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Novel mutations in avian PA in combination with an adaptive mutation in PR8 NP exacerbate the virulence of PR8-derived recombinant influenza A viruses in mice

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ABSTRACT

The polymerase complex of the low-pathogenic avian influenza virus [A/chicken/Korea/KBNP-0028/2000] (0028) has previously been characterized, and novel amino acid residues present in the polymerase acidic protein (PA) that likely contribute to pathogenicity toward mammals have been identified. In the present study, our aims were to generate A/Puerto Rico/8/34 (PR8)-derived recombinant viruses containing the *0028-PA* gene with a single amino acid mutation and to test their pathogenicity and replication ability. We found that the recombinant viruses acquired additional single mutations in the nucleoprotein (NP). Because the additional mutations in NP did not affect viral pathogenicity, but rather attenuated viral replication and polymerase activity, the incompatibility of the avian *PA* gene within the PR8 backbone may have induced an adaptive mutation in *NP*. To minimize the differences due to NP mutations, we generated 0028-PA mutants with an E375G mutation, not affecting viral replication and pathogenicity, in the *NP* gene. The PR8-PA(0028)-E684G mutant showed significantly higher viral replication in mammalian cells as compared to PR8-PA(0028) and led to 100% mortality in mice, with significantly increased interferon β expression. Thus, the E684G mutation in the *PA* gene may play an important role in viral pathogenicity in mice by increasing viral replication and the host immune response.

1. Introduction

Influenza A virus (IAV) has been continually circulating in aquatic birds, and its transmission to mammals has long been considered a worldwide public health threat (Webster et al., 1992). Genetic rearrangement in IAVs has occasionally led to the establishment of novel lineages that are capable of causing pandemic influenza virus infections (Scholtissek et al., 1978). In addition to genetic rearrangements, single amino acid substitutions in viral genomes can lead to changes in host preference, replicative efficiency, and pathogenicity of IAVs (Connor et al., 1994; Subbarao et al., 1993).

The IAV genome is divided into eight segments, and the evolutionary pathway of each genome segment in terms of pathogenicity to mammals is different. Previously, we reported the existence of prototypic nonstructural (*NS*) and polymerase genes that are derived from nonpathogenic avian IAVs (AIVs), and next identified novel mutations in nonstructural protein 1 (NS1) and polymerase basic protein 2 (PB2), which are involved in the pathogenicity to mammals (Kim et al., 2014, 2015; Lee et al., 2017). The mutations identified are shared by most AIVs and represent the minimum requirement for efficient replication in mammalian hosts (Kim et al., 2014, 2015; Lee et al., 2017).

Polymerase acidic protein (PA) performs crucial functions in the life cycle and host adaptation of IAVs (Hu and Liu, 2015). To date, various mutations in PA that increase the pathogenicity of AIVs toward mammals have been reported, and these mutations can be categorized according to their location in different functional motifs and domains (M21I, F35L, V44I, T97I, V127A, K142N, P224S, C241Y, L336M, A343T, K356R, S421I, T552S, I573V, and S616P,) (Bussey et al., 2011;

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Kim et al., 2010; Mehle et al., 2012; Sakabe et al., 2011; Seyer et al., 2012; Song et al., 2009; Sun et al., 2014; Xu et al., 2016; Yamaji et al., 2015). PA can be cleaved into N-terminal (1–257, PA-N) and C-terminal domains (277–716, PA-C) by tryptic proteolysis (Hara et al., 2006). The cap-snatching endonuclease and protease are located in the PA-N domain (Hara et al., 2006; Yuan et al., 2009). The PA-N domain also contains an IRF3-binding site and two nuclear localization signals (NLS I and NLS II) composed of amino acid residues 124–139 and 186–247 (Nieto et al., 1994; Yi et al., 2017). The PA-C domain also contains a loop at amino acid positions 350–355 that can affect pathogenicity toward mammals, and two RNA polymerase II-binding sites, which are composed of K635 and R638 for site 1, and K289 and R454 for site 2 (Lukarska et al., 2017; Xu et al., 2016). In addition, amino acid residues in the terminal regions of PA-C interact with the 15 amino acid residues at the N terminus of polymerase basic protein 1 (PB1) (He et al., 2008; Obayashi et al., 2008). Therefore, mutations arising in the vicinity of these functional motifs and domains may affect viral polymerase activity as well as the interaction with host factors.

In the present study, we investigated the effects of potential viral pathogenic PA mutations (T129I, G351E, M628V, and E684G) identified by comparing the nonpathogenic *PA* gene from A/chicken/Korea/KBNP-0028/2000 (H9N2) (0028) with that of other AIVs possessing mammalian pathogenicity (Kim et al., 2014). We generated PR8-derived recombinant viruses carrying these parent and mutated *0028 PA* genes with a single amino acid substitution. Of note, these recombinant viruses carried an additional single mutation in the *NP* gene. Among the different NP mutations, we selected a neutral mutation (E375G), which did not affect viral replication and pathogenicity to mice, and successfully generated recombinant viruses possessing single mutations in *0028-PA* in concert with the *E375G NP* mutation. By comparing polymerase activity, replication efficiency in mammalian hosts, and pathogenicity toward mice, we found that the E684G mutation in PA increases viral replication efficiency and pathogenicity in mice along with a significantly high induction of IFN-β expression.

2. Materials and methods

2.1. Viruses, eggs, and cells

The *PA* gene from A/chicken/KBNP-0028/2000 (H9N2) (0028) and other genes from A/Puerto Rico/8/34 (H1N1) (PR8) were used to rescue the recombinant PR8 virus using a bidirectional reverse genetics system (Hoffmann et al., 2000). Rescued viruses were propagated three times in 10-day-old Specific Pathogen-Free (SPF) embryonated chicken eggs (ECEs) (Charles River, Wilmington, CA, USA). To estimate the virus titer, each virus was serially diluted from 10^{-1} to 10^{-9} in 10-fold increments, and each dilution was injected into five 10-day-old SPF ECEs and inoculated into MDCK cells. The 50% chicken embryo infectious dose (EID_{50}) and 50% tissue culture infectious dose ($TCID_{50}$) were calculated by the Spearman–Karber method. MDCK and 293T cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). MDCK and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (Life Technologies Co., Carlsbad, CA, USA).

2.2. Construction of reverse-genetics plasmids

The *0028 PA* gene was cloned into Hoffmann's bidirectional transcription vector pHW2000 (Hoffmann et al., 2000). The insert sequence was confirmed by sequencing with the primers cmv-SF (5′-TAAGCAG AGCTCTCTGGCTA-3′) and bGH-SR (5′-TGGTGGCGTTTTTGGGG ACA-3′). Site-directed mutagenesis involving specific amino acid (codon) substitutions in the *PA* genes from the 0028 viruses was achieved by means of the Muta-direct Site-Directed Mutagenesis Kit (iNtRON, Daejeon, Korea). The primers for the site-directed mutagenesis are listed in Table S1. PA or NP plasmids containing changes in the specific amino acids of interest, and seven genome segments of PR8, were transfected into 293 T cells using Hoffmann's eight reverse-genetics plasmids, as previously described (Hoffmann et al., 2000; Lee et al., 2017). All mutant viruses were confirmed by RT-PCR and sequencing.

2.3. Mini-genome assay

To evaluate the polymerase activity of the PA mutants, we used a viral mini-genome assay based on luciferase activity, as described elsewhere (Lee et al., 2017). Briefly, 293T cells in 12-well plates were cotransfected with 100 ng of pHW-NP-Luc and mutant 0028 PA and/or PR8 NP with *PR8 PB1*, *PB2* genes. Additionally, 100 ng of the *Renilla* luciferase plasmid pRL-TK (Promega, USA) was cotransfected, which served as an internal control to normalize variations in transfection efficiency and sample processing. After that, at 24 h after transfection, luminescence was assessed by means of a Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) on a TECAN Infinite200 pro machine according to the manufacturer's instructions (Tecan Benelux, Giessen, Netherlands). The results are reported as the average from triplicate experiments, and the standard deviation (SD) was calculated.

2.4. Viral growth kinetics

To measure these kinetics, MDCK cells were seeded in 12-well plates. After 24 h, the confluent cells were washed twice with phosphate-buffered saline (PBS). Next, mutant viruses at multiplicity of infection (MOI) of 1.0 were inoculated into each well with DMEM supplemented with 1% of bovine serum albumin (BSA, fraction V) (Roche, Basel, Switzerland), 20 mM HEPES, an antibiotic-antimycotic solution (Life Technologies Co., Carlsbad, CA, USA), and 1 μg/mL TPCK-treated trypsin (Sigma-Aldrich, Saint Louis, MO, USA). Supernatants from the virus-infected cells were collected at 12, 24, 48, and 72 h postinoculation (hpi), and the viral titers were measured by $TCID_{50}$. The values are presented as the average of three independent experiments ± SD.

2.5. The mouse experiment

Six-week-old female BALB/c mice (KOATEC, Pyeongtaek, Korea) were used for the analysis of pathogenicity in mice. Five mice were anesthetized by intraperitoneal injection with 15 mg/kg Zoletil 50 (Virbac, Carros, France), after which the mice were inoculated intranasally with 10^6 EID_{50}/50 μL of one of the viruses, as previously described (Kim et al., 2014). Negative control (mock) mice were injected with the same volume of sterilized PBS. Mortality and weight loss were measured for 10 days. Mice that lost more than 25% of their original weight were euthanized and recorded as deaths. To measure virus replication in the lungs of infected mice, three mice from each group were injected with PBS (mock) or 10^6 EID_{50}/50 μL of a mutant virus. The lungs were collected at 3 days postinoculation (dpi) and then stored at −70 °C until further use. The lungs were ground up using a TissueLyzer 2 (Qiagen, Valencia, CA, USA) with 5 mm stainless steel beads in a suspension with a volume of PBS equal to 10% of the lung weight. Next, 10 volumes of PBS were mixed with the ground tissues. After centrifugation at 2000 × *g* for 10 min, the supernatants were subjected to determination of viral titers, which were measured *via* the $TCID_{50}$ method in MDCK cells. Lung supernatants at 3 dpi were used to measure the levels of interferon beta (IFN-β) by means of the VeriKine IFN-β ELISA kit (PBL Assay Science, Piscataway, NJ, USA). All mouse experiments were conducted at BioPOA Co. (Yongin, Korea) following a protocol that adhered to the National Institutes of Health's Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of BioPOA Co. (BP-2014-0004-2, BP-2016-006-2).