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Newcastle disease virus induces G_0/G_1 cell cycle arrest in asynchronously growing cells

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ABSTRACT

The cell cycle, as a basic cellular process, is conservatively regulated. Consequently, subversion of the host cell replication cycle is a common strategy employed by many viruses to create a cellular environment favorable for viral replication. Newcastle disease virus (NDV) causes disease in poultry and is also an effective oncolytic agent. However, the effects of NDV infection on cell cycle progression are unknown. In this study, we showed that NDV replication in asynchronized cells resulted in the accumulation of infected cells in the G_0/G_1 phase of the cell cycle, which benefitted the proliferation of NDV. Examination of various cell cycle-regulatory proteins showed that expression of cyclin D1, was significantly reduced following NDV infection. Importantly, the decreased expression of cyclin D1 was reversed by inhibition of CHOP expression, indicating that induction of the PERK-eIF-2a-ATF4-CHOP signaling pathway was involved in the G_0/G_1 phase cell cycle arrest observed following NDV infection.

1. Introduction

Appropriate regulation of the cell cycle is important for cell proliferation, differentiation, and cell homeostasis. The primary regulatory proteins controlling cell cycle progression are the cyclins and the cyclin-dependent kinases (CDKs). The cyclins and CDKs form complexes (Cook et al., 2000) that, upon activation, are responsible for phosphorylation of downstream targets, including cell cycle repressors.

During cell cycle progression, the expression levels of specific cyclins vary. For example, cyclins D and E are crucial in the G_1 phase, cyclin A is required in the S phase, and both cyclin B and cyclin A are important in the G_2 and mitotic phases (Obaya and Sedivy, 2002). Cyclin D1-Cdk4/6 complexes regulate G_1-phase progression by phosphorylation of the downstream retinoblastoma (Rb) protein (Blomen and Boonstra, 2007; Shafer, 1998). Later in the G_1 phase, cyclin E is induced and it associates with Cdk2 to form active complexes that phosphorylate Rb. Hyper-phosphorylation of Rb deactivates it, causing the release of E2F transcription factors and facilitating the transcription of genes essential for DNA synthesis, so that the cells then progress into the S phase (Lundberg and Weinberg, 1998; Vermeulen et al., 2003; Bagga and Bouchard, 2014). The activities of cyclin-Cdk complexes are regulated by cellular Cdk inhibitors (CKIs) (Vermeulen et al., 2003; Harper and Brooks, 2005), which can be grouped into two families. The CKIs of the INK4 family bind to Cdk4 and Cdk6 and block cyclin D-Cdk4/6 activities (Harper and Brooks, 2005; Sherr, 1994). The CKIs of the Cip/Kip family, which include P21Cip1 and P27Kip1, are potent inhibitors of cyclin E-and A-dependent Cdk2 (Sherr and Roberts, 1999).

Many viruses, including DNA, RNA, and retroviruses, modulate the cell cycle to benefit their own replication. DNA viruses, whose primary site of replication is the nucleus, have been studied most extensively in terms of cell cycle control. Some small DNA tumorigenic viruses, including human papillomavirus (Reinson et al., 2015), simian virus (Rohaly et al., 2010; Lin and Lamb, 2000), and adenovirus, encode proteins that promote entry into the S phase, thereby avoiding competition for cellular DNA replication resources (Jiang et al., 2015). This virus-mediated alteration to the cell cycle may ultimately contribute to cancer progression (Hanahan and Weinberg, 2000). By contrast, some large DNA viruses (for example, herpesviruses) are able to elicit cell cycle arrest in the G_0/G_1 phase to limit the competition between the virus and the host for cellular DNA replication resources. Retroviruses, which also replicate in the nucleus, are also associated with cell cycle perturbations. For example, the Vpr protein of human immunodeficiency virus type 1 is responsible for inducing cell cycle arrest at the G_2/M phase, when the expression of the viral genes is optimal

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(Hrimech et al., 2000; He et al., 1995). Increasingly, RNA viruses, whose primary site of replication is the cytoplasm, have also been observed to alter the host cell cycle. In the coronavirus family, infectious bronchitis virus (IBV) induces a G_2/M-phase arrest in infected cells that favors viral replication (Dove et al., 2006), while mouse hepatitis virus (MHV) replication and some severe acute respiratory syndrome coronavirus (SARS-CoV) proteins induce cell cycle arrest at the G_0/G_1 phase (Chen and Makino, 2004; Yuan et al., 2005).

Newcastle disease virus (NDV) belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. It is a single-stranded, negative-sense, enveloped RNA virus and causes respiratory diseases and death in poultry. NDV has also attracted much interest in cancer viro-therapy, as it can selectively infect and kill human cancer cells (Mansour et al., 2011). NDV induces apoptosis in cancer cells by activating the mitochondrial pathway (Elankumaran et al., 2006; Molouki et al., 2010). Cross talk between apoptosis and the cell cycle occurs as a result of the overlap in their regulatory mechanisms; however, the effects of NDV infection on the cell cycle are unknown.

In this study, we examined the potential effects of NDV infection on cell cycle progression. NDV replication induced cell cycle arrest in the G_0/G_1 phase, and this ability was shared among different strains of NDV. We also analyzed viral protein expression and viral titers to evaluate whether cell cycle arrest in the G_0/G_1 phase produces favorable conditions for viral replication. The findings reported here indicate that cell cycle regulation may be a common strategy exploited by NDV during infection to promote virus proliferation.

2. Materials and methods

2.1. Virus and cells

The NDV velogenic strain Herts/33 and the lentogenic strain La Sota were obtained from the Chinese Institute of Veterinary Drug Control (IVDC) (Beijing, China). Viral titers were determined by plaque assay titration on DF-1 cells and were expressed as the tissue culture infective dose of 50 ($TCID_{50}$) per milliliter. The viruses were inactivated with UV light irradiation (0.36J).

2.2. Infection

For cell cycle analysis, HeLa cells were infected with NDV at a multiplicity of infection (MOI) of one. After 1 h, the cells were cultured in complete medium at 37 °C and harvested at various times post infection (p.i.) for cell cycle and western blot analyses. For comparison of viral protein expression and progeny virus production in different cell cycle phases, cells were infected with NDV at an MOI of 0.1. After 1 h, a medium was added to maintain cells in different cell-cycle phases. Sixteen hours after infection, the cells were harvested and nucleocapsid protein (NP) protein expression was detected by western blotting. The viral titer in the supernatant was determined by the plaque forming assay on DF-1 cells.

2.3. Synchronization of cells

Cell cultures at 80% confluency were synchronized in the G_0 phase by serum deprivation. Approximately 5×10^5 cells/well were plated in a six-well plate and maintained in FBS-free medium for 48 h. For G_1 phase arrest, cells were seeded at approximately 5×10^5 cells/well in six-well plates and treated with N-butyrate (B5887; Sigma, Saint Louis, MO, USA) at 3 mM for 20 h. For G_2 phase arrest, cells were seeded at 5×10^5 cells/well and treated with 100 μM genistein (G6649; Sigma, Saint Louis, MO, USA) for 48 h. For M phase arrest, cells were seeded at 5×10^5 cells per well in six-well plates and treated with nocodazole (M1404; Sigma, Saint Louis, MO, USA) at 50 ng/ml for 10 h.

2.4. BrdU incorporation and flow cytometry analysis

For cell cycle analysis, two-color flow-cytometric analysis was used for accurate determination of the cell cycle profile. Mock-infected and infected cells were pulsed with bromodeoxyuridine (BrdU [B5002; Sigma, Saint Louis, MO, USA] 10 μM to approximately 1×10^6 cells) for 1 h prior to harvesting with trypsin. Cells were fixed with ice-cold 70% ethanol at 4 ℃ overnight and then treated with 2 N HCl containing 0.5% Triton X-100 for 30 min. Residual acid was neutralized by incubating the cell suspension with 0.1 M sodium borate (pH 8.5) for 2 min at room temperature. Cells were then incubated with anti-BrdU-FITC solution (anti-BrdU-FITC antibody [556028; BD Biosciences Pharmingen, San Diego, CA, USA] in a 1:5 dilution) at 4 ℃ overnight. The cell suspension was incubated with propidium iodide (PI) staining solution in phosphate buffered saline (PBS) (50 μg/ml PI [Sigma, Saint Louis, MO, USA] and 200 μg/ml RNase [Beyotime, Shanghai, China]) for 30 min at 37 ℃ and then analyzed with a FACSCalibur Flow Cytometer (Beckman, Mississauga, ON, Canada) and FlowJo software.

2.5. Transfection and plasmid, small interfering RNA

When the cells were grown to 70–80% confluent, plasmid DNA was transfected using Lipofectamine 3000 reagent according to the manufacture's protocol. 16 h post-transfection, cells were infected with NDV. PXJ40F plasmid was constructed and preserved in out lab (Liao et al., 2016). Specific sets of small interfering RNA (siRNA) for CHOP as well as nonsense sequence used as scrambled siRNA were purchased from GenePharma (Shanghai China).

2.6. Western blot analysis and antibodies

Cells were lysed in sodium dodecyl sulfate (SDS) buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue), and the amount of protein in each sample was determined with the bicinchoninic acid assay (Beyotime, Shanghai, China). Ten to twenty micrograms of total cellular protein from each sample were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) and proteins were detected with corresponding primary and secondary antibodies. Blots were performed using an enhanced chemiluminescence detection kit (Thermo Scientific, Inc., Waltham, MA, USA). Primary antibodies were: anti-phospho-Rb (Ser 795) (Sigma-Aldrich, MO, USA), anti-cyclin D1 (Cell Signaling Technology [CST], MA, USA), anti-cyclin E1 (CST, MA, USA), anti-P21 (Santa Cruz Biotechology, CA, USA), anti-P27 (Santa Cruz Biotechology, CA, USA), anti-P53 (CST, MA, USA), anti-PERK/ p-PERK (Abcam, MA, USA), anti- eIF2α/ p- eIF2α, anti-ATF4 and anti-CHOP (CST, MA, USA). A mouse monoclonal antibody against the NDV NP protein was prepared in our laboratory for use in these experiments. Actin was detected with a mouse anti-actin monoclonal antibody (Sigma-Aldrich, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or -mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

2.7. Statistical and densitometry analysis

Statistical probabilities were determined using the Student's *t*-test. Results are presented as means and standard deviations (SD) of three experiments. P values of < 0.05 were considered statistically significant. Representative western blots are shown for one of the three independent experiments. Band density was evaluated and quantified for each protein using Image J software.