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Productive replication of avian influenza viruses in chicken endothelial cells is determined by hemagglutinin cleavability and is related to innate immune escape

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ABSTRACT

Endotheliotropism is a hallmark of gallinaceous poultry infections with highly pathogenic avian influenza (HPAI) viruses and a feature that distinguishes HPAI from low pathogenic avian influenza (LPAI) viruses. Here, we used chicken aortic endothelial cells (chAEC) as a novel in vitro infection model to assess the susceptibility, permissiveness, and host response of chicken endothelial cells (EC) to infections with avian influenza (AI) viruses. Our data show that productive replication of AI viruses in chAEC is critically determined by hemagglutinin cleavability, and is thus an exclusive trait of HPAI viruses. However, we provide evidence for a link between limited (i.e. trypsin-dependent) replication of certain LPAI viruses, and the viruses' ability to dampen the antiviral innate immune response in infected chAEC. Strikingly, this cell response pattern was also detected in HPAI virus-infected chAEC, suggesting that viral innate immune escape might be a prerequisite for robust AI virus replication in chicken EC.

1. Introduction

Avian influenza (AI) is a worldwide spread and highly contagious disease of birds caused by type A influenza viruses. Influenza A viruses are classified according to the antigenic properties of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins that are inserted in the viral envelope. To date, 16 HA and 9 NA subtypes have been detected in various combinations in wild aquatic birds of the orders Anseriformes and Charadriiformes, the natural reservoir for influenza A viruses (Fouchier et al., 2005; Olsen et al., 2006). Occasionally, these avian-origin influenza A viruses –commonly referred to as AI viruses– cross-over from their natural reservoir to gallinaceous birds or mammals, and new viral genotypes may become established in these accidental spill-over hosts (Alexander, 2000; Franca and Brown, 2014). Depending on their pathotype in the chicken host and a molecular signature (mono- or multibasic cleavage site) in their HA glycoproteins, AI viruses are categorized as either low pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI) viruses. LPAI viruses generally cause mild or subclinical respiratory and gastrointestinal tract infections in galliform birds (Mo et al., 1997). However, once established in gallinaceous poultry, LPAI viruses of the H5 and H7 subtypes may evolve into HPAI viruses causing fatal systemic infections with mortality rates of up to 100% in affected flocks (Swayne, 2007).

Cellular tropism of influenza A viruses is critically determined by the viruses' ability to attach to and efficiently enter a specific host cell type. This process is initiated by binding of the viral HA protein to galactose (Gal)-linked sialic acid (SA) moieties of the membranous cellular cognate receptors. AI viruses generally prefer binding to HA receptors in an α2,3 Gal-linkage SA configuration, which are abundantly expressed in most avian cell types or tissues (Costa et al., 2012; Franca et al., 2013; Kimble et al., 2010). Following viral attachment and subsequent receptor-mediated endocytosis, the HA protein mediates the fusion of viral and endosomal membranes at low pH, leading to the release of viral ribonucleoprotein complexes into the cytoplasm (Bottcher-Friebertshauser et al., 2013). However, to exert its fusogenic function, HA must first be cleaved and activated by cellular proteases. LPAI viruses possess a monobasic cleavage site in their HA precursor

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(HA_0) proteins that requires cleavage by trypsin-like proteases to permit proteolytic activation (Garten and Klenk, 2008; Klenk and Garten, 1994). As such proteases are predominantly found in the epithelia of the avian respiratory and gastrointestinal tract, productive replication of LPAI viruses in gallinaceous poultry is generally restricted to these organ sites (Wood et al., 1995). In contrast, HPAI viruses invariably display a multibasic cleavage site (MBCS) in their HA_0 proteins which can be cleaved by ubiquitously expressed proprotein convertases such as furin and PC5/6 (Garten and Klenk, 2008; Horimoto et al., 1994). This molecular configuration allows HPAI viruses to spread systemically and to productively replicate in a large variety of cell types and organ sites in gallinaceous birds, eventually leading to organ-specific disease manifestations and/or multi-organ failure (Swayne, 2007).

Previous studies have shown that endotheliotropism is a hallmark of gallinaceous poultry infections with HPAI viruses (Short et al., 2014b; Swayne, 2007). Endothelial cells (EC) form a single layer of squamous cells that lines the interior surface of blood and lymphatic vessels. However, EC are not only forming the barrier between the vessel lumen and surrounding tissue, they also play a critical role in the maintenance of vascular and immune homeostasis (Michiels, 2003). Consequently, endothelial cell dysfunction (ECD) is a central phenomenon in a large variety of pathologic conditions, including various viral diseases (Lee and Liles, 2011; Steinberg et al., 2012). The pathological consequences of virus-induced ECD are generally severe and may include vascular barrier breakdown, edema, thrombosis, consumptive coagulopathy, hemorrhages, malfunctioning innate immune responses, and profuse inflammatory cell recruitment (Mackow et al., 2014). Strikingly, all these ECD-associated disorders have previously been described to be distinctive disease manifestations of HPAI virus infections in chickens (Chaves et al., 2014; Kobayashi et al., 1996; Kuribayashi et al., 2013; Muramoto et al., 2006; Suarez et al., 1998; Suzuki et al., 2009). Among gallinaceous poultry, the chicken is described as the species in which the tropism of HPAI viruses for EC is most prominent –and likely the cause of the typical rapid-onset of mortality (Bertran et al., 2017; Perkins and Swayne, 2001, 2003; Swayne, 2007). In other gallinaceous species, especially certain game birds including the common pheasant, red-legged partridge, and Chukar partridge, endotheliotropism of HPAI viruses is less pronounced and fatalities are rather due to the infection of neuronal cells, glial cells, cardiomyocytes, and adrenocorticotrophic cells (Bertran et al., 2014, 2017, 2011; Perkins and Swayne, 2001). Strikingly, compared to the situation in chickens, the progression of clinical HPAI manifestations and/or the onset of death is usually delayed in these gallinaceous species (Bertran et al., 2014; Perkins and Swayne, 2001). However, despite a wealth of data pointing to a central role of EC in the pathobiology of HPAI virus infections in chickens or other gallinaceous birds, the detailed mechanisms of the interaction of HPAI viruses with EC in galliform hosts are still largely unknown. Notably, the mechanisms that dictate the apparent restriction of EC permissiveness to HPAI viruses in chickens or other gallinaceous species still remain to be elucidated. This shortcoming is likely due to the present lack of an adequate EC line and/or the difficulty to prepare well-defined cultures of primary avian-origin EC as indispensable tools for in-depth in vitro infection studies.

In the present paper, we report the development of two novel chicken EC culture systems –primary chicken aortic endothelial cells (pchAEC) and a pchAEC-derived cell line (chAEC)– and their use in the assessment of the susceptibility, permissiveness, and host response of chicken EC to infections with LPAI and HPAI viruses. Our data show that productive replication of AI viruses in chicken EC is critically determined by the presence of a multibasic HA_0 cleavage site, and is thus an exclusive trait of HPAI viruses. However, we provide evidence that the replication fitness of HPAI viruses and certain LPAI viruses in chicken EC might also be related to effective viral innate immune escape mechanisms, as was previously shown for H5N1 HPAI viruses in other chicken cell culture models (Baquero-Perez et al., 2015; Liniger et al., 2012a). Furthermore, we present data that point to a potential role of limited EC infection and ECD in the pathogenesis of severe LPAI virus infections in gallinaceous poultry.

2. Materials and methods

2.1. Cell culture

Madin-Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (EMEM) (Gibco) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C and 5% CO_2. Chicken lung epithelial cells (CLEC213) (Esnault et al., 2011) were cultured in Dulbecco's modified Eagle medium/Nutrient F-12 Ham (DMEM F12) (Gibco) supplemented with 7.5% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 40 °C and 5% CO_2.

Primary chicken aortic endothelial cells (pchAEC) were prepared from specific-pathogen-free 18-day-old chicken embryos by adapting the aortic explant methodology first described by McGuire and Orkin (1987). Chicken embryos were killed by decapitation and the ascending aortas were dissected. Aortas were cut into small pieces, suspended in complete macrovascular EC growth medium (EGM-2 BulletKit) (LONZA), and transferred in gelatin-coated 100 mm diameter Petri dishes. Following 2 days of incubation at 40 °C and 5% CO_2, the cultures were inspected under an inverted light microscope to confirm the outgrowth of pchAEC from the adherent explants. The cultures were washed with phosphate-buffered saline (PBS) to remove non-adherent tissue pieces, overlaid with fresh EC growth medium, and incubated for additional 2 days at 40 °C and 5% CO_2. To further propagate pchAEC, the cultures were washed with PBS, dispersed by treatment with 0.05% trypsin-EDTA (Gibco), and split at a ratio of 1:5–1:6. A non-immortalized chAEC line with a limited replicative lifespan was established by serial passaging of pchAEC until passage 70–75. For all experiments in this study, pchAEC and chAEC were used at passage 3 and 50–67, respectively.

2.2. Viruses

The following AI viruses were used in this study: LPAI virus A/Mallard/Marquenterre/Z237/83 (H1N1-Ma83) (kindly provided by N. Naffakh, Institut Pasteur, Paris, France) was derived by reverse genetics (Munier et al., 2010), propagated in 10-day embryonated chicken eggs (2 passages), and further amplified in MDCK (2 passages). LPAI virus A/Mallard/Netherlands/3/99 (H5N2-Ma99) was rescued by reverse genetics as described previously (de Wit et al., 2004), and propagated in MDCK (2 passages). LPAI virus isolates A/Chicken/Italy/22 A/98 (H5N9-Ck98) (kindly provided by I. Capua, IZSVe, Padua, Italy) and A/Duck/France/05057a/05 (H6N2-Dk05) (kindly provided by V. Jestin, ANSES, Ploufragan, France) were propagated in 10-day embryonated chicken eggs (2 passages). LPAI viruses A/Mallard/Sweden/81/02 (H6N1-Ma02) and A/Guinea fowl/Honk Kong/WF10/99 (H9N2-Gf99) were rescued by reverse genetics (de Wit et al., 2004), propagated in MDCK (2 passages), and further amplified in 10-day embryonated chicken eggs (1 passage). LPAI virus A/Turkey/Italy/977/99 (H7N1-Tk99 –a direct HPAI virus progenitor) (kindly provided by R. Volmer, INRA-ENVT, Toulouse, France) was derived by reverse genetics (Soubies et al., 2010) and propagated in MDCK (2 passages) or embryonated chicken eggs (2 passages). HPAI viruses A/Turkey/Turkey/5/05 (H5N1-Tk05) and A/Chicken/Netherlands/1/03 (H7N7-Ck03) were rescued by reverse genetics (de Wit et al., 2004) and propagated in MDCK (2 passages). HPAI virus A/Netherlands/219/03 (H7N7-Hu03 –an isolate from a fatal human case) was rescued by reverse genetics (de Wit et al., 2004), propagated in 10-day embryonated chicken eggs (1 passage), and further amplified in MDCK (2 passages). Isogenic mutants of the H5N1-Tk05 and H7N7-Ck03 viruses in which the MBCS was deleted (H5N1-Tk05$_{\Delta MBCS}$ and H7N7-Ck03$_{\Delta MBCS}$) were rescued by reverse genetics (de Wit et al., 2004), propagated in MDCK (2 passages),