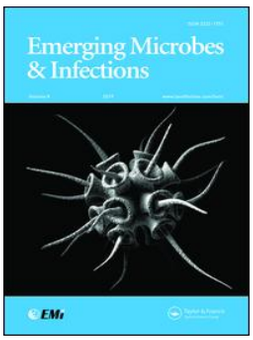

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REVIEW

Receptor binding and transmission studies of H5N1 influenza virus in mammals

Hanjun Zhao[1,2], Jie Zhou[1], Shibo Jiang[3,4] and Bo-Jian Zheng[1]

The H5N1 influenza A virus that is currently circulating in Asia, Africa and Europe has resulted in persistent outbreaks in poultry with sporadic transmission to humans. Thus far, it is believed that H5N1 does not possess sufficient ability for human-to-human transmission and subsequent pandemic infection. Both receptor binding specificity and virus infectivity are key factors in determining whether influenza A virus becomes pandemic. The use of human viral isolates in various studies has helped to illustrate the changes in receptor binding specificity and virulence as a result of adaptation in humans. In this review, we highlight the important amino acids and domains of viral proteins related to receptor binding specificity that have been reported for humans and avians using mammalian models. Thus, this review will consolidate findings from studies that have shed light on the receptor binding and transmission characteristics of the H5N1 influenza virus, with the goal of improving our ability to predict the transmission efficiency or pandemic potential of new viral strains.
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INTRODUCTION

The highly pathogenic avian influenza (HPAI) H5N1 virus was first isolated from sick geese in southern China in 1996.[1] As of October 8th, 2013, outbreaks of H5N1 influenza across the globe have resulted in millions of deaths in birds and 377 deaths (mortality rate of 59.3%) in humans.[2] The first reported cases of H5N1 virus infection in humans occurred in Hong Kong in 1997, which coincided with an outbreak of the virus in the territory's chicken farms.[3,4] More than 15 years after this outbreak, other avian H5N1 virus strains have not acquired the level of transmissibility and replication required to cause a human pandemic. Nonetheless, in anticipation of this possibility, much attention has been focused on transmission and adaptation of avian H5N1 virus in humans and the potential increase in virulence caused by amino acid mutations in viral proteins.

Recently, the geographic distribution of avian H5N1 virus infection has expanded to Kazakstan, Mongolia, Djibouti, Egypt, Turkey and Russia, indicating that more of the world's population is at risk.[5] However, the determinants of infection and susceptibility of humans to H5N1 virus have not been fully elucidated. To become a pandemic virus, the viral strain should be able to transmit efficiently between humans, which is determined by factors such as whether the virus grows to high enough titers in the human lungs.[6,7] Fortunately, the current H5N1 virus strains have not acquired such abilities in humans. However, two recent experimental studies have shown that reassortant H5N1 viruses with four mutations in hemagglutinin (HA) were capable of droplet transmission in a ferret model.[8,9] Therefore, the potential risk does exist for a natural H5N1 virus to evolve into a pandemic virus after continuous circulation between avian and human hosts, which will provide opportunities for the acquisition of the necessary amino acid mutations in viral proteins, particularly HA.

This review will focus on studies reporting on the receptor binding of H5N1 virus in humans and other mammals, as well as the amino acid mutations in viral proteins related to transmission of the virus in humans and experimental animals. In virus binding studies, the attachment of viruses can be directly measured by labeling viruses and then applying them to tissue sections in a method coined virus histochemistry, which has been used to study the pattern of virus attachment in different tissues. By comparing virus binding and transmission in different animal models, this knowledge will help to elucidate potential transmission routes and will provide the genetic basis for predicting whether a mutated virus strain may evolve into a pandemic virus.

VIRAL RECEPTOR BINDING AND TRANSMISSION IN HUMANS

One important aspect of influenza virus infection is the interaction between the viral surface glycoprotein HA and the corresponding receptor on host cells. To infect host cells, influenza virus utilizes HA to bind to complex glycans on the host cell surface via a terminal sialic acid (SA). Influenza viruses have different preferences for SAs with different linkages. For example, human influenza virus prefers sialic acid linked to galactose via an α-2,6 bond (SAα-2,6Gal), whereas avian influenza virus prefers the terminus with sialic acid linked to galactose via an α-2,3 bond (SAα-2,3Gal).[10] SAα-2,6Gal is the major linkage for vicinal galactose in the human upper respiratory epithelium.[11] Epithelial cells in the paranasal sinuses, pharynx, trachea and bronchi mainly express SAα-2,6Gal, which is also expressed in ciliated

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and goblet cells in the human lung.[12,13] Apart from SAα-2,6Gal, the human respiratory tract also expresses SAα-2,3Gal on non-ciliated cuboidal bronchiolar cells, which are situated at the junction between the respiratory bronchiole and alveolus.[11] Highly pathogenic avian H5N1 virus labeled by fluorescein isothiocyanate (FITC) was shown to preferentially attach to type-II pneumocytes, alveolar macrophages, and non-ciliated cuboidal epithelial cells in the terminal bronchioles of the human lower respiratory tract.[14] The binding of H5N1 virus rarely occurs at the trachea and upper respiratory tract, which is consistent with pathological findings observed at autopsy, such as diffuse alveolar damage, interstitial pneumonia, focal hemorrhage, and bronchiolitis.[15,16]

Some strains of the highly pathogenic H5N1 virus, such as A/Hong Kong/486/97 (HK486) or A/Duck/Hong Kong/200/01, show high binding affinity to SAα-2,3Gal but not SAα-2,6Gal. However, some other strains isolated from patients, such as A/Hong Kong/212/03 and A/Hong Kong/213/03 (HK213), can recognize both SAα-2,3Gal and SAα-2,6Gal.[17,18] These findings implicate the ability of H5N1 virus, which preferentially binds to SAα-2,3Gal, to switch to SAα-2,6Gal if the virus is transmitted and begins to adapt to humans. However, efficient replication of H5N1 virus can only occur in the lower, rather than upper, respiratory tract, implicating that the virus may not be readily spread by sneezing and coughing.[11,19] This may account for the inefficient human-to-human transmission of H5N1 virus. The H5N1 virus was also shown to infect nasopharyngeal and oropharyngeal epithelia, which do not express SAα-2,3Gal, suggesting that other binding sites on nasopharyngeal and oropharyngeal epithelia may be able to mediate virus entry.[20]

Among the 16 HA subtypes, only the H1, H2 and H3 subtypes have become adapted to humans.[21,22] These three virus subtypes caused the pandemics of 1918, 1957, 1968 and 2009. The particular mutations within the HA viral protein of these pandemic viruses, which contributed to the adaptation of avian influenza virus to humans, have been identified. H1 of the 1918 pandemic H1N1 virus switched its binding from SAα-2,3Gal to SAα-2,6Gal via the E190D and G225D mutations (H3 numbering) in HA.[23] The H2N2 and H3N2 pandemic viruses were avian-human reassortant viruses.[24] The 1957 and 1968 pandemic H2 and H3 viruses were able to switch their binding specificity from SAα-2,3Gal to SAα-2,6Gal via two amino acid changes of Q226L and G228S (H3 numbering) within HA.[25] When these mutations were introduced into H5 HA of A/Vietnam/1203/04 (VN1203),[22] the E190D and G225D mutations abolished H5 HA binding to all glycans, while the Q226L and G228S mutations afforded H5 HA binding to natural human SAα-2,6Gal. However, a complete shift from SAα-2,3Gal to SAα-2,6Gal binding preference was not observed.

Further studies showed that these two mutations (Q226L and G228S) combined with a loss of glycosylation at 158N[26] and the K193R[27] mutation could enhance the binding affinity to SAα-2,6Gal. Some other HA mutations in the rHK486 and rVN1203 viruses were shown to result in a binding shift from SAα-2,3Gal to SAα-2,6Gal.[28] Although receptor preferences of the viral mutants were changed by HA mutations in this study, almost all virus mutants exhibited attenuated infection efficiency *in vitro* and *in vivo*. These studies indicated that switching the binding preference of H5N1 virus from SAα-2,3Gal to SAα-2,6Gal by mutation in HA was possible; however, a single mutation in HA was unable to benefit the H5N1 virus because these artificial virus mutants could not replicate and transmit efficiently, either *in vitro* or *in vivo*. In nature, some H5N1 isolates from human patients have acquired the ability to increase their binding to SAα-2,6Gal while maintaining a decreased binding capability for SAα-2,3Gal.[18,29] These viruses were highly pathogenic, even though efficient transmission was not observed. In addition, the A134V mutation emerged during the course of virus replication in a fatal case of human infection, and this mutation was shown to increase the binding ability to SAα-2,6Gal.[30] Thus, with continual circulation of H5N1 viruses between avian and human hosts, a new pandemic virus may emerge after the virus obtains suitable binding and efficient replication ability.

VIRAL RECEPTOR BINDING IN MICE

Mice are the most common animal species used in experimental influenza research, although most human virus strains require adaptation in mice through serial passages and transmit inefficiently from mouse to mouse.[31] H5N1 virus can replicate robustly in BALB/c mouse lungs to reach high titers and cause lethal disease without prior adaptation. Studies of FITC-labeled H5N1 virus binding in the lung tissue of mice showed that H5N1 virus mainly binds to the tracheal epithelia and becomes progressively weaker in binding toward the alveoli,[32] a pattern of binding opposite to that of the human respiratory tract. This is consistent with the observation that mouse tracheal epithelia preferentially express SAα-2,3Gal but not SAα-2,6Gal.[13] Thus, the mouse model is suitable for studying the transmission and pathogenicity of influenza viruses that preferentially bind to the receptor via SAα-2,3Gal linkage[33] but is inappropriate for the investigation of H5N1 virus transmission in humans.

The HK213 virus exhibits binding affinity to both SAα-2,3Gal and SAα-2,6Gal. When the HA and NA of this virus were substituted with the HA and NA of VN1203, the recombinant virus demonstrated excellent binding affinity for synthetic α-2,3-linked SA receptor, resulting in expanded organ tropism and increased lethality in mice. In contrast, recombinant VN1203 virus, in which HA was substituted with the HA of HK213, showed reduced lethality and systemic spread in mice.[34] In addition, a human H5N1 virus isolate with the mutation HA222E was mainly confined to the mouse lung, although virus with the HA222K mutation could be isolated from the mouse brain. In particular, the HA222E variant showed a reduced binding affinity for synthetic α-2,3Gal-linked SA receptor analogues compared to that of HA222K.[35] These studies show that HA plays an important role in viral tropism, which can be attributed to the HA binding preference for SAα-2,3Gal- or SAα-2,6Gal-linked receptors as well as the virulence of avian influenza virus in mice. However, because it has been demonstrated that other viral proteins also play a role in viral organ tropism in mice, HA is not the only element affecting viral organ tropism.[19,35–37]

VIRAL RECEPTOR BINDING AND TRANSMISSION IN FERRETS

Ferrets demonstrate preferential expression of SAα-2,6Gal and a lesser amount of SAα-2,3Gal on epithelial cells of the respiratory tract. A receptor binding study of H5N1 virus in ferrets showed that H5N1 virus rarely attaches to the trachea and bronchi, occasionally attaches to non-ciliated cuboidal cells in the bronchioles, and predominantly attaches to type II pneumocytes in the alveoli.[32] These animals were shown to be an ideal model for transmission studies of H5N1 virus because H5N1 virus preferentially attaches to the lower respiratory tract in ferrets, a binding pattern similar to humans.[11,14] Thus far, the transmission of H5N1 virus from avians to humans or humans to humans has been inefficient; instead, patients have contracted the disease primarily through close contact with birds or poultry or the consumption of raw infected duck blood.[16] It remains unclear whether humans can be infected through contact or inhalation of aerosols from H5N1 virus-infected patients.

Using ferrets as an animal model, the transmission ability of H5N1 virus was analyzed in recent studies.[29,38,39] In these studies, efficient-transmission H3N2 and poor-transmission H5N1 virus strains were reassorted to generate mutant viruses. H3N2 reassortant virus (containing HA and NA of human H3N2 virus and the internal protein genes of avian H5N1 virus) exhibited efficient infection but inefficient transmission in ferrets by contact. H5N1 reassortant virus (containing HA and NA of avian H5N1 virus and the internal protein genes of human H3N2 virus) exhibited reduced infection and no transmission by contact.[38] In addition, native reassortants were simulated by coinfecting ferrets with avian H5N1 and human H3N2 viruses; the results revealed various reassortants in airways extending toward and within the upper respiratory tract of ferrets, but only parental H5N1 virus was found in lung tissues. In addition, the H5 reassortants showed reduced titers in nasal secretions compared to the parental H5N1 virus, and no transmission was detected by direct contact between infected and native ferrets.[39] However, in a recent study, a reassortant virus containing H5 HA and a human influenza virus NA with an H5N1 virus background demonstrated respiratory droplet transmission in one of two contact ferrets.[40] In addition, a genetically modified H5N1 virus with four amino acid substitutions at H103Y, T156A, Q222L, and G224S (H5 numbering) in HA and one in PB2 (E627K) was shown to be an airborne-transmissible virus in ferrets.[9] Furthermore, an H5 HA/H1N1 reassortant virus, which was comprised of H5 HA with the four mutations N158D, N224K, Q226L and T318I (H3 numbering) and the remaining seven gene segments from a 2009 pandemic H1N1 virus, was capable of droplet transmission in a ferret model.[8]

These reassortant viruses could preferentially recognize human-type receptors and resulted in efficient infection in ferrets, although they did not cause mortality in infected animals. These results suggest that only surface proteins of human virus are unable to confer H5N1 virus with transmission ability between ferrets, even though these reassortants could attach and replicate in the upper respiratory tract. Moreover, the reassortant viruses with mutant H5 HA could cause droplet transmission in ferrets, suggesting that H5N1 virus with pandemic potential may emerge with continuous viral circulation and evolution.

VIRAL RECEPTOR BINDING AND TRANSMISSION IN CATS

Previous studies have shown that cats can be infected by human or avian influenza A viruses but do not show any signs of disease.[41,42] However, during the outbreaks in Thailand, avian influenza A H5N1 virus was found to infect and caused many fatalities in tigers and domestic cats.[43] After the susceptibility of cats to human isolates of the H5N1 viruses was proven,[44,45] the scientific community began to realize the importance of understanding the transmission and pathogenicity of H5N1 viruses in cats. Based on experiments conducted on virus attachment, H5N1 virus does not bind to the trachea of cats, but rather preferentially binds to type II pneumocytes as well as alveolar macrophages, which is similar to the binding pattern in humans.[14,32] This indicates that cats can be a useful animal model for studying H5N1 viral pneumonia in humans.[14] Both inhalation and ingestion seem to be possible routes for feline infection by H5N1 virus.[44,46] In addition, indirect viral transmission to cats through contact with contaminated bird feces and horizontal transmission from experimentally inoculated cats to other healthy cats represent possible routes of infection.[47,48] Although no evidence has shown that cats can transmit the virus to other species by contact, the epidemiological role of cats requires additional study because pet cats can roam freely among birds, poultry and humans, thus potential linking transmission through hosts when an influenza pandemic occurs.

VIRAL RECEPTOR BINDING IN PIGS

Pigs have been traditionally been considered efficient mixing vessels for avian and human influenza A viruses, and both SAα-2,6Gal and SAα-2,3Gal can be detected in the porcine trachea by lectin histochemistry.[49] Moreover, some avian-human reassortant viruses have been isolated from pigs.[50] A recent study showed that only human influenza A virus strains have the ability to attach to the porcine trachea, whereas H5N1 virus cannot attach to the trachea of pigs.[32] One possible reason for this distinction is that the lectin histochemistry technique is only an indirect measure of influenza A virus binding to host tissues and thus cannot account for other variables that may influence binding between the virus and host cells. Intranasal inoculation with H5N1 virus or the consumption of infected chicken meat was unable to cause severe influenza virus infection in pigs. Moreover, replication of H5N1 virus was restricted to the lower respiratory tract, mainly to the bronchiole and alveoli.[51] These results are consistent with the binding study of H5N1 virus in pigs, which showed that H5N1 virus can only bind to the alveolus and not the trachea, bronchus or bronchiole.[32]

VIRAL RECEPTOR BINDING AND TRANSMISSION IN GUINEA PIGS

Guinea pigs, like ferrets, have been widely used as animal models for transmission studies of influenza virus.[52] Guinea pigs have both avian and mammalian types of airway receptors, and the SAα-2,6Gal and SAα-2,3Gal receptors are present in the nasal and tracheal areas of the guinea pig.[53] Transmission studies of H5N1 virus in guinea pigs showed that mutations D701N within PB2 and T160A within HA were critical for the transmission of A/duck/Guangxi/35/2001 H5N1 virus (DKGX/35) in guinea pigs. In addition, the mutation T160A was responsible for HA binding to human like-receptors. The mutated DKGX/35 virus was also shown to contain the Q226L and G228S (H3 numbering) substitutions in HA, which resulted in binding to only SAα-2,6Gal and decreased transmission among guinea pigs.[54] More recently, the PA gene of SC/09(H1N1) alone was shown to make DK/35(H5N1) transmissible by respiratory droplets between guinea pigs.[52] Thus, guinea pigs may provide a mammalian model for H5N1 transmission studies aiming to assess the pandemic potential of H5N1 isolates.

VIRAL RECEPTOR BINDING AND TRANSMISSION IN MACAQUES

Macaques have been studied as a non-human primate for H5N1 virus infection in some experiments,[14,55–57] and the pattern of H5N1 virus binding in cynomolgus macaques is similar to the viral binding pattern in humans. However, no H5N1 virus can be detected in the trachea of macaques, essentially because the virus preferentially binds to type I, but not type II, pneumocytes in macaques, which is different from the binding of H5N1 virus in humans.[14] Moreover, the precise cell types to which H5N1 virus can bind in the respiratory tract remain controversial. For instance, it was reported that H5N1 virus could also be detected in the epithelium of the trachea and bronchi of macaques,[55] and these differences may have resulted from the different techniques used in these studies. Indeed, although macaques may provide a model for H5N1 receptor binding and transmission studies, ethical constraints, availability and cost may limit the utility of these animals for infection studies of H5N1 virus.

CONCLUSIONS

The currently available data show that mutations in HA of H5N1 virus can increase the binding capacity of this viral protein for SAα-2,6Gal.[58]

Furthermore, these binding and transmission studies have shown that the binding properties of HA can affect virulence, organ tropism and transmission in mammals. Receptor binding studies of H5N1 virus have suggested that the H5N1 virus preferentially binds to SA via the linkage of SAα-2,3Gal, which is mainly located on type II alveolar epithelial cells and alveolar macrophages in the human lower airway. Therefore, the distribution of SAα-2,3Gal may account for the limited transmission between humans. Studies in ferrets have suggested that human transmission of natural H5N1 viruses by contact is not efficient. However, viruses isolated from ducks can be transmitted from inoculated guinea pigs to other guinea pigs by contact, although these animals show no signs of disease.

Due to the circulation of H5N1 virus strains such as HK212, HK213 and DKGX/35, these viruses can recognize both SAs in α-2,3Gal and α-2,6Gal linkages. In addition, certain amino acid substitutions in HA can switch the binding specificity of a virus from SAα-2,3Gal to SAα-2,6Gal, which can affect organ tropism and even enable transmission between ferrets. In contrast to H5N1 virus, the 2009 H1N1 pandemic virus preferentially binds to the SAα-2,6Gal receptor,[59] although viruses with a D222G mutation in HA switch the viral receptor binding preference from SAα-2,6Gal to SAα-2,3Gal and were frequently observed in the lower respiratory tracts of patients with severe clinical outcomes.[60–62] Therefore, the binding properties of influenza virus HA to glycan receptors affect interspecies transmission, organ tropism and virulence in the host. Thus, when regions with H5N1 in circulation are continually surveyed, it should be noted if amino acid mutations exist that are related to binding affinity, as this may provide early evidence for the genesis of a pandemic virus and should contribute to future pandemic prevention efforts.

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