Published in final edited form as:

Graefes Arch Clin Exp Ophthalmol. 2015 October ; 253(10): 1721–1728. doi:10.1007/s00417-015-3073-4.

The Use of Human Cornea Organotypic Cultures to Study Herpes Simplex Virus Type 1 (HSV-1) Induced Inflammation

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Abstract

Purpose—To determine the utility of human organotypic cornea cultures as a model to study HSV-1-induced inflammation and neovascularization.

Methods—Human organotypic cornea cultures were established after retrieving the cornea with intact limbus from donated whole globes. One cornea culture was infected with HSV-1 (10⁴ plaque forming units) while the other cornea from the same donor was mock-infected. Supernatants were collected at times post culture with and without infection to determine viral titer (by plaque assay) and pro-angiogenic and pro-inflammatory cytokine concentration by suspension array analysis. In some experiments, the cultured corneas were collected and evaluated for HSV-1 antigen by immunohistochemical means. Another set of experiments measured susceptibility of human 3-dimensional cornea fibroblast constructs in the presence and absence of TGF-β1 to HSV-1 infection in terms of viral replication and the inflammatory response to infection as a comparison to the organotypic cornea cultures.

Results—Organotypic cornea cultures and 3-dimensional fibroblast constructs were susceptible to HSV-1 with varying degrees. Fibroblast constructs were more susceptible to infection in terms of infectious virus recovered in a shorter period of time. There were changes in the levels of select pro-angiogenic or pro-inflammatory cytokines that were dictated as much by the cultures producing them as whether they were infected with HSV-1 or treated with TGF-β1.

Conclusion—The organotypic cornea and 3-dimensional fibroblast cultures are likely useful in the identification and short-term study of novel anti-viral compounds and virus replication but are limited in the study of the local immune response to infection.

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Conflicts of Interest

The authors have no conflict of interest to declare.

Keywords

cornea; HSV-1; cytokines; pro-angiogenic factors; inflammation

Introduction

Herpes simplex virus type one (HSV-1) is one of eight human herpes viruses in the family herpesviridae; a family of pathogenic, enveloped, and double-stranded DNA viruses. HSV-1 is among the most well-known, invoking the commonly observed image of a lesion on the vermillion border of the labia. HSV-1 has a population seroprevalence rate of 65.3% by midlife, indicating a highly prolific and efficient pathogenic behavior[1]. However, HSV-1 is not limited to its relatively harmless labial location and strays into other bodily systems with far more potential for lasting damage; the most serious being HSV-1 encephalitis and HSV-1 stromal keratitis (HSK).

HSK is among the most prevalent and damaging ocular infections, and is the leading cause of blindness in the developed world with the reported prevalence of ocular herpetic manifestations of 11.8 per 100,000 [2,3]. The virus is a major cause of primary graft failure following cornea transplantation as well as the original cause for up to 10% of corneal transplants to be performed [4,5]. Of additional note, viral transmission from donor to host during corneal transplant has been reported, further highlighting the importance and risks of HSV-1 in ophthalmic patient care [6].

HSV-1 infection is primarily spread through saliva and direct contact, with primary infections often occurring as subclinical. The virus is thought to travel via retrograde transport using the lingual branch of the trigeminal nerve and subsequently establish a latent infection in the trigeminal ganglion [7]. The virus will then periodically reactivate in response to environmental cues (e.g., stress), normally producing “cold” sores along the vermillion border. However, upon reactivation, the virus may travel in an anterograde fashion down the ophthalmic branch of the trigeminal nerve and replicate locally within the innervated cornea epithelium and stroma. Episodic reactivation of the virus yields tissue pathology as a direct result of lytic virus infection, the host immune response to the virus, or a combination of both. Poorly managed patients or individuals that experience frequent reactivation of latent virus develop HSK and likely require corneal transplantation.

While HSK is a significant clinical disease, there are relatively few human models in which to study the pathophysiology and resulting natural course of infection. Mouse models are common but they do not allow for the study of uniquely human patterns of pathophysiology. The purpose of this study was to evaluate which cytokines and pro-angiogenic factors are produced by resident corneal cells in response to HSV-1 infection. To this end, we reproduced the ex-vivo organotypic culture method presented by Alekseev et al. as a model to study the expression of various factors of interest as possible biomarkers produced in the cornea upon infection with HSV-1 [8]. This method allows for responses that are intrinsic to the cornea and independent of the larger circulatory and immune system. In addition, HSV-1 infection elicits lymphangiogenesis via the up-regulation of angiogenic growth factors including VEGF-A [9]. Thus, this model provides a means to measure the expression of pro-

inflammatory and pro-angiogenic cytokines in a human system, and more specifically, describe the intrinsic corneal expression of pro-inflammatory and pro-angiogenic cytokines in response to HSV-1 infection. In a similar manner, the use of 3-dimensional fibroblast constructs [10,11], confirmed susceptibility to HSV-1 infection, as well as inflammatory and pro-angiogenic responses to infection.

Materials and Methods

Cornea Organotypic Culture System

Seven sets of whole globes were received from Minneapolis Lyons Eye Bank (Minneapolis, MN). Only samples ruled out for corneal conditions that could interfere with the study were used. As depicted by the provider, the primary cause of death (PCD) and eye history (EH) for each donor were as follows: 1) PCD: pneumonia; EH: none, 2) PCD: pulmonary embolism; EH: none, 3) PCD: cancer; EH: none, 4) PCD: acute cardiac event; EH: macular degeneration, 5) PCD: cardiovascular arrest; EH: glaucoma, cataracts, 6) PCD: leukemia/lymphoma ; EH: none, 7) PCD: sepsis; EH: glaucoma. Upon receipt, cornea sclera buttons were isolated by cutting a 0.5 cm scleral margin around the corneal button and placed into culture media as previously described [8]. Cornea pairs were divided into one control, one experimental per pair. Corneas were then placed with epithelial side down and warmed 1% agarose solution in culture media was added to the endothelial aspect for support. Once the agarose solidified, the corneas with supporting agarose scaffold were placed in separate cornea culture dishes with the epithelium side up, covered with 6 ml of culture media consisting of minimal essential medium (MEM) supplemented with nonessential amino acids (1X), L glutamine (2mM), penicillin (200 U/ml), and streptomycin (200 μ M) [8], and placed in the incubator at 37° C and 5% CO₂ atmosphere for 15 minutes. Next, the culture media was removed, and the corneas were maintained in the plates with epithelial side down. One cornea was then infected by adding 1.0 ml of culture media containing 10⁴ plaque forming units (pfu) of HSV-1 (strain McKrae) in culture media. The matching cornea served as the mock-infected control receiving 1.0 ml of culture media. Both corneas were then incubated at 37°C and 5% CO₂ for one hour, with gentle rocking every 15 minutes. One hour post infection (pi), both corneas were rinsed three times with sterile phosphate buffered saline (PBS). After rinsing, the corneas were returned to the epithelial side up position, 6 ml of culture media was added, and placed in incubator at 37°C and 5% CO₂. After this step, cornea tissue and culture supernatant were harvested at 1 day, 2 day, 4 day, 6 day, and 8 day time points. Media was replaced with fresh media at 1, 2, 4, and 6 days pi. Harvested tissue was cut into four pieces and placed into -80°C freezer for storage or used on the day harvested. Harvested culture supernatant was placed into two sterile conical tubes and spun down (300 × g, 5 minutes) with cell-free supernatant placed into two additional 15 ml sterile conical tubes before being placed in storage (-80°C freezer) for future analysis. This study was exempt from Institutional Review Board approval.

Isolation and culture of Human corneal fibroblasts (HCFs)

Corneas of healthy donors without any ocular disease were used for the study. Briefly, corneas were processed as per the previous optimized protocol [10,12] by scraping of the epithelium and endothelium. The stromal tissues were cut into small pieces of approximate

size 2×2 mm and placed into T25 culture flasks and allowed to adhere at 37° C for about 30 minutes. The explants were then supplied with Eagle's Minimum Essential Medium (ATCC, Manassas, VA) containing 10% fetal bovine serum (Atlantic Biological's; Lawrenceville, CA) and 1% Antibiotic (Life technologies, Grand Island, NY) as culturing medium and were allowed 1-2 weeks of cultivation. The cultures were then passed upon 80-100% confluence and further used in our culture system.

Assembly of 3D constructs for HCFs

Human corneal fibroblast cells (HCFs) were used to set up 3D constructs as per our previously optimized protocol [11,12]. 1×10^6 cells/well were plated on polycarbonate membrane inserts with 0.4 μ m pores (Corning Costar; Charlotte, NC) and were cultured in Eagle's Minimum Essential Medium containing 10% fetal bovine serum and 1% Antibiotic and stimulated with 0.5mM 2-O- α -D-Glucopyranosyl-L-Ascorbic Acid (American Custom Chemicals Corporation, San Diego, CA). Two conditions were tested here: a) Controls (C) - cultures were grown in EMEM containing 10% FBS and 1% antibiotic/antimycotic culture media supplied with vitamin C, and b) TGF β 1 (T1) - cultures were grown for 4 weeks in regular culture media supplied with vitamin C and T1 (0.1 ng/ml). Fresh media was supplied every other day during the entire study time point.

Viral Titer Plaque Assay

Viral titers were assessed by plaque assay of supernatants from organotypic cornea cultures at 1, 2, 4, 6, and 8 days pi and from human corneal fibroblast constructs at 1 and 3 days pi. Plaque assays were conducted as previously described [13].

Immunohistochemical staining

Following 1 and 8 days of culture, corneas were harvested and portions of the tissue processed for immunohistochemical staining. Briefly, non-fixed tissue was embedded in OCT media, frozen in liquid nitrogen, and cut into sagittal section (20 μ m thick) using a cryostat. Frozen sections were placed on slides, permeated with 1% Triton X-100 in 1X PBS, and blocked 1 hour with 10% donkey serum (Jackson Immuno Labs, West Grove, PA) in 1X PBS, followed by incubation with polyclonal rabbit anti-HSV1 antibody diluted 1:100 (Dako, Carpinteria, CA) in blocking solution. Following three washes using 1X PBS, the tissue was incubated with FITC-conjugated donkey anti-rabbit antibody (Jackson Immuno Labs) at a 1:100 dilution for 1 hour. Nuclei were counterstained with the dye DAPI, and following washes with 1X PBS, samples were mounted with 50% glycerol/1X PBS, and analyzed using Olympus confocal laser scanning microscope (FV500, version 5.0, Olympus, Center Valley, PA).

Suspension array

Pro-inflammatory cytokine and pro-angiogenic growth factor levels in the supernatants of organotypic cornea cultures and fibroblast constructs infected with or without HSV-1 were assessed using two separate Milliplex Map Kits (Millipore, Billerica, MA). The human cytokine/chemokine magnetic bead panel analyzed levels of IL-1 β , IL-6, IL-8, IL-17, TNF- α , CCL2, and CXCL10 (HCYTOMAG-60K). The second examined human angiogenesis/

growth factors via magnetic bead panel (HAGP1MAG-12K), which detected angiopoietin, fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, and hepatocyte growth factor (HGF). Bioplex analysis was run using standard protocol as provided by the instructional booklet.

Statistics

Statistical analysis was performed using the GBSTAT program (Dynamic Microsystems, Silver Springs, MD). ANOVA and Tukey's post hoc T-test were performed comparing uninfected and infected culture systems for analyte concentrations.

Results

Susceptibility of organotypic cornea cultures to HSV-1

Initially, we evaluated the time course of virus replication in cultures following infection with HSV-1. In a time-dependent manner, we could detect virus within the supernatant of organotypic cultures peaking at day 6 pi (**Fig. 1A**). Contrary to what was predicted, there was no detectable virus found in the supernatant 24 hours pi. To further investigate the topography of HSV-1 infection in the organotypic cornea cultures, corneas were removed from culture at day 1 or day 8 pi and analyzed for viral antigen expression by immunohistochemical means. Uninfected corneas served as controls. Whereas there was no HSV-1 antigen detected in the uninfected cornea (**Fig. 1B**) or in infected corneas following 24 hours in culture (data not shown), there was detectable antigen expression throughout the cornea tissue with pronounced expression in the epithelial and endothelial layers at day 8 pi (**Fig. 1B**). The epithelial layers were not consistently intact but loosely affiliated with the remainder of the cornea by day 8 pi. By comparison, there was punctate staining of keratocytes in the intact stroma at day 8 pi. These results suggest there is a slight delay in virus replication in the organotypic cultures but all layers are eventually infected and susceptible to virus replication.

CXCL10 expression parallels HSV-1 infection in organotypic cornea cultures

HSV-1 is known to elicit pro-inflammatory and pro-angiogenic factors following infection of the mouse cornea [14–19]. Therefore, we determined the profile of pro-angiogenic and pro-inflammatory cytokine levels in the supernatants over time following infection. Except for IL-17 (data not shown), with levels below the limit of detection, all cytokines surveyed were detectable within the first 24 hours post culture. CCL2, FGF2, and IL-6 levels did not differ throughout the culture period whether or not cultures were infected with virus (**Fig. 2**). Whereas VEGF D levels were expressed equally in infected and uninfected culture supernatants at day 1 post culture, the analyte was undetectable at later time points in these cultures (**Fig. 2**). In a similar fashion, IL-1 β expression peaked in infected and uninfected cultures at similar levels at day 2 post culture and then precipitously declined (**Fig. 2**). Angpt2 and TNF- α levels peaked in the infected cultures at day 4 post culture with a modest but significant increase of TNF- α content in the infected organotypic cornea culture supernatant compared to the uninfected group at this time point (**Fig. 2**). By comparison, Angpt2 levels in the infected organotypic cornea culture supernatant significantly dropped in comparison to the uninfected group by day 8 post culture (**Fig. 2**). In the uninfected cultures,

Angpt2 and TNF- α levels were maintained from day 2 through day 8 post culture suggesting the virus does impact these two factors at different time points. Similar to IL-8, VEGF A levels were maintained from day 2 through day 8 post culture in the supernatant from infected and uninfected cultures (**Fig. 2**). Two analytes were found to be somewhat unique in comparison to the other factors measured. VEGF-C and CXCL10 levels progressively rose over time through day 8 post culture (**Fig. 2**). Whereas there was no significant difference in the level of VEGF C in the supernatants of organotypic cultures infected with or without HSV-1, CXCL10 levels steadily rose only in the HSV-1-infected tissue becoming significantly elevated to the uninfected group by day 8 post culture. Collectively, CXCL10 is the only soluble factor measured in the cornea organotypic culture model that is selectively induced by HSV-1 infection in a time-dependent manner consistent with the notion it is a useful biomarker to mark cornea HSV-1 infection consistent to what has been reported in the mouse cornea [14].

TGF- β 1 exposure enhances HSV-1 replication in 3-dimensional corneal keratocyte cultures

Next, we wished to compare the human cornea organotypic culture susceptibility to that of 3-dimensional primary cornea keratocyte cultures grown in the presence or absence of TGF- β 1 to HSV-1. In comparison to the delay in infectious virus detected in the supernatants of the organotypic cultures, HSV-1 was readily detectable in the culture supernatants from the primary keratocyte 3-dimensional cultures at day 1 pi (**Fig. 3**). Corneal keratocytes grown in the presence of TGF- β 1, a cytokine associated with corneal wound healing that drives cornea keratocyte differentiation into myofibroblasts [20], were found to be more susceptible to HSV-1 infection compared to mock-treated keratocytes (**Fig. 3**). In contrast to the corneal organotypic cultures, the 3-dimensional keratocyte culture HSV-1 titers peaked much earlier at day 3 pi suggesting these cultures to be more susceptible to HSV-1 infection (**Fig. 3**).

HSV-1 and TGF- β 1 independently alter pro-angiogenic and pro-inflammatory cytokine production in 3-dimensional corneal keratocyte cultures

Similar to what was measured in the cornea organotypic cultures, we determined the profile of pro-angiogenic and pro-inflammatory cytokine levels in the supernatants following infection. In the absence of infection, TGF- β 1 was found to significantly augment CXCL10 and IL-6 levels but reduce HGF levels in the keratocyte cultures (**Fig. 4**). In the mock-treated cultures, HSV-1 was found to significantly reduce FGF2 levels in the keratocyte cultures (**Fig. 4**). All other pro-angiogenic and pro-inflammatory cytokine levels were similar comparing infected to uninfected conditions.

Discussion

In the present study, the cornea organotypic culture system was evaluated for its use to assess HSV-1 susceptibility and as a possible model to study pro-angiogenic conditions leading to hem- or lymph-angiogenesis. We found the organotypic cultures to be susceptible to infection but the release of infectious virions from the tissue was delayed in comparison to a uniform primary 3-dimensional fibroblast culture system. A possible explanation for this differential susceptibility to infection is that while the thick collagenous matrix of the

human stroma acts a diffusion barrier to the virus slowing out its spread between “distant” cells in the in vivo and explanted human cornea, the infection of fibroblasts in the human cell culture can be easily propagated due to easier access for viral spread and abundance of targets. Furthermore, the production of soluble pro-inflammatory and pro-angiogenic growth factors was, for the most part, unremarkable comparing infected to uninfected cultures in the cornea organotypic cultures. Specifically, most of the analytes measured had similar levels comparing infected to uninfected cultures at all the time points surveyed. CXCL10 was the only soluble factor that showed a distinct change in the levels following infection as HSV-1 elicited an increased production of CXCL10 over time. These results are consistent with the pro-inflammatory nature of this chemokine in the cornea of HSV-1-infected mice [14,21]. In comparison to CXCL10, Angpt2 and TNF- α levels peaked in the HSV-1-infected cultures at day 4 post infection with a significant loss of Angpt2 levels at day 8 pi compared to the uninfected group. This loss is specific to Anpt2 as other analytes showed either no change or an increase in levels in HSV-1-infected cultures over time. Anpt2 is reportedly generated by macrophages and lymphatic endothelial cells in the cornea [22]. It is tempting to speculate the loss of Angpt2 levels may be due to the depletion of macrophages and/or lymphatic endothelium of which the latter is specifically targeted in the presence of high viral content [23] and the former has been found in the anterior stroma of human corneas [24]. At no time was IL-17 detected which suggests that the influx of inflammatory cells including neutrophils [25] are a likely source of this pro-inflammatory cytokine in response to HSV-1 infection.

In comparison to the cornea organotypic cultures, the 3-dimensional keratocyte cultures were more susceptible to HSV-1 infection in terms of recoverable virus in the culture supernatant within 24 hr pi and peak virus output by day 3 pi. As a result of the rapid loss of the 3-dimensional keratocyte culture, the measurement of soluble factors in the culture supernatant was conducted only at day 1 pi. HSV-1 infection resulted in the significant loss of FGF2 but no other pro-inflammatory cytokine or pro-angiogenic factor in non-treated cultures. However, in the presence of TGF- β 1, HSV-1 resulted in the significant reduction in CXCL10 expression. A previous study has found an inverse correlation between CXCL10 expression and TGF- β 1 levels [26] as TGF- β 1 suppresses this and other chemokines through the Smad3 pathway [27]. TGF- β 1 was found to significantly augment IL-6 and HGF in uninfected cultures of which these levels were not modified in the presence of HSV-1. TGF- β 1 has previously been reported to stimulate IL-6 production by human fibroblasts [28]. Other reports suggest TGF- β 1 increases or decreases HGF expression likely dependent upon the time of exposure to the cytokine and the tissue under study [29,30]. Consequently, these 3-dimensional keratocyte cultures may provide a useful model to more fully study the molecular mechanisms that transpire in the induction of pro-angiogenic factors and other cytokines in response to TGF- β 1 exposure.

We originally sought to use the cornea organotypic culture system to study those factors and cells responsible for lymphatic vessel genesis in response to HSV-1. However, we were unable to detect any neovascularization in our model which we speculate is due to the level of virus replication which restricted the duration of the study. In the human patient, cornea angiogenesis is evident after many weeks of repeated reactivation of the virus. Therefore,

this culture system will unlikely serve as a model to identify factors and cells that contribute specifically to lymphangiogenesis.

Acknowledgements

The authors would like to thank Akhee Sarkernag, Min Zheng, and Meghan Carr for their technical help in establishing/maintaining cultures and in the analysis of data included in this paper. This work was supported by NIH R01 EY021238-05 (DJJC), NEI R01 EY023568-02 (DK), an unrestricted grant from Research to Prevent Blindness, and NEI core grant EY021725.

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Figure 1. HSV-1 replicates in human cornea organotypic cultures

(A) Summary of organotypic culture method used in this study. The cornea sclera buttons were isolated from seven sets of human globes. Upon placing in culture media, the explants were mock infected or infected with 10^4 PFU/culture HSV-1 and maintained for up to eight days in culture. (B) At 1, 2, 4, 6, and 8 days, samples of culture media were assayed for infectious virus by plaque assay. Scatter plots represent the distribution of individual measurements and depict the mean log PFU/ml HSV-1 $\pm$ SEM. (C) Cornea organotypic cultures infected with or without HSV-1 were collected at 8 days pi. The tissue was

sectioned and stained for HSV-1 antigen expression (green). Sections were placed in mounting medium containing DAPI to stain the nucleus of cells (blue) prior to mounting on slides. Images were captured by confocal microscopy. The bar represents X magnification.

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Figure 2. HSV-1 drives the expression of CXCL10 in human cornea organotypic cultures

Fifty microliters of the culture media collected in Fig. 1A was analyzed using a multiplex suspension array platform to measure the level of angiopoietin 2 (AngPT2), fibroblast growth factor (FGF)2, tumor necrosis factor (TNF)- α , vascular endothelial growth factor (VEGF) A, VEGF C, VEGF D, interleukin (IL)-1 β , IL-6, IL-8, CCL2, and CXCL10. Each scatter plots represent the mean $\pm$ SEM, n=3-4/group/time point repeated twice. **p<.01, *p<.05 comparing the infected to the uninfected cultures at the designated time point. Note: At day 1 pi, replicates for IL-8 and IL-6 measurements showed values beyond the detection limit (the total 3 replicates assayed for IL-8, and 2 out of 3 replicates assayed for IL-6). For such reason, day 1 was not taken into account for statistical analysis of IL-6 and IL-8 concentrations in the culture supernatants.

Figure 3. Three dimensional fibroblast constructs are highly sensitive to HSV-1 infection

Human 3-dimensional constructs were generated from donated corneas in the presence or absence of transforming growth factor (TGF)- β 1 for 4 weeks. Culture media was removed and replaced with fresh media containing 10^4 PFU HSV-1. At day 1 and 3 pi, 200 μ l of supernatant was removed and assayed for infectious virus by plaque assay. The bars represent the mean log PFU/ml HSV-1 $\pm$ SEM, n=3-4/group repeated twice. $8p < .05$ comparing the TGF- β 1-treated to mock-treated group.

Figure 4. TGF- β 1 modifies the expression of CXCL10, HGF, and IL-6 in 3-dimensional fibroblast constructs

The remaining supernatant of fibroblast constructs collected for the plaque assay in figure 3 were analyzed for the indicated pro-angiogenic growth factors and pro-inflammatory cytokines using a multiplex suspension array platform. The bars represent the mean concentration $\pm$ SEM/ml for the indicated analyte, $n=2-3$ /group repeated twice. * $p<.05$ comparing the uninfected (UI) treated with or without TGF- β 1 or in the case of FGF2, comparing the UI to HSV-1 infected groups.