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Human trachea primary epithelial cells express both sialyl(α2-3)Gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl(α2-6)Gal receptor for human influenza viruses

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Abstract We reported previously that the dominant receptors of influenza A and B viruses, and human and murine respiroviruses, were sialylglycoproteins and gangliosides containing monosialo-lactosamine type I- and II-residues, such as sialic acid-α2-3(6)-Galβ1-3(4)-GlcNAcβ1-. In addition, the Siaα2-3Gal linkage was predominantly recognized by avian and horse influenza viruses, and human parainfluenza virus type 1 (hPIV-1), whereas the Siaα2-6Gal linkage was mainly recognized by human influenza viruses (Paulson JC in "The Receptors" [Conn M Ed] 2, 131–219 (1985); Suzuki Y, *Prog Lipid Res* **33**, 429–57 (1994); Ito T, *J Virol* **73**, 6743–51 (2000); Suzuki Y, *J Virol* **74**, 11825–31 (2000); Suzuki T, *J. Virol* **75**, 4604–4613 (2001); Suzuki Y, *Biol. Pharm. Bull.* 28, 399–408 (2005)). To clarify the distribution of influenza virus receptors on the human bronchial epithelium cell surface, we investigated a primary culture of normal human bronchial epithelial (NHBE) cells using two types of lectin (MAA and SNA), which recognize sialyl linkages (α2-3 and α2-6), using fluorescence-activated cell-sorting analysis. The results showed that both α2-3- and α2-6-linked Sias were expressed on the surface of primary human bronchial epithelial cells. The cells infected by hPIV-1 bound to MAA, confirming that cells targeted by hPIV-1 have α2-3-linked oligosaccharides. We also compared the ability of hPIV-1 and human influenza A virus to infect primary human bronchial epithelial cells pre-treated with Siaα2-3Gal-specific sialidase from *Salmonella typhimurium*. No difference was observed in the number of sialidase pre-treated and non-treated cells infected with human influenza A virus, which binds to Siaα2-6Gal-linked oligosaccharides. By contrast, the number of cells infected with hPIV-1 decreased significantly upon sialidase treatment. Thus, cultured NHBE cells showed both α2-3-linked Sias recognized by hPIV-1 and avian influenza virus receptors, and α2-6-linked Sias recognized by human influenza virus receptors.

Keywords Bronchial epithelial cells · Influenza virus · Lectin · Parainfluenza virus · Sialic acid · Sialidase

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Introduction

Sialic acid (Sia) is a generic term for nine-carbon acidic amino sugars (5-amino-3,5-dideoxy-D-glycero-D-galacto-

nonulosonic acids). The amino group is always substituted with either an *N*-acetyl or an *N*-glycolyl group, yielding *N*-acetylneuraminic or *N*-glycolylneuraminic acid, respectively, whereas the hydroxyl groups can be substituted by acetyl, lactoyl, methyl, sulfate or phosphate residues [1]. The distribution of specific Sias varies among animal species.

Influenza viruses specifically recognize oligosaccharides containing a terminal Sia, and the availability of receptors, Sia linked to galactose by an α2,6 linkage (Siaα2-6Gal) and Siaα2-3Gal moieties, in host animals correlates with the receptor specificity of influenza viruses isolated from these species [2]. For example, avian influenza viruses preferentially bind to Siaα2-3Gal-linked oligosaccharides, whereas human influenza viruses preferentially bind to α2-6, rather than α2-3, linkages [3–5]. Thus, target cells for human influenza A virus replication in the human airway epithelium are predicted to express high concentrations of Siaα2-6Gal-containing receptors. Extensive expression of α2-6-linked Sias has been reported on the apical surface of human tracheal epithelial cells [6,7]. By contrast, little is known about the presence of α2-3-linked Sias on these cells.

The human parainfluenza virus type 1 (hPIV-1) preferentially recognizes oligosaccharides containing branched *N*-acetyllactosaminoglycans with terminal Siaα2-3Gal as receptors, and not Siaα2-6Gal-containing sialyloligosaccharides [8]. The target airway cells for hPIV-1 are also thought to express Siaα2-3Gal-linked oligosaccharides on the cell surface as a receptor. Furthermore, human bronchial epithelial cells are reported to consist of two or more groups [9].

In the present study, in order to clarify the distribution of influenza virus and respirovirus receptors on the human bronchial epithelium cell surface, we investigated a primary culture of normal human bronchial epithelial (NHBE) cells with two kinds of lectins, MAA and SNA, using fluorescence-activated cell sorting (FACS) analysis. In addition, we studied the Sia linkages of primary human bronchial epithelial cells infected with human influenza A virus and hPIV-1, using sialidase that originated from *Salmonella typhimurium* LT2, which only cleaves α2-3 linkages.

Materials and methods

Cells

Primary human bronchial epithelial cells were purchased from Cambrex (Walkersville, MD). The culture medium was bronchial epithelial growth media (SAGM; Cambrex) containing bovine pituitary extract (BPE; 30 μg/ml), hydrocortisone (0.5 μg/ml), human epidermal growth factor (hEGF; 0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), triiodothyronine (6.5 ng/ml), bovine serum albumin-fatty-acid free (BSA-FAF; 50 μg/ml), retinoic acid (RA; 0.1 ng/ml), gentamycin (30 μg/ml) and amphotericin B (15 ng/ml). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air.

Lewis lung carcinoma-monkey kidney (LLC-MK2) cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air.

Viruses

The hPIV-1 strain C35 (ATCC VR-94) was obtained from the American Type Culture Collection (Manassas, VA). Confluent monolayers of LLC-MK2 cells were infected with the virus (approximately 10 PFU per cell) in serum-free MEM containing acetylated trypsin (1 μg/ml). Three days after infection, virions were collected from the culture medium. The virus was purified by sedimentation using 50% sucrose gradients [10,11].

The A/Memphis/1/71(H3N2) strain of influenza virus was grown for 2 days in the chorioallantoic cavities of 10-day-old embryonated eggs, and was purified by sucrose-density gradient centrifugation, as described previously [12].

Antibody

Rabbit anti-hPIV-1 antibody was raised by subcutaneous immunization, as described previously [13], and purified using the HiTrap Protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden). Rabbit anti-influenza A virus antibody was raised by subcutaneous immunization, as described previously [14], and was dialyzed.

Detection of α2-6- and α2-3-linked Sias

Primary NHBE cells were harvested and suspended in phosphate-buffered saline (PBS). The cells were incubated for 1 h on ice with digoxigenin-labeled lectins, *Sambucus nigra* agglutinin (SNA; 4 μg/ml; Roche Diagnostics, Mannheim, Germany) [15] specific for α2-6-linked Sias or *Maackia amurensis* agglutinin (MAA; 20 μg/ml) (Roche Diagnostics) [16] specific for α2-3-linked Sias. The cells were incubated for 1 h on ice with fluorescein isothiocyanate (FITC)-labeled anti-digoxigenin antibodies from the digoxigenin-glycan differentiation kit (Roche Diagnostics). FACS analysis of the cells stained with lectins was performed using a FACS Calibur fluorospectrometer (Becton Dickinson, Mountain View, CA).

Sialidase treatment

NHBE cells were cultured in 6 well Multiple well plates (Asahi Techno Glass). Confluent monolayer of NHBE cells in each well in 1.0 ml of medium described above was pretreated with α2-3-sialidase [17] cloned from *S. typhimurium* LT2 (Takara Bio, Shiga, Japan) for 2 h at 37°C by addition of 4 μl of the sialidase solution of 50 U/ml to each well.

Detection of viruses

Sialidase-treated or non-treated primary human bronchial epithelial cells were infected with hPIV-1 or human influenza A viruses. After 16 h, the cells were harvested and suspended in PBS. The cells were incubated for 1 h on ice with anti-hPIV-1 or anti-influenza antibodies. The cells were incubated for 1 h on ice with phycoerythrin (PE)-labeled goat anti-rabbit immunoglobulin G (IgG) antibody (Biogenesis, England, UK). FACS analysis of the stained cells was performed using a FACS Calibur fluorospectrometer (Becton Dickinson).

Results and discussion

Analysis of sialyl-linkage expression on the primary human bronchial epithelial cell surface using lectins

Human influenza viruses isolated from Madin-Darby canine kidney (MDCK) cells bind the Siaα2-6Gal sequence, whereas avian and equine viruses predominantly bind α2-3 linkages [18–20]. hPIV-1 also preferentially binds α2-3-linked oligosaccharides, and not α2-6 linkages [8]. By contrast, swine viruses bind both α2-6 and α2-3 linkages, either equally or with a preference for α2-6 linkages [18–20]. Human trachea cells express Siaα2-6Gal [6] and pig trachea cells express both α2-3 and α2-6 linkages, whereas cells of the horse trachea and duck intestinal mucosa express α2-3 linkages [1,18,19,21,22].

In 1997, an H5N1 avian influenza A virus was transmitted directly from chickens to humans in Hong Kong, China, killing six of the 18 people infected [23–27]. Human-to-human transmission of this virus appeared to be rare; isolates from this strain contained only avian genes, which might have been one reason for the ineffective transmission to humans. The hemagglutinin and receptor-binding specificity of this highly pathogenic H5N1 avian influenza virus was reported to be due to an avian type (Siaα2-3Gal-specific) gene [28]. Since December 2003, the virus has spread throughout many Asian countries, including China, South Korea, Japan, Thailand, Vietnam, Indonesia, Malaysia, Cambodia and Laos [29]. By November 2004, at least 12 and 20 people had died in Thailand and Vietnam, respectively, as a result of direct exposure to high concentrations of the virus. These results suggest that the human airway epithelial cells express not only α2-6- but also α2-3-linkage-containing receptors. In addition, Matrosovich *et al.* [30] and our group [31] demonstrated that some strains of the H9N2 influenza virus isolated from quail had acquired a preference for binding to Neu5Ac2-6Gal receptors, similar to the human strain, indicating that this virus has the potential for human-to-human transmission, although no such case has yet been reported.

In order to examine the distribution of these two structures on primary human bronchial epithelial cells using FACS analysis, we employed two sialyl linkage-specific lectins: the SNA lectin, which recognizes Sias that are linked to galactose by α2-6 linkages [15], and the MAA lectin, which is specific for Sias with α2-3 linkages [16]. FACS analyses of the Sia linkages on primary human bronchial epithelial cell surfaces using these lectins showed a distinct right-shift in the median of the distributions compared with the controls (Figure 1). These data demonstrate that both α2-3- and α2-6-linked Sias are expressed on the surface of primary human bronchial epithelial cells. The staining of the primary cells with FITC-conjugated SNA and MAA was also confirmed using fluorescent microscopy (data not shown).

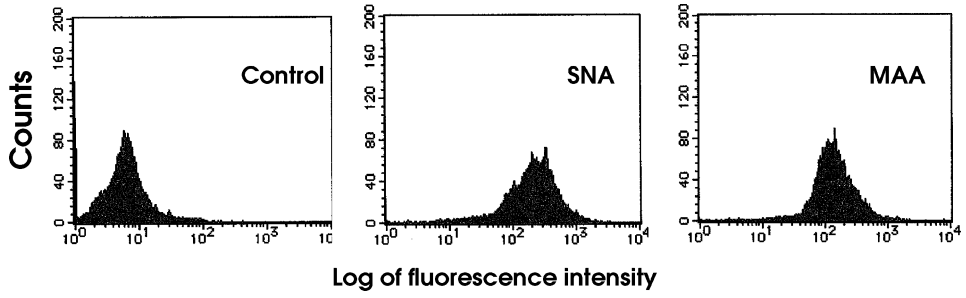

Fig. 1 Comparison of the relative amounts of α2-6 and α2-3 linkages on the surface of NHBE cells. Digoxigenin-labeled lectins (SNA specific for α2-6 linkages and MAA specific for α2-3 linkages) were incubated with the cells for 1 h. The cells were then incubated with anti-digoxigenin antibody conjugated to fluorescein isothiocyanate for 1 h, and analyzed for fluorescence intensity using a FACSCalibur fluorospectrometer. The profile shows cell number as a function of log fluorescence intensity of α2-6- and α2-3-specific lectin-reactive oligosaccharides on the cell surface

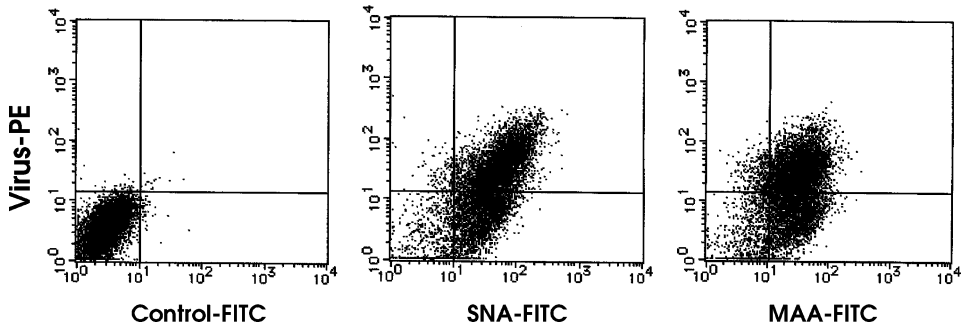

Fig. 2 Expression of viral antigens, α2-6 and α2-3 linkages on the surface of NHBE cells after infection with hPIV-1. NHBE cells were infected with hPIV-1 for 16 h. The cells were incubated for 1 h with anti-hPIV-1 antibody and then incubated for 1 h with phycoerythrin (PE)-labeled goat anti-rabbit IgG antibody. The cells were then incubated with digoxigenin-labeled lectins, SNA or MAA, followed by FITC-labeled anti-digoxigenin antibodies. FACS analysis of the cell staining was performed using a FACSCalibur fluorospectrometer. The intensity of the virus staining is shown on the *y*-axis and the intensity of the Sia staining is shown on the *x*-axis

Double-fluorescent analysis of sialyl-linkage expression on the cells targeted by hPIV-1 was performed using FACS (Figure 2). All of the cells that were infected with hPIV-1 were stained with MAA; therefore, the cells that were targeted by hPIV-1 had α2-3-linked oligosaccharides. These cells also bound to SNA, indicating that they had not only α2-3- but also α2-6-Sia linked to oligosaccharides.

FACS analysis using the double-staining method revealed that the primary NHBE cells stained for both human influenza A virus and hPIV-1 when they were simultaneously infected with both viruses (data not shown).

A recent study involving cultures of differentiated human airway epithelial cells demonstrated that influenza viruses entered the airway epithelium through specific target cells, and revealed striking differences between human and avian viruses. During the course of a single-cycle infection, human viruses preferentially infected non-ciliated cells, whereas avian viruses (as well as an egg-adapted human virus variant with an avian virus-like receptor specificity) mainly infected ciliated cells. This pattern correlated with the preferential localization of receptors for human viruses (α2-6-linked Sias) on non-ciliated cells, and that of receptors for avian viruses (α2-3-linked Sias) on ciliated cells [32]. Our data indicate that primary human bronchial epithelial cells have both types of sialyl sugar chains, as receptors for hPIV-1 and avian influenza viruses (α2-3-linked Sias), and human influenza A viruses (α2-6-linked Sias).

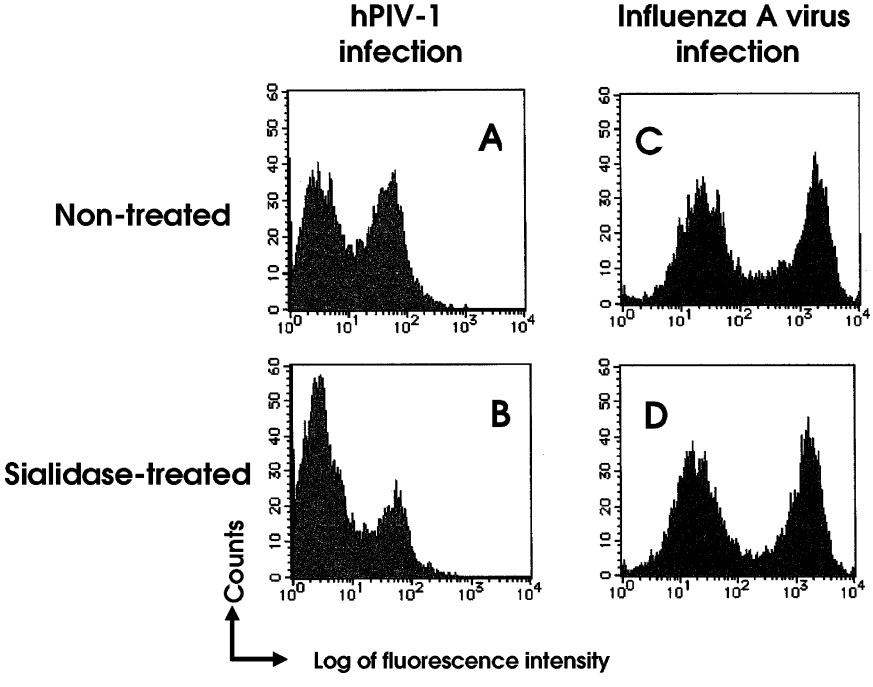

Fig. 3 Infectivity of hPIV-1 and influenza A virus to sialidase-treated NHBE cells. NHBE cells were treated with *S. typhimurium* sialidase. The sialidase-treated cells (B and D) were inoculated with hPIV-1 or influenza A virus for 12 h. As controls, native cells (A and C) were also inoculated with each virus. The anti-virus antibodies were added and incubated on ice for 1 h. The cells were incubated with anti-rabbit Igs conjugated with PE on ice for 1 h. The fluorescence intensities of the cells were analyzed using FACS

Effect of pre-treatment of sialidase originating from *S. typhimurium* LT2 on hPIV-1 infection in a primary culture of NHBE cells

hPIV-1 preferentially recognizes oligosaccharides containing branched *N*-acetyllactosamino-glycans with terminal Siaα2-3Gal as receptors, but not those with α2-6-linked sialyl sugar chains [8]. In order to investigate whether α2-3-linked oligosaccharides on the surface of primary human bronchial epithelial cells are involved in hPIV-1 infection, we compared the infection rate of hPIV-1 and human influenza A virus in primary human bronchial epithelial cells that were pre-treated with α2-3-specific sialidase from *S. typhimurium*. No difference was observed in the number of cells infected with human influenza A virus, which is known to bind to α2-6-linked oligosaccharides, between sialidase pre-treated and non-treated cells (Figure 3). However, the number of cells infected with hPIV-1 decreased significantly upon treatment with α2-3-specific sialidases. These results indicate that primary human bronchial epithelial cells have α2-3-linked Sias that are recognized as receptors by hPIV-1.

In conclusion, primary NHBE cells have Siaα2-3Gal-linked oligosaccharides, to which hPIV-1, avian and horse influenza viruses bind as receptors. In addition, they also have α2-6-linked receptors for human influenza A viruses.

Despite the apparent common origin of influenza A viruses, their host range is clearly restricted. In experimental infections, avian influenza viruses replicated poorly in primates [32–35], and human isolates did not replicate efficiently in ducks [36–38]. However, the direct transmission of a highly pathogenic avian influenza virus, H5N1, to humans was reported in Hong Kong in 1997 [24,25], and also in Thailand and Vietnam during 2004–2005 [33,39]. The results of the present paper suggest that direct exposure of human trachea epithelial cells to high concentrations of H5N1 can mediate direct infection of the avian virus in humans though Siaα2-3Gal receptors in the trachea.

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