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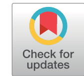
Susceptibility of porcine IPEC-J2 intestinal epithelial cells to infection with porcine deltacoronavirus (PDCoV) and serum cytokine responses of gnotobiotic pigs to acute infection with IPEC-J2 cell culture-passaged PDCoV



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ABSTRACT

The porcine small intestinal epithelial cell line, IPEC-J2, is useful to characterize the interactions of enterocytes with enteric viruses *in vitro*. We investigated whether IPEC-J2 cells are susceptible to porcine deltacoronavirus (PDCoV) infection. We conducted quantification of infectious virus or viral RNA, immunofluorescent (IF) staining for the detection of PDCoV antigens, and TUNEL assay in IPEC-J2 cells inoculated with the strain OH-FD22-P8 grown in LLC-PK cells, and supplemented with 10 μg/ml of trypsin in the cell culture medium. Cytopathic effects (CPE) that consisted of enlarged and rounded cells followed by cell shrinkage and detachment, were identified by the 3rd viral passage in the IPEC-J2 cells. PDCoV antigen was detected in the cells showing CPE. By double IF and TUNEL staining, most PDCoV antigen-positive IPEC-J2 cells failed to show TUNEL-positive signals, indicating that PDCoV-infected IPEC-J2 cells may not undergo apoptosis, but rather necrosis, similar to necrotic cell death of infected enterocytes *in vivo*. There was increased interleukin-6 in PDCoV-infected IPEC-J2 cell culture supernatants at post-inoculation hour (PIH) 48–96, as evaluated by ELISA, concurrent with increased titers of PDCoV at PIH 24–72. The susceptibility of IPEC-J2 cells to PDCoV infection supports their usefulness to characterize the interactions of enterocytes with PDCoV. We also demonstrated that IPEC-J2 cell culture-passaged PDCoV (OH-FD22-P8-I-P4) was enteropathogenic in 10-day-old gnotobiotic pigs, and induced systemic innate and pro-inflammatory cytokine responses during the acute PDCoV infection.

1. Introduction

Porcine deltacoronavirus (PDCoV), a member of the genus *Deltacoronavirus* in the family *Coronaviridae* of the order *Nidovirales*, causes acute diarrhea, vomiting, dehydration and mortality in nursing pigs (Jung et al., 2015b; Lau et al., 2012). PDCoV has been successfully isolated and propagated in two epithelial cell lines of swine origin, LLC porcine kidney (LLC-PK) and swine testicular (ST) cells (Hu et al., 2015). The optimal cell culture conditions to isolate and propagate PDCoV in LLC-PK and ST cells required supplementation of 10 μg/ml of trypsin and 1% pancreatin in the cell culture maintenance medium, respectively (Hu et al., 2015). The addition of trypsin and pancreatin in the PDCoV-inoculated LLC-PK and ST cells, respectively, resulted in similar cytopathic effects (CPE) that consisted of enlarged, rounded, and densely granular cells that occurred singly or in clusters, followed by cell shrinkage and detachment as a result of apoptotic cell death (Jung et al., 2016). On the other hand, infected enterocytes *in vivo* appeared to acutely undergo vacuolar degeneration and exfoliated extensively from the villous epithelium, followed by villous atrophy (Chen et al., 2015; Jung et al., 2015b). This process might be associated with necrosis of the infected enterocytes (Jung et al., 2016).

The porcine enterocyte cell line, IPEC-J2, is a non-transformed, stable small intestinal columnar epithelial cell line (Brosnahan and Brown, 2012; Vergauwen, 2015). The cells were originally isolated from the mid-jejunal epithelium of a neonatal unsuckled piglet in 1989 at the University of North Carolina (Brosnahan and Brown, 2012; Vergauwen, 2015). Because of the significant physiologic and morphologic similarities to enterocytes *in vivo*, this primary cell line has been used increasingly to characterize the interactions of intestinal epithelial cells with enteric bacteria and viruses *in vitro*. IPEC-J2 cells

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have been infected with porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) (Lin et al., 2017; Shi et al., 2017; Xia et al., 2017; Zhao et al., 2014). Compared with PEDV-infected Vero (African green monkey kidney) cells (Guo et al., 2016), the proteomic data from PEDV-infected IPEC-J2 cells appeared to better coincide with the physiologic and pathologic outcomes in PEDV-infected enterocytes *in vivo* (Lin et al., 2017).

In our previous study, IPEC-J2 cells were also evaluated in parallel with LLC-PK and ST cells (Hu et al., 2015). However, IPEC-J2 cells failed to efficiently support the isolation and propagation of PDCoV, despite the fact that IPEC-J2 cells originate from villous enterocytes in the small intestine that are the main site of PDCoV infection *in vivo*. We hypothesized that the lower susceptibility of IPEC-J2 cells to initial infection with PDCoV, compared with LLC-PK or ST cells, could have been increased if virus of higher titer, than that contained in the original fecal samples (Hu et al., 2015), was used as inoculum in IPEC-J2 cells. Therefore, we used the cell culture-adapted PDCoV to investigate: i) whether porcine IPEC-J2 cells are susceptible to infection with cell culture-adapted PDCoV; ii) the cell death mechanism, necrosis or apoptosis, by which PDCoV causes death of infected IPEC-J2 cells *in vitro*; iii) if the cell death of PDCoV-infected IPEC-J2 cells *in vitro* resembles necrosis of infected enterocytes *in vivo*; and iv) whether PDCoV infection induces altered or increased pro-inflammatory or innate cytokine responses in supernatants of IPEC-J2 cells *in vitro* and in the serum of infected gnotobiotic (Gn) pigs *in vivo*. For the *in vivo* study, we inoculated Gn piglets with the IPEC-J2 cell culture-passaged PDCoV to examine the enteropathogenicity and the induction of innate and pro-inflammatory cytokines in the sera during the acute PDCoV infection.

2. Materials and methods

2.1. Virus

The PDCoV OH-FD22-P8 (passage 8) virus was serially passaged in LLC-PK (ATCC CL-101) cells supplemented with trypsin (10 μg/ml) in the cell culture medium for a total of 8 passages, as described previously (Hu et al., 2015). After the 6th passage, the virus was purified once by a plaque assay and then further serially passaged (Hu et al., 2015). The viral RNA titer of the OH-FD22-P8 used in this study was 10.5 log_{10} genomic equivalents (GE)/ml, and the infectious titer was 8.6 log_{10} plaque forming units (PFU)/ml.

2.2. Porcine IPEC-J2 cells

The thirty-second passage of IPEC-J2 cells was kindly provided by Dr. Helen Bershneider at the University of North Carolina, and they were passaged 7 more times in our laboratory. In this study, the IPEC-J2 cells were further passaged up to 18 times (total passages 40–58) before use. Virus was inoculated onto 3–4 day-old confluent cell monolayers. The cells were propagated and passaged in the following growth medium: Dulbecco's modified eagle medium/F12 (DMEM/F12) (Gibco, USA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco), 1% insulin-transferrin-sodium selenite (Roche), and 5 ng/ml of human epidermal growth factor (Invitrogen), as recommended by Dr. Helen Bershneider.

2.3. Infection of IPEC-J2 cells with PDCoV

The cell culture conditions tested to infect IPEC-J2 cells with PDCoV OH-FD22-P8 during each passage of the virus are described in detail in the Results section. During the 1st [multiplicity of infection (MOI), 2.5] to the 2nd passage of the virus, they were as follows: Washing of cells with maintenance medium (DMEM/F12 supplemented with 1% penicillin/streptomycin) (MMT) twice to remove FBS, virus adsorption for one hour, and then washing (with MMT) once and the addition of MMT with 10 μg/ml of trypsin (Gibco). During the 3rd to 5th serial passage of OH-FD22-P8 virus (estimated MOI, 0.1 for the 4th and 5th passages), however, the wash steps were omitted after virus adsorption. Viral CPE was monitored frequently in the inoculated IPEC-J2 cells.

2.4. Periodic-Acid-Schiff or immunofluorescent staining for the detection of the neutral sialylated mucin, serotonin, or leucine-rich repeat-containing G protein-coupled receptor 5 in IPEC-J2 cells

Prior to the infection assays, we attempted to confirm that no other types of cells were present in the IPEC-J2 cells, such as the mucus-secreting goblet cells or the neutrotransmitter serotonin-secreting enterochromaffin cells, and also tested if the IPEC-J2 cell monolayers express leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) (intestinal stem cell marker) antigen by Periodic-Acid-Schiff (PAS) or immunofluorescent (IF) staining at post-inoculation hour (PIH) 24, 48, and 72. IPEC-J2 cells were fixed with 100% ethanol at 4 °C overnight and tested by IF staining, as described previously (Hu et al., 2015; Jung et al., 2015a, 2016), for the detection of serotonin or LGR5 antigen, using monoclonal antibodies against human serotonin or human LGR5 (Novus Biologicals, Littleton, CO, USA). Monoclonal antibodies were diluted 1 in 50 in phosphate-buffered saline. PAS staining was used for the detection of the neutral sialylated mucin-secreting goblet cells, according to the manufacturer's instructions and as described previously (Jung and Saif, 2017).

2.5. Analysis of PDCoV RNA or infectious virus titers in cell culture supernatants

Cell culture supernatants were collected from PDCoV-inoculated and non-inoculated IPEC-J2 cells at the time-points as indicated in the Results section. The RNA was extracted from 200 μl of centrifuged (2000 × *g* for 30 min at 4 °C) cell culture supernatants using the Mag-MAX Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. PDCoV RNA titers in cell culture supernatants were determined, as described previously (Jung et al., 2015b). Infectious-virus titration was conducted by a TCID50 assay, as described previously (Hu et al., 2015). IPEC-J2 cells were seeded into 96-well plates, and after confluence, the monolayers were washed once with MMT. One hundred microliters of 10-fold dilutions of PDCoV were inoculated onto the cell monolayers in eight replicates per dilution. After virus adsorption for one hour, another 100 μl of MMT with 10 μg/ml of trypsin was added to each well. Viral CPE was monitored daily for 4 to 6 days, and IF staining was also done for the detection of PDCoV antigen in IPEC-J2 cells as described below. Virus titers were calculated by using the Reed-Muench method and expressed as $TCID_{50}$ per ml.

2.6. Immunofluorescent staining for the detection of PDCoV antigen in IPEC-J2 cells

When PDCoV-inoculated IPEC-J2 cells showed more than 50% CPE, they were fixed with 100% ethanol at 4 °C overnight. The PDCoV-infected cells were prepared in duplicate and tested by IF staining for the detection of PDCoV antigen, using hyperimmune Gn pig antiserum against PDCoV OH-FD22 strain, as described previously (Jung et al., 2015b; Jung et al., 2016). Trypsin (10 μg/ml) alone-treated IPEC-J2 cells were tested as negative controls for IF staining.

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay in IPEC-J2 cells

The IF-stained IPEC-J2 cells were prepared as described above and evaluated by a TUNEL assay kit (Roche Applied Science, Mannheim, Germany) for apoptosis according to the manufacturer's instructions and as described previously (Jung et al., 2016). The IF-stained IPEC-J2 cells were double-stained by TUNEL assay (Jung et al., 2016). Trypsin

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