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Research Article

Expression of Factor X in BHK-21 Cells Promotes Low Pathogenic Influenza Viruses Replication

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A cDNA clone for factor 10 (FX) isolated from chicken embryo inserted into the mammalian cell expression vector pCDNA3.1 was transfected into the baby hamster kidney (BHK-21) cell line. The generated BHK-21 cells with inducible expression of FX were used to investigate the efficacy of the serine transmembrane protease to proteolytic activation of influenza virus hemagglutinin (HA) with monobasic cleavage site. Data showed that the BHK-21/FX stably expressed FX after ten serial passages. The cells could proteolytically cleave the HA of low pathogenic avian influenza virus at multiplicity of infection 0.01. Growth kinetics of the virus on BHK-21/FX, BHK-21, and MDCK cells were evaluated by titrations of virus particles in each culture supernatant. Efficient multicycle viral replication was markedly detected in the cell at subsequent passages. Virus titration demonstrated that BHK-21/FX cell supported high-titer growth of the virus in which the viral titer is comparable to the virus grown in BHK-21 or MDCK cells with TPCK-trypsin. The results indicate potential application for the BHK-21/FX in influenza virus replication procedure and related studies.

1. Introduction

Influenza is one of the most economically important viral respiratory diseases of human as well as avian and animal species worldwide. The causative agent is belonging to Orthomyxoviridae, negative-sense, single-stranded RNA, which encodes at least eleven proteins. Type A viruses are subtyped on the basis of the two main surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) and further classified as low pathogenic (LP) or highly pathogenic (HP) on the basis of specific molecular and pathogenesis criteria [1, 2]. Avian influenza viruses replicate mostly in the intestine while human influenza strains replicate in the upper respiratory tract [3, 4]. The infection cycle is initiated by the specific binding of viral HA to a terminal sialic acid-capped glycosylated molecule present on the surface of the host cells. Upon attachment to the cell, receptor-mediated endocytosis occurs either by α2–6Gal or by α2-3Gal linkage [5, 6]. Cellular tropism and the infectivity of influenza viruses are primarily determined by the distribution of these receptors in the cell surface. Also the presence of specific host cellular protease (s) for posttranslational cleavage of HA0 precursor protein is essential for viral infectivity, pathogenicity, and tissue tropism [7, 8]. Influenza A viruses target a wide spectrum of tissues so replication of the viruses has been examined in a variety of cells [9–13]. The results demonstrated that susceptibility to the viruses varied significantly between the cells; and in particular the tracheal epithelial, MDCK, and A549 have been described as suitable permissive cells for the replication of both human and avian influenza viruses. Replication of influenza viruses can be attributed to expression of both 2–6 and 2-3 linked sialic acid receptors on the surfaces of the cells. Beside the viral tropism determined by virus-receptor interactions, local density of receptors, lipid raft microdomains, and host cell proteases activating the viral surface glycoproteins play major roles in influenza infectivity [14, 15]. The viruses have the ability to exploit a host virus-activating protease system to support own replication. Cellular host proteases such as transmembrane serine proteases (TMPRSS), an analogous protease from chicken allantoic fluid to the blood clotting factor 10 (FX), and plasmin were involved in the postentry stages of influenza A virus infection

[8, 16]. In particular, there are growing interests in the role of hemostasis during influenza virus infection lately. The fact that factor X might play a role in viral replication suggests that indeed hemostasis and coagulation might be deleterious for the host [17]. Avian influenza viruses reach high titers when grown within chicken-origin cells; however, the efficient replication and infectivity of LP viruses are achieved in the presence of supplemental trypsin [4]. The enzyme enhances the internalization of influenza virus into cells by cleavage of HA but did not improve the ability of the host cells to internalize the virus [18]. It is well documented that the LP viruses cannot be cleaved by ubiquitous intracellular proteases while they replicate efficiently in eggs because of the presence of a protease in allantoic fluid that can cleave HA [19]. Previous studies reveal that FX, a vitamin K-dependent serine protease in the prothrombin family, was induced upon virus infection. The viral activating protein cleaves the fusion proteins of Sendai virus, Newcastle disease virus, and influenza virus at a specific single arginine-containing site and plays a key role in the viral spreading in the allantoic sac [16, 17]. In this study, we cloned the FX mRNA in BHK-21 cell and evaluated the impact of the established BHK-21/FX cell on susceptibility and virus replication kinetics of a LP influenza virus strain to provide insights into the development of future influenza virus diagnostic approaches.

2. Materials and Methods

2.1. Cells and Virus Infection. MDCK and BHK-21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 100 U/mL penicillin, and 100 mg/mL streptomycin, at 37°C with 5% CO_2. With the use of a dose-response test, the optimal L-(tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK) trypsin concentration was determined for each culture. The BHK-21 cell line had greater susceptibility to trypsin toxicity and received 0.1 mg/mL, while MDCK cell line was the most resistant to toxicity caused by trypsin and received 0.45 mg/mL. Monolayers of the cells at a concentration of 1×10^6 cells/mL were infected with a local isolate influenza H9N2 virus A/chicken/Iran SS8 (2011) at a multiplicity of infection (MOI) of 0.01 in the presence of supplemental trypsin. Following adsorption for 1 h at 37°C, the inoculum was removed and washed before DMEM was replaced. The cultures were incubated up to 72 hours post infection (hpi) and controlled by inverted light microscopy for cytopathic effect (CPE). For each cell, four different sets of tissue culture flasks were infected. Mock virus infected cells served as controls.

2.2. Expression Vector pcDNA3.1-FX and Transfection of BHK-21 Cells. The cDNA clone of FX in the pcDNA3.1 expression vector (Invitrogen) was constructed. The open reading frame of FX located in peptidase S1 domain (241–473 nt) isolated from chorioallantoic membrane of embryonated eggs was amplified with primers 5-GGATCCGATGAGTGTCGTCCTGGTGA-3 and 5-AAGCTTAGCCACGCCACTACTACTTT-3, containing restriction sites for *Bam*HI and *Hin*dIII (underlines), respectively. BHK-21 cells were transfected with pcDNA3.1-FX plasmid using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The transfected cells were selected and cultivated in the presence of 10% FBS and 800 g/mL Geneticin (G418; Invitrogen). After two weeks, cells surviving the selection were pooled, passaged three times in 1.5 mg of G418/mL, and frozen in aliquots. The G418-resistant colonies were isolated and subjected to RT-PCR to verify the expression of FX at 232 bp lengths using the extracted RNA (High Pure RNA Extraction Kit, Roche, Germany) and the one-step RT-PCR (iNtRON Biotechnology, Korea).

2.3. BHK-21/FX Cell Screening during Multiple Cell Passages. The growth property and plating condition for the BHK-21/FX cells were assessed prior to virus infection. The cells were seeded into two 48-well cell culture plates and incubated overnight. When the monolayers were between 90% and 95% confluent, they were inoculated with the virus at MOI 0.01. One plate received trypsin-supplemented media and one plate received plain media. The cells were incubated for 4 days at 37°C with 5% CO_2 and checked microscopically for the presence of CPE. Mock cells were included in each experiment as controls. On days 1 and 4 post infection (pi), eight wells in each plate were immunostained using influenza NP specific antibody to assess the expression of the viral internal protein. The cells were examined for virus replication kinetics quantification at different times pi. Afterwards, infectious viral particles were quantified and expressed in $TCID_{50}$. Finally, the remaining harvested supernatant was used as inoculum for ten subsequent passages. The infectivity of influenza virus in each passage was determined using immunoassay procedure. Briefly, cells were washed once with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were washed and immunostained with mouse anti-influenza NP monoclonal antibody followed by FITC-labeled goat anti-mouse IgG (Dako, Glostrup, Denmark). Immunostained cells were examined under Nikon Eclipse E600 fluorescence microscope.

To evaluate the impact of producing influenza virus in BHK-21/FX cell, we have assessed the sensitivity assay by infecting an amount of 10^5 cells with the H9N2 virus for ten subsequent viral passages. At each passage, virus titer was estimated and the HA and NA nucleotide sequences were determined. In this case, the extracted virus RNA was amplified using the one-step RT-PCR for HA and NA segments at full-lengths [12]. The PCR products of viral genes at different passages were cloned and sequenced subsequently in both directions. Sequences were analyzed using the CLUSTAL W alignment method of the BioEdit sequence alignment editor version 7.0.9 software.

2.4. Statistical Analysis. The data are expressed as mean ± SD. Statistical correlation of data was checked for significance by ANOVA and Student's t-test. Differences with $P < 0.05$ were considered significant.

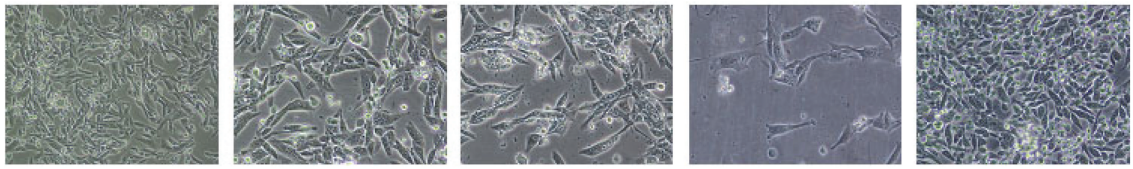

Figure 1: Cytopathogenicity of BHK-21/FX cells to influenza virus infections (MOI 0.01) at interval hours post infection (200x magnification). The BHK-21 cells infected with influenza virus did not manifest cytopathic effects.

3. Results

3.1. Cell Lines Validation. The ability of H9N2 influenza virus to infect the MDCK and BHK-21 cell lines was assessed in the absence and presence of supplemental trypsin. The H9N2 virus replicated in both cells and moderate CPEs were evident 48 hpi only in the presence of trypsin. The virus failed to produce CPE in the absence of trypsin because the HA remains uncleaved and virus replication did not occur.

3.2. BHK-21/FX Cell Establishment and Screening. BHK-21/FX was established by transfection of plasmid encoding FX. Two weeks continuously under antibiotic selection, the surviving cultivated BHK-21/FX cell was assessed systematically for the sensitivity to primary influenza virus infection and permittivity for virus replication and spread. Specific band amplified from total RNA of BHK-21/FX cell in RT-PCR has confirmed the presence of FX gene in the cell. The morphology of BHK-21/FX cells was not different from BHK-21 cells. Following infection of BHK-21/FX cells with influenza virus, visible CPE was observed by 24 hpi with giant cell formation and massive detaching from the culture flask compared to BHK-21 cell (Figure 1). The BHK-21 cells infected by the virus developed a very light CPE at 72 hpi. Both cells showed an increase in virus titer from $10^{3.0}$ $TCID_{50}$ on the first passage which remained constant during serial passages for BHK-21 cell ($10^{5.5}$ $TCID_{50}$), while the H9N2 virus infected-BHK-21/FX cell exhibited the highest viral titer. The amount of infectious virus yield at the first passage in BHK-21/FX cells was increased ~2500 times ($10^{7.5}$ $TCID_{50}$) in fifth passage which was maintained up to seventh passage. This indicates clearly that the cell permits the production of infectious influenza virus particles (Figure 2). Production and spread of the virus were also monitored by detecting NP expression in immunofluorescence assay up to 72 hpi (Figure 3). From 8 hpi, the numbers of NP-expressed cells increased with time elapses. The virus infected-BHK-21 cells have exhibited the same panel in the presence of supplemental trypsin, while virus replication was not observed in the absence of trypsin. It may be due to protease activation of viral HA cleavage site which supports virus entry and replication.

Nucleotide sequences of the HA and NA genes of the virus passaged seven times in BHK-21/FX cells were analyzed and compared with the parental virus genome sequences. No amino acid change was observed at the cleavage site, in the receptor binding pocket, and within the N-glycosylation sites of HA protein. All of them showed conservation of residues

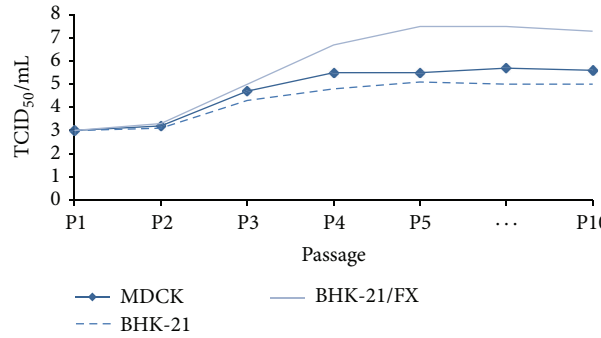

Figure 2: Replication of influenza virus in BHK-21/FX cells. The titer of virus in BHK-21/FX cell supernatants was assayed by $TCID_{50}$ in ten subsequent passages compared to the virus infected MDCK and BHK-21 cells supplemented with trypsin.

H^{183}, L^{190}, L^{226}, Q^{227}, and G^{228} in the receptor binding pocket and RSSR motif in cleavage site. The comparative sequence analysis of NA indicated that the amino acid sequences at the active sites of NA protein at positions ^{366}IRKDSRAG^{373}, ^{399}DSDNRSGY^{406}, and ^{431}PQE^{433} were conserved. Three simultaneously nucleotide substitutions were found at the fifth passage which did not lead to amino acid changes. In the consequent passages of the virus on BHK-21/FX cell, mutation in the nucleotide sequences of viral genes that resulted in change in amino acid codon was not detected.

4. Discussion

Since 1997, several cases of human infections with different subtypes of avian influenza viruses have been identified and raised the pandemic potential of avian influenza virus in human population. Thus, early detection is very important to initiate efficient control programs. Virus isolation in embryonated eggs or in cell culture followed by subtyping is considered as the standard protocol for detecting avian influenza virus. Since previous decade some isolates have low growth properties in MDCK cells line [20]. Avian influenza viruses generally grow efficiently on embryonated eggs or primary chick embryo-originated cells due to the specific receptor distribution and host cell proteases. It has been demonstrated that blood-derived proteases promote influenza A virus replication outside the respiratory tract.

Here, we developed BHK-21 cell line that expresses FX, which could affect influenza virus HA activation in absence of supplemental trypsin. The main focus of our project was the

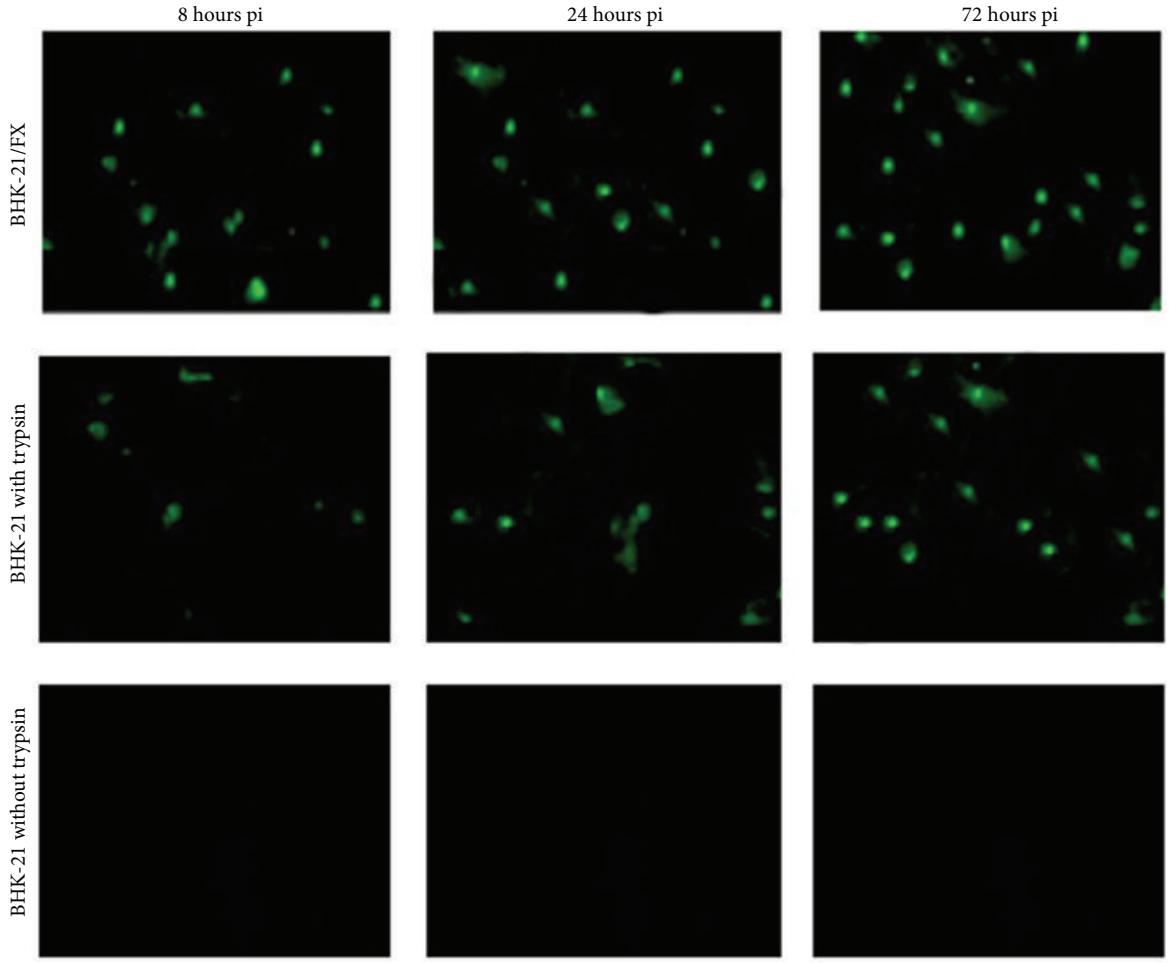

Figure 3: Immunofluorescent detection of influenza virus NP in infected-BHK-21/FX cells (×100). Fluorescence emission was detected in the infected cells at 24 and 72 hours post infection compared to the virus infected-BHK-21 cells supplemented with trypsin and without trypsin.

characterization of LP avian virus propagation in the genetically manipulated BHK-21 cell and potential use of BHK-21/FX cells for the isolation and replication of the viruses. RT-PCR confirmed the presence of the target gene expression in ten serially passaged cells. Growth kinetics of H9N2 virus on BHK-21/FX, BHK-21, and MDCK cells were evaluated by titrations of virus particles in each culture supernatant. The growth kinetic of the virus in the manipulated cell was comparable to those with MDCK or BHK-21 plus TPCK-trypsin ($P < 0.01$). Monitoring of virus titers and release of progeny viruses during multiple cycle infections is of great importance for process characterization and optimization. Based on the length of the interval between the virus inoculation and increasing viral infective titer, the duration of reproductive cycle of influenza virus was estimated as 5-6 hours. Other studies have shown that a single virus progeny production cycle of influenza virus requires 8–10 hours in MDCK cell, the same time in A549 cell, compared to 20 hours in Chang's conjunctival cell [10, 12, 21]. We detected progeny virus release in BHK-21/FX at 8, 18, and 24 hours pi, respectively. The overexpression of 2,6 sialic acid receptors in MDCK cells transfected by type II transmembrane serine proteases members showed sufficient titer recommended for cell-based human influenza vaccine production [7]. Enhancing the virus receptor interaction leads to increasing the membrane fusion, the virion production, and binding rates. Viral entry and initiation infection procedure were also confirmed by detection of viral NP expression in BHK-21/FX cells in absence of exogenous trypsin. The internal viral protein potentially plays an important role in the early stages of the virus life cycle including encapsidating the segmented viral genome into ribonucleoproteins, vRNA synthesis, and interactions with the viral polymerase. The NP protein is an indicator for switching from transcription to replication. Thus, according to the immunofluorescence assay of the protein, the BHK-21/FX cell could cleave the HA protein of H9 subtype in absence of exogenous trypsin. The cleavage is a cell-associated process that leads to virus replication and production of high titer comparable to those in either MDCK or BHK-21 cells plus trypsin. Full-length amplification and sequencing patterns of the HA and NA genes at intervals passages demonstrated that the H9N2 virus at ten passages

remained similar to the parent virus. These data indicate that BHK-21/FX is a permissive cell which is capable of optimizing avian influenza virus replication to obtain high titers.

Characterization of a modified cell line during growth and infection will provide the basis for cell culture-based influenza vaccine development, virus isolation, and diagnosis. In this study, we use FX, a Ca^{2+}-dependent serine protease to explore host factor involved in the influenza virus replication. FX is synthesized in liver, circulated in body as plasma protein, and activated into factor Xa via intrinsic and extrinsic pathways. The active site of factor Xa is divided into four subpockets as S1–S4. The S1 subpocket determines the major component of selectivity and binding [16]. Activation and binding of the protease at the surface of infected cells are an alternative mechanism for the proteolytic cleavage of HA which is perquisite for viral replication and pathogenicity [22]. Recent progress in understanding virus replication at the molecular level has revealed that many enveloped viruses, including influenza A viruses, incorporate cellular surface proteins into viral particles during virus-cell fusion and bud from the plasma membrane of their host cells following replication [23]. Among the host-encoded proteins in influenza virus particles, the annexin family is well represented. Annexins are a family of Ca^{2+}/lipid-binding proteins and act as membrane-membrane or membrane-cytoskeleton linkers. The proteins have been implicated in Ca^{2+}-regulated exocytotic events and certain aspects of endocytosis. Tissue specific expression of annexins suggests highly specialized function which correlates well with the ability of the proteins to interact with cellular membranes [24]. The role of annexin members as cellular interactant viral proteins in replication of influenza virus has been studied. As a virus-host interaction, expression of cellular protein annexin 2 (Anx2) is increased in response to influenza infection due to dysregulation of Ca^{2+} homeostasis. The upregulation of cell-surface Anx2 allows the recruitment of plasminogen which is activated by virion. Subsequently, HA cleaves through Anx2 which is incorporated into virus particles [25]. Anx6 expression significantly increased virus production, while its overexpression could reduce the titer of virus progeny, suggesting a negative regulatory role for Anx6 during influenza A virus infection [26]. Anx13 stimulates apical transport of influenza virus HA in MDCK cells [25]. It is assumed that Ann2 and Anx13 promote the Ca^{2+}-dependent association of lipid raft [24]. The rafts as platforms for intracellular sorting and signal transduction events [27] play a decisive role at several steps during virus replication including intracellular transport of viral proteins, assembly and budding of progeny virus at the plasma membrane, environmental stability of the virus particles, and fusion of viral and host cell endosomal membrane upon virus entry [28–30]. The lipid raft is used as a platform to concentrate HA binding receptors; thus, the virus exploits the signalling capacity of raft domains by mediating efficient fusion. Viral NA is concentrated in rafts for its normal incorporation into virions and budding [23, 28]. The raft association is also mediated by the binding of Anx5 to the plasma membrane. Anx5 is incorporated into influenza envelope during the budding process and a substantial proportion of the protein is present in lipid rafts, the site of virus budding [30].

The interactions between viral and cellular factors determine host susceptibility to influenza infection so the absence of remarkable virus replication in some cells could obviously be due to the absence of specific proteases. On the bases of host-pathogen interactions the BHK-21/FX was developed using a cellular protease factor to support high-titer LP influenza virus replication. The impact of the cell on viral titers and sensitivity at subsequent viral passages at defined cell density and virus MOI were analyzed. Distribution of the specific influenza virus receptor, specificity to other subtypes, sensitivity to higher MOIs, cell stability, and the annexin and lipid raft cellular proteins interactions need to be investigated in future studies to establish influenza virus diagnosis or vaccine manufacturing platforms based on the cellular serine protease and engineered BHK-21 cells.

Disclosure

Shahla Shahsavandi as corresponding author of the paper would like to assure that neither the submitted materials nor portions have been published previously or are under consideration for publication elsewhere.

Conflict of Interests

All of the authors declare that they have no conflict of interests.

Authors' Contribution

Shahla Shahsavandi and other authors participated meaningfully in the study and have approved the final paper.

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