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ORIGINAL RESEARCH

Impact of chicken-origin cells on adaptation of a low pathogenic influenza virus

Shahla Shahsavandi · Mohammad Majid Ebrahimi · Ashraf Mohammadi · Nima Zarrin Lebas

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Abstract Understanding the growth dynamics of influenza viruses is an essential step in virus replication and cell-adaptation. The aim of this study was to elucidate the growth kinetic of a low pathogenic avian influenza H9N2 subtype in chicken embryo fibroblast (CEF) and chicken tracheal epithelial (CTE) cells during consecutive passages. An egg-adapted H9N2 virus was seeded into both cell culture systems. The amount of infectious virus released into the cell culture supernatants at interval times post-infection were titered and plaque assayed. The results as well as cell viability results indicate that the infectivity of the influenza virus was different among these primary cells. The egg-adapted H9N2 virus featured higher infectivity in CTE than in CEF cells. After serial passages and plaque purifications of the virus, a CTE cell-adapted strain was generated which carried amino acid substitutions within the HA stem region. The strain showed faster replication kinetics in cell culture resulting in an increase in virus titer. Overall, the present study provides the impact of cell type, multiplicity of infection, cellular protease roles in virus infectivity and finally molecular characterization during H9N2 virus adaptation procedure.

S. Shahsavandi (✉) · M. M. Ebrahimi · A. Mohammadi · N. Zarrin Lebas
Razi Vaccine & Serum Research Institute, P.O.Box 31975-148, Karaj, Iran
e-mail: shahsavandi_s@yahoo.com

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Introduction

Since the late 1990s, low pathogenic H9N2 avian influenza viruses (AIVs) had been isolated in multiple avian species throughout the Middle and Far East, Europe and Africa. Up to date the AIVs of the H9N2 subtype continue to circulate widely in Eurasia and have frequently been isolated from poultry flocks (Alexander 2003; Brown et al. 2006). Embryonated chicken egg and cell cultures such as Madin Durby Canine Kidney (MDCK) cell are used for isolating and characterizing of the virus (OIE 2008). Currently, it has been reported that some samples positive for AIV by real-time reverse transcriptase–polymerase chain reaction failed to grow in eggs (Moresco et al. 2010). Surveillance of poultry populations for H9N2 virus is essential to investigation of potential cell culture systems for higher isolation rates of AIV.

In general, AIVs enter into target cells by specific binding to a terminal sialic acid-capped glycosylated molecule, followed by fusion of the viral and cellular membranes. The viral hemagglutinin (HA) protein mediates both cellular receptor specificity and membrane fusion. AIVs preferentially recognize α2-3Gal SA receptors (Matrosovich et al. 2004; Thompson et al. 2006) but a few of them like H9N2 that prefer α2-6Gal

SA linkeage have been transmitted directly to humans (Butt et al. 2005; Matrosovich et al. 2001). HA0 precursor protein is cleaved by host proteases into HA1 and HA2 subunits to gain its fusion ability. This causes a conformational change which exposes a hydrophobic fusion domain on HA2 to initiate the infection process. Cleavage of HA is also essential for viral pathogenicity and tissue tropism (Böttcher et al. 2006; Steinhauer 1999). The HA cleavage site of H9N2 viruses is a monobasic motif possessing a single arginine amino acid residue, R(K)SS/R, which is cleaved extracellularly by trypsin, trypsin-like, and membrane fusion virus-activating proteases—the type II transmembrane serine proteases (TMPRSS) (Bugge et al. 2009; Chaipan et al. 2009; Klenk et al. 2007)—which activate the entry of influenza viruses into the target cells. The embryonic chicken cells are often the choice for AIV propagation but the advantages and limitations of the different cell types for virus replication have not been discussed. In this study we investigated the ability of an egg-adapted H9N2 to adapt to the new host cells after several passages. The replication and spread of the virus in homogeneous and heterogeneous primary cells with focus on multiplicity of infection, host cell proteases and molecular changes in HA, neuraminidase (NA), and non structural (NS) genes of the virus were investigated.

Materials and methods

Virus

An egg-adapted H9N2 virus (Shahsavandi et al. 2012) was used as a seed for infection of CEF and CTE cells. The virus was titrated according to Swayne et al. (2008).

Cell culture/virus growth dynamics

To determine the influence of cell type on influenza virus adaptation, CEF and CTE cells were used. CEF cells were prepared from 10-day-old embryonated SPF embryos, carefully chopped with sterile scissors and 3–4 times washed with PBS. Then the tissue fragments were digested in 0.25 % trypsin in dissociation medium at 37 °C for 1 h. The cells were plated into cell culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) and antibiotics including 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml antimycotic solution. To prepare CTE, tracheas of one-day-old SPF chickens were rinsed in DMEM under sterile condition. The tracheal sections were incubated for 2 h at 37 °C in 20 ml dissociation buffer consisting of DMEM, 1 % penicillin/streptomycin, 0.2 % type XIV protease, and 0.02 % DNase. The procedure for removing epithelial sheets from the tracheas was performed according to Shen et al. (2011). The disrupted tracheal epithelial sheets were homogenized and FBS was added to the digesting solution to stop the reaction. Following centrifugation at 1,000 rpm for 5 min the cell pellets were resuspended in ATE medium (Shen et al. 2011). CEF and CTE cells were infected with the H9N2 virus at varying multiplicity of infection (MOI) of 0.001, 0.1, and 10 PFU/cell in 1 ml in DMEM containing 10 % FBS, antibiotics solution at concentration 1× with and without supplemental trypsin. Following adsorption for 1 h at 37 °C, the inoculum was removed and DMEM replaced. Up to 96 h post-infection (hpi) and at 16-h intervals, culture supernatants were collected and stored at −70 °C until use. Impact of cell passage on virus replication dynamics was determined by six consecutive passages. In this case, supernatant of each passage served as the virus seed for the next passage. The growth dynamic of the virus was evaluated by virus titration of cell culture supernatants at different hpi by plaque assay and hemagglutination (HA) testing. Titers obtained from three independent experiments were analyzed statistically ($p < 0.01$).

Plaque assay

Each of CEF and CTE cells (6×10^5 cells/well) incubated overnight in six-well tissue culture plates were seeded with 0.2 ml of a viral suspension serially diluted from 10^{-1} to 10^{-8} and incubated 1 h at 37 °C. Then the viral suspensions were replaced with overlay medium (DMED antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 0.3 μg/ml trypsin and 0.3 % agarose) and incubated for 3 days. A second agar overlay containing 0.14 mg/ml neutral red was added to facilitate plaque counting and the plates were incubated for two additional days to visualize plaques in wells.

HA test

The HA test was used to screen for the potential presence of H9N2 virus in cell culture in microtiter plates by using 0.5 % chicken RBCs. The reactions

were performed in PBS at room temperature (OIE 2008).

MTT assay

The metabolic activity of primary cells following H9N2 virus infection was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, each well containing CEF and CTE cells seeded in 96-well culture plates (approximately 5,000 cells per well) were washed three times with PBS and incubated with culture supernatants in each passage at 37 °C for 96 h. Mock-treated cells served as control. The cells were washed with PBS and incubated with DMEM and 50 µl/well of MTT solution (5 mg/ml) for 3 h at 37 °C, then 200 µl of 0.04 N HCl in isopropanol was added to each well and the plate incubated for 1 h at room temperature. Optical density (OD) value was measured at 540 nm using an ELISA reader.

Fusion assay

The permissivity of the CEF and CTE cells for entry of H9N2 virus and also HA cleavage efficiency of the cells were evaluated using a fusion assay. The H9N2 infected cells were exposed to a low-pH treatment (DMEM buffer, pH 5.0) for 5 min at 37 °C. Then the buffer was removed and then cells incubated for 8 h at 37 °C in fresh neutral DMEM containing 5 % FBS. Cells were fixed with ethanol and stained with Giemsa solution. Fusion activity was determined by counting the fused cells in an entire field.

Determination of virus and cell nucleotide sequences

To investigate the impact of adaptation to CEF and CTE cells the molecular features of the H9N2 virus in serial cell passages were characterized by comparative sequence analysis. Nucleotide sequences of HA, NA, and NS genes in defined passages (P1, P3, and P6) were compared to the virus seed (P0). The nucleotide and the deduced amino acid sequences were aligned using ClustalW with default parameters and analyzed using MEGA4. The expression of viral activating protease (VAP), chicken TMPRSS13 (chTMPRSS13), and β-actin mRNAs in virus infected and mock cell cultures were determined using the one-step RT-PCR mixture (Maxime RT-PCR premix; iNtRON Biotechnology, Sungnam, Korea). Six primer sets listed in Table 1 were designed for detection of the genes. The RT-PCR reaction consisted of 1 cycle of 42 °C for 60 min and 95 °C for 5 min and 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, followed by a final elongation step of 72 °C for 10 min. The annealing temperature for amplification of viral genes was adjusted to 57.5 °C. The purified PCR products of viral genes at different passages were sequenced in both directions (use of Sanger's sequencing method).

Results

The H9N2 virus at 7 HAU was used to infect the chicken cells. The virus replicated well in CEF and CTE cells and the cytopathic effect (CPE) was seen at low and high MOI only in the presence of trypsin. This was evident as an increase in the number of cells becoming detached and floating in the medium compared to mock-infected cells. Infection of the primary cells revealed that cell death was induced rapidly, and to a greater degree, in CEF than in CTE cells within 48–72 hpi. Impact of cell passage on virus replication dynamics was determined by six serial passages of the H9N2 virus in both cells. In the absence of trypsin a decrease in HA titer was found from 7 to 3 at the first passage and to undetectable at the sixth passage. The plaque assay results revealed H9N2 virus was not replicated without trypsin activation. In the presence of trypsin, virus replication was affected by the cell type. Both cells showed a decrease in HA titer in the first passage which remained constant in CEF cell up to the end of experiment. Higher HA titers were observed in infected CTE cell compared to CEF cells. Furthermore, an increase in virus titer from 3 to 5 was seen for CTE cells in the fifth passage. There were no variations in HA titer from the fourth passage to the next passages. Development of countable plaques required 2 days of incubation for CTE and 3–4 days for CEF cells. The H9N2 virus exhibited the greatest rate of plaque formation in CTE cells. Change in size of the plaque was not detected. In both cell systems virus yield did not increase by varying the MOI, indicating that it is not critical for the infection process. However, average yields of virus declined for serial passages performed at MOI 10 without affecting HA titer.

To quantify the observed difference in growth kinetics of virus between the cell systems, cell viability

Table 1 The primer sets sequences used for amplification of the studied genes

Gene	Forward primer	Reverse primer	Length (bp)	Accession No.
HA	CTCGAGCAAAAGCAGGG GAATTTCT	AAGCTTTTATATACAAATGTTGCACCT	1,761	FJ794817
NA	CTCGAGAGCAAAAGCAGGA GTAAAAATG	AAGCTTAGTAGAAACAAGGAGTTTTTT	1,431	JX456183
NS	CTCGAGAGCAAAAGCAGGGT GACAAAAAC	GGATCCAGTAGAAACAAGGGTGTTTTTA	890	JX308782
VAP	TGCTGCTCATTGCATAAACC	GGGACTTCGAGCACTTTCAG	330	NM205022
chTMPRSS13	TTCTGGCTCCCTGACAGTCT	CACCAATCCCTAGCACCTGT	265	NJ030336
β-actin	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTTCA	150	L08165

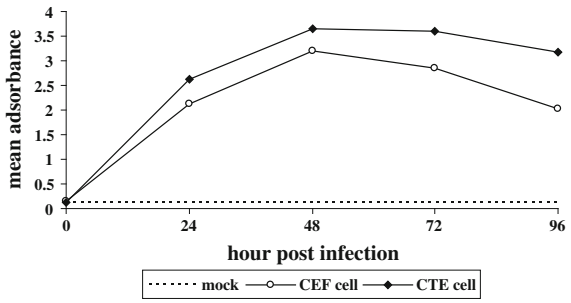

Fig. 1 Metabolic activity of CEF and CTE cells following H9N2 influenza virus infection. Measurement of cell viability in the cells using an MTT assay at different times post infection with a MOI = 0.1. Data points are mean ± SD of three independent experiments

was measured using the MTT assay (Fig. 1). CEF cells consistently showed lower metabolic activity than CTE cells over a range of MOI ($p < 0.01$). The differences in the metabolic activity were also examined by fusion assay 6–8 h following virus exposure. Cell fusion activity was not detected in infected CEF and CTE cells without supplemental trypsin. The average number of fused cells per field for both cells was not significantly different (40.2 for CTE cells vs. 38.67 for CEF cells), confirming that these cells supported virus entry following trypsin treatment, however, their permissivity for the influenza virus may be different. In agreement with growth curves in Fig. 1, the H9N2 virus spreads more efficiently within the epithelium than fibroblast cells. The HA cleavage efficiency of the cell systems was also evaluated by fusion activity. Expression of the host protease mRNAs in the cells was assessed by RT-PCR with reference to the house keeping gene β-actin. Both VAP and chTMPRSS13 proteases were expressed in CTE cells, whereas VAP was not detected in CEF. Sequence analyses of HA indicated two simultaneous amino acid substitutions (R^{311} to G and S^{496} to A) which were definitively established within the HA2 subunit at the third passage in CTE cells. The HA protein in the passages showed conservation of residues H^{183}, L^{190}, L^{226}, Q^{227} and G^{228} in the receptor binding pocket and RSSR motif in the cleavage site. No changes in the number and location of potential N-glycosylation sites were found. Similar results were found in analysis of stalk length, hemadsorbing site and potential glycosylation sites of NA protein, and also in the PDZ domain of NS protein.

Discussion

The HA protein which is composed of receptor-binding and fusogenic subunits mediates both binding of influenza virus to the cell surface and the subsequent fusion of viral and cellular membranes. The HA cleavage site of low pathogenic AIV is activated only in the respiratory and intestinal tract where trypsin-like enzymes are secreted or an addition of exogenous trypsin is required for the efficient replication of the virus in target cells (Klenk et al. 2007). In this study the replication efficiency of an egg-adapted strain of H9N2 AIV was evaluated in CEF and CTE cells. Both cells supported growth of the virus when trypsin was supplemented although after six passages, however, the viral titers were lower than in embryonated chicken eggs. HA values suggest that infection at a high MOI decreases virus release without affecting HA titer. Average yields of virus declined more rapidly for passages performed at MOI = 10. Higher MOI may lead to defective interfering particles (DIPs) formation

(Thompson and Yin 2010). DIPs are intrinsically noninfectious virions that appear after several consecutive passages of infectious influenza virus at high MOI. At this condition more virus and more DIPs are transferred from one generation to the next one, enabling more rapid accumulation of DIPs and greater inhibitory effects on virus growth. In comparison to embryonated chicken egg the release of virus particles and decrease in HA-titers during passages one to six indicate that some factors are involved in cell adaptation process, such as chicken cyclophilin A and use of FBS in cell culture. It has been suggested that cyclophilin A which is widely distributed in a variety of chicken tissues and localized in the cytoplasm of CEF cells inhibits virus replication by interfering with the translocation of newly synthesized M1 protein into nucleus in the early stage of infection (Xu et al. 2010; Liu et al. 2009). FBS contains a variety of inhibitory components, termed β-inhibitors, that bind to N-linked glycans on HA and NA and inhibit receptor-binding, hemagglutinating activities and the infectivity of influenza virus (Matrosovich and Klenk 2003).

Despite of similarities of virus replication dynamic, difference in HA titer was observed between both cells. The growth kinetic analysis showed that replication of the virus was somewhat delayed in CEF compared with CTE cells and the peak HA titer was accessed 24 h later in CEF cells. On the other hand, the rapid cell death in CEF cells was not due to higher levels of virus entry or early replication. It may reflect cell tropism of the virus due to the distribution of appropriate SA receptors on primary CTE cell surface. Shen et al. (2011) demonstrate that chicken tracheal cells express both α2-3 and α2-6 linked SA and its ciliated cells are the primary target cell for AIV. This SA distribution also correlated with AIV infection in vitro and ex vivo (Scull et al. 2009). It has been shown that H9N2 strains isolated from land-based poultry possess a receptor specificity that was similar to that of human isolates (Matrosovich et al. 2001; Saito et al. 2001). Such receptor specificity can explain the increased infectivity of the H9N2 virus in CTE compared to CEF cells. Similar results on susceptibility of the primary tracheal epithelial chicken cells to infection with H7N3 and H6N1 influenza subtypes have been reported (Zaffuto et al. 2008; Shen et al. 2011) suggesting that the ciliated cells support the influenza virus growth. However, other host factors such as glycan topology, concentration of invading viruses, local density of receptors, lipid raft microdomains, co-receptors or SA-independent receptors, may also be important determinants of AIV infectivity (Zhang 2009).

The failure of the H9N2 to produce CPE in cell cultures in the absence of trypsin correlates with virus pathogenicity because HA remains uncleaved. Cleavage of HA is a cell-associated process mediated by the cells in which the virus is replicating, but expression of VAP and chTMPRSS13 genes in CTE cells was not enough to cleave HA protein required to membrane fusion and replication of the H9N2 virus. Okumura et al. (2010) have reported that chTMPRSS13 is a novel serine protease for H5N1 virus with polybasic cleavage motif. In the present study, we show that chTMPRSS13 can not activate the monobasic cleavage motif of the low pathogen AIV and support its replication in the cell cultures. Study for whether the expression of these HA-processing proteases affects cleavage of H9N2 HA, is currently in progress.

The adaptation to a cell allows the influenza virus to optimize its replication in a given host system (Webster et al. 1992). In our study viral HA titer raised from 3 to 5 in CTE cells following four subsequent passages in a shorter time. The faster replication kinetics of the virus in the cell resulted in an increase in virus titer and also fixed simultaneous amino acid substitutions in the HA stem region occurred indicating that a CTE cell-adapted H9N2 strain may have been generated. It has been demonstrated that due to high prevalence of avian receptors a chicken fibroblast cell line able to support the growth of a broad range of avian influenza viruses comparable to that in CEF primary cells (Lee et al. 2008). The use of continuous cell lines for the replication of influenza viruses offers the opportunity to use standardized cell culture conditions. Our study illustrates a cell-specific variation in the replication dynamics of H9N2 influenza virus in chicken fibroblast and epithelial cell types and the cell tropism of the virus to the CTE cells. Unfortunately, the CTE primary culture must be generated fresh from the appropriate day-of-age chick for each usage. For this reason, developing an immortalized chick CTE cell line adapted to serum-free growth conditions which is capable of replicating of AIVs and of supporting high viral titers would be ideal.

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