JM Nicholls 黎國思
JSM Peiris 裴偉士
Y Guan 管　軼

Sialic acid and receptor expression on the respiratory tract in normal subjects and H5N1 and non-avian influenza patients

Key Messages

1. Influenza virus binds to cell receptors via sialic acid (SA)–linked glycoproteins. An accurate knowledge of SA expression is important in understanding influenza pathogenesis. The influenza viruses circulating in Hong Kong bind to the SAα2,6Gal receptor whilst avian influenza (such as H5N1) bind to SAα2,3Gal receptors.
2. SAα2,3Gal and SAα2,6Gal are widely distributed in the upper and lower respiratory tract as determined by lectin binding experiments.
3. Expression of SAα2,3Gal in the respiratory tract was correlated with infection of lung and nasopharyngeal tissues by a range of H5N1 viruses. H5N1 infection of both the upper and lower respiratory tract may have important implications on the spread of H5N1 influenza virus from patient to patient.
4. Ex vivo tissue culture models should be used more widely to determine the ability of tissues to be infected with H5N1 viruses.

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Department of Pathology, The University of Hong Kong
JM Nicholls
Department of Microbiology, The University of Hong Kong
JSM Peiris, Y Guan

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Principal applicant and corresponding author:
Dr JM Nicholls
Department of Pathology, The University of Hong Kong, Queen Mary Hospital, Hong Kong SAR, China
Tel: (852) 2855 4883
Fax: (852) 2872 5197
E-mail: nicholls@pathology.hku.hk

Introduction

Cells of the respiratory tract have a surface covering a number of glycan-containing conjugates, many of which terminate with *N*-acetylneuraminic (sialic acids [SAs])—a series of 9-carbon sugars. Influenza virus infection of humans involves binding of the virus haemagglutinin (HA) to these sialyoligosaccharides on the surface of cells of the respiratory tract. In addition, the virus neuraminidase (NA) cleaves the SA on the host cell, which is important in releasing virus from the cell after virus replication so that newly formed virions can spread out and infect other cells. Since respiratory mucus is also rich in SA, the influenza virus NA facilitates the virus spread through this mucus layer.[1] Thus the balance between the binding affinity of the virus and cell receptor, as well as the virus-releasing activity of the NA are critical to virus replication in a host species. Human influenza A strains preferentially attach to cells with SAα2,6Gal linkages and avian strains to SAα2,3Gal.[2] In the human respiratory tract, the SAs most abundantly expressed are those linked with SAα2,3Gal and SAα2,6Gal.[3] Other data suggest that the inner portions of the carbohydrate chains may also play a role in binding influenza viruses.[4,5]

The affinity of the attachment of the HA is considered an important component in the species barrier that keeps avian influenza viruses from readily infecting humans. The pig respiratory epithelium contains both avian and human-like SA, and can be infected with both human and avian influenza viruses. Therefore pigs are regarded as a hypothetical 'mixing' vessel where re-assortment of avian and human viruses can take place, potentially leading to the emergence of pandemic influenza.[6] Given the presumed importance of the affinity of the influenza virus for its receptor, the distribution of SAα2,6Gal and SAα2,3Gal expression in the human respiratory tract is important for the understanding of influenza pathogenesis, particularly the transmission of the avian influenza A H5N1 virus to humans.

The H5N1 viruses of the 1997 Hong Kong bird flu outbreak had affinity for avian SAα2,3Gal, whereas the virus associated with human disease in Hong Kong in 2003 had affinity for both avian-like SAα2,3Gal and human-like SAα2,6Gal.[7] H5N1 disease in humans differs from conventional human influenza (caused by H3N2 or H1N1) in that the lower (rather than upper) respiratory tract is the major site for virus replication.[8]

This study had three aims: (1) to investigate if antigen unmasking or retrieval affects lectin-ligand expression in histological tissues; (2) to compare findings in the same tissue samples using lectin fluorescence versus cytochemistry for lectin-ligand analysis; and (3) to optimise methods to re-evaluate the distribution of the SAα2,6Gal and SAα2,3Gal in human respiratory tissues and correlate findings with the presence or absence of influenza infection.

Methods

This study was conducted from October 2005 to September 2006. Biopsy

samples were collected from archives of the Histopathology Department of Queen Mary Hospital, Hong Kong SAR. Twelve surgically removed lungs from children with a congenital cystic adenomatoid malformation (CCAM) and seven bronchial biopsies from patients with possible malignancy were selected. Eight nasopharyngeal samples from patients with suspected nasopharyngeal carcinoma were also used. One lung biopsy sample from a 20-week abortus was also used. Seven biopsy tissues of CCAM were also retrieved from the Department of Histopathology, Adelaide Women's and Children's Hospital, Adelaide, Australia. All tissues were fixed in 10% neutral buffered formalin, processed into paraffin and stored at room temperature. Four duck intestines were obtained from the Agriculture and Fisheries Department, Hong Kong and used as positive controls. In addition, four adult pig trachea, bronchus and lung samples were obtained from the Laboratory Animal Unit of the University of Hong Kong. The research was approved by the ethics committees of the University of Hong Kong and Hospital Authority West Cluster.

Lectin histochemistry and fluorescence was used to detect SA linkages.[9] Lectin analysis was performed using the fluorescein-labelled lectins *Sambucus nigra* agglutinin (SNA-I) that primarily detects 6-linked SAs, and *Maackia amurensis* agglutinin (MAA) that primarily identifies 3-linked SAs. Both fluorescein isothiocyanate-labelled (FITC) and tetramethyl rhodamine isothiocyanate-labelled (TRITC) were used as fluorochromes (EY Laboratories, San Mateo, CA, US). Additional FITC conjugated MAA was purchased from Vector Laboratories (Burlingame, CA, US). When peroxidase or biotin conjugation was used, the conjugates were purchased from EY Laboratories (SNA-I and MAA) and Vector (MAA1 and 2).

For the initial trial of optimisation for antigen retrieval methods, one lung block from a case of CCAM was used. The tissues were sectioned at 5 μm and deparaffinised. Control sections had no retrieval. Two different methods were used for retrieval: microwave and enzyme digestion. For microwaving, an Energy Beam Sciences H2800 (East Granby, CT, US) microwave processor was used together with two types of buffer: 10 mM citrate buffer (in which the sections were microwaved for 10, 15, 20 and 25 min at 95ºC) and 0.1 M EDTA buffer (in which sections were microwaved for 15 min at 95ºC). Two enzyme digestion methods were used: trypsin (0.1%) and pronase (1 mg/mL). Sections were incubated with either enzyme for 15 min at 37ºC.

For single fluorescent studies, the sections were microwaved in 95ºC citrate buffer at pH 6.0 for 15 minutes, washed with 0.05 M Tris Buffer Saline (TBS) at pH 7.6 and then incubated with either 1/100 FITC conjugated SNA-I or 1/100 FITC conjugated MAA for 1 h at room temperature in the dark. Double fluorescent studies were performed according to previously published methods.[10] Briefly, sections were microwaved in 95ºC citrate buffer at pH 6.0 for 15 min, washed with 0.05 M TBS at pH 7.6 and then incubated with 1/100 TRITC conjugated SNA-I and 1/100 FITC conjugated MAA for 1 h at room temperature in the dark. The sections were washed 3 times for 5 min each with TBS and the nuclei stained with 5 μg/mL DAPI for 4 min, followed by three 5-min washes with TBS and mounting with DAKO fluorescent mount (Dako, Glostrup, Denmark). Fluorescent examination was with a Nikon Eclipse microscope with SPOT Pursuit Camera (Sterling Heights, MI, US) and Image-Pro Plus software (MediaCybernetics, MD, US).

For lectin horseradish peroxidase (HRP) detection, sections were microwaved in 10 mM citrate buffer at pH 6.0 for 15 min, blocked with 3% H_2O_2 in TBS for 12 min, washed with TBS (3x5 min), and then incubated with 1/50 HRP conjugated SNA-I and 1/50 HRP conjugated MAA at room temperature for 1 h. After three further washes in TBS the sections were developed with AEC substrate kit (Vector Laboratories) at room temperature for 30 min followed by counterstaining with Mayer's hematoxylin and mounting with DAKO aqueous mount (Dako Cytomation). Control sections were pre-treated with NA from *Vibrio cholerae* to ensure that SAs were targeted.

For lectin biotin detection, sections were microwaved in 10 mM citrate buffer at pH 6.0 at 95°C for 15 min, blocked with 3% H_2O_2 in TBS for 12 min and with avidin/biotin blocking kit (Vector Laboratories). Blocked sections were then incubated with 1/100 HRP conjugated SNA-I and 1/200 biotinylated MAA1 (or 1/100 biotinylated MAA2) for 1 h at room temperature, blocked with 1% bovine serum albumin for 10 min at room temperature, and then incubated with strep-ABC complex (Dako Cytomation) diluted 1/100 for 30 min at room temperature. Development was performed using the AEC substrate kit at room temperature for 10 min, the nuclei were counterstained with Mayer's haematoxylin and then the sections were dried and mounted with DAKO aqueous mount. Duck intestine sections were used as controls with and without pre-treatment with SAα2,3 specific NA from Glyko to ensure that SAs were specifically targeted.

Results

In the absence of unmasking techniques, only weak SAα2,3Gal and minimal SAα2,6Gal expression was detectable in the basal epithelium and epithelial cells of the bronchial mucosa of children. All forms of retrieval enhanced the SA staining of the surface epithelial cells and mucus-containing cells for mainly MAA binding (Fig 1). Trypsin and pronase digestion resulted in a mild increase in binding and a tendency for more surface epithelial denudation. When microwave heating was used, the increase in staining was maximal after 15 minutes. As citrate buffer heating appeared to be no more detrimental than other forms of retrieval, this was chosen as the preferred method

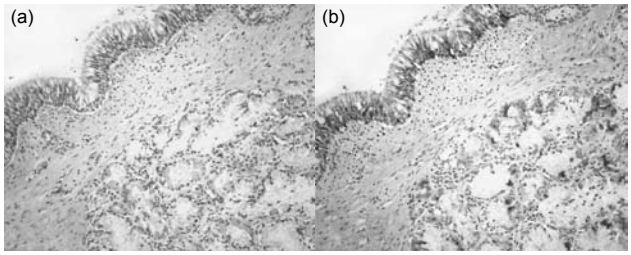

Fig 1. Serial sections of paediatric bronchial mucosa showing the effect of tissue unmasking on lectin expression
(a) With no unmasking, there is minimal binding of *Maackia amurensis* agglutinin (MAA) which detects SAα2,3 in the epithelium and stroma. (b) Following 15 minutes of microwave unmasking, the ciliated and basal epithelium shows increased binding and there is also increased binding detected in the submucous glands (horseradish peroxidase–conjugated MAA with AEC detection; haematoxylin counterstain 200x magnification)

of unmasking for future work. Tissue sections of duck intestine showed SAα2,3Gal expression on the surface of the columnar cells as detected by MAA binding.

The 20-week foetal tissue showed strong bronchial expression of SAα2,3Gal in the bronchial epithelium with cells expressing both SAα2,6Gal and SAα2,3Gal. There was also a low level of alveolar epithelial expression of both SAα2,3Gal and SAα2,6Gal, with greater expression of SAα2,6Gal. Because of greater binding of MAA in the respiratory tract using the MAA from EY Laboratories, we tested the MAA from Vector Laboratories to verify the results. The MAA from EY Laboratories is a combination of two isoforms of MAA—MAA1 and 2. Though both identified SAα2,3Gal, they had different recognition patterns for the inner fragments. While MAA2 is specific towards SAα2,3Galß1-3GalNAc and has been accepted as the traditional avian influenza receptor (Y Kawaoka, personal communication), MAA1 is more specific towards SAα2,3Galß1-4GlcNAc. When an analysis of sequential sections from the upper and lower respiratory tract was performed, several consistent findings were noted. First, SNA-I binding was more abundant in the upper than lower respiratory tract and this was more pronounced in adult tissues (Fig 2a, d, g, j, m) and mucus-secreting and ciliated cells. In the adult lungs the pneumocytes were only weakly bound to SNA-I (Fig 2g), but in the paediatric lungs there was strong binding (Fig 2m). Second, MAA1 was strongly bound throughout the respiratory tract and did not vary with age (Fig 2b, e, h, k, n). It was bound in the ciliated and mucus-secreting cells. In the paediatric bronchus it also highlighted the basal cells (Fig 2k). Third, MAA2 binding appeared to be limited only to pneumocytes and was not strongly bound to the nasopharynx or the bronchial epithelium (Fig 2f, o).

When dual labelling was performed on the adult and paediatric bronchial tissues and compared with single

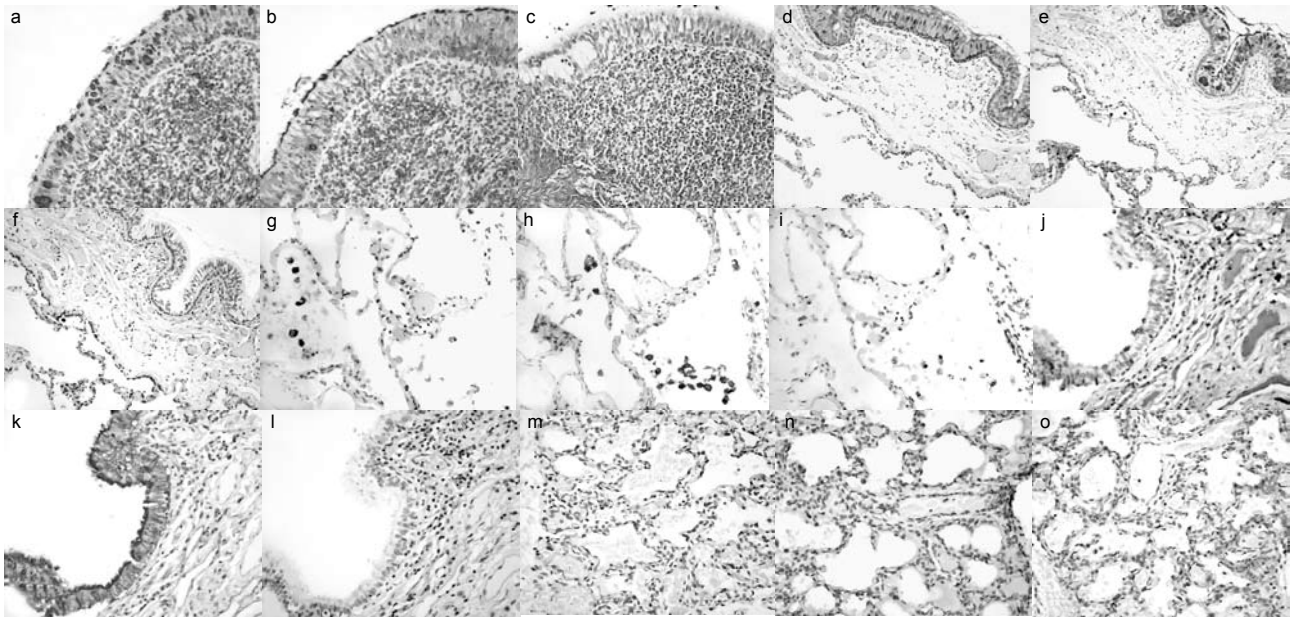

Fig 2. Tissue distribution of *Sambucus nigra* agglutinin (SNA) for SAα2,6, *Maackia amurensis* agglutinin 1 (MAA1), and *Maackia amurensis* agglutinin 2 (MAA2) for SAα2,3 in the adult and paediatric respiratory tract
Serial sections of nasopharynx (a-c), adult bronchus (d-f), adult lung (g-i), paediatric bronchus (j-l), and paediatric lung (m-o) are shown and stained with SNA (a, d, g, j, m), MAA1 (b, e, h, k, n) and MAA2 (c, f, i, l, o). The adult nasopharynx shows SNA and MAA1 binding in the epithelium but no MAA2 binding. A similar pattern is also present in the adult bronchus, and the pneumocytes show MAA1 and MAA2 binding (e, f). Alveolar macrophages (g-i) demonstrate minimal SNA and no MAA2 binding but are positive for MAA1 binding. The paediatric bronchus shows a greater binding of the epithelium with MAA1 (k) than the adult (e). The pneumocytes (m) also show more SNA binding than the adult (g). Staining using horseradish peroxidase–conjugated SNA and biotin conjugated MAA1 and MAA2. (a-f) and (j-l) at 200x magnification and (g-i) and (m-o) at 400x magnification

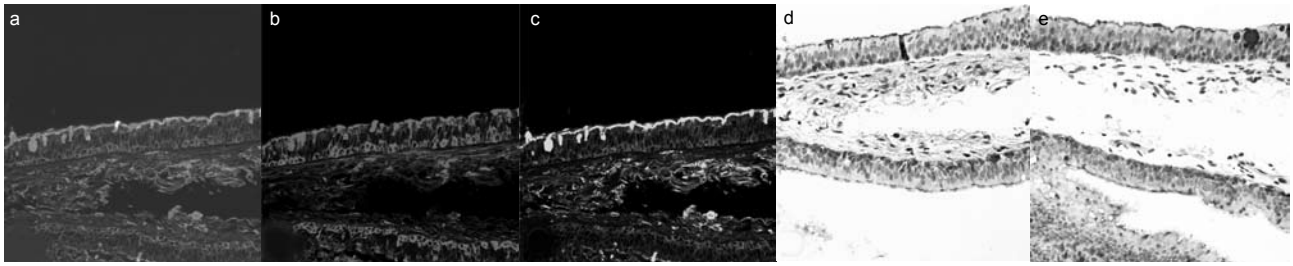

Fig 3. Serial sections for comparison of detection methods for lectin binding of *Sambucus nigra* agglutinin (SNA) for SAα2,6 and *Maackia amurensis* agglutinin (MAA) for SAα2,3 in adult bronchial epithelium
Double fluoresecence for fluorescein isothiocyanate–labelled SNA and tetramethyl rhodamine isothiocyanate–labelled MAA shows a heterogeneous pattern (a), with more binding to the basal epithelium with MAA (b) than SNA (c). Horseradish peroxidase labelling of MAA (d) and SNA (e) shows a similar pattern of binding to the fluorescent stains (x200)

staining, the most significant finding in the bronchial biopsies of adults was a heterogeneous distribution of epithelial SAα2,6Gal and SAα2,3Gal positivity, with no clear difference in the cellular distribution. There was a mixed and occasionally dual expression of both SAα2,3Gal and SAα2,6Gal in the ciliated cells, goblet cells and basal cells (Fig 3). We also found a similar staining pattern when non-fluorochromes were used (Fig 3b-e). We did not find significant expression of SNA-I in endothelial cells in contrast to other reports.[3] When we analysed lectin binding to the trachea, bronchus and alveoli of the sampled pig tissues, there was widespread binding of SNA-I to the tracheal, bronchial and bronchiolar epithelium but only weak binding to the alveolar epithelium. In contrast to a previous report,[6] there was weak binding of either MAA1 or MAA2 in the trachea or bronchus.

Discussion

The difference between our studies and those published previously on SA expression in respiratory epithelial cells can be partially explained by the methods used for lectin analysis as well as the type of lectin conjugate used. Antigen retrieval or unmasking did not become an established procedure in many laboratories until the mid-1990s, and the earlier publications used paraffin embedded tissues without retrieval.[2,10] Later studies on pigs, primates and ducks also did not use retrieval procedures.[6,11] Whilst the precise mechanism of retrieval is unknown, the general consensus is that heating or the use of enzymes serves to unmask antigenic sites that have become cross-linked through formalin fixation.

MAA1 binding in the upper and lower respiratory tract has implications for the pathogenesis of avian influenza infection, as the current H5N1 viruses recognise SAα2,3 motifs detected with MAA1. Because of the varied distribution of MAA1 and MAA2 throughout the respiratory tract, we hypothesised that this may shed new light on the distribution and binding of H5N1 viruses to the upper and lower respiratory tract. We therefore used ex vivo cultures of the upper and lower respiratory tract and infected them with different H5N1, H1N1 and H3N2 viruses. Contrary to previous suppositions, H5N1 viruses were able to replicate in the upper respiratory tract—a region which lacked MAA2 staining but had abundant MAA1 expression. This indicated that the virus was binding to SAα2,3Galß1,3/4GlcNac motifs or even to non-sialyated receptors.

The findings of the in vitro model with cultured cells showed more correlation with the results from children in our studies. It is therefore possible that the human tracheobronchial epithelial culture represents a more primitive model of the human respiratory tract than that in adults.

Conclusions

There was an overall greater detection of SAα2,3Gal in the respiratory tract when microwave unmasking was used. Therefore the routine use of this method was advocated for future investigations on the distribution of receptors for influenza viruses in the respiratory tract as well as attention to the supplier of lectins. These results imply a need to re-evaluate findings reported in previous studies on the tissue distribution of SA receptor types. It appears that paediatric tissues have a greater expression of SAα2,3Gal than previously described. Although fluorescent detection methods require a fluorescent microscope and slide preparations fade with time, they should be used in conjunction with histochemical techniques, as they enable better assessment of dual SA expression. We were able to use the lectin histochemical findings to re-evaluate the tissue tropism of H5N1 infection of the respiratory tract and shed new light on the cells infected by this emerging virus.

Acknowledgements

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