Susceptibility of porcine cytomegalovirus to antiviral drugs

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Objectives: Re-activation of porcine cytomegalovirus (PCMV) in the xenograft has been reported in pig-to-baboon models of xenotransplantation and is associated with invasive disease and consumptive coagulopathy. If xenotransplantation of porcine organs into human recipients is to proceed, donor organs will have to be free from a wide range of infectious agents including PCMV. However, it is prudent to characterise the antiviral susceptibility of this virus. We therefore investigated the effect of selected antiviral agents, currently licensed for the treatment of human herpesvirus infections, on PCMV replication.

Methods: Antiviral susceptibility was determined using real-time PCR and indirect immunofluorescence measurements in a porcine fallopian tube cell line infected with PCMV.

Results: PCMV replication was significantly inhibited by ganciclovir and cidofovir (both EC_{50} < 1 mg/L) and to a lesser extent by foscarin (EC_{50} within range 25–50 mg/L) and aciclovir (EC_{50} > 25 mg/L).

Conclusions: These results show that, if it proves necessary, ganciclovir and cidofovir should be considered as first-line drugs to treat PCMV infections in xenograft recipients.

Keywords: xenotransplantation, ganciclovir, cidofovir

Introduction

Xenotransplantation of porcine tissues and organs has been proposed to alleviate the current shortfall in human donor organs available for transplant. While advances have been made in overcoming immune rejection, xenotransplantation has raised considerable safety concerns regarding the potential for transmission of porcine infectious agents, particularly viruses, to humans. Viruses of particular concern are those transmitted in the germline or in utero, such as members of the retrovirus and herpesvirus families. Porcine cytomegalovirus (PCMV) is a β-herpesvirus showing sequence homology to human herpesviruses 6, 7 and human cytomegalovirus (HCMV). It causes generalized infection in newborn piglets, with 90% of pigs being seropositive in the UK. PCMV represents a potential risk in xenotransplantation since its human counterpart, HCMV, is frequently transmitted from the organ donor and re-activates post-transplant, where it is associated with invasive disease and consumptive coagulopathy. If xenotransplantation of porcine organs into human recipients is to proceed, donor organs will have to be free from a wide range of infectious agents including PCMV. However, it is prudent to characterise the antiviral susceptibility of this virus. We therefore investigated the effect of selected antiviral agents, currently licensed for the treatment of human herpesvirus infections, on PCMV replication.

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In this study, we have investigated the effect on PCMV replication of antiviral drugs including ganciclovir, foscarin, cidofovir and aciclovir, currently licensed for use in the treatment and/or prophylaxis of human herpesvirus infections. The antiviral agents used in this study are all nucleoside/nucleotide analogue inhibitors of the viral DNA polymerase, apart from foscarin, which is a pyrophosphate analogue. Antiviral susceptibility is conventionally determined by plaque reduction assay relying on the development of cytopathic effect (CPE). There is very little information at present on the ability to culture PCMV in vitro. The virus is slow growing in pig fallopian tube (PFT) cells, an adherent fibroblast-like cell line, replicating to produce CPE~11 days post-infection. It has otherwise only been shown to grow in porcine lung macrophages, which are difficult to obtain and potentially compromised by other infectious agents. We have therefore applied real-time PCR to determine the antiviral inhibition of PCMV DNA production by PFT cells using quantification of viral DNA in cell culture supernatant. In addition, PCMV
indirect immunofluorescence (IIF) was used to determine the effect of each antiviral on PCMV-infected cells. We show that ganciclovir, the first choice therapy of HCMV infection, is also effective against PCMV. In addition, cidofovir was active against PCMV replication, with foscarnet and aciclovir demonstrating less activity.

Materials and methods

In vitro culture of PCMV

PCMV infection was established in PFT cells. PCMV (laboratory-adapted strain) and the PFT cell line were kindly provided by Dr Clive Patience and Dr Jay Fishman. Infected and uninfected cells were maintained in MEM (Invitrogen Ltd, Paisley, UK) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (both Sigma-Aldrich Company Ltd, Irvine, UK) and 10% FCS (Labtech International, Ringmer, UK) in a 5% CO2 incubator at 37°C.

Monitoring antiviral susceptibility in supernatant

Chronically infected PFT–PCMV cells were divided between four 25 cm2 cell culture flasks; one flask for each of three concentrations of antiviral drug tested, and one control flask containing no antiviral drug (day –2). Unattached cells were removed from cultures after 2 days by total medium replacement (day 0), and real-time PCMV PCR of culture supernatant—harvested on day 2—was used to confirm equal quantities of PCMV-infected cells in each flask. The effect of each antiviral drug on PCMV replication was investigated by monitoring PCMV viral load in supernatant every 2 days from day 2 (the first day that antiviral drug was added) until day 14. On these days, supernatant was collected prior to total medium replacement including the appropriate antiviral drug concentration. Ganciclovir (Roche Products Ltd, Welwyn Garden City, UK) and cidofovir (Moravek Biochemicals Inc., Brea, CA, USA) were diluted to a final concentration of 1, 5 and 10 mg/L, whereas foscarnet (AstraZeneca UK Ltd, Luton, UK) was diluted to a final concentration of 25, 50 and 100 mg/L. Aciclovir (Foulding Pharmaceuticals Plc., Royal Leamington Spa, UK) was diluted to a final concentration of 5, 10 and 25 mg/L. Supernatant at each time point was centrifuged (400g, 6 min) to remove detached cells and 5 µL of cell-free supernatant was tested by real-time PCR. On day 14, all cultures were harvested for the preparation of IIF slides and DNA extraction.

Determining antiviral susceptibility by IIF assay

IIF slides were prepared from cells harvested at day 14. Cells were washed twice in PBS and 1.5 × 10^4 spotted onto each well of a polystyrene-coated slide. IIF assays were performed using serum from PCMV-seropositive pigs. Porcine serum samples were diluted 1/20 or 1/40 in PBS, spotted onto slides and incubated in a humidified chamber at 37°C for 40 min. Bleeding was used as a negative control in wells containing infected and uninfected cells. Slides were washed once in PBS containing 1% BSA (Sigma-Aldrich Company Ltd) and twice in PBS alone for 5 min each. Slides were then incubated with rabbit anti-swine FITC-labelled IgG antibody (Dako Cytomation Ltd, Ely, UK), diluted 1/40 in PBS, at 37°C for 40 min. After incubation, slides were washed as before, mounted in Vectashield fluorescent preservative (Vector Laboratories Ltd, Peterborough, UK) and visualized under an Olympus BX60 fluorescence microscope. The number of fluorescing (PFT–PCMV) cells in each well was counted. Photographs were taken using an Olympus DPCI digital camera.

Determining antiviral susceptibility by real-time PCR of DNA extracted from cells at day 14

DNA was extracted from cells using the Wizard Genomic DNA Purification Kit (Promega UK Ltd, Southampton, UK). DNA concentration was determined by spectrophotometry and 100 ng was analysed by real-time PCR, with uninfected PFT cellular DNA tested as a negative control.

Real-time PCR for PCMV DNA

Target sequences were quantified by real-time PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). Primers PCMVpolF (5’-GCTGCGTGTCCTCCCTCTAG-3’) and PCMVpolR (5’-ATTGTGATAAAGTCACCTGCTGC-3’) and probe (5’-6FAM-CCATCACCAGCATAGGCGGGAC-TAMRA-3’) were designed using Primer Express software (Applied Biosystems, Warrington, UK), from a region of the DNA polymerase gene of PCMV. Primers and probe show 100% nucleotide identity to the DNA polymerase nucleotide sequences of three PCMV strains (55b, B6 and OF-1) deposited in GenBank, suggesting their ability to amplify different isolates. Each reaction contained 100 ng of each primer, 5 pmol of probe, 200 µM of each dNTP (Promega UK Ltd), 2.5 U of HotStarTaq (Qiagen Ltd, Crawley, UK) and target DNA, in 10 × PCR buffer (containing 15 mM MgCl2) (Qiagen Ltd), in a final volume of 25 µL. Cycle parameters were: initial cycles of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C denaturation for 1 s at 60°C annealing/extension for 1 min. All samples were tested in triplicate. The threshold cycle values for the PCR amplification of the standards were used to generate a standard curve for the quantification of target DNA, and the three viral loads was calculated.

Determining cytotoxicity

The cytotoxic effect of each antiviral on PFT–PCMV cells was determined by counting the total number of remaining adherent cells, harvested from each culture at day 14, following removal of culture supernatant. An aliquot of cells was diluted in an equal volume of Trypan Blue (0.4%) and formaldehyde (37%) (1:1) (both Sigma-Aldrich Company Ltd), and counted using a haemocytometer.

Results

Monitoring antiviral susceptibility by the measurement of PCMV loads in supernatant and infected cells

PCMV load was monitored in supernatant from day 2 of culture and then every second day until day 14 by real-time PCR (Figure 1). PCMV cultures were sub-confluent at the start of the experiment, replicating to confluency during the 14 day period. Antivirals at the highest concentrations used in this study did not inhibit the activity of the Taq polymerase in the real-time PCR assay (data not shown). The results in Figure 1 show that in all cultures containing no antiviral drug, PCMV viral load in supernatant increased at a steady rate over 14 days. At the lower concentrations of ganciclovir, foscarnet and aciclovir tested, viral load increased at a lesser rate over the 14 days. At the highest concentrations of these antivirals tested there was little or no evident increase in viral load over 14 days. At all concentrations of cidofovir tested there was no increase in PCMV viral load in supernatant over 14 days.

The effect of each antiviral on PCMV in PFT cells was determined by quantifying the viral load in DNA extracted from cells after the 14 day period. Results in Figure 2 show a dramatic reduction in
Susceptibility of PCMV to antivirals

PCMV viral load in cells incubated with all concentrations of cidofovir tested, and the higher concentrations of ganciclovir and foscarnet tested, compared with no-drug controls. There was a slight reduction in PCMV viral load in PFT cells incubated with the higher concentrations of aciclovir tested; however, overall aciclovir and foscarnet had a much less dramatic effect on PCMV replication than the other drugs.

EC₅₀ values were calculated from the real-time PCR results of DNA extracted from PFT–PCMV cells following the 14 day period. Ganciclovir and cidofovir both had EC₅₀ values of <1 mg/L (<4 µM), whereas foscarnet had an EC₅₀ value within the range of 25–50 mg/L (83–167 µM). Aciclovir had an EC₅₀ value of >25 mg/L (>111 µM).

IIF results

In order to complement the results determined by real-time PCR, IIF was used to assess the number of PCMV-infected cells at day 14, following treatment with the antiviral agents. Consistent with the results shown in Figure 2, the IIF data show a dramatic reduction in the number of fluorescing PFT–PCMV cells in those cultures containing cidofovir and the highest concentrations of ganciclovir and foscarnet (Figure 3). There was a lesser reduction in the number of fluorescing cells in cultures containing varying concentrations of aciclovir tested, compared with the no-drug control. A representative example of these results is shown in Figure 4.

Cytotoxicity

Toxicity of the antiviral drugs used in this study to PFT–PCMV cells was determined by counting the total number of adherent cells harvested from cultures at day 14. Less than 5% of adherent cells were excluded by Trypan Blue. A significant reduction (50%) in total cell count was only found in the culture of PFT–PCMV cells incubated with 10 mg/L cidofovir. No significant toxicity was noted at the lower concentrations of cidofovir tested, or at any concentration of the other antivirals tested (data not shown).
HCMV is an important pathogen in the human allograft recipient, where viral transmission from the donor and re-activation are common occurrences, and a major cause of disease and graft failure. Similarly, in xenotransplantation, re-activation of PCMV is a concern, as it has been shown to cause invasive disease and possibly to contribute to consumptive coagulopathy in pig-to-baboon models.\textsuperscript{10,11} Whether PCMV would be transmitted to human cells outside the xenograft is unclear, as previous studies have failed to provide evidence for PCMV infection of human cells either \textit{in vivo} or \textit{in vitro},\textsuperscript{16} although only two cell lines were tested. In order to avoid potential zoonotic transmission of porcine viruses in xenotransplantation, it is ideal to eliminate as many potential pathogens as possible from pigs being bred for xenograft organs. For this, defined breeding methods to eliminate infectious agents from pigs and stringent testing of donor animals are required. It is possible to exclude most pathogens by caesarian delivery and barrier-rearing of donor animals; indeed, we have shown that PCMV could potentially be eliminated from pigs by these methods.\textsuperscript{13} If zoonotic transmission could not be prevented, through failure of screening methods, knowledge of antiviral susceptibility of PCMV would be essential, as PCMV would undoubtedly be re-activated under the strong immunosuppressive conditions.\textsuperscript{10-12}

In this study, we determined the efficacy of anti-herpetic agents, currently licensed for the treatment of human herpesvirus infections, to prevent PCMV replication \textit{in vitro}. Real-time PCR was used to calculate the effect of each antiviral, at three different concentrations, on PCMV viral load in supernatant over a 14 day period, and in DNA extracted from cells at day 14. In this cell culture system, ganciclovir and cidofovir (EC\textsubscript{50} < 1 mg/L) were most effective against PCMV replication; however, some toxicity was associated with the highest concentration of cidofovir tested. Foscarnet (EC\textsubscript{50} within range 25–50 mg/L) and aciclovir (EC\textsubscript{90} > 25 mg/L) were the least effective antivirals.

The activity of ganciclovir and to a lesser extent aciclovir suggests the presence of an HCMV UL97 gene homologue in the PCMV genome, as these drugs require initial phosphorylation by viral-encoded protein kinases. Such a gene has yet to be identified and characterized in PCMV.

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It is possible that the long intracellular half-life of cidofovir contributed to its antiviral activity against PCMV in this study. The long half-life of cidofovir\textsuperscript{13} permits the drug to be administered once weekly in induction therapy, and twice weekly for maintenance therapy for HCMV infection. In this study, the replacement of cidofovir in the cell culture flasks every 2 days meant that high levels of the
Susceptibility of PCMV to antivirals

Drug may have built up in the cells thus exaggerating its antiviral effect, and potentially contributing to the toxicity found at the highest concentration tested. Ganciclovir and aciclovir have much shorter intracellular half-lives of \( \sim 1 \) day and \( 1–2 \) h, respectively,\(^{18}\) and are therefore less likely to accumulate within the cells.

In a recent study, similar antiviral susceptibilities for PCMV to those we have identified, were reported.\(^{12}\) In that preliminary investigation, the level of PCMV infection in the culture system was presented only as a percentage reduction in viral load. Our results confirm and extend those from this previous study.

Comparison of the antiviral EC\(_{50}\) values for PCMV determined in this study with those previously obtained for HCMV is difficult, as differing methodologies have been used. Several studies have tried to compare the antiviral susceptibilities of different viruses obtained by plaque reduction assays and modern molecular methods,\(^{19,20}\) but have produced varying results suggesting that there is not a consistent relationship between the two methods.

In the case of antiviral agents that interfere with DNA replication, there is a delicate balance between cytotoxicity and efficacy. Cytotoxicity \textit{in vitro} is often determined by Trypan Blue exclusion. However, in this study, since PFT cells are adherent, and the culture medium was replaced at regular intervals throughout the study, this method was not feasible. Cytotoxicity was therefore best determined by counting the total number of adherent cells harvested following incubation with antiviral agents. Cytotoxicity results were confirmed in a parallel study using cellular DNA extracted at day 14 from uninfected adherent cells, cultured with each concentration of drug, as a marker of the total cell count (data not shown).

Although we have only tested one isolate of PCMV, it is highly probable that it is representative of the antiviral activity of agents against PCMV. In particular, the DNA polymerase genes of PCMV strains B6 and OF-1, and isolate 55b are \( >99.3\% \) identical at the amino acid level.\(^{4}\)

In solid-organ allograft recipients either ganciclovir prophylaxis or pre-emptive therapy is used to treat HCMV infections post-transplant. Our results suggest that ganciclovir may also be used to treat any potential PCMV infections that may arise, with cidofovir providing an alternative drug for management.

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References


