รายงานวิจัยฉบับสมบูรณ์

โครงการ
“การปรับตัวของไวรัสไข้หวัดนก H5N1 ข้ามเขตกั้น ระหว่างสัตว์ปีกและมนุษย์”

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สัญญาเลขที่ **BRG5180008**

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กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับการสนับสนุนโดยสำหนักงานกองทุนสนับสนุนการวิจัย และความเห็นในรายงานฉบับนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป

บทคัดย่อ

ไวรัสไข้หวัดนก H5N1 เป็นไวรัสที่มีความรุนแรงสูงและทำให้เกิดความเสี่ยงที่จะก่อให้เกิดการระบาดใหญ่รุนแรงทั่วโลก ปัจจุบันแม้ไวรัสจะสามารถติดเชื้อในมนุษย์แต่ก็ยังไม่ได้ปรับตัวให้เข้ากับมนุษย์ได้อย่างสมบูรณ์ การแพร่เชื้อจากคนไปคนยังเกิดได้ยาก มีความกังวลกันว่าไวรัสอาจสามารถปรับตัวให้เข้ากับคนได้ดีขึ้นและสามารถแพร่เชื่อจากคนไปคนได้อย่างมีประสิทธิภาพ ผู้วิจัยจึงทำการศึกษากลไกที่น่าจะเกี่ยวข้องกับการปรับตัวนี้และกลไกของ inter-species barrier ที่ขัดขวางไม่ให้ไวรัสไข้หวัดนกแพร่เชื้อในคนได้ดี การศึกษาในโครงการนี้พบว่าระดับการแสดงออกของ receptor ของไวรัสในเยื่อบุทางเดินหายใจของมนุษย์มีความแตกต่างกันในแต่ละบุคคล และอาจมีผลต่อประสิทธิภาพในการติดเชื้อไวรัสไข้หวัดนกและไข้หวัดใหญ่ โดยพบว่ากลุ่มผู้ป่วยที่มีอาการภูมิแพ้ในจมูกมีระดับ receptor ของไวรัสสูงขึ้น และเยื่อบุโพรงจมูกที่มีระดับ receptor สูงขึ้นนี้สามารถติดเชื้อไวรัสไข้หวัดใหญ่และไข้หวัดนกได้ดีขึ้น นอกจากนี้ผู้วิจัยยังได้ทำการศึกษาฤทธิ์ของโปรตีน neuraminidase ของไวรัสไข้หวัดนก H5N1 และพบว่ามีรูปแบบของการออกฤทธิ์แบบเดียวกับไวรัสไข้หวัดนกอื่นๆ และพบการกลายพันธุ์ที่ทำให้รูปแบบความจำเพาะของการออกฤทธิ์มีลักษณะคล้ายไวรัสไข้หวัดใหญ่ ซึ่งอาจเป็นส่วนหนึ่งของการปรับตัวของไวรัสเข้าสู่มนุษย์

Keywords: ไวรัสไข้หวัดนก; เขตกั้นระหว่าง species; sialic acid; neuraminidase; RNA polymerase; temperature sensitive

Abstract

H5N1 avian influenza virus is a highly virulent virus that causes a serious threat of severe pandemic influenza. Although, the virus can infect and cause severe disease in humans, it has not fully adapted to human host. Human-to-human transmission of the current H5N1 virus is still inefficient. It is feared that the virus may eventually adapt to transmit efficiently from human to human. In general, avian influenza viruses do not infect human because of interspecies barriers. We are investigating some mechanisms as well as genetic determinants of the viral properties that are likely to be involved in the inter-species barriers. We found that expression of viral receptor on human nasal mucosa can be variable and affected by allergic and inflammatory conditions, and that the level of receptor availability can affect infection of seasonal and avian influenza viruses. These data suggest that the receptor expression may be an important determinant of susceptibility to infection and may compromise the inter-species barrier. We also characterize the substrate specificity of neuraminidase (NA) of H5N1 avian influenza viruses, and found their activity to be higher for α2,3-linked sialic acid. We found a mutation (P320H) that was associated with reduced NA activity against α2,3-linked sialic acid. This reduction in the NA activity is considered to be an adaptation to human hosts.

Keywords: avian influenza virus; inter-species barrier; sialic acid; neuraminidase; RNA polymerase; temperature sensitive

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Rationale:

H5N1 avian influenza virus is a major threat for emergence of a pandemic virus. In contrast to most avian influenza viruses, which do not readily infect human, highly pathogenic H5N1 avian influenza strains can transmit directly from avian species to human and cause severe diseases. Despite the ability to infect and cause severe disease in human, most H5N1 viruses has not yet fully adapted to human and efficiently transmitted from human to human. If the H5N1 virus is adapted to an efficient person-to-person transmission, it could cause a pandemic. The mechanisms responsible for the inefficient transmission of avian influenza viruses in human or the “interspecies barriers” are far from fully defined. A well-documented mechanism of this interspecies barrier is the

receptor-binding preference. Avian influenza viruses preferentially recognize α2,3-linked sialic acid, whereas human influenza viruses recognize α2,6-linked sialic acid, which is present in more abundance in human upper airway. However, it is likely that receptor adaptation of HA alone will not be sufficient for the H5N1 virus to become a pandemic virus.

Objectives:

This study explored various mechanisms involved in the interspecies barrier of H5N1 avian influenza viruses.

Approaches:

We used in vitro experimental models to study various properties of H5N1 avian influenza viruses, which may be involved in the interspecies barrier such as interaction with cellular factors, infectivity and cytokine induction in human cells and substrate specificity of NA, and compared them to those of seasonal influenza viruses. We also looked for variability of those properties among viral isolates and identified responsible mutations.

Results

Interaction with host cellular factors

The viral non-structural protein 1 (NS1) is believed to play crucial roles in the viral pathogenesis. A screening for human macrophage genes interacting with H5N1 NS1 by yeast two-hybrid system was performed. Seven clones were obtained and identified as interleukin-6 receptor, MHC class I HLA-B, cathepsin B, ubiquitin and ADAR1. As these genes play important roles in immune responses, protein targeting for proteosomal

degradation and RNA editing, H5N1 virus may use NS1 to manipulate these host proteins for their own benefits thereby causing the unusual severity of human H5N1 diseases.

Induction of proinflammatory cytokines

While Hong Kong 1997 isolates of H5N1 viruses were previously shown to hyper-induce proinflammatory cytokines from human macrophage and the cytokine hyperinduction is believed to be a virulence mechanism, we show that current H5N1 strains induce variable levels of TNF-α, which were not necessarily higher than those induced by seasonal influenza viruses. The result suggests that hyper-induction of TNF-α in human macrophages is not always associated with a highly pathogenic phenotype.

Role of receptor expression on the interspecies barrier

Influenza viruses bind and infect respiratory epithelial cells through sialic acid on cell surface. Differential preference to sialic acid types contributes to host- and tissue-tropism of avian and seasonal influenza viruses. Although the highly pathogenic avian influenza virus H5N1 can infect and cause severe diseases in humans, it is not efficient in infecting human upper respiratory tract. This is because of the scarcity of its receptor, α2,3-linked sialic acid, in human upper airway. Expression of sialic acid can be influenced by various factors including inflammatory process. Allergic rhinitis and nasal polyp are common inflammatory conditions of nasal mucosa and may affect expression of the sialic acid and susceptibility to influenza infection. We show that mucosal surface of nasal polyp expressed higher level of α2,3- and α2,6-linked sialic acid than normal nasal mucosa. Accordingly, both H5N1 avian influenza viruses and seasonal influenza viruses replicated more efficiently in nasal polyp tissues explants. Our data suggest a role of nasal inflammatory conditions in susceptibility to influenza infection, especially by

avian influenza viruses, which is generally inefficient in infecting human upper airway. This may contribute to the gradual adaptation of the virus to human population.

Effects on cellular gene expression

We analyzed expression profile of lungs from two fatal H5N1 avian influenza cases using microarray. We identified 3435 genes with higher than 2 fold changes in mRNA levels in both cases as compared to those of normal lung. Of these, 1019 genes showed increased mRNA levels, while 2416 genes showed decreased mRNA levels. Gene ontology analysis identified several ontology terms with significant association with these genes, most of which are related to cellular metabolism and regulation of cellular process including apoptosis and chemotaxis. Pulmonary surfactant protein D (SPD), which has been shown to contain influenza inhibitory activity, was found to be down-regulated. The levels of SPD mRNA in the H5N1 infected lungs were confirmed by a quantitative RT-PCR to be lower than those of normal lungs and lungs from patients with respiratory failure from other causes. SPD plays multiple roles in respiratory innate defense against various pathogens, regulation of inflammatory responses, and maintenance of alveolar integrity. Reduction of SPD in H5N1 avian influenza may therefore play important roles in the pathogenesis in this fatal disease.

Substrate specificity of NA

We analyzed NA activity of avian influenza H5N1 strains on α2,3- and α2,6-linked sialic acid. While seasonal and pandemic H1N1 influenza viruses showed low activity on both substrate, NA of H5N1 viruses showed variable degree of activity on α2,3-linked sialic acid and low activity on α2,6-linked sialic acid. NA of some human isolates of H5N1 virus had low activity on α2,3-linked sialic acid, giving a pattern

similar to that of seasonal influenza viruses. A mutation, P320H, was identified to be the genetic determinant of the change. These data suggest that some diversity of NA activity of H5N1 viruses may contribute to the viral adaptation to human hosts.

Conclusions:

While the current H5N1 avian influenza viruses have not yet fully adapted to human hosts, some evidences of gradual adaptation were identified. We identified a mutation in NA that caused a change in NA activity to that resembles seasonal influenza viruses. Although the virus still has receptor preference of avian influenza type, altered sialic acid expression in upper airway of allergic individual may compromise the interspecies barrier and allow efficient infection of this virus.

We also characterize other properties of H5N1 avian influenza, which may be involved in the viral pathogenesis and adaptation to human hosts. We identified a number of human genes interacting with the viral NS1 and we show that current H5N1 strains varied in their ability to induce proinflammatory cytokine and did not necessarily induce higher levels of cytokine in comparison to seasonal influenza viruses.

Output

Publication

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ภาคผนวก

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Identification of cellular partners of Influenza A virus (H5N1) non-structural protein NS1 by yeast two-hybrid system

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Summary. – Influenza A virus (IAV) subtype H5N1 is associated with the re-emergence of severe human influenza. The virus is highly virulent and viral non-structural protein 1 (NS1) is believed to play a crucial role in the viral pathogenesis. A screening for human proteins interacting with NS1 was performed by a yeast two-hybrid system (Y2H). Two bait plasmids that expressed DNA binding domain (BD) fused to either RNA binding domain or to effector domain of NS1 were constructed and transformed into yeast. The bait yeast was mated with a prey yeast containing human macrophage cDNA library fused to DNA activation domain. Obtained clones were interacting with interleukin-6 receptor (IL-6R), MHC class I HLA-B, cathepsin B, ubiquitin, and adenosine deaminase acting on RNA (ADAR1). These proteins play important role in the immune response, targeting for proteosomal degradation, and RNA editing. Thus, IAV H5N1 may use NS1 to manipulate these host proteins for its own benefit and in that way confer an unusual severity to the infection.

Keywords: Influenza A virus; H5N1 subtype; NS1; protein-protein interaction; yeast two-hybrid system; ADAR1; IL-6 receptor; HLA-B; cathepsin B; ubiquitin

Introduction

IAV (the family *Orthomyxoviridae*) is a negative-stranded RNA virus. The virus genome consists of eight RNA segments encoding 10 proteins. IAVs are divided into different subtypes based on the antigenic differences of the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Currently, 16 different HA subtypes and 9 NA subtypes of IAV isolated from avian species have been identified.

The "bird flu" outbreak in Hong Kong in 1997 caused by IAV (H5N1/97) was first documented as a solely avian influenza virus causing respiratory disease and death in humans. It was associated with an overall mortality rate of 33%. The clinical symptoms featured viral pneumonia progressing to acute respiratory distress and multiple organ dysfunction syndromes associated with lymphopenia and hemophagocytosis (Abdel-Ghafar *et al*., 2008; Chan *et al*., 2002; Claas *et al*., 1998). Since 2001, the precursor of H5N1 viruses has continued to re-assort and has given rise to novel virus genotypes. One of these H5N1 genotypes was associated with the re-emergence of human influenza in 2003 that eventually led to a widespread outbreak in Asia with transmission to humans in Vietnam and Thailand (Allen *et al*., 2006; Peiris *et al*., 2004). This outbreak posed a significant threat to human health and a potential for the onset of IAV pandemic.

Presently, it is still not clear why H5N1 IAV is so highly virulent. However, the IAV-encoded NS1 is suggested as one of the putative contributing factors (Li *et al*., 2006). The results obtained with recombinant mutant viruses indicated that NS1 protein contributed to the virulence of IAV during infection primarily by allowing the viruses to disarm the interferon (IFN)-based defense system of the host cell

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Abbreviations: ADAR1 = adenosine deaminase acting on RNA; BD = binding domain; ED = effector domain; HA = hemagglutinin; HDV = Hepatitis D virus; IAV = Influenza A virus; IF = immunofluorescence; IFN = interferon; IL-6 = interleukin-6; IL-6R = IL-6 receptor; NA = neuraminidase; NS1 = non-structural protein 1; PKR = protein kinase R; RD = RNA-binding domain; TNF = tumor necrosis factor; Y2H = yeast two-hybrid system

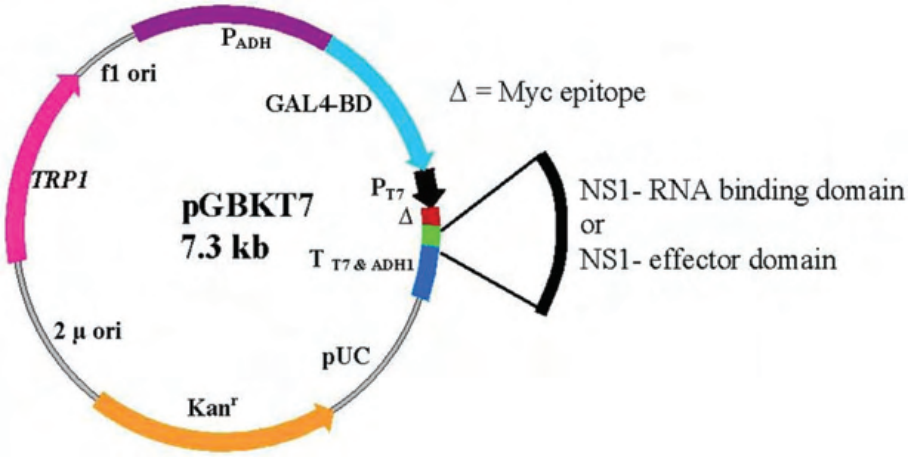

Fig. 1

Schematic diagram of the bait plasmids

GAL4-BD = GAL4-DNA binding domain; T7 = T7 RNA polymerase promoter; P_{ADH} = transacted *Saccharomyces cerevisiae* ADH1 promotor; pUC = plasmid replication origin; Kan^r = kanamycin resistance gene; 2 μ ori = yeast 2μ replication origin; TRP1 = tryptophan coding sequence; f1 ori = f1 bacteriophage origin of replication.

(Lipatov *et al*., 2005; Seo *et al*., 2004). Additional studies reported that the NS1 gene was associated with the virulence of IAV in a mouse model. On the other hand, IAV with the deleted NS1 gene exhibited an attenuated phenotype in the mice and pigs. Amino acid Glu92 of NS1 was shown to confer high virulence to the infecting virus together with a resistance to antiviral cytokines (Solorzano *et al*., 2005; Seo *et al*., 2002). NS1 protein has two functional domains e.g. N-terminal dsRNA-binding (RD) and dimerization domain and C-terminal effector domain (ED).

The multifunctional NS1 protein is widely regarded as a virulence factor that contributes to the viral pathogenesis by modulating many viral and host cellular processes (Cheung *et al*., 2002; Falcon *et al*., 1999). However, detailed mechanism of the NS1 function as well as the influence of cellular factors interacting with NS1 is not completely understood.

The aim of the present study was the search for cellular proteins interacting with IAV (H5N1) NS1 protein using Y2H system, nucleotide sequencing, and sequence identification. Furthermore, the relevant proteins that interacted with NS1 protein were confirmed by co-localization in the infected cells using immunofluorescence (IF) assay.

Materials and Methods

Cells and virus. MDCK (Madin-Darby canine kidney) cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco). The H5N1 viral isolate was A/Thailand/1(KAN-1)/04. Virus infectivity was assessed by 50% tissue culture infectious dose titration in MDCK cells. The experiments of H5N1 virus were performed in a biosafety level-3 facility**.**

Bait plasmids construction. The NS1 gene of H5N1 IAV was amplified by RT-PCR using forward and reverse primers that contain *Eco*RI and *Bam*HI restriction sites as follows: H5N1NS1-*Eco*RIF (5'-CCGGAATTCATGGATTCCAACACTGTGTC-3') and H5N1NS1-*Bam*HIR (5'-GCGGGATCCTCAAACTTCTGACT CAAT-3'). A bait plasmid was initially constructed by inserting full-length NS1 in-frame into *Eco*RI and *Bam*HI site of pGKBT7 containing GAL4 DNA-binding domain (BD) (Clontech Laboratories). To eliminate the auto-activation of NS1 protein full range, NS1 was separated into RNA binding domain (RD) by H5N1NS1-*Eco*RIF and H5N1NS1-*Bam*HIR 341-357 primers (5'-GCGGGATCCTTA CATTATTGCCTGGTCC-3') and effector domain (ED) by primers H5N1NS1-*Eco*RIF 358-380 (5'-CCGGAATTCGATAAAGTCGT CATATTGAAAGC-3') and H5N1NS1-*Bam*HIR. Each domain was individually cloned into pGKBT7 (Fig. 1). The plasmids were subjected to DNA sequencing and individually transformed into Y187 yeast (Clontech Laboratories).

Construction of macrophages cDNA library. Peripheral blood mononuclear cells were separated by gradient centrifugation through Ficoll-Hypaque (Pharmacia). The cells were washed twice and viable cells were brought to a final concentration of 5×10^5 cells/ml in 100 mm tissue-culture plates (Corning) for 90 mins at 37°C. The monocytes were enriched by adsorption onto tissue-culture plates in RPMI-1640 (Invitrogen). Cultures was washed 10 times with medium to remove unattached lymphocytes, while adherent monocytes were cultured in RPMI-1640 medium supplemented with 10% normal human serum (Sigma) and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems). The cells were allowed to differentiate for 10 days. Differentiated macrophages were identified by a typical morphol-

ogy. Total RNA was prepared from macrophages by using TRIzol reagent (Invitrogen). From this total RNA, mRNA was purified by NucleoTrap® mRNA purification (BD Biosciences). cDNA was synthesized using the MATCHMAKER Library Construction and Screening Kit (Clontech Laboratories) and co-transformed with the plasmid pGADT7-Rec carrying GAL4 activation domain (Clontech Laboratories) into AH109 yeast. The transformation mixture was spread on 150 mm SD/-Leu agar plates for a total of 40 plates. The plates were incubated at 30°C for 5 days. After colonies appeared, the plates were chilled at 4°C for 4 hrs. Subsequently, 5 ml of a freezing medium (YPD medium with 25% glycerol) was added to each plate. The transformants were swirled and all liquid was combined in a sterile flask, and then stored at -80°C for using as prey plasmid cDNA library.

Screening of NS1-binding protein by Y2H. To screen for NS1-binding proteins from the human macrophage cDNA library, bait yeast (Y187 containing pGBKT7-NS1) was mated with prey yeast (AH109 transformed with human macrophage cDNA library). Bait yeast in log phase growth was mixed with prey yeast carrying human macrophage cDNA library and cultured in 50 ml of YPDA broth contain 50 μg/ml kanamycin with gentle swirling (30–50 rpm) for 24 hrs. After mating, the culture was centrifuged and cell pellet was resuspended in 10 ml of 0.5 x YPDA containing 50 μg/ml kanamycin and spread onto 150 mm on SD/-Trp/-Leu/-His/-Ade plates. The library screening plates were incubated at 30°C until yeast colonies appeared. After 2–3 days, the colonies were visible on plates. The plates were incubated for at least 2 weeks to allow slower growing colonies to appear. During the incubation, 2 mm yeast colonies were harvested and re-streaked on SD/-Trp, -Leu agar and incubated at 30°C for 2 days before keeping at 4°C. To select yeast clones with bait and prey fusion protein interactions, the mated diploid yeast clones were re-cultured on SD/-Trp/-Leu/-Ade/-His agar and SD/-Trp/-Leu/-Ade/-His agar plus X-α-gal at 30°C for 2 days. The positive yeast clones could grow on this SD medium plate and showed blue colonies on the SD medium plate with X-α-gal. Plasmid pGADRecT7-cDNA plasmids were isolated from the positive yeast transformants grown in a Leu-deficient medium using lithium acetate method and transformed into *Escherichia coli* (Gietz *et al*., 2002). cDNA inserts were PCR amplified and the PCR products were digested with *Alu*I restriction enzyme and analyzed by gel-electrophoresis. Plasmids giving similar restriction patterns were considered as repetitive clones.

Specificity of the interactions of positive yeast clones. Plasmid shuffling in yeast was performed to test specific interactions between human and viral protein. Plasmids from positive clones were individually transformed into Y187 yeast. The pGBKT7-NS1-RD or -ED plasmid was transformed into AH109 yeast. Y187 containing pGADRecT7/SV40 T-Ag was mated with AH109 containing pGBKT7/p53 and pGBKT7/laminC as positive and negative controls, respectively. The specific interaction assay was then performed by mating of individual transformed Y187 yeast and AH109 containing pGBKT7-NS1-RD or -ED, pGBKT7, pGBKT7/p53 or pGBKT7/laminC. Specific interaction was indicated by growth of mated yeast on SD/-Trp/-Leu/-Ade/-His dropout medium and appearance of blue colony on X-α-Gal plates. cDNA sequences from the positive clones were identified through BLAST search on the NCBI GenBank and EMBL databases.

IF assay. MDCK cells grown on 22 mm glass base dishes were infected with A/Thailand/1(KAN-1)/04. At 8 hrs post infection, the cells were fixed with 80% cold acetone in PBS for 30 mins and permeabilized with 0.1% Triton X-100 for 10 mins. NS1 was detected by incubation of cells with anti-NS1 antibody for 1 hr and subsequently with Alexa 488-labeled secondary antibody (Invitrogen) for 1 hr. NS1-binding proteins were detected by incubating cells with anti-ADAR1 or anti IL-6R antibodies for 1 hr and with Cy3-labeled secondary antibody (Invitrogen) for 1 hr. Nuclei were visualized with Hoechst 33258 dye (Invitrogen). Cellular localization of NS1 and NS1-binding protein was observed by using a laser scanning confocal Zeiss LSM 510 microscope.

Results

Bait plasmid expression in yeast

Y187 yeast transformed with pGKBT7-NS1 could grow on SD/-Ade/-His/-Leu/-Trp medium showing auto-activation by NS1. Therefore, it could not be used in the screening of NS1-binding protein in the yeast two-hybrid system. In order to eliminate this auto-activation, NS1 was firstly separated into RD and ED. Each domain was individually cloned into pGKBT7. The constructs expressed fusion protein at high level and grew on SD/-Trp medium but not on SD/-Ade/-His/-Leu/-Trp medium (Fig. 2). Thus, these transformed yeasts could be used in Y2H analysis. The fusion proteins were expressed in the bait yeast showing expected M_r of RD and ED at 33.5 K and 31.5 K, respectively.

Y2H screening of NS1-binding proteins

Positive colonies (250) from mating of the pGKBT7-NS1-RD-transformed yeast and positive colonies (60) from mating of the pGKBT7-NS1-ED-transformed yeast with the prey yeast grew on SD/-Ade/-His/-Leu/-Trp plates containing X-α-Gal. In these plates, α-galactosidase enzyme was secreted by the MEL1$^+$ positive colonies, which could catalyze a shift of X-α-Gal to the blue pigment. Positive results showed appearance of strong blue colony from X-α-Gal plate (Fig. 3a). The plasmids in the clones were screened by restriction length analysis and duplicate clones were eliminated. After checking for repetitive clones and specificity by re-transformation and mating, 7 clones remained. Of these, 4 clones interacted with H5N1 NS1-RD and 3 clones interacted with H5N1 NS1-ED.

Specificity of the interaction of positive yeast clones

To test the specificity of interaction between H5N1 NS1-RD or H5N1 NS1-ED (bait plasmids) and H5N1 NS1-binding proteins (prey plasmids), individual prey yeast colony was mated with bait yeast including yeast containing the

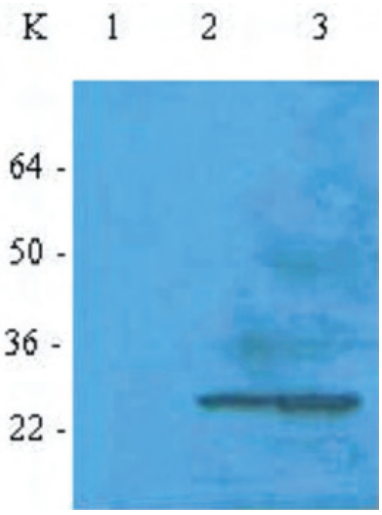

Fig. 2

Expression of NS1-RD and NS1-ED fusion proteins in bait yeast

Y187 cells, negative control (lane 1), Y187 cells containing pGBKT7H5N1-RD (lane 2), Y187 cells containing pGBKT7H5N1-ED (lane 3). M_r standards on the left.

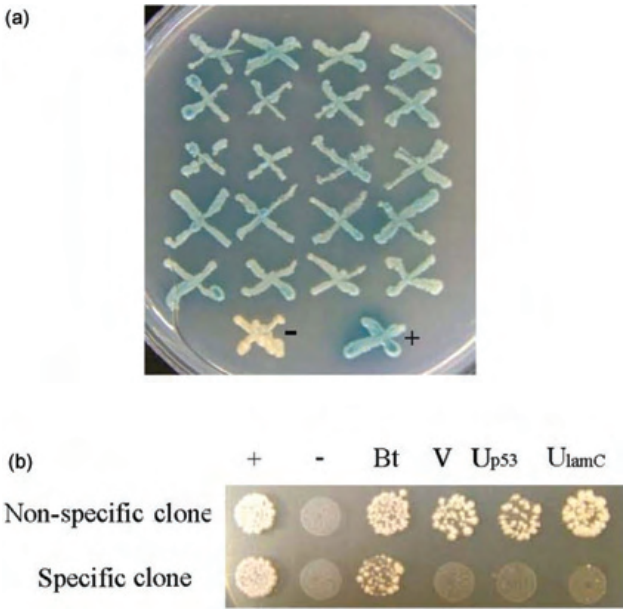

Fig. 3

Screening of yeast clones expressing NS1-binding proteins

(a) Plating of yeast AH109 transformed with pGBKT7NS1 containing macrophage cDNA library on the SD/-Trp/-Leu/-His/-Ade +X-α-Gal medium. (b) Verification of the specificity of interaction. Non-specific and specific prey yeast clone mated with a specific bait clone (Bt) and nonspecific bait yeasts (V, U_{p53}, U_{lamC}). Positive (+) and negative (-) control.

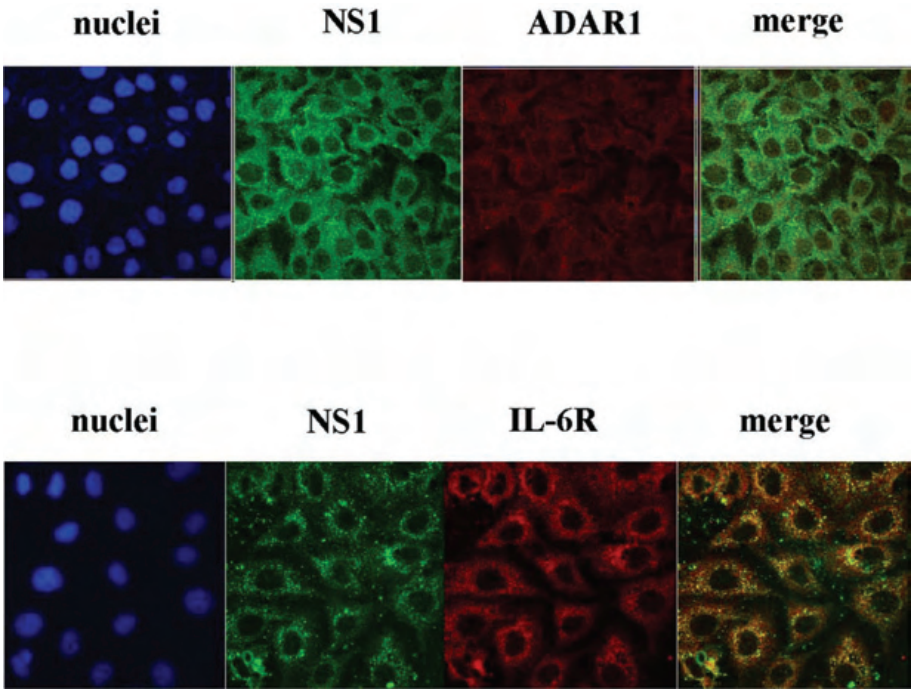

Fig. 4

Cellular co-localization of NS1 with ADAR1 (upper panel) and IL-6R (lower panel) in the infected MDCK cells

Staining for NS1 (green), ADAR1 or IL-6R (red) and cell nuclei (blue).

original bait plasmid, an empty bait vector pGBKT7 and unrelated bait pGBKT7/p53, and pGBKT7/lamin C plasmids. The last two plasmids expressed two unrelated proteins that should not interact with H5N1 NS1-binding proteins. Only bait and prey colony could grow on SD/-Trp/-Leu/-His/-Ade plate, what is considered as a specific clone. For positive control, AH109 yeast containing pGBKT7/p53 was mated with Y187 yeast containing pGADRecT7-SV40-T Ag and a negative control was set up by a mating between AH109 yeast containing pGBKT7/lamin C and Y187 yeast containing pGADRecT7-SV40-T Ag. The specific interaction between H5N1 NS1-RD and H5N1 NS1-binding protein is shown as selective growth of only mated yeast carrying the specific interacting partners (Fig. 3b). In contrast, a non-specific clone allowed the growth of yeast colonies, when mated with specific NS1 bait or non-specific baits pGBKT7, pGBKT7/p53, and pGBKT7/lamin C (Fig. 3b).

Sequencing and identification of NS1-binding proteins

To identify the NS1-binding proteins, cDNA from 7 positive clones were sequenced and subjected to BLAST search in NCBI GenBank <http://www.ncbi.nlm.nih.gov> and EMBL databases <http://www.ebi.ac.uk>. Of 4 clones interacting with NS1-RD, 2 clones contained IL-6R, 1 clone contained ADAR1 and 1 clone cathepsin B. Of 3 clones interacting with NS1-ED, 2 clones contained MHC class I HLA-B and 1 clone contained ubiquitin.

Co-localization of NS1 and NS1-binding proteins in infected MDCK cells

MDCK cells were infected with A/Thailand/1 (KAN-1)/2004 (H5N1). At 8 hrs post infection, the localization of NS1 and binding proteins ADAR1 or IL-6R were analyzed by IF using confocal microscope. The results showed co-localization of NS1 and both ADAR1 and IL-6R in the cytoplasm of infected cells (Fig. 4).

Discussion

The Y2H system provides a way of identification for the interaction between two proteins. In the present study, human macrophage proteins interacting with IAV NS1 RD and IAV NS1 ED were identified. Subsequently, a co-localization of H5N1-NS1 with ADAR1 and IL-6R were confirmed by double IF staining and confocal microscopy. These putative NS1-binding proteins may contribute to the viral pathogenesis. These proteins play an important role in the immune response, protein targeting for proteosomal degradation, and RNA editing. The interaction between NS1 and those proteins may contribute to the unusual severity of human H5N1 influenza. However, a confirmation of these interactions requires further studies.

ADAR1 is an RNA editing enzyme targeting dsRNA that plays an important role in post-transcriptional regulation of cellular RNA (Keegan *et al*., 2004; Bass *et al*., 2002). It edits a Hepatitis D virus (HDV) RNA during viral replication what is required for production of a large delta antigen. Therefore, this enzyme is essential for HDV replication. On the other hand, over-expression of ADAR1 could lead to a hyper-editing of HDV genome and inhibition of the viral replication (Jayan *et al*., 2002). Recently, ADAR1 was shown to up-regulate Human immunodeficiency virus-1 expression by a post-transcriptional mechanism (Phuphuakrat *et al*., 2008). NS1 of IAV can inhibit host cellular mRNA expression post-transcriptionally. Because both ADAR1 and NS1 are dsRNA binding proteins and are involved in the post-transcriptional regulation of RNA, interactions between these two proteins may be involved in the inhibition of host mRNA expression by NS1 (Hale *et al*., 2008; Fernandez-Sesma *et al*., 2007). In addition, ADAR1 was shown to inhibit dsRNA-activated protein kinase (PKR) activity, which is an important component of the interferon-induced antiviral state (Nie *et al*., 2007). NS1 has also been shown to counteract the antiviral function of IFN inducible PKR (Min *et al*., 2007). Recently, ADAR1 has been shown to suppress IFN signaling and to block a premature apoptosis in hematopoietic cells (Hartner *et al*., 2009; Iizasa *et al*., 2009). The ability of ADAR1 to suppress IFN signaling makes it a good partner for NS1 to mediate viral evasion of the host innate antiviral mechanisms.

IL-6 is a pleiotropic cytokine that regulates an immune reaction. IL-6R is the binding component specific to IL-6. IL-6R binds to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130. Signal transduction involves the activation of JAK (Janus kinase) tyrosine kinase family members leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. Another major signaling pathway for IL-6R is the MAPK (mitogen-activated protein kinase) cascade (Heinrich *et al*., 2003; Akira *et al*., 1997). IL-6 is the predominate inducer of the acute-phase response, an innate immune mechanism, which is triggered by the infection and inflammation. IL-6 also plays a multiple role during subsequent development of acquired immunity against incoming pathogens, stimulation of antibody production by B cells, regulation of macrophage and dendritic cell differentiation, and response of regulatory T cells to microbial infection (Rose-John *et al*., 2007). In addition, IL-6 can inhibit TNF (tumor necrosis factor) production *in vitro* and *in vivo* (Benigni *et al*., 1996). Therefore, IL-6 may play an important role in a down-regulation of innate response in transition to the specific immune response. Increased amounts of TNF were detected in the human macrophages infected with highly virulent H5N1 viruses and TNF hyperinduction is believed to be a key virulence factor

(Cheung *et al.*, 2002). Interaction of NS1 to IL-6R may interfere with signaling of IL-6R and the immune regulatory function of IL-6. This may contribute to the hyperactive responses and severe inflammation in H5N1 infection.

Cathepsin B is a lysosomal cysteine protease of the papain family. It is found in lysosomes and also associated with the plasma membrane or secreted, what indicates its role in the digestion of extracellular matrix component (Chwieralski *et al.*, 2006). Cathepsin B has been shown to be involved a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis, and cancer progression. In addition, cathepsin B has been implicated in an apoptotic pathway that involves the TNF signaling pathway (Chwieralski *et al.*, 2006; Canbay *et al.*, 2003). Finding a solution to the problem of NS1 contribution to airway inflammation and apoptosis through its interaction with cathepsins B requires further studies.

Antigen presenting cells present virus-derived antigenic peptides in association with MHC class I antigens to prime antiviral CTL (cytotoxic T-lymphocyte). Viruses interfere with this antiviral response by down-regulation of the expression of MHC class I molecule on the surface of antigen presenting cells. The virus-specific CTL recognizes virus-derived antigenic peptides in association with MHC class I antigens (Martin *et al.*, 2002; Bodmer *et al.*, 1987). A decreased expression of these antigens on the surface of virus-infected cells prevents their recognition and killing by the CTL. Interaction of NS1 protein with HLA-B may interfere with the antigen presentation by HLA-B providing an immune escape mechanism for the virus.

Ubiquitin is a small protein that is composed of 76 amino acids. Ubiquitin modification and protein degradation by the ubiquitin-proteasome pathway is a mechanism for controlling the function and availability of regulatory proteins in the cell. It also provides for many different viruses to achieve successful viral infection. Many viruses have been reported to involve different strategies to utilize the ubiquitin-proteasome pathway for their own benefits (Gao *et al.*, 2006; Corbin-Lickfett *et al.*, 2003; Dantuma *et al.*, 2003; Eom *et al.*, 2003; Kalejta *et al.*, 2003; Luo *et al.*, 2003; Andrejeva *et al.*, 2002; Strack *et al.*, 2000; Didcock *et al.*, 1999). IAV requires ubiquitin-proteasome activity at an early stage of the infection. It has been recently shown that IAV requires the proteasome for endosomal progression (Ros *et al.*, 2004; Khor *et al.*, 2003). Interaction of NS1 protein with the ubiquitin may direct ubiquitination to certain cellular or viral targets, thereby regulating cellular functions required for the effective viral replication.

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ORIGINAL ARTICLE

Induction of TNF-α in human macrophages by avian and human influenza viruses

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Abstract The highly pathogenic avian influenza virus H5N1 is known to induce high level of tumor necrosis factor α (TNF-α) from primary macrophages. However, it is still unclear whether current H5N1 strains also induce high TNF-α production, as most of the data were derived from extinct clade 0 H5N1 strain. Here, we show that current clade 1 and 2 H5N1 strains induce variable levels of TNF-α that are not necessarily higher than those induced by seasonal influenza viruses. The result suggests that hyper-induction of TNF-α in human macrophages is not always associated with a highly pathogenic phenotype. We further tested the contribution of the NS gene segment from H5N1 isolates to TNF-α induction by using reverse genetics. While NS conferred some variation in TNF-α induction when incorporated into an H1N1 virus genetic background, it did not affect TNF-α induction in an H5N1 virus genetic background, suggesting that other viral genes are involved.

Introduction

Highly pathogenic H5N1 avian influenza viruses can be transmitted directly from avian species to humans. Their virulence is extremely high, producing 100% mortality in

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some species [14]. The overall mortality in reported human cases worldwide is about 60% [1]. This virulence is due, in part, to the highly cleavable hemagglutinin, which contains polybasic amino acids at the cleavage site [4, 9, 17]. In addition, the inflammatory responses in the human lung may be excessive for the level of viral replication detected [13], suggesting that immunopathogenesis may be involved in viral pathogenesis. Strong induction of proinflammatory cytokines has been detected in patient blood and tissue samples [3, 8, 13], leading many investigators to hypothesize that hyper-induction of proinflammatory cytokines or the induction of a cytokine storm may play an important role in the pathogenesis of H5N1 avian influenza viruses [5, 10, 13, 15]. Indeed, some H5N1 viruses have been shown to induce higher tumor necrosis factor α (TNF-α) production in human macrophages in vitro than do some human influenza viruses and low-pathogenic avian influenza viruses [3, 8]. These reports, however, were mostly based on Hong Kong 1997 strains, which carry several internal genes, especially the NS gene, that are of different lineages from most current H5N1 strains. NS1 is critical to the host innate antiviral response and contributes to cytokine hyper-induction [3]. Here, we investigated whether clade 1 and clade 2 viruses recently found circulating in Southeast Asia induce a high level of TNF-α in primary human macrophages relative to currently circulating human influenza viruses, and whether the NS gene of the H5N1 virus is responsible for this property.

Materials and methods

Primary human macrophages

Peripheral blood mononuclear cells were separated from the buffy coat of donated blood units by centrifugation on a Ficoll density cushion (Amersham Biosciences) or by using a Lymphoprep Tube (Axis-shield PoC AS) (the former was used for the experiments of Fig. 1, the latter for those of Figs. 2, 3). Cells were washed twice, and viable cells were resuspended at a final concentration of 5×10^5 ml^{-1}. Monocytes were enriched by adsorption onto 24-well tissue culture plates (Corning or Falcon, Becton-Dickinson Labware) for 2 h at 37°C in RPMI 1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate, non-essential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Invitrogen). Cultures were then washed ten times with medium to remove unattached lymphocytes. Attached cells were allowed to differentiate for 10 days in the presence of normal human serum and granulocyte–macrophage colony-stimulating factor (GM-CSF). Our research protocol was approved by the Research Ethics Review Committee of the Institute of Medical Science, the University of Tokyo (approval number: 18-15-0129), and the Institutional Review Board of the Faculty of Medicine Siriraj Hospital (SiEC protocol number: 004/2551).

Virus strains

Viral isolates used in this study are shown in Table 1. The viral stocks were prepared in Madin–Darby canine kidney (MDCK) cells. Virus infectivity was assessed by tissue culture infectious dose (TCID50) titration in MDCK cells and calculated by the method of Reed and Muench [16].

Infection and cytokine production

Macrophages were infected at a multiplicity of infection (MOI) of 0.1, 1 or 2 as determined with MDCK cells. After 90 min of virus adsorption, the virus inoculum was removed and incubated in RPMI with 10% normal human serum (GIBCO BRL or heat-inactivated autologous serum). At specific time points after infection, samples of culture supernatant were collected for TNF-α analysis. Concentrations of TNF-α were determined by using a TNF-α enzyme-linked immunosorbent assay screening kit (Pierce).

Detection of NP by using ELISA

Infected cells were washed and permeated four times with washing buffer (PBS pH 7.2 + 0.05% Tween-20) or with 70% acetone, and cellular endogenous peroxidase was then blocked with 500 μl well^{-1} of freshly prepared 3% H_2O_2 for 30 min at room temperature. After the washing step, 300 μl of mouse monoclonal antibody to influenza A nucleoprotein (Chemicon International Inc., California, USA or 2S-347/3) was added at a dilution of 1:1,000 in blocking buffer (PBS pH 7.2 with 1% BSA and 0.1% Tween-20). The plate was then incubated at 37°C for 1 h and washed four times with washing buffer. Goat anti-mouse antibody conjugated to horseradish peroxidase (Southern Biotech Associates Inc., Birmingham, USA) or anti-mouse IgG horseradish-peroxidase-linked whole antibody from sheep (GE Healthcare) was added at a dilution of 1:2,000 in blocking buffer. After incubation at 37°C for 1 h, the plate was washed four times with washing buffer. Then, 300 μl of TMB peroxidase substrate system (KPL, Gaithersburg, USA or eBioscience) was added and incubated at room temperature in the dark for 10 min. Finally, the reaction was stopped with 500 μl of 1 M H_2SO_4 or stop solution (Thermo Scientific), and optical densities (ODs) were measured at 450/630 nm.

Plaque assay

MDCK cells were seeded in 12-well plates 1 day before virus inoculation. Confluent MDCK cells were washed

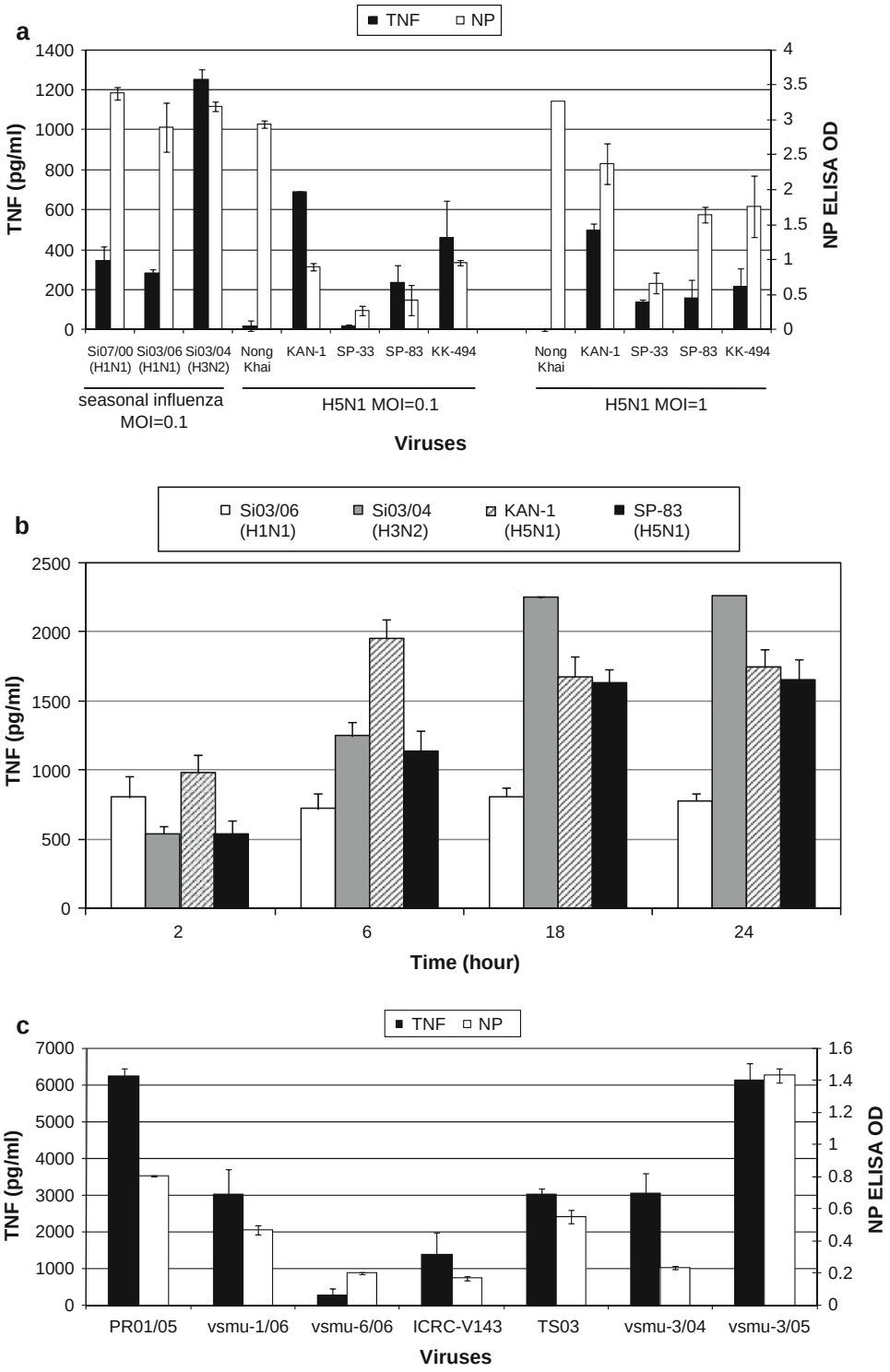

Fig. 1 TNF-α production by primary human macrophages after infection with H5N1, H3N2, and H1N1 human influenza virus isolates. The TNF-α levels in the culture supernatant at 24 h postinfection are shown by *solid bars*. The levels of infection of macrophages by these viruses were monitored by measuring the amounts of intracellular NP by ELISA, as shown by *open bars*. The data shown are representative of three independent experiments performed in duplicate (**a**). A time course experiment using selected viruses at an MOI of 0.1 confirmed the observed TNF-α induction pattern at 24 h. The data were derived from an experiment performed in triplicate using one donor (**b**). A similar experiment using non-human H5N1 isolates showed variable levels of infectivity and TNF-α induction. The data shown are representative of three independent experiments performed in duplicate (**c**)

once with serum-free medium and then inoculated with 100 µl of a tenfold dilution of virus. Cells were incubated in a 5% CO_2 incubator with shaking every 10 min for 1 h. The inoculums were removed and overlaid with MEM (Minimum Essential Medium) (Gibco/BRL, Grand Island, NY, USA) containing 1% low-melting-point agarose. After being incubated for 48 h, the cells were fixed for 1 h with 10% formalin, washed with tap water and then stained with 0.5% crystal violet.

Generation of recombinant viruses

Recombinant viruses containing the NS gene of A/chicken/Bangkok/vsmu-6/06 (vsmu-6/06) (low TNF-inducer) or

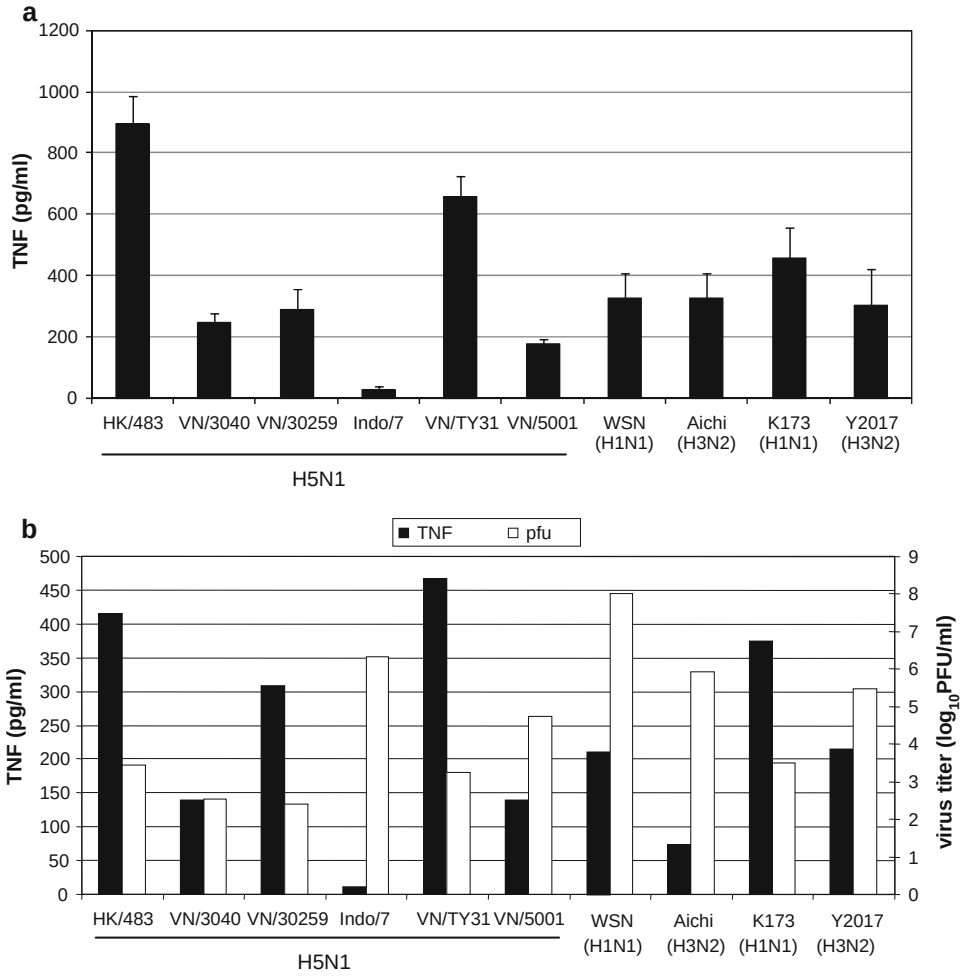

Fig. 2 TNF-α induction in primary human macrophages by another set of influenza viruses, including H5N1 avian influenza viruses of clade 0, 1 and 2, as well as H1N1 and H3N2 human influenza viruses. The TNF-α levels in the culture supernatant at 24 h postinfection are shown by *solid bars*. The data were derived from three independent experiments performed in duplicate using three donors (**a**). In one experiment, the levels of viral replication were measured by virus titers in the culture supernatant, as shown by *open bars* (**b**)

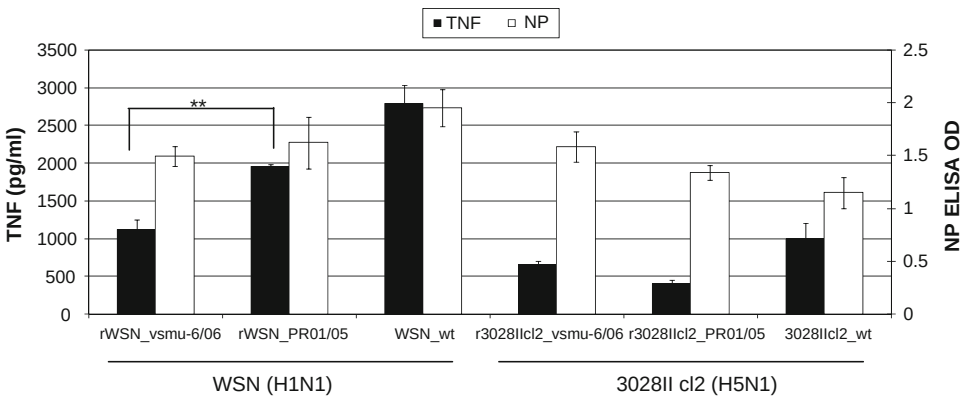

Fig. 3 TNF-α production by primary human macrophages upon infection with recombinant viruses. The viruses examined possessed genes from WSN (H1N1) or 3028II cl2 (H5N1), with the exception of NS, which came from either a low TNF-α inducer (vsmu-6/06) or a high TNF-α inducer (PR01/05). Wild-type WSN and 3028II cl2 were also assessed. Cells were infected at an MOI of 2, and the levels of TNF-α (*solid bars*) and NP (*open bars*) at 18 h postinfection were determined. The data shown are representative of three independent experiments performed in duplicate using four different cell donors. The difference between TNF-α levels induced by WSN viruses carrying NS from vsmu-6/06 and PR01/05, marked by *asterisks*, is statistically significant (*t* test, $P < 0.01$)

Table 1 Influenza A viruses used in this study

Name of virus	Abbreviation	Virus clade
H5N1 influenza viruses		
A/Hong Kong/483/97	HK/483	0
A/Thiland/1(KAN-1)/04	KAN-1	1
A/Thailand/2(SP-33)/04	SP-33	1
A/Thiland/5(KK-494)/04	KK-494	1
A/Thailand/SP-83/04	SP-83	1
A/Vietnam/UT3040/04	VN/3040	1
A/Vietnam/UT30259/04	VN/30259	1
A/Indonesia/UT3006/05	Indo/7	2.1
A/Laos/Nong Khai 1/07	Nong Khai	2.3.4
H5N1 influenza viruses from animals		
A/great barbet/Thailand/PR01/05	PR01/05	1
A/chicken/Bangkok/vsmu-1/06	vsmu-1/06	1
A/chicken/Bangkok/vsmu-6/06	vsmu-6/06	1
A/chicken/Thailand/ICRC-V143/07	ICRC-V143	1
A/chicken/Thailand/TS03/06	TS03	1
A/chicken/Bangkok/vsmu-3/04	vsmu-3/04	1
A/green peafowl/Chonburi/vsmu-3/05	vsmu-3/05	1
A/chicken/Vietnam/TY31/05	VN/TY31	1
A/duck/Vietnam/5001/05	VN/5001	1
H1N1 influenza viruses		
A/Thailand/Siriraj 07/00	Si07/00	
A/Thailand/Siriraj 03/06	Si03/06	
A/Kawasaki/173/01	K173	
A/WSN/33	WSN	
H3N2 influenza viruses		
A/Thailand/Siriraj 03/04	Si03/04	
A/Yokohama/2017/03	Y2017	
A/Aichi/2/68	Aichi	

A/great barbet/Thailand/vsmu-2/05 (PR01/05) (high TNF-inducer) with either an A/WSN/33 (H1N1) or an H5N1 A/Vietnam/3028II/03 (clone 2; H5N1) background were generated by using reverse genetics as previously described [12].

Results

First, we tested a number of influenza viruses that were isolated from humans in Thailand for their ability to induce TNF-α in human macrophages. These isolates comprised four clade 1 viruses from the central region of Thailand and one clade 2.3.4 virus from Laos. The H5N1 viruses were isolated from fatal human cases, whereas the seasonal influenza H1N1 and H3N2 isolates were from patients who experienced a normal influenza-like illness without any unusual severity. We observed a wide variation in the levels of TNF-α induced by the various avian and human influenza virus strains. There was a substantial variation among donors in the levels of TNF-α production, but the patterns of difference among isolates were similar among cells from different donors (data not shown). In contrast to previously published data showing TNF-α hyper-induction by the Hong Kong 1997 H5N1 strains, we did not observe any significant difference in the levels of TNF-α induced by the H5N1 and the human H1N1/H3N2 viruses (Fig. 1a). The average level of TNF-α induced by the H5N1 viruses did not differ significantly from that induced by the seasonal influenza viruses (t test, $P = 0.197$). To control for infection efficiency, we performed an ELISA to quantify intracellular viral antigen. ELISA ODs of seasonal influenza-infected cells were higher than those of H5N1-infected cells, indicating that the seasonal influenza strains were more successful in infecting human macrophages. Moreover, at a tenfold higher MOI and comparable ELISA ODs, the TNF-α levels from macrophages infected with H5N1 avian influenza virus were still not higher than those infected with seasonal influenza virus (Fig. 1a). We also performed a time course experiment using some of the viral strains and showed that the TNF-α induction patterns were consistent at all time points and did not show higher TNF-α induction by H5N1 viruses (Fig. 1b). We further tested a number of H5N1 animal isolates from Thailand. There was also a wide variation in the levels of TNF-α induction by these viruses (Fig. 1c). ELISA ODs of infected cells suggested that variable efficiency of infection may have contributed to these levels of TNF-α induction. Nevertheless, some viruses with comparable infectivity, as indicated by NP expression, still induced different levels of TNF-α; for example, A/chicken/Thailand/ICRC-V143/07 and A/chicken/Bangkok/vsmu-3/04 induced much lower levels of TNF-α than did A/chicken/Bangkok/vsmu-6/06.

To validate our finding of this strain variability in TNF-α induction in human macrophages, we tested another set of H5N1 isolates. This set of viruses comprised nine H5N1 avian influenza viruses: one clade 0 Hong Kong 1997 isolate, two clade 1 Vietnam viruses from humans, two clade 1 Vietnam poultry isolates, one clade 2.1 human isolate from Indonesia, and four human influenza viruses. As observed in our previous experiment, the levels of TNF-α induced by the H5N1 isolates were highly variable (Fig. 2). While the clade 0 Hong Kong 1997 virus and some H5N1 viruses induced high levels of TNF-α compared to those induced by human influenza viruses, other clade 1 and clade 2 H5N1 viruses did not induce high levels and, in some cases, induced much lower levels of TNF-α than did the human influenza viruses. In these experiments, the infection levels were determined by virus output, as measured by plaque-forming units (pfus), and were found to be highly variable. However, the extent of viral replication and TNF-α induction were not correlated.

Because the NS gene segment has previously been shown to contribute to the induction of high levels of TNF-α from human macrophages, we generated reassortant viruses carrying NS genes from H5N1 isolates that are known to induce either high or low levels of TNF-α. The role of NS in TNF-α induction was clearly observed in reassortant viruses with an H1N1 human influenza virus background. A reassortant virus possessing A/WSN/33 genes and its NS gene from either a virus that induces low level TNF-α (vsmu-6/06) or a virus that induces high level TNF-α (PR01/05) produced TNF-α at low and high levels, respectively (Fig. 3). In contrast, reassortant viruses possessing genes from an H5N1 virus (A/Vietnam/3028II/03) and their NS gene from either vsmu-6/06 or PR01/05 induced comparable levels of TNF-α (Fig. 3), indicating that the effect of the NS gene on TNF-α induction is dependent on other viral genes.

Discussion

In vivo data suggest that increased proinflammatory cytokine production may play a role in the pathogenesis of H5N1 viruses [5]. The hyper-induction of TNF-α by the 1997 strains of H5N1 avian influenza viruses supported this immunopathogenesis hypothesis [3, 7]. However, despite their highly virulent phenotype, the clade 1 H5N1 viruses did not induce higher levels of TNF-α than H1N1 or H3N2 human influenza viruses. This indicates that the hyper-induction of TNF-α in primary macrophage may not correlate with viral virulence. Although we did not directly test the virulence of the viruses, all of our H5N1 viruses were obtained from humans or animals with severe disease. On the other hand, the H1N1 and H3N2 viruses were isolated from patients who experienced mild infections. This difference in disease severity despite similar levels of TNF-α induction indicates that the level of TNF-α induction, as measured in this system, is not a good marker for predicting virulence. Some clade 1 H5N1 viruses from Vietnam have been shown to induce high levels of proinflammatory cytokines from primary bronchial epithelial cells and alveolar cells [2]. It is not clear whether this finding was specific to certain viral isolates or whether the cytokine hyper-induction differed between macrophages and respiratory epithelial cells.

NS1 is known to play a role in the cytokine hyper-induction phenotype [3]. Recent H5N1 viruses have acquired different NS gene segments and therefore differ in this regard from Hong Kong 1997 viruses [11]. It is unlikely that all of the NS1 genes carry similar determinants for cytokine hyper-induction [11]. Cytokine induction is more likely to be variable and to, at best, represent one of many virulence factors that contribute to viral pathogenesis. Our results suggest that NS1 may only partially contribute to the level of cytokine induction and that the effect of NS1 may depend on the viral genetic background. The viral determinants for cytokine hyper-induction are therefore multi-factorial, and the relative contributions of each factor may be variable.

The H5N1 avian influenza viruses showed variable infectivity in human macrophages. This cellular tropism may contribute to viral pathogenesis in humans. Findings from autopsy cases have shown that alveolar macrophages, as well as other macrophages such as microglia, Kuffer's cells, and Hofbauer cells, are important targets of H5N1 avian influenza virus in humans [6]. Variable infectivity together with variable cytokine induction may contribute to the severity and level of the inflammatory response. This, in turn, may involve the interaction of several viral genes. Further studies are required to determine the precise role of infection in macrophages and of cytokine induction in H5N1 avian influenza pathogenesis.

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PLoS one

Enhanced Susceptibility of Nasal Polyp Tissues to Avian and Human Influenza Viruses

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Abstract

Background: Influenza viruses bind and infect respiratory epithelial cells through sialic acid on cell surface. Differential preference to sialic acid types contributes to host- and tissue-tropism of avian and seasonal influenza viruses. Although the highly pathogenic avian influenza virus H5N1 can infect and cause severe diseases in humans, it is not efficient in infecting human upper respiratory tract. This is because of the scarcity of its receptor, α2,3-linked sialic acid, in human upper airway. Expression of sialic acid can be influenced by various factors including inflammatory process. Allergic rhinitis and nasal polyp are common inflammatory conditions of nasal mucosa and may affect expression of the sialic acid and susceptibility to influenza infection.

Methodology/Principal Finding: To test this hypothesis, we detected α2,3- and α2,6-linked sialic acid in human nasal polyp and normal nasal mucosal tissues by lectin staining and infected explants of those tissues with avian influenza viruses H5N1 and seasonal influenza viruses. We show here that mucosal surface of nasal polyp expressed higher level of α2,3- and α2,6-linked sialic acid than normal nasal mucosa. Accordingly, both H5N1 avian influenza viruses and seasonal influenza viruses replicated more efficiently in nasal polyp tissues explants.

Conclusions/Significance: Our data suggest a role of nasal inflammatory conditions in susceptibility to influenza infection, especially by avian influenza viruses, which is generally inefficient in infecting human upper airway. The increased receptor expression may contribute to increased susceptibility in some individuals. This may contribute to the gradual adaptation of the virus to human population.

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Introduction

The viral surface protein, hemagglutinin, of influenza viruses can bind to various types of sialic acid molecules on cell surface glycans [1]. The sialic acid serves as main receptor for influenza virus binding and entry into target cells. Preference to the two major receptor types, α2,3- and α2,6-linked sialic acid, is a pivotal difference between avian and human influenza viruses [2]. The main target cell of H5N1 avian influenza viruses (AIVs) in humans is type II alveolar epithelial cells [3], which express the α2,3-linked sialic acid abundantly [4]. In contrast to alveoli, epithelia of human upper airway express mainly α2,6-linked sialic acid and lack α2,3-linked sialic acid [4]. Using α2,3-linked sialic acid, AIVs including the highly pathogenic H5N1 viruses, do not infect human upper airway efficiently. However, in vitro explants of tissues from human nasopharynx and tonsil have been shown to be susceptible to infection by H5N1 AIVs [5]. It is not known how H5N1 AIV establishes infection in humans. Possibilities are direct invasion of lung via aerosols and minimal infection in upper airway with spreading into lung via minor aspiration. The presence of virus in nasopharyngeal aspirates and throat swabs suggest that infection of upper airway exists and may precede lung infection.

Expression of sialic acid on cell surface can be affected by multiple factors, including cellular differentiation, oncogenesis, and inflammation [6,7]. Cell surface sialic acid was shown to affect histamine release in allergic conditions [8]. Sialic acid content in mucin produced by nasal mucosa can be altered by allergic reaction [9]. Nasal polyp is a common condition caused by chronic allergic or inflammatory process. We asked whether mucosal surface of nasal polyps contained an altered level of sialic acid. Because availability of suitable receptor can determine efficiency of infection, altered levels of cell surface sialic acid may affect the susceptibility to influenza viruses, especially for α2,3-linked sialic acid, which is scarce in upper airway and if upregulated may enhance susceptibility to H5N1 AIV.

Materials and Methods

Nasal polyp and mucosal tissues

Six nasal polyposis patients schedule for turbinate reduction procedures (turbinoplasties) were recruited for the study. All

polyposis patients had the skin prick test positive for the common allergens in Thailand. For the comparison group, four patients with the diagnosis of inferior turbinate hypertrophy and scheduled for partial inferior turbinectomies were recruited. Informed consents were signed by the patients and the study was approved by the Institutional Review Board of Faculty of Medicine Siriraj Hospital. During the surgeries (polypectomies or turbinoplasties), small pieces of tissues (polyps vs. mucosa) were cut and immediately sent to the laboratory.

Tissue culture and viral infection

The tissues were extensively washed and immediately placed into culture medium (F-12K nutrient mixture with L-glutamine, and antibiotics) (Gibco BRL, USA) in 24-well tissue culture plates. The tissues were infected with 1×10^6 tissue culture infectious doses 50% ($TCID_{50}$) of influenza A viruses of subtypes H5N1 [A/Thailand/3 (SP-83)/04] or H1N1 [A/Thailand/Siriraj-3/06 (H1N1)] within three hours after collection. After two hours infection the unattached virus was removed by washing twice with PBS. The tissues were incubated at 37°C in 5% CO_2 incubator for 0, 20, 24 and 48 hours before the supernatants were collected for virus titrating by plaque assay. The tissues were then fixed in 10% neutral buffered formalin and processed for histological sections.

Lectin staining

Tissue section were deparaffinized with xylene for 5 minutes then sequentially hydrated with 100, 95 and 80% alcohol for 5 minutes at each step. The tissues were then blocked for non-specific binding with 3% bovine serum albumin (Sigma, USA) in phosphate buffer saline (PBS) for 1 hour. After discarding blocking solution, tissues were incubated with 1 µg of FITC-conjugated Maackia amurensis I lectin (MAA I) or Sambucus nigra lectin SNA (Vector Laboratories, USA) in blocking solution for 1 hour at room temperature, then washed twice with PBS and finally counterstained with Evan's blue for 10 minutes. The slides were mounted and visualized under fluorescence microscopy. In some experiments, fresh tissue were pre-digested with neuraminidase from *Clostridium perfringens* (Sigma, USA) at a concentration of 1U/ml in PBS for 1 hour at 37°C before performing paraffin section and the lectin staining in order to confirm the specificity. Six lectin-stained sections of nasal polyps and nasal turbinates from six patients were counted for epithelial cells with positive and negative staining in three fields of the slide to calculate average percentages of sialic acid-positive cells. About 200 cells were counted in each slide.

RNA extraction & Real time RT-PCR

Fresh tissues were cut into small pieces with a sterile surgical blade. Total RNA was isolated from small quantities of tissue (1 to 10 mg) using 800 µl TRIZOL reagent (Invitrogen, USA) and then purified using Qiagen RNAeasy kit according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega, USA) and a random hexamer. The amplification was then performed in a Sybr green dye detection format (LightCycler; Roche, USA). The amplification reactions contained 1×LightCycler Fast Start DNA Master Sybr Green dye I (LightCycler; Roche, USA) and 0.4 mM of each forward and reverse primer. Melting-curve analyses were performed from 65°C to 95°C. The following primers were used to detect the expression of specific genes: ST3GAL1 forward and reverse [10]; ST3GAL4 forward and reverse [11]; E14134 and E14135 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA [12]. Reactions were performed in triplicates. All quantitations (threshold cycle [C_T] values) were normalized to that of GAPDH to generate ΔC_T, and the difference between the ΔC_T value of the nasal polyp tissue and that of the reference (nasal mucosa tissue) was calculated as $\Delta\Delta C_T$. The relative level of gene expression was expressed as $2^{-\Delta\Delta CT}$.

Detection of viral infection in vitro

Paraffin-embedded, infected and non-infected control tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 15 minutes at room temperature. Tissue sections were treated with pre-warmed (37°C) 200 µg/mL proteinase K in TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) for 15 minutes at 37°C. A biotinylated anti-sense probe for in situ hybridization was prepared by in vitro transcription from a full-length viral nucleoprotein clone. Tissue sections and probes were heated in hybridization buffer (50% formamide, 3X SSC, 1X Denhardt's solution, 200 µg/mL Yeast tRNA, 50 mM sodium phosphate pH 7.4, and 1 mg/mL of dextran sulfate in diethyl pyrocarbonate-treated water) for 10 minutes at 90°C, placed on ice for 10 minutes, then mixed and left to hybridize overnight at 37°C in humidified chamber. The hybridization signal was developed with conjugated Streptavidin-Horseradish Peroxidase (PIERCE, USA) and diaminobenzidine. The sections were then counterstained with hematoxylin.

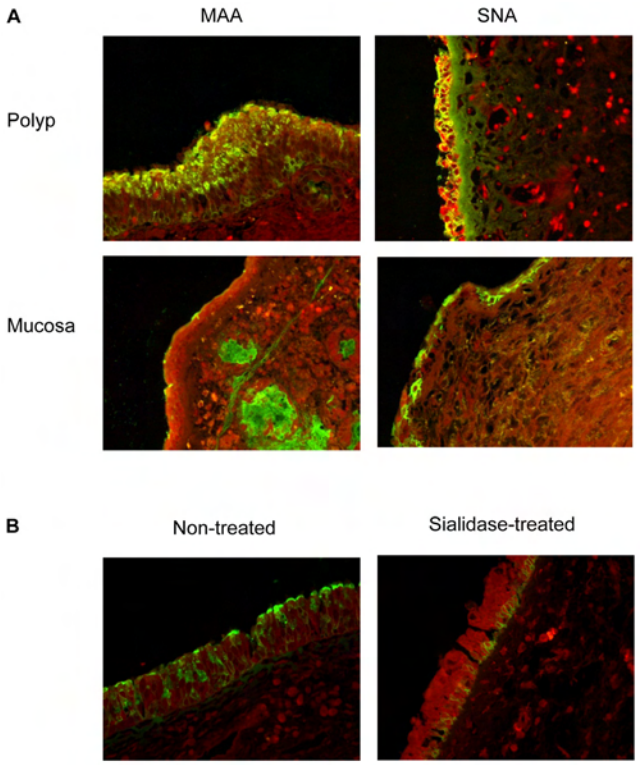

Figure 1. Representative micrographs of nasal polyp and nasal turbinate mucosa showing distribution of α2,3- and α2,6-sialic acid. Tissue sections were stained with FITC conjugated lectin MAA I (left panel) or SNA (right panel), specific toward α2,3- or α2,6-linked sialic acid, respectively (a). To confirm the specificity and the presence of α2,3-linked sialic acid on nasal polyp, tissues were digested with 1U/ml of sialidase before MAA I staining. The sialidase-treated tissue (right) lost the staining signal on the apical surface, while non-treated section was positive (left) (b).
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Statistical analysis

Correlations between percentages of lectin-stained cells and viral titers produced the same tissues were determined using Pearson correlation analysis and linear regression analysis. All statistical computations were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL).

Results

Expression of sialic acid on nasal polyps

Initially four nasal polyp and four nasal turbinate mucosa samples were examined and tissue sections were stained with the lectins. Histological examination showed normal intact nasal mucosa in the turbinate specimens and submucosal infiltration of eosinophils, lymphocytes, and plasma cells in the polyp specimens typical of allergic reaction. The SNA and MAA I staining in polyp tissues were more intense and covered more cells than that observed in normal mucosal tissues. Both SNA and MAA I lectins stained mucosal surface of polyps, whereas normal nasal mucosa showed positive staining with SNA on the mucosal surface and positive staining with MAA I in submucosal glands (Figure 1a). In order to provide a quantitative measurement, we counted cells with positive staining on their apical surface. While 79.93±10.06% and 71.63±22.21% of epithelial cells on nasal polyps expressed α2,3- and α2,6-linked sialic acid, respectively, only 17.94±16.83% and 35.64±12.51% of epithelial cells on normal nasal mucosa expressed the receptors. These differences are statistically significant at $p<0.01$ for α2,3-linked sialic acid and $p<0.05$ for α2,6-linked sialic acid, by t-test. This indicates that the α2,3-linked sialic acid, which is the receptor for AIV, is present on mucosal surface of nasal polyps but not on normal nasal mucosa. The lectin staining pattern of normal nasal mucosa is in agreement with what has been previously reported [4]. In order to confirm the expression of α2,3-linked sialic acid on the nasal polyps, we digested fresh tissue of nasal polyp by 1U/ml sialidase before performing the lectin staining. The sialidase digestion eliminated the MAA I staining signal from the apical surface of the epithelial cells confirming the specificity. Staining in basal cells was not eliminated probably because they were not accessible to the

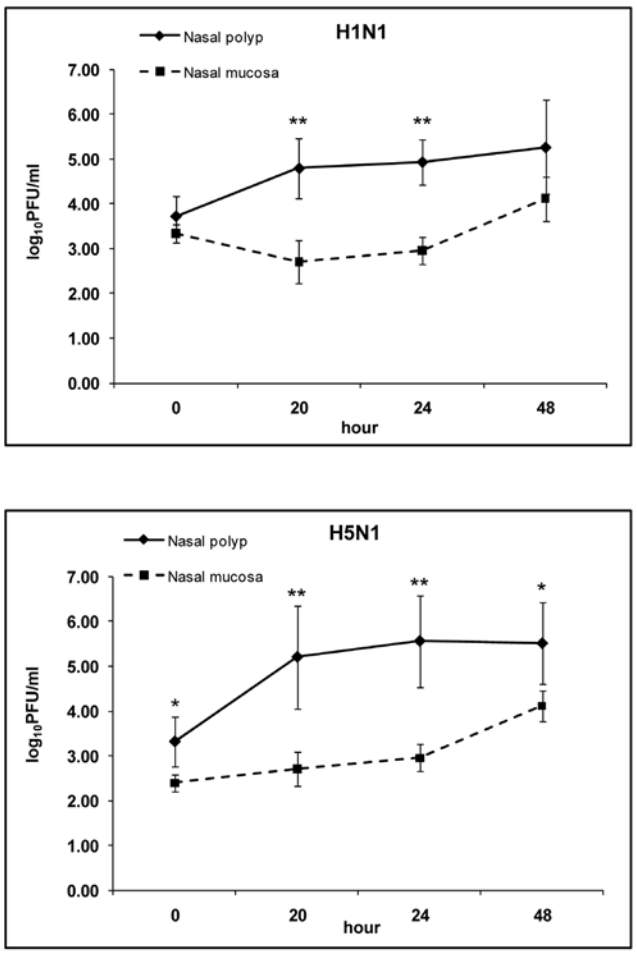

Figure 2. Infection of human and avian influenza viruses in nasal polyp and nasal turbinate mucosal explants. Two pieces of tissue of about 3–4 mm in diameter from each specimen were cultured in a 24-well tissue culture plate. Each tissue explant was infected with 1×10^6 TCID50 of H5N1 AIV [A/Thailand/3 (SP-83)/04] or seasonal influenza virus H1N1 [A/Thailand/Siriraj-3/06]. After two hours infection the unattached virus was removed by washing twice with PBS. At 0, 20, 24 and 48 hr post-infection, the culture supernatants were collected for virus titrating by plaque assay. The data were derived from two nasal polyps and two nasal mucosa. One or two asterisks indicate a statistical significance at a *P* value of <0.05 or <0.01, respectively, as determined by a t test for a comparison between viral titers from nasal polyp and nasal mucosa at the same time point.
doi:10.1371/journal.pone.0012973.g002

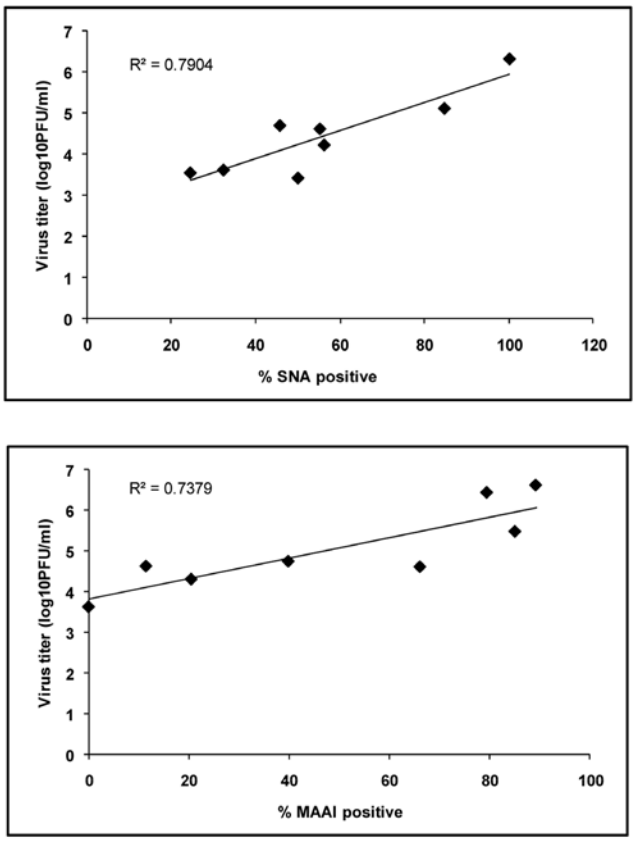

Figure 3. Correlation between the percentages of lectin-positive cells and the viral titers. Dot plots of percentages of lectin-positive cells versus maximum viral titers produced from the same tissue samples show linear correlation with Pearson correlation coefficient of 0.889 for SNA ($p=0.003$) and 0.859 for MAA I ($p=0.006$). The data were derived from the same experiments shown in Figure 1, 2 and 4.
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enzyme as the digestion was done on an intact piece of tissue (Figure 1b).

Infection of tissue explants by influenza viruses

To test whether the increased sialic acid expression on nasal polyps would result in enhanced susceptibility to influenza infection, tissue explants were infected with influenza viruses. Tissue explants were derived from 2 nasal polyps and 2 normal nasal mucosal tissue samples. Both the seasonal influenza virus [A/Thailand/Siriraj-3/06 (H1N1)] and the highly pathogenic AIV [A/Thailand/3 (SP-83)/04 (H5N1)] could infect normal nasal mucosa explants as indicated by the increase of viral titer in the culture supernatant. The titers of H5N1 AIV were comparable to or even somewhat higher than those of seasonal H1N1. This may reflect the higher replication efficiency of H5N1 as seen in various cell lines (data not shown). Interestingly, both the seasonal influenza and the H5N1 AIV replicated to higher titers in nasal polyp explants (Figure 2). The maximum viral titers showed

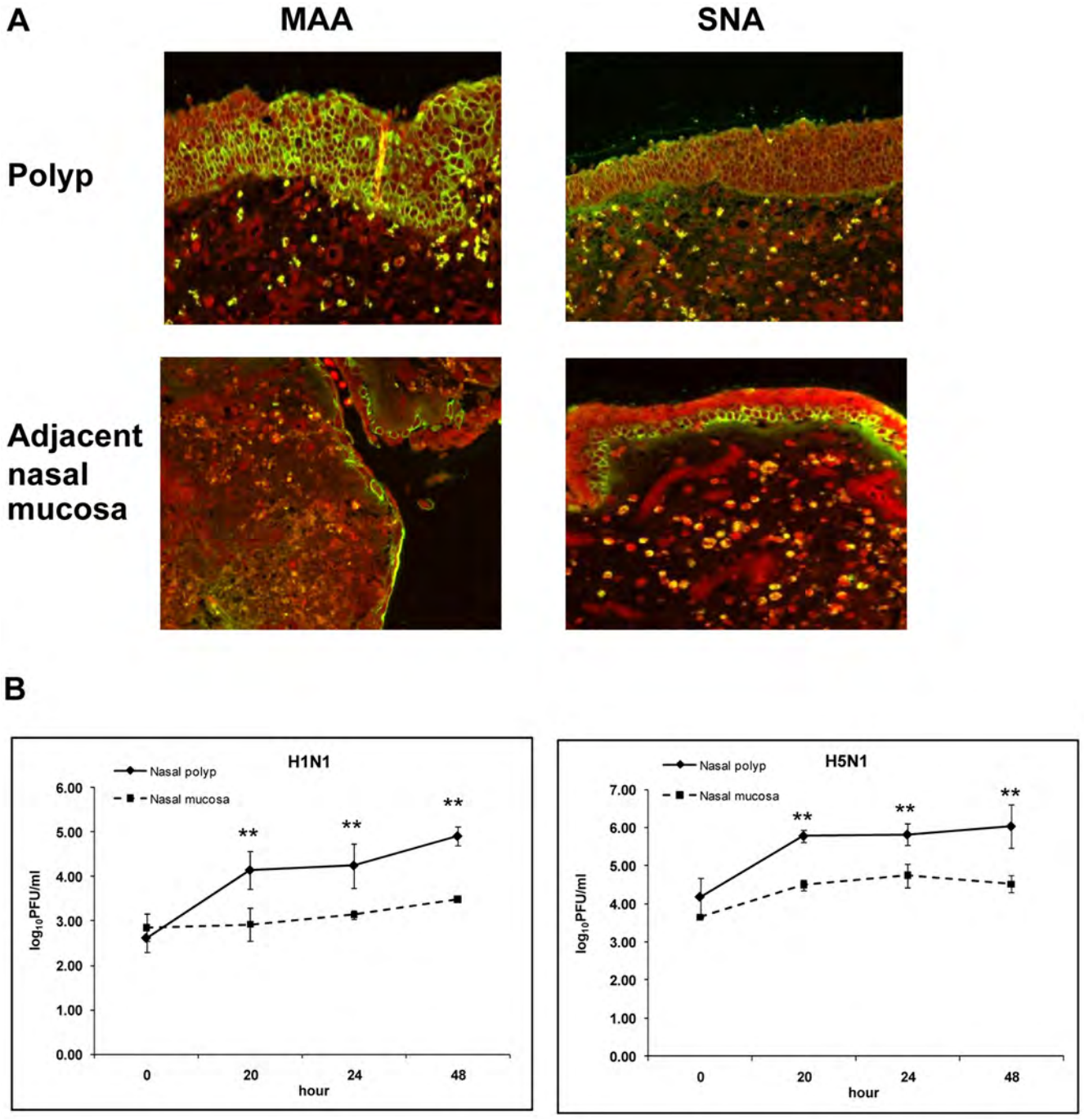

Figure 4. Distribution of receptor and infection of nasal polyps and adjacent normal mucosa. Distribution of sialic acid receptors (a) and outputs of viral infection (b) of tissue samples from nasal polyps and adjacent normal mucosa from the same patients. The data were derived from experiments using nasal polyps and adjacent normal nasal mucosal tissue samples from two patients.
doi:10.1371/journal.pone.0012973.g004

correlation with the percentages of sialic acid-positive cells on the same tissue samples (Figure 3).

To ensure that the difference between nasal polyps and normal nasal mucosa was not because of normal variation among individuals, we repeated the experiments using tissue samples from nasal polyps and adjacent normal mucosa from the same patients, and similar results were observed (Figure 4). This indicated that nasal polyps were indeed more efficiently infected by seasonal and avian influenza viruses.

We performed in situ hybridization on sections of infected and non-infected polyp tissues. Positive hybridization signal was observed on the mucosal surface of infected polyp, while the non-infected polyp was negative (Figure 5). This indicates that the infection occurred in the epithelial cells, where the viral receptor was present, and further supports the association between the presence of viral receptor and the infection.

Expression of ST3GAL1 and ST3GAL4 mRNA

In order to explore the mechanism of sialic acid upregulation, we measured mRNA levels of ST3GAL1 and ST3GAL4, which are the major enzymes responsible for adding sialic acid to galactose through the α2,3-linkage [13]. Both of the enzymes showed higher levels of mRNA in nasal polyp (Figure 6). This suggests that up-regulation of the enzymes is responsible for the observed sialic acid up-regulation.

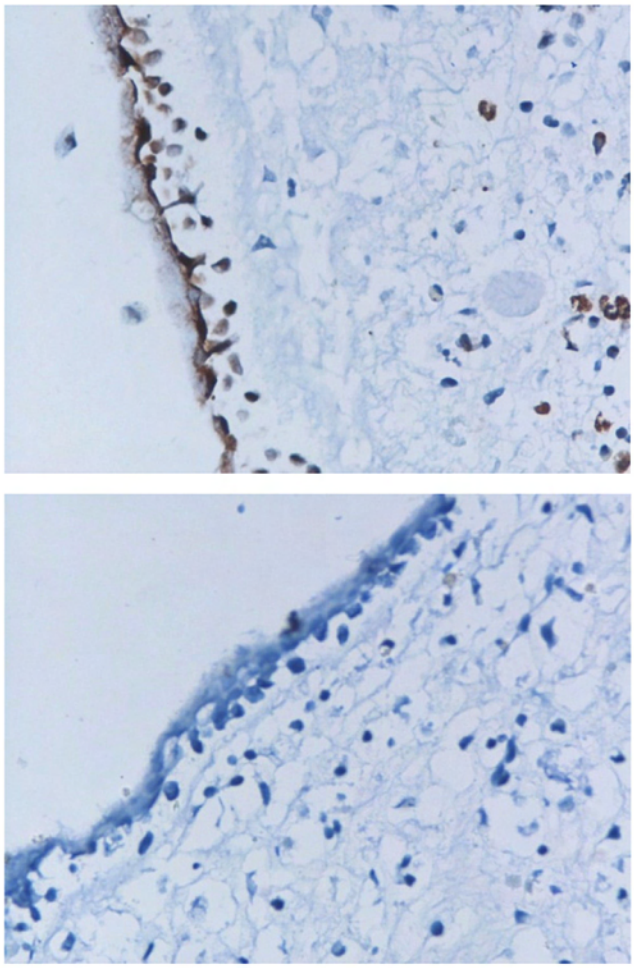

Figure 5. In situ hybridization of H5N1 AIV-infected (upper) and non-infected (lower) polyp tissues. The hybridization signal is shown in red-brown color in the epithelial cells on the mucosal surface of infected tissue.
doi:10.1371/journal.pone.0012973.g005

Discussion

Previous reports showed that nasal polyp tissue explants could be infected by influenza viruses [14,15]. The authors suggested that nasal polyp tissue can be used as an in vitro model for influenza infection. These reports are in agreement with our data showing more efficient infection in nasal polyp explants as compared to normal nasal mucosa. Furthermore, our data show a correlation between cell surface sialic acid availability and the efficiency of infection, suggesting that receptor availability may be a major determinant for efficient infection in respiratory epithelium.

In addition to sialic acid upregulation, allergic and inflammatory conditions can cause a number of changes including upregulation of various cytokines, such as IL-1, IL-4, IL-5, IL-8, and TNF-α [16]. Whether these changes contribute to the sialic acid upregulation and the increased efficiency of influenza infection is not clear. NF-κB is required for influenza virus replication [17], and it has been shown to be upregulated in nasal polyp [18]. It is possible that upregulation of NF-κB may also contribute to the enhanced influenza infection in the nasal polyp tissue.

H5N1 AIV transmission in humans is inefficient [19]. Only a small fraction of exposed individuals became infected [20]. Clusters of infected individuals are often within blood-related individuals [21], suggesting host factors contributing to the susceptibility to the infection. Variability of sialic acid expression on mucosa of upper respiratory tract is likely to be one of the factors. Here, we show that sialic acid on nasal mucosa and the efficiency of H5N1 AIV infection can be upregulated by allergic and inflammatory conditions and that the upregulated α2,3-linked sialic acid can support H5N1 AIV infection [22,23,24]. This suggests that conditions capable of upregulating α2,3-linked sialic acid in human upper airway may contribute to susceptibility to H5N1 AIV infection in exposed individuals. This variation in sialic acid expression may provide an opportunity for AIVs to infect certain individuals as entry points into human population, which

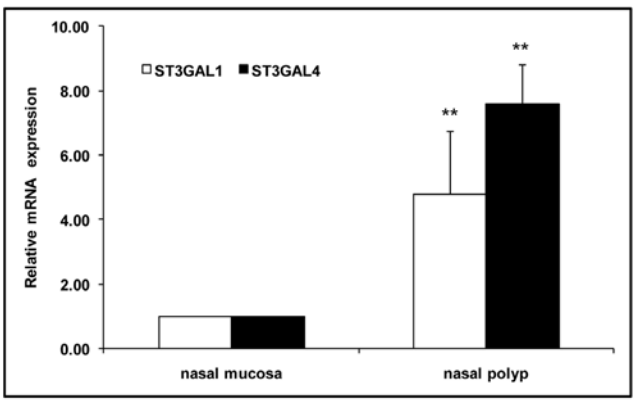

Figure 6. Expression of ST3GAL1 and ST3GAL4 mRNA measured by quantitative real time RT-PCR. The amount of total RNA was normalized using GAPDH mRNA. The data are shown as the mean ± SD from an experiment done in triplicate. The data were derived from two pieces of tissues from the same patient. The difference between mRNA expression in nasal polyp and nasal mucosa, marked by asterisks, is statistically significant (t test, $P<0.01$).
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provide a chance for further adaptation, which will enable the virus to gain full access into human population. Understanding this may be crucial for preventing emergence of a pandemic virus.

Field evidences linking nasal polyp and increased influenza susceptibility are lacking. However, allergic conditions such as asthma, which is associated with allergic nasal conditions, have been shown to be associated with upper respiratory tract infection [22,23,24]. Most investigators consider this relationship as triggering of asthmatic attack by viral infection. Nevertheless, the association exists and whether allergic persons are more susceptible to viral infection or influenza has not been fully explored. As for nasal allergy, the signs and symptoms of the allergy can be confused with those of viral infection, which further complicates the observation of association between the two conditions. Carefully designed clinical studies are needed to explore this possible relationship.

Although our data are derived from in vitro experiments, our experimental system involves only minimal in vitro handling of the tissue and is likely to resemble what could happen in vivo. Nevertheless, whether incidence of seasonal influenza and the risk of H5N1 AIV infection in exposed individuals can be indeed influenced by allergic conditions and levels of cell surface sialic acid requires further exploration and clinical investigations.

Author Contributions

Conceived and designed the experiments: OS PT PA. Performed the experiments: OS PT CB JL MU. Analyzed the data: OS PT CB JL MU PP PA. Contributed reagents/materials/analysis tools: PP. Wrote the paper: PA.

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Manuscript

Decreased expression of surfactant protein D mRNA in human lungs from fatal H5N1 avian influenza cases

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Abstract

Microarray analysis of gene expression profile of lungs from two fatal H5N1 influenza cases identified 3435 genes with higher than 2 fold changes in mRNA levels as compared to those of normal lung. 1019 genes and 2416 genes were commonly up-regulated and down-regulated, respectively. Gene ontology analysis identified several ontology terms with significant association with these genes, most of which are related to cellular metabolism and regulation of cellular process including apoptosis and chemotaxis. Pulmonary surfactant protein D (SP-D) was found to be down-regulated. Quantitative RT-PCR confirmed the levels of SP-D mRNA in the H5N1 infected lungs to be lower than those of normal lungs and lungs from patients with acute respiratory distress syndrome. SP-D plays multiple roles in respiratory innate defense against various pathogens, regulation of inflammatory responses, and maintenance of alveolar integrity. Reduction of SP-D in H5N1 influenza may play important roles in the pathogenesis of the disease.

Keywords: *Influenza; H5N1; microarray, gene expression; Pulmonary surfactant protein D; SP-D*

Introduction

Avian influenza H5N1 virus is highly unusually in its extremely high virulence with a fatality of about 60% in human and almost 100% in many avian and mammalian species [WHO, 2008]. The highly cleavable hemagglutinin precursor (HA0) enabling the virus to disseminate outside respiratory and digestive tracts independently of tryptase-like enzymes is one of the major virulence factors [Walker et al., 1992]. Nevertheless, pathogenesis of avian influenza H5N1 virus in human is far from clearly elucidated. In vitro experiments using Hong Kong 1997 isolates in primary human macrophages suggested that avian influenza H5N1 viruses might induce higher levels of proinflammatory cytokine as compared to low pathogenic avian influenza H5N1 virus or human influenza viruses [Cheung et al., 2002]. This and the observation of hemophagocytosis, a sign of excessive pro-inflammatory cytokine, in autopsy samples from the Hong Kong 1997 outbreak lead to a hypothesis of "cytokine storm" as a major course of severe inflammation and tissue destruction [Chan, 2002]. Expression microarray data from experimental infection in monkeys infected with 1918 H1N1 Spanish influenza virus [Kobasa et al., 2007] and ferrets infected with avian influenza H5N1 virus [Cameron et al., 2008] showed some up-regulation of many genes in innate immune responses including chemokines, interferon response genes, and genes in complement cascade. However, it is still unclear whether these animal data are relevant to human disease. Despite some indications of increased cytokine levels in patients' sera, the role of aberrant innate immune response in human lungs infected by avian influenza H5N1 virus has not been explored and direct evidence for inappropriate innate responses in lungs of avian influenza H5N1 virus infected patients is lacking. In order to obtain evidences for the proposed role of aberrant innate immune response in avian influenza H5N1 virus

pathogenesis in human, we analyzed expression profile of lung tissues from two fatal cases of avian influenza H5N1 virus infected patients in comparison to normal human lung using an expression microarray.

Materials and Methods

Patients and tissue samples

Patient A was a 48-year-old man who had a progressive viral pneumonia. He had fever, cough, running nose, myalgia and chest pain at the onset of illness. He developed dyspnea on day 2 of illness, and a chest radiograph showed interstitial infiltrations at right upper and left middle lung fields and a mass-like infiltration at right middle lung field. The diagnosis of avian influenza was suspected on day 4 of illness after a history of direct contact with dying chicken was revealed. Respiratory secretion was then sent to the national laboratories and confirmed to be positive for H5N1 avian influenza virus. The patient died on day 6 of illness.

Patient B was a 6-year-old boy who had a progressive viral pneumonia that led to acute respiratory distress syndrome and death 17 days after onset of illness. He was treated initially with multiple broad-spectrum antibiotics. The virological diagnosis of H5N1 infection was made on day 7 of illness. He was treated with oseltamivir from the 15th day of illness, once this antiviral became available in Thailand. He was also treated with methylprednisolone from day 15 of illness until death and with G-CSF for leukopenia from day 5 to day 10 of illness.

Virological diagnosis was done by antigen detection, viral culture, and RT-PCR on a nasopharyngeal wash specimen as described [Chokephaibulkit et al., 2005] and was confirmed by a seroconversion of neutralizing antibody against H5N1 virus. The virus was identified to be avian influenza virus (H5N1) by sequencing.

Autopsy was carried out by standard techniques using precautions to minimize risk of transmission of infection. The tissue obtained was prepared for routine histology and part was kept in -70^{o}C. The study was approved by the Siriraj Ethics Committee.

Normal lung specimens were obtained at the Department of Forensic Medicine, Faculty of Medicine Siriraj Hospital during routine autopsy examination of cases died in traffic accidents. All the normal lung specimens showed normal macro- and microscopic morphology. Archival lung specimens with acute respiratory distress syndrome (ARDS) were from a preterm male baby with hyaline membrane disease (ARDS1), a systemic lupus erythematosus (SLE) patient with cytomegalovirus pneumonitis (ARDS2), and a patient with acute myocarditis and multi-organ failure (ARDS3).

Microarray analysis

Total RNA was extracted by using Trizol from formalin-fixed paraffin-embedded (FFPE) blocks of lung tissue samples and then purified using Qiagen RNAeasy kit according to the manufacturer's instructions. As the RNA samples extracted from FFPE tissues were not optimal for the cRNA amplification method recommended by Illumina. Instead, the total RNA from FFPE tissues were used with WT-Ovation FFPE System (NuGen, San Carlos, CA) according to the manufactuere's instructions to synthesize the biotin-labeled cDNA. The Sentrix BeadChip Array for Gene Expression Human-6 V2 (Illumina, San Diego, CA) was hybridized to the biotin-labeled cDNA, with the change in the hybridization temperature from 58˚C to 48˚C to accommodate the altered hybridization kinetics of cDNA/DNA pairs relative to cRNA/DNA pairs, washed and scanned according to the manufacturer's instructions.

Raw intensity values were normalized against the normal lung dataset using quantile normalization in GeneSpring software (Agilent Technologies). Probes with low signals (<20% percentile) in all samples and control were excluded from the

analysis. Genes with $\geq$ 2-fold change in at least one sample were identified. Gene ontology (GO) analysis was performed on these gene lists using Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) to identify significantly enriched GO terms among those genes. Database and accession numbers.

Real time RT-PCR

The levels of SP-D mRNA were confirmed by real-time RT-PCR, using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics Gmbh, Mannheim, Germany) on a LightCycler 2.0 Instrument (Roche Diagnostics). The following primers were used to detect the expression of specific genes: SP-D forward [Kim et al., 2007] and reverse (CCAGTTGGCTCAGAACTCGCA); and E14134 and E14135 [Nishimori et al., 1997] for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The amplification reactions contained 1×LightCycler Fast Start DNA MasterPLUS SYBR Green I and 0.4 mM of each forward and reverse primer. Melting-curve analyses were performed from 65°C to 95°C. RNA extracted from normal lung tissue was serially diluted and used for standard curve setting of SP-D. Target gene expression levels were normalized with respect to GAPDH expression.

Immunohistochemistry

Paraffin-embedded lung sections were stained with primary antibodies against surfactant protein D. Briefly, the paraffin sections were deparaffinized with xylene, rehydrated with ethanol and pretreated in 10 mM sodium citrate buffer, pH 6.0, in a water bath at 95°C for 40 min. The sections were allowed to cool down in the buffer for 20 minutes at room temperature, washed in 10 mM Tris-HCl, pH 7.2, containing 140 mM NaCl and 7.5 mM NaN3 (TBS) and preincubated in 3% hydrogen peroxide

at room temperature for 10 minutes to block endogenous peroxidase activity. The sections were further digested with 20ug/ml Proteinase K for 5 minutes at 37°C and the reaction was stopped by placing the sections in distilled water for 5 minutes. The sections were then incubated with 2% (w/v) BSA in TBS for 30 min; washed with TBS; incubated overnight with SP-D specific primary antibody (Abcam, Cambridge) diluted 1:50 at 4°C; washed with TBS; incubated for 1 hr with biotin-labeled goat anti-mouse Ig (Dako, Carpinteria, CA) diluted 1:100 in TBS; washed with TBS; incubated for 1 hr with HRP-coupled streptavidin (Dako) diluted 1:200 in TBS without NaN3; washed with TBS and water; incubated for 5 min in DAB; counterstained with Mayer's hematoxylin; dehydrated with ethanol and acetone before being mounted. Positive signals of SPD protein were observed under light microscope.

Results

The two patients died at different stages in the course of disease. Histopathological findings reflected this difference and showed markedly different pathological features between the two cases. In the lung of patient A, who died on day 6 of illness, acute inflammatory infiltration and alveolar damage were prominent, while in the lung of patient B, who died on day 17, infiltration of inflammatory cells was less prominent and marked proliferation of type II pneumocytes was observed. This probably reflects acute destructive phase and regenerative phase of the disease in patient A and B, respectively. Despite this difference in pathological findings, overall expression profiles of the two cases are quite similar. In patient A, 1037 genes were found to be more than 2-fold up-regulated as compared to normal human lung, and 2566 genes were found to be more than 2-fold down-regulated. In patient B, 1169 genes were found to be more than 2-fold up-regulated as compared to normal human lung, and 2434 genes were found to be more than 2-fold down-regulated. Of these, 1019 genes and 2416 genes were commonly up-regulated and down-regulated, respectively, in both patients. Gene ontology (GO) analysis revealed several significantly enriched functional GO terms with up- or down-regulated genes (Fig. 1). Most of these GO terms are related to cellular metabolism and regulation of cellular processes including apoptosis and chemotaxis. Chemokines and related genes are of particular interest because their potential role in the pathogenesis. In this group of genes, we found up-regulation of CCL1, CCL7, CCR2, CXCR1, XCR1, IL-21R, and IL-18; and down-regulation of IL-8, CCL2, CCR1, CXCR4, CYR61, CKLF, CKLF2, CKLF5, CKLF6, FPR1 and SP-D. Short descriptions of these genes are shown in Table 1. The pulmonary surfactant-associated protein D (SP-D) is of particular interest for the pathogenesis of avian influenza H5N1 virus because of its

inflammatory modulating and anti-influenza activities. Moreover, the level of SP-D expression was shown to be altered most markedly in comparison to the levels of up- or down-regulation of other genes listed above. SP-D mRNA was down-regulated by 61 folds and 87 folds in the lungs of patient A and patient B, respectively. SP-D was shown to inhibit influenza infection by binding to the viral HA. In addition, another related protein, pulmonary surfactant-associated protein C (SP-C) was also down-regulated by 44 folds and 37 folds in the lungs of patient A and patient B, respectively.

In order to confirm the down-regulation of SP-D mRNA, we performed quantitative RT-PCR using SP-D-specific primers. The RT-PCR results showed that the levels of SP-D mRNA in the lungs of both H5N1 cases were lower than those of normal lungs and lung of a patient died of respiratory failure from other causes (Fig. 2). It is relevant to mention that one of the controls, ARDS2, had underlying systemic lupus erythematosus (SLE), and that patients with SLE were showed to have lower levels of circulating SP-D [Hoegh et al., 2009]. Nevertheless, the SPD mRNA levels in the H5N1-infected lungs were even lower than this control.

The SPD-mRNA data was confirmed with protein data analysis of the lung tissue using immunohistochemistry method. Immunohistochemistry results (Fig. 3) show localization of SP-D in normal human lung (3a) and lung of patient B infected with avian influenza H5N1 virus (3b). SP-D was found in pneumocyte type I cells in the normal human lung and in pneumocyte type II cells in the lung of avian influenza H5N1 infected patient. The immunohistochemistry showed higher intensity of SP-D protein staining in the normal lung in comparison to lung of patients infected with avian influenza H5N1 virus.

Discussion

In contrast to the previously published microarray data in monkeys and ferrets [Cameron et al., 2008; Kobasa et al., 2007], our data did not show any evidence of excessive innate immune response in the H5N1 infected human lungs. It is still not clear whether this was due to host species difference or a difference in disease phases. The lack of appropriate interferon response could be a reason for overwhelming viral replication. Influenza virus is known to have mechanisms for evading interferon response [Seo et al., 2002]. The possibility that avian influenza H5N1 virus replication is not blocked by interferon response in human as a viral virulence factor deserves further investigation.

The similarity in the expression profiles between the two cases despite the difference in the pathological findings and disease phases is remarkable and suggests that the expression profile was directly related to the avian influenza H5N1 virus infection and pathogenesis. Our findings showing down-regulation of many genes related to cellular metabolism are in accordance with the previously published data in ferret infected by avian influenza H5N1 virus showing down-regulation of many genes related to cell proliferation [Cameron et al., 2008]. These expression profiles may reflect the general deteriorating condition of the lungs.

Pulmonary surfactant proteins play important roles in inflammatory modulation and maintenance of alveolar integrity [Boggaram, 2003]. Pulmonary surfactant, which comprises many proteins and lipid components, reduce the alveolar surface tension and prevent collapsing of alveolar wall during expiration [Johansson and Curstedt, 1997]. The down-regulation of SP-D and SP-C could have important implication to the H5N1 viral pathogenesis in different aspects. SP-D has been shown to contain influenza inhibiting activity via its binding to the viral hemagglutinin

[Hartshorn et al., 2000]. Reduced SP-D levels in the lungs might compromise the innate anti-viral mechanism and contribute to the high level of viral replication in the lungs. It has been recently shown that H5N1 viruses were less sensitive to SP-D than recent seasonal influenza viruses and required higher concentration of SP-D for the inhibition [Hartshorn et al., 2008]. However, since the local concentration of SP-D in alveoli is not known, whether SP-D can inhibit H5N1 infection in the lung is not clear. SP-D is a lectin that can bind to carbohydrate moieties of various microorganisms. Reduction of SP-D in H5N1 infection may therefore enhance superinfection by other pathogens [Hartshorn et al., 1994]. Moreover, SP-D has been shown to modulate inflammation [Haczku, 2008]. Genetic disorder of SP-C is associated with an inflammatory lung disease [Cameron et al., 2005; Hamvas et al., 2007; Hamvas et al., 2004; Nogee et al., 2001]. Therefore, a deficiency in SP-D and SP-C may result in an uncontrolled inflammatory process and contribute to the severe inflammation found in H5N1 infected lungs. Therapeutic approaches using surfactant components have been proposed and studied in animal models and patients with acute respiratory distress syndrome. However, effectiveness of this therapeutic approach is still controversial [Anzueto et al., 1996; Spragg et al., 2004; Sun et al., 2009]. This suggests that in some cases of acute respiratory distress syndrome, surfactant deficiency may not play the leading role in the pathogenesis. In contrast, patients with acute respiratory distress syndrome from avian influenza H5N1 virus may have prominent deficit in surfactant components. Surfactant is produced by type II pneumocytes, and this cell type has been shown to undergo massive apoptosis in the same autopsy cases [Uiprasertkul et al., 2007]. Massive death of surfactant-producing cells may result in a deficiency in surfactant, which may lead to acute respiratory distress syndrome. It is possible that the mechanism of acute respiratory distress

syndrome in H5N1 infection may be different from acute respiratory distress syndrome caused by other types of injury. Although surfactant replacement as a therapeutic approach did not show impressive efficacy in other types of acute respiratory distress syndrome, it should be tested in avian influenza H5N1 virus infected patients and may yield a better efficacy.

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Legends

Fig. 1. Enriched GO terms of up- and down-regulated genes in lungs of H5N1-infected patient A and patient B. Raw intensity values were normalized against the normal lung dataset using quantile normalization in GeneSpring software (Agilent Technologies). Probes with low signals (<20% percentile) in all samples and control were excluded from the analysis. Genes with $\geq$ 2-fold change in at least one sample were identified. Gene ontology (GO) analysis was performed on these gene lists using Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) to identify significantly enriched GO terms among those genes.

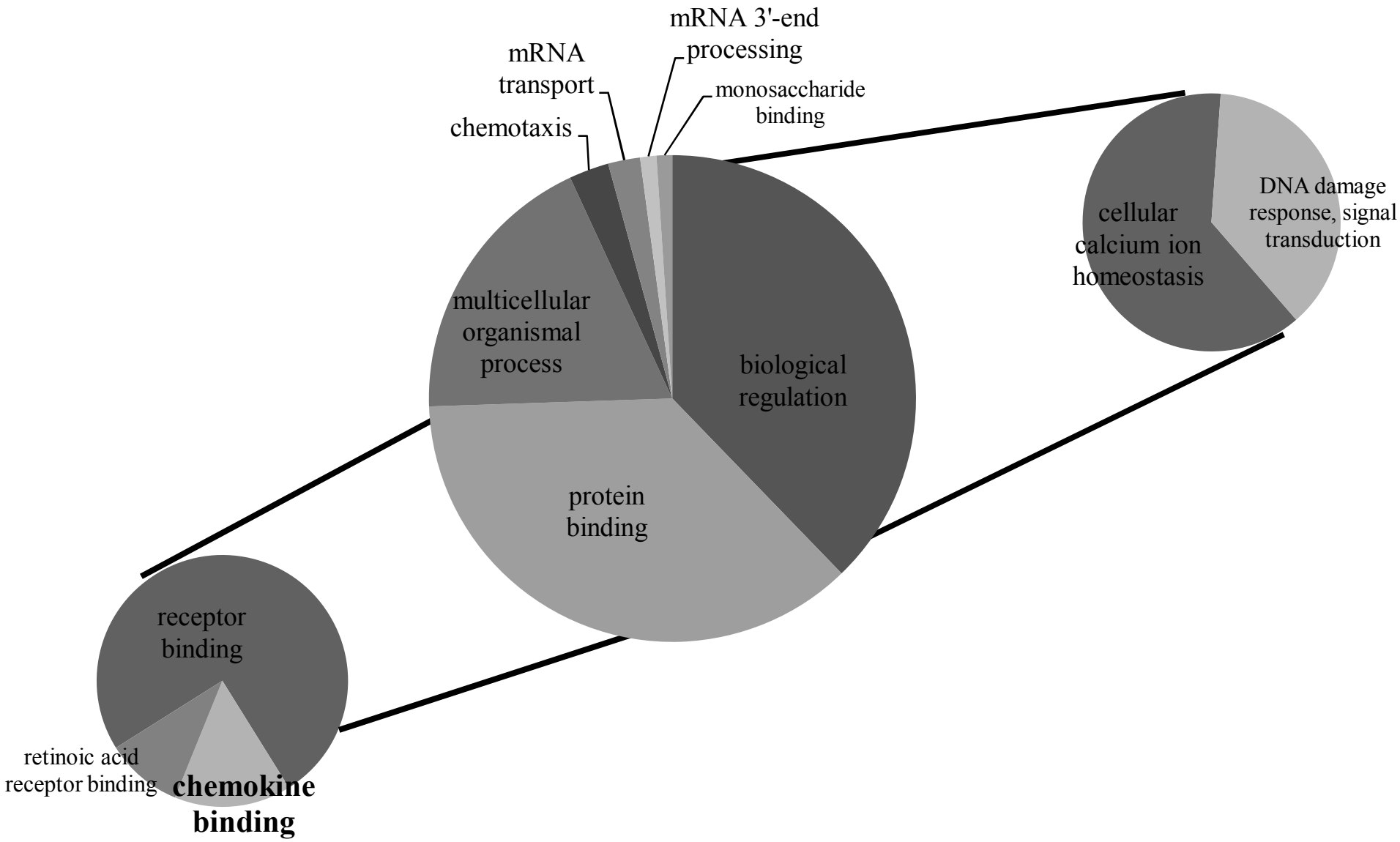
Fig. 2. RNA extracts from normal lung tissues (NL1 and NL2), lung tissues from patients with acute respiratory distress syndrome (ARDS1, ARDS2 and ARDS3), and H5N1-infected lung tissues of patient A and patient B (A and B) were analyzed for the expression of SP-D mRNA by real-time RT-PCR. Results are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; compared with normal lung tissues.

Fig. 3. Immunohistochemical localization of SP-D in normal human lung (a) and lung of patient B infected with avian influenza H5N1 virus (b).

Table 1 Short description of genes

Gene	Protein name	Function	Fold Change	
			Patient A	Patient B
Up-regulated genes				
CCL1	C-C chemokine 1	- Cytokine that is chemotactic for monocytes but not for neutrophils. Binds to CCR8.	2.53	2.24
CCL7	C-C chemokine 7	- Chemotactic factor that attracts monocytes and eosinophils, but not neutrophils. Augments monocyte anti-tumor activity. Also induces the release of gelatinase B. This protein can bind heparin. Binds to CCR1, CCR2 and CCR3.	4.71	3.05
CCR2	C-C chemokine receptor 2	- Receptor for the MCP-1, MCP-3 and MCP-4 chemokines. Transduces a signal by increasing the intracellular calcium ions level.	2.46	2.18
CXCR1	High affinity interleukin-8 receptor A	- Receptor to interleukin-8, which is a powerful neutrophils chemotactic factor. Binding of IL-8 to the receptor causes activation of neutrophils. This response is mediated via a G-protein that activate a phosphatidylinositol-calcium second messenger system. This receptor binds to IL-8 with a high affinity and to MGSA (GRO) with a low affinity.		
XCR1	Chemokine XC receptor 1	- Receptor for chemokines SCYC1 and SCYC2. Subsequently transduces a signal by increasing the intracellular calcium ions level.	8.71	2.56
IL-21R	Interleukin-21 receptor	- Receptor for IL-21, which is a cytokine with immunoregulatory activity and may promote the transition between innate and adaptive immunity.	6.2	4.25
IL-18	Interleukin-18	- Augments natural killer cell activity in spleen cells and stimulates interferon gamma production in T-helper type I cells.	2.8	2.5
Down-regulated genes				
CCL2	C-C chemokine 2	- Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils. Augments monocyte anti-tumor activity.	4.93	2.43
CCR1	C-C chemokine receptor 1	- Binds to MIP-1-alpha, MIP-1-delta, RANTES, and MCP-3 and, less efficiently, to MIP-1-beta or MCP-1 and subsequently transduces a signal by increasing the intracellular calcium ions level. Responsible for affecting stem cell proliferation.	2.12	2.84
CXCR4	C-X-C	- Receptor for the C-X-C chemokine CXCL12/SDF-1. Transduces a signal by increasing the	21.31	2.53

1a


mRNA 3'-end processing
mRNA transport
monosaccharide binding
chemotaxis
cellular calcium ion homeostasis
DNA damage response, signal transduction
multicellular organismal process
biological regulation
protein binding
receptor binding
retinoic acid receptor binding
chemokine binding

1b

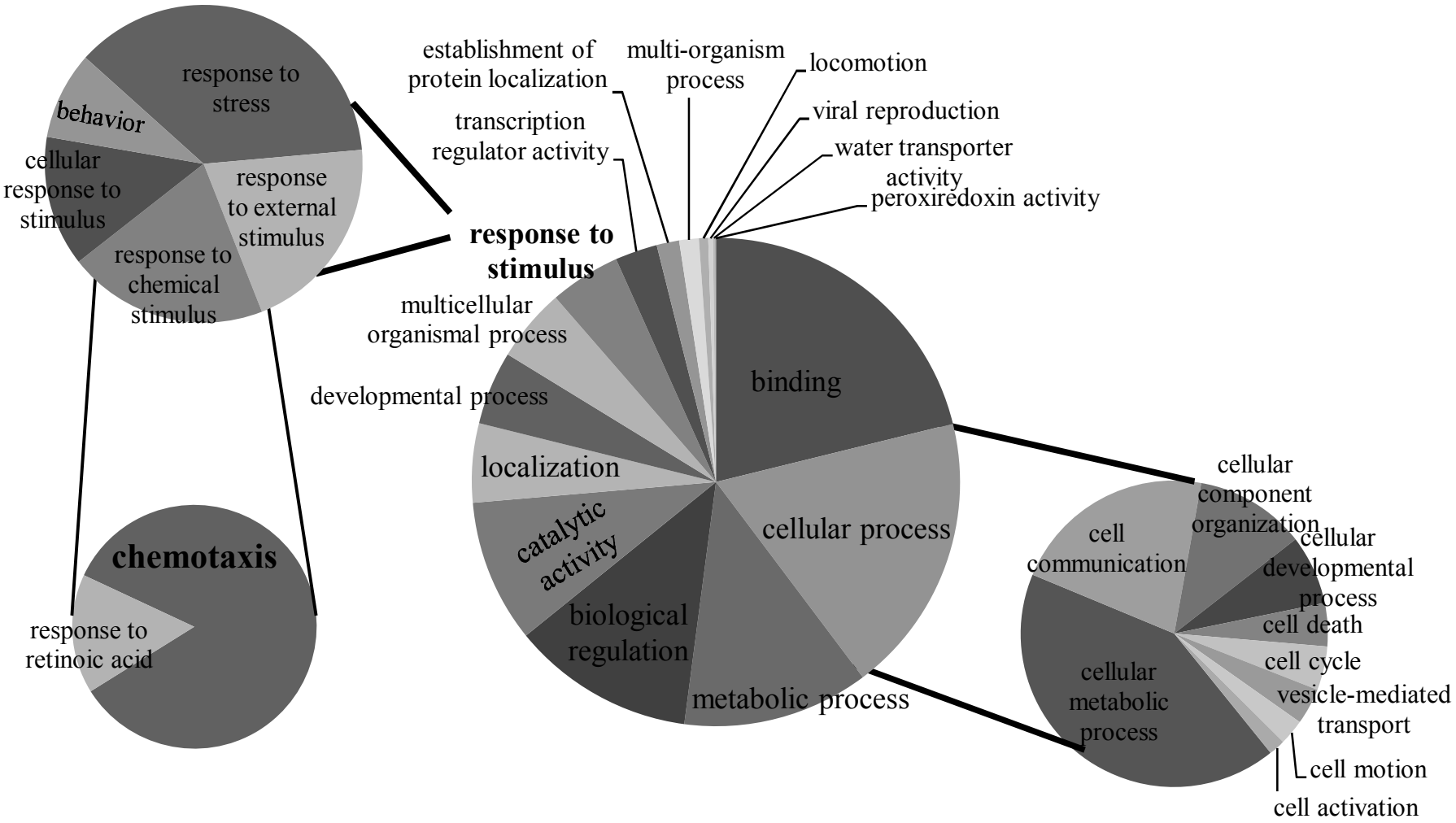


Fig. 1. Enriched GO terms of up- (a) and down-regulated (b) genes in lungs of H5N1-infected patient A and patient B. Raw intensity values were normalized against the normal lung dataset using quantile normalization in GeneSpring software (Agilent Technologies). Probes with low signals (<20% percentile) in all samples and control were excluded from the analysis. Genes with $\geq$ 2-fold change in at least one sample were identified. Gene ontology (GO) analysis was performed on these gene lists using Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) to identify significantly enriched GO terms among those genes.

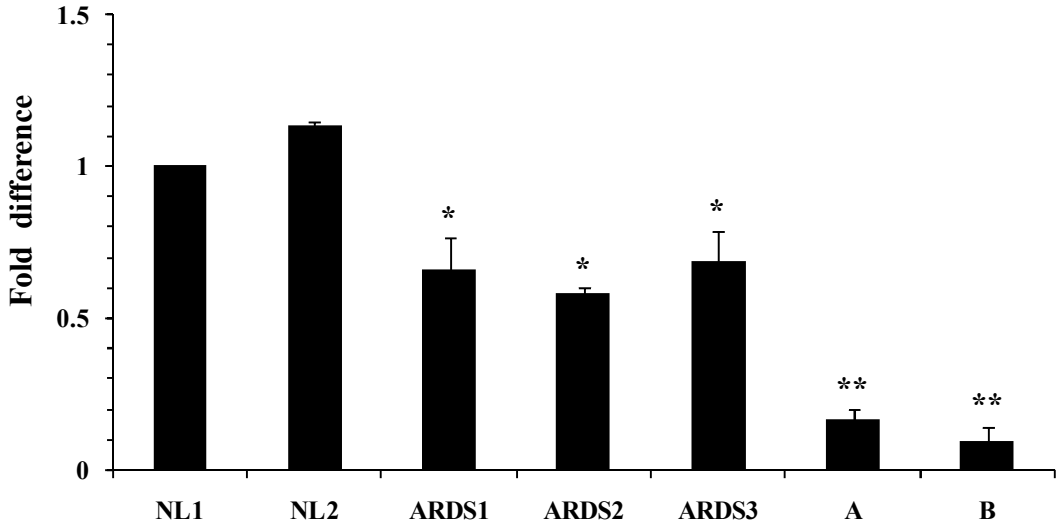


Fig. 2. RNA extracts from normal lung tissues (NL1 and NL2), lung tissues from patients with ARDS (ARDS1, ARDS2 and ARDS3), and H5N1-infected lung tissues of patient A and patient B (A and B) were analyzed for the expression of SP-D mRNA by real-time RT-PCR. Results are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; compared with normal lung tissues.

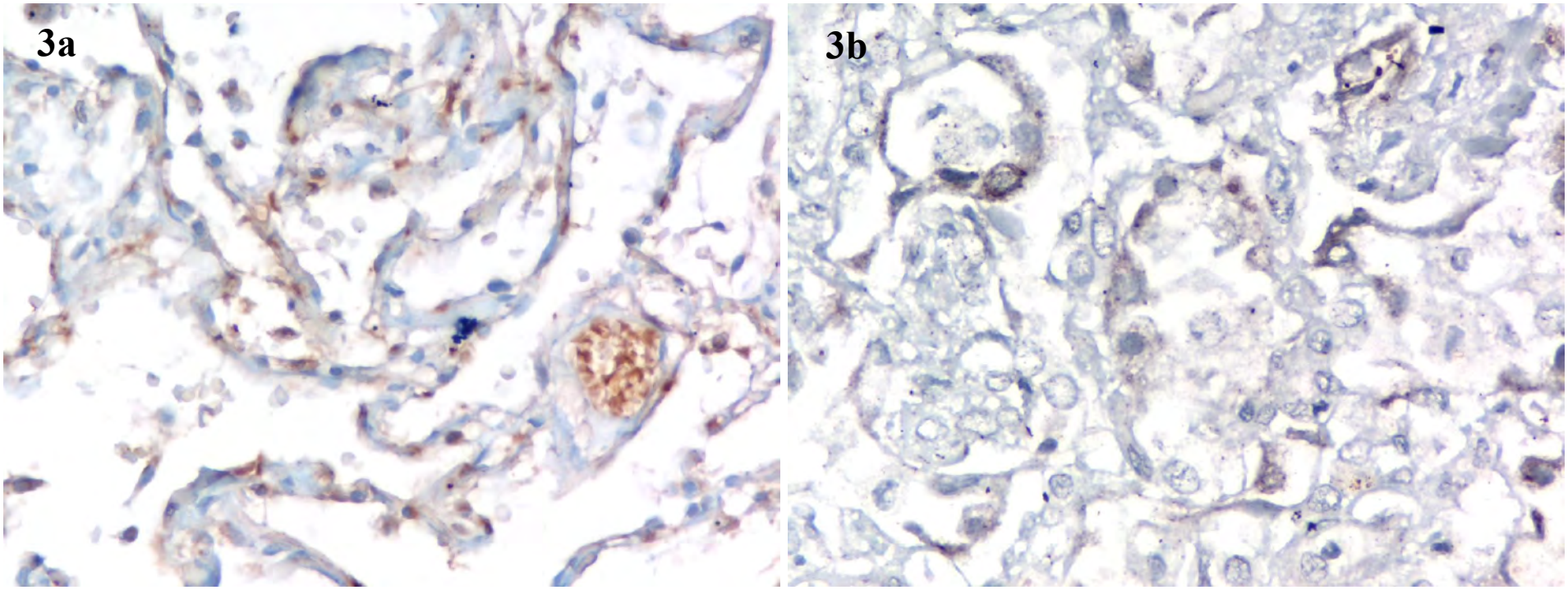


Fig. 3. Immunohistochemical localization of SP-D in normal human lung (a) and lung of patient B infected with avian influenza H5N1 virus (b). SP-D was found in pneumocyte type I cells in the normal human lung and in pneumocyte type II cells in the lung of avian influenza H5N1 infected patient B.

Manuscripts: Substrate specificity of avian influenza H5N1 neuraminidase

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Abstract

While preference of influenza virus hemagglutinin (HA) on the usage of α2,3- or α2,6-linked sialic acid as viral receptor is a well-defined difference between avian and human influenza viruses, less is known about substrate specificity of the viral neuraminidase (NA). As HA and NA counteract each other, their activities have to be balanced. Adaptation of NA activity may therefore accompany a change in the HA receptor preference and contribute to adaptation of avian influenza viruses to human hosts. We analyzed NA activity of avian influenza H5N1 strains from human and animals on α2,3- and α2,6-linked sialic acid using two different assays. While seasonal and pandemic H1N1 influenza viruses showed low activity on both substrate, NA of H5N1 viruses showed variable degree of activity on α2,3-linked sialic acid and low activity on α2,6-linked sialic acid. NA of a human isolate of H5N1 virus had low activity on α2,3-linked sialic acid, giving a pattern similar to that of seasonal influenza viruses. NA of this isolate contained only one mutation at position 139 (leucine to valine). This mutation and the change in NA activity may contribute to the adaptation of H5N1 avian influenza viruses to human host.

Introduction

NA is a tetrameric type II transmembrane glycoprotein on envelope of influenza virus. NA molecule consists of three domains: globular head , stalk and transmembrane domains [1-3]. The function of NA is to cleave terminal bound sialic acid on carbohydrate chains of glycans on cell surface and viral envelope in order to release of newly budded virions from host cells [2]. If the function of NA is impaired, sialyl residues on the surface of virus particles and infected cells can be bound by the HA, which leads to virus aggregation at the cell surface preventing the dissemination of infection [4, 5]. Moreover, NA can bind to plasminogen[6] and have a possible role in apoptosis of host cells[7, 8].

HA of avian and human influenza viruses bind preferentially to α2,3- and α2,6-linked sialic acid, respectively. This difference is believed to play an important role in the interspecies barrier of influenza transmission between avian species and human. A change in the receptor preference is required for emergence of a new pandemic strain from avian influenza viruses [9]. HA and NA counteract each other, and their activities need to be balanced for the virus to replicate efficiently [4]. NA activities on α2,3- and α2,6-linked sialic acid have been previously characterized for some avian and seasonal influenza viruses [10-12]. N2 NA isolated from human and avian had been studied for substrate specificity [10, 12] Avian and early human isolated N2 showed much more activity on α2,3-linked sialic acid than α2,6-linked. However, late human N2 isolation trended toward increase substrate specificity for α2,6-linked whereas maintaining the α2,3-linked activity. The N1 substrate specificity had also been studied [11]. Similar to N2 activity, N1 isolated from avian hosts showed much higher activity on α2,3- than α2,6-linked substrate, while human viruses showed reduced activity to α2,3- and increased activity to α2,6-linked sialic acid. Highly pathogenic H5N1 avian influenza virus is causing a wide-spread epidemic in poultry with occasional transmission to humans and poses a serious pandemic threat, while receptor preference of H5N1 HA has been extensively studied [13], data on their NA substrate specificity are scarce. We therefore

characterized NA activity of H5N1 viruses in comparison to NA of seasonal and pandemic influenza viruses.

There are several methods for detection of NA activity, i.e. colormetric, fluoromeric and chemiluminescent methods [14-19]. The colormetric assay used fetuin, a natural glycoprotein with α2,3- and α2,6-linked sialic acid, as the NA substrate. Free sialic acid was released after the cleavage and convented to β-formol pyruvic acid by periodate oxidation. β-formol pyruvic acid was then incubated with thiobarbituric acid (TBA) to form a chromophore (pink to red color). This chromophore was extracted and detected by spectrophotometer at 549 nm [20, 21]. Although TBA assay is the gold standard to detect NA activity, it is time-consuming and sensitive to interference by complex culture media[19]. This method could not distinguish activities on α2,3- and α2,6-linked sialic acid. Similar to TBA assay, NA Star which is a chemiluminescent assay could not discriminate the NA substrate specificity because it used derivative of neuraminic acid substrate as the substrate. Although, the BODIPY-label assay[16] which is a fluorometric assay can be used for studying the NA substrate specificity by label α2,3- and α2,6-linked sialic acid substrate with BODIPY FLSE, this assay was complex and time consuming. In this report, we devised a number of assays capable of measuring substrate-specific NA activity.

Material & Methods

Cell line and Virus: Madin-Darby canine kindney (MDCK) cells were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) in the present of Gentamicin, Penicillin G and Fungizone. 293T cell were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, antibiotics and antifungal. Viruses used in this study are show in Table 1. Viruses were cultured in MDCK cells in MEM without phenol red[17].

Hemagglutination (HA) test: Fifty microlitres of 0.5% goose (*Anser cygnoides*) red blood cell suspension in phosphate-buffered saline (PBS) were add into 50 µl of two-fold serially diluted virus in PBS, then, the mixture was incubated at 4°c for 30 min. The HA titre was calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination

NA substrate specificity by neuraminidase assay (Amplex red): NA activity was assayed following instruction provided by manufacturer (Molecular Probe, Inc.). This assay utilizes Amplex Red to detect H_2O_2 generated by oxidation of desialiated galactose which is the end product of neuraminidase action. In the presence of HRP, H_2O_2 reacts with 1:1 stoichimetry with Amplex Red reagent, then, generate resorufin, the red-fluorescent oxidation product, and detected at 640 nm. The method had been modified in order to study the substrate specificity by using 2 types of glycopolymer instead of fetuin. The substrates which were applied for this assay was Neu5Acα2,3LacNAcb-pAP and Neu5Acα2,6LacNAcb-pAP which contained α2,3-linked sialic acid and α2,6-linked sialic acid, respectively [13, 22]. Briefly, 10 µl of 64 HA unit of virus was mix with 10 µl of either Neu5Acα2,3LacNAcb-pAP or Neu5Acα2,6LacNAcb-pAP mixture. The NA activity on each substrate was detected at 640 nm after incubated at 37°C for 90 min.

Elution assay: The ability of NA to elute virus from red blood cells was previously described[23]. To assess the ability of NA to elute virus bound on substrate specificity, 2 types of RBC which are Horse and Goose RBC were applied. While HRBC only expressed α2,3-linked sialic acid on

their surface, GRBC expressed both α2,3- and α2,6-linked sialic acid on their surface. In order to obtain RBC with only α2,6-linked sialic acid, an α2,3-specific sialidase was used to eliminate the α2,3 -linked sialic acid on the surface of GRBC without damaging α2,6-linked sialic acid [24]. For sialidase treatment, a 100-µl of 10% suspension of GRBC in PBS was treated with 1.25 U of a α2,3-sialidase cloned from *Salmonella thyphimurium* LT2 (Takara, Japan) for 1 hr at 37°C [25]. The treated GRBC was washed twice and adjusted to 0.5% GRBC with PBS. The elution assay was done by incubation of fifty microlitres of two-fold dilution of virus containing the HA titre on HRBC and α2,3-sialidase treated GRBC of 1:16 with 50 µl of 1% HRBC and 0.5% α2,3-sialidase treated GRBC respectively in U-bottom microtitre plate at 4°C for 1 hr. The U-bottom microtitre plate was then stored at 37°C. The reduction of HA titres was recorded after 1, 2, 3 and 24 hr.

Sequencing : RNA was extracted from culture supernatant of infected MDCK cells using High Pure Viral Nucleic acid kit (Roche diagnostic, Germany). NA gene was then amplified by using One step RNA PCR kit (Takara,Inc, Japan) and BA NA 1F and NA1314R as the forward and reverse primers, respectively [26] (table 2). The PCR product was visualised by 1% agarose gel electrophoresis with SYBR staining and purified by using QIAquick® Gel Extraction Kit (QIAGEN, USA) according to the manufacturer's instructions. The purified amplicon was then sequenced.

Result

The NA activity by Amplex Red:

The N1 substrate specificity from H5N1 and H1N1 was shown in figure 1. The N1 activity was 10-30 fluorescence unit on α2,3-linked sialic acid and 5-10 fluorescence unit on α2,6-linked sialic acid depend on subtype and host. Compare between two substrate, both H5N1 and H1N1 showed more activity on α2,3-linked sialic acid than α2,6-linked (*p value* < 0.0001 and *p value* = 0.0005 respectively). The average activity of NA isolated from H5N1 virus showed significantly higher activity on α2,3-linked sialic acid than H1N1 (*p value*< 0.05), whereas there are no difference on

these two groups of virus on α2,6-linked sialic acid (*p value* > 0.05). To assess the host affect on the substrate specificity, NAs of H5N1 viruses isolated from patient and animal were compared. NA of a human isolate of H5N1 virus showed significantly lower activity than avian isolate (*p value* <0.05). This isolate, KK494, showed lowest activity on α2,3-linked sialic acid . It showed significantly lower activity when compared to Kan-1A which is the human isolate H5N1 virus (figure2). To identify the genetic determinant of this change in the NAs activity, NAs of the H5N1 viruses were sequenced. The sequences are shown in figure3.

There is one mutation on KK494: L139V (N1 numbering). This position is 10 amino acids from an active site residue (position 149). We searched the Influenza Resource Database and did not find any NA sequence with this L139V mutation.

The NA activity by elution assay:

In order to confirm the results by the Amplex Red assay, virus elution assay was used. It is the method for monitoring NA activity by observing the reduction of HA titre which means observing the NA activity for cleaving the virus bound from the erythrocyte. In this experiment, HRBC which only expressed α2,3-linked sialic acid and α2,3-linked sialic acid- sialidase treated GRBC were applied. Only H5N1 isolated viruses could be bound and eluted from HRBC (Fig 4). Whereas, human influenza virus both seasonal flu and 2009 strain could not bind to the HRBC (data not show).

Although NA activity from human-isolated H5N1 virus showed similar pattern of cleavage on α2,3-linked which is rapidly released from HRBC then slowly cleaved from HRBC. Similar to Amplex Red assay, Kan-1A/2004 showed more activity than others because its NA could cleave all virus bound from HRCB whereas others still had some virus bound RBC (Fig 7A). KK494/2004 also was the lowest NA activity in the human isolated H5N1. KK494/2004 had shown the constant HA titre although it showed the same HA titre as SP83. Comparing to human isolates, avian-isolated H5N1

were classified into 2 groups (Fig7B) similar to Amplex Red assay. Group 1 showed very slow kinetics of cleavage, which started to show the reduction in HA titer after 2-3 hours. This group consisted of VSMU-1/2006 and VSMU-4NSN/2004. The other group showed a faster kinetics comparable to those of the human isolates.

Discussion

Similar to previous studies [11, 16, 27, 28], NA from human seasonal influenza virus had similar activity on α2,3-linked sialic acid and α2,6-linked sialic acid . This might be because 1.)they are transmitted from avian in decades ago and still have the avian gene[29]. 2).Self protection of virus by releasing from the mucus which is rich of α2,3-linked sialic acid[30].

To detect the NA activity by Amplex Red assay, all viruses had been cultured in phenol red free medium to avoid the interference of fluorescent assay as studied previously [14].

Although the NA activity by elution assay showed similar result to Amplex Red assay in each group of H5N1 isolate, some of the NA of animal isolated H5N1 virus seemed to have lower activity than human isolated virus because some of the NA of animal isolated H5N1 virus shown higher HA titre than human isolated H5N1 which means these NA of animal isolated H5N1 could not cleave the virus bound RBC. This is might because of the limitation of the assay which is related to the binding between HA and receptor on erythrocyte. This also affected the reduction of HA titre. Faster reduction may not mean higher NA activity, but they might be the effect of not appropriate binding. Therefore, low NA activity virus can cleave itself from the erythrocyte.

Interestingly, KK494/2004 had lowest NA activity on α2.3-linked sialic acid. The mutation that was found on this isolate had not been found on database and also first isolation from patient. This might be the effect of subpassage of the virus. L139V affect the α2.3-linked sialic acid activity

may be because this position is near the active site and 150 cavalry of NA. This might leads to inappropriate folding of the active site. However, it needs further investigation.

Although the conclusion was similar to the previous studies that virus had more activity on α2,3-linked sialic acid than α2,6-linked sialic acid. This paper was the first study on substrate specificity of N1 from H5N1, H1N1 2009 and seasonal flu strain. The substrate specificity is needed to monitor to prevent and aware of the severe outbreak which can lead to pandemics.

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Figure legend

Figure1 The N1 activity from avian isolated H5N1, human isolated H5N1, H1N1 2009 and H1N1 seasonal flu on substrate specificity by Amplex Red assay.

N1 from H5N1 (□) and H1N1 (■) had been studied the NA activity on α2,3-linked sialic acid (Fig 1A) and α2,6-linked sialic acid (Fig 1B). The activity was assayed at 37°C for 90 min and detected at 640 nm.

Figure2 The NA activity from human-isolated H5N1 virus on α2,3-linked sialic acid by Amplex Red assay. NA from human isolated H5N1 viruses which are A/Thailand/ Kan-1A/2004, A/Thailand/3(SP-83)/2004 and A/Thailand/5(KK-494)/2004 had been assayed the activity on α2,3-liniked sialic acid by Amplex Red. The statistic was calculated by one way ANOVA.

Figure3 The sequence alignment of NA from human isolated H5N1 virus.

Amino acid sequence of NA from human isolated H5N1 virus by viral culture were aligned with human isolated H5N1 virus on flu genome database.

Figure4 NA activity by Elution assay

NA from human isolated H5N1 (Fig 4A) and animal isolated H5N1 virus (Fig 4B) had been observed the activity in order to cleave the virus bound α2,3-linked sialic acid on HRBC. 16 HAU virus which released from 1% HRBC at 37°C had been observed. The reduction of HA titre were monitored at 1, 2, 3 and 24 hr respectively.

Table 1 Virus strain and source of isolation

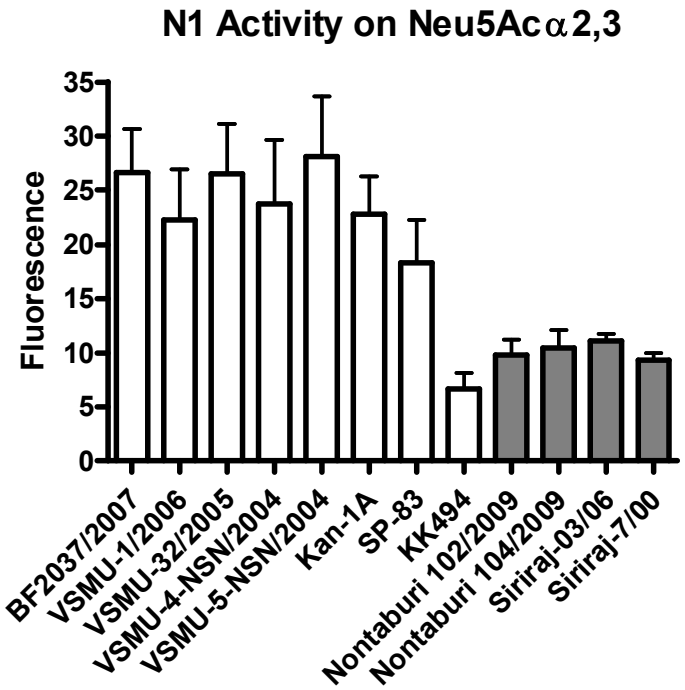
Virus	Subtype	Passage	Source
A/Thailand/KAN-1A/2004	H5N1	MD8	Human
A/Thailand/5(KK-494)/2004	H5N1	MD8	Human
A/Thailand/3(SP-83)/2004	H5N1	MD8	Human
A/Openbill stork/Thailand/VSMU-4-NSA/2004 (H5N1)	H5N1	MD4	Avian
A/Openbill stork/Thailand/VSMU-5-NSA/2004 (H5N1)	H5N1	MD4	Avian
A/Chicken/Bangkok/VS-MU-1/2006	H5N1	MD4	Avian
Lab strain BF2037/50	H5N1	MD4	Avian
A/Openbill stork/Thailand (Nakhonsawon)/VSMU-32/2005 (H5N1)	H5N1	MD4	Avian
A/Thailand/Nontaburi102/2009	H1N1 2009	MD4	Human
A/Thailand/Nontaburi104/2009	H1N1 2009	MD4	Human
A/Thailand/Siriraj-7/00 (A/New Caledonia/20/99-like virus)	H1N1	MD7	Human
A/Thailand/Siriraj-03/06	H1N1	MD7	Human

Table2 Universal Neuraminidase gene primer for RT-PCR [26]

Primer	Sequence	Expected size (bp)
Ba-NA-1	TATTGGTCTCAGGG**AGCAAAAGCAGGAGT**	1413
BA-NA-1413R	ATATGGTCTCGTATT**AGTAGAAACAAGGAGTTTTTT**	1413

Figure1 The N1 activity from avian isolated H5N1, human isolated H5N1, H1N1 2009 and H1N1 seasonal flu on substrate specificity by Amplex Red assay.

A



B

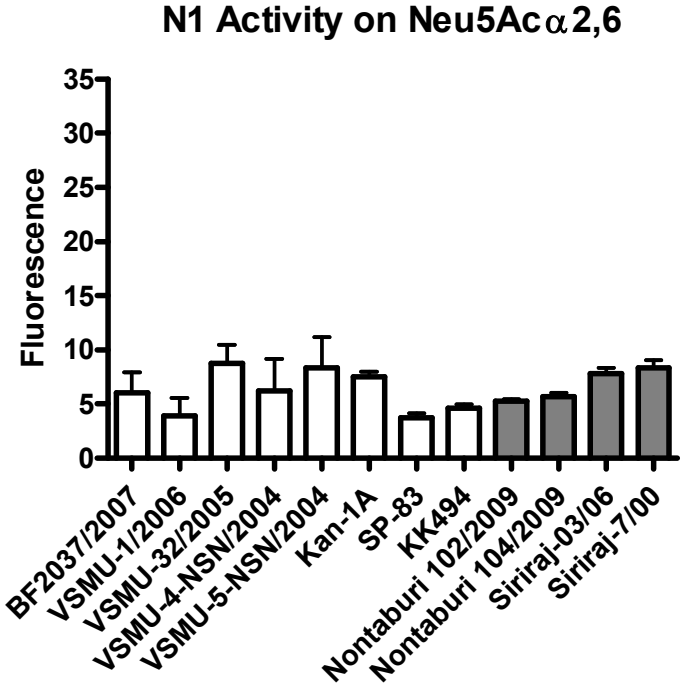


Figure2 The NA activity from human isolated H5N1 virus on α2,3-linked sialic acid by Amplex Red assay

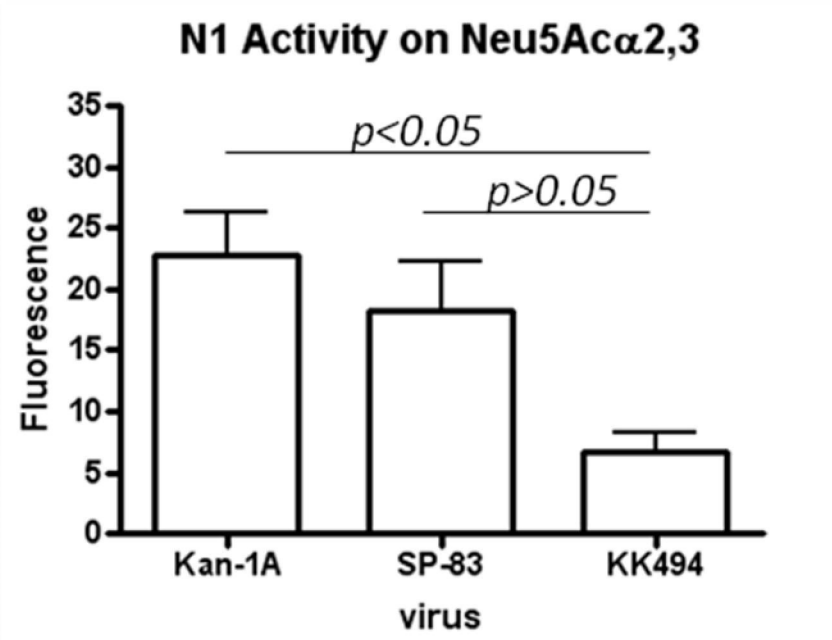


Figure3 The sequence alignment of NA from human isolated H5N1 virus

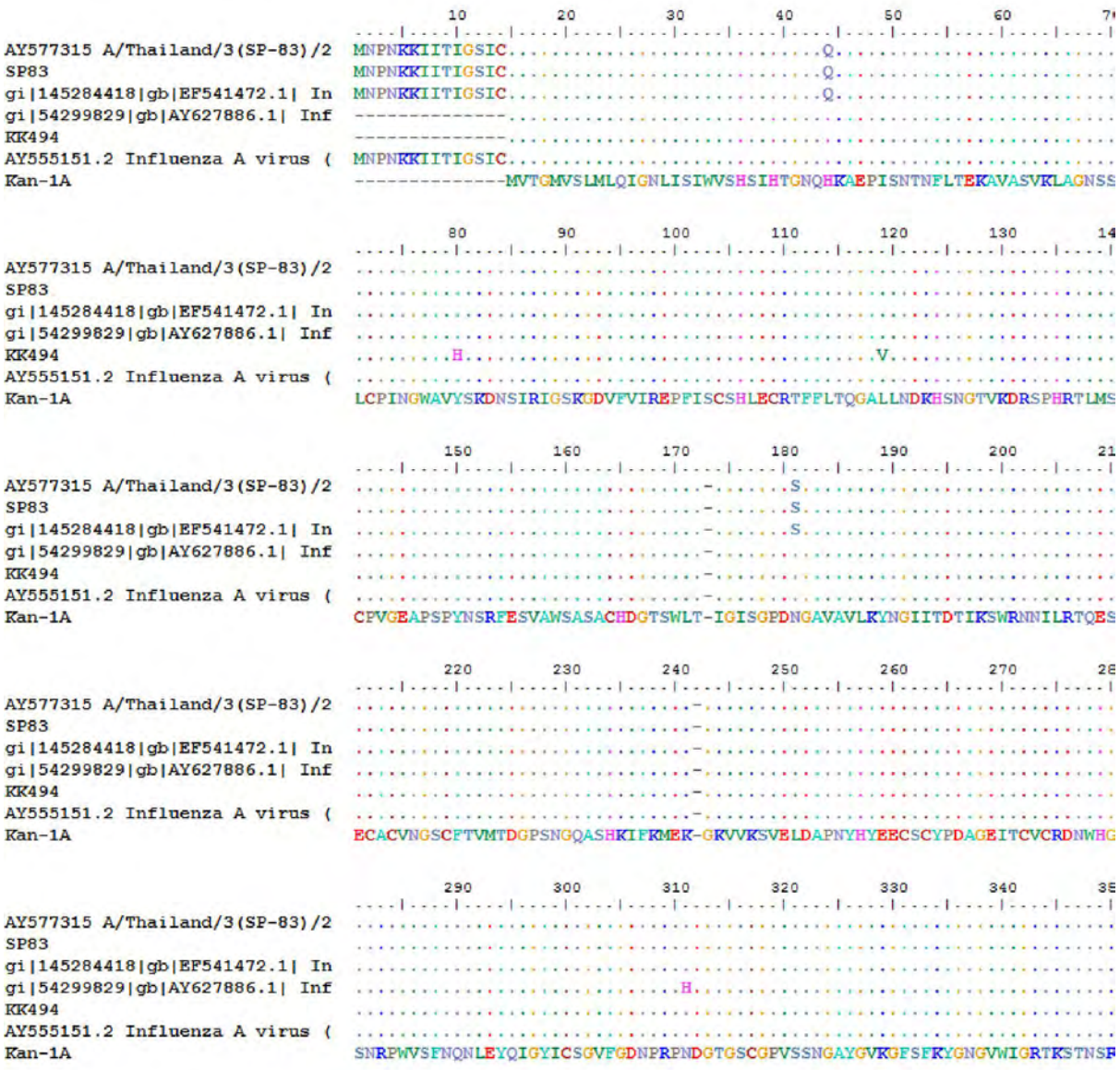
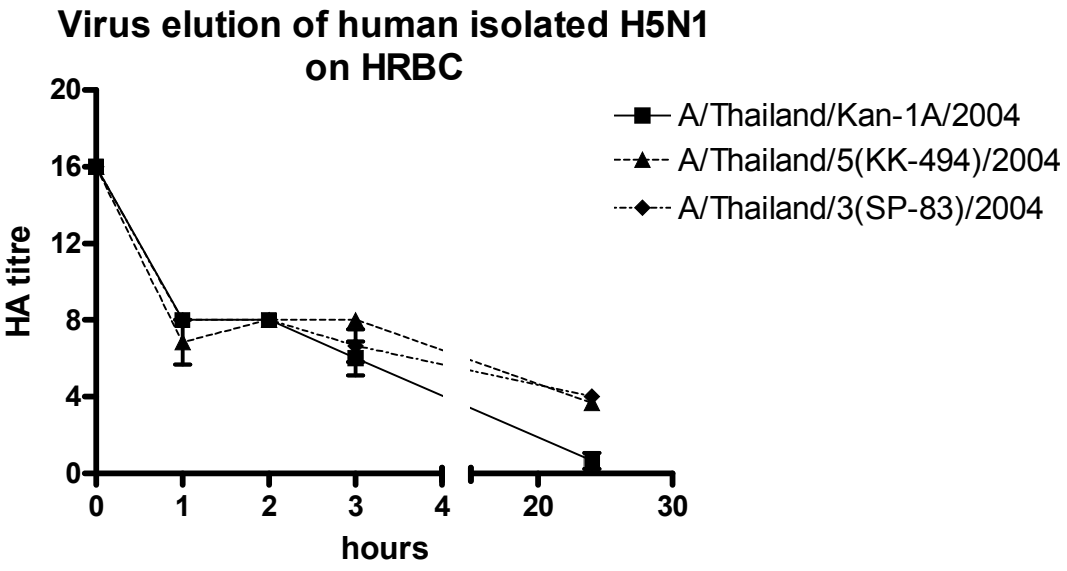


Figure4 NA activity by Elution assay

A



B