	Identical to isolate 13D	Identical to isolate 13D	**481**	451	ND	ND	FJ655111
R14	A	7.6	T16G, (A24G), T125C, (T171C), A412G, **ins437G**, (C513T, T694C, G719A, A801G, & A1065C)	C6G, L42P, I138V, & **146Fs→228St**	280	276	B	A	EU541367
	B	<0.2	T16G, (A24G), T125C, (T171C), A412G, (C513T, T694C), G719A, (A801G, & A1065C)	C6G, L42P, I138V, & G240E	**324+346**	321	B	A	FJ655112
R15	A	<0.2	A412G, (T694C, A801G, T915C), & G1042A	I138V & V348I	370	367	B	B	FJ655113
	B	12.5	**ins548C**	**185Fs→228St**	370	367	B	B	FJ655114
	C	10.3	Identical to isolate 15B	Identical to isolate 15B	370	367	B	B	FJ655114

NOTE. TK, thymidine kinase; Fs, Frame shift; St, Stop; Del, indicated nucleotides deleted; and ND, not done.

a. IC50 was defined as the concentration of ACV that reduced the viral copies by 50% compared to the control infected cells in the absence of ACV. Cut-off value for resistance ≥1 μM ACV.

b. TK DNA sequences of the first HSV-1 isolate (isolate A) from each patient were compared to a reference HSV-1 TK sequence (KOS strain). The nucleotides shown for isolates B and C isolates represent nucleotide changes compared to the preceding isolates A and B, respectively. Nucleotide substitutions, insertions and deletions resulting in a modified TK protein conferring resistance to ACV are underlined and silent nucleotide changes are between brackets.

c. Predicted TK protein sequences of the first HSV-1 isolate (isolate A) from each patient were compared to a reference HSV-1 TK sequence (KOS strain). The amino acids shown for isolates B and C isolates represent amino acid changes compared to the preceding isolates A and B, respectively. Amino acid substitutions, deletions, and insertions potentially conferring resistance to ACV are underlined. Several nucleotide changes in the TK gene resulted are frameshift mutations leading to premature stop codon (e.g. 146Fs→228St) or abrogation of the wild-type stop codon and subsequently a longer TK protein (e.g. 296Fs→>376).

d. Amplicon length is in base pairs. Occasionally, two amplicons were detected (e.g. US12 genotype of isolate A of patient R1).

e. Genbank accession numbers of the complete TK gene sequences of the indicate isolates are provided.

Table 3. Proportion of acyclovir resistant viruses in sequential corneal HSV-1 isolates

Patient	Isolate	ACV IC_{50} (μM)[a]	No. of pfu/ml with:[b] ACV	No ACV	ACV^R variant frequency[c]
R2	A	<0.2	3.5×10^5	4.0×10^6	8.8×10^{-2} (8.8%)
	B	1.9	6.0×10^3	1.4×10^4	4.3×10^{-1} (43%)
R8	A	9.6	2.7×10^7	2.9×10^7	9.3×10^{-1} (93%)
	B	<0.2	1.3×10^2	2.2×10^6	5.9×10^{-5} (<0.1%)
R10	A	6.9	1.1×10^8	1.2×10^8	9.2×10^{-1} (92%)
	B	<0.2	1.0×10^3	5.0×10^6	2.0×10^{-4} (<0.1%)
R12	A	<0.2	5.3×10^1	4.5×10^6	1.2×10^{-5} (<0.1%)
	B	<0.2	1.4×10^3	6.6×10^7	2.1×10^{-5} (<0.1%)
R13	A	<0.2	7.0×10^2	1.9×10^6	3.7×10^{-4} (<0.1%)
	B	8.8	1.0×10^8	1.3×10^8	7.7×10^{-1} (77%)
R14	A	7.6	5.0×10^5	7.0×10^5	7.2×10^{-1} (72%)
	B	<0.2	1.0×10^1	7.5×10^4	1.3×10^{-4} (<0.1%)

NOTE. ACV, acyclovir; No., number; and pfu, plaque forming units.

a. IC50 was defined as the concentration of ACV that reduced the viral copies by 50% compared to the control infected cells in the absence of ACV. Cut-off value for resistance was 1 μM ACV.

b. Corneal HSV-1 isolates were cultured in medium supplemented with 0 μM ACV or 5 μM ACV for 4 and 10 days, respectively. Upon incubation the virus titers were determined.

c. Frequency of ACV-resistant (ACVR) viruses was calculated as follows: virus titer with 5 μM ACV divide by the virus titer without ACV. The percentages of ACVR viruses in each isolate are shown between brackets.

First, the sequential isolates were cultured in the absence or presence of ACV (5 μM) for 3 and 10 days, respectively, to determine the proportion of ACV^R variants (Table 3). The ACV concentration and culture period was chosen to ensure that only pre-existing ACV^R variants formed plaques (data not shown). ACV^R variants were not only detectable in ACV^R but also in ACV^S corneal isolates, albeit at lower frequencies in ACV^S isolates (Table 3). Second, the ACV^R variants were genetically characterized by determining both the TK gene sequence and US1/US12 genotype of the plaques (16-25 plaques/isolate). Except for patient R2, all ACV^S isolates contained several HSV-1 variants with specific TK aa mutations potentially conferring ACV^R (Table 4). Some of the TK protein alterations were identified in multiple isolates. For example, nucleotide insertions at codons 146 and 185 leading to a frameshift and premature stop codon at residue 228 in patients R8, R10, and R12-R14 (Table 4).

Table 4. Characteristics of acyclovir resistant viruses in corneal HSV-1 isolates

Patient; isolate	Amino acid polymorphisms in HSV-1 TK protein (number of virus plaques with indicated polymorphism)[a]	Amplicon length[b]	
		US1	US12
R2; A	**G56S (22)**	341	334
R8; B	7Fs→54St (1), 93Fs→120St (1), M128T (1), 139Fs→182St (1), **146Fs→228St (8)**, S181R (1), 185Fs→228St (1), G206W (2), R222H (2), 261St (1), 342 St (1), & M347R (1)	326	318
R10; B	79Fs→111St (1), P82S (2), R89W (1), G200D (1), G206W (1), R216H (1), R220H (4), 146Fs→228St (4), & 281St (1)	215	211
R12; A	D55G (1), 115Fs→228St (1), 146Fs→182St (1), **146Fs→228St** (13), 185Fs→228St (2), & R220H (1)	254	242
R12; B	**146Fs→228St (12)**, A189V (9), & T287M (1)	254	242
R13; A	G59R (1), E83K (1), L107P (1), 146Fs→228St (2), 185Fs→228St (1), **185Fs→>376 (1)**, 342St (2), & 374Fs→>376 (1)	460	451
	R51W (1)	460	486
	H58P (1), 146Fs→182St (1), & 296Fs→>376 (1)	413	427
	M128I (1)	413	404
	185Fs→>376 (1), & 310St (1)	413	486
	Del36E & D286E (1)	371	362
	T245M(1)	NA	NA
R14; B	R51W (2), 146Fs→182St (1), **146Fs→228St (9)**, 185Fs→228St (1), T287M, 296Fs→>376 (1), & C336Y (3)	324	321

NOTE. Fs, frameshift; St, stop codon; and Del, indicated amino acid deleted.

a. The TK gene sequence of each HSV-1 plaque, obtained upon culture of the indicated acyclovir sensitive (ACVS) corneal isolate in the presence of 5 μM ACV, was compared with published TK sequences of ACVS and acyclovir resistant (ACVR) HSV-1 strains. Amino acid substitutions and frameshift mutations, including premature stop codon (e.g. 146Fs→228St) or abrogation of the wild-type stop codon (i.e. 296Fs→>376), potentially conferring ACVR are shown. Plaques with a TK gene sequence and US1/US12 genotype identical to the preceding (patients R8, R12, R14, and R10) and succeeding (patients R2, R12 and R13) ACVR isolates are underlined (see also Table 2).

b. Amplicon length is in base pairs.

Next, the virus plaques were genotyped to determine if the ACV^R variants shared the same US1/US12 genotype. If not, this would suggest that the ACV^R variants with distinct TK sequences are different HSV-1 strains. Notably, several ACV^R variants in the ACV^S isolates of 3 of 5 patients had a TK sequence and US1/US12 genotype identical to the preceding (patient R8 and R14) and consecutive (patient R2) ACV^R isolate (Table 2 and 4). The ACV^R variants recovered from ACV^S isolate A of patient R13 displayed 6 different US1/US12 genotypes. Only 1 of 19 ACV^R variants shared both the same US1/US12 genotype and TK gene sequence with the consecutive ACV^R isolate (i.e. isolate R13C). In case of patient R10, neither ACV^R variant recovered from ACV^S isolate A had a TK sequence identical to the succeeding isolate B (Tables 2 and 4). Finally, both ACV^S isolates of patient R12 contained a genetically identical ACV^R variant, although present at frequencies too low to confer an ACV^R phenotype.

Discussion

To our knowledge, this is the first study to systematically evaluate the ACV susceptibility and genetic characterization of sequential HSV-1 isolates in immunecompetent patients. Three main findings are reported. First, corneal HSV-1 isolates

are heterogeneous mixtures of ACV^S and ACV^R viruses, in which the proportion of ACV^R variants determines both the isolate's ACV^R phenotype and ACV-treatment refractory disease of the patient sampled. Second, corneal ACV^S HSV-1 isolates contain multiple ACV^R variants that express the same patient-specific US1/US12 genotype, but have different TK sequences. Third, genetically identical ACV^R HSV-1 strains were recovered from consecutive corneal lesions of the same patient.

Twenty-five of 38 (66%) corneal isolates analyzed were ACV^R. Consistent with previous reports, all isolates contained ACV^R variants, albeit at low frequencies in the ACV^S isolates ($1.6 \pm 1.7 \times 10^{-4}$), and isolates were defined as ACV^R when >20% of infectious HSV-1 present were ACV^R [13,18,22]. Consistent with our previous ACV study, the ACV susceptibility phenotype correlated with the clinical response to ACV treatment [14]. Notably, 22 of 26 (85%) ACV^R isolates were obtained at time of ACV treatment, or closely related TK-dependent antivirals like valacyclovir and famcyclovir, whereas 8 of 13 (62%) ACV^S isolates were obtained from ACV non-treated patients (Table 1). The association between ACV treatment and recovery of ACV^R isolates (p=0.008; Fisher's exact test) suggests selective pressure of ACV to the enrichment of corneal ACV^R HSV-1. This phenomenon is relatively more common among immunocompromised non-HK patients and neonates during prolonged ACV treatment, which underscores the pivotal role of local immune responses to clear the virus from infected tissues [23-26].

The cornea is an immuneprivileged site. It lacks lymphatic drainage and corneal resident cells inhibit local inflammatory responses to preserve the integrity of the cornea [3,27]. Thus, the cornea may provide the optimal environment for the ACV-driven enrichment of ACV^R HSV-1 and subsequent refractory disease in ACV-treated HK patients. Selection of ACV^R HSV-1 variants can occurs within a few days after start of ACV therapy [10,26]. A switch to different antivirals like TFT and foscarnet was beneficial to control the HK episode in several ACV refractory patients. Nonetheless, disease deteriorated in about two-third of the patients. The differential clinical outcome did not correlate with the ACV susceptibility phenotype of the patients' isolates, suggesting that recovery of ACV^R HSV-1 does not predispose to disease deterioration in rHK patients. However, the majority of the patients had HSK at inclusion of the study. HSK is an HSV-1-induced T-cell-mediated chronic inflammatory disease disrupting the corneal stromal layer [3,27]. Although the prolonged retention of ACV^R virus in ACV-treated rHK patients may have played an additional role, the local inflammatory response initiated during each recurrence was most likely central to the deterioration of disease in the patients studied [3].

Acyclovir resistance is commonly conferred by mutations in the HSV-1 TK gene, and incidentally the viral DNA polymerase gene [11-13]. To determine the genetic determinants of ACV susceptibility, the entire TK ORF of 13 ACV^S and 154 ACV^R corneal HSV-1 were sequenced. Alignment of the ACV^S and ACV^R TK sequences revealed extensive polymorphisms. In addition to presumed natural polymorphisms, shared by ACV^S and

ACV^R isolates [11-13], numerous TK mutations were selectively detected in ACV^R HSV-1. About two-third of the ACV^R-associated mutations were located in the conserved and functional regions of the TK protein (Fig. 1). Twenty-nine of 48 (60%) ACV^R-related mutations found have not been described previously and need to be validated for their role in ACV^R by confirmatory testing using site-directed mutagenesis (Table 5).

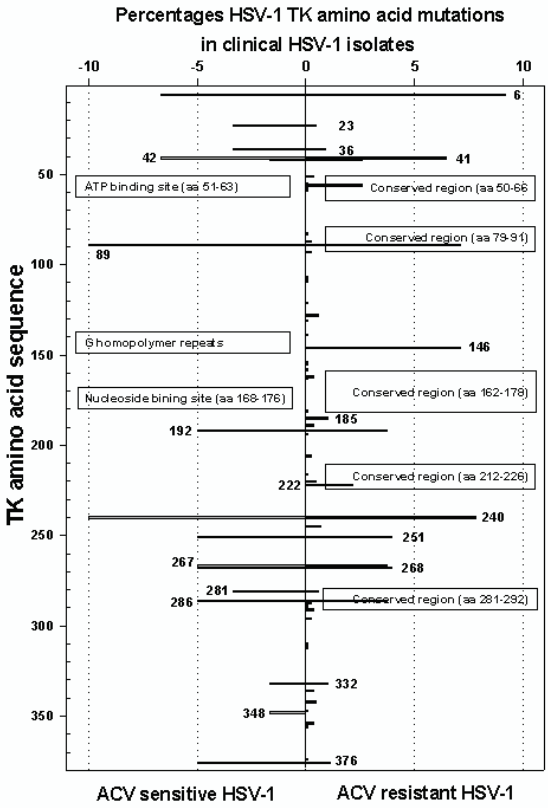


Figure 1. Schematic presentation of HSV-1 thymidine kinase protein polymorphisms identified in cornea HSV-1 of patients with herpetic keratitis. The y-axis and x-axis show the amino acid (aa) numbering of the HSV-1 TK protein and the percentage of ACV sensitive (left panel, white bars) and ACV resistant HSV-1 isolates (right panel; black bars) containing the aa alterations at the indicated positions, respectively. The functional and conserved domains of HSV-1 TK boxed and prominent polymorphisms are numbered referring to the respective aa changed. Data presented are obtained from 13 ACV sensitive and 160 ACV resistant cornea HSV-1 described in the current study.

The sequential HSV-1 isolates expressed 4 different ACV susceptibility profiles. Patients had either uniform ACV^R or ACV^S sequential isolates, or the ACV susceptibility phenotype changed in time from ACV^S→ACV^R and ACV^R→ACV^S. Combined analyses of the viral TK sequence and genotype provided detailed information on the composition of the corneal isolates. Sequential isolates of two-third of the rHK patients had identical US1/US12 genotypes, indicating possible reactivation of the latent virus. The overall TK sequence homology between sequential isolates with a similar ACV susceptibility phenotype supports this notion (patients R3, R4, R9, R11, R12, and R15). In contrast, within the same group of patients, TK sequences of sequential isolates with a disparate ACV susceptibility phenotype were different (patients R2, R3, R7, R8, R10, R12, and R15) (Table 2). This, combined with the presence of multiple TK mutants sharing the same

US1/US12 genotype in ACV^S isolates, indicates that corneas of rHK patients are infected with different ACV^R variants of the same HSV-1 strain during consecutive HK episodes (Tables 2 and 4).

ACV^R HSV-1 variants may represent transient viruses that emerge spontaneously during corneal infection or signify viruses that reactivated from the innervating sensory ganglion [11-13]. Studies in mice have demonstrated that the pathogenesis of HSV-1 depends in part on the ACV^R phenotype of the virus. Whereas TK is dispensable for HSV-1 replication in dividing cells, TK activity is essential for the virus to replicate in resting cells like neurons and to reactivate from latency in mice [28-30]. These features are thought to contribute to the low prevalence of ACV^R HSV in the human population compared to other drug-treated viral infections. Indeed, the majority of the studies on recurrent HSV infections report on transient ACV^R viruses [11-13]. Hitherto, only a few studies, mainly case reports on immunocompromised patients, have described recovery of genetically identical ACV^R HSV strain from recurrent skin lesions [31-35]. Recently, the Straus' lab has shown that sensory ganglia of a severely immunocompromised patient were infected with multiple ACV^R TK variants in addition to wildtype HSV-1, implicating that ACV^R HSV-1 do establish latency [36]. The current study extends the observations in the aforementioned studies to immunecompetent patients. Genetically identical ACV^R viruses were re-isolated from the same cornea of several rHK patients and ACV^R variants were identified in ACV^S isolates that caused ACV refractory disease during a preceding or consecutive recurrence. Furthermore, identical ACV^R variants were identified at low frequencies in ACV^S sequential isolates of patient R12 responsive to ACV treatment. Thus, ACV^R HSV-1 viruses establish latency and may reactivate several months to years later to cause recurrent corneal lesions.

In conclusion, the data presented demonstrate that clinical resistance to ACV therapy is determined by the percentage of ACV^R HSV-1 variants in corneal HSV-1 isolates. ACV treatment appears to contribute to the enrichment and persistence of ACV^R HSV-1 in corneal lesions of immunecompetent rHK patients. The recovery of a genetically identical ACV^R virus during consecutive HK episodes warrants prompt ACV susceptibility analyses of corneal isolates recovered from patients with a history of ACV refractory HK. In case of ACV^R, switching treatment to other antivirals, preferably TK-independent drugs like foscarnet, and tapering steroid treatment is recommended to avert corneal morbidity due to rHK.

Table 5. Potential acyclovir resistance-associated HSV-1 TK protein polymorphisms detected in acyclovir resistant corneal HSV-1 isolates and plaques

ACV^R isolate[a]	ACV^R plaques (N)[a]	TK seq. (N)[b]	Conserved region TK[c]	TK amino acid polymorphism	Published [references][d]	Genbank accession No.[e]
	R8B (1)	1		7Fs→54St	No	FJ655115
R5A, R1A	R13A (1)	3		del36E	Yes [1]	FJ655155
	R13A (1), R14B (2)	3	CR1 (aa 51-63) and ATP-BS (aa 50-66)	R51W	Yes [2]	FJ655151, FJ655157
	R12A (1)	1		D55G	No	FJ655135
R2B	R2A (22)	23		G56S	No	FJ655095
		1		H58P	No	FJ655152
	R13A (1)	1		G59R	No	FJ655143
	R10B (1)	1	CR2 (aa 79-91)	79Fs→111St	No	FJ655126
R9B, R9C		2		Y80N	No	
	R10B (2)	2		P82S	No	FJ655128
	R13A (1)	1		E83K	Yes [2]	FJ655144
R1B, 1C		2		Y87H	No	
	R10B (1)	1		R89W	Yes [2]	FJ655129
	R8B (1)	1		93Fs→120St	No	FJ655116
	R13A (1)	1		L107P	No	FJ655145
	R12A (1)	1		115Fs→228St	No	FJ655136
R7C		1		R123S	No	
	R13A (1)	1		M128I	No	FJ655156
R9A, R9B, R9C		3		M128L	No	
	R8B (1)	1		M128T	No	FJ655117
	R8B (1)	1		139Fs→182St	No	FJ655125
	R12A (1), R13A (1), R14B (1)	3	7G homopolymer repeats (aa 144-146)	146Fs→182St	Yes [3]	FJ655138, FJ655153, FJ655158
R8A, R14A	R8B (8), R10B (4), R12A (13), R13A (2), R14B (9)	38		146Fs→228St	Yes [3]	FJ655104, FJ655127, FJ655137, FJ655146, FJ655112
R3C		1		155Fs→182St	Yes [3]	
R11A		1	CR3 (aa 162-178) and NBR (aa 168-176)	L178R	Yes [1]	
	R8B (1)	1		S181R	Yes [1]	FJ655123
R13B, R13C, R13D, R13E	R13A (2)	6	6C homopolymer repeats (aa 183-185)	185Fs→>376	No	FJ655111
R15B, R15C	R8B (1), R12A (2), R13A (1), R14B (1)	7		185Fs→228St	Yes [4]	FJ655124, FJ655139, FJ655147, FJ655159
	R13B (9)	9		A189V	Yes [4]	FJ655141
R10A		1		del194I	Yes [1]	
	R10B (1)	1		G200D	No	FJ655130
	R8B (2), R10B (1)	3		G206W	No	FJ655118, FJ655131
	R10B (1)	1	CR4 (aa 212-226)	R216H	Yes [5]	FJ655132
	R10B (4), R12A (1)	5		R220H	No	FJ655133, FJ655140
	R8B (2)	2		R222H	Yes [2]	FJ655119
R4B, R4C		2		T245P	No	
	R8B (1)	1		261St	No	FJ655120
	R10B (1)	1	CR5 (aa 281-292)	281St	Yes [2]	FJ655134
	R13A (1)	1		D286E	No	FJ655155
	R12B (1), R14B (1)	2		T287M	Yes [3]	FJ655142, FJ655160
R1B, R1C		2		L291R	No	
	R13A (1), R14B (1)	2		296Fs→>376	Yes [4]	FJ655150, FJ655161
	R13A (1)	1		310St	No	FJ655154
	R14B (3)	3		C336Y	Yes [2]	FJ655162
	R8B (1), R13A (2)	3		342St	Yes [2]	FJ655121, FJ655148
R4B, R4C		2		T354P	No	
	R8B (1)	1		M347R	No	FJ655122
R4A		1		T354P	No	
	R13A (1)	1		374Fs→>376	No	FJ655149

NOTE. ACV, acyclovir; TK, thymidine kinase; CR, conserved region; ATP-BS, ATP-binding region; NBR, nucleotide- binding region; aa, amino acid; Fs, frameshift; St, stop codon; and Del, indicated amino acid deleted.

a. Recovery of the respective TK variant in the indicated corneal ACVR HSV-1 isolates and plaques obtained upon culture of the indicated acyclovir sensitive (ACV^S) corneal isolate in the presence of 5 μM ACV. (N), indicates the number ACV^R plaques expressing the respective TK mutation.
b. Number of corneal ACV^R HSV-1 isolates and plaques expressing the respective TK mutation.
c. Localization of the conserved and functional domains of the HSV-1 TK protein.
d. TK mutations were compared to published TK sequences. References of studies describing an identical TK mutant are shown between brackets.
e. Genbank accession numbers of the respective HSV-1 TK mutants are indicated

References

1. Roizman B, Knipe DM, Whitley RJ. Herpes simplex viruses. In: Fields Virology, eds Knipe DM and Howley P. Lippincott Williams & Wilkens, Philadelphia: **2007**: 2502-76.
2. Liesegang TJ. Herpes simplex virus epidemiology and ocular importance. Cornea **2001**;20:1-13.
3. Remeijer L, Osterhaus AD, Verjans GM. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul Immunol Inflamm **2004**;12:255-85.
4. Remeijer L, Maertzdorf J, Buitenwerf J, Osterhaus AD, Verjans GM. Corneal herpes simplex virus type 1 superinfection in patients with recrudescent herpetic keratitis. Invest Ophthalmol Vis Sci **2002**; 43:358-63.
5. Herpetic Eye Disease Study Group. Oral acyclovir for herpes simplex virus eye disease: effect on prevention of epithelial keratitis and stromal keratitis. Arch Ophthalmol **2000**; 118:1030-6.
6. Rezende RA, Bisol T, Hammersmith K, et al. Efficacy of oral antiviral prophylaxis in preventing ocular herpes simplex virus recurrences in patients with and without self-reported atopy. Am J Ophthalmol **2006**; 142:563-7.
7. Naesens L, De Clercq E. Recent developments in herpesvirus therapy. Herpes **2001**; 8:12-6.
8. Bacon TH, Levin MJ, Leary JJ, Sarisky RT, Sutton D. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. Clin Microbiol Rev **2003**; 16:114–128.
9. Stránská R, Schuurman R, Nienhuis E, et al. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. J Clin Virol **2005**; 32:7-18.
10. Danve-Szatanek C, Aymard M, Thouvenot D, et al. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. J Clin Microbiol **2004**; 42:24–9.
11. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. J Clin Virol **2003**; 26:29-37.
12. Chibo D, Druce J, Sasadeusz J, Birch C. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Res **2004**; 61:83-91.
13. Frobert E, Cortay JC, Ooka T, Najioullah F, Thouvenot D, Lina B, Morfin F. Genotypic detection of acyclovir-resistant HSV-1: characterization of 67 ACV-sensitive and 14 ACV-resistant viruses. Antiviral Res **2008**; 79:28-36.
14. Duan R, de Vries RD, Osterhaus AD, Remeijer L, Verjans GM. Acyclovir-resistant corneal HSV-1 isolates from patients with herpetic keratitis. J Infect Dis **2008**; 198:659-63.
15. Liesegang TJ. Classification of herpes simplex virus keratitis and anterior uveitis. Cornea **1999**;18:127-43.
16. Holland EJ, Schwartz GS. Classification of herpes simplex virus keratitis. Cornea **1999**;18:144-54.
17. Thi TN, Deback C, Malet I, Bonnafous P, Ait-Arkoub Z, Agut H. Rapid determination of antiviral drug susceptibility of herpes simplex virus types 1 and 2 by real-time PCR. Antiviral Res **2006**; 69:152-7.
18. Shin YK, Cai GY, Weinberg A, Leary JJ, Levin MJ. Frequency of acyclovir-resistant herpes simplex virus in clinical specimens and laboratory isolates. J Clin Microbiol. **2001**; 39:913-7.
19. Norberg P, Bergström T, Liljeqvist JA. Genotyping of clinical herpes simplex virus type 1 isolates by use of restriction enzymes. J Clin Microbiol **2006**; 44:4511-4.
20. Duan R, van Dun JM, Remeijer L, Siemerink M, Mulder PG, Norberg P, Osterhaus AD, Verjans GM. Prevalence of herpes simplex virus type 1 glycoprotein G (gG) and gI genotypes in patients with herpetic keratitis. Br J Ophthalmol **2008**; 92:1195-200.
21. Maertzdorf J, Remeijer L, Van Der Lelij A, Buitenwerf J, Niesters HG, Osterhaus AD, Verjans GM. Amplification of reiterated sequences of herpes simplex virus type 1 (HSV-1) genome to discriminate between clinical HSV-1 isolates. J Clin Microbiol **1999**;37:3518-23.
22. Sarisky RT, Nguyen TT, Duffy KE, Wittrock RJ, Leary JJ. Difference in incidence of spontaneous mutations between Herpes simplex virus types 1 and 2. Antimicrob Agents Chemother **2000**;44:1524-9.
23. Chen Y, Scieux C, Garrait V, et al. Resistant herpes simplex virus type 1 infection: an emerging concern after allogeneic stem cell transplantation. Clin Infect Dis **2000**;31:927-35.
24. Levin MJ, Bacon TH, Leary JJ. Resistance of herpes simplex virus infections to nucleoside analogues in HIV-infected patients. Clin Infect Dis **2004**;39 :S248-57.
25. Oram RJ, Marcellino D, Strauss D, et al. Characterization of an acyclovir-resistant herpes simplex virus type 2 strain isolated from a premature neonate. J Infect Dis **2000**;181:1458-61.
26. Levin MJ, Weinberg A, Leary JJ, Sarisky RT. Development of acyclovir-resistant herpes simplex virus early during the treatment of herpes neonatorum. Pediatr Infect Dis J **2001**;20:1094-7.
27. Lepisto AJ, Frank GM, Hendricks RL. How herpes simplex virus type 1 rescinds corneal privilege. Chem Immunol Allergy **2007**;92:203-12.

28. Jamieson AT, Gentry GA, Subak-Sharpe JH. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J Gen Virol **1974**;24:465-80.
29. Tenser RB, Ressel S, Dunstan ME. Herpes simplex virus thymidine kinase expression in trigeminal ganglion infection: correlation of enzyme activity with ganglion virus titer and evidence of in vivo complementation. Virology **1981**; 112:328-41.
30. Chen SH, Pearson A, Coen DM, Chen SH. Failure of thymidine kinase-negative herpes simplex virus to reactivate from latency following efficient establishment. J Virol **2004**;78:520-3.
31. Shimada Y, Suzuki M, Shirasaki F, et al. Genital herpes due to acyclovir-sensitive herpes simplex virus caused secondary and recurrent herpetic whitlows due to thymidine kinase-deficient/temperature-sensitive virus. J Med Virol. 2007 Nov;79(11):1731-40.
32. Palù G, Gerna G, Bevilacqua F, Marcello A. A point mutation in the thymidine kinase gene is responsible for acyclovir-resistance in herpes simplex virus type 2 sequential isolates. Virus Res. 1992 Sep 1;25(1-2):133-44.
33. Kost RG, Hill EL, Tigges M, Straus SE. Brief report: recurrent acyclovir-resistant genital herpes in an immunocompetent patient. N Engl J Med. 1993 Dec 9;329(24):1777-82.
34. Saijo M, Suzutani T, Itoh K, et al. Nucleotide sequence of thymidine kinase gene of sequential acyclovir-resistant herpes simplex virus type 1 isolates recovered from a child with Wiskott-Aldrich syndrome: evidence for reactivation of acyclovir-resistant herpes simplex virus. J Med Virol **1999**;58:387-93.
35. Morfin F, Thouvenot D, Aymard M, Souillet G. Reactivation of acyclovir-resistant thymidine kinase-deficient herpes simplex virus harbouring single base insertion within a 7 Gs homopolymer repeat of the thymidine kinase gene. J Med Virol **2000**; 62:247-50.
36. Wang K, Mahalingam G, Hoover SE, Mont EK, Holland SM, Cohen JI, Straus SE. Diverse herpes simplex virus type 1 thymidine kinase mutants in individual human neurons and ganglia. J Virol **2007**;81:6817-26.

Chapter 7

Summarizing Discussion

Among the eight human herpesviruses known today, herpes simplex virus type 1 (HSV-1) is the most frequent cause of primary and recurrent eye disease. Despite the availability of effective antiviral and immune-suppressive drugs, recurrent cornea infections with HSV-1 continue to be the leading cause of corneal morbidity in the industrialized world. The clinical presentation of HSV-1-induced keratitis, also referred to as herpetic keratitis (HK), varies from superficial self-limiting epithelial lesions to a potential sight-threatening chronic inflammation of corneal stroma known as herpetic stromal keratitis (HSK). HK is a complex disease that involves the interaction between the initiating virus and the immune system of the host [1]. The availability of experimental animal models, including HSV-1 infection of mice and rabbits, has provided invaluable insights into virus- and host-mediated mechanisms involved in the pathology of corneal HSV-1 infections. In contrast, limited data are available on the pathogenesis of HK in humans. The evolutionary co-existence of HSV-1 and man has inevitably resulted in the mutual adaptation of the virus and its natural host. Consequently, studies on the virus-host interactions involved in human HK are pivotal to the development of new diagnostic tools and therapeutic intervention strategies aiming to prevent the deleterious effect of corneal HSV-1 infections in humans. In the present thesis, we aimed to decipher the role of the intra-corneal host immune response and genetic characteristics of the triggering virus involved in pathology of human HK.

Interaction between corneal resident cells and neutrophils: a role of GM-CSF

In general the clinical effects of corneal HSV-1 infections can be divided into two entities: acute and chronic HK. While the acute phase is transient in the majority of the infected individuals, some patients will develop a chronic inflammatory response in the stroma of the infected cornea leading to irreversible corneal damage [2]. Consequently, therapeutic intervention of HK should be focused on blocking the transition from the acute to the chronic corneal disease.

The development and maintenance of a chronic inflammatory response involves a disrupted balance between inflammatory cell recruitment, retention and proliferation on the one hand and cell death and efflux on the other hand. Several cell types have been shown to be pivotal in the pathogenesis of HSK. Dendritic cells initiate the disease by presenting viral antigens to T-cells in the draining lymph node. In response, T-cells enter the infected cornea, recognize virus infected cells and secrete a variety of cytokines, leading to perpetuation of the local inflammatory response [3-7]. Neutrophils have a dual role. During the initiation phase cornea infiltrating neutrophils clear the virus and undergo apoptosis.

The second wave of neutrophils, entering the cornea just before the clinical phase of HSK, represents the major cell type actively involved in corneal pathology [8]. However, the role of cornea resident cells, including fibroblasts and epithelial cells, have largely been neglected in studies on the immunopathogenesis of HSK.

Previous work from our group has demonstrated the presence of the T-cell cytokine IL-17 in human HSK corneas. IL-17 was found in HSK corneas but not in control corneas and the IL-17 receptor proved to be constitutively expressed on human corneal fibroblasts (HCF). It was shown that IL-17 stimulation increased the TNF-α and IL-1 induced secretion of various neutrophil and T-cell chemotactic cytokines and chemokines, suggesting the additional role of HCF in the immunopathology of HSK [9]. In Chapter 2, we have continued to explore the possible keratogenic role of HCF and human corneal epithelial cells (HCE). Our results indicate that IL-17 selectively enhances TNFα- and IL-1β-induced granulocyte macrophage colony-stimulating factor (GM-CSF) secretion by both corneal cell types. GM-CSF activates human neutrophils and delays their apoptosis. Moreover, GM-CSF stimulated neutrophils kill HSV-1-infected HCF in the presence of immune serum and secrete the neutrophil chemo-attractant IL-8. These data are consistent with other studies suggesting a role of IL-17 in chronic neutrophil-mediated immunopathological diseases , like rheumatoid arthritis and asthma [10-15]. Since IL-23 is documented as an essential factor to promote the expansion and survival of pathogenic IL-17 producing T-cells (Th17 cells) [16-18], IL-17 and IL-23 knock-out mice have been used to study the role of the respective cytokines in the immunopathogenesis of HSK [19, 20]. Unexpectedly, the absence of IL-17 signaling resulted in a transient decrease of pro-inflammatory mediators, neutrophil migration, and cornea pathology. Mice lacking IL-23 showed enhanced cornea lesions. These observations demonstrate the intriguing roles of the IL-17/IL-23 pathway in the development of HSK. Due to the differences between species and the different route of ocular HSV-1 infection between mice and humans, additional studies are needed to address the role of both cytokines in the immune-pathogenesis of human HSK.

HSK patients benefit from topical treatment with corticosteroids, as is currently standard in clinical practice. However, topical administration of corticosteroids onto the corneal surface may enhance viral replication when not covered by appropriate antiviral treatment. Furthermore it induces thinning of the epithelial layer and subsequently increases the risk to opportunistic bacterial infections, and may cause secondary glaucoma and cataract [21]. Therefore, approaches designed to modify cytokine and chemokine expression within the microenvironment of HSV-1 infected corneas may be of therapeutic value in HSK patients. GM-CSF is a major regulatory cytokine that orchestrates the effector function of both mature macrophages and neutrophils. As shown in Chapter 2 and in previous studies, GM-CSF delays the apoptosis and promotes activation of neutrophils by inducing the release of proteolytic enzymes and free oxygen radicals [22]. Therefore,

GM-CSF is a potential therapeutic target for HSK. Two strategies may be employed to neutralize GM-CSF in corneas. The first strategy involves RNA interference (RNAi), which has been described as a highly conserved mechanism of sequence-specific post-transcriptional gene silencing. It has been applied to neutralize the GM-CSF receptor in a mouse model [23]. The second option is to neutralize the GM-CSF protein itself using monoclonal antibodies (mAbs). Plater-Zyberk *et al.* have successfully used an anti-GM-CSF mAb to reduce the severity of experimentally induced arthritis in mice [24]. Because the function of the first neutrophil wave in HSK is virus elimination, the major objective of GM-CSF neutralization will be to reduce the anti-apoptotic and activating effects of GM-CSF on the second wave of cornea-infiltrating neutrophils, thereby preventing the transition of the acute to chronic phase of HSK.

HSV-1 US4 and US7 genotypes in human herpetic keratitis

Besides the intra-corneal immune response, the genetic make-up of both the triggering virus and host are considered as important factors that determine the outcome of HK [1]. The susceptibility to HSV-1 corneal infections differs between inbred mouse strains [25, 26], and several genes have been shown to predispose to severe HSV-1 induced disease in humans [27-32]. In contrast, there are only few studies addressing the role of the viral genotype in HSV-1 diseases. Studies in mice have shown that cornea infection with different HSV-1 isolates results in disparate disease severities[26, 33, 34]. Recently, Norberg and colleagues have described that clinical HSV-1 isolates can be divided into three distinct genotypes, arbitrarily designed as genotype A, B and C. DNA sequences of three genes, US4, US7 and US8, located in unique short (US) region, were used to define the viral genotypes. These genes encode the viral glycoproteins G (gG), gI, and gE, respectively [35]. Since gE and gI proteins are involved in cell-to-cell spread of the virus and the binding of IgG to the Fc receptor, and because gG is essential for virus entry through the apical surface of polarized cells [36], we explored the prevalence and disease contribution of the gG (US4) and gI (US7) genotypes in HK patients (Chapter 3).

The prevalence of the three HSV-1 genotypes was determined in a large cohort of HK patients. The genotypes of 178 corneal HSV-1 isolates were defined by a validated PCR-RFLP method. Among the 3 genotypes, genotype B was more frequent than A and C (Chapter 3). This genotype frequency in HK patients was similar to data presented in an analogous study on 28 Swedish clinical isolates obtained from patients with various diseases including genital and oral HSV-1 infections [37]. Overall, the data indicate that US4 and US7 HSV-1 genotypes do not predispose to HK. The majority of the 178 HSV-1 corneal isolates displayed the same genotype of the US4 and US7 genes. However, about one-third (34%) of the isolates showed differences in US4 and US7 genotypes, indicating that these cornea isolates represent intergenic recombinants. Genotype A and B recombinants predominated, whereas genotype A and C intergenic recombinants were

rarely observed. These differences may in part be due to the genetic distance between genotype A and B compared to C. The overall mutation rate of HSV-1 have been estimated to be 3.5×10^{-8} mutations/site/year [38]. Consequently, HSV-1 superinfection is most likely central to the development of intergenic HSV-1 recombinants [39, 40]. Indeed, it has been demonstrated that recombination of two different HSV-1 strains infecting the same anatomical site, can give rise to new and even more virulent strains [41]. These data support the concept that genotypes A and B may have been introduced earlier than genotype C, enabling genotypes A and B to recombine over a longer time frame.

Because mouse studies have demonstrated HSV-1 strain-dependent differential HK pathology, we intended to identify associations between the identified viral genotypes and the patients' clinical parameters. Several clinical parameters appeared to be significantly associated with a specific viral genotype. However, these associations were all controversial (e.g. HK in the right eye was associated with genotype C). Since this was an explorative study, no correction for multiple testing was applied. Therefore, the significant associations could easily have arisen by chance. Based on the data presented in Chapter 3, we cannot draw the conclusion that a distinct US4 or US7 genotype predisposes to the clinical outcome of HK.

Previous studies in mice suggest that specific polymorphisms within the HSV-1 genes UL9, UL33, UL36, UL41, UL42 and US1 may affect virulence and pathology in the cornea. The aforementioned polymorphisms are potential candidates to be analyzed in a large cohort of human cornea HSV-1 isolates. Alternatively, genomes of a large selection of cornea HSV-1 strains recovered from patients with differential disease outcome could be sequenced to identify individual genes or "constellations" of genes predisposing to the severity of disease [1].

Human alpha-herpesviruses in post-PKP corneal tissues

Herpetic stromal keratitis is a sight-threatening disease. Five to 10% of corneal transplantations, also referred to as penetrating keratoplasty (PKP) are performed on HSK patients to restore sight [42-45]. Graft failure occurs more frequently in HK patients, compared to those without a history of HK (non-HK patients). The frequency of post-PKP infectious keratitis in HK patient varies from 1.8-7.4% [46-49]. The development of post-PKP HK could be due to the reactivation of the endogenous latent virus strain or graft-to-host transmission. Since the visual prognosis of patients with post-PKP HK is poor, the identification of PKP patients at risk is of major importance.

In Chapter 4, a retrospective cross-sectional study was performed on corneal buttons obtained after PKP of 83 HK patients and 367 non-HK patients. In addition, cornea-scleral rims of 273 transplanted cornea donors and 84 clear eye bank corneas were analyzed. The prevalence of α-HHVs in human cornea tissues was determined by qualitative or real-time PCR (qPCR). In accordance with previous studies, we demonstrated that HSV-1 was the

most prevalent α-HHV detected in cornea tissues and was more frequently identified in corneas of HK patients. The low prevalence of HSV-2 and VZV is most likely due to the difference in anatomical location of latent HSV-2 compared to HSV-1, and the low frequency of HSV-2 and VZV reactivation compared to HSV-1 in immunocompetent individuals, respectively [36].

Forty-eight percent of the corneas from HK patients and 4% of the corneas from non-HK patients were HSV-1 DNA positive. The detection of α-HHV positive corneas from non-HK patients is puzzling. This may be due to cross-contamination, or less likely misdiagnosis of the corneal disease. Asymptomatic virus shedding could be an alternative explanation. It has been demonstrated that one-third of the healthy adults asymptomatically shed virus in their tears at least once in 30 days [50]. Compared with HK corneas, the HSV-1 DNA load in non-HK corneas is 2 10-log lower. This suggests that the presence of α-HHV in non-HK corneas is most likely irrelevant to the disease and represents asymptomatic shedding of reactivated virus in the respective patients [51, 52].

Previous studies have advocated that, in addition to the innervating sensory ganglion, HSV-1 establishes latency in the cornea [53-55]. HSV-1 DNA has been detected in healed corneas of HK patients [56-58]. Alternatively, the viral DNA detected may simply represent viral remnants that have been incompletely cleared by the local immune response. In our study, several corneas of HK and non-HK patients, who did not show signs of active disease at time of surgery, were investigated for relevant HSV-1 transcripts. During a lytic infection HSV-1 genes are expressed in a temporal cascade. One immediate early gene (α) ICP127, two late (γ) genes gG and gC, and the latency associated transcript (LAT) gene were selected in the study [36]. Since LAT is transcribed both during lytic infection and latency, expression of ICP127, gG and gC would indicate whether the virus is lytic or latent [54]. The data obtained demonstrate that all four HSV-1 genes are transcribed, implicating a lytic HSV-1 infection in the corneas analyzed. Furthermore, the cornea HSV-1 DNA load decreased gradually with increasing recurrence free interval, and high viral loads correlated with fulminant disease and pre-operative steroid treatment. Thus, HSV-1 DNA persists in human corneas for extensive periods of time, and the data do not support the hypothesis that HSV-1 establishes true latency in corneal tissues.

Statistical analyses showed that neo-vascularization was inversely correlated with corneal HSV-1 DNA load. In active HK lesions, corneas are densely infiltrated with lymphocytes [59]. Deep neo-vascularization will facilitate the egress of inflammatory cells into the affected tissue [8, 60]. These studies along with our data provide evidence that local inflammatory responses are involved in clearing the virus from HSV-1 infected cornea tissues. Another interesting finding is that the intra-corneal HSV-1 DNA load correlated with the patients' age in both HK and non-HK groups. It has been reported that the incidence of HK and the associated morbidity is more common in the elderly [61, 62]. Similar observations have been described in mouse studies, in which adult mice are more

susceptible to severe HSV-1 induced cornea inflammation compared to young mice [63]. Aging is associated with a declining function in various organs and physiological systems, including the immune system. The age-related changes of the immune system may contribute to an increased susceptibility to infectious diseases in the elderly[64]. Thus, the correlation between the patient's age and the intra-corneal HSV-1 DNA load is most likely due to the age-related decline in immune control of latent HSV-1.

There are several risk factors that will influence the cornea graft survival. Corneal neo-vascularization, and a history of graft rejection or HK are known high risk factors for graft failure [44, 59, 65]. An additional risk identified in our study is the presence and particularly the HSV-1 DNA load in excised corneas of HK patients. This parameter correlated with graft survival independently from the aforementioned risk factors. Implementation of HSV-1 qPCR on excised corneas of transplanted HK patients are recommended to identify those patients at risk to develop graft rejection.

Transplantation of donor tissues poses the risk to transmit infectious diseases. The anecdotal detection of HSV-1 in donor corneas and the documented graft-to-host transmission of HSV-1 after PKP, argues to determine the prevalence and clinical relevance of α-HHV DNA in a large cohort of donor cornea tissues [44, 59, 65]. In Chapter 4 this issue was addressed by αHHV PCR analyses of 273 corneal-scleral rims and 84 clear eye bank corneas. αHHV DNA could only be detected in the corneascleral rims: 2 out of 273 corneal-scleral rims contained HSV-1 DNA. The HSV-1 DNA load was very low and neither PKP patient who received the central part of these donor corneas developed post-PKP HK. Thus, α-HHV qPCR analyses on donor cornea tissues do not seem to have diagnostic value to predict post-PKP HK and newly acquired HK in PKP patients.

Acyclovir resistant HSV-1 in herpetic keratitis patients

In acute ocular infections like herpes epithelial keratitis (HEK), application of antiviral drugs accelerates the elimination of replicating virus from the cornea epithelium [66]. In case of HSK, a balanced combination treatment with steroids and antivirals is essential [21]. Among the currently available antiviral drugs, acyclovir (ACV) therapy is the gold standard to treat HK patients, largely because of its high specificity for HSV-1 and limited corneal toxicity [67]. ACV is a pro-drug, converted by the viral protein thymidine kinase (TK) into its active component. Resistance to ACV is largely attributed to mutations in the TK gene that alter or block TK activity [68]. Early upon the introduction of ACV in the early 80s of the last century, ACV resistant (ACV^R) HSV-1 have been detected in clinical isolates. Several studies performed on the ACV^R surveillance showed that the prevalence of ACV^R HSV-1 ranges from 0.1-0.6% in immune-competent patients, to 4.3-14% among immunocompromised individuals with HSV-1 disease [69-71]. Hitherto, the prevalence and clinical relevance of corneal ACV^R HSV-1 in HK patients has not been studied in detail.

In Chapter 5, the prevalence and molecular characteristics of corneal ACV-resistant HSV-1 isolates was determined in 173 of immunecompetent HK patients. While, the HK corneal morbidity is largely due to the recurrent HSV-1 infections, about 20-61% of the patients with recurrent herpetic keratitis (rHK) eventually develop HSK [80]. This evidence conduct us to extend our research to uncover the incidence and clinical significance of ACV^R HSV-1 in rHK patients.

In Chapter 6, the ACV susceptibility and genetic characteristics of 38 sequential corneal HSV-1 isolates from 15 immunecompetent rHK patients were determined. We analyzed the ACV sensitivity of 173 corneal HSV-1 isolates. Eleven of 173 (6.4%) HSV-1 isolates were characterized as ACV^R, and all patients included in this study were immuno-competent. The data demonstrate an unprecedented high prevalence of ACV^R in otherwise healthy patients. This could be due to the unique properties of the cornea, being an immune-privileged site, contribute to this relatively high prevalence of ACV^R HSV-1. The cornea lacks blood and lymphatic vessels and corneal resident cells effectively inhibit local inflammatory responses to preserve the corneal integrity. These factors may enable the enrichment of ACV^R HSV-1 strains in corneas under long term ACV pressure [72].

Recent surveys have shown that the prevalence of ACV resistance has remained the same during the last 2 decades, even though the use of ACV has increased extensively [73-75]. However, the results from our studies showed an opposite situation at least in the ACV treatment of corneal HSV-1 infections. At the Rotterdam Eye Hospital, where all HK patients were recruited, prophylactic ACV treatment has greatly increased after 1994. Interestingly, all corneal ACV^R HSV-1 isolates identified in this study were from patients who presented after 1994. Moreover, in the sequential HSV-1 isolates of rHK patients, ACV susceptibility patterns correlated with ACV treatment. Twenty-five of the 38 (66%) sequential corneal isolates were defined as ACV^R. Among 25 ACV^R HSV-1 corneal isolates, 22 isolates (88%) were obtained at time of ACV treatment, and 8 (62%) of 13 ACV^S isolates were recovered from treatment naïve patients. This suggests that the increased ACV prescriptions for HK contributed to the emergence of resistant corneal HSV-1 strains, thereby alerting the effectiveness of ACV therapy in time.

In the study described in Chapter 5, several mutations in the HSV-1 TK gene were detected in both the ACV^S and ACV^R isolates. These TK mutations represent natural TK polymorphisms that do not alter TK activity. Additionally, TK mutations were identified solely in ACV^R isolates, which potentially confer the ACV^R phenotype of the respective isolates. One particular TK mutation, an arginine to leucine substitution at residue 178, is of particular interest because of its location within the nucleoside-binding site of TK and had been found twice in the corneal ACV^R isolates analyzed [76, 77]. An insertion in the G-repeat region at nucleotide position 430 was found in two ACV^R isolates, resulting in a TK protein truncation. Although the molecular and *in vitro* sensitivity data suggests that these newly identified TK mutations described in Chapter 5 are significant, their roles in

ACV resistance still need to be confirmed by functional studies such as site-directed mutagenesis.

Ganciclovir (GCV) is an alternative drug that can be applied in the treatment of HK. Like ACV, GCV depends on TK activity to be converted into its active compound [78]. Notably, 5 of 11 corneal ACV^R HSV-1 isolates showed cross-resistance to GCV. The TK mutations identified included the above-described frame-shift, two amino acid deletions and one amino acid substitution. Compared with the ACV^R/GCV^S isolates, these mutations may induce more severe functional loss of TK activity. Foscarnet (FOS) is a third antiviral treatment with a different mechanism of action. FOS is a pyrophosphate analogue that inhibits the viral DNA polymerase directly and is TK-independent. However, due to its nephrotoxicity, FOS administration requires slow intravenous infusion, extensive prehydration and careful dose adjustment [79]. Therefore, FOS is only prescribed to patients who are not responding to ACV and GCV treatment. One of the 11 ACV^R corneal isolates showed cross-resistance to both GCV and FOS. No obvious mutations were found in the TK gene of this isolate, suggesting that mutations in the DNA polymerase gene are involved [76, 77]. In this particular patient, a switch to a more toxic antiviral therapy such as cidofovir would have been more appropriate to preserve the cornea.

Since HSV-1 is capable of establishing latency in the peripheral nervous system[86], rHK may be caused by reactivation of the endogenous virus or by reinfection with an exogenous virus, known as HSV-1 super-infection [87]. In the study presented in Chapter 6, the genotypes of 5 of 15 and 10 of 15 sequential isolates were different or identical to the successive isolates from the same patient, respectively. Whereas the first group represents corneal HSV-1 superinfection, the genotype data of the latter group of rHK indicate that the same virus had reactivated to cause recurrent disease.

It has been demonstrated that clinical HSV-1 isolates are a mixture of viruses, which contain both ACV^S and ACV^R viruses [81-83]. These different virus populations are TK variants of the same strain or represent a collection of different strains with disparate ACV susceptibility phenotypes [38]. In Chapter 6, the ACV^R mutation frequencies were tested in 14 isolates recovered from 7 patients. The ACV^R variant frequencies of ACV^S and ACV^R isolates ranged from 1.2×10^{-5} to 8.8×10^{-2}, and 4.3×10^{-1} to 9.3×10^{-1}, respectively. Consistent with previous reports, corneal HSV-1 isolates were characterized ACV^R when the population of ACV^R variants was higher than 20% [84, 85]. The combined analyses of the TK sequence and US1/US12 genotype of ACV^R variants in ACV^S isolates showed that the majority of the ACV^R variants are TK mutants of the same HSV-1 strain.

Several studies have demonstrated that TK is not essential for HSV-1 replication [88], but is possibly involved in HSV-1 pathogenicity and reactivation in mice [89, 90]. Since the majority of the ACV^R mutations are located within the HSV-1 TK gene, it is considered that ACV^R strains are deficient to establish latency in humans. However, in 7 of the 10 rHK patients studied, a genetically identical ACV^R HSV-1 strain reappeared during a

consecutive recurrence. This indicates that ACV^R HSV-1 does establish latency in immune-competent individuals and reactivates intermittently to cause recurrent disease, which in our cohort was associated with ACV-treatment refractory disease.

The medical records of the HK patients with ACV^R corneal HSV-1 isolates in Chapter 5, were reviewed for evidence of clinical resistance. Nine out of 11 patients were clinically resistant to ACV therapy, and 7 of the 9 had switched to other antiviral therapies during the course of treatment. Although all patients initially responded to the antiviral therapies at the beginning, 7 of these 11 patients eventually developed corneal blindness. Furthermore, the clinical outcome of the 15 rHK patients did not correlate with the recovery of ACV^R HSV-1 in the study presented in Chapter 6 . These could be due to the fact that the majority of the patients had already developed HSK at inclusion of the studies. So the local T-cell mediated responses have been more detrimental to cornea than the prolonged persistence of ACV^R HSV-1 during ACV therapy [59].

Notably, recovery of ACV^R HSV-1 isolates correlated with ACV-treatment refractory disease of the respective HK patient. This observation argues in favor of the implementation of diagnostic ACV susceptibility assays on corneal HSV-1 isolates of HK patients who are refractory to ACV therapy or rHK patients with a history of ACV refractory HK. Prompt identification of ACV^R HSV-1 in HK patients will save therapy time and may prevent the development of more severe disease in the end. In addition, the second and third classes of anti-HSV drugs, like FOS and cidofovir, are of limited use due to severe side effects including corneal toxicity [79]. Thus, there is an unmet need for the discovery of new antivirals to treat HK patients non-responsive to ACV and GCV therapy. If newly developed drugs with working mechanisms different from nucleoside antiviral drugs and low toxicityl, a combination of the new drug and ACV could be prescribed to HK patients at start of the disease. This would lead to a more efficient way of treating HK patients.

Conclusions

The work presented in this thesis addresses novel aspects of the pathogenesis of HSV-1 infections of the cornea as well as novel therapeutic and prognostic modalities for HK patients.

First, it was shown that corneal resident cell play important roles in modulating the host immune responses by secreting various pro-inflammatory cytokines and chemokines. The elucidation of the identity and role of these factors, as well as their regulation locally, may lead to novel therapeutic means to treat HSK patients at risk to develop corneal blindness. For example, due to strong anti-apoptotic and activation effects on PMNs, GM-CSF would be an excellent target to prevent the transition from the acute to the chronic phase of HK.

Second, although it was demonstrated in Chapter 3 that the genotype of gG and gI gene do not associated with HK development, the identifications of virulent HSV-1 genes or combinations thereof is warranted. Corneal HSV-1 isolates can be tested for the presence of these HK-inducing HSV-1 genes to identify those patients at risk to develop severe HK and to start appropriate treatment regimens.

Third, the correlation between HSV-1 DNA load and corneal graft rejection warrants the implementation of HSV-1-specific qPCR on explanted cornea buttons to improve the long-term visual prognosis of transplanted HK patients.

Fourth, the relatively high prevalence corneal ACV^R HSV-1, as well as the ability of ACV^R HSV-1 to establish latency and to reactivate intermittently leading to ACV-treatment refractory disease in HK patients, argue for the implementation of HSV-1 susceptibility assays on corneal HSV-1 isolates recovered from HK patients with a clinical history, or those clinically suspected, of ACV refractory disease. A switch to HSV-1 TK-independent antivirals, like FOS, will improve the outcome of disease. Given the corneal toxicity of FOS, the development of a new generation of antivirals to treat corneal ACV^R HSV-1 infections is indicated.

In conclusion, the data presented in this thesis provide more insight into the pathology of HK and describe new diagnostic tools to improve the diagnosis and treatment of HK patients at risk to develop severe corneal disease.

References

1. Brandt C. The role of viral and host genes in corneal infection with herpes simplex virus type 1. Exp Ye Res 2005;80:607-21
2. Carr DJ, Harle P and Gebhardt BM. The immune response to ocular herpes simplex virus type 1 infection. Exp Biol Med (Maywood) 2001;226:353-66
3. Bauer D, Mrzyk S, van Rooijen N, Steuhl KP and Heiligenhaus A. Macrophage-depletion influences the course of murine HSV-1 keratitis. Curr Eye Res 2000;20:45-53
4. Hendricks RL, Tumpey TM and Finnegan A. IFN-gamma and IL-2 are protective in the skin but pathologic in the corneas of HSV-1-infected mice. J Immunol 1992;149:3023-8
5. Keadle TL, Usui N, Laycock KA, Miller JK, Pepose JS and Stuart PM. IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis. Invest Ophthalmol Vis Sci 2000;41:96-102
6. Niemialtowski MG, Rouse BT. Predominance of Th1 cells in ocular tissues during herpetic stromal keratitis. J Immunol 1992;149:3035-9
7. Streilein JW, Dana MR and Ksander BR. Immunity causing blindness: five different paths to herpes stromal keratitis. Immunol Today 1997;18:443-9
8. Biswas PS, Rouse BT. Early events in HSV keratitis--setting the stage for a blinding disease. Microbes Infect 2005;7:799-810
9. Maertzdorf J, Osterhaus AD and Verjans GM. IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts. J Immunol 2002;169:5897-903
10. Chabaud M, Garnero P, Dayer JM, Guerne PA, Fossiez F and Miossec P. Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. Cytokine 2000;12:1092-9
11. Laan M, Cui ZH, Hoshino H, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J Immunol 1999;162:2347-52
12. Linden A. Role of interleukin-17 and the neutrophil in asthma. Int Arch Allergy Immunol 2001;126:179-84
13. Luzza F, Parrello T, Monteleone G, et al. Up-regulation of IL-17 is associated with bioactive IL-8 expression in Helicobacter pylori-infected human gastric mucosa. J Immunol 2000;165:5332-7
14. Molet S, Hamid Q, Davoine F, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol 2001;108:430-8
15. Miyamoto M, Prause O, Sjostrand M, Laan M, Lotvall J and Linden A. Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. J Immunol 2003;170:4665-72
16. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ and Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003;278:1910-4
17. Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 2003;421:744-8
18. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005;201:233-40
19. Kim B, Sarangi PP, Azkur AK, Kaistha SD and Rouse BT. Enhanced viral immunoinflammatory lesions in mice lacking IL-23 responses. Microbes Infect 2008;10:302-12
20. Molesworth-Kenyon SJ, Yin R, Oakes JE and Lausch RN. IL-17 receptor signaling influences virus-induced corneal inflammation. J Leukoc Biol 2008;83:401-8
21. Wilhelmus KR, Gee L, Hauck WW, et al. Herpetic Eye Disease Study. A controlled trial of topical corticosteroids for herpes simplex stromal keratitis. Ophthalmology 1994;101:1883-95; discussion 1895-6
22. Fleetwood AJ, Cook AD and Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. Crit Rev Immunol 2005;25:405-28
23. Scherr M, Battmer K, Dallmann I, Ganser A and Eder M. Inhibition of GM-CSF receptor function by stable RNA interference in a NOD/SCID mouse hematopoietic stem cell transplantation model. Oligonucleotides 2003;13:353-63
24. Plater-Zyberk C, Joosten LA, Helsen MM, Hepp J, Baeuerle PA and van den Berg WB. GM-CSF neutralisation suppresses inflammation and protects cartilage in acute streptococcal cell wall arthritis of mice. Ann Rheum Dis 2007;66:452-7
25. Grau DR, Visalli RJ and Brandt CR. Herpes simplex virus stromal keratitis is not titer-dependent and does not correlate with neurovirulence. Invest Ophthalmol Vis Sci 1989;30:2474-80
26. Williams LE, Nesburn AB and Kaufman HE. Experimental Induction of Disciform Keratitis. Arch Ophthalmol 1965;73:112-4

27. Bochud PY, Magaret AS, Koelle DM, Aderem A and Wald A. Polymorphisms in TLR2 are associated with increased viral shedding and lesional rate in patients with genital herpes simplex virus Type 2 infection. J Infect Dis 2007;196:505-9
28. Hobbs MR, Jones BB, Otterud BE, Leppert M and Kriesel JD. Identification of a herpes simplex labialis susceptibility region on human chromosome 21. J Infect Dis 2008;197:340-6
29. Hurme M, Haanpaa M, Nurmikko T, et al. IL-10 gene polymorphism and herpesvirus infections. J Med Virol 2003;70 Suppl 1:S48-50
30. Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B and Jamieson GA. Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. Lancet 1997;349:241-4
31. Lekstrom-Himes JA, Hohman P, Warren T, et al. Association of major histocompatibility complex determinants with the development of symptomatic and asymptomatic genital herpes simplex virus type 2 infections. J Infect Dis 1999;179:1077-85
32. Zhang SY, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. Science 2007;317:1522-7
33. Brandt CR, Grau DR. Mixed infection with herpes simplex virus type 1 generates recombinants with increased ocular and neurovirulence. Invest Ophthalmol Vis Sci 1990;31:2214-23
34. Wander AH, Centifanto YM and Kaufman HE. Strain specificity of clinical isolates of herpes simplex virus. Arch Ophthalmol 1980;98:1458-61
35. Norberg P, Bergstrom T, Rekabdar E, Lindh M and Liljeqvist JA. Phylogenetic analysis of clinical herpes simplex virus type 1 isolates identified three genetic groups and recombinant viruses. J Virol 2004;78:10755-64
36. Roizman B KD, Whitley RJ. Herpes simplex viruses. In: Knipe DM HP, Griffin DE, et al., ed. Fields virology. 5th ed. Vol. 2: Philadelphia: Lippincott Williams & Wilkins, 2007:2007:2501-2603.
37. Norberg P, Bergstrom T and Liljeqvist JA. Genotyping of clinical herpes simplex virus type 1 isolates by use of restriction enzymes. J Clin Microbiol 2006;44:4511-4
38. Sarisky RT, Nguyen TT, Duffy KE, Wittrock RJ and Leary JJ. Difference in incidence of spontaneous mutations between Herpes simplex virus types 1 and 2. Antimicrob Agents Chemother 2000;44:1524-9
39. Lingen M, Hengerer F and Falke D. Mixed vaginal infections of Balb/c mice with low virulent herpes simplex type 1 strains result in restoration of virulence properties: vaginitis/vulvitis and neuroinvasiveness. Med Microbiol Immunol 1997;185:217-22
40. Wildy P. Recombination with herpes simplex virus. J Gen Microbiol 1955;13:346-60
41. Kintner RL, Allan RW and Brandt CR. Recombinants are isolated at high frequency following in vivo mixed ocular infection with two avirulent herpes simplex virus type 1 strains. Arch Virol 1995;140:231-44
42. Beekhuis WH, Renardel de Lavalette JG, van Rij G and Schaap GJ. Therapeutic keratoplasty for active herpetic corneal disease: viral culture and prognosis. Doc Ophthalmol 1983;55:31-5
43. Foster CS, Duncan J. Penetrating keratoplasty for herpes simplex keratitis. Am J Ophthalmol 1981;92:336-43
44. Koay PY, Lee WH and Figueiredo FC. Opinions on risk factors and management of corneal graft rejection in the United kingdom. Cornea 2005;24:292-6
45. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group. Ophthalmology 1994;101:1536-47
46. Lomholt JA, Baggesen K and Ehlers N. Recurrence and rejection rates following corneal transplantation for herpes simplex keratitis. Acta Ophthalmol Scand 1995;73:29-32
47. Panda A, Kumar TS. Prognosis of keratoplasty in viral keratitis. Ann Ophthalmol 1991;23:410-3
48. Sterk CC, Jager MJ and Swart-vd Berg M. Recurrent herpetic keratitis in penetrating keratoplasty. Doc Ophthalmol 1995;90:29-33
49. Vajpayee RB, Sharma N, Sinha R, Agarwal T and Singhvi A. Infectious keratitis following keratoplasty. Surv Ophthalmol 2007;52:1-12
50. Kaufman HE, Azcuy AM, Varnell ED, Sloop GD, Thompson HW and Hill JM. HSV-1 DNA in tears and saliva of normal adults. Invest Ophthalmol Vis Sci 2005;46:241-7
51. Asimakis P, Kirkness CM. Storage of donor corneas, surgery, outcome, and complications of penetrating keratoplasty. Curr Opin Ophthalmol 1996;7:35-40
52. Toma HS, Murina AT, Areaux RG, Jr., et al. Ocular HSV-1 latency, reactivation and recurrent disease. Semin Ophthalmol 2008;23:249-73
53. Cook SD, Brown SM. Herpes simplex virus type 1 persistence and latency in cultured rabbit corneal epithelial cells, keratocytes, and endothelial cells. Br J Ophthalmol 1986;70:642-50
54. Cook SD, Hill JM, Lynas C and Maitland NJ. Latency-associated transcripts in corneas and ganglia

of HSV-1 infected rabbits. Br J Ophthalmol 1991;75:644-8
55. Gordon YJ, Romanowski E, Araullo-Cruz T and McKnight JL. HSV-1 corneal latency. Invest Ophthalmol Vis Sci 1991;32:663-5
56. Kaye SB, Lynas C, Patterson A, Risk JM, McCarthy K and Hart CA. Evidence for herpes simplex viral latency in the human cornea. Br J Ophthalmol 1991;75:195-200
57. Perng GC, Zwaagstra JC, Ghiasi H, et al. Similarities in regulation of the HSV-1 LAT promoter in corneal and neuronal cells. Invest Ophthalmol Vis Sci 1994;35:2981-9
58. Zheng X. Reactivation and donor-host transmission of herpes simplex virus after corneal transplantation. Cornea 2002;21:S90-3
59. Remeijer L, Osterhaus A and Verjans G. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul Immunol Inflamm 2004;12:255-85
60. Guess S, Stone DU and Chodosh J. Evidence-based treatment of herpes simplex virus keratitis: a systematic review. Ocul Surf 2007;5:240-50
61. Butler TK, Spencer NA, Chan CC, Singh Gilhotra J and McClellan K. Infective keratitis in older patients: a 4 year review, 1998-2002. Br J Ophthalmol 2005;89:591-6
62. van der Meulen IJ, van Rooij J, Nieuwendaal CP, Van Cleijnenbreugel H, Geerards AJ and Remeijer L. Age-related risk factors, culture outcomes, and prognosis in patients admitted with infectious keratitis to two Dutch tertiary referral centers. Cornea 2008;27:539-44
63. Turner J, Turner OC, Baird N, Orme IM, Wilcox CL and Baldwin SL. Influence of increased age on the development of herpes stromal keratitis. Exp Gerontol 2003;38:1205-12
64. Kumar R, Burns EA. Age-related decline in immunity: implications for vaccine responsiveness. Expert Rev Vaccines 2008;7:467-79
65. Panda A, Vanathi M, Kumar A, Dash Y and Priya S. Corneal graft rejection. Surv Ophthalmol 2007;52:375-96
66. Wilhelmus KR. Therapeutic interventions for herpes simplex virus epithelial keratitis. Cochrane Database Syst Rev 2008:CD002898
67. Ohashi Y. Treatment of herpetic keratitis with acyclovir: benefits and problems. Ophthalmologica 1997;211 Suppl 1:29-32
68. Hill EL, Hunter GA and Ellis MN. In vitro and in vivo characterization of herpes simplex virus clinical isolates recovered from patients infected with human immunodeficiency virus. Antimicrob Agents Chemother 1991;35:2322-8
69. Christophers J, Clayton J, Craske J, et al. Survey of resistance of herpes simplex virus to acyclovir in northwest England. Antimicrob Agents Chemother 1998;42:868-72
70. Gilbert C, Bestman-Smith J and Boivin G. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist Updat 2002;5:88-114
71. Nugier F, Colin JN, Aymard M and Langlois M. Occurrence and characterization of acyclovir-resistant herpes simplex virus isolates: report on a two-year sensitivity screening survey. J Med Virol 1992;36:1-12
72. Lepisto AJ, Frank GM and Hendricks RL. How herpes simplex virus type 1 rescinds corneal privilege. Chem Immunol Allergy 2007;92:203-12
73. Bacon TH, Levin MJ, Leary JJ, Sarisky RT and Sutton D. Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy. Clin Microbiol Rev 2003;16:114-28
74. Danve-Szatanek C, Aymard M, Thouvenot D, et al. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. J Clin Microbiol 2004;42:242-9
75. Stranska R, Schuurman R, Nienhuis E, et al. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. J Clin Virol 2005;32:7-18
76. Chibo D, Druce J, Sasadeusz J and Birch C. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Res 2004;61:83-91
77. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. J Clin Virol 2003;26:29-37
78. Faulds D, Heel RC. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs 1990;39:597-638
79. Naesens L, De Clercq E. Recent developments in herpesvirus therapy. Herpes 2001;8:12-6
80. Green LK, Pavan-Langston D. Herpes simplex ocular inflammatory disease. Int Ophthalmol Clin 2006;46:27-37
81. Gupta R, Hill EL, McClernon D, et al. Acyclovir sensitivity of sequential herpes simplex virus type 2 isolates from the genital mucosa of immunocompetent women. J Infect Dis 2005;192:1102-7
82. Parris DS, Harrington JE. Herpes simplex virus variants restraint to high concentrations of acyclovir exist in clinical isolates. Antimicrob Agents Chemother 1982;22:71-7
83. Shin YK, Cai GY, Weinberg A, Leary JJ and Levin MJ. Frequency of acyclovir-resistant herpes

simplex virus in clinical specimens and laboratory isolates. J Clin Microbiol 2001;39:913-7
84. Frobert E, Cortay JC, Ooka T, et al. Genotypic detection of acyclovir-resistant HSV-1: characterization of 67 ACV-sensitive and 14 ACV-resistant viruses. Antiviral Res 2008;79:28-36
85. Langlois M, Allard JP, Nugier F and Aymard M. A rapid and automated colorimetric assay for evaluating the sensitivity of herpes simplex strains to antiviral drugs. J Biol Stand 1986;14:201-11
86. Efstathiou S, Field HJ, Griffiths PD, et al. Herpes simplex virus latency and nucleoside analogues. Antiviral Res 1999;41:85-100
87. Remeijer L, Maertzdorf J, Buitenwerf J, Osterhaus AD and Verjans GM. Corneal herpes simplex virus type 1 superinfection in patients with recrudescent herpetic keratitis. Invest Ophthalmol Vis Sci 2002;43:358-63
88. Jamieson AT, Gentry GA and Subak-Sharpe JH. Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J Gen Virol 1974;24:465-80
89. Efstathiou S, Kemp S, Darby G and Minson AC. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J Gen Virol 1989;70 (Pt 4):869-79
90. Jacobson JG, Ruffner KL, Kosz-Vnenchak M, et al. Herpes simplex virus thymidine kinase and specific stages of latency in murine trigeminal ganglia. J Virol 1993;67:6903-8

Acknowledgement

This thesis presents the result of the four and a half year's research work in Rotterdam Erasmus Medical Center. Without the support from so many people who have helped me in different ways in different stages of conducting the research, it would be impossible to accomplish this thesis. It is the right moment for me to express my deep gratitude to them and acknowledge their contributions.

I would like to express my appreciation to my promoter, Prof. Ab Osterhaus, his trustful support. I still remember the first time we might each other at the 17th flour. He looks so critical and seldom to have a smell on your face. I was scared at that moment, how strict will this person be? Indeed he is more like a seniors, like a beacon, lighting up my way forward and guide me through so many difficulties. My deep gratitude goes to my co-promoter dr. Geroges Verjans. He is the person, who ushers me to the fascinating research field of virology and immunology, who devotes enormous amount of time and intelligence to guide me to develop research ideas, who takes great pains to help me sharpen my views and arguments, who continuously gives me line-by-line comments for my papers and teaches me how to prepare for presentations. I am greatly indebted to him, for his day-to-day support, his excellent supervision, his kindness, and his patience. I would like to express my appreciation to another co-promoter Dr. Lies Remeijer. She guided me in to the wonderful clinical area. Her advice, ideas, and critical opinion are invaluable in my research practice and thesis writing. Moreover, this project would not success without the great collaboration of Rotterdam Eye Hospital. I wish to thank Lies also for her nice coordination for the collaboration and well arrangement for the clinical data and samples.

My gratitude also goes to Jessica, Rory and Freek, for their valuable advices in experiments designs and contributions in data collecting of Chapter 3, 4, 5 and 6. Many thanks to all my colleagues from the Virology Department for their friendship. If I want detail into the innumerable help that I got from them in both my experiments and daily life, then I will need to write another book to list them down. In particular all of my old and new roommates in Ee1720, your friendship make our office a home, give me so much laughter and happiness. Special thanks to Simone, Wim, and Lydia for helping me to ideal with all the complicated official documents and providing me many practical and useful information which enable me to survive well in this city. Monique and Rory, my paranymphs, exhausting my vocabulary cannot express my appreciation to you. Thank you for the inspiring discussions, critical suggestions for this thesis, the well arrangement for my promotion and all the things you did for me.

Furthermore, I wish to thank my chums, Xingnan Li, Bo Yang, Yan Jing and Shanshan

Wang for their carefully reading, checking of my thesis and their encouragements to pull me out of the frustrations. There is a Chinese saying:“ At home by parents, to go out on a friend.” I would like to give my deep thanks to all of my friends for their friendship, help, and all other kinds of sharing. My old friends, Wenlong Zhao and Nong Chen, picked me up at Schiphol airport seven years ago, and helped me to settle down in this country. It’s like a dream to see old friends in such a beautiful place. From then on I acquainted more and more friends. To name but a few, Fan Su, Pu Ran, Xueyuan Zhang, Ze Zhu, Xiaohua Lu, Aihua Xiong, Zaijun Li, Tao Jiang, Ying Shi, Ruofei Zhao, Liubai Ruan, Yugang Yu, Yan Xiong, Juan Pang, Yuan Ju, Cuicui Shi, Jing You, Steven Nijenkamp, I-Ching Lin, Tai'an Liu, Ting Li, Hailiang Mei, Defeng Lang, Xue Zhao, Yeming Gong, Zhangrong Guo, Yan Chen, Chao Wang, Ying Zhu, Yongping Chen, Kemei Chen, Huanling Wang, Hong Tang, Zhuhang Li, Baoli Yang, Hong Chen, Li Chen, Younan Li and Ming Wu. Their precious friendship make my life colorful and alive, being with them is a great memory of my life. My Special thanks go to Yamei Hu, Mingsheng Tian, and their son Eric. No one fully understand how lucky I am to meet this happy family. They offered me so much help from the first day we know each other, and never stop no matter when and where.

I reserve my most profound thanks to my family. 老爸老妈，感谢你们用那无止尽的爱为我撑起一片天空，给我勇气去面对挑战。无论悲伤还是快乐总有老妈和我分享，无论我面临怎样的窘境总有老爸为我引导方向，没有你们的鼓励，信任和爱护我的人生将不会如此绚丽多彩！做为女儿，即使穷尽一生也不可能报答你们为我所付出的一切。My greatest thank is due to Xu, my husband, and Tommy, my son. They are the persons who sacrificed the most during my PhD study. Their constant love and support enabled me to complete this work. They are the source of my happiness.

Samenvatting (Dutch Summary)

Herpetische stromale keratitis (HSK), met name veroorzaakt door herpes simplex virus type 1 (HSV-1), is de meest voorkomende oorzaak van non-traumatische cornea blindheid in de wereld. In tegenstelling tot herpetische epitheliale keratitis (HEK), waarbij de cornea epitheel lesies veroorzaakt worden door het cytopathologisch effect van het virus, wordt de pathologie van de chronische hoornvliesontsteking HSK veroorzaakt door infiltrerende onstekings-cellen in het stroma en de sub-epitheliale cellaag van de cornea. Herpetische keratitis (HK) is een complexe ziekte, waarbij zowel het virus als de gastheer bepalend zijn voor het verloop van de ziekte.

Het ontstekingshormoon Granulocyt Macrofaag Kolonie Stimulerende Factor (GM-CSF) speelt een belangrijke rol bij chronische ontstekingsprocessen zoals reuma en astma. In **hoofdstuk 2** is de rol van GM-CSF in de immunopathogenese van HSK onderzocht. De studie laat zien dat menselijke hoornvlies epitheel cellen en fibroblasten GM-CSF uitscheiden na stimulatie met zowel macrofaag- als T lymphocyt-specifieke ontstekings-hormonen (o.a. tumor necrosis factor alfa, interleukine 1 en 17). GM-CSF zorgt dat granulocyten langer blijven leven en dat ze geactiveerd worden, waardoor ze HSV-1-geïnfecteerde hoornvlies fibroblasten kunnen doden. Blokkering van GM-CSF in cornea's van HSK patiënten dient te worden overwogen om de prognose van HSK patiënten in de toekomst te verbeteren.

Voorgaande studies in muizen hebben aangetoond dat de mate van HK afhankelijk is van de virus stam waarmee de muis geïnfecteerd wordt. In **hoofdstuk 3** is een groot aantal hoornvlies HSV-1 kweken van HK patiënten onderzocht om te bepalen wat de prevalentie is van de drie bekende HSV-1 glycoprotein G (gG) en gI varianten en of een variant geassocieerd is met het HK ziektebeeld. De HSV-1 variant B kwam het meeste voor. Er werden geen klinisch relevante associaties gevonden tussen een HSV-1 variant en de ziekteverschijnselen in de bestudeerde HK patiënten. De resultaten duiden dat gG of gI varianten niet predisponeren voor een veranderd klinisch beeld van HK.

Hoofdstuk 4 beschrijft de prevalentie en klinische conse-quenties van humane alfa-herpesvirussen in hoornvliesweefsels van hoornvlies donoren en patiënten die een hoornvlies transplantatie (HT) hebben ondergaan. HSV-1 was het meest voortkomende herpesvirus en de hoeveelheid HSV-1 DNA in hoornvlies biopten van getransplanteerde HK patiënten correleerde met de leeftijd van de patiënt, de ziekte-vrije periode, hoornvlies neovascularisatie, pre-HT steroïden behandeling en de ernst van de ziekte. Deze bevindingen pleiten voor de implementatie van real-time HSV-1 PCR op hoornvlies biopten van HK patiënten om de post-HT diagnose en behandeling te verbeteren.

De introductie van acyclovir (ACV) als anti-HSV-1 medicijn heeft de prognose van HK patiënten drastisch verbeterd. Echter, het veelvuldig gebruik van ACV kan leiden tot ACV resistentie (ACV^R), met ernstige gevolgen voor de behandelde patiënt. In **hoofdstuk 5** zijn de prevalentie en genetische eigenschappen van ACV^R HSV-1 hoornvlies kweken in een groot cohort van HK patiënten onderzocht. De resultaten laten een onverwacht hoog percentage aan ACV^R zien: 11 van de 173 onderzochte HSV-1 hoornvlies kweken waren ACV^R. Het merendeel van deze patiënten reageerde niet op ACV therapie. Dit benadrukt het nut van het monitoren van de ACV gevoeligheid van HSV-1 hoornvlies kweken wanneer een klinische ACV^R vermoed wordt. Wanneer ACV^R HSV-1 gevonden wordt, kan foscarnet of cidovovir behandeling gestart worden om de HSV-1-geïnduceerde hoornvlies infectie te controleren.

Hoofdstuk 6 beschrijft het onderzoek aan de incidentie en klinische relevantie van ACV^R in patiënten met recidiverende HK (rHK). Hiervoor werden 38 sequentiële HSV-1 hoorvlies kweken van 15 rHK patiënten in detail bestudeerd. Hoornvlies kweken blijken te bestaan uit zowel ACV sensitieve (ACV^S) als ACV^R varianten van hetzelfde virus. In patiënten die ongevoelig zijn voor ACV therapie werden hoofdzakelijk ACV^R virussen geïsoleerd. De herhaaldelijke isolatie van ACV^R HSV-1 uit hoornvliezen van rHK patiënten geeft aan dat deze ACV^R virussen latent aanwezig kunnen blijven en op een later tijdstip aanleiding kunnen geven tot een klinisch ACV^R recidief. Bij patiënten bekend met een ACV resistente HK wordt aangeraden de ACV gevoeligheid van HSV-1 hoorvlies kweken bij elk recidief zo snel mogelijk te bepalen.

中文摘要 (Chinese Summary)

单纯疱疹病毒（HSV-1）引起的角膜感染称为单纯疱疹病毒性角膜炎(HK)，它的转归取决于 3 种相互作用的因素，即宿主固有免疫的基因组成、适应性免疫、病毒的种属。HSV-1 感染性角膜上皮炎（HEK）主要由病毒感染所造成的细胞坏死、溶解、脱落所致，大多数患者可在短时间内自行消退。而 HSV-1 感染引起的免疫性角膜基质炎（HSK）则是一种由病毒感染所诱导的免疫性疾病，其发病率、致盲率均居各种非创伤性眼科疾病之首，是当今世界上危害最严重的感染性眼病之一。

由于人粒细胞巨噬细胞集落刺激因子（GM-CSF）在慢性炎症性疾病（如类风湿，哮喘）的致病机理中占有重要位置，在本论文的第二章中我们研究并讨论了其在 HSK 的免疫病理学中所起到的作用。人眼角膜细胞可以在 T 细胞和巨噬细胞所分泌的细胞因子的刺激下产生大量 GM-CSF，可以延长多形核白细胞的寿命并使之得到活化。本研究证明，局部减弱或消除 GM-CSF 的作用将会给 HSK 病人的治疗带来积极的作用。

先前的小鼠研究显示，用不同的 HSV-1 病毒株感染小鼠可以诱发不同程度 HK。本论文的第 3 章对于从 HK 患者眼角膜所提取的 HSV-1 样本进行了基因型分析，以找到不同的 gG 和 gI 基因型与不同 HK 临床表征之间的关系。178 个样本的数据显示，HSV-1 的 gG 和 gI 基因型与 HK 的临床表征之间并无显著关联。

在第四章中，我们对在穿透性角膜移植术（PKP）中被移除角膜的 α 疱疹病毒检出率及病人术后的临床表征进行了分析。在 HSV-1，HSV-2 和 VZV 三种 α 疱疹病毒中，HSV-1 的检出率最高。HSV-1 在被移除角膜中的滴度与角膜的无复发生存期，角膜新生血管的形成，以及 HK 的临床表征等紧密相关。这些数据显示，通过实时聚合酶链式反应检测 HK 病人被移除角膜的 HSV-1 滴度将会提高 HK 病人 PKP 后的预后和治疗质量。

阿昔洛韦（ACV）药物的发现在很大程度上提高了 HSV-1 病毒感染所致眼科疾病的治疗效果和预后，但它的广泛应用也不得不面对耐药 HSV-1 病毒株的出现。在第五章中，通过对 173 个 HK 病人样品的耐药性及基因型的特征分析，我们发现 ACV 耐药性的检出率在 HK 病人中是相当高的。因此，长期对那些患有难治性 HK 病人的病毒样本进行耐药性检测是十分必要的。耐药病毒的检测对于病人治疗方法的及时调整将起到至关重要的作用。在第六章中，我们进一步对复发性 HK 病人的连续性样本的耐药性和病人的临床表征进行了分析。通过基因型分析发现，每一个 HK 病人的 HSV-1 样品都是很多病毒株的混合体，它们大都起源于同一祖先，却拥有不同突变的 TK 基因。这个

混合体中也含有耐药病毒株，而整个样本的耐药性则取决于耐药病毒株在此混合体中所占的比例。耐药病毒存同样在于病人的复发样本中，说明这些病毒可以在宿主中建立它们的潜伏状态并复发。这一事实说明，对复发性 HK 病人的病毒样本进行耐药性检测也是同样重要的。

本论文中对 HK 的免疫学致病机理的一些分析，及耐药病毒株的检测手段和分析结果都经对 HK 的诊断及更有效治疗提供了有力的依据。

ABBREVIATIONS

7AAD	7-amino actinomycin D
ACV	Acyclovir
ADCC	Antibody dependent cytotoxicity
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CI	confidence interval
CM	Conditioned medium
CMV	Cytomegalovirus
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxy ribonucleic acid
DNA pol	DNA polymerase
dpi	days post infection
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopic
FACS	Flow cytometer
FBS	Fetal bovine serum
FOS	Foscarnet
gB	Glycoproteins B
GCV	Ganciclovir
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HCE	Human corneal epithelial cell
HCF	Human corneal fibroblast
HE	Hematoxylin and Eosin
HEK	Herpes epithelial keratitis
HHV	Human herpes virus
HK	Herpetic keratitis
HLA	Histocompatibility Leukocyte Antigen
HMBS	*Homo sapiens* hydroxyl-methyl-bilane synthase
HPMPC	Cidofovir
HR	hazard ratio
HSV	Herpes simplex virus
HSK	Herpes stromal keratitis
IEK	Infectious epithelial keratitis
Ig	Immunoglobuline
IHC	Immunohistochemistry
IL	Interleukin
INF	Interferon
ISK	Immune stromal keratitis
kb	kilo-base pairs
LAT	Latency-associated transcript
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NK cell	Nature killer cell
NSK	Necrotizing stromal keratitis
ORF	Open reading frame
PCR	Polymerase chain reaction
PCV	Panciclovir
PMN	Polymorphonuclear cells
PKP	Penetrating keratoplasty
RE	Restriction enzyme
RFI	Recurrence free interval
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
RNAi	RNA interference
SEM	Standard error mean
SFM	Defined keratinocyte serum-free
TGF	Transforming growth factor
TK	Thymidine Kinase
TNF	Tumor necrosis factors
US	Unique short sequence
UL	Unique long sequence
UV	Ultraviolet
VZV	Varicella zoster virus

Curriculum vitae

The author of this thesis was born on August 18th, 1977 in Beijing, China. She obtained her Bachelor of Science degree from China Agricultural University, Beijing, in 1999. In 2000, she studied as a master student of botany at the same university for one year. From 2002, she started the master program of Biotechnology at Wageningen University in the Netherlands, and obtained her master degree in 2004. In the same year she started her Ph.D research at the Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands under the supervision of Prof.dr. A.D.M.E Osterhaus and dr. G.M.G.M Verjans.

Publications

Duan R, de Vries RD, van Dun JM, van Loenen F, Osterhaus AD, Remeijer L, Verjans GM.. Acyclovir Sensitivity and Genetic Characteristics of Sequential HSV-1 Corneal Isolates in Patients with Recurrent Herpetic Keratitis. *Submitted to Journal of Infectious Disease.*

Remeijer L, **Duan R**, van Dun JM, Bettink MA, Osterhaus AD, Verjans GM. Prevalence and Clinical Consequences of Herpes Simplex Virus Type 1 DNA in Human Cornea Tissues. *Journal of Infectious Disease. Accepted.*

Duan R, de Vries RD, Osterhaus AD, Remeijer L, Verjans GM. Acyclovir-resistant corneal HSV-1 isolates from patients with herpetic keratitis. *J InfectDis. 2008 Sep 1;198(5):659-63.*

Duan R, van Dun JM, Remeijer L, Siemerink M, Mulder PG, Norberg P, Osterhaus AD, Verjans GM. Prevalence of herpes simplex virus type 1 glycoprotein G (gG) and gI genotypes in patients with herpetic keratitis. *Br J Ophthalmol. 2008 Sep;92(9):1195-200.*

Duan R, Remeijer L, van Dun JM, Osterhaus AD, Verjans GM. Granulocyte Macrophage Colony Stimulating Factor Expression in Human Herpetic Stromal Keratitis (HSK): Implications for the Role of Neutrophils in HSK. *Investigative Ophthalmology & Visual Science 2007 Jan;48(1):277-84.*

Wang ZB, Li WJ, **Duan R**, Le Y. Expression of a novel rice PHGPX gene in E.coli and it's antioxidative activity. *Federation of Asian and Oceanic Biochemistry and Molecular Biologists 15th symposium, Beijing, 2000.*

Patent

Liu JY, Li WJ, Zhao NM, **Duan R**, Fan JH. Rice PHGPx gene，protein and its application. *China Patent, Patent No.: 00109313.4*

PhD Portfolio Summary

Rui Duan
Department of virology
Research School: Post-graduate School Molecular Medicine
PhD period: 2004-2009
Promotor: Prof. Dr. A.D.M.E Osterhaus
Co-promotors: G.M.G.M Verjans
L. Remeijer

In-depth courses

2008	Course on Basic and translational Oncology. One week course in General Oncology provided by the Post-graduate School Molecular Medicine, Erasmus MC and the research school MGC, Leiden.
2007	Symposium Post-infectious Diseases. Two days symposium in post-infectious disease organized by the Post-graduate School Molecular Medicine, Erasmus MC.
2006~2007	Course in English Biomedical Writing and Communication. Five month course provided by the Post-graduate School Molecular Medicine, Erasmus MC.
2005	Course on Laboratory Animal Science. Two weeks training course in laboratory animal science (Article 9 Certificate) provided by Utrecht University.
2004	Course in virology. One-week international training course in General Virology provided by the Post-graduate School Molecular Medicine and the Department of virology, Erasmus MC.
2004	Course in immunology. Two-week international post-doctoral training course in immunology, provided by the Leiden Institute for Immunology.
2004~present	International seminar series in Virology, Immunology, Cell biology, and Molecular Medicine, provided by the Post-graduate School Molecular Medicine and the Department of Virology, Erasmus MC
2004~present	Internal and external presentations at the department of Virology twice a week.

Propositions

Accompanying the thesis:

"**Herpetic Keratitis in Humans: Interaction between Virus and Host**"

1. GM-CSF secreted by corneal resident cells prolongs the survival and modulates the effector functions of neutrophils (this thesis).

2. Performing HSV-1 specific real-time PCR on cornea explants is pivotal for the follow-up of transplanted HK patients (this thesis).

3. The clinical outcome of HK is independent of HSV-1 US4 and US7 genotypes (this thesis).

4. Corneal HSV-1 isolates from HK patients consist of multiple TK mutants from the same virus strain (this thesis).

5. ACV-resistant HSV-1 strains establish latency and reactivate to cause HK refractory to ACV treatment (this thesis).

6. Studies on the immune control of HSV-1 and VZV latency in humans are important to prevent recurrent herpetic disease. (Verjans, GMGM. et al 2007)

7. Vaccines against herpesviruses will not induce sterile immunity.

8. A study on 100 BALB/c mice equals one human case report.

9. While waiting for the next influenza pandemic, HSV-1 is responsible for the simmering pandemic of oral and ocular herpes that has plagued humanity for millennia.

10. Mycoplasma infection in tissue cultures is an underestimated problem.

11. To raise a child is harder than to obtain a PhD.