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Long-term infection passaging of Human Adenovirus 36 in monkey kidney cells

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ABSTRACT

Human Adenovirus 36 (HAdV-36) has been related to diverse effects on metabolism and may attenuate the lipid accumulation in kidneys with increased adiposity. Some of these effects would be related to viral persistence. However, until now, a model of persistent *in vitro* infection by HAdV-36 is unknown. In this study, we examined the cells of the Vero lineage to explore their permissiveness to long-term HAdV-36 infection. HAdV-36 was productively replicated in Vero cells and maintained long-term infection for up to 35 cell passages. A subculture was obtained from the cells that survived the primary infection at a low MOI (0.5). The production of the extracellular infectious virus with titers ranging from 10^4 to 10^6 $TCID_{50}$/mL and DNA-bearing cells was detected. In long-term infected cells, the intracellular distribution of viral antigen was demonstrated by performing immunolocalization (IFI) and expression of cell-viral antigen in 50% of cells by flow cytometry, using anti-HAdV-36 hyperimmune rabbit serum. Furthermore, *E1a* and *E4orf1* genes in long-term infected passages showed a decreasing trend. Our preliminary results reveal that renal epithelial monkey cells are permissive for the productive infection of HAdV-36. Vero cell culture long-term infection might be a promising model for addressing the fundamental aspects of the HAdV-36 biology that cannot reveal broadly-used cultures, which do not maintain long-term infection in primary or transformed cells.

KEYWORDS: HAdV-36. Ad36. Vero cells. HAdV-36 long-term *in vitro* infection.

INTRODUCTION

HAdVs were first recognized and isolated as pathogens approximately 70 years ago. HAdVs are non-enveloped dsDNA viruses that belong to the *Adenoviridae* family and the *Mastadenovirus* genus. Over 80 types have been described and grouped into seven species (A-G). Species D contains the most types, including HAdV-36, first described in 1980, with genomic characterization in 2010[1]. The HAdV-36 E4orf1 protein has been proposed as a biological factor that can improve glucose and lipid metabolism, even with weight gain and adiposity[2]. Furthermore, E4orf1 might attenuate the lipid accumulation in kidneys[3].

Additionally, HAdV-36 can spread to most organs (liver, kidneys, lungs, brain, and adipose tissue) of naturally or experimentally infected animals[4,5], which suggests a link to HAdV-36 persistence[6]. However, the molecular mechanism through which HAdV-36 can establish a persistent infection is unknown. The viral replication and capacity for adenovirus production have been examined using renal epithelial monkey cell culture (Vero cell lineage)[7].

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MATERIALS AND METHODS

HAdV-36 (VR-1610) was obtained from ATCC (Manassas, VA, USA). The stock of HAdV-36 was purified, propagated and titrated as described by Dhurandhar *et al.*[8] A viral stock at a 10^{12} $TCID_{50}$/mL concentration was used in the experiments. Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% of fetal bovine serum (FBS) under standard conditions.

Cell culture infection: acute and long-term infected cultures

Confluent monolayers of Vero cells were inoculated with HAdV-36 stock at a multiplicity of infection (MOI) of 1.0 for acute infection and 0.5 for long-term infection. Both were grown in DMEM with 2% FBS in T25 flasks in triplicate. The acute infection was considered until 14 days post-inoculation (dpi). On the other hand, for long-term infected cultures, incubation was maintained for eight days, and then the supernatant (SN) was removed and replaced with a fresh culture medium. The incubation continued for another seven days. After this incubation, the surviving infected cells formed confluent monolayers (at 20 dpi) in order to obtain long-term infected cultures that were subcultured (henceforth, passaged) usually every week for 35 passages, which is equivalent to eight months. In a similar way, non-infected Vero cells, used as control, were incubated. Two-thirds of these were used to determine the cells bearing viral DNA, cells expressing viral antigen and the mRNA expressing *E1a* and *E4orf1* genes. Also, the final third was subcultured.

$TCID_{50}$ assay

We analyzed HAdV-36 infectivity or productive infection in the passages based on the production of extracellular virions released in the SNs using a $TCID_{50}$ assay. As described briefly by Dhurandhar *et al.*[8], four wells of 96 well-plate were inoculated with a ten-fold dilution series, and the Spearman–Karber's method[9] was used to calculate the viral titer.

HAdV-36 DNA detection

In the long-term infected passages cells, the infection by HAdV-36 was proven using conventional PCR. The primers used were F (5´-TGAGCAGCAGATGGCTCTAATCTC-3´) and R (5´-GGTCTTCTTCTGAGGGTGATGACTC-3´), as previously described[10], to amplify a fragment of the *E1a* gene yielding an amplicon size of approximately 320 bp. Non-infected and acutely infected cells were included as controls. An Applied Biosystems Veriti thermocycler (CA, USA) was used. The PCR products were separated by agarose gel electrophoresis (1.5%), visualized using ethidium bromide staining (0.5 µg/mL) and analyzed using a 212 PRO Carestream photo documentation system (Carestream 212 PRO, Gel Logic, Rochester, USA). The amplification products of PCR were purified with a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced using a commercial supplier to confirm the genetic identity of the virus.

For antigen detection by immunostaining assays, a hyperimmune rabbit serum against HAdV-36 (from now on referred to as pAb) was produced in two New Zealand white rabbits by immunization with concentrated and purified HAdV-36, following the protocols described by Sarmiento *et al.*[11].

Viral antigen detection by indirect immunofluorescence (IFI)

Cells from long-term infected passages were trypsinized, seeded and grown until they reached the confluence on the wells chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). Similarly, non-infected and acutely infected cells were seeded as controls. Fluorescence staining was performed as described by Sarmiento *et al.*[11] with slight adaptations. We used pAb (1:300 in blocking solution) as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, W. Baltimore Pike, USA) (1:500 in PBST 0.1%) as the secondary antibody. Nuclei were counterstained with DAPI (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Moreover, the cells were viewed using a fluorescence microscope (Nikon, Eclipse E600, Japan).

Viral antigen detection by flow cytometry

We used 5×10^5 cells of cultures of the long-term infected, acutely infected and non-infected to detect the viral antigen. We used pAb diluted 1:300 in PBST 0.1% as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, W. Baltimore Pike, USA) (1:500 in PBST 0.1%) as the secondary antibody. Subsequently, the cells were resuspended in one mL of PBS and 1×10^4 cells were analyzed using flow cytometry. Data were then collected using a FACSCanto II cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using the FACSDIVA software (version 6.1.3, Becton Dickinson). For each sample, at least 10,000 events were acquired.

Fluorochrome-labeled isotype-matched control pAb was used to determine background staining.

Expression of E1A and E4orf1 mRNA in long-term cultures

Cells were washed three times with PBS and lysed in Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The total RNA extracted after treatment with DNase was used to synthesize cDNA using Oligo dT and MMLV RT (Promega, Madison, WI, USA). The amplification was done using the SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA) and QIAquant 96 systems (Qiagen Ca, USA); the primers used were: α-tubulin (Fw 5'-CAGATGCCAAGTGACAAGAC-3' Rv 5'-ACTCCAGCTTGGACTTCTTG-3'), E1A (Fw 5' AGATGCCCATGATGACGACC-3' Rv 5' GCCTTGTACTGGCTCGGTAG3') and E4ORF1 (Fw 5' CCAGGAAGGGGCTAGCAATA3' Rv 5' AATCACTCTCTCCAGCAGCAGG3'). Relative gene expression was calculated using the comparative ΔΔCt method (where C_T is the threshold cycle) and was used to determine the fold increase in gene expression of long-term infected cells and acutely infected cells normalized to α-tubulin expression.

RESULTS AND DISCUSSION

In this study, the infection of Vero cells by HAdV-36 resulted in productive infection, with viral DNA-bearing cells and expression of the HAdV-36 antigen in almost 50% of infected cells in the long term, from 20 dpi to 200 dpi or higher, which is consistent with a persistent carrier-state infection.

The persistence of HAdV-36 is plausible based on Dhurandhar *et al.*[5] and Krishnampuram *et al.*[4], whose independent studies showed that experimentally infected monkeys and mice harbored the HAdV-36 in the liver, lungs, and kidneys for at least four months after inoculation.

Using Vero cells that mimic the entry cells of adenoviruses, we managed to replicate HAdV-36 in them, as was done for HAdV-5[7]. Furthermore, we obtained long-term infected cultures by infecting Vero cells with a low MOI (0.5). Similarly, Tirado *et al.*[12] used this to establish a persistently infected culture. Our control of the infection acute phase at eight dpi allowed the surviving cells to multiply until the confluence at 14 dpi. For instance, the study by Alidjinou *et al.*[13] used this strategy to establish persistent infection by Coxsackievirus *in vitro*.

Afterwards, we performed the first passage and obtained a $10^{10.5}$ $TCID_{50}$/mL viral titer. Nevertheless, we found that some cells grew with this level of the infectious virus and multiplied to the confluence at 20 dpi. Similarly, in research by Kopliku *et al.*[14], this strategy allowed the establishment of a model of persistent infection in bovine kidney epithelial cells with FMDV.

In other words, the strategies mentioned allowed for a balance between the presence of the virus and cell multiplication, which are characteristics of viral persistence in culture[15].

Additionally, in the subsequent passage at 20 dpi, we obtained confluent monolayers 72h after the passage; this phenomenon occurred in the following passages, unlike non-infected cells that reached confluence between 24 to 48 h. In this sense, it has been shown that surviving cells that are regularly passaged for a long time become steady-state persistently infected cells[16].

Notably, we assessed the infectivity of HAdV-36 in long-term infected passages quantifying an extracellular infectious virus. From 20 dpi until after 200 dpi, cultures continuously released infectious viruses with titers ranging from 10^4 to 10^6 TCID50/mL, almost half of the titer obtained in the infection at 14 dpi ($10^{10.5}$ $TCID_{50}$/mL) (Figure 1A). These results are interpreted as active viral replication, as revealed by the infectivity that has been evaluated in other *in vitro* persistence models[13]. Indeed, we chose it here because other methods, such as PCR, have low specificity for detecting infectious viruses[17]. However, we used PCR to obtain a 320 bp amplicon, corresponding to a fragment of the *E1a* gene, as an indicator of HAdV-36 entry into Vero cells (Figure 1B). E1A was tested because these immediate-early proteins drive cells into the S phase and induce the expression of all other early viral genes. Thus, we viewed positive signals for the HAdV-36 antigen using immunostaining-IFI (Figure 2, A to I). However, the cells did not show a homogeneous pattern in viral antigen expression (cells with different levels of green fluorescence brightness) (Figure 2, F and I). Therefore, the quantitative expression of the viral antigen was determined using flow cytometry assays and the expression antigen was found in ~50% of the long-term infected cells (Figure 2L), which was lower than the expression in the acutely infected cultures (~80%) (Figure 2K).

As mentioned earlier, the carrier-state persistence feature shows a heterogeneous pattern of virus-infected cells[15]. Alternatively, it has been shown that there is an unstable dynamic phase at an early stage of the process to establish a persistent infection in a steady-state in cultured cells, for which the viruses capable of establishing a persistent infection would be selected[16], especially to control Vero cell infection by HAdV-36 for a longer time.

Long-term infections have been documented in other adenovirus species and other cell lineages[18]. However, none of

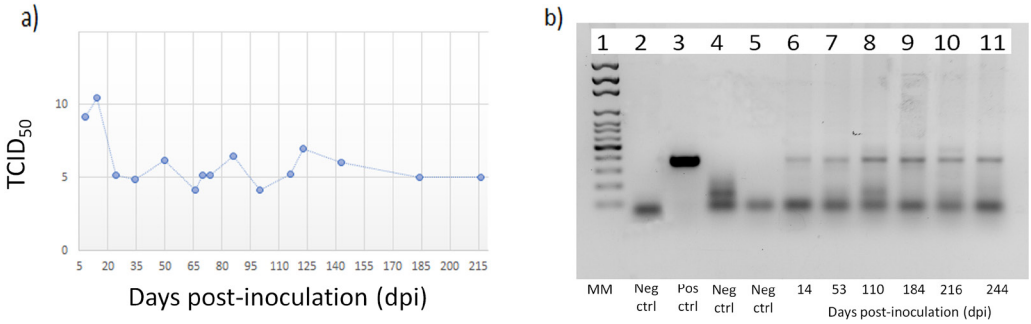

Figure 1 - The persistent presence of HAdV-36 in long-term infected cultures: A) In long-term infected passages (more than 20 dpi), the production of extracellular virions was found with titers ranging between 10^4 to 10^6 $TCID_{50}$/mL, which were lower than the titer found in acutely infected cultures ($10^{10.5}$ $TCID_{50}$/mL at 14 dpi or less). Representative experiments throughout the passages; B) Presence of HAdV-36 DNA-bearing cells in long-term infected cultures were revealed by obtaining the 320 bp length amplicons corresponding to a fragment of the *E1a* gene using conventional PCR. Representative experiments of acutely infected cells at 14 dpi, in lane 6, and long-term infected passages at 53-, 110-, 184-, 216- and 244-days post-inoculation, respectively, in lanes 7-11.

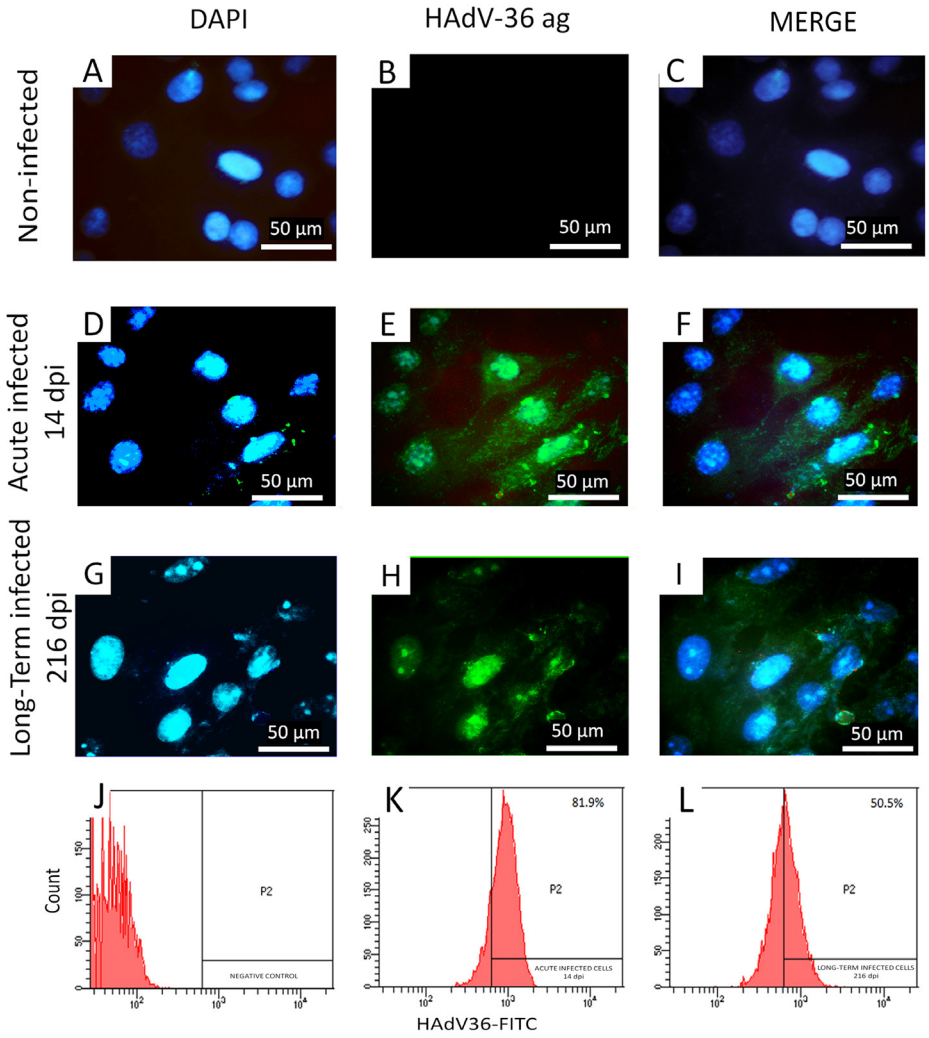

Figure 2 - The HAdV-36 antigen was expressed in long-term infected Vero cells. Non-infected cells as control (A, B, C). Immunostaining of antigen in acutely infected cells at 14 days post-inoculation (D, E, F). Representative images of immunostaining for HAdV-36 antigen in long-term infected cells (216 days post-inoculation) (G, H, I) show the pattern of positive immunolocalization of the viral antigen (green fluorescence). Nuclei were counterstained with 4´, 6-diamidino-2-phenylindole (blue fluorescence). Microphotographs at 400X magnification. Images were captured using fluorescence microscopy. Scale bar 50 µm; Quantitative expression of HAdV-36 antigen in Vero cell cultures. Long-term infected Vero cell cultures expressed the viral antigen in 50% of the cells (L) compared to acutely infected cells in which 80% expressed the viral antigen (K), analyzed through flow cytometry. Non-infected cells were included as control cells (J). Representative experiments are shown at 14- and 216-days post-inoculation.

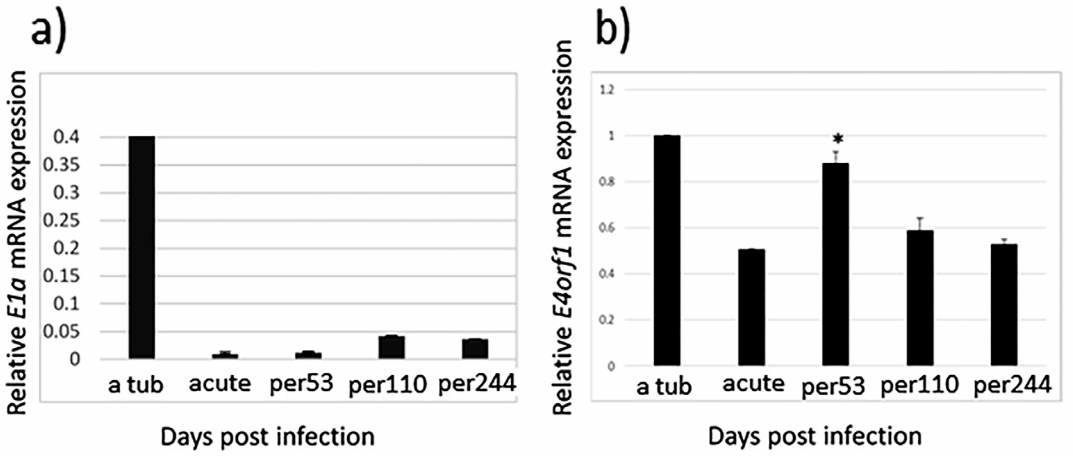

Figure 3 – Expression of *E1a* and *E4orf1* were induced at detectable levels since the first long-term infected passages, in decreasing tendency. Relative expression of *E1a* (A) and *E4orf1* (B) mRNA was determined in passages of long-term and acutely infected Vero cells through normalization of the amount of mRNA of α-tubulin in the housekeeping gene qRT-PCR using the ΔΔ Ct method. Representative experiments are shown at 14-, 53-, 110-, and 244-days post-inoculation. The data represent the average of three independent experiments. Significant differences for α-tubulin are indicated by (*) $p < 0.05$.

these infections have been with HAdV-36. In addition, we have achieved long-term infections in murine macrophages in our lab, but they have not been sustained as much as in Vero cells.

In another sense, several studies illustrate that E4orf1 and E1A are essential for viral replication and interaction with each other. In addition, these proteins have been related to the reprogramming effect of cellular metabolism caused by adenovirus infection[2]. Consequently, we analyze the relative expression of *E1a* and *E4orf1* in HAdV-36 long-term infection versus acute infection using α-tubulin as a housekeeping gene (Figure 3). In long-term infected cells, we found low relative expression of *E1a* (Figure 3A) and a decrease in the relative expression of E4orf1 after 53 dpi (Figure 3B). However, we did not find a significant difference in gene mRNA ratios in acutely and long-term infected cell passages beyond 53 dpi. In contrast, this was observed on an *in vitro* model of persistent HAdV-C5 infection (in interferon-expressing cells) that resulted in the repression of *E1a* expression[19].

Interestingly, the expression of E4orf1 mRNA in the long-term infected passage at 53 dpi showed a significant difference, although, after this time, the expression was not significant with respect to acutely infected cells (Figure 3B). This finding leads to further investigation of whether E4orf1 can stimulate the secretion of growth factors that subvert apoptosis, as found by Jerebtsova *et al.*[20] in renal glomerular endothelial cells, knowing that the mechanisms that favor cell survival play an essential role in establishing viral persistence. The relative expression level of both viral genes was lower than the expression level of the housekeeping gene. Equally, Dickherber and Garnett-Benson[21] studied this in their model of persistent infection by Ad5dl309 in lymphocytes. Moreover, in persistently infected cells, it has been found that viral gene transcription can be repressed. On the other hand, the viral gene expression pattern has been studied in murine fibroblasts through an *in vitro* HAdV-36 infection in the short term (1–2 weeks post-infection)[10,22]. However, nothing is known about expression during long-term infection in renal epithelial cells. Indeed, our findings suggest that HAdV-36 was established as a persistent carrier-state infection in Vero cells.

CONCLUSION

Finally, our results prelude investigating genes involved in HAdV-36 persistence in epithelial cells. In addition, we could have a model to study the effect of HAdV-36 and its proteins on the expression of molecules related to the entry of LCFA, such as CD36 and FATP4, potential targets to attenuate lipid accumulation in the kidneys.

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AUTHORS' CONTRIBUTIONS

MGRV conceived and designed the research. JGSB and FMH contributed to the planning of the experiments; FMH, JGSB and MGRV performed the data analysis; PAV performed the culture viral infectivity assays, the immunocytochemical assays and the flow cytometry assays; PAV and FSA performed the PCR assays; MGRV, MP, ODA and PAV wrote the manuscript. All authors have read, commented on and approved the final manuscript.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

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