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Susceptibility of the upper respiratory tract to influenza virus infection following desialylation

J Nicholls *, M Chan, D Kwong

KEY MESSAGES

1. In general, human and swine viruses bind to host respiratory tract surface molecules that have an α2-6 linkage between sialic acid (Sia) and adjacent sugar molecules. Avian viruses preferentially bind to host receptors with an α2-3 linkage. Information on controlling influenza virus infection by removing Sia from the host surface is limited.

2. Lectin histochemistry was used to identify the Siaα2-6 and Siaα2-3 linkage and this lectin binding was re-examined after topical (surface) sialidase treatment. The presence of sialylated glycans in tissues was analysed using mass spectrometry. The presence of infection of human upper and lower respiratory tract tissue was tested after sialidase treatment with avian and human viruses.

3. There was a diffuse expression of Siaα2-6 throughout the upper and lower respiratory tract. Siaα2-3 varied according to site with more susceptible of the upper respiratory tract to influenza virus infection following desialylation.

4. Both prophylactic as well as therapeutic sialidase treatment was able to prevent infection with avian and human influenza viruses. Sialidase therapy offers a potentially useful clinical option and is now in phase II clinical trial.

Introduction

The specificity of influenza for a particular host is mediated by the interaction of haemagglutinin (HA), a viral cell surface glycoprotein with host glycoconjugate receptors that contain terminal sialic acid (Sia) residues. The HA of influenza A strains that infect humans attaches preferentially to cells with Sia linked α2-6 residues to galactose, whereas avian strains preferentially bind Sia linked α2-3 to galactose.1 As an example, the H5N1 viruses of the bird flu outbreak in Hong Kong in 1997 had an affinity for binding to avian α2-3 linked Sia.

On the basis of binding by the lectin, *Maackia amurensis* agglutinin (MAA), Siaα2-3 (and thus H5N1 infection) appears to be restricted mainly to the human lower respiratory tract, whereas by *Sambucus nigra* agglutinin (SNA) binding, Siaα2-6 linkages in both the upper and lower respiratory tract have been identified. This presumed inability of the H5N1 virus to establish infection in the upper respiratory tract has significant clinical and epidemiological implications. For example, H5N1 transmission is unlikely to occur via droplets or ingestion, and will likely require aerosol transmission to reach the lower respiratory epithelium.

Previously we used different isoforms of the lectin MAA and demonstrated that MAA2 (which binds O-linked Siaα2-3) had limited binding in the upper respiratory tract, but there was widespread binding of MAA-I (which binds N-linked Siaα2-3) in the nasopharynx, adenoid and trachea of adults and children.2 We further went on to demonstrate that H5N1 viruses with only Siaα2-3 binding specificity could replicate in the upper respiratory tract of humans.2 There was widespread binding of MAA-I—the lectin that identifies Siaα2-3Gal—a binding preference of H5 viruses. This isoform of MAA did detect non-Sia termini such as sulphated galactose.

A new pharmaceutical agent—DAS181—utilises the *Aspergillus viscosus* sialidase coupled together with an epithelial cell anchoring domain.4 This fusion protein effectively degraded receptor Sia for both human and avian influenzas and potentially conferred protection against a broad range of influenza viruses. As MAA-I identified non-Sia residues, fresh tissues were also submitted for mass spectrometric analysis to determine residual glycoprotein profiles following DAS181 treatment.

If Sia are the sole receptors for influenza virus infection, then we proposed there would be an absence of influenza virus (IFV) infection as...
determined by 50% tissue culture infectious dose (TCID₅₀), enzyme-linked immunosorbent assay and immunohistochemistry following pre-incubation of normal upper respiratory tract tissues with neuraminidase (NA) and DAS181. Nonetheless, two reports have suggested that influenza virus infection may occur in the absence of NA, although these have been mainly confined to non-human or tumour cell lines. This warrants investigation of the alternate receptor hypothesis by exploring whether tissues can still be infected by influenza viruses despite NA or DAS181 treatment.

**Methods**

This study was conducted from January 2009 to December 2010. Fresh biopsies were obtained from the normal nasopharynx and bronchus of individuals screened for nasopharyngeal carcinoma, and from healthy volunteers under a study approved by the Institutional Review Board of The University of Hong Kong and Hospital Authority (Hong Kong West). Lung tissue excised for pulmonary malignancy or other surgical procedures was also obtained.

Viruses used included A/Hong Kong/54/98 (H1N1), A/Hong Kong/1174/99 (H3N2), A/Qu/HK/G1/97 (H9N2), A/Dk/HK/Y280/97 (H9N2), A/Hong Kong/213/03 (H5N1), A/Vietnam/3046/04 (H5N1).

Infection was carried out using the viruses at a titre of 1×10⁶ TCID₅₀/ml, and ultraviolet light inactivated viruses as controls. Following incubation of the tissue fragments with the virus, the supernatant was removed and replaced with fresh medium. The tissue fragments of each biopsy were incubated at 37°C for 48 hours at which time they were fixed in 10% neutral buffered formalin and processed for influenza nucleoprotein immunohistochemistry.

**Lectin binding profiles**

Sections were microwaved in 10 mM citrate buffer pH 6.0 at 95°C for 15 minutes then blocked with 3% H₂O₂ in TBS for 12 minutes and with avidin / biotin blocking kit (Vector Labs). They were then incubated with biotinylated MAA-I and MAA-II (Vector Labs) and horseradish peroxidase (HRP)-labelled SNA and LFA (EY Labs) for 1 hour at room temperature, blocked with 1% bovine serum albumin for 10 minutes at room temperature, and then incubated with strep-ABC complex (Dako Cytomation, K-0377) diluted 1/100 for 30 minutes at room temperature.

**Neuraminidase and DAS181 treatment**

Fresh and paraffin-embedded tissues were incubated with three different neuraminidases from Glyko (Glyko S – Siaα2,3 specific; Glyko N – Siaα2,3 and Siaα2,8 specific and Glyko A – Siaα2,3 and Siaα2,6 specific) as well as DAS181 obtained from NexBio Pharmaceuticals. Incubation was performed at 37°C for 2 hours before influenza virus infection or lectin binding assessment.

**Mass spectrometric analysis**

Frozen bronchus and lung tissue biopsies before and after DAS181 treatment were washed with PBS to remove any excreted mucus. Glycoproteins were solubilised from the tissues by homogenisation in a detergent extraction buffer. Extracted glycoproteins were reduced and carboxymethylated prior to tryptic protease digestion. N-linked glycans were enzymatically cleaved from the peptide backbone by digestion with peptide N-glycosidase F and subsequently purified on a Sep-Pak C18 reverse-phase cartridge. O-glycans were chemically released from glycopeptides by reductive elimination. GC-MS linkage data were performed on glycan samples both before and after digestion with linkage specific sialidases (α2-3 specific from *Streptococcus pneumoniae* and α2-3, 6, 8,9 specific from *Arthrobacter ureafaciens*). MALDI-TOF MS profiles of permethylated glycans after sialidase digestion were recorded to assess the degree of desialylation.

**Results**

Sialidase treatment using Glyko A sialidase (which cleaves both α2-3 and α2-6 Sia) was able to remove binding of SNA and MAA to the epithelium, and this was more prominent in tissues from the bronchus and lung.

Using fresh bronchial and tracheal biopsies, the dosage of DAS181 necessary to lead to desialylation was determined. A concentration of 5 µg/cm² was suitable to remove Sia from the surface epithelium, as shown by a decrease in SNA binding compared with the control. As DAS181 was not internalised there was no change in the intracellular Sia that remained in the goblet cells. Ten minutes exposure to DAS181 was sufficient to lead to significant desialylation at 10 µg/cm², although by 30 minutes there was an equal level of desialylation at both 5 µg/cm² and 10 µg/cm². After a single treatment there was desialylation up to 48 hours with increased intracellular binding of SNA seen in bronchial epithelium associated with weak binding to the epithelium at 72 hours. This finding was in accord with an in vitro human airway epithelium finding: DAS181 at 5-10 µg/cm² was able to stop lectin binding indicating removal of extracellular Sia, and this effect lasted for 72 hours.

Accordingly, if Sia was the sole receptor for influenza, then removal of the Sia from glycans should diminish infection with influenza. Because H5N1 is associated with a higher mortality than seasonal influenza, we initially focused on the prophylactic and therapeutic effect of DAS181 in H5N1 infection. After 2 hours incubation with DAS181, there was a...
reduced binding of SNA and MAA and increased binding with PNA (which detects the exposed galactose). In a repeat experiment of the previously published mass spectrometric analysis, there was reduced sialylation after 2 hours incubation of lung tissues with DAS181. Infection experiments with H5N1 showed that control tissues had positive influenza nucleoprotein cells in the tissue sections indicating infection and this was reduced by a single dose of DAS181. Continuous exposure to DAS181 significantly abolished infection. When influenza M-gene analysis was performed, control H1N1, H3N2 and H5N1 tissues showed increased gene expression that was abolished after DAS181 treatment.

During the duration of the project, a new pandemic virus H1N1pdm emerged. We studied whether DAS181 would be able to abolish this infection. Pre-treatment with DAS181 prevented influenza virus replication as demonstrated by TCID₅₀ and influenza M gene analysis.

**Discussion**

This project aimed to investigate interaction of the HA of different influenza viruses with the Sia present on the respiratory epithelium and how interfering with this interaction could affect influenza replication in the normal respiratory tract. We demonstrated that sialidase therapy was able to reduce lectin binding to epithelia from various sources (ie removal of both α2-3 and α2-6 linked glycans) and confirmed that different isoforms of the lectin *Maackia amurensis* should be used for detecting α2-3 type glycans. We then demonstrated that a single dose of DAS181 was sufficient for desialylation and that the effect of desialylation could last for up to 3 days and that ciliated epithelial cells were the preferred target for desialylation in the bronchial epithelium. In addition, DAS181 treatment was able to effectively block H5N1 and other seasonal virus infection in *ex vivo* tissue.

Overall, Sia was the main receptor for influenza, and removal of this Sia would be sufficient to abolish influenza virus infection. Nonetheless, how our findings correlate with findings in other studies in which influenza infection occurred in the presence of desialylation remained unclear. We therefore tested the effects of DAS181 on a wider range of influenza viruses (including H7N7 viruses, H1N1pdm, H3N2, H5N1 and H1N1 viruses) and used MDCK cells and CHO cells. As expected, DAS181 treatment markedly reduced infection in CHO cells with all viruses tested, but in MDCK cells despite DAS181 treatment there was still definite evidence of infection with most H5N1 viruses—A/Vietnam/3046/04 (H5N1) and A/HongKong/54/98 (H1N1). The two possibilities (which are not mutually exclusive) are (1) there are glycans present on MDCK cells that are not present on CHO cells and are resistant to sialidase treatment and (2) these two viruses have distinct binding profiles that allow binding to non-Sia glycans.

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**References**