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Porcine epidemic diarrhea virus through p53-dependent pathway causes cell cycle arrest in the G0/G1 phase

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV), an enteropathogenic *Alphacoronavirus*, has caused enormous economic losses in the swine industry. p53 protein exists in a wide variety of animal cells, which is involved in cell cycle regulation, apoptosis, cell differentiation and other biological functions. In this study, we investigated the effects of PEDV infection on the cell cycle of Vero cells and p53 activation. The results demonstrated that PEDV infection induces cell cycle arrest at G0/G1 phase in Vero cells, while UV-inactivated PEDV does not cause cell cycle arrest. PEDV infection up-regulates the levels of p21, cdc2, cdk2, cdk4, Cyclin A protein and down-regulates Cyclin E protein. Further research results showed that inhibition of p53 signaling pathway can reverse the cell cycle arrest in G0/G1 phase induced by PEDV infection and cancel out the up-regulation of p21 and corresponding Cyclin/cdk mentioned above. In addition, PEDV infection of the cells synchronized in various stages of cell cycle showed that viral subgenomic RNA and virus titer were higher in the cells released from G0/G1 phase synchronized cells than that in the cells released from the G1/S phase and G2/M phase synchronized or asynchronous cells after 18 h p.i.. This is the first report to demonstrate that the p53-dependent pathway plays an important role in PEDV induced cell cycle arrest and beneficially contributes to viral infection.

1. Introduction

Viral infection disrupts cell cycle by inducing activation of a series of signal transduction pathways, which play an important role in viral life cycle by promoting replication of progeny viruses after viral infection (Davy and Doorbar, 2007). Examples can be found among DNA viruses, retroviruses, and RNA viruses. For example, the cell proliferation induced by Reovirus δ1 s nonstructural protein is blocked in the G2/M phase cell cycle (Poggioli et al., 2000); Influenza A virus A/WSN/33 (H1N1) causes cell cycle arrest in G0/G1 phase, which is beneficial to the expression of viral protein and generation of progeny virus (He et al., 2010); In the coronavirus family, mouse hepatitis virus (MHV) replication and some severe acute respiratory syndrome coronavirus (SARS-CoV) proteins are able to induce cell cycle arrest at G0/G1 phase (Chen and Makino, 2004; Yuan et al., 2005; Yuan et al., 2006; Yuan et al., 2007); Infectious bronchitis virus (IBV) infection causes cell arrest in G2/M phase to favor viral replication (Dove et al., 2006); Transmissible gastroenteritis virus (TGEV) infection induces cell cycle arrest at S and G2/M phases (Ding et al., 2013); PEDV M protein and ORF3 protein can block S-phase progression in mammalian cells (Xu et al., 2015; Ye et al., 2015); However, the effects of PEDV infection on the cell cycle in Vero cells and the significance of PEDV replication in cell cycle regulation remains to be further studied.

The cell cycle can be divided into a number of separate events (Pines, 1999): DNA replication (S phase), nuclear division (mitosis [M]), and cell division (cytokinesis), separated by two gap periods (G1 and G2). Quiescent cells are described as being in G0. Progression in each phase of the cycle and from one phase to the next is tightly regulated and highly orchestrated, controlled by cyclins and cyclin-dependent kinases as well as other factors. In the G1 phase, cells express three kinds of Cyclin D (cyclin D1, D2 and D3), combining with and activating cdk4/6, which is necessary for cells to enter the G1 phase from G0 phase (Besson et al., 2008). Cyclin E is also expressed in the G1 phase, which combines with cdk2 to make the cells complete the G1/S phase transition. The forward progression of S phase requires the kinase complex formed by cyclin A and cdk2 (Duronio, 2012). At the late stage of G2 and early M, the combination of Cyclin A and cdk1 (cdc2) initiate cells into M phase (Enomoto et al., 2009). It has been shown that these cell cycle regulatory molecules are regulated by some upstream pathways, such as p53 signaling. p53 monitors genome integrity during cell

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cycle G1/S and G2/M. If cell DNA is damaged, the cells are arrested in the G1 phase and cannot enter the S phase, or the cells are stagnated in the G2 phase and cannot enter the M phase, p53 is able to bind to the damaged DNA and promote its Repair (Schlereth et al., 2010). p21 is the down-stream signaling molecule of tumor suppressor gene p53, because the promoter of p21 gene contains p53 binding domain, so p53 can activate p21 gene by transcription (Roy et al., 2009). p21 can extensively act on cdk-Cyclin complexes and inhibit their activity, especially G1 phase cdk4/6-Cyclin D complexes (Starostina and Kipreos, 2011). In the present study, we investigated the effects of PEDV replication on the cell cycle progression, the roles of p53 signaling activation in regulation of cell cycle progression in PEDV-infected cells, and the significance of cell cycle regulation in PEDV replication. The results showed that PEDV infection may affect the cell cycle and promote replication of viral genes.

2. Materials and methods

2.1. Viruses, cells and antibodies

Vero cells (ATCC, CCL-33) were grown in Dulbecco Minimal Essential Medium (D-MEM) (Gibco BRL, MD, US) supplemented with 10% heat-inactivated fetal bovine serum (PAN), 1% penicillin/streptomycin (Invitrogen) at 37℃ in 5% CO_2 atmosphere incubator. In the present study, the CH/SXYL/2016 strain of PEDV (GenBank accession number is MF462814) was isolated from intestinal tract contents of PEDV infected piglets in Shaanxi province in China. 0.5 MOI PEDV (containing 10 μg/ml trypsin) infection solution was incubated for 1 h, then the virus was removed and washed 3 times with PBS, then DMEM containing 2% FBS was added, and cell samples were taken at the indicated time points for detection. Viral titers were determined by median tissue culture infectious dose($TCID_{50}$) (Reed and Muench, 1938).

Antibodies against cdc2, cdk2, cdk4, cdk6, Cyclin B1, Cyclin D1, Phospho-p53 (Ser15 and Ser20), p21 Waf1/Cip1 and β-Actin were purchased from Cell Signaling Technology. Antibodies against Cyclin A, Cyclin E, p53 were purchased from Santa Cruz. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Bioss. FITC-conjugated antirabbit IgG was purchased from Molecular Probes.

2.2. Measurement of cell proliferation and viability

MTT assay was carried out using the cell proliferation kitI(MTT) according to the manufacturer's recommended procedures (Roche Applied Science). Briefly, Vero cells plated in 96-well plates at approximately 90% confluence were infected with PEDV at an MOI of 0.5, 1.0, 2.0, 5.0, 10.0. At various times postinfection, 20 μl of MTT labeling reagent was added to each well and incubated at 37℃ for 4 h. After 150 μl of solubilization solution was added, the absorbance was measured at 550 nm in an enzyme-linked immunosorbent assay reader.

2.3. BrdU incorporation and flow cytometry analysis

2×10^6 PEDV-infected Vero cells were treated with a concentration of 10 μm bromodeoxyuridine (BrdU) (Sigma), incubated at 37℃ for 30 min to allow BrdU incorporation, rinsed with phosphate-buffered saline (PBS) twice and added with 2 ml EDTA/trypsin at 37℃ for 1 min. Vero cells were pelleted by centrifugation at 1000 × g for 5 min and then fixed in 1 ml of 75% ethanol for 4 h at 4℃. BrdU-labeled cell samples in 75% ethanol solution were pelleted by centrifugation at 1500 × g for 5 min, rinsed with 1 ml PBS, and then incubated in 1 ml of 0.1 M HCl in PBS at 37℃ for 10 min before addition of 3 ml PBS. Samples were then pelleted by centrifugation at 1000 × g for 5 min before addition of 100 μl anti-BrdU solution (anti-BrdU antibody [BD Biosciences] diluted 1:5 in PBS, 0.5% Tween 20, and 1% FBS) and incubated for 60 min at 25℃. Samples were rinsed with PBS twice, pelleted by centrifugation at 1000 × g for 5 min before addition of 100 μl anti-mouse fluorescein isothiocyanate (FITC)-labeled solution (antibody diluted 1:10 in PBS, 0.5% Tween 20, and 1% FBS) and incubated for 30 min in the dark at 25℃. Samples were rinsed twice in PBS before addition of 1 ml of PI staining solution (PBS, 50 μg/ml PI, 20 μg/ml RNase A). Labeled cells were analyzed for PI staining and BrdU incorporation using a FACSCalibur analyzer (Becton Dickinson), and percentages of cells in the G0/G1, S, and G2/M phases in each sample were determined by gating using CellQuest software (Becton Dickinson).

2.4. G0/G1, G1/S, and G2/M synchronization in Vero cells

Vero cells were synchronized at G0/G1 phase using FBS-free DMEM medium treatment. Vero cells were cultivated for 48 h using DMEM medium without FBS supplementation. Synchronized cells were mock infected or infected with 0.5 MOI of PEDV. After virus adsorption for 1 h and PBS rinse, cells were treated with medium and harvested at different times for RT-qPCR and flow cytometric analysis.

Vero cells were synchronized at G1/S phase using double-thymidine method. Vero cells were cultivated with DMEM medium containing 2 mM thymidine (Sigma) for 16 h, then rinsed with PBS for three times. After maintained for 12 h, cells were treated as described above once again and infected with 0.5 MOI of PEDV eventually. At 18 h post infection (p.i.), cells were harvested for RT-qPCR and flow cytometric analysis.

Vero cells were synchronized at G2/M phase using nocodazole (Sigma) treatment. Cells were treated with DMEM medium containing 60 ng/ml thymidine (Sigma) for 16 h, then rinsed with PBS for three times and infected with 0.5 MOI of PEDV eventually. At 18 h p.i., cells were harvested for RT-qPCR and flow cytometric analysis.

2.5. Western blot analysis

Cells in the experimental group were harvested and treated with RIPA Buffer (Sigma). Protein concentrations were determined using the BCA Protein Assay Kit. Equal amounts of proteins were transfer to polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Atlanta, GA, US) by 12% SDS-PAGE followed by wet-transfer cell (Bio-Rad, Trans-blot SD). The membranes were blocked with 2.5% nonfat dry milk in PBS'T (0.5% Tween-20 in PBS) at room temperature for 1 h and then membranes were probed with primary antibodies over night at 4℃, followed by HRP-conjugated secondary antibodies incubation at room temperature for 1 h. Western blotting was performed using enhanced chemiluminescence ECL (Pierce, Rockford, IL, US) as described in the manufacturer's instructions.

2.6. RNA analysis by real-time quantitative PCR (RT-qPCR)

Total RNAs were extracted from the PEDV-infected Vero cell by using the RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentrations for all samples were determined, and equal amounts of 1 μg RNA for each sample went through reverse transcription (RT) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) in an TL988-IV Real Time PCR Thermocycler. The primers for RT-qPCR in this study were described in Table 1. The real-time PCR was carried out in a final volume of 10 μl, sense and anti-sense primers (0.4 μmol/l) and target cDNA (4 ng). The reaction consists of a 10 min initialization at 95℃ and 35 cycles of 3s-denaturation at 95℃, 15s-annealing & elongation at 60℃. A negative control was included in each run and the specificity of amplification reaction was checked by melting curve (Tm value) analysis. Genomic equivalent normalization of each sample was