Reducing Chorioretinal Viral Counts with Intravitreal Foscarnet Injections in a Rabbit Model of Herpes simplex Virus Type-1 Retinitis

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ABSTRACT

The efficacy of intravitreal foscarnet injections was evaluated in a rabbit model of Herpes simplex virus type-1 (HSV-1) retinitis. In untreated infected animals, viral titration revealed that the optic chiasm, vitreous and chorioretina were positive for HSV-1. On the other hand, foscarnet treatment significantly decreased the viral count in the chorioretina when compared to the untreated group. Immunolocalization of HSV in untreated infected animals clearly showed infected cells in the outer and inner layers of the retina and also in the ciliary body of the eye. Clinical examination by indirect ophthalmoscopy indicated an absence of optic nerve congestion and a lower level of vitritis in foscarnet treated animals compared to the untreated group. It is concluded that intravitreal injections of foscarnet reduced the viral titer in the chorioretina in a rabbit model of HSV-1 retinitis. This route of administration might be valuable for the treatment of CMV retinitis in AIDS patients with sight threatening lesions or intolerance to intravenous anti-CMV drugs.

INTRODUCTION

Cytomegalovirus (CMV) is an ubiquitous human herpes virus which causes one of the most important life-threatening opportunistic infections in individuals with impaired immune system. Although CMV infection is asymptomatic in most healthy individuals, it could be reactivated in immunosuppressed hosts, such as organ transplant recipients and patients with AIDS, causing significant morbidity and mortality. CMV retinitis has become the most common infectious ocular disease associated with AIDS, affecting 12 to 46% of AIDS patients (1). It induces a necrotic retinitis that quickly progresses to total retinal destruction and blindness in less than six months if untreated.
(2). The major life-threatening nature of CMV-related diseases in high risk individuals denotes the need for a safe and appropriate treatment of CMV infections.

Foscarnet (phosphonoformic acid, PFA) is a pyrophosphate analogue which prevents the replication of human herpes viruses, including CMV, by inhibiting viral DNA polymerases through noncompetitive interaction with viral enzymes at the pyrophosphate binding sites (3). This drug has been approved by the Food and Drug Administration (FDA) for the treatment of CMV retinitis in patients with AIDS (4). It can also inhibit human immunodeficiency virus (HIV) reverse transcriptase (5-7). Combined with zidovudine (AZT), foscarnet is synergistic against HIV (8). In contrast to nucleoside analogues, foscarnet does not have to be phosphorylated intracellularly to be active against HIV or CMV. This difference is of importance as one of the major mechanisms of resistance developed by microorganisms, such as CMV or other members of the herpes family, is their capacity to mutate and modify or eliminate phosphorylation of the antiviral agents within infected cells. Because of the poor bioavailability of foscarnet when taken orally and its low cellular permeability, large doses of foscarnet have to be administered intravenously to patients to be active. Induction treatments with intravenous foscarnet can stop the progression of the infection for a while, but reactivation occurs in a high percentage of cases. This is most probably due to the poor penetration of the drug into the eye cavity through the blood retinal barrier (9) which can account for antiviral resistance. Foscarnet is not metabolized, and 83% of a cumulative intravenous dose is recovered unchanged in the urine during the first 36 hours after stopping the infusion (4).

An important problem associated with prolonged maintenance therapy of foscarnet is its toxicity. The most frequent dose-limiting adverse effects associated with intravenous foscarnet therapy are renal impairment and alterations in plasma minerals and electrolytes (4,10,11). A 2- to 3-fold increase in serum creatinine has been observed in 20-66% of AIDS patients receiving 130 to 230 mg/kg/day of foscarnet as a continuous infusion (4,10,12); hemodialysis may be required in severe cases of renal impairment. This nephrotoxicity is believed to be due to acute tubular necrosis or interstitial tubular nephritis which tends to be reversible on foscarnet withdrawal. Reversible calcium and phosphorus abnormalities (both increases and decreases were reported) have been observed in foscarnet recipients (11,13-15). Therapy with this antiviral is also associated with anemia in 20-50% of AIDS patients (13,16,17). Because of the severe toxicity of foscarnet, treatment is commonly stopped, resulting in a retinitis relapse.

A well-defined animal model would be very useful to evaluate the efficacy of antiviral agents against CMV retinitis. Unfortunately, human CMV is a species-specific virus which is not transmissible to animals (18). Presently, no successful infection with human CMV has been reported in animal models. Therefore, we have chosen the human Herpes simplex virus type-1 (HSV-1)-KOS strain to induce retinitis in the rabbit model. This model has been previously used to evaluate the clinical efficacy of antiviral drugs (19,20). The present paper describes the efficacy of foscarnet administered intraocularly in a rabbit model of acute HSV-1 retinitis.

MATERIAL AND METHODS

Virus Culture

The HSV-1, KOS strain was cultured on confluent African green monkey kidney cells (VERO cells, CCL 81, American Type Culture Collection, Rockville, MD) maintained in minimal essential medium with Earle's salts (Gibco BRL, Burlington, Ontario, Canada) supplemented with 2% fetal bovine serum and non-essential amino acids, 2 mM L-glutamine, 100 units/ml of penicillin G and 100 μg/ml of streptomycin. The viral stock was obtained by centrifugation (300 × g for 10 min at 4°C) of the supernatant of cells showing a cytopathogenic effect of approximately 80%. The supernatant obtained was then filtered through 0.45 μm filters (Millipore, Bedford, MA), titered by plaque assay and stored at -80°C until use. The viral titer was 7 × 10² pfu/ml.
IC Determination

Determination of the foscarinet inhibitory concentrations of 50, 90 and 99% of HSV-1 (IC$_{50}$, IC$_{90}$ and IC$_{99}$) was carried out by the standard plaque reduction assay using VERO monolayers as previously described (21).

Animal Treatments

New-Zealand rabbits (1.5-2.0 kg, Charles River Breeding Laboratories Inc., St-Constant, Quebec, Canada) were divided into two experimental groups and injected intravitreally in the left eye (OS) with saline or foscarinet (Foscavir™, obtained from our local pharmacy at the hospital) followed by inoculation with HSV-1 KOS strain. In brief, the left eye of 18 animals was topically anesthetized with 0.5% proparacaine-HCl (Alcaine®, Alcon, Mississauga, Ontario, Canada), disinfected with 0.05% chlorhexidine gluconate and diluted with 1% tropicamide and cyclopentolate-HCl (Mydriacyl® and Cyclogyl®, Alcon, Mississauga, Ontario, Canada) before the procedure. A 50 µl anterior chamber paracentesis was performed in order to lower intraocular pressure before the injection. The intravitreal injection was given 2 mm from the limbus, at the superior pole of the eye, and was given over ~ 15 sec period using a 30-gauge needle coupled to a tuberculeine syringe. Saline or 1.2 mg of foscarinet (100 µl) was injected in the middle of the vitreous cavity, whereas the HSV-1 inoculum (10$^6$ pfu/100 µl/OS) was delivered, two hours later, 1-2 mm in front of the optic nerve head. The dose of foscarinet was chosen based on clinical experience with some patients who received intraocular injections of the drug. Preliminary experiments were also carried out to select the optimal virus inoculum showing an acute progression of the retinitis. The whole procedure was carried out under indirect ophthalmoscopic visualization. Treatments were repeated on day 2 post-infection. On day 4, the animals were sacrificed with intravenous Euthanyl (24 mg/kg), and tissues were processed for viral titration or histopathology. We chose the prophylactic approach because the HSV-1 replication cycle is more rapid than that of CMV, and the infection is more aggressive.

Viral Titration

Samples (eyes and brain) were aseptically removed from the rabbits for viral titration (six animals per group). In brief, the aqueous humor was removed with a syringe, and volume was measured. The cornea and lens were then excised, and the vitreous was withdrawn. The eyeball was then cut in four equal parts, and the chorioretina was delicately removed and weighed. Afterwards, the optical nerve was cut, and the optic chiasm was entirely removed and weighed. Regarding the brain samples, a cortical biopsy of each cerebral hemisphere was performed in the occipital region which corresponds to the cortical projection of visual areas. A biopsy was also performed at the posterior lobe of the cerebellum. Samples were diluted in culture medium supplemented with 2% fetal bovine serum. They were sonicated 3 times for 10 sec (W-375, Heat-Systems-Ultronics, Farmingdale, NY), centrifuged (760 × g for 10 min at 4°C), and the supernatant was frozen at -80°C until the viral titration was performed. Five hundred microliters of diluted supernatant were added to confluent VERO cells in each well (24 wells plate), and the plate was centrifuged (750 × g for 45 min at 20°C). Cells were then washed with culture medium to remove non-adherent virus and were incubated with a layer of agarose Sea Plaque® (Mandel, Guelph, Ontario, Canada) 1.2%:culture medium 2X (1:1). After 48 h of incubation, cells were fixed with 10% formaldehyde and colored with 0.05% methylene blue. The lysed plaques were counted in each well, and results were reported as number of pfu per ml or per g of sample.

Histology

At the time of sacrifice, eyes (three animals per group) were removed, and a small incision was done at the limbus. Untreated uninfected rabbits were also used as controls. Eyes were then fixed in
6% buffered glutaraldehyde for 24-48 h at room temperature. After fixation, eyes were washed in running tap water overnight and placed in 70% ethanol to harden for 24 h before cutting (22). Pieces were processed manually for dehydration in series of ethanol and chloroform and embedded in paraffin (Paraplast X-TRA®, Fisher Scientific, Montreal, Quebec, Canada).

**Immunolocalization of HSV-1**

Following deparaffinization, 4 μm-thick tissue sections were placed in 1% hydrogen peroxide solution (in absolute methanol) to neutralize endogenous peroxidase. Tissue sections were hydrated in series of graded ethanol to water and then to a TRIS buffer. Immunolocalization of HSV was carried out using a modified protocol from Kuppermann et al. (19). Tissue sections were incubated for 1 h with a rabbit anti-HSV polyclonal antibody conjugated with peroxidase (DAKO Immunoglobulins, Dimension Lab. Inc., Mississauga, Ontario, Canada) at a dilution of 1/50. This antibody reacts with antigens common to HSV types 1 and 2. It binds to all the major glycoproteins present in the viral envelope and at least to one core protein. The substrate for the peroxidase (3-amino-9-ethyl carbazole, AEC from Sigma, St. Louis, MO) was added and incubated for 1 h to reveal HSV-infected cells. Negative controls were performed by omission of the antibody in all three groups. The hematoxylin counterstaining was performed only on the negative controls.

**Clinical Examination**

Clinical development of ocular disease was assessed on days 0, 2 and 3 by slit lamp examination of the anterior segment, including conjunctiva, cornea, anterior chamber, iris and anterior vitreous changes. Indirect ophthalmoscopic examination of the fundus through a dilated pupil was also performed. Scores, on a 0 to 4+ scale, were given for severity of anterior chamber inflammation (flare and cells), vitritis and optic nerve head edema induced by HSV retinitis. Anterior chamber flare and cells were graded from 0 to 4+, depending on the density of the proteinaceous exudate and the number of cells per field. Vitritis and inflammatory scoring was as follows: 0, no abnormalities; 1+, isolated strands or cells; 2+, mild to moderate haze and infiltration with most of the retina visible; 3+, moderate to severe haze and infiltration with most of the retina obscured; 4+, severe haze and infiltration with the retina totally obscured. Optic nerve head edema was scored as 0 to 4+, ranging from no abnormality to severe vascular engorgement and nerve head swelling (23).

**Statistical Analysis**

All statistical analyses were performed on Stat View™ SE + Graphics © 1988 (Abacus Concepts Inc., Berkeley, CA). Difference between groups was tested using analysis of variance (ANOVA). Group comparisons were performed using the Fisher’s PLSD test and a difference at p< 0.05 or less was considered to be statistically significant (p values are given in the figure legends).

**RESULTS**

**Inhibitory Concentrations of Foscarnet In Vitro**

The inhibitory concentrations (IC) of foscarnet against HSV-1 (KOS strain) in VERO cells were determined by plaque reduction assay. The IC_{50}, IC_{90} and IC_{99} were 37, 61 and 67 μg/ml of PFA, respectively. The KOS strain is well-characterized and widely used as a control to test the susceptibility to many antiviral drugs. Moreover, this strain is easily grown in high titer on VERO cells. Our IC values were comparable with other published values (24).
Effect of Intravitreal Foscarnet on Viral Titers

The viral titration in tissues of untreated infected and foscarnet-treated rabbits is shown in figure 1. Results clearly showed a significant reduction in the HSV-1 counts in the infected chorioretinae (Figure 1A) of the foscarnet treated group as compared to the untreated infected group (the difference

FIGURE 1. Viral Titration of HSV-1 (KOS Strain) in the Left Eye of Infected Rabbits (A) chorioretina, (B) vitreous humor and (C) optic chiasm. Results are expressed as individual values (n=6). The bar represents the mean of each group. The dotted line is the detection limit of the assay. *Significantly different from untreated infected group (ANOVA, Fisher PLSD test, p<0.01).
was 3.44 logs, p<0.0001, with 3 values below the detection limit). In the vitreous (Figure 1B), no significant difference was observed between the two groups, with 2/6 and 1/6 eyes below the detection limit for the untreated infected and infected+PFA groups, respectively. In the optic chiasm (Figure 1C), the difference was also not significant between the two groups, with 3/6 and 4/6 eyes below the detection limit for the untreated infected and infected+PFA groups, respectively. Although no significant difference was observed, the virus titer was lower in the foscarnet treated group compared to the untreated infected one. The virus was not found in the hemispheres or the cerebella.

![Histopathology and Immunolocalization](Image)

**FIGURE 2.** Histopathology (Left Panels: A, C and E) and Immunolocalization (Right Panels: B, D and F) of HSV-1 in Chorioretinae of Rabbits i) control uninfected rabbits (A, B), ii) untreated infected rabbits (C, D) and iii) infected rabbits treated with foscarnet (E, F). Results on untreated infected rabbits show inflammation of the outer nuclear layer (ONL) and inner nuclear layer (INL) regions (panel C) and arrows indicate virus-infected cells in the same region (panel D); GCL, ganglion cell layer. Magnification 200X.
of any of the animals in either treatment group. The limits of detection for the viral titration were 1.82 log pfu/ml (and pfu/g) for the chorioretina and optic chiasm, and 1.52 log pfu/ml for the vitreous.

Histopathologic Examination and Immunolocalization

The whole retina of each group was used for histopathologic examination and immunolocalization. Figure 2 shows typical micrographs of chorioretinae of each group. The retina of uninfected rabbits (Figure 2A) showed that the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL) were not damaged. On the other hand, the histology of the retina of infected rabbits (Figure 2C) demonstrated a massive destruction. It was indeed difficult to identify the GCL, and there was clear infiltration of inflammatory cells in the INL and ONL. In contrast, there was no damage in infected rabbits that were treated with foscarnet (Figure 2E). The retinal layers were well preserved and were comparable to that of uninfected controls. However, we observed some inflammatory cells in the vitreous. The immunolocalization of HSV in the retina clearly showed infected cells in the INL and ONL as well as in the GCL of untreated infected rabbits (Figure 2D). No virus was immunolocalized in the retina of the uninfected group (Figure 2B) nor in the infected group treated with foscarnet (Figure 2F).

Clinical Examination

Clinical evaluation of the different treatment groups was performed by an ophthalmologist who was not aware of the specific treatment for each group. Clinical examination results correlated well with the viral titration and immunolocalization experiments. In contrast, there was only a very mild anterior chamber inflammation and vitritis in the infected group treated with foscarnet. There was a moderate anterior chamber inflammation, vitritis and optic nerve head edema with vascular congestion and small splinter-like hemorrhages in the untreated infected group (Table 1). Retinitis was difficult to assess by indirect ophthalmoscopy because of the unpigmented retina of the albino rabbits used in this experiment.

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<th>Day</th>
<th>Anterior chamber inflammation</th>
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* Scores: 0 to 4+ as described in the methods section.
DISCUSSION

Since the introduction of highly active antiretroviral therapy (HAART) for the treatment of AIDS patients (a combination therapy including two nucleoside analogue reverse transcriptase inhibitors and one protease inhibitor), there has been a significant decrease in the incidence of CMV retinitis. Some have tried, successfully, to stop the maintenance therapy for CMV a few weeks after HAART introduction (25). It has also been proposed to stop the maintenance therapy in patients undergoing HAART treatment when the following criteria are fulfilled: 1) CD4 count is higher than 150/mm³, 2) there is a negative CMV-PCR and 3) the HIV viral load is lower then 200 copies/ml (26). However, others have reported that there are still patients under HAART therapy who have a high risk of CMV retinitis relapse (27,28). The issue on whether or not to stop maintenance therapy for CMV disease is yet to be defined. In addition, the compliance of patients under triple therapy is poor because of the large number of pills that have to be taken at different times daily. It is clear that there is still a need for an efficient CMV treatment with less systemic side effects, and a more convenient way of administration than daily restricting i.v. perfusions with catheter-related complications.

It is now well recognized that antivirals injected intravenously do not reach the eye in sufficient concentrations to inhibit the viral replication (9,29). This observation might explain why there are retinitis relapses in AIDS patients, with a shorter time to progression between each reactivation (30). The low drug concentrations obtained in the eye might also be an important factor for antiviral resistance. In addition, toxicity of foscarnet following systemic treatments is a major concern. The main toxicity associated with foscarnet treatment is renal impairment mainly manifested by an increase in serum creatinine (4,11). Other toxicities caused by foscarnet include reversible calcium and phosphorus abnormalities (11,13-15) and anemia (13,16,17). In many cases, treatments have to be stopped to eliminate those side effects which lead to persistence or recurrence of retinitis. The current dosing regimen for foscarnet is intravenous infusion of 60 mg/kg three times a day or 90 mg/kg twice a day, for 2-3 weeks (as induction therapy) and 90-120 mg/kg daily thereafter (as maintenance therapy). It is also recommended to hydrate before and during the infusion of the drug to reduce the renal toxicity of foscarnet. Finally, it is necessary to infuse the drug over a period of 2 hours at each treatment interval which is not convenient to patients.

Oral ganciclovir is an alternative for the treatment of CMV retinitis as a maintenance therapy. Such treatment might contribute to a better quality of life for patients as compared to those treated with intravenous antivirals (31-33). However, the bioavailability of oral ganciclovir in the eye cavity is still not sufficient to inhibit viral replication for a prolonged period of time. There is also a local treatment option, approved in 1996, for CMV retinitis: the ganciclovir eye-implant (Vitrasure™, Chiron Corporation). It is a sustained-release device containing ganciclovir, which is surgically placed in the vitreous cavity and can deliver ganciclovir for up to 8 months (34). However, despite promising results, this therapy still represents a delicate surgical intervention with major associated risks such as vitreous hemorrhage, uveitis, endophthalmitis, retinal detachment, and decrease in visual acuity (35). Intraocular injections of antivirals represent another interesting alternative for the treatment of CMV retinitis. Case reports of patients receiving intravitreal foscarnet or ganciclovir were published with promising results (1,36-38). The advantages of using this route of administration are that it is simple to perform, and high concentrations of drug can be obtained in the vitreous cavity (39,40). This might help to reduce the drug resistance observed after a few weeks or months of therapy. Furthermore, there is no significant systemic or retinal toxicity when ganciclovir or foscarnet are given by the intraocular route even at a dose higher than the one used in the present study. However, we must note that the half-life of intravitreal foscarnet is relatively short (about 34 hours) (41) and, clinically, this means that the drug has to be administered once or twice a week which can increase the risks of endophthalmitis associated with the injections. Furthermore, this treatment does not prevent future systemic infection or infection of the contralateral eye.

The experimental protocol used in this study is characterized by a rapid onset of retinitis within 4 days in untreated infected animals. This is mainly due to the high inoculum of HSV-1 injected 10⁶ pfu). Lower inocula (1×10⁵ and 5×10⁵ pfu) did not result in an extensive retinitis in rabbits (data not shown). In our model, we have used a prophylactic regimen to evaluate the efficacy of intravitreal
foscarnet injections. We have observed a significant decrease in the viral titer in the chorioretinae of animals treated with foscarnet compared to the saline-treated group. This is an indication that an active replication of the virus occurred in the retinal cells for which foscarnet was able to inhibit. Since foscarnet is not active against free virus (as other antivirals) this could explain why there was no significant difference in the vitreous virus titer between the two groups. In the optic chiasm, the viral titer was not significantly different between the saline-treated and foscarnet-treated groups which can be due to the low amount of virus found in this tissue (3/6 and 4/6 under the detection limit, respectively). It is well known that HSV-1 intraocular administration in rabbits leads to encephalitis, but this process takes up to 8-10 days to be established, depending mainly on the viral inoculum injected (42). This can explain why we did not detect HSV-1 in the brain tissue at the time of sacrifice (4 days post-infection).

The histopathology of eyes of animals treated with foscarnet showed an impressive protection of the retina against HSV-1 infection as compared to the saline treatment group. There was no detectable virus by immunolocalization with a specific antibody in the foscarnet-treated group compared to the saline-treated one. On the other hand, there was a massive infection of the retinal cells (ganglion cell layer, inner and outer nuclear layers) in this latter group. We could observe some inflammatory cells near the retina of the foscarnet-treated group, most probably because of a reaction due to the presence of free HSV-1 particles in the vitreous.

The ophthalmologic examination of the two groups of rabbits was consistent with the low viral titer and the preservation of the retina in the foscarnet-treated group as compared to the saline-treated group. There was a very mild anterior chamber inflammation and vitritis in the foscarnet group that can be explained by an inflammatory reaction due to the residual presence of some viral particles. No optic nerve head edema was seen in this treated group compared to the untreated infected group.

In conclusion, we have demonstrated that intravitreal foscarnet is efficient in an acute model of HSV-1 retinitis in rabbits. We believe that this model can be helpful to evaluate the efficacy of new antiviral drugs or slow release drug systems against viral retinitis. It can also be helpful to avoid antiviral resistance because of the high concentrations achieved in the eye. Intracocular administration of foscarnet in combination with oral ganciclovir might be an interesting solution to improve the actual maintenance treatment of CMV retinitis. It can be performed easily on an outpatient basis and is less restrictive than i.v. daily perfusions.

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REFERENCES


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