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Efficient replication of the arginine-glycine-aspartic acid (RGD) lacking Human Parechoviruses in 3D Human Airway Epithelial Cell models

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Abstract

Human parechovirus (HPeV) infections are mainly found in children, associated with a broad range of symptoms, including respiratory symptoms. The respiratory epithelium is the initial tissue exposed to HPeVs since transmission of HPeVs is supposed to be oral-fecal, suggesting a role for respiratory epithelium in HPeV entry and replication. Difficulties have been shown in detection of specific HPeV types in cell culture, most pronounced for HPeV3 and the more recently detected HPeV7-16, which have never been successfully propagated in cell culture. HPeV3 and the newer genotypes HPeV7-16 lack the arginine-glycine-aspartic acid (RGD) motif in the VP1 capsid region. This region is essential for HPeV1 receptor binding and entry.

In this study we used primary well-differentiated human airway epithelium (HAE) cell cultures from different origin: an in house bronchial HAE as well as commercial bronchial and nasal HAE cells MucilAir™ (Epithelix). We showed efficient replication of HPeV3 and to a lesser extent of HPeV1 in contrast to what has been shown before using standard cell culture systems. The new RGD-lacking genotypes HPeV9 and HPeV14 and a recombinant HPeV3 strain, which were not able to replicate on standard cell cultures, could be propagated on HAE. Therefore, the HAE cell culture system provides a new tool for further studies on tropism and pathogenesis of RGD-less HPeV types, which could lead to the further characterization of receptors for the RGD-lacking HPeVs and other human picornaviruses.
Introduction

The human parechoviruses (HPeVs) belong to the family Picornaviridae, a large and diverse group of positive sense RNA single stranded viruses (1). HPeVs are widespread, causing disease mainly in young children, with clinical symptoms ranging from mild respiratory and gastrointestinal symptoms to severe neonatal sepsis and infections of the central nervous system (CNS) (2). The genus Parechovirus contains 16 HPeV genotypes based on sequencing of the VP1 region (3). The VP1 region encodes for one of the three capsid proteins VP0, VP3 and VP1. The C-terminus of the VP1 capsid protein of HPeV1, 2, 4, 5 and 6 contain the arginine- glycine- aspartic acid (RGD) motif, which has been shown to be essential for HPeV1 receptor binding and entry (4–6). HPeV3 and the more recently identified types HPeV7-16 lack this RGD motif suggesting an alternative receptor or entry route for these viruses. Indeed, several studies showed difficulties to detect HPeV3 in cell cultures regularly used for HPeVs because only a low induction of cytopathic effect (CPE) could be scored (7, 8). Similarly, the newer HPeV7-16 were all detected by PCR in patient derived samples and could not be cultured in standard cell lines (9–11). For HPeV3 we showed that when virus replication in cell culture was monitored by RT-PCR instead of CPE, most cell lines supported replication of HPeV3 (12, 13). Still several HPeV strains, mostly lacking the RGD motif, were unable to replicate in our standard cell lines. We discovered an HPeV1 strain (HPeV1-652281) that was closely related to the prototype HPeV1-Harris strain but lacked the RGD motif, despite its essential role for HPeV1 in utilising the αvβ3, αvβ6 integrins as receptors (5, 6, 14, 15). Furthermore, we discovered an RGD-less HPeV5 (HPeV5-652444). Both strains showed a specific sequence; Isoleucine- Serine- Asparagine (ISN)/ Threonine- Serine- Asparagine (TSN) at the position of the RGD motif where HPeV3 has a deletion (9). Previously we described an interesting HPeV3 strain that could not be cultured (16). This HPeV3-651689 is the only HPeV3 strain that does not exclusively cluster in phylogenetic trees with HPeV3 in both structural as non-structural genomic regions. In the non-structural regions HPeV3-651689 mainly clustered with HPeV7 PAK5045 as shown by phylogenetic analysis of whole genomes (11, 16). Of the newer HPeV genotypes, HPeV14 (HPeV14-451564) was first discovered in our laboratory by PCR screening in faeces (9), while HPeV9, first described by Oberste et al., (Oberste et al., unpun., http://www.picornaviridae.com), was found once in our samples in 2010 (HPeV9-1051908). An RGD-containing strain that could not be cultured was HPeV2-950912, showing distinct features from the HPeV2-Williamson strain; HPeV2 is hardly detected in the Netherlands (9, 17). HPeV strains lacking the RGD motif show differences in in vitro growth characteristics compared to RGD containing HPeVs such as HPeV1 (13) thereby implying use of an (unknown) different cellular receptor and thus a potentially different cell tropism.
The respiratory epithelium is the initial tissue exposed to HPeVs since transmission of HPeVs is supposed to be oral-fecal, thus suggesting a role for respiratory epithelium in HPeV entry and replication. Recently, primary human airway epithelial (HAE) culture systems have become available, closely mimicking the in vivo human respiratory environment (18–20). Several viruses that could not be propagated in standard cell culture indeed have successfully been cultured in these HAE culture system, such as rhinovirus C, a picornavirus species belonging to the Enterovirus genus (19, 21, 22). Therefore we used the HAE cell culture as a primary culture system to study replication of RDG-less HPeV strains.

Materials and Methods

Virus strains

The following HPeV strains were used as prototypes: HPeV1A Harris, provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, HPeV2-2008, HPeV4-251176, HPeV5-552322 and HPeV6-550389 and passaged to obtain a sufficient virus stock; HPeV3-150237 (23) isolated from stool and passaged two to three times to obtain sufficient virus stocks. HPeV1, 2, 4, 5 and 6 were cultured on HT29 cells, HPeV3 on Vero cells; both cell lines were maintained in Eagle’s Minimum Essential Medium (EMEM) supplemented with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 μg/ml) and ampicillin (0.1 μg/ml). The virus working stocks were stored in aliquots at -80°C. The virus concentration was determined in standard cell culture by the median tissue culture infective dose (TCID50) and calculated by the Reed and Muench method (24). Stool samples containing a selection of different HPeVs which could not be cultured in standard cell culture, HPeV1-652281, HPeV2-20950912, HPeV3-651689 (16), HPeV5-652444 (9), HPeV9-1051908, HPeV14-451564 (9) were used for further culture on HAE.

HAE cell culture.

Normal primary human bronchial epithelial cells (HBEpC) were derived as described previously (18, 21), the HBEpC were maintained at 37°C in a 5% CO2 incubator for one or two serial passages as a monolayer in bronchial epithelial cell serum-free growth medium (BEGM) (20), medium was refreshed at 2- or 3-day intervals. At 75% confluence, cells were dissociated with 2 ml of TrypLE Express enzyme (Invitrogen). HTEpC were diluted in air-liquid interface (ALI) medium (20). A total of 2*10^5 HTEpC were seeded on 12-well or 7*10^4 were seeded on 24- well ThinCerts with a 0.4-μm pore size (Greiner Bio-One). The inserts were pre- coated with type IV collagen (Sigma). Medium was renewed every 2 or 3 days, the apical medium was removed when reaching 100% confluence, creating an air...
liquid interface. Medium from the basolateral compartment was renewed every 2 or 3 days and the apical surface was washed every week with Hanks balanced salt solution (HBBS) (invitrogen). The cultures were maintained for 30 days to let the cells differentiate into well-differentiated pseudostratified human airway epithelium.

For comparison of our in house bronchial HAE, bronchial and nasal epithelial cells from MucilAir™ (Epithelix) were used. MucilAir™ ready-to-use culture medium (Epithelix) from the basolateral compartment was renewed every 2 or 3 days.

**HPeV infection of HAE**

The HAE cells were infected with the different HPeVs, 150 μl of 100 TCID50/50 μl virus stock in 12 well inserts or 50 μl of 100 TCID50/50 μl in 24 well inserts. In case of the clinical HPeV strains 100 μl of 0.22 μm filtered clinical specimen was used for inoculation of the nasal or bronchial 24 well inserts. The virus samples were added apically and after 3 hours incubation at 37°C in a 5% CO2 incubator, the inoculum was removed and the cells were washed with HBSS for 10 min at 37°C, this was considered as timepoint 0. Samples were collected from the apical and the basolateral side every 24 hours post infection.

**RNA isolation and detection of HPeV by real-time reverse transcription-PCR**

From all harvested apical wash and basolateral supernatant 25 μl was removed for RNA extraction and quantitative RT-PCR detection at 0, 24, 48 and 72 hours post infection. The supernatant was extracted by automatic extraction using the total nucleic acid isolation kit with the MagnaPure LC instrument® (Roche Diagnostics). The RNA was eluted in 50 μl elution buffer and reverse transcribed as described previously (7). Five μl of cDNA was used for real-time PCR using the LC480 (Roche Diagnostics). The virus copies per PCR were calculated with a standard curve as described previously (25).

**Immuno- fluorescence assay**

Polyclonal antibodies (Abs) used in fluorescence assays against HPeV1 and HPeV3 were obtained by immunization of rabbits (Harlan Laboratories). For detection of viral infection the cells were fixed apical and basolateral with 4% para-formaldehyde (PFA)/ PBS for 30 minutes. The PFA was removed and the cells were washed consecutively 3 times with PBS. To avoid unspecific binding cells were blocked with PBS supplemented with 50 mM NH4Cl, 0.1% saponin and 2% BSA (confocal staining buffer (CB)). After incubation with CB for 60 min apical and basolateral, the apical side is incubated for 1 hour at RT with the primary antiHPeV1 or antiHPeV3 polyclonal Abs and with anti-β-tubulin IV for staining the ciliated cells. Cells are washed 3 times (5’) with CB and incubated with the secondary anti-rabbit secondary goat Abs - fluorescein isothiocyanate (FITC) labelled IgG
(Jackson Immuno Research), antimouse Cy5 labelled IgG and 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) were added and incubated for 1hr 37°C. After washing, the membrane containing the cells was cut out and mounted on a glass slide. The cells were analysed on a Leica SP8 confocal microscope.

Results

Efficient HPeV3 replication in bronchial HAE cells

Well-differentiated human airway epithelial cells isolated from an adult donor were infected with the prototype HPeV strains HPeV1A Harris and HPeV3-150237 to determine replication on primary human cells. Replication was measured by determining the virus RNA load by real-time PCR on time samples from apical washes and the basolateral medium.

![Image](image.png)

**Figure 2.** Confocal images of HPeV1 and HPeV3 infected HAE cells. Virus was detected 72 hours post infection with rabbit polyclonal antiHPeV1 or antiHPeV3 polyclonal Abs, visualized by anti-rabbit secondary goat Abs – FITC (green). Ciliated cells were visualized with anti-β-tubulin IV, anti-mouse Cy5 labelled IgG (Yellow) and the nucleus was stained with DAPI (blue).

The infected HAE cultures did not show recognizable CPE, but viral RNA could be measured after 24 hrs. HAE cells from 5 different adult donors were infected to study reproducibility and donor variation. In 4 out of 5 donors, replication of HPeV1 and 3 could be detected (Fig. 1). In donor 5 no replication could be detected within 72 hrs post infection (data not shown). In contrast to what we have found previously using standard cell culture, replication
of HPeV3 on HAE was more efficient compared to HPeV1 in 3 out of 4 donors, (Fig. 1, donors 2, 3 and 4). Peak infection of HPeV3 was observed after 24 hr in donor 2, 3 and 4 (between 10^5 and 10^6 viral copies/PCR), whereas HPeV1 reached the highest titers after 48 or even 72 hr post infection. Next to comparison of HPeV1 and HPeV3 in HAE we infected the bronchial cells with HPeV2, 4, 5 and 6. HPeV2 replicated on HAE with maximum titers of 10^6 viral copies/PCR 72 hr post infection while replication of HPeV4, 5, and 6 was very low or even absent (n=1, data not shown).

To determine which type of cells from the well-differentiated epithelial culture are infected, viral antigen was identified in the HAE cells by immunofluorescence against HPeV1 and 3, while ciliated cells were distinguished from non-ciliated cells by β-tubulin IV staining. Viral antigen was detected in both ciliated and non-ciliated HAE cells infected with HPeV1 or HPeV3 (Fig 2).

**Figure 1.** Replication of HPeV1 and HPeV3 on well-differentiated bronchial epithelial cells obtained from different donors. The viral replication was determined by measuring viral RNA in the apical wash and the basolateral medium at 24, 48 and 72 hours post infection. HPeV1: orange line- apical, dotted orange line- basolateral, HPeV3: black line- apical, dotted black line- basolateral.

**HPeVs replication in MucilAir™**

To compare replication of HPeV1 and 3 in upper and lower respiratory tract cells, commercially available well-differentiated cells from nasal and bronchial origin (MucilAir™, Epithelix isolated from Caucasian elderly women), were infected with HPeV1 and 3. HPeV1 and HPeV3 showed similar replication patterns; in nasal cells replication could be measured after 24 hr post infection, but the highest titer was reached after 72 hr post infection (10^6 copies/PCR). In bronchial cells even higher titers were reached, up to 10^8 copies/PCR. The replication curves were different from our in house HAE system (Figures 1 and 3). Notable is the dip in bronchial cells after 48 hr where no replication could be measured for HPeV3 and lower replication for HPeV1. This pattern could be reproduced in bronchial cells from two different donors.
Propagation of RGD-less HPeVs in MucilAir™

We subsequently infected both nasal and bronchial (MucilAir™, Epithelix) cells with diluted and filtered stool samples containing the RGD-less HPeV1-652281, HPeV5-652444, HPeV3-651689, HPeV9-1051908 and HPeV14-451564 and the RGD containing HPeV2-950912. HPeV3-651689 replicated on both nasal and bronchial epithelial cells showing a dip around 48 hr post infection, and reaching high titers (10^5 copies/PCR) in bronchial cells but not on nasal cells (10^2 copies/PCR after 96 hr post infection) (Fig 4). HPeV14-451564 showed a similar pattern as HPeV3-651689 in bronchial cells, reaching even higher titers around 10^6 copies/PCR 96 hr post infection. HPeV9-1051908 was able to replicate on bronchial cells, but the first positive samples were obtained only after 72 hr post infection. The HPeV1, 2 and 5 strains could not be propagated from their original sample on MucilAir™.
Figure 4. Infection of HAE bronchial and nasal cells (MucilAir™) with different clinical isolates containing HPeV1-652281, HPeV5-652444, HPeV3-651689, HPeV2-20950912, HpeV9-1051908, HPeV14-451564. Viral replication was followed by detection of viral RNA 24, 48, 72 and 96 hours post infection in de apical wash.

Discussion

In this study efficient replication of HPeV3, and to a lesser extent of HPeV1, was demonstrated using primary well-differentiated human airway epithelial cell cultures in contrast to what is previously shown in standard cell culture systems (7, 8, 26). In addition, with the MucilAir™ HAE system, we were able to propagate patient-derived HPeV9 and HPeV14, as well as the recombinant HPeV3-651689, none of which we could propagate in standard cell culture. Unfortunately, our HPeV1, HPeV5 (which lack the RGD motif) and the outlier HPeV2 strain were not able to replicate. This is most likely due to a low infectious virus present in the faecal samples.

The HAE system seems to be suitable for propagation of RGD-less HPeV types, with higher replication in the bronchial cells, indicating that an as yet unidentified RGD-independent receptor is expressed on these cells. For HPeV1 it has been shown that it utilizes αvβ3 and αvβ6 integrins as its receptor, and that the interaction with the RGD motif in VP1 is essential (5, 14, 15). For the other HPeVs, the receptor is unknown, but the RGD containing strains (HPeV2, 4, 5, and 6) are likely to use the same integrins. Previous studies showed that the vibronectin αvβ3 and αvβ6 receptors are only expressed at low or even undetectable levels in normal healthy airways in vivo and in vitro (27, 28). This could explain the less efficient replication of HPeV1 we observed compared to HPeV3, and the low or even absent replication of HPeV4, 5, and 6 in HAE. However, HPeV1 is still able to efficiently replicate in HAE, suggesting that an αvβ3 and αvβ6 integrin independent entry pathway might be used in healthy airways. Apparently, the unknown receptor for HPeV3 and the other RGD-less
HPeVs is expressed on HAE cells. Respiratory cells may reflect the initial port for entry of HPeV3 and even for a subsequent CNS infection. For EV71 it has been shown that infection of rhesus monkeys with EV71 via the respiratory tract resulted in more severe neurological lesions compared to the orally infected monkeys (29). The efficient infection of HPeV3 of human airway might therefore give a good portal to the CNS. HPeV7-16 have only recently been isolated and have not been detected frequently up to now, therefore only little is known of their clinical outcome and thus involvement in severe HPeV infections.

A relationship between clinical manifestations and in vitro replication characteristics of EV71, CAV9 and Echo9 has previously been suggested (30–37). However, this data is inconsistent; not all Echo 9 strains isolated from patients with paralysis were able to cause paralysis in mice, while some strains isolated from non-symptomatic children did cause paralysis in mice (38). From our previous study we were not able to draw a clear relationship between pathogenicity and replication in cell lines in vitro. HPeV3 strains could replicate efficiently in neural cells, in correlation with their in vivo CNS involvement. However, this correlation could not be shown for all HPeV3 strains and not for HPeV1 (12). All these studies used continuously growing cell lines obtained from (human) tumours or mice, which do not necessarily represent the tissue infected during human infection. Primary cell culture systems provide a much better alternative for pathogenicity studies, in which cells are used representing the tissue which is exposed to viral infection, and containing the receptors expressed in human tissues. During this study we saw variation in replication between different donors as well as between the systems used. This indicates the importance of testing multiple donors for primary cell culture systems as a tool for further research of cell tropism and the RGD-independent entry pathway.

In this study, cells derived from adult tissue were used showing a surprisingly efficient replication of HPeV3. HPeV3 infects mainly young children, adults with symptomatic HPeV3 infection have only been reported in Japan, showing symptoms of myalgia, muscular weakness, sore throat and orchidynia (39, 40). Despite large screenings studies, only a few HPeV3 infected adults have been described elsewhere (13). From our study it can be concluded that HPeV3 can infect adult HAE cells, indicating that entry is not the bottleneck explaining few adult infections in Caucasians. However, to investigate the differences in HPeV3 infection of respiratory tissue between children and adults, more studies are needed including infection of tissue obtained from of children.

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References


