Acta virologica **55**: 147 – 153, 2011 doi:10.4149/av_2011_02_147

Overexpression of α-2,6-sialyltransferase stimulates propagation of human influenza viruses in Vero cells

N. LI[1], Y. QI[1], F.Y. ZHANG[2], X.H. YU[1], Y.G. WU[1], Y. CHEN[1], C.L. JIANG[1*], W. KONG[1*]

[1]National Engineering Laboratory of AIDS vaccine, Jilin University, Changchun 130021, P.R. China; [2]Changchun BCHT biotechnology Co., Ltd., Changchun 130021, P.R. China

Received December 16, 2010; accepted May 9, 2011

Summary. – Human influenza viruses are major concern as the leading cause of global pandemics. In infecting cells, they preferentially bind to sialyloligosaccharides containing terminal *N*-acetyl sialic acid linked to galactose by an α-2,6-linkage (NeuAcα2,6Gal). The amount of NeuAcα2,6Gal in Vero cells, which are predominantly used for production of influenza vaccines over the past 30 years, may not be as high as that in epithelial cells of human respiratory tract, what leads to the suboptimal virus growth in Vero cells. In this study, we stably transfected Vero cells with cDNA of human α-2,6-sialyltransferase (SIAT1), an enzyme catalyzing α-2,6-sialylation of galactose on glycoproteins. Overexpression of SIAT1 in the transfected Vero cells (Vero-SIAT1 cells) was confirmed by Western blot analysis and immunofluorescence microscopy. Vero-SIAT1 cells expressed 7 times higher amounts of NeuAcα2,6Gal, but 3 times lower amounts of NeuAcα2,3Gal as compared to parental Vero cells. Furthermore, the influenza viruses A (H1N1 and H3N2) and B grew in Vero-SIAT1 cells to the higher titers than in Vero cells. Taken together, these results imply that Vero-SIAT1 cells are useful not only for the propagation of human influenza viruses, but also for the preparation of influenza vaccines.

Keywords: influenza virus; α-2,6-sialyltransferase; Vero cells

Introduction

Influenza epidemics continue to impose a significant threat on the world's population in terms of hospitalizations and deaths (Harper *et al*., 2004; Thompson *et al*., 2004). So, choice of a cell line for propagation of the influenza viruses is an essential tool for the production of influenza vaccine (Youil *et al*., 2004). Historically, embryonated chicken eggs have been used to propagate influenza viruses and they are still used by most manufacturers to produce influenza vaccine (Belsey *et al*., 2006). Infection of continuous cell line (CCL) with influenza virus has provided an alternative to the embryonated egg inoculation (Govorkova *et al*., 1999). In addition, propagation of influenza viruses in the cell culture generally results in a homogeneous virus preparation with respect to hemagglutinin (HA) gene compared to the virus propagation in eggs (Katz *et al*., 1990; Robertson *et al*., 1990).

Influenza A and B viruses bind to sialyloligosaccharides present on the host cell surface glycolipids or glycoproteins. Human influenza viruses preferentially bind to the NeuAcα2,6Gal, while avian influenza viruses mainly bind to the NeuAcα2,3Gal (Connor *et al*., 1994). Human epithelial cells in respiratory tract mainly contain NeuAcα2,6Gal and duck intestine epithelial cells mainly possess NeuAcα2,3Gal (Couceiro *et al*.,1993; Matrosovich *et al*., 2004). Thus, enhanced α-2,6-linked receptor levels should increase the number of interactions between human influenza virions and the cell surface and in this way to increase the avidity

*Corresponding authors. E-mail: weikong@jlu.edu.cn, jiangcl@jlu.edu.cn; fax: +86-431-85195516.

Abbreviations: BCIP = 5-bromo-4-chloro-3-indolyl-phosphate; CCL = continuous cell line; HA = hemagglutinin; MAA = Maackia amurensis agglutinin; NBT = 4-nitroblue tetrazolium chloride; NeuAcα2,6Gal = *N*-acetyl sialic acid linked to galactose by an α-2,6-linkage; NeuAcα2,3Gal = *N*-acetyl sialic acid linked to galactose by an α-2,3-linkage; SIAT1 = human 2,6-sialyltransferase; SNA = Sambucus nigra agglutinin

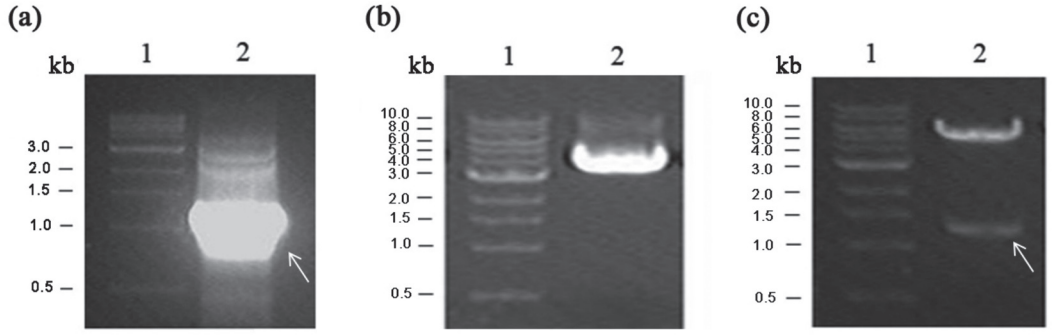

Fig. 1

Construction of plasmid pcDNA3.1-SIAT1-Myc expressing SIAT1

Agarose electrophoresis. (a) PCR products with pSIAT1-Myc (lane 2), (b) PCR products with pcDNA3.1-SIAT1-Myc (lane 2), (c) products of *Not*I and *Bam*HI digestion of pcDNA3.1-SIAT1-Myc (lane 2). 1 kb ladder (lanes 1).

of virus binding (Matrosovich *et al*., 2003; Hatakeyama *et al*., 2005).

Vero cell line is the most widely accepted CCL by regulatory authorities and has been used for over 30 years for the production of polio and rabies virus vaccines. Vero cell-based vaccines are licensed in Europe and currently, Vero cell-based H5N1 vaccine is undergoing clinical trials. These developments have illustrated the value of this cell culture platform in the rapid development of vaccines against a range of virus diseases (Barrett *et al*., 2009).

Although NeuAcα2,6Gal is present in Vero cells, its amount may not be as high as that of epithelial cells in human respiratory tract leading to a suboptimal growth of virus in these cells (Govorkova *et al*., 1996). To test whether overexpression of NeuAcα2,6Gal in cells could make them more effective for the propagation of human influenza viruses, we permanently transfected Vero cells with the gene of SIAT1, an enzyme that catalyzes the α-2,6-sialylation of *N*-acetyllactosamine moieties of glycoproteins and glycolipids (Matrosovich *et al*., 2003).

Based on previous reports on successful overexpression of SIAT1 in mammalian cells, we assumed that the SIAT1 overexpression would increase α-2,6-sialylation, but decrease α-2,3-sialylation due to a competition between respective enzymes for the same substrate (Dall'Olio *et al*., 1995; Breen *et al*., 1998; Fukuta *et al*., 2000; Lin *et al*., 2002; Matrosovich *et al*., 2003).

In this study, we attempted to establish a Vero cell line stably expressing SIAT1, to determine its α-2,6-sialylation and α-2,3-sialylation activities, and to examine its capacity to propagate influenza A (H1N1 and H3N2) and B viruses. The obtained results confirmed our expectations by showing that the established Vero cell line stably expressing SIAT1 was able (i) to produce 7 times higher amounts of NeuAcα2,6Gal, but 3 times lower amounts of NeuAcα2,3Gal and (ii) to propagate influenza viruses A (H1N1 and H3N2) and B to the higher titers as compared to parental Vero cells.

Materials and Methods

Cells. Vero cells were purchased from the American Type Culture Collection and passaged using standard laboratory technique. The cells were maintained in MEM containing 10% FCS at 37°C in a humidified tissue-culture incubator equilibrated with 95% air and 5% CO_2.

Viruses. The following egg-derived influenza virus vaccine strains were kindly provided by the National Institute for Biological Standards and Control (NIBSC, UK): A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004 (B).

Plasmid construct. The cDNA clone of human SIAT1 in the pBluescript II SK(+) vector was synthesized by ShineGene (Shanghai, China). The full-length ORF of SIAT1 was PCR amplified with primers 5′-GCGGCCGCGCCACCATGATTCACACCAACCTG-3′ and 5′-GGATCCTTAAAGATCTTCTTCTGATATGAGTTTTTG TTCGCAGTGAATGGTCCG-3′, containing restriction sites for *Not*I and *Bam*HI, respectively, and Myc epitope sequence downstream of the *Bam*HI site. The PCR product was cloned into the pcDNA3.1(-) expression vector (Invitrogen) carrying neomycin resistance gene between *Not*I and *Bam*HI restriction sites resulting in pcDNA3.1-SIAT1-Myc (Fig. 1).

Stable transfection of Vero cells. Vero cells were transfected with pcDNA3.1-SIAT1-Myc plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer′s instructions. Briefly, Vero cells were transfected and incubated in MEM with 10% FCS for 4 hrs before replacing cultivation medium with a MEM supplemented with 10% FCS containing 1 mg/ml of antibiotic G418 sulfate (Promega). The cells were fed with selective medium every 3–4 days until G418-resistant foci can

be identified. Then G418-resistant clones were isolated and transferred to 24-well plates. Further, each clone was passaged 3–5 times in the presence of 1 mg/ml of G418. Final plating of the cells for infection experiments and lectin staining were performed in medium without G418. The cells in these experiments were used at passage levels from 5 to 30 counted from the frozen cell stock.

Western blot analysis. Protein extract of Vero cells was prepared by a modified RIPA buffer (50 mmol/l Tris/HCl, pH 8.0, 250 mmol/l NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, complete mini protease inhibitors (Roche)). Then, PAGE and blotting to the Immobilon Hybond-C membranes (Amersham Biosciences) together with immunodetection were performed using standard techniques. Anti-tubulin (Covance) and anti-Myc (Millipore) antibodies were used in the immunodetection. To examine the levels of NeuAcα2,3Gal and NeuAcα2,6Gal, we used digoxigenin-labeled lectins as Sambucus nigra agglutinin (SNA) specific for NeuAcα2,6Gal and Maackia amurensis agglutinin (MAA) specific for NeuAcα2,6Gal (digoxigenin-glycan differentiation kit, Roche). Anti-digoxigenin alkaline phosphatase-conjugated antibody was used as a secondary antibody. Immunoreactive complexes were visualized using NBT/BCIP reaction. Densitometric analysis was assessed by BandScan 4.30 software.

Immunofluorescence microscopy. Vero cells were seeded on glass coverslips and grown for 48 hrs. Then, they were fixed in 4% paraformaldehyde for 30 mins at RT and permeated with 0.5% Triton X-100 in PBS for 30 mins. The coverslips were washed 3 times in PBS for 5 mins, incubated in 1% BSA in PBS for 30 mins to block unspecific reaction and stained with rhodamine-conjugated phalloidin (1 μg/ml) for 2 mins. After 3 washes with PBS, the coverslips were mounted and observed with a Nikon Eclipse E600 fluorescence microscope (40× magnification).

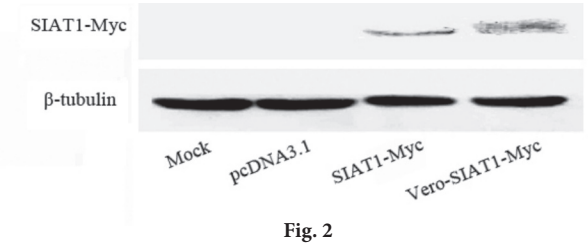

Fig. 2

Western blot analysis of SIAT1 expression in Vero-SIAT1 cells

Vero cells transfected with pcDNA3.1-SIAT1-Myc or empty vector pcDNA3.1 and mock-transfected Vero cells. SIAT1-Myc represents transiently transfected Vero cells and Vero-SIAT1-Myc represents stably transfected Vero cells. β-tubulin was used as an internal loading control.

Plaque titration. After virus infection of Vero cells, once plaques were clearly visible, the agar overlay was carefully removed and the cells stained with 0.1% neutral red solution. Plaque titers were determined as PFU/ml (Youil *et al.*, 2004).

Statistical analysis. Each experiment was performed at least three times. All values were presented as means ± SD. Differences between experimental groups and controls were assessed by Student's t-test using Excel software. $P \leq 0.05$ was considered statistically significant.

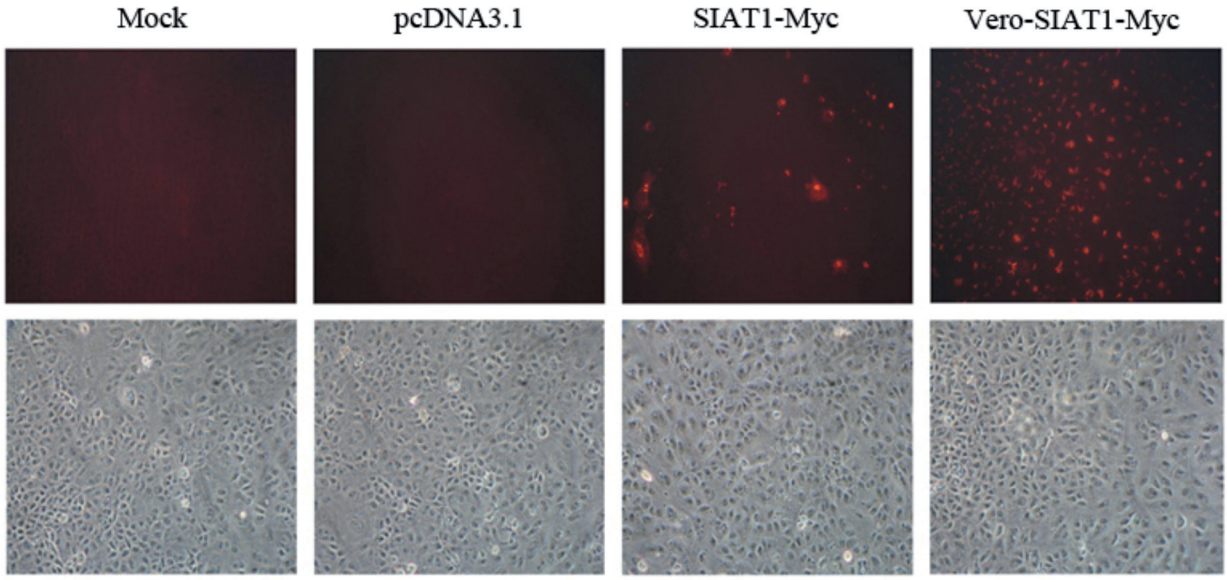

Fig. 3

Fluorescence of SIAT1 expression in Vero-SIAT1 cells

Fluorescent (upper row) and light microscopy (lower row). Description of samples – see legend to Fig. 2. Representative images of three independent experiments are shown.

Results

Establishment of Vero-SIAT1 cell line stably expressing SIAT1

To obtain a cell line constitutively expressing a high level of SIAT1 protein, we stably transfected Vero cells with pcDNA3.1-SIAT1-Myc (Vero-SIAT1-Myc) and selected the cell clones resistant to the G418 sulfate. In order to investigate the consequences of SIAT1 expression, we transiently transfected Vero cells for 48 hrs with blank control MEM (mock), negative control pcDNA3.1(-) vector (pcDNA3.1), and positive control pcDNA3.1-SIAT1-Myc (SIAT1-Myc) . The positive control SIAT1-Myc could only transiently express SIAT1 protein. All these cells were analyzed by Western blot and immunofluorescence microscopy using an anti-Myc antibody (Figs 2, 3). The results showed that one cell clone was able to stably express SIAT1 protein at a high level compared to the mock and pcDNA3.1 transfected cells.

α-2,6- and α-2,3-sialylation activities of Vero-SIAT1 cells

To compare the expression of NeuAcα2,3Gal and NeuAcα2,6Gal on the surface of transfected and parental cells, we used the linkage-specific lectins SNA and MAA. Western blot analysis indicated that transfected Vero-SIAT1 cells expressed 7 times higher amounts of NeuAcα2,6Gal and 3 times lower amounts of NeuAcα2,3Gal than parental Vero cells (Fig. 4). By contrast, in the positive control SIAT1-Myc cells, the reactivity with SNA and MAA did not show any difference in the amount of of NeuAcα2,6Gal and NeuAcα2,3Gal receptors compared to the mock or pcDNA3.1 transfec-

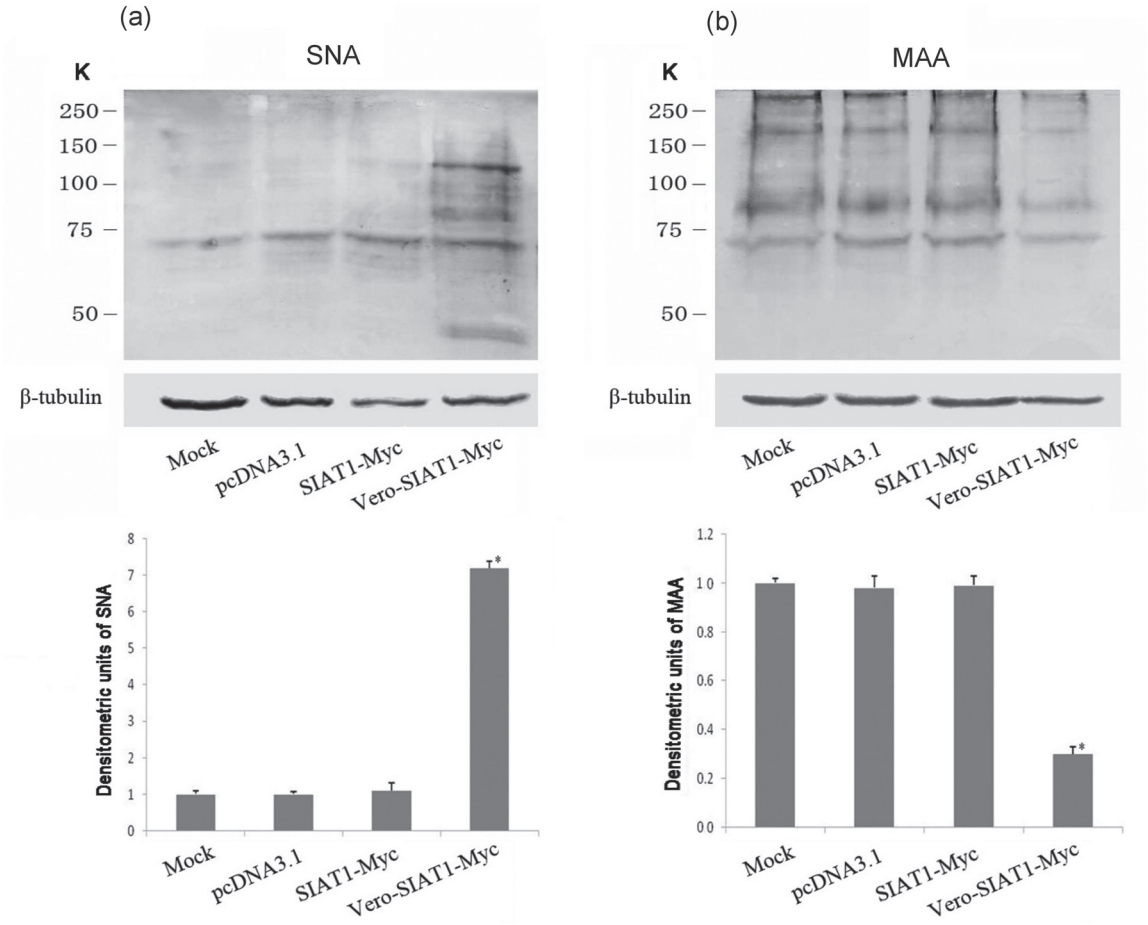

Fig. 4

α-2,6- and α-2,3-sialylation activities of Vero-SIAT1 cells

Western blot analysis of α-2,6-(SNA) and α-2,3-(MAA) sialylation activities in Vero-SIAT1 cells. Description of samples – see legend to Fig. 2. Protein size markers are on the left. Densitometry of the SNA and MAA bands in Western blot analysis (lower row). Data are means of three separated experiments ± SD. *Significant difference (P <0.05).

tion in Vero cells. This outcome was conceivable, since the cells expressed a low level of SIAT1 protein after 48 hrs of transient transfection (Figs 2, 3). The presented pattern of reactivity with lectins remained unchanged after 20 passages of Vero-SIAT1 cells in the presence of G418 indicating that the sialylation phenotype of the cells was stable (data not shown).

Propagation of influenza viruses in Vero-SIAT1 cells

To test the influence of a higher expression level of NeuAcα2,6Gal receptor on the cell susceptibility to the human influenza viruses, we performed a plaque assay using 3 human influenza viruses H1N1, H3N2, and type B that were previously egg-passaged. All of these viruses multiplied to the higher titers in Vero-SIAT1 cells than in the parental Vero cells (Table 1). For example, H1N1 virus showed 4.9 times higher titer in the Vero-SIAT1 cells than in Vero cells (Fig. 5). These data indicated that Vero-SIAT1 cells were more suitable than parental Vero cells for the propagation of human influenza viruses previously passaged in embryonated eggs.

Table 1. Growth of human influenza viruses in Vero-SIAT1 and Vero cells

Virus	Titer (PFU/ml) [a] in cells		Ratio of titers Vero-SIAT1/ Vero
	Vero-SIAT1	Vero	
A/New Caledonia/20/99 (H1N1)	5.4×10^7	1.1×10^7	4.9
A/Wisconsin/67/2005 (H3N2)	3.7×10^7	9.0×10^6	4.1
B/Malaysia/2506/2004	3.3×10^6	1.2×10^6	2.8

[a]The virus titer in each cell line was determined by the plaque assay.

Discussion

Influenza is a serious infectious disease that threatens human health. It affects individuals of all ages and is responsible for the recurrent seasonal epidemics as well as periodic global pandemics of varying severity (Harper *et al.*, 2004; Thompson *et al.*, 2004). Human influenza virus has drawn our attention due to its high mortality. An effective way to prevent influenza pandemics is the vaccination of human

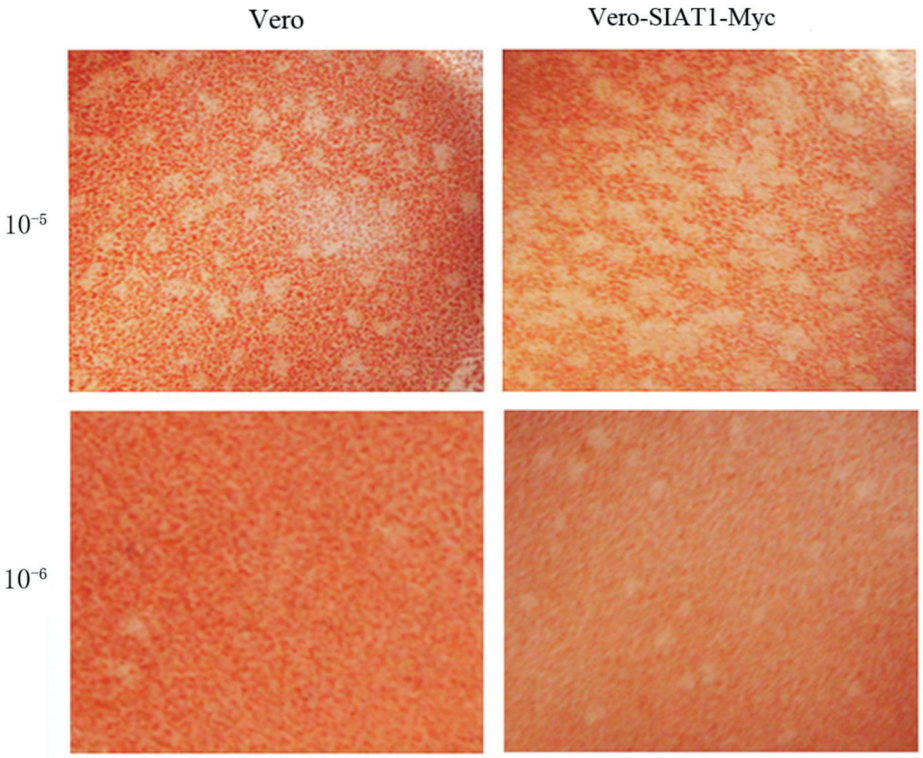

Fig. 5

Plaques of human influenza A virus (H1N1) on Vero and Vero-SIAT1 cells

10^{-5} and 10^{-6} – dilutions of virus inoculum.

population. Successful production of influenza vaccines depends on the selection of cell substrate that provides a high viral yield. Over the past 30 years, Vero cells have been widely used for a human vaccine production and they are the only CCL fully accepted by regulatory authorities for the production of whole virus vaccines.

Human influenza virus receptor is NeuAcα2,6Gal. Although this receptor is present in Vero cells, its amount may not be as high as that of epithelial cells in human respiratory tract, what leads to a suboptimal virus growth in Vero cells (Govorkova *et al.*, 1996). This study was undertaken to examine, whether a genetically engineered increase in the density of human influenza virus receptors on the cell surface can result in the increased virus attachment to these cells. To address this question, we established Vero-SIAT1 cell line stably expressing SIAT1.

This is the first study to show that stable transfection with the SIAT1 gene increased the concentration of NeuAcα2,6Gal receptors on the surface of Vero cells about 7 times. This effect is consistent with the enhanced α-2,6-sialylation proceeding in several other mammalian cell lines transfected with SIAT1 (Dall´Olio *et al.*, 1995; Breen *et al.*, 1998; Fukuta *et al.*, 2000; Lin *et al.*, 2002; Matrosovich *et al.*, 2003). Stable cells lines do not adequately mimic epithelial cells *in vivo,* since they maintain a lower concentration of the NeuAcα2,6Gal and a higher concentration of the NeuAcα2,3Gal receptors. However, as we expected, the concentration of NeuAcα2,3Gal in Vero-SIAT1 cells decreased by about 70%.

We speculate, therefore, that all human influenza viruses irrespective of their type and subtype will likely display an enhanced binding to the Vero-SIAT1 cells. Here, we showed that Vero-SIAT1 cells were able to support higher levels of virus growth of influenza A (H1N1 and H3N2) and B viruses than the parental Vero cells. This effect is consistent with enhanced α-2,6-sialylation in MDCK cell line after its transfection with SIAT1. Previous studies have indicated that human influenza virus was bound more strongly to the transfected MDCK cells than to the parental MDCK cells (Matrosovich *et al.*, 2003; Hatakeyama *et al.*, 2005). This result confirmed that overexpression of SIAT1 enhanced the concentration of NeuAcα2,6Gal receptors accessible to the human influenza A and B viruses on the cell surface (Gambaryan *et al.*, 1997; Matrosovich *et al.*, 2003).

In summary, we were able to create a spectrum of the sialic acid receptors present on the surface of Vero cells by the transfection of cells with SIAT1 gene. Consequently, the transfected cells were more similar to the epithelial cells of human respiratory tract. This study demonstrated that Vero-SIAT1 cells are a promising system for the propagation of human influenza viruses and production of influenza vaccine. Thus, these cells provide a promising alternative to the currently used egg-based influenza vaccine production. Furthermore, this system might facilitate further studies of the virus-host interactions and viral pathogenesis.

Acknowledgement. This study was supported by the grant 2008ZX10001-012 from the National Grand program on Key Infectious Disease.

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