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Standardized large-scale H-1PV production process with efficient quality and quantity monitoring

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The promising anticancer properties of rodent protoparvoviruses, notably H-1PV, have led to their clinical testing. This makes it necessary to produce highly pure, well-characterized virus batches in sufficient quantity. The present work focused on developing standardized production, purification, and characterization procedures as a basis for exploiting H-1PV both preclinically and in clinical trials for anticancer virotherapy. Two infection and two virus purification strategies were tested and the resulting virus preparations compared for their purity and full-, infectious-, and empty-particle contents. The adopted production process, which involves culturing and infecting NB-324K cells in 10-layer CellSTACK® chambers (1×10^3 infectious units per infected cell), is simple, scalable, and reproducible. Downstream processing to eliminate contaminating DNA and protein includes DNAse treatment, filtration, and two Iodixanol density-gradient centrifugations, the first gradient being a step gradient and the second, either a step (1×10^{10} PFU/ml) or a continuous gradient (3×10^{11} PFU/ml). A procedure was also developed for obtaining infectious particle-free preparations of empty virions for research purposes: cesium chloride density gradient centrifugation followed by UV irradiation (1×10^{14} physical particles/ml). For quick, sensitive determination of physical particles (and hence, particle-to-infectivity ratios), a "Capsid-ELISA" was developed, based on a novel monoclonal antibody that specifically targets assembled capsids.

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1. Introduction

H-1PV belongs to the genus Protoparvovirus within the Parvovirinae subfamily of Parvoviridae (Cotmore et al., 2014). It consists of a non-enveloped icosahedral capsid 25 nm in diameter and contains a single-stranded DNA genome about 5 kb long, encoding non-structural proteins – notably NS1 (83 kDa) and NS2 (25 kDa) – and the capsid proteins VP1 (81 kDa) and VP2 (65 kDa). Another capsid protein, VP3 (63 kDa), is generated by post-translational cleavage of VP2 (Faisst et al., 1995; Halder et al., 2012; Hanson and Rhode, 1991; Toolan et al., 1960). Protoparvoviruses replicate in an S-phase-dependent fashion and undergo a lytic cycle after infection of permissive cells (Burnett et al., 2006). While the natural host of H-1PV is the rat, this virus has recently raised much interest because it replicates preferentially in transformed cells, including a number of human tumor cells. The virus thus has oncolytic and oncosuppressive properties that have been demonstrated in various cell cultures and animal models (Nuesch et al., 2012; Rommelaere et al., 2010). In xenograft models, H-1PV has been shown to suppress a number of human tumors, including cervical tumors (Faisst et al., 1998; Li et al., 2013), pancreatic tumors (Angelova et al., 2009b; Grekova et al., 2011), mammary carcinomas (Dupressoir et al., 1989), gliomas (Geletneky et al., 2010; Kiprianova et al., 2011), and lymphomas (Angelova et al., 2009a). On the basis of these preclinical proofs of concept, a first clinical trial (phase I/IIa) of H-1PV ParvOryx01 (clinical trial identifier NCT01301430) was launched in 2011 in patients with recurrent glioblastoma multiforme (Geletneky et al., 2012),

To test and eventually exploit the therapeutic potential of H-1PV, it is necessary to develop an efficient, simple, and reproducible virus production process and reliable assays for the quantitative and qualitative characterization of final virus batches. Methods have been published for small-scale production of H-1PV in NB-324K cells and its purification by cesium chloride (Halder et al., 2012; Paradiso, 1981) or Iodixanol (Wrzesinski et al., 2003; Zolotukhin et al., 1999) density gradient centrifugation. The literature also describes titration of infectious virions by plaque assay

Abbreviations: CPE, cytopathic effect; CS, CellSTACK®; CsCl, cesium chloride; EU, endotoxin unit; ELMI, electron microscopy; GP, genome containing viral particle; HAU, hemagglutination unit; HCP, host cell protein; IOD-PBS, Iodixanol-PBS; IU, infectious unit; MOI, multiplicity of infection; PFU, plaque forming unit; PP, physical particle; Q-PCR, quantitative real time PCR; RI, refraction index; RT, room temperature; VIS-Ringer, Visipaque-Ringer.

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(Tattersall and Bratton, 1983), of H-1PV physical particles by electron microscopy (Halder et al., 2012) or hemagglutination assay (Kongsvik and Toolan, 1972), and of genome-containing particles by quantitative PCR (Lacroix et al., 2010). However, no systematic, comparative analyses have been conducted to determine and optimize virus yield and purity or the sensitivity of analytical methods for virus quantification and quality monitoring. The aims of the present study were thus to standardize and optimize H-1PV production, purification, and characterization. This has led us to introduce major innovations for large-scale virus production, with elimination of unwanted contaminants through improved virus batch clarification and infectious particle purification. We have also focused on obtaining infectious-virion-free empty-particle batches for use as non-infectious controls in preclinical studies. To detect assembled viral capsids, we have developed a quick, convenient ELISA exploiting an in-house-generated monoclonal antibody (BL-H1) that might further be used for basic investigation of protoparvovirus assembly and to monitor viremia and seroconversion in virus-treated patients. Altogether, these standardization and development efforts have yielded reliable up- and downstream processes for the reproducible and efficient preparation of H-1PV stocks meeting the quantity and quality requirements for clinical-grade virus batches.

2. Materials and methods

2.1. Producer cell line, H-1PV virus stock

NB-324K human newborn kidney cells transformed with simian virus 40 (SV40) (Tattersall and Bratton, 1983) were cultured at 37 °C in minimum essential medium (MEM, Sigma, Germany) with 5% heat-inactivated fetal bovine serum (FBS, Biowest, France) in a 5% CO_2 atmosphere. The medium was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Germany). For production, NB-324K cells propagated in 175-cm^2 Y-flasks (Nunc, Denmark) were seeded into a 10-layer CellSTACK® culture chamber (Corning, Germany) with a 6360 cm^2 growth area. Cell density and viability were measured by staining living cells with 0.4% trypan blue (Invitrogen™, Germany). Cells were counted with a Countess® Cell counter (Life Technologies, Germany). An in-house purified H-1PV virus stock was used to infect the cells.

2.2. H-1PV production

A 10-layer CellSTACK® (CS) was chosen as a convenient single-use production system. For simultaneous cell seeding and infection, NB-324K cells were seeded at 3.6×10^4 cells/cm^2 into the 10-layer CS and infected immediately with H-1PV at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU) per cell. The pH during infection was 7.0 ± 0.1. The infected cells were incubated for 4 days at 37 °C under 5% CO_2 until the cytopathic effect (CPE), measured as the percentage of dead and detached cells observed under a microscope, reached at least 30%. For non-simultaneous seeding and infection, NB-324K cells were seeded at 7.9×10^3 cells/cm^2 into a 10-layer CS and allowed to grow for three days, by which time they had reached a density of approximately 3.6×10^4 cells/cm^2, as measured on a control-flask culture. These anchored cells were then infected at a MOI of 0.01 PFU/cell and incubated for 4 days as described above. For harvesting, the medium was aspirated and infected cells were treated with PBS/1 mM EDTA. The medium supernatant and detached cells were centrifuged for 5 min at $5000 \times g$. The pellet was washed with PBS, resuspended in Virus Tris/EDTA lysis buffer, pH 8.7 (VTE) containing 0.05 M Tris–HCl, 0.5 mM EDTA, and subjected to three freeze–thaw cycles. After centrifugation for 5 min at $5000 \times g$, cell debris were discarded. The cell lysate was then sonicated at 48 W for 1 min in a Sonorex Super 10 P ultrasonic homogenizer (Bandelin, Germany) and treated with DNAse (50 U/ml, Sigma, Germany) for 30 min at 37 °C to eliminate the non-encapsidated viral DNA and contaminating host cell DNA.

2.3. H-1PV purification

The DNase-treated virus harvest was clarified by filtration through a 0.2-μm Sartolab® P20 Plus filter (Sartorius, Germany). Two different methods were used to purify the virus, either two consecutive Iodixanol gradients or a cesium chloride density gradient followed by dialysis against VTE buffer. The consecutive Iodixanol gradients consisted of a first step gradient of Iodixanol-PBS (IOD-PBS) followed by either a step or a continuous gradient of Visipaque-Ringer (VIS-Ringer).

2.3.1. IOD-PBS and VIS-Ringer density step gradients

To eliminate proteins, a step density gradient centrifugation was done as described by Zolotukhin (Zolotukhin et al., 1999). For this, 25 × 89 mm Quickseal tubes (Beckmann, Germany) were filled with 20 ml virus suspension. This suspension was underlaid with four layers of Iodixanol (Axis-Shield, Norway) in PBS (Iodixanol concentrations: 15%, 25%, 40%, and 60%). Ultracentrifugation was performed for 2 h at 4 °C in a 50.2 Ti rotor at 50,000 rpm (Beckmann, L870 M, Germany). Usually, 3.5 ml virus suspension was collected from the 40% Iodixanol layer. Afterwards, a second density gradient centrifugation was performed with Visipaque (GE Healthcare, Norway) diluted in Ringer solution (B. Braun, Germany) for further protein elimination and separation of full from empty particles. For this, 25 × 89 mm Quickseal tubes were filled with virus suspension from the IOD-PBS density gradient, diluted at least 1:2.5 in Ringer solution. Then 5 ml of 25%, 4 ml of 40%, and 4 ml of 55% Visipaque in Ringer solution were underlaid. For detection of the 40% layer, a reference gradient was made, where the 25% and 55% Visipaque/Ringer phases were colored with phenol red. Additionally, the location of the 40% phase was marked on the outside of the sample tube. Ultracentrifugation was performed for 2 h at 4 °C in a 50.2 Ti rotor at 50,000 rpm. After centrifugation two fractions were collected in the region of the original 40% phase using a syringe and hollow needle: a 2.5 ml lower band and a 800 μl upper band corresponding to the full and empty-particle fractions. The refraction index of a 5 μl sample was measured with a refractometer (AR200, Reichert Analytical Instruments, Germany) and the densities of the regions from which the fractions were taken were calculated using a reference table for Iodixanol (Axis-Shield, Norway).

2.3.2. Continuous VIS-Ringer gradient

For the continuous VIS-Ringer gradient, Quickseal tubes were filled with virus suspension diluted in Ringer solution to a refraction index of 1.3815 (corresponding to 30% Visipaque). The virus suspension was underlaid with 0.5 ml of 65.2% Visipaque cushion, and the tube was completely filled with 30% Visipaque/Ringer solution. Ultracentrifugation was performed for 10 h at 4 °C in a 70.1 Ti rotor at 63,000 rpm. Fractions of about 500 μl were collected from the bottom under controlled dripping.

2.3.3. Cesium chloride density gradient and dialysis

A CsCl density gradient was established as described previously (Paradiso, 1981). For this, 14 × 95 mm polyallomere centrifuge tubes (Beckmann, Germany) were filled with 5 ml CsCl at 1.4 g/cm^3 density and overlaid with 1 ml 1 M saccharose followed by 5 ml virus suspension. Ultracentrifugation was performed at 15 °C for at least 20 h at 39,000 rpm in an SW41 rotor. Different fractions were collected from the bottom (fr# 1: 500 μl, fr# 2: 300 μl, fr# 3–20: 200 μl) and the capsid (physical particle, PP) content was measured

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