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H3N2 Influenza A Virus Replicates in Immortalized Human First Trimester Trophoblast Cell Lines and Induces Their Rapid Apoptosis

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Abstract

Problem—Epidemiological data suggested that pandemic influenza increased the risks of spontaneous abortion and premature labor, while seasonal influenza also increased the risk of schizophrenia in adolescence. However, their pathogenesis is so far unknown.

Method of study—The first trimester trophoblast cell lines, namely, Swan71 and HTR8 cells were challenged with A/Udm/72 influenza virus (H3N2). At indicated time points, cells were examined for expression of influenza proteins. Viral replication in culture media, apoptosis and the expression of human leukocyte antigen (HLA)-G were also examined.

Results—Intracellular localization of viral proteins was observed. Twenty-four hours after inoculation, virus was detected in culture media while most cells fell into apoptosis. During apoptosis, expression of HLA-G was unchanged.

Conclusion—We revealed replication of low pathogenic influenza virus in the first trimester trophoblast cell lines. Placental damages are likely to be induced by direct cytopathic effects of influenza virus and subsequent apoptosis rather than down regulation of HLA-G expression and subsequent rejection by maternal immune system.

Keywords

Apoptosis; first trimester trophoblast; influenza A virus

Introduction

Influenza is a highly contagious, acute viral febrile respiratory infection that results in global morbidity and mortality. During pregnancy poor outcome have been reported following the

previous influenza pandemics.¹ During the influenza pandemic of 1918, remarkably high rates of spontaneous abortion and preterm birth were reported.^{2,3} Asian influenza pandemic during 1957–58 also increased the rates of central nervous system defects and several other adverse outcomes, including birth defects, spontaneous pregnancy loss, fetal death, and preterm delivery,^{4–6} while seasonal influenza did not increase the rates of pregnancy complications.⁷ Recent studies have suggested the association of maternal influenza infection during pregnancy with three to seven-fold increase of schizophrenia in the offspring.^{8–10} Brown et al. reported a statistically significant association noted between elevated concentrations of cytokines or antibodies to influenza antigens in maternal serum and the incidence of schizophrenia in the offspring. They speculated that 4–21% of schizophrenia cases would have been prevented if the maternal infection had not occurred. Experimental rodent models have suggested that maternal influenza infection can influence the physiology, behavior, and neuropathology of adult offspring.¹¹ As viral RNA has not been detected in the fetal brain of influenza infected animals, these changes are speculated to be secondary to the maternal inflammatory responses, rather than results of direct viral effects.¹² To the best of our knowledge, the susceptibility and kinetics of influenza viruses in the early gestational trophoblasts have not been studied, while replication of influenza virus in freshly prepared cells from the term fetal membranes have been studied extensively by Uchide et al.¹³

In this study, we have hypothesized that invasive trophoblasts may be targets of influenza virus *in vivo* and *in vitro*. Taking into account the difficulty to obtain first-trimester human trophoblast primary cultures, we utilized the HTR8/SVneo and Swan71 (Sw.71) cell lines, both were derived from human first trimester extravillous trophoblast (EVT)¹⁴ and have been employed as models of EVT cell proliferation, migration and invasiveness *in vitro*.^{15–18} In this study, we observed that both cell lines were susceptible to non-pandemic influenza A (H3N2) virus and fell into apoptosis without remarkable reduction of human leukocyte antigen (HLA)-G expression.

Materials and methods

Cell Lines

The human first trimester trophoblast cell lines Swan71 (Sw.71, derived by telomerase-mediated transformation of a 7-week cytotrophoblast isolate described by Straszewski-Chavez)^{19,20} and HTR8 (H8, originally were obtained from human first-trimester placenta and immortalized by transfection with a cDNA construct that encodes the simian virus 40 large T antigen)¹⁴ were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sw.71) or RPMI 1640 (H8) normal growth medium (Gibco-Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 0.1 mM minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, 100 nM of penicillin and streptomycin (Gibco-Invitrogen). Cells were cultured in monolayer at 37°C and 5% CO₂.

Virus

The influenza virus A/Udorn/307/72 (H3N2) maintained in Nihon University School of Medicine was propagated either in 10-day-old embryonic chicken eggs or in Madin-Darby canine kidney (MDCK) cells. MDCK cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% FBS. Viral titers were checked with plaque formation assay before inoculation.

Viral Infections

Cells cultured in 6-well plates (2×10^5 /well) were washed with phosphate-buffered saline (PBS) and infected with the influenza A/Udorn/72 at a multiplicity of infection of five (MOI

5) for 40 min at 37°C. After a 40-min adsorption, the inocula were removed, and cells were incubated with RPMI (for H8 cells) or DMEM (for Sw.71 cells) for the indicated times. Non-infectious influenza A/Udorn/72 was generated by incubating the virus at 56°C for 30 min, and inactivation was confirmed by the lack of cytopathic effect or replication on MDCK cell. For apoptosis examination, positive control was performed by incubating the cells with Actinomycin D (Nakarai Tesque, Inc., Kyoto, Japan), 1 μ L of stock solution (1 mg/mL) per 10^5 cells.

Immunofluorescence Assay

H8 and Sw.71 cells grown on glass cover slips in 6-well plates were infected with the A/Udorn/72 at an MOI of 5. At the indicated time after infection, the cells were fixed with 4% paraformaldehyde solution for 10 min, washed with PBS, and incubated with rabbit anti-Udorn serum (1:1000 dilution), which had been prepared by immunizing rabbits with the purified virions as described previously,²¹ for 1 hr at room temperature. Negative controls were placed by mock-virus treatment, heat-inactivated virus treatment as well as staining with rabbit sera without virus immunization. After washing with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG solution, prepared using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes, Invitrogen, Tokyo, Japan), for 40 min at room temperature. After washing, the cells were mounted with Fluoromount G (SouthernBiotech, Birmingham, AL, USA), and the fluorescent images were collected using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).²² Each experiment was repeated at least three times, and Fig. 1 shows typical influenza viral protein staining.

Hemagglutination Assay

Serial twofold dilutions of specimens were made in 50 μ L of PBS on 96-well U-bottom plates. To each well, 50 μ L of 0.5% chicken erythrocytes in PBS was then added. The plates were kept at 4°C for 1 hr, then the hemagglutination (HA) patterns were read and HA titers were determined from the last dilution showing complete HA. Each experiment was repeated three times and the average values of the three independent measurements are shown.

RNA Isolation, cDNA Synthesis and Real Time Polymerase Chain Reaction

Cultured trophoblasts in 6-well plates and supernatant were solubilized with 1mL of TRIZOL[®] (Invitrogen). RNA was first extracted with chloroform, precipitated with absolute ethanol. Next, after washing with 75% ethanol, RNA was re-dissolved in RNase-free water. The quantity and quality of the RNA were determined by ultraviolet absorbance at 260 nm. Then, DNase digestion step was performed using DNase I (TaKaRa Bio Inc., Shiga, Japan).

For cDNA synthesis, reverse transcription (RT) was performed using PrimeScript[™] RT Reagent Kit (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol, in which, the primer T7-Uni12 was used for influenza examination.

Real-time polymerase chain reaction (PCR) was conducted with the SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Bio Inc.) according to the manufacturer's protocol with appropriate annealing temperature. For quantification of viral RNA copy numbers, dilutions of an external standard corresponding to 10^6 copies down to 1 copy of an influenza viral RNA solution were subjected to PCR in parallel using the primer pair T7 and vPB2qR3.²³ All reactions were performed on the ABI Prism[™] 7500 sequence detection system (Perkin-Elmer Applied Biosystems, Tokyo, Japan). The primers were obtained from TaKaRa Co.Ltd (Tokyo, Japan). Oligonucleotide sequences of the primers used in this study are shown in Table I.

Detection of Apoptosis

The early apoptosis was measured using Apo-Strand™ ELISA apoptosis detection kit (BIOMOL, Plymouth Meeting, PA, USA). This detection system employs monoclonal antibody to single-stranded DNA (ssDNA) which occurs in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis.²⁴ Tests were performed in cells grown in 96-well plates. For influenza virus inoculation Sw.71 and H8 cells were seeded (5000 cells/well) and cultured overnight before treatment. Cells were incubated with an MOI of 5 of influenza A/Udorn/72 and incubated for 18 hr. In brief, cells were fixed for 30 min with fixative indicated by the manufacturer and dried by incubating at 56°C for 20 min. Formamide was then added to the cells, and they were heated at 56°C for 30 min to denature DNA in apoptotic cells. Blocking solution was then added to all wells to block non-specific binding sites. Next, cells were incubated with antibody mixture for 30 min and rinsed with 1X wash buffer. After washing, cells were incubated with 100 µL of peroxidase substrate for 45 min, and absorbance was read using an ELISA plate reader at 405 nm. Reaction (color development) was stopped by the addition of 100 µL of 1% sodium dodecyl sulfate. Negative controls without viral inoculation, heat inactivated viruses and positive control with actinomycin D treatment were placed.

Flow Cytometric Analysis of HLA-G

For determination of the surface expression of HLAG molecules, the cells (2×10^5) were washed twice with cold PBS containing 3% bovine serum albumin and then stained with FITC-conjugated mouse antihuman HLA-G monoclonal antibody (Clone MEMG/11) (Exbio, Praha, Czech Republic) for 30 min at 4°C. After centrifugation, the cells were suspended in 0.5 mL of 1% paraformaldehyde and then subjected to flow cytometry analysis. Gated events were collected using the FACScalibur cytometer and analyzed with CellQuest software (Becton-Dickinson Biosciences, Tokyo, Japan).

Statistical Analysis

Analysis of variance was used for statistical analysis of the results. The resulting *P*-value <0.05 using Fisher's exact test was considered statistically significant.

Results

Immunofluorescence Detection of Influenza Virus Derived Antigens in H8 and Sw.71 Cells

H8 and Sw.71 cell lines were inoculated with nonpandemic influenza A Udorn/72 virus (H3N2). Intracellular localization of the virus in the infected cells was examined using immunofluorescence staining of the cells at indicated time points of 8 and 24 hr post infection (h p.i.) using rabbit anti-Udorn serum targeting with viral proteins of hemagglutinin protein (HA), nucleoprotein (NP) and matrix protein (M1). Non-immunofluorescence was detected in the mock-infected or heat-inactivated virus-treated cell (Fig. 1, panels 1A–F, and 2A–F) while strong intracellular localization of the virus was detected in the both cell lines at 8 and 24 h p.i. (Fig. 1, panels 1I, 1M for H8 cells and 2I, 2M for Sw.71 cells). Negative controls employing rabbit sera showed no immunofluorescence (Fig. 1, panels 1N–Q and 2N–Q). In addition, as seen in the Fig. 1, chromatin condensation and apoptotic nuclear fragmentation were observed by Hoechst staining (panels 1H and 2L) in influenza virus infected cells.

Apoptosis of the Cells Infected

To confirm the presence of apoptosis and to make quantitative analysis, we examined the amount of ssDNA using ELISA at 18 h p.i.. At the 18 h p.i., the absorbance of ssDNA at 405 nm was 2.1 ± 0.05 for H8 cells and 1.95 ± 0.15 for Sw.71 cells. For mock treated cells

and inactivated virus-treated cells, the absorbances of ssDNA were 0.28 ± 0.08 and 0.29 ± 0.11 , respectively for H8 cells; and in the case of Sw.71 cells, they were 0.30 ± 0.07 and 0.38 ± 0.13 , respectively. The absorbance values of negative and positive controls were 0.81 ± 0.01 and 2.68 ± 0.16 , respectively. The amount of ssDNA which suggests presence of apoptosis induced by influenza infection of the two cell lines were significantly higher than those of the corresponding mock-treated cells and inactivated-virus treated cells ($P < 0.0001$ for all cases) (Fig. 2).

Viral Replication in Infected Cells into Culture Media

By HA assay, we observed the evidence of exocytotic release of the viral progeny from both cell lines. Although at 8 h p.i., HA titers were under detectable levels in both cell lines, they were detected at 24 h p.i. (Fig. 3). We observed almost identical results with plaque formation assay (data not shown). Real time RT-PCR detected viral RNA at 8 h p.i. in the both cell lines and significantly increased at 24 h p.i. (Fig. 4).

Expression of HLA-G

HLA-G is a non-classical major histocompatibility antigen with a restricted pattern of expression.²⁵ HLA-G is selectively expressed on cytotrophoblasts and invasive extravillous trophoblast at the fetomaternal interface where it may play a major role in maternal-fetal tolerance.²⁶ Its down-regulation caused by viral infections is considered to be a major cause of miscarriage.²⁷ We examined the expression of HLA-G using flow cytometry. Both in H8 and Sw.71 cells, we detected HLA-G in protein level. We observed no remarkable changes of HLA-G expression of the both cell lines at 8 and 24 h p.i. (Fig. 5). Forty-eight h p.i., we could detect no HLA-G protein because of cytopathic effects (data not shown).

Discussion

In this study, we revealed that immortalized human trophoblast cell lines mimicking invasive trophoblasts are susceptible to H3N2 influenza virus. We consider that this finding is important because the human placenta is characterized by extensive invasion of cytotrophoblasts into the uterus wall, allowing their direct contact with the maternal blood, and by the extent of hormonal production.²⁸ In other words, trophoblastic invasion during the first trimester is a critical step to establish human pregnancy. Thus, insufficient invasion caused by various reasons including genetic abnormalities, disrupted maternal immune tolerance as well as viral infections results pregnancy failures. Productive infection and possible pathogenic roles of cytomegalovirus (CMV),²⁹ adenovirus,³⁰ adeno-associated virus-2 (AAV-2)³¹ in early human trophoblasts have been reported while infection of influenza virus have not been studied despite its clinical importance. The most plausible explanation is that non-pandemic influenza is a relatively localized disorder in respiratory organs. Viremia is believed to occur infrequently in influenza.³² A number of studies searching for influenza viremia after the onset of illness have failed to detect virus, supporting the notion that influenza viremia is at most a rare event in the post-symptomatic period and if it exists, it is not generally sustained for long periods. However, recent studies employing highly sensitive PCR suggested transient viremia before onset of respiratory is not rare.^{33,34}

More importantly, viral RNA was detected from extrapulmonary sites including autopsy specimens of heart, kidney, brain, spinal cord, spleen, and liver of a pregnant 19-year-old woman who died as a result of A2/HongKong/8/68 infection.³⁵ Another case of a 24-year-old pregnant woman infected with influenza A/Bangkok (H3N2) showed positive results in fetal tissues and amniotic fluid.³⁶

Taken together, these findings suggest fetoplacental tissues have a chance to be infected with influenza virus delivered by maternal systemic circulations. Limited viremia could be controlled with neutralizing antibodies evoked with the previous influenza infection or vaccinations.

However, in a case of pandemic, it is a completely different story. An influenza pandemic can develop with the emergence of a new virus with high transmission capability, and that harbors a novel HA that has not circulated for decades. In the 20th century, there were three overwhelming pandemics with influenza A, in 1918, 1957 and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu) and H3N2 (Hong Kong flu), respectively. During pandemics, pregnant subjects might have a higher risk of viremia and subsequent transplacental infection because of lack of previous immune exposures.

Gu et al. reported an autopsy case of pregnant woman who was killed by H5N1 influenza.³⁷ Employing in situ hybridization and immunohistochemical methods, they observed positive staining of influenza virus in placenta and fetal organs. They speculated that in addition to the lungs, H5N1 influenza virus disseminates to other organs including the brain and could also be transmitted from mother to fetus across the placenta.

Taken together, our findings suggest not only pandemic H5N1 influenza virus but also H3N2 and possibly other seasonal influenza viruses might replicate effectively in the invasive trophoblasts and subsequently induce placental apoptosis which might cause congenital anomalies as well as pregnancy failures. This cytopathic effect is independent from reduced HLA-G expression often observed in placental viral infections.

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Fig. 1.

Immunofluorescent images of H8 and Sw.71 cells infected with the A/Udorn/72 at an MOI of 5. At 8 h p.i. (panels 1G–I and 2G–I) and 24 h p.i. (panels 1K–M and 2K–M), the cells were fixed and stained for viral proteins using immunofluorescence with rabbit anti-Udorn serum. (1G, 1K, 2G, and 2K) differential interference contrast images; (1H, 1L, 2H, and 2L) the nuclei were counterstained with Hoesch stain (blue); (1I, 1M, 2I, and 2M) immunofluorescence staining of influenza viral proteins. Immunofluorescence (right) and corresponding brightfield (left) images are presented of the cells stained with antibodies for the viral proteins. Mock-infected cells and the cells incubated with the inactivated virus were also examined at 8 and 24 h p.i. (panels 1A–F and 2A–F). (Panels 1N–Q and 2N–Q), Udorn-infected H8 and Sw.71 cells stained with rabbit serum as negative controls.

Fig. 2.

ELISA detection of single-stranded DNA present in apoptotic cells. Each of the cell suspensions was prepared in a 96-well flat-bottom plate as specified by the manufacturer. Then the cells were inoculated with the influenza A/Udorn/72 H3N2 at an MOI of 5. Single-stranded DNA provided in the ELISA kit was used as a control. Results are expressed as the means and standard deviations of three determinations.

Fig. 3. Kinetics of influenza virus replication measured using the HA assay. H8 and Sw.71 cells were infected with the A/Udorn/72 at MOI 5. At the indicated time points post-infection (8 and 24 h p.i.), HA activity was measured in the culture supernatants.

Fig. 4. Results of quantification of viral RNA copies in culture media using real-time PCR. y-axis: viral RNA copy numbers. The experiment was repeated three times. Each data point represents the mean $\pm$ S.D.

Fig. 5. Flow cytometry analysis of the non-classical class I MHC molecule HLA-G of H8 and Sw. 71 cells by staining of HLA-G with FITC-conjugated mouse anti-human HLA-G monoclonal antibody. There was no shift in the histogram upon infection with the virus at 8 and 24 h p.i.

Table I

Oligonucleotide sequences of the primers used in this study

Primer	Sequence 5' to 3'	Sense
T7	TAA TAC GAC TCA CTA TAG G	+
T7-Uni12	TAA TAC GAC TCA CTA TAG GAG CAA AAG CAG G	+
vPB2qR3	TAG TAT CTC GCG AGT GCG AGA CT	–