Establishment and characterization of an air-liquid canine corneal organ culture model to study acute herpes keratitis

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Structured abstract

1. Abstract

Despite the clinical importance of herpes simplex virus (HSV)-induced ocular disease, the underlying pathophysiology of this disease remains poorly understood, in part due to the lack of adequate virus-natural host models in which to study the cellular and viral factors involved in acute corneal infection. We developed an air-liquid canine corneal organ culture model and evaluated its susceptibility to canine herpesvirus type 1 (CHV-1) in order to study ocular herpes in a physiologically relevant natural host model. Canine corneas were maintained in culture at an air-liquid interface for up to 25 days and no degenerative changes were observed in the corneal epithelium during cultivation using histology for morphometric analyses, TUNEL assays and transmission electron microscopy (TEM). Next, canine corneas were inoculated with CHV-1 for 48 hours and at that time point post infection, viral plaques could be visualized in the corneal epithelium and viral DNA copies were detected in both the infected corneas as well as in culture supernatants. In addition, we found that canine corneas produced proinflammatory cytokines in response to CHV-1 infection similarly to what has been described for HSV-1. This emphasizes the value of our model as a virus-natural host model to study ocular herpesvirus infections.

2. Importance

This study is the first to describe the establishment of an air-liquid canine corneal organ culture model as a useful model to study ocular herpesvirus infections. Advantages of this physiologically relevant model include that it (i) provides a system in which ocular...
herpes can be studied in a virus-natural host setting and (ii) reduces the number of experimental animals needed. In addition, this long-term explant culture model may facilitate research in other fields also, where non-infectious and infectious ocular diseases of dogs and men are being studied.
Introduction

Alphaherpesvirus infection is an important cause of disease in humans and many animal species. Characteristics of the members of this Herpesviridae subfamily include short replication cycles, induction of lifelong latency and a narrow host range (1). Infection occurs via mucosal surfaces of the respiratory and genital tracts or via epithelial surfaces such as the cornea. In humans, a primary ocular infection with herpes simplex virus type 1 (HSV-1) typically results in conjunctivitis, which can then advance to keratitis. A recrudescent keratitis occurs when HSV-1 reactivates from trigeminal ganglia, or possible other sites, and it is a leading infectious cause of visual impairment and blindness in humans (2, 3). Likewise, it is increasingly recognized that alphaherpesviruses such as canine herpesvirus type 1 (CHV-1) and feline herpesvirus type 1 (FHV-1) are an important cause of ocular disease in small companion animals. Based on the strong similarities between HSV and CHV-1/FHV-1 regarding ocular pathogenesis and the lesions induced during primary and recurrent infections, dogs and cats represent useful natural host models for studies on pathogenic mechanisms and viral factors involved in alphaherpesvirus infection in the eye (4, 5). Unfortunately, and despite the clinical importance of herpesvirus-induced ocular disease, the underlying pathophysiology of this disease remains poorly understood, in part because of the lack of adequate in vitro models in which to study the cellular and viral factors involved in acute corneal infection.

In general, knowledge of the pathogenesis of ocular herpes is limited to HSV-1 and, to a lesser extent, HSV-2 (6) and corneal HSV infection is traditionally studied in two types of experimental models. The first is the in vitro model in which cultured monolayers of
corneal epithelial cells are infected with HSV (7). This system offers simplicity, a high level of reproducibility, and requires relatively little time and costs. The other is the in vivo model in which animals such as rabbits or mice are inoculated directly with HSV by corneal scarification (8). This model provides a more physiologically relevant (in vivo) system, but examines disease in non-natural hosts, is costly and time consuming, requires animal experimentation, and has a greater degree of variability. More recently, an organotypic corneal model of acute HSV-1 infection has been reported using rabbit and human corneas (9).

For CHV-1, no in vitro corneal epithelial cell culture systems have been developed to date and only a few in vivo experimental models of primary ocular CHV-1 infections in dogs have been described (10, 11). For FHV-1, a primary corneal cell culture model has been developed, but in general, FHV-1 ocular pathogenesis studies are almost exclusively done in live animals (4, 12). However, the practical use of these animals for in vivo studies is hampered by high animal purchase and maintenance costs as well as ethical questions on the use of dogs and cats as experimental animals. Therefore, there is a critical need for the development of physiologically relevant in vitro models that are inexpensive and minimize the number of experimental animals, as outlined by the 3R concept (13). Organ explant models prove a valuable alternative for in vivo systems, since three-dimensional structures and normal cell-cell contacts are maintained (14). Indeed, these models are useful stepping-stones bridging in vitro and in vivo models because they allow for accurate validation of cell culture results and limit the amount of animal experimentation needed.
Over the years, a variety of human corneal organ culture systems have been established to study corneal wound healing or corneal transplantation and to evaluate ophthalmic drugs (15, 16). More specifically, the use of an air-liquid human corneal organ culture system was shown to be a valuable *in vitro* system for long-term maintenance of epithelial and endothelial integrity. In this model, corneas are placed epithelial side up in a fixed position and culture medium is added to a level that exposes the epithelium intermittently to air-liquid environments while rocking on a tilting platform (17, 18). Despite the successful use of this air-liquid model to study corneal wound healing processes, its use for the study of infectious diseases has not been documented so far.

Therefore, the aim of the present study was to establish and characterize an air-liquid model using canine corneas and to evaluate its usefulness to study acute ocular herpevirus infection in a virus-natural host setting using CHV-1.

**Materials and Methods**

*Collection of canine corneas*

Canine corneas were obtained from research Beagles, euthanized for reasons not related to this study. Ophthalmic examination, including slit-lamp biomicroscopy of the anterior segment, was performed prior to euthanasia and all eyes appeared clinically normal. Both corneas were collected from each dog within 1h after euthanasia, wrapped in a gauze strip, and soaked in sterile phosphate buffered saline (PBS) for transport to the lab.

*Cornea culture medium and Virus*
The cornea culture medium consisted of Dulbecco’s minimal essential medium (DMEM, Corning Cell Grow, Manassas, VA) containing 10% fetal bovine serum (FBS, Atlanta Biological Flowery Branch, GA) and supplemented with 1% non essential amino acids, 1% sodium pyruvate, L-glutamine (300μg/ml), and penicillin (200U/ml)/streptomycin (200μg/ml), all from Life Technologies (Grand Island, NY).

The CHV-1 isolate used in this study was CHV-1 duk, a strain obtained from a dog diagnosed with dendritic ulcerative keratitis (19).

**Air-liquid culture model**

The air-liquid culture model for canine corneas is based on previously described human cornea models, with some modifications (9, 17). Briefly, canine corneas were rinsed thoroughly with PBS and placed epithelial side down in a well of a sterile ceramic spot plate (Avogadro’s Lab Supply). A 1% low melting point agarose (Invitrogen, Grand Island, NY) solution in cornea culture medium was prepared and used to fill the endothelial concavity of the cornea. Upon solidification of the agarose solution, the cornea was placed epithelial side up on a Keflar® ring (Cometic Gasket, Concord, OH), affixed with sterile paraffin to a 100 x 15 mm glass petri dish containing 15-17ml cornea culture medium (Figure 1A). Culture dishes were placed on a rocker platform (Bellco Glass Inc., Vineland, NJ) and rocked at 10 cycles/min in a humidified incubator at 37°C with 5% CO₂ (Figure 1A). Every other day of culture, medium was gently removed and replaced with fresh cornea culture medium.

Canine corneas were collected before culture (day 0) and at days 5, 10, 15, 20 and 25 of culture in the air-liquid system. At the time of collection, corneas were removed from the culture dishes and bisected. One half was fixed with 4% paraformaldehyde (PFA) for
4 hours, followed by dehydration through ethanol and embedded in paraffin for sectioning and histology. The other half was embedded in optimal cutting temperature (OCT, VWR, West Chester PA) and snap frozen at -80°C for cryosectioning and TUNEL and antibody-based immunofluorescence (IF).

**Viral inoculation of the canine corneal organ cultures**

Canine corneas were infected by placing the corneas epithelial surface down in 6-well culture plates (Corning, Grand Island NY) in the presence of 2ml of cornea culture medium containing different concentrations of CHV-1 duk. After 1h of incubation at 37°C with 5% CO₂, medium was removed and corneas were gently rinsed 3 times with PBS to remove any unbound virus. Corneas were then transferred to the air-liquid culture model, exactly as described above, and corneas were incubated at 37°C with 5% CO₂ for 48 hours.

At 48 h post infection (p.i.), culture medium was collected and frozen at -80°C for viral titrations and DNA extraction. In addition, corneas were bisected with one half embedded in OCT for cryosectioning, the other half snap frozen at -80°C for DNA extraction.

**Histology**

Paraffin-embedded corneas were cut into 4 μM sections and mounted on glass slides. Slides were deparaffinized, stained with hematoxylin-eosin (H&E), and changes in epithelial morphology were evaluated using light microscopy. Quantitative analyses of the epithelial cell layer were performed as follows: images of tissue sections were captured with a digital camera mounted on an Olympus BX51 light microscope (Center Valley, PA). Micro Suite Basic Edition software was used to measure and record...
epithelial thickness at 9 places on each of 4 cornea sections collected per time point. Average thickness calculations were based on 36 measurements for each time point. In addition, average numbers of nuclei per $1 \times 10^3 \, \mu m^2$ epithelium were determined by counting nuclei in 3 areas of $1 \times 10^3 \, \mu m^2$ per time point (Figure 1B).

**Immunofluorescence (IF)**

OCT-frozen corneas were cut into 6 $\mu M$ sections, mounted on Superfrost slides (Erie Scientific, Portsmouth NH), fixed with cold acetone for 10 min, air dried and stored at -20°C until analyses. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI, VWR, West Chester, PA) was used at 0.5 $\mu M$ for 5 min incubation at room temperature (RT) to visualize the nuclei. Slides were washed twice with PBS and coverslips were mounted with a glycerol-based medium (Dako, Carpinteria CA). Slides were analyzed using a Zeiss LSM confocal microscope (Oberkochen, Germany) and images were captured with an attached camera controlled by ZEN imaging software.

To analyze epithelial cell viability, the In Situ Cell Death Detection Kit (Fluorescein) (Roche) was used and the Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) reaction was performed as per manufacturer’s instructions. A positive control cornea, incubated with 1mg/ml DNAse I (Sigma-Aldrich, Saint Louis, MO) in 50mM Tris-HCl pH = 7.5, 10mM MgCl$_2$, and 1mg/ml BSA, for 30 min at 37°C, was included.

To analyze CHV-1-infection, slides were incubated FITC-conjugated anti-CHV-1 antibodies (Abs) (VMRD, Pullman, WA) for 1 h at RT. Slides were washed 3 times with FA rinse buffer, consisting of 0.09M Na$_2$CO$_3$, 0.4M NaHCO$_3$, and 0.15M.
of a confirmed CHV-1-infected liver, kind gift from Dr. Dubovi, Cornell Diagnostic
Center, were included as a positive control.

**Immunohistochemistry (IHC)**

Immunohistochemistry (IHC) was performed, as described previously (20). To detect
apoptosis, polyclonal rabbit anti-caspase-3 Abs or rabbit IgG isotype control Abs
(Abcam, Cambridge MA), diluted 1:100 in PBS, were used as primary Abs and
horseradish peroxidase (HRP)-conjugated goat-anti rabbit IgG (Jackson Immunoresearch,
West Grove PA), diluted 1:100 in PBS, was used as a secondary Ab. To detect CHV-1
positive cells, hyperimmune serum from an experimentally infected Beagle (kind gift
from Dr. Leland Carmichael, Baker Institute for Animal Health, Cornell University) was
biotinylated using the EZ-link Sulfo-NHS-LC-LC-biotin kit, according manufacturer’s
instructions (Thermo Scientific). A biotin-conjugated control antibody was also included.
These primary antibodies were used at a final concentration of 1mg/ml in PBS and HRP-
conjugated streptavidin (BD Biosciences, San Jose CA) was diluted 1:100 in PBS. Slides
were fixed to coverslips using Glycergel mounting medium (Dako, Carpinteria CA) and
images were captured with a digital camera mounted on an Olympus BX51 light
microscope. For each staining, cryosections of CHV-1-infected liver were included as a
positive control.

**Transmission electron microscopy (TEM)**

Corneas for transmission electron microscopy (TEM) were fixed and prepared, exactly as
previously described (21) and analyzed on an FEI T12 Twin TEM (FEI, Delmont, PA)
operating at 80kV.
Quantitative polymerase chain reaction (qPCR) and reverse transcriptase PCR (qRT-PCR)

Total cellular DNA was extracted from snap frozen corneas using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). For CHV-1 detection, the following primers targeting glycoprotein E (gE) were used: forward primer, 5’-CACCCTCATGCA AACTCTTG-3’; reverse primer 5’-AGTGATGTTGCCCTCAAAGG-3’ – amplicon length 77 base pairs (bp). To normalize the quantity of input DNA across cornea samples, beta2-microglobulin (B2M) DNA was amplified using the following primers: forward primer, 5’-CCTTGCTCCTCATCCTCCTC-3’; reverse primer 5’-TGGGACACCTGACGTA CGTAGC-3’ – amplicon length 133 bp. To normalize the quantity of input DNA across supernatant samples, samples were spiked with 2 x 10^5 Marek’s disease virus (MDV) copies before DNA extraction, as previously described (22), and MDV gD was amplified using the following primers: forward primer, 5’- TGGGACGACGCAATATGATG-3’; reverse primer 5’- AATGGTTCATTAGTAGA CAGTTGGC-3’ – amplicon length 108 bp (23).

Total RNA was extracted from snap frozen tissues using a Qiashredder column, followed by a RNeasy Plus Micro Kit (all from Qiagen, Valencia CA) and cDNA was synthesized from mRNA using reagents from Invitrogen (Life Technologies, Grand Island NY). Primers used for housekeeping and cytokine cDNA amplification are described in Table I.

For each sample, 30 ng DNA or 10 ng cDNA was added to triplicate wells of a 96 well reaction plate, with SYBR Green Master Mix (Life Technologies, Grand Island NY) and 0.3 μM forward and reverse primers. The qPCR was performed using an ABI 7500
Ct method \(2^{-\Delta Ct}\) was used to quantify gene expression levels where \(\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference})\). The reference consisted of DNA or cDNA from uninfected
corneas, respectively and data were reported as DNA fold change.

Canine gene expression array

Total RNA was extracted from snap frozen tissues using the Qiagen RNeasy mini
plus kit. RNA quality and concentrations were initially quantified using a Nano-Drop
spectrophotometer, and then assayed on a Fragment Analyzer using ProSize software
(Advanced Analytics, Ames, IA). All samples had an RNA quality number (RQN score)
above 8.7. One µg of total RNA from each sample was linearly amplified and Cy3
labeled using the Agilent Low-Input QuickAmp Labeling kit (Agilent, Santa Clara, CA).
Labeled samples were hybridized to an 8x60K Canine Gene Expression Microarray
(Agilent), containing a total of 43,708 probes, and after hybridization, slides were
scanned on the Agilent DNA Microarray Scanner (G2505B) using one color scan. Dye
channel was set to Red & Green and both the Red and Green PMT was set to 100%. The
scanned images were analyzed with Feature Extraction Software (Agilent) to obtain
background subtracted and spatially detrended Processed Signal intensities. Samples
were then further quantile normalized and filtered using GeneSpring GX.

Results

Canine corneas can be maintained in the air-liquid model for at least 3 weeks
Canine corneas were cultured for 25 days in the air-liquid system, as described in the
Materials & Methods section (Figure 1A). Histological analyses of the epithelium
showed uniform cell layers (between 5 and 7 layers) horizontal across the corneal surface and no rounded, sloughed cells were observed over the entire period of cultivation (Figure 2A). Although there was some variation in epithelial thickness over time, particularly at day 25 of culture (Figure 2A), no significant differences in epithelial thickness were noted and the thickness averaged approximately 50 μm at all time points tested (Figure 2B). This is consistent with the average epithelial thickness of human corneas (24). The slightly thicker epithelium noted at day 25 of culture might be explained by the columnar-shaped morphology of the basal epithelial cells, which may be an indication of reduced cell proliferation. With the exception of a drop in number of nuclei after initiating the culture system, no further significant changes were found in nuclei numbers through day 25 of culture (Figure 2B). Transmission electron microscopy (TEM) was used to further evaluate the integrity of the corneal epithelium, and epithelial cells maintained their cell-to-cell contact up to day 25 of culture, as indicated by the presence of intact desmosomes (Figure 3).

The viability of the cultured corneas was also assessed and no TUNEL-positive cells were detected over the period of cultivation (Figure 2C). TUNEL stainings of DNase I-treated corneas were included as positive controls and TUNEL-positive cells were readily detected, indicating our negative results were not due to a technical error (Figure 2C).

Canine corneas in the air-liquid model are susceptible to CHV-1 infection

To evaluate the susceptibility of our air-liquid canine corneal organ culture model to CHV-1 infection, we inoculated canine corneas with different concentrations of the CHV-1 strain CHV-1 duk and analyzed samples at 48h post infection (pi). At this time point, CHV-1 positive epithelial cells (Figure 4A) as well as CHV-1-induced
subepithelial plaques (Figure 4B) were readily observed at all virus concentrations tested, using immunohistochemistry (IHC) and immunofluorescence (IF), respectively. For the corneas infected with $1 \times 10^6$ PFU/ml of CHV-1 duk, 300 consecutive cryosections of 6 \( \mu \)m were analyzed by fluorescence microscopy and it was calculated that approximately $6 \times 10^3$ plaques/cornea were present with an average plaque size of 150 $\mu$m$^2$. However, since this approach is extremely labor intensive, we developed a qPCR assay to detect CHV-1 DNA copies by targeting gE. Using qPCR we were able to detect viral DNA by calculating the fold change in infected corneas compared to the uninfected control corneas (Figure 4C). The observed fold change was dependent on the viral input indicating that our cornea model is suitable to evaluate and compare different levels of infection (Figure 4C).

In addition, we collected culture media from infected corneas and performed qPCR. To normalize the quantity of input DNA across samples, supernatants were spiked with Marek’s disease virus (MDV) copies before DNA extraction and the amount of these DNA copies were detected by targeting MDV gD. This approach was developed previously to detect copies of equine herpesvirus type 1 (EHV-1) in nasal swab samples (22). CHV-1 DNA copies were detected by targeting gE, as described above, and expressed as the fold change compared to uninfected control corneas. In agreement with what we observed in infected cornea tissue, CHV-1 DNA copies could be detected in the collected culture media and the observed fold change was dependent on the viral input (Figure 4C). To assess whether the viral DNA detected in the supernatants was infectious, A72 cells, a canine tumoral fibroblast cell line (ATCC), were inoculated with culture media from CHV-1 or mock-infected corneas and cytopathic effect (CPE) was
evaluated by light microscopy 2 days later. A72 cells inoculated with supernatants from CHV-1 infected corneas showed morphological changes including ballooning of cells and the formation of small cytoplasmic tails (Figure 4D). No such morphological changes were observed in A72 cells inoculated with supernatants from mock-infected cells (Figure 4D).

CHV-1 does not cause apparent corneal damage at 48 h pi

To evaluate CHV-1 induced corneal damage in our culture model, histology was performed on mock-infected and CHV-1-infected corneas at 48 h pi. to study virus-induced changes in the epithelium. Upon CHV-1 infection, no significant changes in epithelial thickness or number of nuclei were observed at 48 h pi. (Figure 5A). Since viral infections, including those with herpesviruses (25), are known to induce apoptosis, we evaluated apoptotic cells in the epithelium of infected canine corneas by IHC using rabbit anti-caspase-3 Abs. No apoptotic cells were detected in infected corneas at 48 h pi. (Figure 5B). As a positive control, we performed caspase 3 antibody binding on cryosections of a CHV-1 infected liver in which apoptotic cells were identified, indicating our negative results were not due to a technical error (Figure 5B).

CHV-1 upregulates cytokines in infected corneas similarly to HSV-1

Global gene expression was compared between CHV-1 infected and mock-infected corneas at 48 h pi. using the single color Agilent Canine Oligo Microarray. Over 1052 gene entities were differentially expressed between infected and non-infected corneas using a filter criteria of at least 10-fold change and overall, more genes were upregulated than downregulated after CHV-1 infection (Figure 6A). We identified 29 genes that showed an up- or downregulation of ≥100-fold and of those genes, 20 were up- and 9
were downregulated in CHV-1 infected corneas. An overview of the 10 genes that showed the highest up- or downregulation are summarized in Table II. Interestingly, three genes, namely CCL20, IL8 and IL1-α, found to be upregulated in CHV-1-infected corneas, have been described previously to be upregulated during ocular HSV-1 infection (26–28). We verified the microarray findings using qRT-PCR and found that all three cytokines showed a significant increase in gene expression in the CHV-1 infected corneas (Figure 6B). Moreover, the fold increase detected by qRT-PCR mirrored the fold increase identified with the microarray perfectly, with CCL20 showing the highest upregulation, followed by IL8 and lastly by IL-1α (Figure 6B). Although further validation of the other genes identified with the canine microarray is required, our present data on these three cytokines indicate that changes occurring during CHV-1 infection in canine corneas are like those documented during HSV-1 infection of corneal epithelial cells.

Discussion

The present study is the first to describe the establishment of an air-liquid canine corneal organ culture model, which mimics the blinking of the eyelids, for studies on the ocular alphaherpesvirus CHV-1. This model system might prove an excellent virus-natural host model to study ocular herpesvirus infection. The air-liquid corneal organ culture model was originally described in the 1990s by the group of P. Binder to study corneal wound healing in humans (17, 18), but has not been previously used to study ocular pathogens such as alphaherpesviruses.

To date, only one paper has been published describing the use of a rabbit (non-natural host) and human (natural host) corneal explant model to study acute HSV-1 keratitis (9).
In this model, the authors cultured corneas, for approximately 7 days, by fully immersing them in culture medium, thereby covering the epithelial surface. It has been previously demonstrated that such corneal culture techniques result in a reduction in epithelial cell thickness, increased epithelial edema, and changes in cellular differentiation (29). In contrast, maintenance of natural morphology and an increase in long-term survival have been noted when human corneas are cultured with their epithelial surface intermittently exposed to air and liquid environments (17). Although in the present study we did not directly compare morphology and viability of canine corneas cultured in the traditional versus the air-liquid system we did not detect any degenerative changes in the epithelial layers of the cornea cultured for up to 25 days in the air-liquid system. To further mimic the *in vivo* situation, corneas were filled with agar allowing them to better maintain their shape. Despite the presence of this supporting gel scaffold, there was an initial drop in number of nuclei/μm² indicative of cell expansion due to mechanical stress release, but the number of nuclei remained constant for the rest of the culture period and was not associated with cell death. Another advantage of using canine corneas in the air-liquid system instead of human corneas in the traditional system is the quality of the tissue. Corneas from human donors that are available for research are often of poor quality since these corneas have been found unsuitable for corneal transplantation and are either from older donors or from donors with severe medical disorders. In contrast, canine corneas are often available from young and/or healthy research or privately owned dogs that have been euthanized for reasons unrelated to corneal problems, and these corneas can often be collected shortly after euthanasia. For these reasons, the quality and structural integrity of
canine corneas available for experimental work are often superior to those of human corneas.

We confirmed the susceptibility of the air-liquid canine corneal organ culture model for CHV-1 infections and optimized all necessary tools to study viral replication and viral-induced corneal damage. As proof of principle, we infected canine corneas with different concentrations of CHV-1 duck and analyzed samples at 48 h pi. Our salient findings were that CHV-1 (i) replicates in the corneal epithelial cells, (ii) spreads in a plaque-wise manner in the corneal subepithelium, (iii) produces viral DNA copies in a infection concentration-dependent manner and (iv) does not induce detectable corneal damage and apoptosis of corneal epithelial cells early after infection. Based on the success of our explant model to support CHV-1 replication kinetics, future experiments are planned including (i) examination of infected corneas at various time points pi, to study CHV-1 replication kinetics and virus-induced corneal damage in more detail, (ii) infecting corneas with clinical isolates of CHV-1 exhibiting different ocular phenotypes, to study potential differences in replication kinetics and/or corneal damage and (iii) infecting corneas with CHV-1 mutants, to study the role of viral ocular virulence factors.

We are currently creating a bacterial artificial chromosome (BAC) of the CHV-1 duck that we can use to create recombinant viruses in which viral ocular virulence determinants are deleted. We plan to focus first on infected cell protein 0 (ICP0), the immediate-early gene US1, and thymidine kinase (TK), as these viral genes have been confirmed to play important roles during HSV keratitis (30–32).

Using a commercially available canine microarray, we began to decipher alterations in corneal cellular gene expression upon CHV-1 infection. Of the 29 genes found to be
up- or downregulated ≥ 100-fold in CHV-1 infected corneas, 3 genes have been described previously in relation HSV-1 keratitis. The expression of the chemokine-ligand 20 (CCL-20) was found to be induced in human corneal epithelial cells upon in vitro infection with HSV-1 and an accumulation of this cytokine was found in murine corneas using a murine herpetic stromal keratitis model (27). Using qPCR, we also confirmed in the present study the upregulation of canine CCL-20 in CHV-1 infected corneas. Another cytokine, interleukin 8 (IL8), has been reported previously to be upregulated after HSV-1 infection of a telomerase-immortalized human corneal epithelial cell line (28) and we confirmed canine IL-8 to be upregulated in CHV-1 infected corneas, albeit to a lesser extent than canine CCL20. A third cytokine, interleukin 1 alpha (IL-1α), has been found to be elevated in the murine model of herpetic stromal keratitis starting at day 2 pi, with peak levels being reached at day 10 pi after which the expression of IL-1α gradually diminished over the next 10 days (26). In our canine cornea explant model we also confirmed an upregulation of IL-1α at day 2 pi and plan to study the expression kinetics of this cytokine long-term using our model. Taken together, our microarray and qRT-PCR data show that canine corneas produce proinflammatory cytokines in response to CHV-1 infection, analogous to what has been described before for HSV-1. This emphasizes the value of our system as a virus-natural host model to study ocular herpesvirus infections. Experiments to study additional innate immune and other genes found to be differentially expressed in CHV-1 infected corneas using the canine gene expression array, will soon be initiated. These studies will further improve our knowledge on host responses during acute ocular herpesvirus infection. A more comprehensive
understanding of the pathogenesis of this disease will improve the potential for rational
development of vaccines and antiviral treatments.

We propose our air-liquid canine corneal organ culture system as a useful model for
studies on ocular herpesvirus infections. Our model provides a physiologically relevant
system in which ocular herpes can be studied in a virus-natural host setting and
minimizes the number of experimental animals needed. Finally, this long-term explant
culture model may also facilitate research in other fields, where non-infectious and
infectious ocular diseases of dogs and men are being studied.

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Michelle Edelman for her technical assistance with cutting and analyzing the CHV-1-
infected cornea slides.

Figure Legends

Figure 1. (A). Set-up of the air-liquid canine corneal organ culture model. The canine
cornea is placed on a Kelfar® ring in a cell culture dish containing cornea culture
medium and is incubated on a rocking platform, creating an air-liquid environment mimicking the natural blinking of the eye. (B). **Representation of the quantitative analyses of the corneal epithelium.** Pictures represent a gross image of a stained cornea section, with ruler added for scale, and a magnified image of a stained cornea section, with boxes indicating the 3 measure areas in which nuclei were counted. Scale bar: 100 μm.

**Figure 2. Epithelial morphometry and viability analyses of cultured canine corneas.** (A). Representative pictures of H&E stained cornea sections at different time points post culture. Scale bar: 20 μm. (B). Quantitative analyses of corneal epithelium showing epithelial thickness in μm and number of nuclei per 1x10³ μm² epithelium. (C). Representative pictures of TUNEL stained cornea sections at different time points post culture. DAPI labeling was used to visualize nuclei and a DNAse-treated positive control section is shown as an inset in the picture representing day 0. Scale bar: 20 μm.

**Figure 3. Transmission electron microscopy (TEM).** Representative TEM pictures of canine corneas cultured for 25 days in the air-liquid model. The nucleolus is indicated by a black arrow and the nuclear membrane by a white arrow. Asterisks indicate desmosomes.

**Figure 4. CHV-1 infection of cultured canine corneas.** (A). Representative pictures of CHV-1 positive epithelial cells at 48 h pi. visualized with biotin-labeled CHV-1 hyperimmune serum, followed by streptavidin-HPR. Slides were counterstained with Gill’s hematoxylin. (B). Representative confocal picture of a subepithelial viral plaque at 48 h pi. visualized with FITC-conjugated anti-CHV-1 Abs. DAPI labeling was used to visualize nuclei. Scale bar: 25 μm. (C). CHV-1 DNA copies in cultured canine corneas
and supernatants of infected canine corneas were determined using quantitative PCR (qPCR) and data are represented as the fold change compared to mock-infected canine corneas. (D). Representative pictures of A72 cells, 2 days post inoculation with supernatants of mock- or CHV-1 infected corneas. Arrows show ballooning of cells, indicative for cytopathic effects (CPE).

**Figure 5.** Epithelial morphometry and apoptosis analyses of CHV-1 infected canine corneas. (A). Quantitative analyses of mock or CHV-1 infected corneal epithelium at 48 h pi. showing epithelial thickness in μm and number of nuclei per 1x10^3 μm^2 epithelium. (B). Representative pictures of mock or CHV-1 infected cornea sections at 48 h pi. incubated with anti-caspase-3 or rabbit IgG isotype control Abs. Sections were counterstained with Gill’s Hematoxylin, and a CHV-1-infected liver was included as a positive control. Black arrows indicate caspase-3-positive cells. Scale bar: 20 μm.

**Figure 6.** Gene expression array of CHV-1-infected canine corneas. (A). Heat map visualization (i) and box plot representation (ii) of canine genes differentially expressed with at least 10-fold changes. Columns represent the CHV-1- and mock-infected corneas at 48 h pi. and rows indicate the genes. Color key indicates gene expression values with green the lowest and red the highest. (B). Heat map of the cytokines CCL20, IL8 and IL1-α in CHV-1- versus mock-infected corneas using excel. Colors range from white (no expression) to red (high expression) (i); qRT-PCR confirming gene expression of these 3 cytokines (ii); fold upregulation of these cytokines in the microarray experiment compared to real-time PCR results (iii).
References


Figure 1. (A). Set-up of the air-liquid canine corneal organ culture model. The canine cornea is placed on a Kelfar® ring in a cell culture dish containing cornea culture medium and is incubated on a rocking platform, creating an air-liquid environment mimicking the natural blinking of the eye. (B). Representation of the quantitative analyses of the corneal epithelium. Pictures represent a gross image of a stained cornea section, with ruler added for scale, and a magnified image of a stained cornea section, with boxes indicating the 3 measure areas in which nuclei were counted. Scale bar: 100 μm.
Figure 2. Epithelial morphometry and viability analyses of cultured canine corneas. (A). Representative pictures of H&E stained cornea sections at different time points post culture. Scale bar: 20 μm. (B). Quantitative analyses of corneal epithelium showing epithelial thickness in μm and number of nuclei per 1x10^3 μm² epithelium. (C). Representative pictures of TUNEL stained cornea sections at different time points post culture. DAPI labeling was used to visualize nuclei and a DNase-treated positive control section is shown as an inset in the picture representing day 0. Scale bar: 20 μm.
Figure 3. Transmission electron microscopy (TEM). Representative TEM pictures of canine corneas cultured for 25 days in the air-liquid model. The nucleolus is indicated by a black arrow and the nuclear membrane by a white arrow. Asterisks indicate desmosomes.
Figure 4. CHV-1 infection of cultured canine corneas. (A), Representative pictures of CHV-1 positive epithelial cells at 48 h pi. visualized with biotin-labeled CHV-1 hyperimmune serum, followed by streptavidin-HPR. Slides were counterstained with Gill’s hematoxylin. (B), Representative confocal picture of a subepithelial viral plaque at 48 h pi. visualized with FITC-conjugated anti-CHV-1 Abs. DAPI labeling was used to visualize nuclei. Scale bar: 25 μm. (C), CHV-1 DNA copies in cultured canine corneas and supernatants of infected canine corneas were determined using quantitative PCR (qPCR) and data are represented as the fold change compared to mock-infected canine corneas. (D). Representative pictures of A72 cells, 2 days post inoculation with supernatants of mock- or CHV-1 infected corneas. Arrows show ballooning of cells, indicative for cytopathic effects (CPE).
Figure 5. Epithelial morphometry and apoptosis analyses of CHV-1 infected canine corneas. (A). Quantitative analyses of mock or CHV-1 infected corneal epithelium at 48 h pi. showing epithelial thickness in μm and number of nuclei per 1x10³ μm² epithelium. (B). Representative pictures of mock or CHV-1 infected cornea sections at 48 h pi. incubated with anti-caspase-3 or rabbit IgG isotype control Abs. Sections were counterstained with Gill’s Hematoxylin, and stainings of a CHV-1-infected liver were included as a positive control. Black arrows indicate caspase-3-positive cells. Scale bar: 20 μm.
Figure 6. Gene expression array of CHV-1-infected canine corneas. (A). Heat map visualization (i) and box plot representation (ii) of canine genes differentially expressed with at least 10-fold changes. Columns represent the CHV-1- and mock-infected corneas at 48 h pi. and rows indicate the genes. Color key indicates gene expression values with green the lowest and red the highest. (B). Heat map of the cytokines CCL20, IL8 and IL1-α in CHV-1- versus mock-infected corneas using excel. Colors range from white (no expression) to red (high expression) (i); qRT-PCR to confirm gene expression of these 3 cytokines (ii); fold upregulation of these cytokines in the microarray experiment compared to real-time PCR results (iii).
### Table I. Overview of primers used in quantitative RT-PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal Protein L32</td>
<td>RPL32</td>
<td>Housekeeping gene</td>
<td>TTGAAGTGCTGCTGATGTGC</td>
<td>GGGATGGTGACTCTGATGG</td>
<td>121 bp</td>
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<tr>
<td>Chemokine (C-C motif) Ligand 20</td>
<td>CCL20</td>
<td>Attraction of lymphocytes (strong) and neutrophils (weak)</td>
<td>TCATGGGCTTCACACAACAG</td>
<td>TTTGGATCTGCACACACAGC</td>
<td>97 bp</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>IL8</td>
<td>Attraction of neutrophils; potent promoter of angiogenesis</td>
<td>CACTCCACACCTTCCATCC</td>
<td>GCCAGGCACACCTCATTTC</td>
<td>120 bp</td>
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<tr>
<td>Interleukin 1 alpha</td>
<td>IL1-α</td>
<td>Epidermal cytokine, important for maintenance of the skin barrier</td>
<td>ACCCACTTCATGAGGACTGC</td>
<td>CATGGCTGCCACCACCTAC</td>
<td>99 bp</td>
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</table>
Table II. Overview of the top 10 genes that were differentially expressed in CHV-1-infected corneas compared to mock-infected corneas, using an 8x60K Canine Gene Expression Microarray (Agilent).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Fold change ([0] versus [1]*)</th>
<th>Up-or down-regulation after CHV-1 infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum amyloid A1</td>
<td>SAA1</td>
<td>1683.6</td>
<td>UP</td>
</tr>
<tr>
<td>S-antigen, retina and pineal gland</td>
<td>SAG</td>
<td>-900.6</td>
<td>DOWN</td>
</tr>
<tr>
<td>Interleukin 33</td>
<td>IL33</td>
<td>528.9</td>
<td>UP</td>
</tr>
<tr>
<td>Synaptobrevin homologue YKT6</td>
<td>YKT6</td>
<td>460.5</td>
<td>UP</td>
</tr>
<tr>
<td>Peripherin 2</td>
<td>PRPH2</td>
<td>-389.4</td>
<td>DOWN</td>
</tr>
<tr>
<td>Gamma transducing activity polypeptide 1</td>
<td>GNGT1</td>
<td>-354.5</td>
<td>DOWN</td>
</tr>
<tr>
<td>Glucose Transporter-like Protein-9</td>
<td>GLUT9</td>
<td>349.9</td>
<td>UP</td>
</tr>
<tr>
<td>WAP four-disulfide core domain 5</td>
<td>WFDC5</td>
<td>317.0</td>
<td>UP</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid A receptor</td>
<td>GABAAR</td>
<td>-262.2</td>
<td>DOWN</td>
</tr>
<tr>
<td>Nucleoporin SEH1</td>
<td>SEH1L</td>
<td>258.6</td>
<td>UP</td>
</tr>
</tbody>
</table>

* [0] represents mock-infected corneas and [1] represents CHV-1-infected corneas