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Short communication

First report of the oncolytic effect of EHV-1 on the non-small lung cancer – *in vitro* studies

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Abstract

In recent years there have been a growing number of reports on applying viruses in oncological treatment. In the present study, we demonstrated for the first time that animal virus EHV-1 productively replicates in the human adenocarcinoma cell line (A549) without the need for adaptation. Real-time PCR analysis and immunofluorescence assay showed that EHV-1 could infect and causes lysis of human lung cancer cells. According to our results, we can assume that EHV-1 has oncolytic potential.

Key words: EHV-1, adenocarcinoma cell line, oncolytic virus

Introduction

Cancer poses a major problem for contemporary medicine. For that reason, new methods based on targeted therapy, such as virotherapy, are under development. Viruses with well-known genome sequences may be genetically modified so that the virus gains the ability to infect cancer cells. The first oncolytic virus (modified human herpesvirus type 1, HHV-1) was registered in 2015 in the USA and was later approved by FDA (Food and Drug Administration). During its replication, the virus produces granulocyte-macrophage colony-stimulating factor (GM-CSF). Applying this therapy led to a marked improvement in survival rates in patients with melanoma (Liu et al. 2003). However, it still comes under consideration whether use or not to use human viruses as the patients may have antibodies

against them before starting therapy (Chodkowski et al. 2017). Consequently, applying animal viruses such as equine herpesvirus type 1 (EHV-1), can be considered.

EHV-1, an important pathogen for horses, causes various clinical disorders ranging from mild respiratory disease, abortion, and neonatal foal death to severe neurological disorders as equine herpesvirus myeloencephalopathy (EHM) (Cymerys et al. 2010). It can infect an extensive range of cell types in the respiratory tract, lymphoid organs, and the nervous system. It is believed that the key receptor that EHV-1 uses to enter cells is MHC class I (Drukker et al. 2002). Even though its ability to infect human cells, EHV-1 is not pathogenic for humans, and therefore its application could be considered in anticancer treatment.

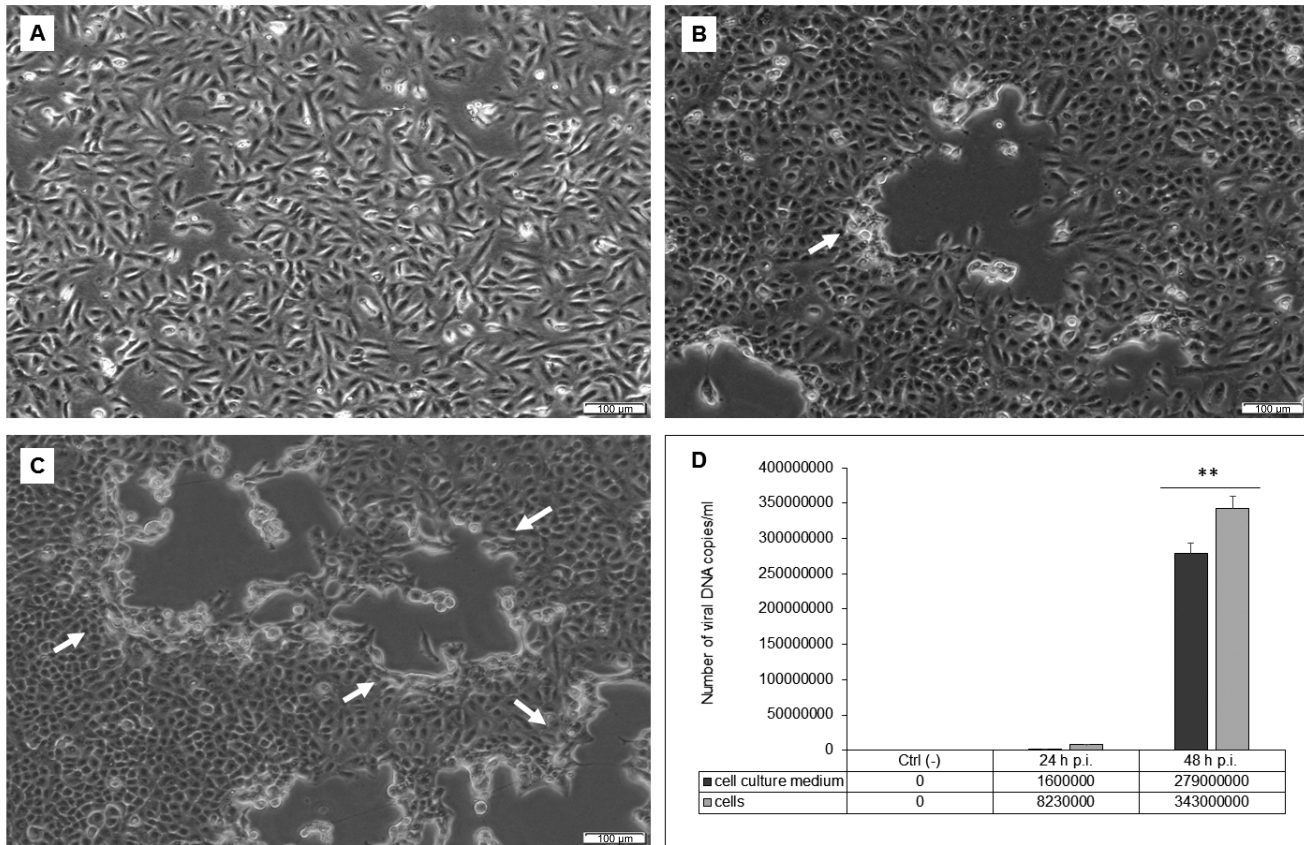


Fig. 1. Morphological changes of A549 cells infected with Jan-E EHV-1 strain (A-C). CPE was manifested by cell destruction and form plaque (white arrows); (A – 0 h p.i.; B – 24 h p.i.; C – 48 h p.i.). Scale bar: 100 μ m. (D) Real-time PCR analysis of viral DNA level during infection. Comparison of viral DNA level (viral gB copy number per mL) in A549 cell line and cell culture medium during 24 and 48 hour post infection. Statistical differences were interpreted as significant at $p \leq 0.05$ (*), highly significant at $p \leq 0.01$ (**) and not significant at $p \geq 0.05$. Bars=100 μ m

Materials and Methods

Cell culture and virus strains

A549 cell line (non-small-cell lung cancer) was cultured in Modified Eagle's medium (MEM), with 2 mmol/l L-glutamine, 10% FBS/FCS, and antibiotics – 10 U/ml penicillin and 100 μ g/ml streptomycin. ED cell line (equine dermal; ATCC CCL57), a homologous cell line, was suspended in Eagle's minimum essential medium (MEM) with Earle's salts, 5% of inactivated foetal calf serum, and 40 mg/ml of gentamicin (Gibco Life Technologies), was used to propagate the virus. Both types of cells were maintained at 37°C with 5% CO₂. In this study, a field non-neuropathogenic strain of EHV-1 (Jan-E) isolated from aborted foetus (neuropathogenicity confirmed by PCR-RFLP neuropathogenic/non-neuropathogenic discrimination test) was used (Cymerys et al. 2016). A549 cells (10⁷ cells per well) were infected with Jan-E EHV-1 strain (MOI = 1.0) and incubated for 24 and 48 hours at 37°C with 5% CO₂.

Real-Time PCR

For the detection and quantification of EHV-1 DNA, a quantitative real-time PCR (qPCR) analysis with fluorescent TaqMan probes (150 nM), complementary for the sequence within the amplified products was performed. Sequences of primers and probe, complementary to the EHV-1 gB gene, were as follows: forward-5' CACGTCTTTAGCGGTGAT3', reverse-5' CAAGCTCGTTCAGGTACAG 3', probe-FAMTGCATTCAGACCTATGCTCTCCAAC-BHQ. The reaction mixture was prepared using TaqMan Master Kit® (Roche Diagnostics). qPCR tests were run on the LightCycler 2.0 instrument (Roche Diagnostics) according to the in-house quantitative method, previously described in Słowska et al. (2016). Fluorescence levels were detected at 530 nm wavelength, specific for FAM fluorophore dye. Data was analyzed with GraphPad InStat version 3 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences were interpreted as significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**).

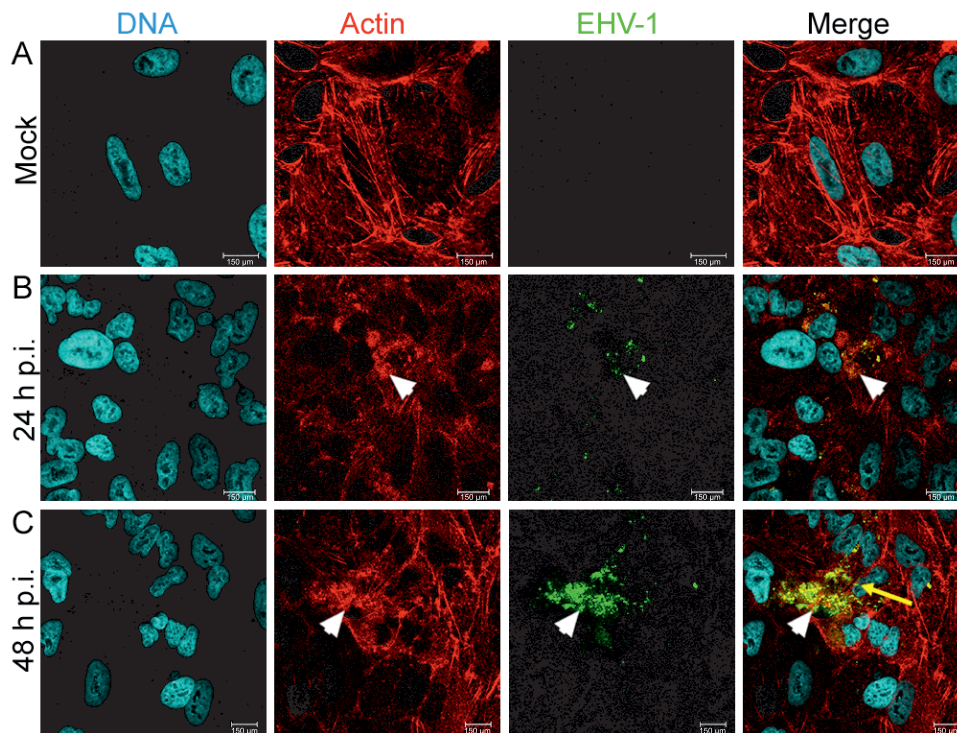


Fig. 2. Immunofluorescence images of Jan-E EHV-1-infected A549 cell line; A – control, uninfected cells; B – 24 h p.i.; C – 48 h p.i. with staining for actin (red fluorescence), EHV-1 (green fluorescence), nuclei (blue fluorescence). Arrowheads indicate accumulation of viral antigen in perinuclear area at 24 and 48 h p.i. Yellow arrow on panel C indicates the presence viral antigen inside the nucleus. Bars=150 µm

Immunofluorescent staining procedures

For direct immunofluorescence assay, cells were grown on microscopic coverslips placed in 6-well plates and infected with Jan-E EHV-1 at the MOI of 1.0. Filament structures of actin were visualized by TRITC-phalloidin conjugate (500 ng/ml; Sigma-Aldrich). The presence of viral antigen was detected by means of direct immunofluorescence, using polyclonal antiserum EHV-1/ERV conjugated to FITC (VMRD, Inc.). Cell nuclei were stained with Hoechst 33258, in compliance with manufacturer's recommendations. Slides were examined using Fluoview FV10i laser scanning confocal microscope (Olympus Poland Sp. z o.o.). Images were captured at a 60x magnification using FV10i software converted to 24-bit tiff files for visualization.

Results and Discussion

During EHV-1 infection a cytopathic effect (CPE) was visible as changes in A549 cells morphology was observed (Fig. 1). After 24 h p.i., CPE was manifested by destruction of cells and focal degeneration (Fig. 1B). Moreover, we observed that the infected cells surrounding the plaque were rounded and fused with the adjacent cells to form syncytia (Fig. 1C, white arrows). These observations indicated that productive replica-

tion cycle of EHV-1 in this cell cultures has occurred. Moreover, in cells which did not undergo lysis, accumulation of viral antigens in the cytoplasm, accompanied by increased density of actin fibres was detected. Viral antigens were accumulated in perinuclear area at 24 h p.i. (Fig. 2 B-C, arrowheads). In addition, viral antigen was also detected in nucleus at 48 h p.i. (Fig. 2C, yellow arrow).

Real-time PCR was applied to detect viral DNA in A549 cells in order to determine the level of virus replication ($CCID_{50}$) (Fig. 1D). For this analysis, viral DNA was isolated separately from cells and cell culture medium. The highest number of viral DNA copies in the cells was noted at 48 h after EHV-1 infection. However, between 24 and 48 h p.i., there was a tenfold increase in the number of viral DNA copies in the cell culture medium, which clearly indicates the viral replication and the release of progeny virions. The level of EHV-1 replication in the A549 line is similar for this observed in equine dermal cell line (natural host) (Cymerys et al. 2016).

Application of EHV-1 as an oncolytic virus in the treatment of glioma cell lines was already proposed by Courchesne et al. (2012), and White and Frampton (2013). In our opinion, it is warranted to investigate its oncolytic potential on other cancer cell lines, such as lung cancer, that is one of the most common mali-

gnancies. The current study is the first to demonstrate that the animal virus EHV-1 can infect and lyse human lung cancer cells. Our preliminary studies indicate that EHV-1 infection in cancer cells is productive. These results open the path of further research to using EHV-1 as a oncolytic virus.

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