Mechanistic study on the assembly and release of lentiviral particles pseudotyped with haemagglutinin of highly pathogenic avian influenza H5N1 viruses: implications for strain-specific pseudotype development

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KEY MESSAGES

1. We have developed and characterised lentiviral particles pseudotyped with avian influenza A virus H5 haemagglutinin (H5pp) from a Cambodian H5N1 isolate, which can be used as a safe tool for high-throughput serological studies without the requirement of biosafety level 3 facilities (BSL-3).
2. Not all H5 haemagglutinins (HA) give rise to efficient production of H5pp. The amino acid residue at position 134 of the influenza A virus HA protein receptor binding domain is a critical molecular switch to control the ability of H5 HA to pseudotype lentiviral vectors.

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The emergence and re-emergence of H5N1 virus in Hong Kong and China

There have been three influenza pandemics in the 20th century: the 1918 Spanish flu (H1N1), the 1957 Asian flu (H2N2), and the 1968 Hong Kong flu (H3N2). These influenza pandemics caused severe illness, especially the 1918 Spanish flu that claimed millions of lives worldwide. Avian influenza A virus (H5N1) is highly pathogenic in wild birds and poultry. Occasionally, it crosses the species barrier to infect humans. The first human outbreak of H5N1 influenza virus occurred in Hong Kong in 1997 following severe outbreaks at three chicken farms. By the end of 1997, 18 people had been confirmed to be infected with H5N1 of whom six died. Luckily, the highly pathogenic avian influenza H5N1 virus has caused only limited infection in humans since 1997. H5N1 virus has not gained efficient transmissibility from poultry to humans or between humans. Nonetheless, the deadly H5N1 viruses persist to re-emerge in the human population.

Since the first human outbreak in 1997, there have been more than 500 documented human cases of H5N1 infection with a mortality rate of about 60%.[1] After the traumatic experience of H5N1 outbreaks in poultry in 1997, the Hong Kong SAR government launched an intensive surveillance system in live poultry markets and conducted mass culling of poultry whenever a severe outbreak was identified. These measures were successful and no human cases of H5N1 infection were reported until 2003. In January 2003, three people in a family of five developed fever and severe respiratory illness after their visit to mainland China. One died before H5N1 infection was confirmed, and two were confirmed to have H5N1 infection based on virological evidence. Human infection with H5N1 virus in China has been detected almost every year since 2003. In light of the increasing human travel between Hong Kong and Mainland China, the threat of H5N1 is unlikely to disappear.

In 2009, a swine-origin pandemic H1N1 virus (S-OIV) emerged in Mexico and the USA. The virus quickly spread globally with high transmissibility between humans. It is known that pigs are the mixing vessels that can be co-infected by swine, human, and avian influenza viruses. The fear is that if the high pathogenicity of H5N1 influenza virus somehow combines with the high transmissibility of S-OIV, it would be devastating. There is an urgent need to speed up research on influenza viruses, including the highly pathogenic H5N1.

Virulence factors for highly pathogenic H5N1 viruses

H5N1 viruses have been characterised in various animal models in order to understand the virulence factors. In birds and poultry, the cleavability of the virus haemagglutinin (HA) surface protein plays a major role in virulence. In the mouse model, a polybasic amino acid cleavage site in HA and 627K of polymerase basic protein 2 are two important virulence factors. Viruses with these features replicate and spread systemically in mice and eventually lead to a lethal outcome. In the ferret model, HA and non-structural genes contribute to the high virulence of H5N1 in these animals.[2]

The HA of H5N1 contains a furin-dependent polybasic cleavage site that is characteristic of highly pathogenic H5N1 viruses.[3] Currently, the spread of H5N1 virus in the human population is limited. However, through mutation and reassortment, the virus may become more easily transmissible from birds to humans and/or between humans, posing a potential pandemic threat to public health worldwide. It is therefore important to fully understand the biology of H5N1 viruses and to develop sensitive and rapid diagnostic methods. Nonetheless, an obstacle to the study of H5N1 viruses is the stringent safety requirements. Thus, retroviral particles pseudotyped with H5-HA (H5pp) have been developed. Similar to the replication-competent virus, H5pp entry requires alpha-2,3 sialic acids, is pH-dependent, and can be neutralised by sera-containing anti-H5N1 antibodies,[4] thus making it a very useful and safe tool.

H5 haemagglutinin pseudotyped lentiviral particles are a safe tool for serological/epidemiological studies without the requirement for BSL-3 facilities

Previously, we reported the development of H5 HA pseudotyped lentiviral particles using HA of A/Cambodia/40808/2005 (H5Cam), a H5N1 isolate from a Cambodian patient who died from the infection. The lentiviral backbone is deficient in its original viral envelope protein and the entry of the particle is therefore dependent on the 'foreign' envelope protein that is pseudotyped on the surface of the particles. The H5pp is capable of conducting only a single round of transduction, thus making it a very useful and safe alternative for assays that normally involve the use of real H5N1 viruses. Assays using H5pp can be performed in BSL-2 facilities. This is especially useful when researchers do not have access to BSL-3 facilities. As a mimic of the real H5N1 virus, H5pp can be used in a wide range of applications, including sero-diagnosis, entry mechanism studies, and drug discovery.[5]

The H5 HA pseudotyped lentiviral particles can be engineered to contain only HA or both HA and neuraminidase (NA) [Fig 1]. The pseudotyped particles usually contain a reporter gene: luciferase or green fluorescent protein. Upon transduction of H5pp into permissive cells, H5pp enters the target cells in a sialic acid dependent manner. The reporter gene is subsequently expressed in the target cell and facilitates measurement of the activity of the pseudo-particles. Among the various applications of H5pp, the most widely used is probably sero-diagnosis and sero-surveillance (Fig 2). For this purpose, H5pp has certain advantages over H5N1-pp: it is specifically neutralised only by anti-HA antibodies, avoiding the confounding effect of antibodies against NA1 due to infection with influenza virus subtypes other than H5N1. H5Npp is easy to produce for all four H5N1 virus stains, but the efficiency to generate HA-only H5pp varies a great deal with HAs derived from different H5N1 virus strains. The underlying mechanism is not known.

Novel findings

From January 2009 to December 2010, a study was conducted to analyse the ability of HAs

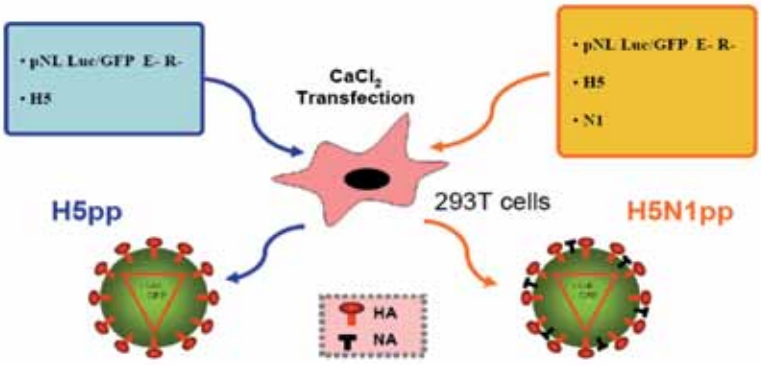

FIG 1. Production of H5pp and H5N1pp in 293T cells

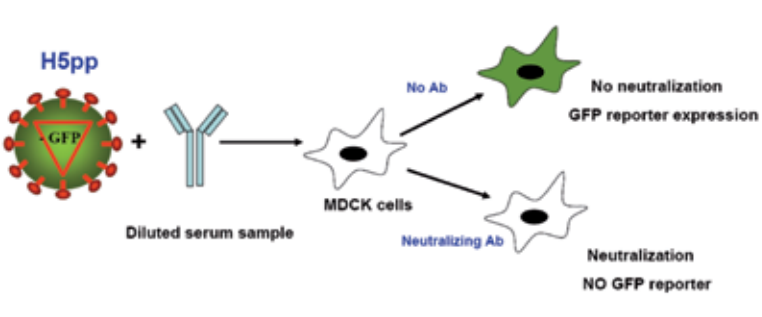

FIG 2. Neutralisation-based serodiagnostic assay using H5pp

from different clades of avian influenza virus to pseudotype lentiviral particles, which do not give rise to the same level of efficient H5pp production as H5Cam. In particular, the expression and cleavage of two H5 HAs (from A/Cambodia/2005/40808 and A/Anhui/2005/01) was compared, as was their ability to pseudotype lentiviral vector in HEK293T cells.

To ascertain the flexibility and adaptability of H5pp production in the event of novel emerging H5N1 virus strains, we developed strain-specific H5pp and compared the ability of three other strains belonging to different clades to pseudotype lentiviral particles. Unexpectedly, we observed significant differences in the efficiency of H5-HAs to generate H5pp in 293T cells. In particular, H5 HA from A/Anhui/2005/01 (H5Anh) was unable to produce H5pp, yielding low luminescence signals after particle transduction in Madin-Darby canine kidney (MDCK) cells, whereas H5-HA from A/Cambodia/40808/2005 (H5Cam) was the most efficient strain.

Through in depth mutagenesis studies, we revealed that when a single A134V mutation was introduced in the receptor-binding site, the ability of the usually inefficient H5Anh to generate H5pp was largely restored.[6] Differences in receptor binding ability and cell surface expression pattern, due to mutations in the receptor-binding domain of HA, may be the underlying mechanism. First, by multiple sequence alignment, we identified a small region around the 130-loop of receptor binding site of HA that seemed to be a 'hot spot' that harboured diverse sequence variations among different H5N1 strains. Then serial H5-HA mutants were generated, and eventually we found that one single A134V mutation was a critical switch to dictate the ability of H5 HA to produce H5pp. Using a cell-based ELISA binding approach, we found that H5Anh that contains 134A displayed strong binding to both MDCK and MDCK-SIAT cells (more alpha-2,6- and less alpha-2,3-sialic acid than parental MDCK); and A134V mutation reduced the binding to a dramatically lower level. It is very likely that strong binding of H5Anh to its cell surface receptors makes it difficult to release H5pp from the producer cells; and A134V mutation reduces the binding thus allowing the release of H5pp. We did not observe an increase in binding to MDCK-SIAT cells that contain more alpha-2,6-sialic acids on the cell surface, suggesting that A134V mutation probably leads to decreased binding to alpha-2,3-sialic acids rather than a switch to alpha-2,6-sialic acid binding.[6]

Implications

Our study has demonstrated the underlying molecular mechanism for the efficient production of H5pp. Through serial mutagenesis of two H5-HAs, we have revealed that differences in receptor binding ability, due to mutations in the receptor-binding domain of HA, may be the underlying mechanism. In addition, A134V mutation has been reported as a naturally occurring mutation in the human host. Our results may have implications for the understanding of human host adaptation of avian influenza H5N1 viruses.

Interestingly, alanine at position 134 is highly conserved in avian H5N1 viruses; to date A134V mutation has been found only in human isolates of H5N1 viruses. All avian H5N1 viruses possess 134A except for one that has 134S instead. Notably for human isolates of H5N1 viruses, more diversity is observed at this position: three H5N1 viruses isolated from human patients have 134T and eleven have 134V. In at least two cases (A/Cam/408008/2005 and A/Thailand/676/2005), viruses found in the original patient specimens were a mixture of both the wild type virus containing 134A in the HA and mutant virus containing 134V. It is possible that some human isolates of H5N1 viruses may actually contain an A134V mutation but were not detected in the process of virus isolation or sequencing of viral genomes. These observations suggest that 134V may be selected as the avian H5N1 viruses adapt for replication in human hosts. It may be of importance to monitor closely mutations in the receptor binding site of H5 HA; H5pp production together with soluble HA protein cell binding analysis may serve as convenient functional assays to monitor for mutations in H5N1 viruses with potential consequences for human host adaptation.

We have found that the A134V mutation not only exerts a critical influence in the determination of pseudotyping efficiency, but has an impact on H5N1 viruses. Both A/Cambodia/408008/2005 and A/Cambodia/V0401301/2011, two different H5N1 isolates carrying the same A134V mutation, could agglutinate human and guinea pig red blood cells (RBCs) but failed to agglutinate horse RBCs; two other strains of H5N1 viruses without the A134V mutation could also agglutinate horse RBCs. These observations indicate that an A134V mutation in H5-HA reduces virus binding to alpha-2,3-sialic acid. The differential RBC binding properties observed at the whole virus level, when both HA and NA are present, support the idea that A134V mutation in H5-HA can be biologically relevant.[6]

Through several independent lines of evidence, we have identified the molecular determinants in HA that enable efficient packaging into H5pp and have defined the underlying molecular mechanism. Our results have been discussed in the context of understanding human host adaptation of avian influenza H5N1 viruses.

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