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ORIGINAL RESEARCH

The expression of essential components for human influenza virus internalisation in Vero and MDCK cells

Maharani Ugiyadi · Marselina I. Tan · Ernawati A. Giri-Rachman · Fawzi R. Zuhairi · Sony H. Sumarsono

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Abstract MDCK and Vero cell lines have been used as substrates for influenza virus replication. However, Vero cells produced lower influenza virus titer yield compared to MDCK. Influenza virus needs molecules for internalisation of the virus into the host cell, such as influenza virus receptor and clathrin. Human influenza receptor is usually a membrane protein containing Sia(α2,6) Gal, which is added into the protein in the golgi apparatus by α2,6 sialyltransferase (SIAT1). Light clathrin A (LCA), light clathrin B (LCB) and heavy clathrin (HC) are the main components needed for virus endocytosis. Therefore, it is necessary to compare the expression of SIAT1 and clathrin in Vero and MDCK cells. This study is reporting the expression of SIAT1 and clathrin observed in both cells with respect to the levels of (1) RNA by using RT-PCR, (2) protein by using dot blot analysis and confocal microscope. The results showed that Vero and MDCK cells expressed both SIAT1 and clathrin proteins, and the expression of SIAT1 in MDCK was higher compared to Vero cells. On the other hand, the expressions of LCA, LCB and HC protein in MDCK cells were not significantly different to Vero cells. This result showed that the inability of Vero cells to internalize H1N1 influenza virus was possibly due to the lack of transmembrane protein receptor which contained Sia(α2,6) Gal.

Keywords Clathrin · MDCK cells · Sialyltransferase · Vero cells

M. Ugiyadi (✉)
Research and Development Division, Bio Farma, Jl. Pasteur No.28, Bandung 40161, Indonesia
e-mail: m.rani@biofarma.co.id

M. I. Tan · F. R. Zuhairi · S. H. Sumarsono
Research Group of Physiology, Developmental Biology, and Biomedical Science, School of Life Science and Technology, Institut Teknologi Bandung, Jl. Ganesha No.10, Bandung 40132, Indonesia
e-mail: marsel@sith.itb.ac.id

F. R. Zuhairi
e-mail: fawzi.zuhairi@gmail.com

S. H. Sumarsono
e-mail: sonyheru@sith.itb.ac.id

E. A. Giri-Rachman
Research Group of Microbiology, Genetics, Biology Molecular, School of Life Science and Technology, Institut Teknologi Bandung, Jl. Ganesha No.10, Bandung 40132, Indonesia
e-mail: erna_girirachman@yahoo.com

Introduction

Vero and MDCK cell lines are the most widely used subtrates for influenza virus replication. These mammalian cells are able to produce a consistent yield of virus and minimize any adventitious agent contamination. Vero cell are considered as a safe cell line for biological production. This cell line was derived from African Green Monkey kidney and has been used extensively for viral vaccine production. Vero cells have

been well characterized and licensed by World Health Organization (WHO) whereas MDCK derived from Madine Darby Canine Kidney has not (Patriarca 2007).

Unfortunately, the normal Vero cells is not a potential substrate to produce a high yield influenza virus compared to MDCK cells (Zuhairi et al. 2012). Therefore it is necessary to observe supporting components for influenza virus internalisation in Vero cells to be able to determine the most essential substances for influenza virus internalisation. Virus receptor is the essential component needed for internalisation by the host cell. Hemaglutinin A (HA) molecule in human influenza binds to transmembrane protein contains Sia(α2,6) Gal (Gambaryan et al. 2002). It has been reported that Vero cells contain two types of influenza binding receptors, i.e.: 93 % Sia(α2, 3) Gal and 21 % Sia(α2, 6) Gal (Govorkova et al. 1996). The enzyme that is responsible for sialic acid transfer to the acceptor oligosaccharides is α2, 6 sialyltransferase (Paulson and Colley 1989).

The influenza virus enters the cells via receptor-mediated endocytosis by constructing formation of clathrin coated pits (CCP) at the site of virus binding. Ninety-four percent (94 %) influenza virus internalisation occurs through CCP (Rust et al. 2004). Generally, CCP is pinched off to form clathrin coated vesicle (CCV). CCV is a component that is responsible for substances transferring and exchanging between specific membranes. Clathrin protein that is involved in CCP and CCV is constructed from a three-arm structure called triskelion. Each triskelion is composed by large polypeptides as heavy chains and small polypeptides as light chains that assemble into basket-like cages (Alberts et al. 2008; McMahon and Boucrot 2011). Cells that have sufficient polypeptides are expected to have an efficient endocytosis.

This study tested the hypothesis that the inability of Vero cells to internalize H1N1 human influenza was due to the lack of SIAT1, light clathrin A (LCA), light clathrin B (LCB) or heavy clathrin (HC) compared to MDCK cells. In the present study we examined the expression of SIAT1, LCA, LCB and HC in MDCK and Vero cells in order to obtain the natural profile of both cell types. Analysis is performed on RNA and protein levels. The objectives of the experiments were to find out whether or not Vero cell had different expression of SIAT1, LCA, LCB and HC compared to MDCK, as a reason for having lower susceptibility to influenza virus.

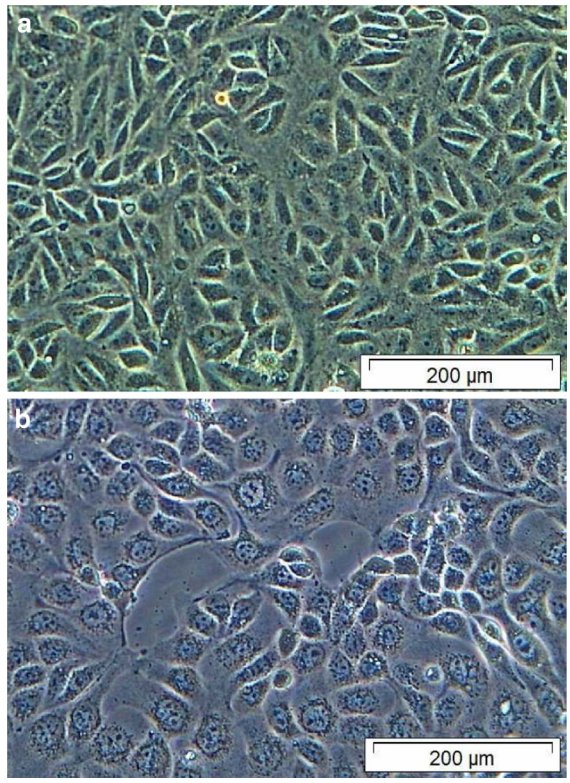

Fig. 1 Monolayered Vero cell culture (**a**) and MDCK cell culture (**b**)

Materials and methods

Cells

The Vero cell line was obtained from the ATCC (Manassas, VA, USA) and the MDCK cell line was obtained from Avian Influenza Research Center (Airlangga University, Surabaya, Indonesia). Vero cells were cultivated in Dulbecco's Modified Eagle Medium (MP Biomedicals LLC, Santa Ana, CA, USA) containing 10 % Foetal Bovine Serum (Life Technologies, Carlsbad, CA, USA) Erythromycin (Meiji, Tokyo, Japan), Kanamycin (Sigma Aldrich, St. Louis, MO, USA) and Sodium bicarbonate (Merck Millipore, Darmstadt, Germany), and incubated at 37 ± 1 °C. MDCK cells were cultivated in Minimum Essential Media (Life Technologies, Grand Island, NY, USA) containing 10 % FBS, Erythromycin, Kanamycin and Sodium bicarbonate, and incubated at 37 ± 1 °C. Vero and MDCK cells used in this study were confluent monolayers of cells that were analyzed five days after plating on cell culture plates (Fig. 1a, b).

RT-PCR

The expression of SIAT1, LCA, LCB, and HC RNA was determined by RT-PCR using Sensiscript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) and HotStarTaq Plus Master Mix Kit (Qiagen GmbH). The RNA from approximately 1.8×10^7 cultured Vero cells and 1.2×10^7 cultured MDCK cells was isolated using an RNeasy minikit (Qiagen GmbH). The RNA amount used for RT-PCR was <50 ng. RT-PCR was conducted in a two-step reaction; reverse-transcription followed by PCR. The mRNA expression levels were identified by the RT-PCR products using gel electrophoresis.

SIAT1, LCA, LCB dan HC primers used for PCR were designed from the similar genes of *Macaca mulatta* from National Center of Biotechnology Informations (NCBI). The forward primer for the SIAT1 gene was (5'-CCTGGTCTTTCTTCTGTTTG-3') and the reverse primer was (5'-CCTCTACCATGGATAC ATTC-3'); these primers amplified a 422 bp gene fragment. The forward primer for the LCA gene was (5'-CAGCAGGAGAGCGAAATTGCGGGCATCGA G-3') and the reverse primer was (5'-CCGAGAATT GGCATCAAGGGCTTCCAAGCG-3'); these primers amplified a 273 bp gene fragment. The forward primer for the LCB gene was (5'-GAGATTGCAGGCATA GAGAACGACC-3') and the reverse primer was (5'-CTTCTCTACTTGTTCACTCTGGCGC-3'); these primers amplified a 348 bp gene fragment. Finally, the forward primer for the HC gene was (5'-CCAGCT CCAGAACCTGGGTATCAAC-3') and the reverse primer was (5'-GGCTGAGACTCTCCTTCCATA CTC C-3'), for a 375 bp amplicon.

Agarose gel electrophoresis of DNA

The PCR products (8 µl each) were subjected to electrophoresis in 1 % agarose (GelPilot LE Agarose, Qiagen GmbH) gels dissolved in 40 ml of 1X TAE buffer (Buffer TAE, Qiagen GmbH) with 1 µl of ethidium bromide (stock 10 mg/ml). The gels were submerged in 1X TAE buffer in the electrophoresis chamber, and a dye solution (Blue/Orange Loading Dye, Promega, Madison, WI, USA) was added. Electrophoresis was carried out in a horizontal electrophoresis chamber (Mini-Sub cell GT cell, Bio Rad, Hercules, CA, USA) at 90 volts for 1 h, and a gel doc station (Gel Doc XR, Bio Rad, Milan, Italy) was used for gel imaging. Linier regression was used for bands analysis.

Dotblot

The protein lysates of MDCK and Vero cell cultures were isolated using Cytobuster Protein Extraction Reagent (Novagen, San Diego, CA, USA). Cell culture flasks were placed in ice and the cells were washed with ice-cold Phosphate-Buffered Saline (PBS). PBS (Life Technologies) was removed and ice cold lysis buffer was added. Cells were scraped and transferred into a pre-cooled microcentrifuge tube. The tubes were centrifuged at $16{,}000 \times g$ for 5 min at 4 °C. Tubes were placed on ice and the supernatant was aspirated. The concentration of the protein was measured by the Lowry method using a Nano Drop 2000c UV VIS Spectrophotometer (Thermoscientific, Wilmington, DE, USA).

Protein lysates were diluted in 1 % PBS sodium azide (NaN_3) (1:4). For each sample, twenty microliters of protein lysate with a concentration of 14 mg/ml protein (for MDCK) and 15 mg/ml protein (for Vero) was dropped onto a nitrocellulose membrane on a dot-blot apparatus. Membranes were stained for one of the four proteins of interest, and PBS was used as a negative control. Membranes were blocked with 5 % PBS + skim milk (Difco, Becton Dickinson & Co, Sparks, MD, USA), and washed with 0.05 % PBS-Tween 20 (Sigma-Aldrich). Each membrane was incubated with one of the following antibodies: (i) anti-SIAT1 rabbit polyclonal antibody (sc-20926, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), (ii) anti-LCA rabbit polyclonal antibody (sc-28276), (iii) anti-LCB (sc-28277), or (iv) anti-HC goat polyclonal antibody (sc-6579). Each antibody was diluted 1:200 in PBS + 5 % skim milk, and membranes were incubated with antibodies for two hours. After washing with 0.05 % PBS-Tween 20, the membranes were incubated with an alkaline phosphatase conjugated secondary antibody (either goat anti-rabbit IgG-AP: sc-2007 or rabbit anti-goat IgG-AP:sc-2771) diluted 1:2500 in PBS + 5 % skim milk, and visualized with Nitroblue Tetrazolium (Merck KgaA, Darmstadt, Germany). The dot blots were densitometrically analyzed using SCION Image software. The intensity of protein was calculated by the difference between sample and negative control.

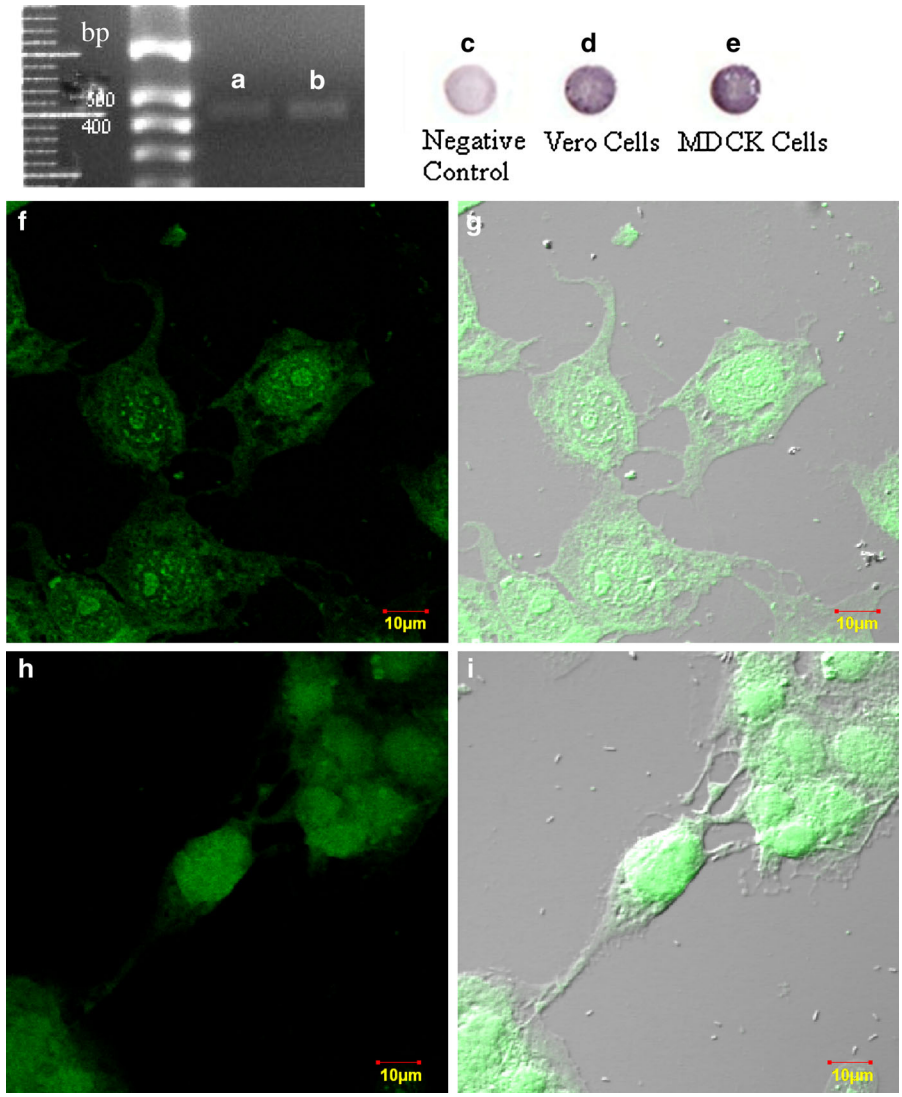

Fig. 2 The expression of SIAT 1 in Vero and MDCK cells. The expression of SIAT1 RNA (422 bp fragment) as a product of RT-PCR on RNA extracts from Vero (**a**) and MDCK cells (**b**). Dot-blot analysis showing protein expression of SIAT1 in Vero (**d**) and MDCK cells (**e**). Protein expression intensity of SIAT1 measured by confocal microscopy (**f**, **h**). Protein location of SIAT1 is shown by confocal microscopy (**g**, **i**)

Immunofluorescence confocal microscopy

Vero and MDCK cells grown on glass slides were fixed with 2.5 % glutaraldehyde (E-Merck, Darmstadt, Germany) and treated with 0.3 % Triton X-100 (Sigma-Aldrich, Steinheim, Germany). Cells were washed with PBS (Life Technologies) and incubated with either SIAT1, LCA, LCB, or HC antibody as mentioned above, followed with a IgG FITC (Santa Cruz, secondary antibody diluted 1:400 in PBS . The fluorescence intensity of SIAT1, LCA, LCB and HC staining was analyzed with an Olympus FluoView FV 1000 confocal laser scanning microscope (Olympus America Inc, Melville, NY, USA).

Photomicrographs of single optical slices were taken, and images were superimposed for quantification of expressed protein. The amount of protein expressed was based on the level of fluorescence, with n = 3 cells for each protein. Protein intensity was analyzed using Olympus Fluoview Ver.1.7a software (Olympus America Inc.).

Statistics

The mean immunofluorescence intensities were compared using independent sample *T*-tests with $\alpha = 5$ % and $df = 4$. Differences were statistically significant when $P < 0.05$.

Table 1 Summary of SIAT1, LCA, LCB and HC protein intensity analyzed and measured by confocal microscope in Vero and MDCK cells

	Vero cells (/cell)	MDCK cells (/cell)	*P* value
SIAT1	642.23 ± 38.07	918.58 ± 134.23	0.027
Light Clathrin A	1,124.83 ± 261.68	747.37 ± 101.09	0.080
Light Clathrin B	2,333.8 ± 249.18	1,620.50 ± 485.57	0.086
Heavy Clathrin	1,095.52 ± 278.00	762.68 ± 132.01	0.134

Results

Expression of SIAT1 in Vero and MDCK cells

SIAT1 was expressed in RNA and protein level in Vero as well as in MDCK cells. Figure 2a, b show the expression of SIAT1 in RNA level in Vero and MDCK cells. DNA sequencing showed that the SIAT1 gene segment (422 bp) we amplified from both cell types was 99 % identical to *Macaca mulatta* ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 (ST6GAL1), mRNA (GI 384475725) fragment 394–815 (data not shown). SIAT1 protein expression levels were detected by dot

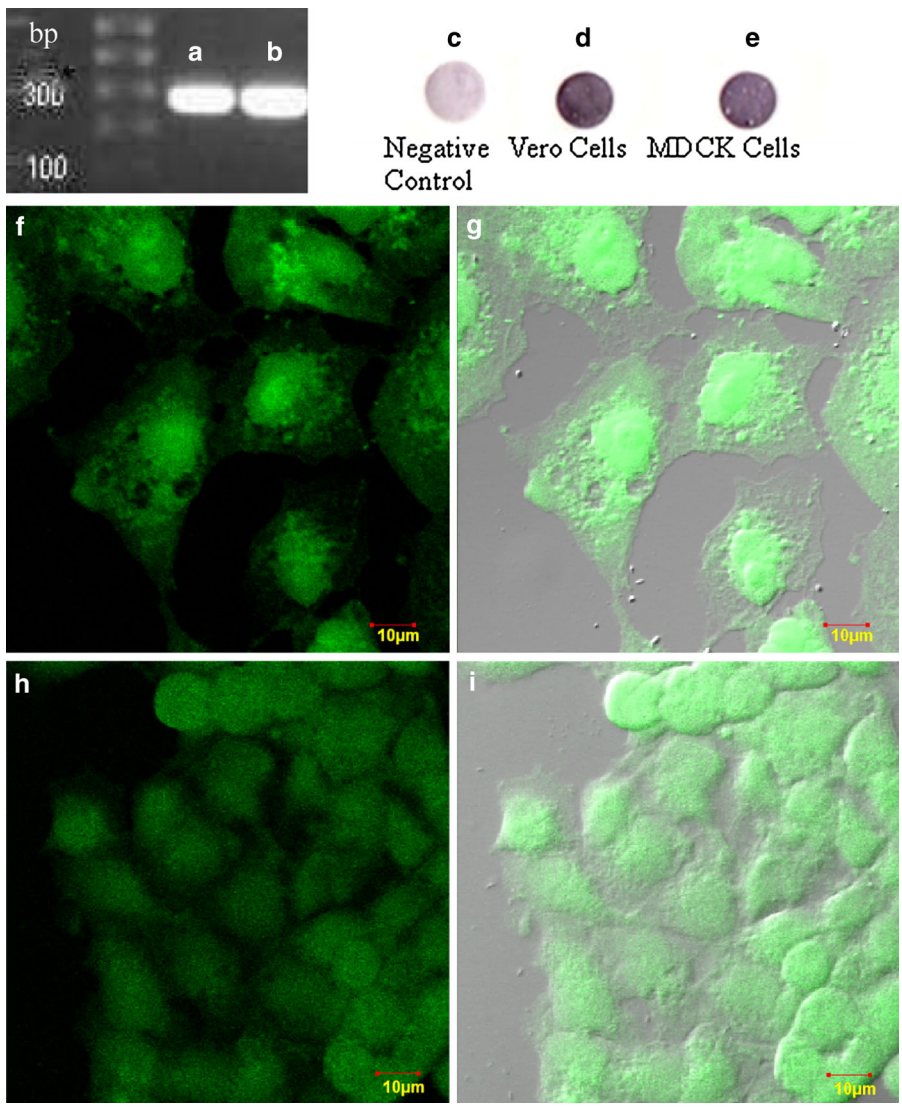

Fig. 3 The expression of Light Clathrin A (LCA) in Vero and MDCK cells. The expression of LCA RNA (273 bp fragment) as a product of RT-PCR on RNA extracts from Vero (**a**) and MDCK cells (**b**). Dot-blot analysis showing protein expression of LCA in Vero (**d**) and MDCK cells (**e**) Protein expression intensity of LCA measured by confocal microscopy (**f**, **h**). Protein location of LCA is shown by confocal microscopy (**g**, **i**)

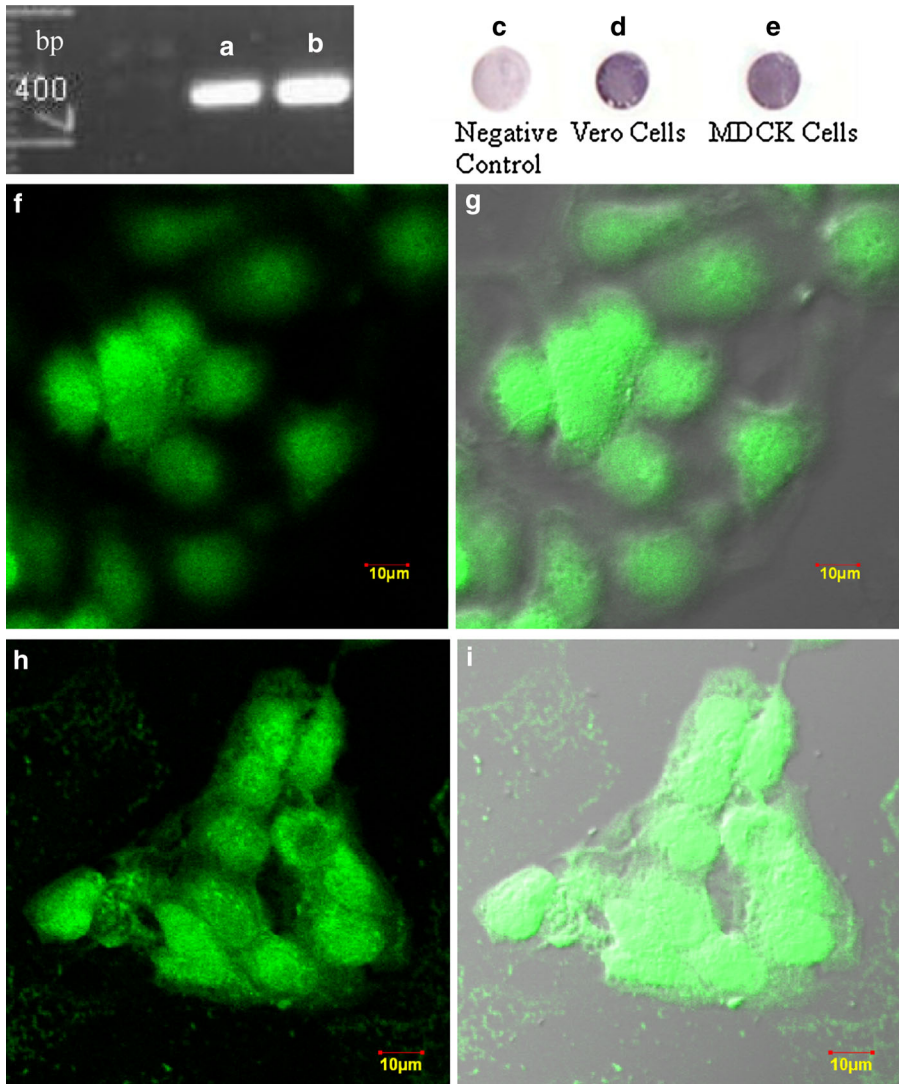

Fig. 4 The expression of Light Clathrin B (LCB) in Vero and MDCK cells. The expression of LCB RNA (348 bp fragment) as a product of RT-PCR on RNA extracts from Vero (**a**) and MDCK cells (**b**). Dot-blot analysis showing protein expression of LCB in Vero (**d**) and MDCK cells (**e**). Protein expression intensity of LCB measured by confocal microscopy (**f**, **h**). Protein location of LCB is shown by confocal microscopy (**g**, **i**)

blot as shown in Fig. 2d, e, with a fluorescence intensity of 4695 in Vero and 5775.5 in MDCK cells. By confocal microscopy, SIAT1 protein was expressed with a fluorescence intensity of 642.23 ± 38.07/cell in Vero cells and 918.58 ± 134.23/cell in MDCK cells (Fig. 2f, h). Fig. 2g, i show that SIAT1 was located in the cytoplasm of both cell types. The expression of SIAT1 protein in Vero cells was significantly lower than in MDCK cells (Table 1) ($P = 0.027$).

Expression of LCA, LCB and (HC) in Vero and MDCK cells

LCA RNA expression levels of both cell types are shown in Fig. 3a and b. DNA sequencing showed that the Vero LCA gene segment (273 bp) we amplified was 97.8 % identical to *Homo sapiens* light chain A clathrin, transcript variant 5, mRNA (GI 296179385) fragment 311–583, and the MDCK LCA gene segment (273 bp) we amplified was 98.9 % identical to the predicted *Canis lupus familiaris* light chain A-like clathrin, transcript variant 4 (LOC100856559) (GI 359320840) mRNA fragment 224–496 (data not shown). Dot-blot analysis showed that LCA protein fluorescence intensity was 6472 in Vero cells and 5797 in MDCK cells (Fig. 3d, e). By using confocal microscope, it was shown that expression of LCA protein intensity level in Vero cells was 1,124.83 ± 261.68/cell, and in MDCK cells 747.37 ± 101.09/cell (Fig. 3f, h). Fig. 3g, i show that LCA was located in

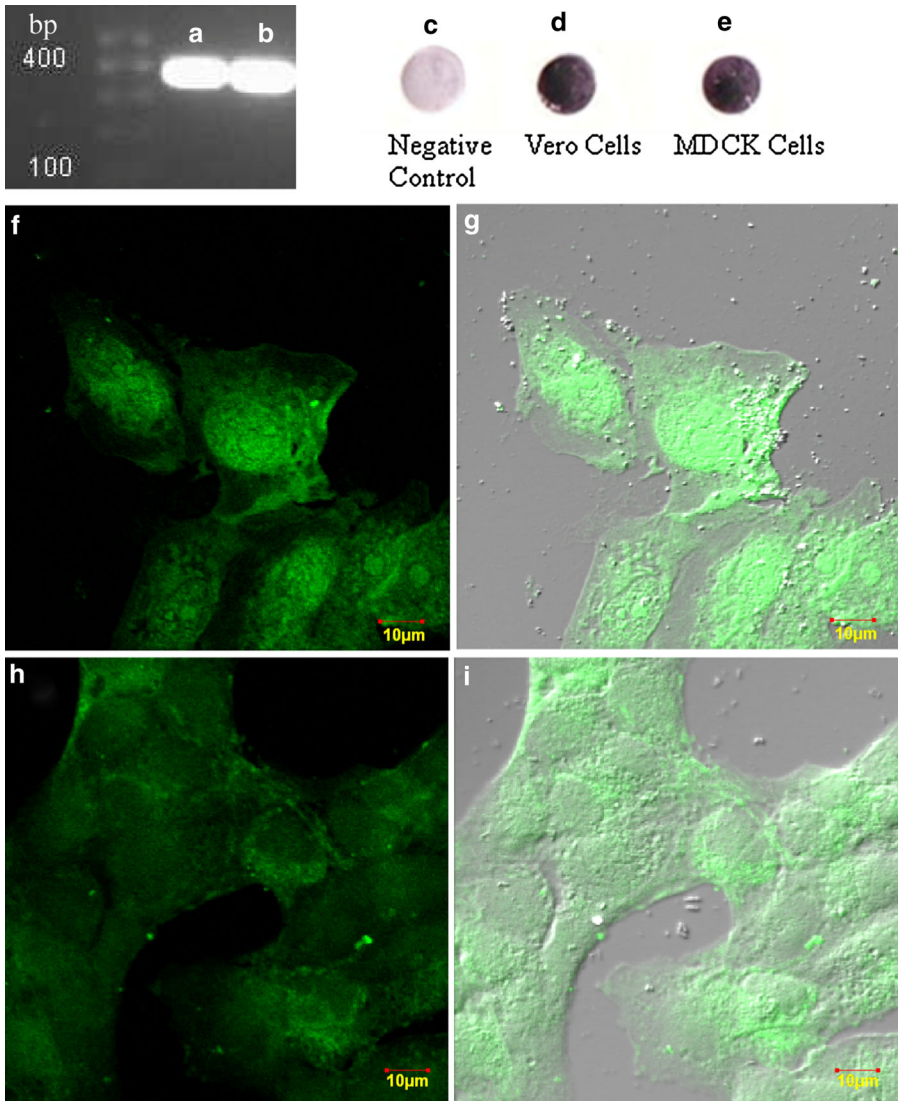

Fig. 5 The expression of heavy clathrin (HC) in Vero and MDCK cells. The expression of HC RNA (375 bp fragment) as a product of RT-PCR on RNA extracts from Vero (**a**) and MDCK cells (**b**). Dot-blot analysis showing protein expression of HC in Vero (**d**) and MDCK cells (**e**). Protein expression intensity of HC measured by confocal microscopy (**f**, **h**). Protein location of HC is shown by confocal microscopy (**g**, **i**)

the cytoplasm of both cell types. The expression of LCA protein in Vero cells was not significantly different to that in MDCK cells (Table 1) ($P = 0.080$). LCB RNA expression levels of both cell types are shown in Fig. 4a, b. DNA sequencing showed that the Vero LCB gene segment (347 bp) we amplified was 97.9 % identical to predicted Macaca mulatta light chain B-like clathrin (LOC702233) mRNA (GI 297295796) fragment 303–649. The MDCK LCB gene segment (347 bp) we amplified was 98.6 % identical to predicted Canis lupus familiaris light chain B clathrin, transcript variant 1 (CLTB), mRNA (GI 345799333) fragment 290–636 (data not shown). Dot-blot analysis showed that LCB protein fluorescence intensity was 7350 in Vero cells and 6498 in MDCK cells (Fig. 4d, e). By confocal microscopy, the protein fluorescence intensity level in Vero cells was 2333.8 ± 249.18/cell and in MDCK cells was 1620.50 ± 485.57/cell (Fig. 4f, h). Fig. 4g, i show that LCB was located in the cytoplasm of both cell types. The expression of LCB protein in Vero cells was not significantly different to MDCK cells (Table 1) ($P = 0.086$).

HC RNA expression levels of both cell types are shown in Fig. 5a and b. DNA sequencing showed that the Vero cell HC gene segment (378 bp) we amplified was 99.4 % identical to Homo sapiens heavy chain clathrin mRNA (GI 115527063) fragment 479–856, and the MDCK HC gene segment (378 bp) we

amplified was 99.2 % homologous to predicted Canis lupus familiaris heavy chain clathrin transcript variant 1 (CLTC), mRNA on fragment 263–641 (GI 345805678) (data not shown). Dotblot analysis showed that the fluorescence intensity of HC was 6888 in Vero and 5365 in MDCK cells (Fig. 5d, e). By confocal microscopy (Fig. 5f, h), the protein fluorescence intensities were 1095.52 ± 278.0/cell in Vero and 762.68 ± 132.01/cell in MDCK cells. Fig. 5g, i showed that HC is located in the cytoplasm in both cell types. The expression of HC protein in Vero cells was not significantly different to those in MDCK (Table 1). ($P = 0.134$).

Discussion

Endocytic vesicles are formed after CCP are pinched off (Conner and Schmid 2003; Lakadamyali et al. 2004).

SIAT1 is an N-linked glycosylation enzyme that catalyzes the addition of α2,6-linked sialic acid to a free β1,4-linked galactose to form a Sia(α2,6) Gal protein receptor (Kim et al. 2009; Dall'Olio et al. 2004). Sia(α2,6) Gal is a specific protein receptor for H1N1 influenza virus (Srinivasan et al. 2008). There are variations of expression levels of specific glycosylation genes among cell lines. The difference in SIAT1 expression determines the amount of glycosylation; thus, the amount of influenza virus entering the cell could be different. Virus entry is inhibited in the cells which lack complex N-linked glycosylation (Hossler et al. 2009; Chu and Whittaker 2004). Compared with MDCK cells, Vero cells had significantly lower expression of SIAT1 protein. Because of this, it is likely that not as many influenza viruses can enter Vero cells compared with MDCK cells, and this entry difference may result in lower titers of influenza viruses grown in Vero cells than in MDCK cells.

Two-third of influenza virus enter host cells through clathrin mediated endocytosis (Rust et al. 2004). Clathrin is needed to form CCP that is induced by viral binding on the viral binding site. Endocytic vesicles are formed after CCP are pinched off (Conner and Schmid 2003; Lakadamyali et al. 2004). CCV as the endocytic vesicle, is formed by light clathrin chain and HC chain which are fundamental components to build the functional unit triskelion (Mellman 1996). Configuration of triskelion is a basket-like structure to form coated pit needed for clathrin mediated endocytosis (Kirchhausen 2000). Our results show that both light and heavy clathrin chains were not expressed at significantly different levels in Vero cells than in MDCK cells. It was demonstrated that Vero cells had adequate clathrin components for clathrin mediated endocytosis mechanism but had less SIAT1 than MDCK. Further research should investigate whether increasing the amount of Sia(α2,6) Gal in Vero cells can increase the internalisation of human influenza viruses compared with normal Vero cells.

Conclusion

The intensity of substantial components for influenza virus internalisation on Vero and MDCK cell was different. SIAT1 intensity in Vero cells was lower than on MDCK cells. This condition might have caused the lack of Sia(α2,6) Gal receptor formation on Vero cell membrane as a reason for having lower number of influenza virus binding ability compared to MDCK cell. However, LCA, LCB and HC intensities as main components for influenza virus endocytosis were not significantly different in Vero cells than in MDCK cells. These results support the hypothesis that the lower expression level of SIAT1 in Vero cells will generate lower internalisation of influenza virus.

References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2008) Intracellular vesicular traffic. In: Anderson M, Granum S (eds) Molecular biology of the cell, 5th edn. Garland Science, New York, pp 754–755

Chu VC, Whittaker GR (2004) Influenza virus entry and infection require host cell N-linked glycoprotein. Proc Natl Acad Sci USA 101:18153–18158

Conner SD, Schmid SL (2003) Regulated portals of entry into cell. Nature 422:37–44

Dall'Olio F, Chiricolo M, D'Errico A, Gruppioni E, Altimari A, Fiorentino M, and Grigioni WF (2004) Expression of β-galactoside α2,6 sialyltransferase and of α2,6-sialylated glycoconjugates in normal human liver, hepatocarcinoma, and cirrhosis. Glycobiology 14:39–49

Gambaryan A, Webster R, Matrosovich M (2002) Differences between influenza virus recepteor on target cells of duck and chicken. Arch Virol 147:1197–1208. doi:10.1007/s00705-002-0796-4

Govorkova EA, Murti G, Meignier B, Taisne C, Webster RG (1996) African green monkey kidney (Vero) cells provide

an alternative host cell system for influenza A & B viruses. J Virol 70:5519–5524. doi:10.1093/glycob/cwp079

Hossler P, Khattak SF, Li ZJ (2009) Review: optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology 19:936–949

Kim PJ, Lee DY, Jeong H (2009) Centralized modularity of N-linked glycosylation pathways in mammalian cells. Plos One Modul Glycosylation Netw 4:e7317. www.plosone.org

Kirchhausen T (2000) Clathrin. Annu Rev Biochem 69:699–727

Lakadamyali M, Rust MJ, Zhuang X (2004) Endocytosis of influenza viruses. Microbes Infect 6:929–936

McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12:517–533

Mellman I (1996) Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 12:575–625

Patriarca PA (2007) Use of cell lines for the productions: an appraisal of techical, manufacturing, and regulatory consideration. Initiative for vaccine research. World Health Organization, Geneva

Paulson JC, Colley KJ (1989) Glycosyltransferase: structure, localization, and control of cell type-specific glycosylation. J Biol Chem 264:17615–17618

Rust MJ, Lakadamyali M, Zhang F, Zhuang X (2004) Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat Struct Mol Biol 11:567–573

Srinivasan A, Viswanathan K, Raman R, Chandrasekaran A, Raguram S, Tumpey TM, Sasesekharan V, Sasisekharan R (2008) Quantitative biochemical rationale for differences in transmissibility of 1918 pandemic influenza A viruses. Proc Natl Acad Sci USA 105:2800–2805

Zuhairi FR, Maharani, Tan MI (2012) The role of trypsin in the internalisation process of influenza H1N1 virus into Vero and MDCK cell. ITB J Sci 44:297–307