Infection of sialyltransferase knockout mouse embryo fibroblasts with H5N1 influenza A virus

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Introduction

Sialic acid is commonly found terminating carbohydrate chains attached to glycoproteins, glycolipids, and proteoglycans, where it plays a role in cell-to-cell recognition. In these carbohydrate chains, sialic acid is most often bound to galactose via a α-2,3 linkage, or an α-2,6 linkage. Pathogens such as influenza A use these sialic acid residues as a means of recognizing and binding to their hosts. In particular, human influenza viruses preferentially bind to α-2,6 linked sialic acid while avian influenza viruses preferentially bind to α-2,3 linked sialic acid. This preference for one form of sialic acid over another is a key factor in determining whether a subtype of influenza will be able to infect a host. For example, while the avian influenza A virus H5N1 has been known to cause serious disease in humans, human infection by avian H5N1 influenza virus is rare. This is likely due to the relative absence of α-2,3 linked sialic acid, the preferred binding substrate for avian influenza virus, in the human upper respiratory tract. As well, there is no observed human-to-human transmission of H5N1, likely for this reason. One major concern is that if the H5N1 flu virus was to be able to mutate to recognize α-2,6 sialic acid linkages, its pathogenicity would increase, as it would be able to spread through human-to-human contact.

Sialic acid is added to its substrate molecules by enzymes known as sialyltransferases, or STs. Although there are a number of STs, we have chosen to focus on 6 of particular importance: ST3Gal1 – ST3Gal6, are responsible for adding α-2,3 linked sialic acid to substrate molecules. At this point in time, the specific substrates of these STs *in vivo* are unknown. Studies have been conducted with soluble enzyme-substrate assays, which may not accurately reflect ST3Gal specificity *in* vivo (Kono, 1997). It is unknown whether one or many ST3Gal enzymes produce the receptors which are required for influenza binding.

Previously, infection studies were done in ST3Gal1, ST3Gal2 and knockout mice with a recombinant H5N1 influenza virus. When compared to wild type (WT) mice, ST3Gal knockout (KO) mice appeared to show more infection as quantified by plaque assay, and also exhibited decreased survival. However, sialic acid is known to play a role in cell-to-cell interactions which mediate immune system responses. Because infection differences between wild type and ST3Gal KO mice may result from immune system interactions, it is therefore necessary to study the effects of ST3 knockout on viral infection in a system free from immune system interactions.

Hypotheses: If ST3Gal4 is responsible for the production of influenza receptors, a decrease in virus infection should be observed in ST3Gal4 KO mouse embryo fibroblasts (MEFs) as compared to WT MEFs.

Objectives: To determine if influenza A virus infection differs in MEFs from ST3Gal4 KO mice and WT controls.

Materials and Methods

Mice and isolation of fibroblasts: KO mice for ST3 Gal4, and WT C57BL/6 controls were obtained from Jackson Labs. Female mice exhibiting vaginal plugs after mating were weighed daily to confirm pregnancy. Mouse embryos were isolated from pregnant female mice at 12-14 days gestation, minced and trypsinized. MEFs were cultured in growth medium (DMEM) containing 10% fetal bovine serum until confluency. MEFs were passaged once and frozen in liquid nitrogen.

Virus: Influenza A/rgHK/213/03xPR/8, containing the H5 and N1 genes of Influenza A/HK/213/2003 on an influenza A/PR/8/34 background was propagated in Caco2 cells . Virus stocks were stored at -80C. Virus titres were calculated from plaque assays performed on Madin-Darby canine kidney (MDCK) cells.

Flow cytometry:
WT and ST3Gal4 KO MEFs were seeded on (100 mm) cell culture dishes and cultured for 24 hours. Passage 2 MEFs were counted with a hemacytometer, infected with H5N1 influenza virus at a multiplicity of infection (m.o.i.) of 3 for 1 hour in serum-free DMEM and incubated for 24 hours in DMEM. The MEFs were trypsinized, washed, fixed and permeabilized, and incubated in the presence or absence of mouse monoclonal antibody specific for the influenza A nucleoprotein (NP)(MAB8257; Millipore) and/or phycoerythrin-conjugated goat anti-mouse immunoglobulin. Analysis was performed with an FC-500 flow cytometer. Parameters were unchanged between each analysis.

ST3Gal 4 KO Embryo Gestation

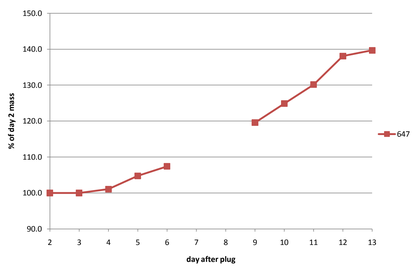

Figure 1. Growth curve of suspected pregnant mouse #647. %weight was measured daily using day 2 mass as a reference point. Mice display a 40%-50% increase in weight approaching the 12th day of pregnancy.

WT and Gal 4 KO Flow Cytometry

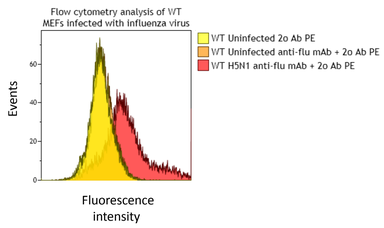

Figure 1. Flow Cytometry analysis of WT MEFs infected with H5N1 as compared to uninfected controls, n = 10000. The peaks for WT uninfected controls are superimposed. Fluorescence intensity is measured on a logarithmic scale.

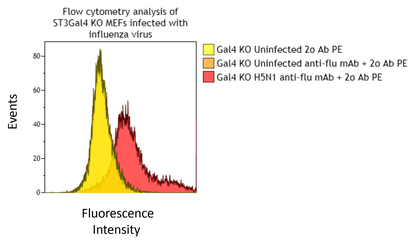

Figure 3. Flow Cytometry analysis of Gal 4 KO MEFs infected with H5N1 as compared to uninfected controls, n = 10000. The peaks for Gal 4 KO uninfected controls are superimposed. Fluorescence intensity is measured on a logarithmic scale.

Observations

1. H5N1 infected cells show a notable increase in fluorescence intensity when compared to the uninfected cells for both WT and ST3Gal4 KO. ST3Gal 4 KO demonstrate a higher MFI, and therefore a higher concentration of influenza proteins, than the WT cells.
2. There is no appreciable difference in fluorescence between uninfected cells stained with only 2° antibody (PE) versus those stained with both αflu mouse antibody + 2° antibody (PE).

Results

Table 1: Flow cytometric analysis of MEF infection by influenza A virus

Experiment Description	M FI	MFI Ratio
WT MEF uninfected 2˚Ab only	4.88	-----
WT MEF uninfected αflu mAB + 2°Ab	5.4	-----
WT MEF H5N1 24hr αflu mAB + 2°Ab	52.4	9.70
Gal 4 KO MEF uninfected 2˚Ab only	4.05	-----
Gal 4 KO uninfected αflu mAB + 2°Ab	4.03	-----
Gal 4 KO MEF H5N1 24hr αflu mAB + 2°Ab	55.02	13.65

Note: Mean fluorescence intensity (MFI) ratios were calculated by dividing the mean FI values of the infected MEFs by the mean FI values of the corresponding uninfected control MEFs.

Overall, the Gal4 KO MEFs expressed more influenza NP than the WT MEFs when both were infected with H5N1. Differences between the MFI of WT and Gal4 KO uninfected controls are insignificant when compared to the increase in MFI in the infected population.

Discussion

The Gal 4 KO MEFs are not refractive to infection and are readily infected, contrary to the original hypothesis. It is possible that the Gal 4 KO MEFs may demonstrate increased infectability, due to the significant increase in MFI ratio of the Gal 4 KO MEFs as compared to the WT MEFs; this would need to be confirmed through additional study. It also is possible that the increased susceptibility to virus and decreased survivability of ST3Gal4 KO organisms is not a result of immune system interactions, but of other cell-to-cell interactions. ST3Gal 4 likely does not sialylate receptors required for influenza binding to the cell.

Supplementary Work

The experiments summarized in table 1 will need to be repeated to confirm the data presented here. By performing flow cytometry on all Gal KO strains under the same conditions, the relative susceptibility of the Gal KO strains can be identified. In order to quantify differences Infectivity studies will continue to be performed in order to examine virus titres in the lungs of infected animals and to generate survival curves for both H5N1 and H1N1 on all mice strains. Notably, the ST3Gal4 MEFs appear to be as susceptible to H5N1 influenza A infection as WT MEFs. This observation suggests that ST3Gal4 is not solely responsible for generating influenza A virus receptors.

References

Kono, M et al. (2007.) Mouse p-galactoside α2,3-sialyltransferases: comparison of in vitro substrate specificities and tissue specific expression. *Glycobiology, 7*(4): 469-479.

Acknowledgements

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