SHORT COMMUNICATION

Decrease of Virus Receptors during Highly Pathogenic H5N1 Virus Infection in Humans and Other Mammals

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Highly pathogenic avian influenza H5N1 virus causes a severe, often fatal, pneumonia in humans. The tropism and pathogenesis of highly pathogenic avian influenza H5N1 virus can partly be explained by the presence of H5N1 virus receptors in the human alveoli, which are the site of inflammation during pneumonia. Although studies on the distribution of influenza virus receptors in normal respiratory tract tissues have provided significant insights into the cell tropism and pathogenesis of influenza viruses, the distribution of influenza virus receptors have not been studied during influenza virus infection. Therefore, we studied the distribution of H5N1 virus receptors, by virus and lectin histochemistry, during highly pathogenic avian influenza H5N1 virus infection in alveolar tissues of humans, macaques, ferrets, and cats. In all species, we observed a decrease of H5N1 virus receptors in influenza virus–infected and neighboring cells. The observed decrease of H5N1 virus receptors was associated with the presence of MxA, a known marker for interferon activity. Taken together, our data suggest that the decrease of H5N1 virus receptors might be part of a defense mechanism that limits viral replication in the lower respiratory tract. (*Am J Pathol* 2013, 183: 1382–1389; <http://dx.doi.org/10.1016/j.ajpath.2013.07.004>)

The most common complication of influenza virus infection in humans is pneumonia. Although all influenza viruses that are able to infect humans can cause pneumonia, the frequency varies largely between different influenza virus subtypes.¹ Highly pathogenic avian influenza (HPAI) H5N1 virus, which is primarily an infectious disease of poultry, causes a severe, often fatal, pneumonia in humans.² The severity of disease caused by HPAI H5N1 virus can partly be explained by the cell tropism of HPAI H5N1 virus for cells in the lower respiratory tract (LRT).^{3–5}

The cell tropism of influenza viruses depends, among other factors, on the ability to attach to host cell receptors, which is the first step in the replication cycle.⁶ Interestingly, human and avian influenza viruses use differently linked sialic acids (SAs) as their receptor. Human influenza viruses attach predominantly to α 2,6-linked SAs, whereas avian influenza viruses attach predominantly to α 2,3-linked SAs. These SAs are differentially expressed throughout the human respiratory tract; seasonal influenza viruses attach abundantly to ciliated epithelial cells of the upper respiratory tract (URT), whereas avian influenza viruses, including HPAI H5N1 virus, attach predominantly to epithelial cells

in the LRT.^{4,5,7,8} In the alveoli, which is the functional part of the lung and the site of inflammation during pneumonia, seasonal influenza viruses and pandemic H1N1 virus attach predominantly to type I pneumocytes, whereas HPAI H5N1 viruses attach predominantly to type II pneumocytes and alveolar macrophages.^{5,9} The attachment of HPAI H5N1 virus to type II pneumocytes and alveolar macrophages in the human alveolus corresponds with infection of these cells in *in vivo*— and *in vitro*—infected human tissues.^{3,9–11}

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Besides human infections, HPAI H5N1 virus is also able to infect and cause a severe pneumonia in many other mammalian species. Mammals in which the pathogenesis of HPAI H5N1 virus has been studied include ferrets, macaques, and cats.^{12–14} A common target for HPAI H5N1 virus in all three species as well as in humans is the Clara cell in the terminal bronchioli and the type II pneumocyte in the alveoli. In addition, HPAI H5N1 virus attaches in the alveoli to type I pneumocytes in macaques and to alveolar macrophages in humans and cats.^{4,5,7}

Studies on the distribution of influenza virus receptors, by virus (VHC) and lectin histochemistry (LHC), have provided significant insights in the pathogenesis of influenza viruses. In humans and other mammals, the tropism of HPAI H5N1 virus for cells deep in the lung seems to be associated with the severe pneumonia caused by this virus.^{3–5,7,9,11} However, all these studies have been performed on uninfected tissues and not in tissues obtained early after infection. During influenza virus infection many cytokines and chemokines are up-regulated, including interferons that are known to activate genes with antiviral activity.^{15,16} Whether such local responses would also influence the receptor distribution is not known. However, such information is important to understand the pathogenesis of influenza virus infections because the ability of progeny virus particles to attach to and infect new susceptible cells determines continuous virus replication and the ongoing disease progression.

To get more insight into the pathogenesis of HPAI H5N1 virus infections in humans and other mammals, we here determined the distribution of H5N1 virus receptors during HPAI H5N1 virus infection. We focused on the alveoli, because this is the site of inflammation and virus replication during pneumonia. Human and cynomolgus macaque lung biopsies were infected *ex vivo* with HPAI H5N1 virus, and the distribution of influenza virus receptors was determined by VHC and LHC. Furthermore, the distribution of HPAI H5N1 virus receptors was determined in lung tissues from ferrets and cats infected *in vivo* with HPAI H5N1 virus. Additional staining was performed to detect the interferon-induced MxA protein as a marker of local interferon response.

Materials and Methods

Ethics

The human lung tissues were obtained within the Erasmus MC. Approval from the Dutch Medical Ethical Committee was obtained to use human respiratory tract tissues for *ex vivo* infections. Experiments with the macaque lung biopsies and the HPAI H5N1 virus inoculation of cats and ferrets were approved by the Dutch Animal Ethical Committee.

Experimental Design

To determine the distribution of HPAI H5N1 virus receptors early after infection, human and cynomolgus macaque lung

biopsies were infected *ex vivo* and cultured for 24 hours. In addition, lung tissues were selected from ferrets and cats infected with HPAI H5N1 virus. The distribution of HPAI H5N1 virus receptors was determined in combination with the expression of influenza virus antigen in all tissues. HPAI H5N1 virus attachment was detected by VHC in combination with the detection of influenza virus antigen by immunohistochemistry (IHC). In addition, the presence of the avian influenza virus receptor, $\alpha 2,3$ -linked SA, was detected by LHC, again in combination with the detection of influenza virus antigen by IHC. To determine local interferon responses serial tissue sections were stained with an antibody directed against MxA by IHC.

Ex Vivo Lung Culture of Human and Cynomolgus Macaque Lung Biopsies

Surgically removed lung tissues from three human donors and four healthy control cynomolgus macaques were used for *ex vivo* infections. Human donors were anonymous, and a part of the lung without any gross lesions was selected for the *ex vivo* cultures. Histologically, no evidence of respiratory disease was found in all tissues used for *ex vivo* infections. Biopsies with a diameter of 3 mm were cultured overnight in F12K medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L glutamine, and 5% fetal calf serum at 37°C in 95% O₂ and 5% CO₂. Before infection, biopsies were washed with serum-free F12K medium. Per donor or macaque, two biopsies were infected with 10⁷ particles/mL HPAI H5N1 virus (A/Vietnam/1194/2004), and two biopsies were sham-infected for 1 hour at room temperature on a rocker. After infection, biopsies were washed and cultured in serum-free F12K medium. After 24 hours, biopsies were collected in 10% neutral-buffered formalin and embedded in paraffin. Most of the alveolar epithelium was intact in all lung biopsies included, as confirmed by evaluation of serial sections stained for keratin by IHC (data not shown).

In Vivo HPAI H5N1 Virus–Infected Cat and Ferret Lung Tissues

Tissues from previous studies in which ferrets and cats had been inoculated experimentally with HPAI H5N1 virus were selected for analysis. Lung tissues from three ferrets infected with HPAI H5N1 virus (A/Indonesia/02/2005) and sacrificed 1 day after inoculation, which contained foci with virus antigen, were included in this study.¹² From three cats, infected with HPAI H5N1 virus (A/Vietnam/1194/2004) and sacrificed 7 days after inoculation, lung tissues with foci of virus antigen were selected.¹⁷ From two uninfected cats and ferrets, lung tissues were included as control.

Double Staining for H5N1 Virus Attachment and Influenza A Virus Antigen

Formalin-fixed, paraffin-embedded tissue sections (3 μ m) were deparaffinized with xylene and hydrated with graded

alcohol. For detection of influenza A virus antigen by IHC, tissue sections of two biopsies per condition per donor were first stained with a primary antibody against the influenza A virus nucleoprotein (clone hb65; ATCC, Wessel, Germany) as described previously.¹⁸ Binding of the primary antibody was detected with an alkaline phosphatase–labeled goat–anti-mouse IgG2a (SouthernBiotech, Birmingham, AL). After washing, HPAI H5N1 virus attachment was detected by VHC as described previously.⁴ Briefly, inactivated, fluorescein isothiocyanate–labeled HPAI H5N1 virus (A/Vietnam/1194/2004) was incubated on tissue sections and detected with a peroxidase-labeled rabbit anti–fluorescein isothiocyanate (Dako, Glostrup, Denmark) and tyramide signal amplification system (PerkinElmer, Boston, MA). Alkaline phosphatase was revealed with the use of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate system (Dako), resulting in a dark blue precipitate. Peroxidase was revealed with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich, St. Louis, MO), resulting in a red precipitate. Tissues were counterstained with hematoxylin. Virus attachment and influenza virus antigen in combination with an isotype control or omission control were performed as negative controls. To quantify the degree of virus attachment, two observers independently counted the number of cells to which HPAI H5N1 virus attached in five arbitrarily selected $\times 400$ high-power fields per tissue of the following categories: i) uninfected biopsies/tissues, ii) parts of infected biopsies/tissues that did not contain any virus antigen, and iii) foci in infected biopsies/tissues that did contain virus antigen. The maximum variation between the two observers was 23% when <10 cells were counted per $\times 400$ high-power field and 10% when >11 cells were counted per $\times 400$ high-power field.

Double Staining for MAL-II and Influenza A Virus Antigen

Formalin-fixed, paraffin-embedded sections (3 μm) were deparaffinized with xylene and were hydrated with graded alcohol. For the detection of $\alpha 2,3$ -linked SAs by LHC, tissue sections were pretreated with 0.1% protease for 10 minutes at 37°C, and endogenous peroxidase was blocked with 3% hydrogen peroxide. Sections were washed with Tris-buffered saline (TBS) and blocked with TBS + 1% bovine serum albumin (BSA) for 1 hour at room temperature. Without washing, slides were incubated with biotinylated *Maackia amurensis* lectin II (MAL-II; Vector Laboratories, Burlingame, CA) in TBS + 1 mmol/L MgCl_2 + 1 mmol/L MnCl_2 + 1 mmol/L CaCl_2 + 1% BSA overnight at 4°C. After washing with TBS, slides were incubated with peroxidase-labeled streptavidin in TBS + 1% BSA for 1 hour at room temperature. Subsequently, slides were washed in PBS and stained for influenza A virus antigen as described in the previous section. Enzymatic activity of peroxidase and alkaline phosphatase was revealed with the use of AEC and BCIP/NBT, respectively. Tissues were counterstained with hematoxylin. MAL-II

and influenza A virus antigen staining in combination with an isotype control or omission control were included as negative controls.

Staining for MxA

For detection of MxA, formalin-fixed, paraffin-embedded, serial sections (3 μm) were deparaffinized with xylene and hydrated with graded alcohol. Antigen retrieval was performed at 100°C for 15 minutes with Tris-EDTA buffer pH 9. After blocking with 5% BSA, sections were incubated with the primary mouse antibody M143 directed against MxA,¹⁹ according to the above-described IHC protocol. Binding of the primary antibody was detected with a peroxidase-labeled goat–anti-mouse IgG2a (SouthernBiotech). Peroxidase activity was revealed with the use of AEC. Sections were counterstained with hematoxylin. An isotype control was included as a negative control.

Statistical Analysis

To determine whether the number of cells to which HPAI H5N1 virus attached differed, significance was determined as follows. A paired *t*-test was used to determine the significance between parts without virus antigen and foci with virus antigen in HPAI H5N1 virus–infected biopsies/tissues from one donor and between infected and uninfected biopsies from one donor. The *U*-test was used to determine significances between infected and uninfected tissues from different donors.

Results

Double Staining for H5N1 Virus Attachment and Influenza A Virus Antigen

The attachment of HPAI H5N1 virus to alveolar epithelial cells in human, macaque, ferret, and cat tissues infected with HPAI H5N1 virus was compared with uninfected tissues. Overall, HPAI H5N1 virus attachment was decreased in foci with virus antigen compared with parts without virus antigen or uninfected tissues (Figure 1).

In human *ex vivo*–cultured uninfected lung biopsies, HPAI H5N1 virus attached predominantly to type II pneumocytes in the alveoli. However, in HPAI H5N1 virus–infected lung biopsies, H5N1 virus attachment was decreased to cells positive for viral antigen and surrounding alveolar epithelial cells (Figure 1). Quantification of the number of cells to which HPAI H5N1 virus attached showed a decrease in the infected biopsies, both in parts with and without virus antigen, compared with the uninfected biopsies which was significant in two of three donors (Figure 2A).

In macaque *ex vivo*–cultured uninfected lung biopsies, HPAI H5N1 virus attached to both type I and type II pneumocytes in the alveoli. In HPAI H5N1 virus–infected lung biopsies, HPAI H5N1 virus did not attach to infected

Figure 1 HPAI H5N1 virus attachment in uninfected and infected human, macaque, ferret, and cat alveolar tissues. Double staining for HPAI H5N1 virus attachment by virus histochemistry (red) and influenza virus antigen in infected cells by immunohistochemistry (dark blue). **Top row:** Noninfected *ex vivo*—cultured human and macaque lung biopsies and control lung tissues from ferret and cat. **Bottom row:** *Ex vivo* HPAI H5N1 virus—infected human and macaque lung biopsies and lung tissues from HPAI H5N1 virus—infected ferret and cat. **Arrows** indicate influenza virus antigen in infected cells. Original magnification, $\times 400$. HPAI, highly pathogenic avian influenza.

cells or surrounding alveolar epithelial cells (Figure 1). The number of cells to which HPAI H5N1 virus attached was decreased in foci with virus antigen compared with parts without virus antigen, which was significant in two of four macaques. Comparison of infected biopsies with uninfected biopsies showed a significant decrease in the number of cells to which HPAI H5N1 virus attached in all macaques for which uninfected biopsies were present (Figure 2B).

In lung sections of uninfected ferrets, HPAI H5N1 virus attached predominantly to type II pneumocytes. In lung sections of HPAI H5N1 virus—infected ferrets, HPAI H5N1 virus attached to type II pneumocytes in parts without any virus antigen. However, in foci with virus antigen, HPAI H5N1 virus did not attach to alveolar epithelial cells. This decrease in virus attachment was observed in infected cells, surrounding alveolar epithelial cells, and alveolar epithelial cells in nearby alveoli, which were negative for virus antigen (Figure 1). The number of cells to which HPAI H5N1 virus attached was decreased in foci with virus antigen compared with parts without virus antigen, which was significant in two of three ferrets (Figure 2C). In lung sections of uninfected ferrets, HPAI H5N1 virus attached to more cells compared with lung sections of infected ferrets in areas with virus antigen, although this decrease was not significant.

In lung sections of uninfected cats, HPAI H5N1 virus attached predominantly to type II pneumocytes. In lung sections of HPAI H5N1 virus—infected cats, HPAI H5N1 virus attached to type II pneumocytes in parts without any virus antigen. However, in foci with influenza virus antigen less attachment to alveolar epithelial cells was found. This decrease in virus attachment was observed in infected cells, surrounding alveolar epithelial cells, and alveolar epithelial cells in nearby alveoli, which were negative for influenza virus antigen (Figure 1). The number of cells to which HPAI H5N1 virus attached was decreased in foci with virus

antigen compared with parts without virus antigen which was significant in two of three cats. In addition, in lung sections of uninfected control cats, HPAI H5N1 virus attached to significantly more cells than in HPAI H5N1 virus—infected cats, both in parts with and without virus antigen (Figure 2D).

Double Staining for MAL-II and Influenza A Virus Antigen

MAL-II attachment was used to directly detect the specific receptor for avian influenza virus. Overall, MAL-II attachment to alveolar epithelial cells was decreased in infected tissues compared with uninfected human, macaque, ferret, and cat tissues (Figure 3). In addition, in HPAI H5N1 virus—infected tissues, MAL-II attachment was reduced in foci with virus antigen expression compared with parts without virus antigen expression (Figure 3). The decrease in MAL-II attachment in foci positive for influenza virus antigen correlated with the reduction of HPAI H5N1 virus attachment in the same parts.

Staining for MxA

To determine whether the observed decrease in virus attachment might be linked to a interferon (Figure 4) response, an IHC staining for a marker for type I and III interferon activity, MxA,²⁰ was performed on serial tissue sections. In general, in all species MxA staining was observed in the cytoplasm of alveolar epithelial cells and alveolar macrophages in HPAI H5N1 virus—infected tissues but not in any of the uninfected tissues. In the human and macaque *ex vivo*—infected lung biopsies, which have only a diameter of 3 mm, MxA expression was high in biopsies with virus antigen expression and absent in uninfected biopsies.

Figure 2 Number of cells to which HPAI H5N1 virus attaches in uninfected and HPAI H5N1 virus—infected alveolar tissues. Number of cells to which HPAI H5N1 virus attaches in human (A), macaque (B), ferret (C), and cat (D) alveolar tissues double stained for HPAI H5N1 virus attachment and influenza virus antigen expression. Each symbol represents an individual count, and each line represents the mean of the counts per tissue. Open circles indicate the number of cells to which HPAI H5N1 virus attaches in uninfected tissues, closed triangles indicate the number of cells to which HPAI H5N1 virus attaches in HPAI H5N1 virus—infected tissues in parts without virus antigen expression, and plus signs indicate the number of cells to which HPAI H5N1 virus attaches in HPAI H5N1 virus—infected tissues in foci with virus antigen expression. * $P < 0.05$, ** $P < 0.01$. HPAI, highly pathogenic avian influenza; hpf, high-power field; ND, not determined; VHC, virus histochemistry.

Furthermore, in the *in vivo*—infected ferret and cat lung tissues MxA staining was most abundant in foci with virus antigen expression and surrounding areas (Figure 4, A–D, F, and G). In general, the presence of MxA was associated with the decrease of HPAI H5N1 virus attachment in all species, although MxA expression seemed more widespread than the decrease of HPAI H5N1 virus receptors (Figure 4). In uninfected ferret and cat lung tissues MxA expression was absent.

Discussion

Here, we show for the first time that HPAI H5N1 virus infection is associated with a decrease of H5N1 virus

receptors on infected cells and on cells that are in close proximity to infected cells. This phenomenon was observed early after infection in the alveoli of *ex vivo*—infected human and macaque lung biopsies and in the alveoli of *in vivo*—infected ferrets. In addition, this decrease was still observed in cat alveolar tissues 7 days after inoculation.

The decrease of HPAI H5N1 virus receptors in the alveoli, which are normally present on type II pneumocytes in all species included in this study, might be a defense mechanism to control virus replication. The decrease of receptors on infected cells could be a direct effect of the neuraminidase expression, as previously shown for H1N1 and H3N2 influenza viruses,²¹ Newcastle disease virus,²² and human parainfluenza virus.²³ This mechanism would

Figure 3 MAL-II attachment in uninfected and infected human, macaque, ferret, and cat alveolar tissues. Double staining for MAL-II binding by lectin histochemistry (red) and influenza virus antigen expression by immunohistochemistry (dark blue). The **top row** consists of noninfected *ex vivo*—cultured human and macaque lung biopsies and control lung tissues from ferret and cat. The **bottom row** consists of *ex vivo* HPAI H5N1 virus—infected human and macaque lung biopsies and lung tissues from HPAI H5N1 virus—infected ferret and cat. **Arrows** indicate examples of influenza virus antigen-positive cells. Original magnification, $\times 400$. HPAI, highly pathogenic avian influenza; MAL-II, *Maackia amurensis* lectin II.

Figure 4 MxA expression in ferret and human lung infected by HPAI H5N1 virus. Serial tissue section of infected ferret lung double stained for influenza virus antigen by immunohistochemistry (A; dark blue; **arrow**) and HPAI H5N1 virus attachment by virus histochemistry (red; **arrowhead**) and stained for MxA antigen by immunohistochemistry (B; red). Infected ferret lung (C–E) double stained for influenza virus antigen by immunohistochemistry (dark blue; **arrow**) and HPAI H5N1 virus attachment by virus histochemistry (red; **arrowhead**), representing a focus with abundant virus antigen expression but no virus attachment (C), an area with neither virus antigen expression nor virus attachment (D), and an area without virus antigen expression but with virus attachment (E). Serial sections (F, G, and H of C, D, and E, respectively) stained for MxA antigen by immunohistochemistry (red). MxA antigen immunohistochemistry (red) in uninfected human lung (I) and HPAI H5N1 virus–infected human lung (J). Original magnification: $\times 200$ (A and B); $\times 400$ (C–J). HPAI, highly pathogenic avian influenza.

prevent superinfection of infected cells, thereby decreasing the chance of reassortments.

A decrease of H5N1 virus receptors on alveolar epithelial cells in close proximity to HPAI H5N1–infected cells has not been described before. Previous studies on the receptor distribution in the lung of HPAI H5N1 virus–infected humans have not described a decrease in the number of HPAI H5N1 virus receptors.^{24,25} However, because these tissues were from fatal cases at the end stage of disease (sometimes even weeks after the onset of clinical symptoms), they might not be representative for events that occur early after infection. Whether the decrease of influenza virus receptors during infection is a common phenomenon of multiple influenza virus subtypes is not known. However, recently the same phenomenon was observed in influenza virus–infected pigs. Infection with either swine influenza virus or avian influenza virus led to a decrease in *Sambucus nigra* lectin, MAL-I, and MAL-II attachment in foci with influenza virus antigen.²⁶

The mechanism behind the decrease of HPAI H5N1 virus receptors on cells in close proximity of HPAI H5N1 virus–infected cells remains to be elucidated. The presence of MxA, which is a marker for type I and type III interferon activity,²⁰ in areas without virus attachment might indicate that the decrease of H5N1 virus receptors is a direct or indirect effect of local interferon. It is unlikely that MxA is directly responsible for the down-regulation of receptors, because its antiviral activity is directed against an early step of viral replication and mainly targets the viral nucleoprotein.²⁷ Besides MxA many other proteins are up-regulated by interferons, such as double-stranded RNA-activated protein kinase and 2',5'-oligoadenylate synthetase that could decrease the receptor expression by blocking cellular gene expression.¹⁵ Another possible mechanism could be that influenza virus receptors are cleaved from the cell surface by the viral neuraminidases of progeny virus particles. More functional studies should unravel the mechanism behind the decrease of receptors on cells in close proximity of infected cells.

It is not known whether the same phenomenon also occurs in other parts of the respiratory tract, such as the URT and the LRT airways (trachea, bronchi). Because HPAI H5N1 virus neither attaches nor replicates efficiently in the URT or LRT airways in humans, macaques, ferrets, and cats,^{4,8,12,17,28} this cannot be studied in a similar experimental approach. Therefore, these studies should be performed with viruses that attach and replicate in the mammalian URT, for example, seasonal influenza viruses that infect the URT of ferrets.^{4,12}

In fatal cases of human HPAI H5N1 virus, the primary lesion is a severe pneumonia, associated with high viral loads in throat swabs and lungs.^{29,30} However, in the lungs of fatal cases of HPAI H5N1 virus, influenza virus antigen expression is observed only in scattered type II pneumocytes in the alveoli.^{10,31,32} The fact that relatively few alveolar epithelial cells are infected, although these cells are exposed to high virus titers, could possibly be explained by the decrease of influenza virus receptors.

In conclusion, we show for the first time that H5N1 virus receptors are decreased on alveolar epithelial cells in humans and three other mammalian species upon virus infection. Possible mechanisms for this decrease in virus receptors are removal of receptors by viral neuraminidase and down-regulation or internalization of receptors mediated by an MxA-induced protein such as interferon.

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