Virus Research xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Identification of morphological differences between avian influenza A viruses grown in chicken and duck cells

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ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form
11 December 2014
Accepted 10 January 2015
Available online xxx

Keywords:
Avian influenza virus
Chicken
Duck
Virus morphology
Filamentous virus

ABSTRACT

Although wild ducks are considered to be the major reservoirs for most influenza A virus subtypes, they are typically resistant to the effects of the infection. In contrast, certain influenza viruses may be highly pathogenic in other avian hosts such as chickens and turkeys, causing severe illness and death. Following *in vitro* infection of chicken and duck embryo fibroblasts (CEF and DEF) with low pathogenic avian influenza (LPAI) viruses, duck cells die more rapidly and produce fewer infectious virions than chicken cells. In the current study, the morphology of viruses produced from CEF and DEF cells infected with low pathogenic avian H2N3 was examined. Transmission electron microscopy showed that viruses budding from duck cells were elongated, while chicken cells produced mostly spherical virions; similar differences were observed in viral supernatants. Sequencing of the influenza genome of chicken- and duck-derived H2N3 LPAI revealed no differences, implicating host cell determinants as responsible for differences in virus morphology. Both DEF and CEF cells produced filamentous virions of equine H3N8 (where virus morphology is determined by the matrix gene). DEF cells produced filamentous or short filament virions of equine H3N8 and avian H2N3, respectively, even after actin disruption with cytochalasin D. These findings suggest that cellular factors other than actin are responsible for the formation of filamentous virions in DEF cells. The formation of elongated virions in duck cells may account for the reduced number of infectious virions produced and could have implications for virus transmission or maintenance in the reservoir host.

1. Introduction

Influenza A viruses show variable morphology, with shapes ranging from spherical or elliptical and about 100 nm in diameter to elongated or filamentous with a length reaching to more than several micrometres; occasionally, they are pleomorphic (Calder et al., 2010). Viruses have three membrane-associated proteins: haemagglutinin (HA); neuraminidase (NA); and a small amount of matrix protein 2 (M2). Beneath the lipid envelope, there is a matrix protein 1 (M1) layer. All these proteins play an important role in virus morphogenesis (Bouvier and Palese, 2008; Palese and Shaw, 2007). Diversity of virus morphology is thought to be a genetic trait; in particular the seventh viral RNA segment (M), which encodes the matrix proteins, plays a dominant role in determining virus shape (Elleman and Barclay, 2004; Roberts et al., 1998). However, the importance of specific M protein residues as determinants of virus morphology appears to differ between influenza viruses of different species (Elton et al., 2013). In addition, the surface glycoproteins (HA and NA) have also been implicated in modulation of virus shape (Jin et al., 1997; Zhang et al., 2000).

Non-viral factors may also determine influenza A virus morphology. Newly isolated clinical strains usually comprise filamentous forms, while laboratory-adapted viruses, especially

Abbreviations: 293T, human embryonic kidney cells; BSA, bovine serum albumin; cDNA, complementary deoxyribonucleic acid; CEF, chicken embryo fibroblasts; Cyt.D, cytochalasin D; DAPI, 4′,6-diamidino-2-phenylindole; DEF, duck embryo fibroblasts; DM, dissociation medium; DMEM, Dulbecco's modified Eagle's medium; EM, electron microscopy; FCS, foetal calf serum; HA, haemagglutinin; HPAI, highly pathogenic avian influenza; HRP, horseradish peroxidase; LC3, microtubule-associated protein 1A/1B-light chain 3; LLC-MK2, rhesus monkey kidney epithelial cell line; LPAI, low pathogenic avian influenza; M, matrix; MDCK, Madin Darby canine kidney cells; MOI, multiplicity of infection; NA, neuraminidase; NP, nucleoprotein; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulphate; TCPK, L-1-tosylamido-2-phenylethyle chloromethyl ketone; VLPs, virus-like particles.

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http://dx.doi.org/10.1016/j.virusres.2015.01.005
0168-1702/

with many passages in eggs or cell culture, typically exhibit spherical morphology (Cox et al., 1980). Cellular factors such as cell polarity and the actin cytoskeleton can play a major role in determining virus morphology (Sun and Whittaker, 2007). Epithelial cells have been shown to produce more filamentous particles than fibroblasts and an intact actin cytoskeleton is important for forming filamentous but not spherical virions (Roberts and Compans, 1998; Simpson-Holley et al., 2002). Furthermore, endocytic trafficking regulator and its effector Rab11-family interacting protein 3 (Rab11-FIP3) are also required to support the formation of filamentous virions (Bruce et al., 2010).

Aquatic birds such as ducks are considered to be the major natural reservoirs of influenza A viruses (Webster et al., 1992). Infection of ducks is usually clinically silent, and virus replication mainly occurs in the epithelial cells of the digestive tract. Large amount of viruses are shed in faeces leading to environmental contamination (Webster et al., 1978). In contrast, when transmitted to domestic poultry such as chickens, turkeys and quail, low pathogenic avian influenza (LPAI) viruses typically cause mild respiratory signs and reduced productivity (Pillai et al., 2010). In addition, in experimentally infected ducks, most highly pathogenic avian influenza (HPAI) virus infections are non-lethal and produce limited or no clinical signs (Kishida et al., 2005; Jeong et al., 2009; Shortridge et al., 1998). In contrast, HPAI viruses infecting chickens (naturally and experimentally) are lethal causing mortality reaching 100%, often within two days. Kuchipudi et al. (2011) observed that duck cells undergo rapid cell death following *in vitro* infection with LPAI H2N3 viruses, while cell death occurs less rapidly after infection in chicken cells. This study also showed that the number of infectious virions produced in chicken cells was significantly higher than in duck cells. However, there was no significant difference between viral M gene RNA production between the two species. We hypothesized that the differences in production of infectious H2N3 virus in chicken and duck cells may be due to altered virus assembly or defects in the viral structure. We therefore examined virus production from chicken and duck cells using transmission electron microscopy and compared the ability of cells to produce filamentous virus after infection with low pathogenic avian H2N3 (which typically has a spherical morphology in cell culture) or a filamentous equine virus, H3N8 (where the M protein sequence determines a filamentous morphology) by immunofluorescence. Additionally, the importance of cellular actin in determining virus morphology was investigated by disruption with cytochalasin D.

2. Materials and methods

2.1. Viruses

Two influenza A subtypes were used in this study: LPAI H2N3 (A/mallard duck/England/7277/06) and equine influenza H3N8 (A/equine/Newmarket/5/03) that were kindly provided by Dr. Ian Brown (Animal and Plant Health Agency) and Dr. Debra Elton (Animal Health Trust), respectively. H3N8 has a filamentous morphology determined largely by amino acid 85 (S) and 231 (D) of the M protein (Elton et al., 2013). Viruses were propagated in the allantoic cavity of embryonated hen's eggs.

2.2. Cells

MDCK cells were maintained in growth media consisting of Dulbecco's modified Eagle's medium containing 10% foetal calf serum (FCS; Invitrogen) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Embryo fibroblast cells were extracted from 8-day-old chicken embryos (eggs provided by Henry Stewart & Co. Ltd., Louth, Lincs, UK) and 10.5-day-old Pekin duck embryos (eggs provided by Cherry Valley Farms Ltd., Rothwell, Lincs, UK). The embryos were minced and digested in 0.25% trypsin in dissociation medium (DM; F12 Hams, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5% amphotericin B) at 37 °C for 1 h. Large undigested tissue pieces were removed using a cell strainer and the remaining suspension was centrifuged at 400 × *g* for 5 min. Cells were seeded into cell culture flasks (Nunc) and maintained in growth media.

2.3. Infection of chicken and duck cells

Monolayers of chicken and duck embryo fibroblast cells were grown in 24-well plates. Cells were infected with LPAI H2N3 in triplicate at multiplicity of infection (MOI) of 1.0 in serum-free medium (infection medium) supplemented with 2% Ultroser G (Pall Biosepra), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and 500 ng/ml TPCK trypsin (Sigma–Aldrich), and incubated for 2 h. After 2 h, the cells were carefully washed three times with PBS, to remove residual virus inoculum, followed by addition of fresh media. Supernatants were collected at 2, 4, 6, 8, 24, and 48 h post infection and were stored at −80 °C until use.

2.4. Virus infectivity assay

Confluent MDCK cells grown in 96-well plates were infected in triplicate with virus collected from chicken and duck cells to determine virus infectivity. Cells were washed after 2 h incubation with virus, incubated for a further 4 h and then fixed with 1:1 acetone:methanol. Viral nucleoprotein expression was detected using a primary mouse monoclonal antibody (Abcam, Cambridge, UK) followed by visualization with Envision+ HRP (DAB; Dako, Ely, UK). Cells expressing viral nucleoprotein were counted and the mean number of positive cells in four fields used to calculate focus-forming units of virus per microlitre of inoculum.

2.5. Quantification of virus production (measurement of M gene copy number)

A one-step reverse transcription-RT-PCR assay using influenza virus M gene-specific PCR primers and hydrolysis probe was performed as previously described (Slomka et al., 2009). In brief, viral RNA was extracted from culture supernatants of infected chicken and duck cells using QIAamp viral RNA purification kit (Qiagen) following the manufacturer's instructions. A one-step absolute quantification of viral M gene expression was performed using SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen). Quantitative RT-PCR conditions and cycling parameters for samples were as follows: one cycle at 50 °C for 30 min, one cycle at 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Threshold cycle (Ct) values were converted to viral gene copy number by a standard curve generated using *in vitro* transcribed M gene RNA using LightCycler 480 software, release 1.5.0 (Roche).

2.6. Western blotting

Polyacrylamide gel electrophoresis using Novex 14% Tris–Glycine mini gels (Invitrogen), followed by western blotting, were used to detect M1 protein in culture supernatants. Samples to be tested, 1 μl of chicken or duck virus supernatant, were suspended in 5 μl of 2× Tris glycine SDS sample buffer (Invitrogen) with 1 μl of 2× reducing agent (Invitrogen) and distilled water (to 10 μl) to lyse viral protein. The mixture was incubated at 95 °C for 5 min, and then cooled and spun briefly. Samples were run on the gel for approximately 1 h then transferred to a 0.2 μm Hybond ECL Nitrocellulose Membrane (GE Healthcare, Life Sciences) by wet blotting. The membrane was treated with

Please cite this article in press as: Al-Mubarak, F., et al., Identification of morphological differences between avian influenza A viruses grown in chicken and duck cells. Virus Res. (2015), http://dx.doi.org/10.1016/j.virusres.2015.01.005