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Short communication

Glycan-specificity of four neuraminidase-sensitive animal rotavirus strains

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ABSTRACT

Group A rotaviruses (RVAs) are divided into neuraminidase (NA)-sensitive and NA-insensitive strains depending upon their binding affinity to the VP8* domain in the terminal sialic acids (SAs) of cell surface carbohydrates. Although NA-sensitive strains are known to use terminal SAs as an attachment factor, the exact nature of this attachment factor is largely unknown. Here we show that the specific linkage of SA-containing glycan to glycoprotein or glycolipid is an attachment factor used by NA-sensitive porcine G9P[7] PRG9121 and G9P[23] PRG942, bovine G6P[1] NCDV, and canine G3P[3] strains. Infectivity of porcine G9P[7] and G9P[23] strains was markedly blocked by α2,3-linkage and α2,6-linkage inhibitors, indicating that these strains bind to both α2,3- and α2,6-linked SAs. However, the infectivity of bovine G6P[1] and canine G3P[3] strains was significantly reduced by α2,6-linkage inhibitor but not by α2,3-linkage blockers, demonstrating a predilection of these strains for α2,6-linked SAs. The infectivity of four NA-sensitive strains was equally reduced by inhibitors of lipid membrane and *N*-linked glycoprotein but not by an inhibitor of *O*-linked glycoprotein, indicating that these strains utilize both glycolipid and *N*-linked glycoprotein. Our study demonstrates that four NA-sensitive animal strains could have a strain-dependent binding preference toward α2,6-linked SAs (P[1] NCDV and P[3] CU-1 strains) or both α2,3- and α2,6-linked SAs (P[7] PRG9121 and P[23] PRG942 strains) to the glycolipid and *N*-linked glycoprotein.

1. Introduction

Viruses must bind to surface receptor(s) on the susceptible host cells in order to initiate infection (Neu et al., 2011). Group A rotaviruses (RVAs), the most important cause of severe gastroenteritis in young children and many animals, also initiate infections through an orderly interactions of the VP8* and VP5* domains of the spike VP4 protein and VP7 protein with different cell surface receptors (Arias et al., 2015). As the first RVA molecule to bind to host cells, the VP8* domains of the spike VP4 protein attach to the cell surface carbohydrate moieties (glycans), terminal or internal sialic acids (SAs), or histoblood group antigens (HBGAs) (Le Pendu et al., 2014; Tan and Jiang, 2014). Following pretreatment with terminal-SA cleaving neuraminidase (NA), a marked reduction of infectivity is found in some animal RVA strains, which were consequently classified as NA-sensitive (Arias et al., 2015). By contrast, when the pretreatment with NA did not show this reduced sensitivity to infection to many animal and most human RVA strains, these strains were classified as NA-insensitive (Arias et al., 2015). A recent study indicates that depending upon the VP8* sequences, RVAs can be classified into five P genogroups (P[I] to P[V]) (Liu et al., 2012).

Viral receptors are important determinants of virus-specific tissue tropism and pathogenesis (Olofsson and Bergström, 2005). Although the P[I] genogroup contains the most animal and human P genotypes (Liu et al., 2012), the nature of their receptor(s) to the VP8* domain is largely unknown. This prompted us to investigate the nature of the cellular attachment factor(s) used by the VP8* domain of four NA-sensitive animal RVA strains (porcine G9P[7] PRG9121 and G9P[23] PRG942, bovine G6P[1] NCDV, and canine G3P[3] strains) in the P[I] genogroup. Using inorganic salt, glycosidases, lectins, glycosylation inhibitors in this study, we determined the specific linkage of SA-containing glycans with glycoprotein and glycolipid as attachment factors in four NA-sensitive animal RVAs strains in the P[I] genogroup.

2. Materials and methods

2.1. Cells and viruses

Monkey kidney MA104 cells, human lung fibroblast WI-38 cells, and

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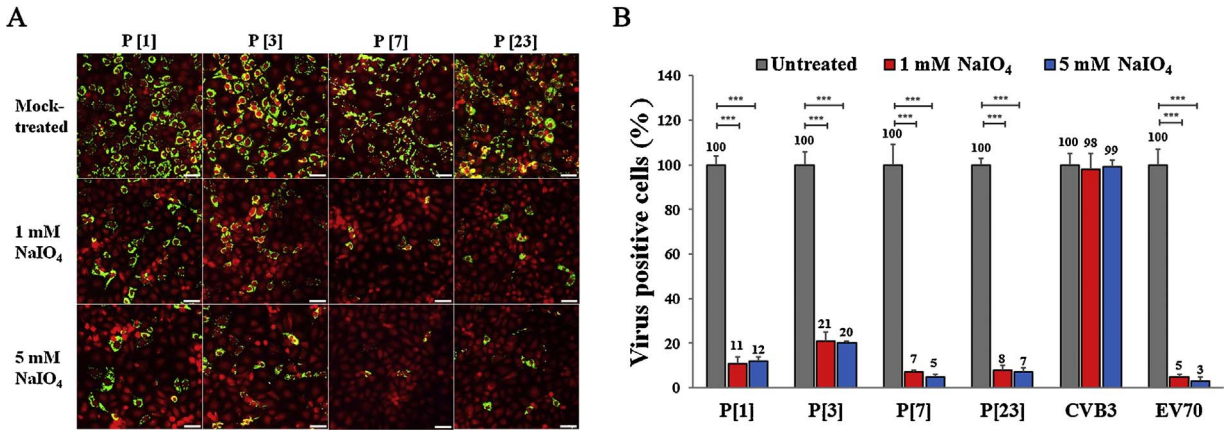

Fig. 1. (A) Infectivity of MA104 cells by four P[I] genogroup animal RVA strains after treatment with $NaIO_4$ as assessed by an immunofluorescence assay. Bars = 50 μm. (B) The levels of RVA antigen-positive cells as well as EV70 and CVB3 strains following $NaIO_4$ treatment are quantified from three independent fields of view and expressed as percentage of mock-treated, virus-infected cells. Error bars indicated standard deviations from triplicate experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

human cervical cancer HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown in each corresponding medium with antibiotics and serum supplements as described elsewhere (Kim et al., 2012, 2014). The bovine RVA G6P[1] strain NCDV-Lincoln and the canine RVA G3P[3] strain CU-1 were purchased from the ATCC. The porcine RVA strains G9P[7] PRG9121 and G9P[23] PRG942 were isolated from piglets with diarrhea in South Korea (Kim et al., 2012). The RVA strains used were propagated in MA104 cells as described elsewhere (Kim et al., 2012). The enterovirus 70 (EV70) J670/71 strain (ATCC) was propagated in WI-38 cells and the coxsackievirus B3 (CVB3) Nancy strain (ATCC) was cultured in HeLa cells (Kim et al., 2014). Each viral titer was assessed by the cell culture immunofluorescence (CCIF) assay using monoclonal antibodies (Mabs) specific to each virus as described below, and was expressed as fluorescence focus units per milliliter (FFU/mL).

2.2. Reagents and antibodies

Sodium periodate ($NaIO_4$; Sigma-Aldrich), *Maackia amurensis* lectin (MAL; Sigma-Aldrich), and *Sambucus nigra* lectin (SNL; Sigma-Aldrich) were dissolved in PBS (pH 7.2). DL-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; Calbiochem) and benzyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (BenzylGalNAc; Sigma-Aldrich) were dissolved in ethanol. Tunicamycin (Sigma-Aldrich) was dissolved in DMSO. Other reagents included NA (Sigma-Aldrich), and sialidase S (SS; Prozyme). Biotin-conjugated HBGAs-polyacrylamides (PAA) were purchased from GlycoTech. Antibodies used in this study were anti-RVA VP6 capsid (Median Diagnostics), anti-EV70 capsid (GeneTex), anti-CVB3 capsid Mabs (Millipore), Mabs against various HBGA molecules, and FITC-conjugated goat anti-mouse IgG-antibody (Santa Cruz). Horseradish peroxidase-conjugated streptavidin was obtained from Jackson Immuno Research Lab.

2.3. Infectivity assay

Chemicals, metabolic inhibitors, and enzymes used in the infection inhibition assay were carried out as described previously (Kim et al., 2014). Infectivity assays for each virus were carried out as described previously with only slight modification (Kim et al., 2014). Briefly, mock- and reagent-treated cells were infected with each virus strain at a multiplicity of infection (MOI) of 1 FFU/cell and incubated at 37 °C for 1 h. The inoculum was removed and then maintenance medium was added. The cells were then incubated for 5 h (CVB3 Nancy), 14 h (EV70, J670/71), or 18 h (each RVA strain) at 37 °C before being fixed with 80% acetone in PBS. Fixed cells in 8 well chamber slides were washed with PBS (pH 7.2). Mabs against RVA VP6, EV70 capsid, or CVB3 capsid were added and incubated at 37 °C for 1 h or 4 °C overnight. Cells were then washed 3 times with PBS (pH 7.2) and FITC-conjugated goat anti- mouse IgG Mab and then incubated for 1 h at 37 °C. Nuclei were stained with 500 nM propidium iodide for 5 min and cells were then examined using fluorescence microscopy.

2.4. Synthetic HBGA binding assay

Recombinant GST-VP8* domains of four animal strains in the genogroup [I] and one human G11P[25] Dhaka6 strain in the genogroup [III], and the P particle of VA387 (genogroup II genotype 4) human norovirus (NV) strain were cloned, expressed, and purified as described previously (Hu et al., 2012; Tan and Jiang, 2005; Tan et al., 2011). Using purified RVA VP8* and NV P particle, the synthetic oligosaccharide-based HBGA binding assay was carried out as described previously (Huang et al., 2012). The signal intensities were displayed using a TMB kit (KOMA BIOTECH, Korea), and the optical density (OD) at 450 nm was read using a microplate reader (Thermo Fisher Scientific).

2.5. Statistical analysis

Statistical analysis was performed by GraphPad Prism 5.03 (USA). A one-way analysis of variance (ANOVA) was used to determine the statistical significance ($P < 0.05$).

3. Results and discussion

P[1]-bearing strains are one of the most important bovine RVAs (Papp et al., 2013), whereas P[3]-carrying RVA strains were most popularly detected in the cats (Dhama et al., 2009). Though porcine RVA strains belonging to P[7], P[6] and P[13] in order are commonly detected pathogens, porcine P[23] strain is relatively common in South Korea (Kim et al., 2012). Therefore, we selected four representative strains: bovine G6P[1] NCDV, porcine G9P[7] PRG9121 and G9P[23] PRG942, and canine RVA G3P[3] CU-1. To confirm whether or not these four strains require carbohydrate moieties for binding and infection, we removed the carbohydrate moieties from the cells by pretreatment with $NaIO_4$. As a result, the infectivity of bovine P[1] NCDV, canine P[3] CU-1, and porcine P[7] PRG9121 and P[23] PRG942 strains was markedly decreased (Fig. 1), confirming that these strains utilize carbohydrate moieties as an attachment factor.

To further confirm or determine the NA-sensitivity of the above selected animal RVA strains, we used NA from *V. cholera*, which cleaves the terminal unbranched α2,3-linked, α2,6-linked, and α2,8-linked SAs from the underlying glycans (Kim et al., 2014). The infection assay