

Expression of Avian Influenza Virus Receptors and H5N1 Virus Infection in Human Respiratory Tract

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Receptor specificity restricts influenza virus cross species transmission, with SA α 2,6 Gal and SA α 2,3 Gal sialic acids recognized by human and avian influenza viruses, respectively. This study investigated the distribution of these two species of sialic acids in the human respiratory tract. The SA α 2,3 Gal species was infrequently detected in the upper respiratory tract, but prevalent in the lower part, while the SA α 2,6 Gal species is more common in the upper respiratory tract. Though alveolus cells are more susceptible than trachea and bronchus epithelial cells to avian influenza H5N1 virus infection in the *ex vivo* experiment, H5N1 virus was found to infect the upper respiratory tract epithelial cells of a human case. It was also found that H5N1 virus infection occurs in the epithelial cells of the respiratory tract which do not express detectable SA α 2,3Gal. These observations may be important to investigate further if the currently-observed limited human to human transmission by H5N1 virus is associated with the differential expression of SA α 2,3 Gal in human upper respiratory tract among individuals.

Introduction

The binding of influenza virus to host cells is mediated via the viral surface protein haemagglutinin, which recognizes cell surface glycoproteins containing terminal sialic acid residues. Haemagglutinin proteins from human and avian influenza viruses differ in their ability to recognize different receptor structures of sialyloligosaccharide molecules on host cell membrane [1, 2]. Host tropism of influenza viruses is restricted by receptor specificity. Human influenza viruses preferentially bind to the terminal sialic acid with an α 2,6 linkage to the underlying galactose (SA α 2,6Gal), whereas avian and equine viruses prefer an α 2,3 linkage to the underlying galactose (SA α 2,3Gal) [2,3], which predominate in their respective target species. In recent years, however, avian influenza virus H5N1 subtype, has repeatedly crossed host barrier and infected humans. Over 300 human cases have been confirmed in various affected countries by the World Health Organization since 2003 when H5N1 virus human infection reemerged in Hong Kong [5]. Genetic analysis has demonstrated that

the H5N1 viruses causing human infections up to now are still of the avian type [6, 7, 8]. The molecular mechanism underlying such cross species transmission is not clear though it is generally believed that the availability (or presence) of α 2,3 linkage terminal sialic acid receptor in human airway may attribute to the susceptibility to avian influenza virus infection. H5N1 virus only showed inefficient human to human transmission up to this stage [9]. It is also suspected that some individuals with high exposure to H5N1 virus do not become infected due to an inherent reduced susceptibility to this virus. Two recent studies demonstrated SA α 2,3Gal expression and influenza virus binding in the bronchioles and alveoli of the human respiratory tract, indicating that avian influenza viruses can replicate relatively efficiently in the lower respiratory tract, providing a possible explanation for inefficient human-to-human transmission of avian influenza viruses [10, 11]. In another study, H5N1 virus was found to be able to infect epithelial cells of the human upper respiratory tract in an *ex vivo* infection model [12]. However, the prevalence and the extent of expression of SA α 2,3Gal and SA α 2,6Gal in the human respiratory tract have still not been fully investigated. In this report we studied the distribution and prevalence of SA α 2,3Gal and SA α 2,6Gal on the airway epithelia of different anatomical areas of the human respiratory tract. Our results demonstrated that SA α 2,6Gal predominates in the upper respiratory tract, while SA α 2,3Gal is patchily expressed in the upper respiratory tract of a portion of individuals but more prevalent in the lower part of the respiratory tract. Examination of *ex vivo* and *in vivo* H5N1 virus-infected human tissues indicated H5N1 virus infection of upper respiratory tract may not completely dependent upon avian influenza virus receptor.

Materials and Methods

Tissue samples. 144 paraffin-embedded human respiratory tissue sections from 88 patients were obtained from the pathological archives in several local hospitals in Guangxi and Guangdong provinces, China. The age of the patients ranged from antenatal to 76 years old. Different anatomical parts of respiratory tract were included based on the availability of tissues, and were then histologically classified as trachea, bronchus, bronchiole and alveolus. Paraffin-embedded respiratory tissue sections from a H5N1 human infection case [4] in Hong Kong in 2003 were also included in this study.

Lectin histochemical staining of human airway tissues. Expression of SA α 2,6Gal and SA α 2,3Gal was examined using biotinylated lectins: *Sambucus nigra* lectin (SNA, EY Laboratories, Inc., California, USA), and *Maackia amurensis* lectin (MAA-I & MAA-II, Vector Laboratories, California, USA). Deparaffinized and rehydrated tissue sections were blocked consecutively for 15 minutes with avidin D and biotin solutions (Vector Laboratories) to reduce nonspecific binding of endogenous biotin and avidin. These sections were then incubated with biotin-labeled MAA-II or SNA at a concentration of 10 μ g/ml or 1 μ g/ml, respectively, for 60 minutes at room temperature, and then with streptavidin/peroxidase complex reagent (Vector

Laboratories) for 30 minutes followed by color development using 3,3'-diaminobenzidine (DAB, Vector Laboratories).

Ex Vivo infection of human respiratory tissue. For the *ex vivo* infection study, one H5N1 isolate from human infection, A/HK/212/03 [4] and two avian H5N1 influenza viruses, DK/GX4444/05 and DK/GX3546/05 [7,8], were passaged once in 10-day embryonated chicken eggs and stored at -80°C until use. Eight cases of surgically-removed human respiratory tract tissues from lung cancer or non-cancer patients were obtained from local hospitals in Guangdong province in accordance to the local Institutional Ethical Review Board's approved protocols. The tissues were cut into small pieces of about 0.2cm^3 and infected with 10^3 TCID₅₀ of each H5N1 virus in a 500 μl inoculum. The virus was removed after a one hour absorption at $37^{\circ}\text{C}/5\% \text{CO}_2$, and tissues were further incubated at $37^{\circ}\text{C}/5\% \text{CO}_2$ for 24 hour in 3ml of serum-free F12K Nutrient Mixture (GIBCO, New York, USA) supplemented with 25mM HEPES buffer solution and 1% Antibiotic-Antimycotic (GIBCO). The infected tissues were then fixed in 10% neutral formalin for 24 hours before being histologically sectioned for immunohistochemical detection of influenza nucleoprotein (NP).

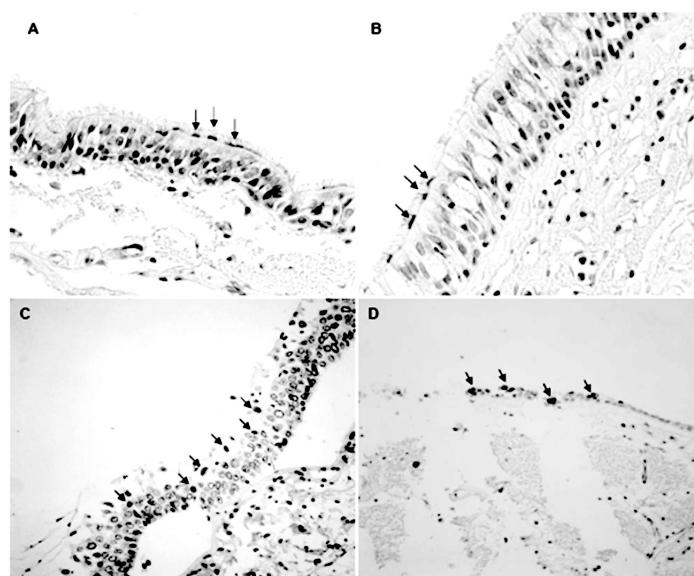
Immunohistochemical staining of tissues. For detection of influenza A nucleoprotein (NP) in human tissues, sections were blocked with 1% bovine serum albumin/PBS, stained with a anti-influenza nucleoprotein monoclonal antibody (17H4 clone) raised with H5N1 strain CK/Yu22/02 [8] at 1:5000 dilution at 4°C for overnight and then incubated with goat anti-mouse IgG, H & L chain specific biotin conjugate (Calbiochem) at 1:2000 dilution for 30 minutes at room temperature. Tissue blocks were then incubated with streptavidin/ peroxidase complex reagent (Vector Laboratories) for 30 minutes at room temperature. Color development and images capture were carried out as described above.

Results and Discussion

Distribution of influenza virus receptors in human respiratory tract. 144 respiratory tissue sections including 10 tracheas, 40 bronchi, 46 bronchioles and 48 alveolar tissues were studied by lectin histochemistry methods. SA α 2,6Gal and SA α 2,3Gal recognized by SNA or MAA-II respectively on the apical epithelial surface were counted as positive in this study, while relative intensity of staining achieved with the lectins was not used as a direct quantitation measure of sialic acid distribution, because the relative affinities of the lectins, and the degree of biotinylation may differ among different preparations. SA α 2,3Gal was only detected in a small portion of the upper respiratory tract tissues, with 20% in trachea and 42.5% in bronchus sections showing positive staining. As shown in Figure 1A and B, only sporadic SA α 2,3Gal positive cells were observed in each positive sample. In contrast, SA α 2,3Gal was more regularly observed in the lower respiratory tract epithelial cells, with 68.7% of the bronchiolar and 79% of alveolar tissues showing positive staining in all cases examined (data not shown). In contrast, expression of human influenza

virus receptor, SA α 2,6Gal, was mainly detected in the upper respiratory tract and to a lesser degree in the alveolar epithelium, with 100% in trachea, 97.5% in bronchus, 80.4% in bronchioles but only 43.8% in alveolar tissues (data not shown). These results, consistent with previous studies, suggested that lower respiratory tract may be more susceptible to avian H5N1 influenza virus infection for its relatively higher expression of SA α 2,3Gal. However, the observation of SA α 2,3Gal expression in some epithelial cells of tissues from the upper respiratory tract argues one possibility that the currently-observed limited human to human transmission by H5N1 virus may be associated with the differential expression of SA α 2,3 Gal in human upper respiratory tract among individuals.

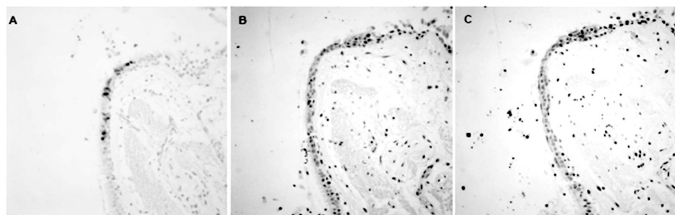
Figure 1. Detection of SA α 2,3 Gal expression in human respiratory tract tissues, trachea (A) and bronchus (B), by immunostaining with lectin probes MAA-II; Detection of influenza viral antigen (nucleoprotein) in trachea (C) from one H5N1 virus infected patient and in *ex vivo* infected bronchus tissue (D) with avian H5N1 virus, DK/GX3546/05, with immunohistochemical staining. Viral nucleoprotein positive cells were shown in brown color.



Avian H5N1 virus infection in human respiratory tract tissues. Previous reports suggested that alveolus cells in the lung represent the major site for avian H5N1 virus replication in human infection [13]. Infection of avian H5N1 influenza virus in human upper respiratory tract is less defined. We examined tissues of upper respiratory tract obtained from a postmortem H5N1 human case in Hong Kong in 2003 [4]. Immunohistochemistry staining of influenza NP protein with a monoclonal antibody raised against H5N1 strain Ck/Yu22/2002 (clone 17H4) showed that influenza viral antigen was present in tracheal (Figure 1C), bronchial and also in alveolar epithelial cells (data not shown). To further explore the possibility of H5N1 virus infection in the upper respiratory tract, we then performed H5N1 virus infection in *ex vivo* cultured human respiratory tissues removed from the respiratory tract of patients suffering non-infectious diseases. The H5N1 human

strain (A/HK212/03) we tested was isolated from a fatal human infection case [4]. A previous receptor binding study found that this strain can bind to both avian and human receptors [14]. These *ex vivo* infection tests further demonstrated that this virus infected both bronchial and alveolar epithelial cells with similar efficiency. In this study, we also tested two avian H5N1 isolates, DK/GX4444/05 and DK/GX3546/05 (data not shown). Genetic analysis of the haemagglutinin gene of these two viruses indicated they are typical avian type [7, 8]. We could also demonstrate that bronchial and alveolar epithelial cells were both infected by these viruses (figure 1D). This evidence supports the notion that upper respiratory tract is also susceptible to avian H5N1 virus infection. It remains to be determined if the presence of SA α 2,3Gal in the upper respiratory tract would predispose towards susceptibility to H5N1 virus infection. It is noteworthy that, when sequential tissue sections from *ex vivo* H5N1 infected bronchus tissues were stained for both virus replication and the expression of influenza receptors, in the area where H5N1 virus infection was observed, staining with the lectin MAA-II failed to detect SA α 2,3Gal species (Figure 2 A, B and C). However, a strong positive staining of SNA and a patchy signal of MAA-I lectin staining were observed in the same region. Previous reports on lectin affinity studies had showed that MAA-I also reacts with other species of sialic acids [15], and the inner fragment of the carbohydrate chain in sialic acid-containing oligosaccharides may also be involved in influenza virus entry into the target cells [16, 17]. However, the exact mechanism of how H5N1 avian influenza virus infection epithelial cells which do not express SA α 2,3Gal remains to be investigated.

Figure 2. H5N1 virus infects bronchus cells without expressing SA α 2,3Gal. Human bronchus tissue infected with DK/GX/4444/05 was stained for viral antigen (NP) expression, consecutive sections of bronchus tissue were stained with monoclonal antibody specific for viral antigen (NP) (A), with lectin MAA-II specific for avian influenza virus receptor SA α 2,3Gal β 1-3GalNAc (B), and with MAA for both SA α 2,3Gal β 1-3GalNAc and SA α 2,3Gal β 1-4GalNAc expression (C), respectively as described in the Materials and Methods.



It is believed that alteration of receptor specificity is essential for the emergence of pandemic viruses from their avian progenitors. Whether this change is now happening to H5N1 is under intensive investigation. This virus may well be becoming less restricted to SA α 2,3Gal sialic acid receptor specificity. If this virus is adapting to become more like a human influenza virus by increasing binding affinity for the SA α 2,6Gal sialic acid receptors which are more widely expressed throughout the human respiratory tract, an imminent pandemic seems likely.

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