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Antiviral effects of glycosylation and glucose trimming inhibitors on human parainfluenza virus type 3

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Abstract

Endoplasmic reticulum (ER) α-glucosidase inhibitors block the trimming of N-linked glycosylation and thus prevent the production of several viruses. The present study investigates the antiviral effects of the α-glucosidase and α-mannosidase inhibitors (castanospermine, 1-deoxynojirimycin, bromoconduritol, deoxymannojirimycin and swainsonine) on human parainfluenza virus type 3 (HPIV3). The α-glucosidase inhibitors (castanospermine, 1-deoxynojirimycin) in recombinant expression systems reduced the surface and intracellular expression of both HPIV3 F and HN proteins. On the other hand, α-mannosidase inhibitors prevented processing of the oligosaccharides on HPIV3 glycoproteins into the complex form. Consequently, α-glycosidase inhibitors (castanospermine and 1-deoxynojirimycin) significantly inhibited viral fusion activity. We demonstrated that the α-glucosidase inhibitors (castanospermine and 1-deoxynojirimycin) reduced the infectivity of newly released viral particles. We postulate that α-glucosidase inhibitors can prevent the first steps of HPIV3 envelope glycoprotein processing and that the inhibition of glucose trimming has antiviral effects.

Keywords: Human parainfluenza virus type 3; Oligosaccharide processing; Glycosylation inhibitors

1. Introduction

Human parainfluenza virus type 3 (HPIV3) encodes the surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) proteins. While HN provides neuraminidase activity and attachment to cell surface receptors, F protein mediates virus–cell fusion. F protein is synthesized as the inactive form, F0, which is then cleaved by an endogenous protease into F1 and F2 subunits with fusion activity. F protein forms homometric oligomers in the endoplasmic reticulum (ER) that are transported along a secretory pathway to the plasma membrane (Cote et al., 1987; Storey et al., 1987). Other studies have shown that both the F and HN proteins are required for syncytia formation (Ebata et al., 1991; Heminway et al., 1994).

HPIV3 F and HN proteins are both modified by N-linked carbohydrates and their N-glycans are not completely Endo H sensitive. Pulse-chase experiments have shown that the N-glycan chains of F and HN proteins appear to contain both high-mannose and complex oligosaccharides with three and four potential N-linked glycosylation sites, respectively (Spriggs and Collins, 1990). These N-linked glycosylation sites probably play important roles in protein folding, intracellular trafficking and infectivity (Tanaka and Galinski, 1995).

During the synthesis of N-linked glycans in mammalian cells, oligosaccharide core $(Glc)_3(Man)_9(GlcNAc)_2$ units are co-translationally added to polypeptides that are synthesized de novo in the ER. The chain of glycan molecules is then biochemically modified within the ER and the golgi apparatus to generate the diversified glycan moieties found in mature glycoproteins. The α-glucosidases I and II of the ER are involved in trimming terminal glucose moieties from oligosaccharides, and the resulting monoglucosylated glycoproteins can bind to ER chaperones, namely, membrane-bound calnexin (CNX) and/or its soluble homologue calreticulin (CRT) (Hammond et al., 1994; Molinari and Helenius, 2000; Peterson et al., 1995). Removal of the last glucose molecule from glycans by glucosidase II releases the

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glycoprotein from CNX and/or CRT. The binding between CNX and/or CRT and glycoprotein increases the folding efficiency of glycoproteins (Zhang et al., 1997).

Enveloped animal viruses often contain one or more viral glycoproteins, and thus α-glucosidase inhibitors block infection by several viruses (Branza-Nichita et al., 2001; Courageot et al., 2000; Dwek et al., 2002; Mehta et al., 1998; Nakhasi et al., 2001; Papandreou et al., 2002; Wu et al., 2002; Zitzmann et al., 1999). The α-glucosidase inhibitor, castanospermine (CS), reduces the surface transport of NA protein on the influenza A virus by 50%, but not that of HA protein (Saito and Yamaguchi, 2000). However, a study of the measles virus (MV) has revealed that CS quantitatively reduces the intracellular production of infectious MV particles (Bolt, 2001). Although recent studies have clarified the antiviral effects of iminosugar derivatives especially on the Flaviviridae family (Courageot et al., 2000; Mehta et al., 1998; Wu et al., 2002; Zitzmann et al., 1999), the inhibitory effects on the Paramyxoviridae family are still poorly understood (Bolt, 2001; Tomita et al., 1999).

To determine whether inhibitors of α-glucosidase and mannosidase block the transport of F and HN glycoproteins on HPIV3 through secretory pathways, we biochemically analyzed the surface expression of HPIV3 F and HN proteins in vitro and examined the effect of the inhibitors upon the production of infectious virus particles.

2. Materials and methods

2.1. Cell lines and virus

The HeLa cell lines, HeLa β-gal and HeLa tat (Tanaka et al., 1996), as well as Cos7 and CV-1 cells were routinely passaged in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Japan) supplemented with 10% fetal bovine serum and maintained as described (Tanaka and Galinski, 1995).

2.2. N-linked glycoprotein processing inhibitors and concentrations

We decided the concentration of each reagent with reference to Wojczyk et al. (1995). The final concentrations of the reagents (Sigma–Aldrich, Japan) were as follows: castanospermine (CS, 260 μM), bromoconduritol (Br, 1 mM), deoxynojirimycin (dNM, 1 mM), deoxymannojirimycin (dMM, 1 mM), swainsonine (SW, 30 μM), and tunicamycin (6 μM).

2.3. Cell viability assay

Cell viability was determined using a colorimetric assay (Cell Counting Kit-8; Dojindo, Tokyo, Japan). HeLa cell lines, Cos7 and CV-1 cells in 96-well plates were incubated with various concentrations of each inhibitor for 2 days and then the WST-8 reagent was added to the medium. Cells were incubated at 37 °C for 3 h and then examined using a Multi-Detection Microplate Reader (Powerscan HT, Dainippon Pharmaceutical, Japan) at 450 nm.

2.4. Plasmids and transfection

The HPIV3 HN and F glycoprotein genes (Tanaka et al., 1996) were subcloned into the pcDL-SR beta 8.2 vector and then transfected into Cos7 cells by electroporation as described (Tanaka et al., 1996). At 48 h post-transfection, the cells were starved in methionine- and cysteine-free DMEM for 1 h and pulsed for 30 min in the presence of 100 μCi/ml of EXPRE^{35}S^{35}S Protein Labeling Mix (NEN, MA). The labeled cells were then either lysed or chased for 0, 1.5 or 3 h in complete DMEM with 2% fetal bovine serum. When investigating inhibition, the inhibitors at the concentrations described above were added to the medium 2 h before the pulse and maintained throughout the chase period. The medium was discarded and the cells were lysed with TNE buffer (50 mM Tris–HCl, pH 7.6, 0.15 M NaCl, 1% NP-40, 1 mM PMSF, 2 mg of aprotinin/ml). All samples were immunoprecipitated with anti-HPIV3 polyclonal antibody (a gift from Takemasa Sakaguchi, Hiroshima University, Japan). Equal portions of the precipitates were incubated with and without endoglycosidase H (Endo H) (Roche-Diagnostics, Japan). Equal volumes of either 100 mM sodium citrate (pH 5.3), 1 mM PMSF, 2 mg aprotinin/ml (mock-treated) or the same buffer containing 5 mU Endo H were mixed and added to the samples, which were incubated for 16 h at 37 °C, and then resolved by 8% SDS-PAGE.

2.5. Indirect immunofluorescence

The inhibitors at the concentrations described above were incubated with the medium for 48 h. The cells were then transfected for 48 h with the HPIV3 HN and/or F genes and then cells on cover slips with or without inhibitors were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+} (+) for 10 min at room temperature for surface staining or with methanol–acetone (1:1) for 7 min at −20 °C for total staining. The cells were rinsed three times with PBS (+), blocked with washing buffer [0.2% gelatin-PBS (+)] and then reacted with anti-HPIV3 F monoclonal antibody (c199/3) or anti-HPIV3 HN monoclonal antibody 13-5-9-6-2 (ATCC, #HB-8935) for 30 min at room temperature. After three rinses with washing buffer, the cells were incubated with anti-mouse IgG conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Invitrogen, CA) for 30 min at room temperature. After three rinses with washing buffer, the cells were mounted with VECTASHIELD (Vector Laboratories Inc., CA) and photographed using a confocal microscope system (LSM510; Carl Zeiss Co., Japan).

2.6. X-gal staining and quantitative colorimetric assay of β-galactosidase activities

The HPIV3 HN and F genes were transfected into HeLa tat and HeLa β-gal cells by electroporation. Cells infected with HPIV3 at a multiplicity of infection (m.o.i.) of 0.01 were maintained in DMEM containing 2% fetal bovine serum and various inhibitors. Syncytia formation by HeLa tat and HeLa β-gal cells was semi-quantified (β-Galactosidase Enzyme Assay System;

Promega, Tokyo, Japan) as described (Tanaka and Galinski, 1995) and visualized by X-gal staining in situ.

2.7. Virus infection and titration

Monolayers of HeLa tat and HeLa β-gal cells in 6-well plates were adsorbed with HPIV3 at an m.o.i. of 0.01 for 1 h at 37 °C. Free viruses were then removed by gentle washing with serum-free medium, and then fresh medium containing various concentrations of inhibitors was added and the cells were further incubated at 37 °C. To determine virus titers, the cells infected with HPIV3 in the presence or absence of inhibitor were lysed by freeze-thawing twice and then we assayed plaque-forming ability in the supernatants clarified by centrifugation at 10,000 × *g* for 5 min. Various dilutions of viruses were added to 80% confluent CV-1 cells and incubated at 37 °C for 1 h. After adsorption, the cells were washed, overlaid with 1% agarose (SeaPlaque; FMC BioProducts, TAKARA Bio Co., Japan) containing DMEM with 2% FCS and incubated for 3 days. Thereafter, the cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet. Differences between cells infected with HPIV3 and treated or not with glycosidase inhibitors were analyzed by Student's *t*-test. *P*-values are stated in the text when relevant.

3. Results

3.1. Influence of various glycosylation inhibitors on HPIV3 F protein processing

Core glycosylation and N-glycan processing of HPIV3 F protein by transfection into Cos7 cells were examined by comparing electrophoretic migration before and after digestion with Endo H. Metabolically labeled HPIV3 F protein was immunoprecipitated with monoclonal antibody from cell lysates, digested with Endo H, and resolved by SDS-PAGE under reducing conditions. The incomplete digestion of F0 with Endo H indicates that F protein did not undergo complete high-mannose modification. Untreated F protein acquired partial Endo H resistance during a 180 min chase (Fig. 1; FWT, F0, and F1). Tanaka et al. discovered that HPIV3 F does not achieve complete Endo H resistance even after a 180 min chase (Tanaka and Galinski, 1995). The

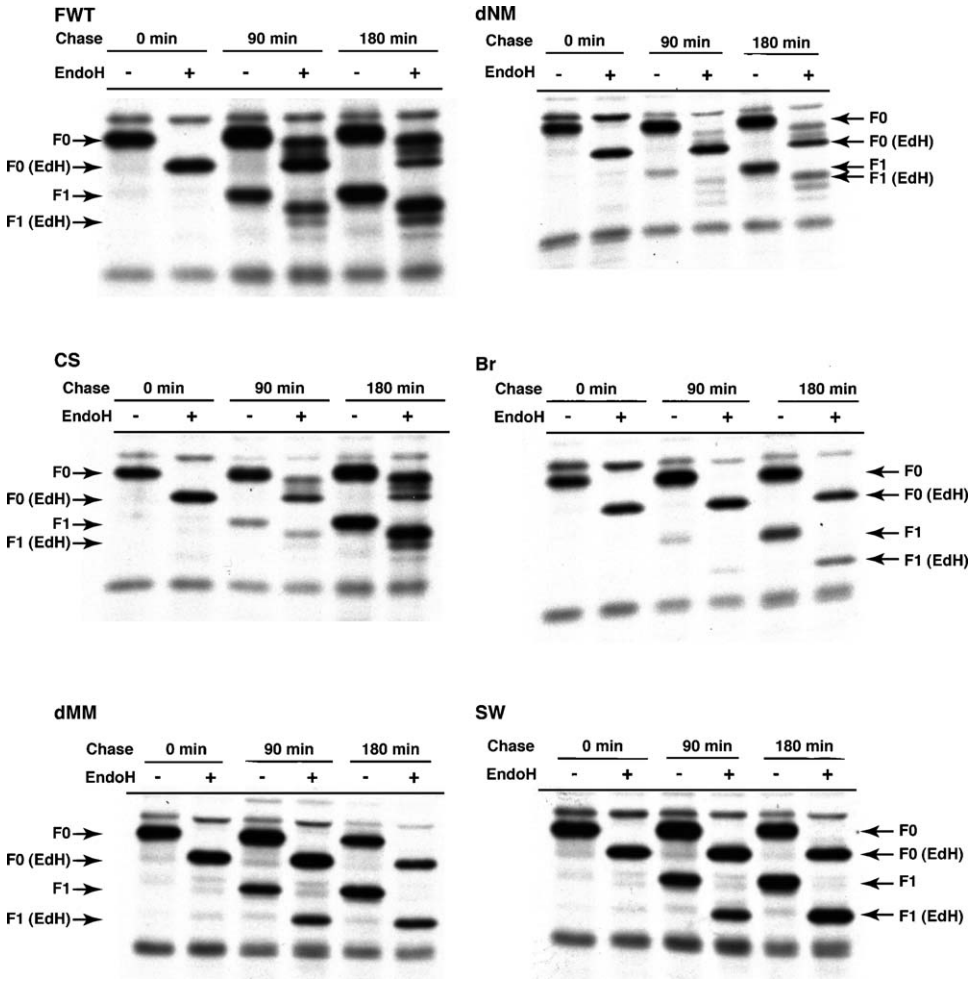

Fig. 1. Processing of HPIV3 F protein in transfected Cos7 cells incubated with glucosidase or mannosidase inhibitors. Mock-treated (FWT) cells or cells incubated each inhibitor (dNM, CS, Br, dMM, and SW) were pulse-labeled and chased as described in Section 2. Cell lysates were immunoprecipitated with anti-HPIV3 antibody. Proteins were separated by 8% SDS-PAGE, immunoprecipitated, incubated in the presence (+) or absence (−) of Endo H and visualized by fluorography.

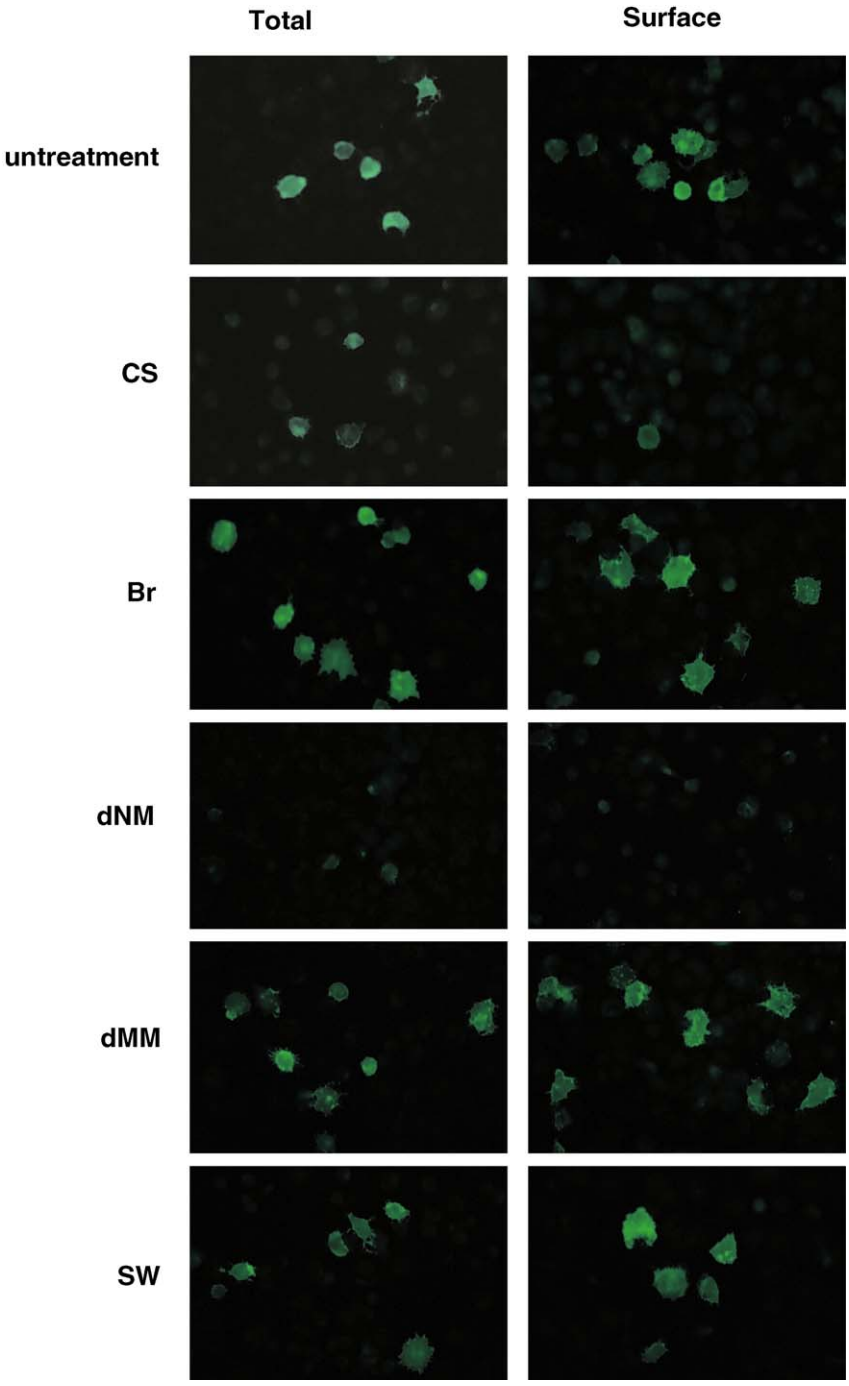

Fig. 2. Effects of each inhibitor in vitro against intracellular and surface expression of recombinant HPIV3 F protein. At 48 h post-transfection, the cells were fixed and stained by indirect immunofluorescence using anti-F monoclonal antibody (c199/3) and Alexa 488 conjugated secondary antibody.

inhibitors, CS and dNM allowed core glycosylation but partially blocked the removal of glucose residues (Fig. 1; F0 and F1 in dNM and CS). These inhibitors retarded the intracellular maturation and reduced the amounts of F protein. However, morphological changes between drug- and mock-treated cells were not obvious (Fig. 2). In contrast, after exposure to each inhibitor (Br, dMM, SW), F protein exposed to Endo H became completely sensitive throughout the 180 min chase. This demonstrated that all of the N-glycans in these proteins are of the high-mannose type.

3.2. Influence on F and HN proteins in presence of glycosylation inhibitors detected by immunofluorescence

We investigated F protein processing by immunofluorescence staining to detect surface and/or intracellular F protein expression. Both CS and dNM, which specifically block both α-glucosidase I and II, significantly reduced surface and intracellular expression (Fig. 2). Among the examined inhibitors, dNM appeared to be the most effective. However, immunofluorescence staining did not reveal any significant differences among

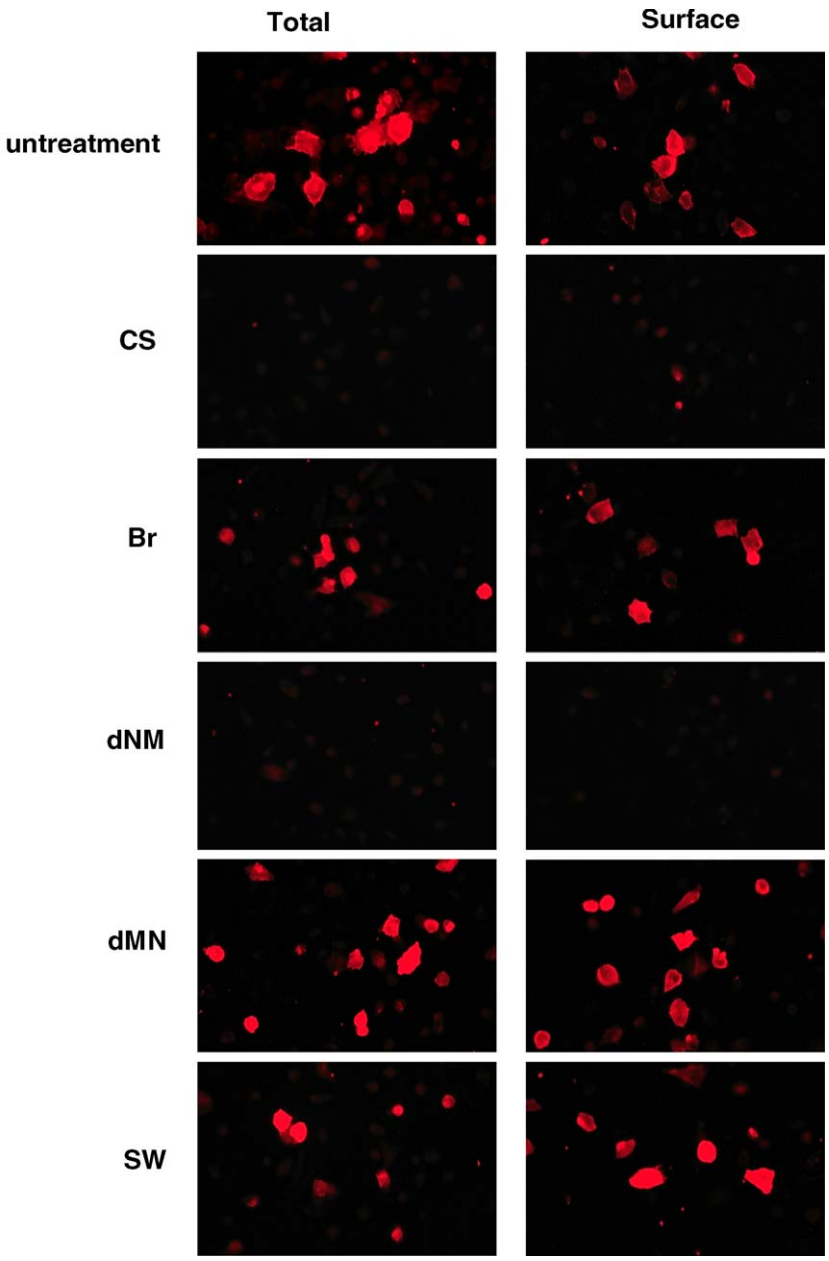

Fig. 3. Effects of each inhibitor in vitro against intracellular and surface expression of recombinant HPIV3 HN protein. Cells transfected with HPIV3 HN gene were fixed and stained by indirect immunofluorescence with anti-HN monoclonal antibody (13-5-9-6-2) and Alexa 594 conjugated secondary antibody.

cells exposed to dMM, SW and Br (Fig. 2). In the presence of CS and dNM, F protein did not accumulate inside the cells, which suggested that F protein is degraded by cellular proteases in the presence of CS and dNM and that the amount accumulated is insufficient for detection by immunofluorescence staining. The antiviral effects differed between the NA and HA proteins of the influenza virus (80% and 97% reduction for HA and NA protein, respectively) as described (Saito and Yamaguchi, 2000). We therefore used immunofluorescence to determine whether these glycosylation inhibitors affect the intracellular transport of HPIV3 HN protein. Fig. 3 shows that not only the intracellular, but also the cell surface expression of HN protein exposed to CS and dNM obviously decreased compared with untreated cells. Taken together, these results show that initial trimming of the glucose residues on N-glycans is a necessary condition for both the secretion and cell surface expression of F and HN proteins.

3.3. *Influence of cell-fusogenic activity in vitro*

Membrane fusion between enveloped viruses and cells is important for viral entry into cells. Cell fusion activity requires the expression of both HPIV3 F and HN proteins on the cell surface (Heminway et al., 1994). Since several glycosylation inhibitors affected the intracellular processing of HPIV3 F and HN proteins (Figs. 1–3), we examined their effects by X-gal staining using a transient expression system with the HPIV3 F and HN genes. The inhibitors dNM and CS significantly

reduced fusion activities whereas Br, SW, and dMM had no obvious effects (Fig. 4A and B). Furthermore, no morphological changes were evident in any of the cells incubated with inhibitors for 48 h after gene transfection (Fig. 4A). We then examined the β-galactosidase activities of cells transiently expressing the F and HN genes in the presence of each inhibitor (Fig. 4B). The fusion activity of the cells treated with CS and with dNM was significantly decreased compared with that of untreated cells ($P < 0.05$ for both). Nevertheless, the β-gal activities of the cells incubated with the other inhibitors (Br, dMM, and SW) did not significantly differ from those of untreated cells (Fig. 4B).

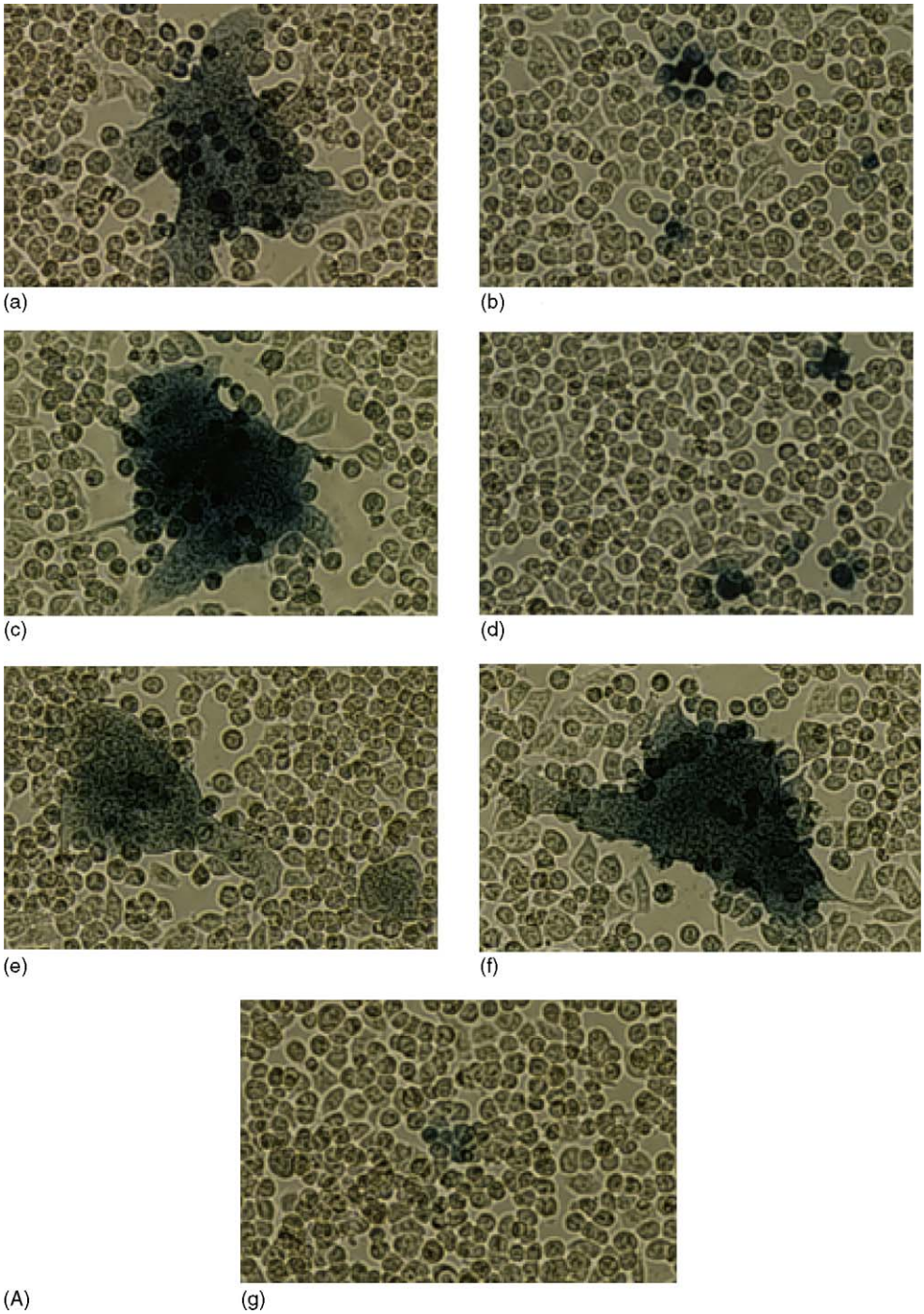

Fig. 4. (A) Inhibitory effects of glycosidase or mannosidase inhibitors on cell fusion. Plasmids containing HPIV3 F and HN genes were transfected into HeLa tat and HeLa β-gal cells by electroporation and then cells were stained with X-gal at 48 h post-transfection. Multinuclear cells were evident among the cells incubated with Br (c), dMM (e), and SW (f) but not with dNM (b) and CS (d). Cells expressing HPIV3 F and HN proteins in the absence of inhibitors are shown in (a) and mock transfected cells are indicated as (g). (B) Inhibitory effects of glycosidase or mannosidase inhibitors on cell fusion in vitro determined by β-galactosidase assays. HeLa tat and HeLa β-gal cells were transfected with HPIV3 F and HN genes and incubated with each inhibitor. At 48 h post-transfection, β-galactosidase activities were measured at 420 nm o.d. as described in Section 2.

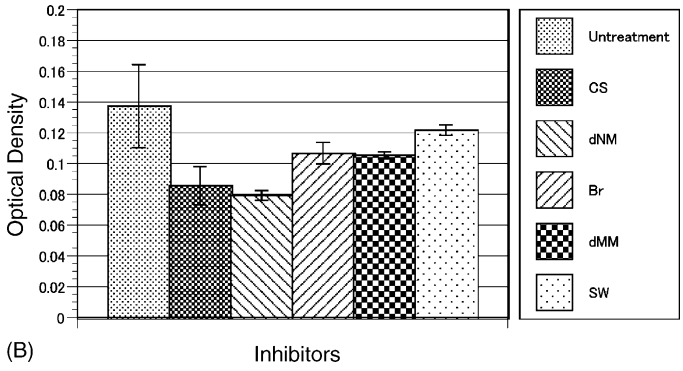

Fig. 4. (*Continued*).

3.4. Glucosidase inhibitors prevent secretion of infectious HPIV3

We examined the influence of glycosidase inhibitors on the HPIV3 titer and found that CS and dNM reduced the production of infectious HPIV3 particles. HeLa cell lines mixed with equal amounts of HeLa tat and HeLa β-gal cells were infected with HPIV3 at an m.o.i. of 0.01 and then 1 h later, the inoculum was removed and replaced with fresh medium with or without glycosylation inhibitors. Forty-eight hours later, the amount of virus was determined by plaque assays. Fig. 5 shows that dNM and CS inhibited the cytopathic effect (CPE) of HPIV3 on HeLa cells. On the contrary, the inhibition of complex glycan formation by dMM and SW did not prevent plaque formation and Br did not affect fusion activities. Table 1 shows that the titer of infected cells treated with CS was significantly decreased compared with that of untreated cells ($P < 0.001$) and that the titers of those incubated with dNM was considerably reduced ($P < 0.001$). In addition, the titer of infected cells incubated with dMM was reduced compared with that of untreated cells ($P < 0.001$) but not to a higher degree than with dNM and CS. These data show that the antiviral effects of dNM and CS are mediated through a step that occurs before glucose residues are completely removed. Consequently, initial

Table 1
Influence of glycosylation inhibitors on HPIV3 titer

Inhibitor	Concentration	Infectivity ($\times 10^7$ p.f.u./ml)
CS	260 μM	0.06 ± 0.0
Br	1 mM	2.60 ± 0.8
dNM	1 mM	0.10 ± 0.0
dMM	1 mM	0.72 ± 0.2
SW	30 μM	2.80 ± 0.7
Tunicamycin	6 μM	ND
Mock treatment	–	6.90 ± 0.5

ND, not detectable. All samples for plaque assay were prepared from infected cells incubated in the presence or absence of inhibitors by freeze-thawing and supernatants were clarified by centrifugation at 10,000 × *g* for 5 min. Titers of samples were determined by plaque assay and are presented as plaque forming units/ml. All values are averages of triplicate assays ± standard deviation (S.D.).

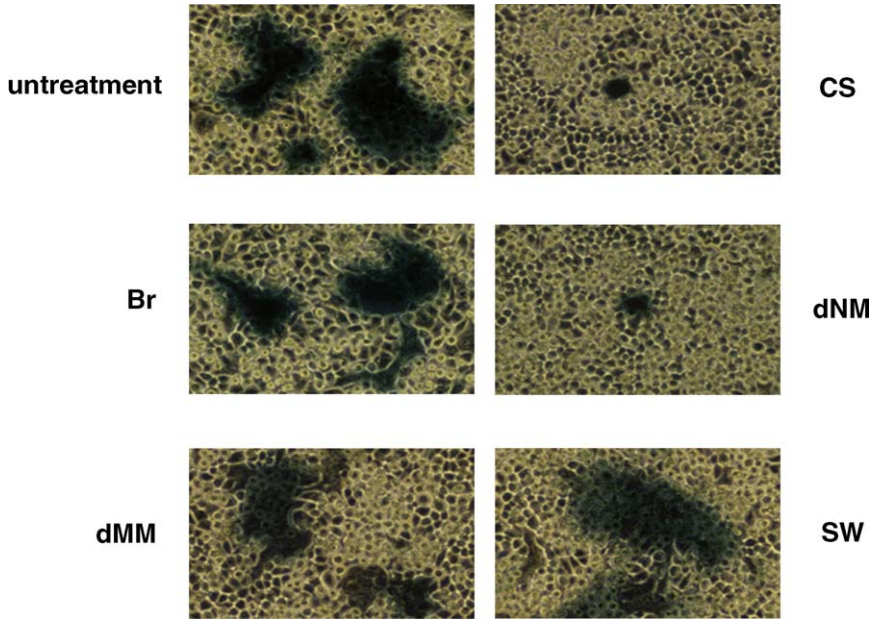

Fig. 5. Inhibitory effects of glycosidase or mannosidase inhibitors on fusion of HPIV3-infected cells. At 48 h postinfection, HeLa tat and HeLa β-gal cells were stained with X-gal. No inhibitor affected morphology of infected cells.

glucosidase processing plays the most important role in HPIV3 infectivity.

4. Discussion

Here, we examined the influence of glycosidase inhibitors on HPIV3 F and HN glycoproteins. The golgi α-mannosidase inhibitors dMM and SW prevented the processing of oligosaccharides on HPIV3 glycoproteins into complex forms, but did not seem to influence viral fusion activity. In contrast, the α-glycosidase inhibitors dNM and CS profoundly affected viral fusion activity, apparently through reducing intracellular and surface expression (Fig. 4A and B). To determine whether the reduced expression was due to the recognition of monoclonal antibody epitopes, we applied a polyclonal antibody to HPIV3 for immunofluorescence staining. The results were similar to those shown in Figs. 2 and 3 results (data not shown). Thus, the envelope glycoproteins underwent an obvious conformational change in the presence of glycosidase inhibitors (dNM and CS), which in turn influenced the secretion pathway. The effects of inhibitors on fusion could thus be attributed to the reduced expression and transport of both F and HN glycoproteins rather than to a direct effect on the fusion function (Figs. 2, 3, 4A and B). We also found that the HPIV3 virus titer of the cells incubated with dMM was decreased compared with untreated cells (Table 1). This could be due to the decreased stability of F and HN proteins induced by the misfolding that results from blocking the glycosidase and mannosidase activities. Inhibition of golgi α-1,2-mannosidase I by dMM might affect F and HN protein folding and/or stability more than α-1,3-glucosidase II and/or golgi α-1,2-mannosidase II inhibition by Br and/or SW. A study of dengue virus has shown that α-glucosidase inhibition affects the folding pathways of the envelope glycoproteins prM and E. The complexes formed between incompletely folded prM and E are apparently unstable, leading to a redundant pathway (Courageot et al., 2000; Wu et al., 2002). Our results support these findings. The formation of properly folded envelope glycoprotein complexes of several viruses requires a lectin chaperone pathway. The molecular chaperones, CNX and CRT, have lectin-like affinity for monoglucosylated N-linked oligosaccharides and interact with the intermediate oligomeric complexes of viral envelope glycoproteins in the ER. Removal of the last glucose residue from glycans by glucosidase II releases the glycoprotein from CNX and/or CRT (Hammond et al., 1994; Molinari and Helenius, 2000; Peterson et al., 1995; Zhang et al., 1997). The MV F and H proteins associated with CNX in the ER and the association of F protein was diminished and delayed in cells incubated with CS. The association between H protein and CRT is more efficient than that between H protein and CNX. The antigenicity and function of F protein is apparently influenced by CS much more than those of H protein in MV (Bolt, 2001). However, whether α-glycosidase reduces the production of viral particles in HPIV3-infected cells and decreases syncytium formation have remained unknown. We did not examine intracellular interactions between HPIV3 glycoproteins and CNX and/or CRT, but interaction might be required for glycoprotein folding to assume a functional conformation. A study of the interaction between the HN and/or the F proteins of Sendai virus and ER chaperones has shown that F protein synthesized de novo undergo rapid maturation in the ER through transient interaction with CNX. In contrast, HN protein requires more complex processes involving prolonged association with BiP, CNX, and CRT for quality control in the ER (Tomita et al., 1999). As described above, proteins might associate with ER chaperones in cells incubated with α-glycosidase, and the slow and incomplete intracellular migration of the HPIV3 glycoproteins might be related to the prolonged retention of a substantial fraction of glycoproteins synthesized by the ER chaperones.

This study indicates that N-linked oligosaccharide processing plays a crucial role in the early stages of HPIV3 morphogenesis. We believe that α-glucosidase inhibitors can prevent the first steps of glycoprotein processing of the HPIV3 envelope and that the inhibition of glucose trimming has antiviral effects. We did not examine the protective ability of the inhibitors used in this study against HPIV3 challenge in vivo. No rodent models have been examined with HPIV3 in vivo because this virus infects only humans and some primates. However, Wu et al. have reported that the oral delivery of *N*N-DNJ, a 9-carbon alkyl derivative of deoxynojirimycin, reduces the mortality rate of mice given a lethal challenge with the Japanese encephalitis virus. Daily oral delivery of a high dose of *N*N-DNJ (200 mg/kg/day), substantially increased the survival rate to 47% ($P = 0.0896$ versus untreated group), compared with 7% and 8% for the untreated and low-dose (20 mg/kg/day) groups, respectively. Sublethal illness did not develop in the surviving mice (Wu et al., 2002, 2005). Inhibitors of glycosylation processing have been applied to as treat human diseases for decades and has attracted recent focus (Alper, 2001a,b). More inhibitory effects should be explored using other α-glucosidase inhibitors to develop new antiviral medications that can be clinically applied against HPIV3 infection.

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