Substrate Specificity of Equine and Human Influenza A Virus Sialidase to Molecular Species of Sialic Acid

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Most equine influenza A viruses (IAVs) show strong binding to glycoconjugates containing N-glycolylneuraminic acid (Neu5Gc) as well as N-acetylneuraminic acid (Neu5Ac). Therefore, the progeny of equine IAV is thought to be released from the infected cell surface through removal of sialic acids by the viral sialidase. In the present study, equine IAV sialidases showed significantly lower substrate affinity than that of human IAV sialidases to artificial and natural Neu5Gc-conjugated substrates. The substrate specificity of equine IAV sialidases is in disagreement with their binding specificity to molecular species of sialic acid. The results suggest that substrate specificity of equine IAV sialidase for Neu5Ac, rather than for Neu5Gc, is important for an advantage at the early infection stage and the process of progeny virus release from the surface of infected cells.

Key words equine influenza A virus; human influenza A virus; N-glycolyl neuraminic acid; Neu5Gc; neuraminidase; sialidase activity

Influenza A virus (IAV) initiates infection through binding of virus hemagglutinin (HA) to terminal sialic acids in glycoconjugates on the host cell surface. In the late infection stage, progeny virus particles are thought to be released from the infected cell surface through removal of sialic acids by sialidase activity of virus neuraminidase (NA). Molecular species of sialic acid are mainly divided into two types, N-acetylneuraminic acid (Neu5Ac) and N-glycolyneuraminic acid (Neu5Gc). Most of the equine and some of the human IAVs can bind to terminal Neu5Gc- as well as Neu5Ac-galactose linkage. Since equine H7 and some H3 IAVs show preferential binding to Neu5Gc, which is mainly expressed in the horse trachea and red blood cells (RBC), Neu5Gc has been thought to be the main receptor of equine IAV. Sialidase activity of equine IAV is therefore thought to be sensitive to Neu5Gc due to facilitation of the virus release process.

It has long been thought that an important factor for efficient replication and transmissibility of IAV is a balance between the functions of HA and NA. For example, strong receptor binding avidity of HA is believed to require strong sialidase activity of NA at a level similar to that of HA for efficient replication and transmissibility of IAV. Conversely, weak binding avidity of HA requires similar weak activity of NA. This hypothesis suggests the importance of a balance between the functions of HA and NA. For the hypothesis, the molecular species of sialic acid has not been validated. HA of most equine IAVs strongly prefers terminal Neu5Gc-galactose linkage to Neu5Ac. If NA of most equine IAVs has much higher substrate specificity for Neu5Gc than that of human IAVs preferring Neu5Ac binding, the hypothesis about a balance between HA and NA would be greatly supported from a new aspect of sialic acid molecular species.

To investigate whether sialidase activity of equine IAV coincides with the viral binding activity in recognition of molecular species of sialic acid, we measured substrate specificities of equine IAV sialidases for Neu5Gc and Neu5Ac by using artificial and natural substrates containing different molecular species of sialic acid and compared them with those of human IAV.

MATERIALS AND METHODS

Cells, Viruses and Substrates Human embryonic kidney 293T cells were maintained in high-glucose Dulbecco’s modified Eagle’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS).


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Neu5Ac) was purchased from Sigma-Aldrich, St. Louis, MO, U.S.A. 2′-(4-Methylumbelliferyl)-α-N-glycolyneuraminic acid (4MU-Neu5Gc) was synthesized by Dr. Tadamune Otsubo and Dr. Kiyoshi Ikeda (Fig. 1).

Measurement of Sialidase Activity Using 4MU-Neu5Ac or 4MU-Neu5Gc  Use of the same protein amount (1.25 µg/mL) of virus showed various absolute sialidase activity (Fig. S1). The protein amount of virus does not necessarily match the absolute sialidase activity among virus strains. Therefore, we measured relative substrate specificity of virus sialidase activity for Neu5Ac and Neu5Gc as follows. Ten microliters of each virus (final protein concentration, 0.3–10 µg/mL) in 100 mM acetate buffer (pH 6.0) was reacted with 2.5 µL of 0.2 mM 4MU-Neu5Ac or 4MU-Neu5Gc at 37°C for 30 min. The protein amounts of each virus were determined within quantitative linear ranges of sialidase activities when the sialidase activities were measured in serial dilutions of virus suspensions. The reaction was stopped with 100 µL of 100 mM carbonate buffer (pH 10.7). Fluorescent intensity was measured at an excitation of 355 nm and emission of 460 nm using an Infinite M200 (Tecan Group Ltd., Männedorf, Switzerland). The substrate specificity (percentage of 4MU-Neu5Gc/4MU-Neu5Ac) in each virus strain is shown as an average calculated from relative percentages of 4MU-Neu5Gc/4MU-Neu5Ac in each dilution of the virus within the ranges used. To measure enzymatic parameters, 4MU-Neu5Ac and 4MU-Neu5Gc were 2-fold serially diluted from 0.4 and 1 mM, respectively. These diluted substrates were reacted with each virus (final protein concentration, 0.6–10 µg/mL) at 37°C for 30–120 min. Values of K_m and V_max were calculated by a Hanes–Woolf plot. When sialidase activities were measured in serial dilutions of viruses and with different reaction times, virus concentrations and reaction times within a linear manner of the activity were used as appropriate. Standard error (S.E.) was calculated from three or four independent values.

Expression of the Viral NA Gene in Cells  Human IAV NA genes were obtained from A/Aichi/2/1968 (H3N2), A/Hong Kong/1/1968 (H3N2), A/Texas/1968 (H2N2) and A/Memphis/1/1971 (H3N2). These NA genes were inserted into the restriction enzyme sites between EcoRI and Xhol of the expression vector pCAGGS/MCS as described previously. 7) Viral RNAs of equine IAVs were obtained by using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.). The NA genes were amplified using TaKaRa RNA PCR Kit Ver. 3.0 (TaKaRa Bio, Shiga, Japan) and inserted into the restriction enzyme sites between EcoRI and Xhol of the expression vector pCAGGS/MCS. Nucleotide sequences of all NA genes were confirmed. The expression vectors containing NA genes (1 µg/well) were transfected into 293T cells at 70% confluence in a 24-well plate using TransIT-293 reagent (Mirus Bio LLC, Madison, WI, U.S.A.). After 24 h, the cells were collected with 0.5 mL of phosphate buffered saline (PBS; pH 7.2, 131 mM NaCl, 14 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl) and centrifuged for 9 min at 4°C (700×g). The pellets were suspended with 1.2 mL of PBS. The cell suspensions were divided into 600.0, 300.0, 150.0, 60.0, 30.0, and 15.0 µL. After centrifugation for 9 min at 4°C (700×g), the pellets were suspended with 170 µL of 100 mM acetate buffer (pH 6.0). A 50-µL aliquot of the cell suspension was reacted with 2.5 µL of 0.4 mM 4MU-Neu5Ac or 4MU-Neu5Gc at 37°C for 30 min. The reaction was stopped with 200 µL of 100 mM carbonate buffer (pH 10.7). The substrate specificity (percentage of 4MU-Neu5Gc/4MU-Neu5Ac) in each NA is shown as an average from respective relative percentages of 4MU-Neu5Gc/4MU-Neu5Ac in two-fold dilutions of cell suspensions within quantitative linear ranges of sialidase activity. S.E. was calculated from three independent values.

Analysis of Neu5Ac and Neu5Gc Using HPLC  One percent of human or equine RBC was fixed with glutaraldehyde (final concentration, 1.5%) at 4°C for 20–22 h to prevent hemolysis under an acidic condition. To measure Neu5Ac and Neu5Gc contents on the RBC, fluorometric determination of sialic acids was conducted by the modified HPLC method using 1,2-diamino-4,5-methylenedioxybenzene (DMB) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described. 8,9 Briefly, 20 µL of 6.25–50% RBC in 100 mM acetate buffer (pH 6.0) was reacted with 180 µL of 25 mM sulfuric acid at 80°C for 12 h (hydrolysis of glyco-chains). The supernatant of the reaction mixture was centrifuged through Microcon YM-3 (MILLIPORE, Billerica, MA, U.S.A.) at 4°C for 180 min (14000×g). One hundred microliters of the flow-through (sialic acids) was reacted with 100 µL of DMB reagent consisting of 7 mM DMB, 1 mM β-mercaptoethanol and 18 mM sodium hydrosulphite (Sigma-Aldrich Corp.) in water. After incubation at 60°C for 2.5 h under protection from light (fluorescent derivatization of sialic acid), the mixture was cooled on ice (derivatization reaction stop). A 100-µL aliquot of the supernatant was injected into the HPLC equipment with a TSKgel ODS-100V 5 µm (4.6×150 mm) column (TOSOH Inc., Tokyo, Japan) at 40°C under a flow rate 1.2 mL/min of methanol–water=25:75 (v/v). Fluorescent intensity of sialic acid derivatives (excitation at 373 nm and emission at 448 nm) was measured by the fluorescent detector FP-2020 Plus (JASCO Corp., Tokyo, Japan). For the establishment of calibration curves, standard mixtures of Neu5Ac and Neu5Gc (0.1, 1, 10 µM) (Sigma-Aldrich Corp.) or water only were used.

Measurement of Sialidase Activity Using Human and Equine RBC  One hundred eighty microliters of 6.25–50% human or equine RBC was reacted with 180 µL of 0.1 mg/mL human (A/Memphis/1/1971, 0.063 units for 4MU-Neu5Ac) or equine IAV (A/equine/Miami/1963, 0.056 units for 4MU-Neu5Ac) at 37°C for 12 h. The reaction mixture was centrifuged through Microcon YM-3 at 4°C for 180 min (14000×g). Neu5Ac and Neu5Gc contents in the flow-through were measured using HPLC as described above.

RESULTS

To investigate substrate specificities of equine and human IAV sialidases for sialic acid species, Neu5Ac and Neu5Gc,
we measured sialidase activities of eighteen equine and nine human IAVs using 4MU-Neu5Ac and 4MU-Neu5Gc (Fig. 1). Neu5Gc/Neu5Ac ratios (%) of equine IAV sialidases were 3.8–13.4 (median, 6.3). On the other hand, Neu5Gc/Neu5Ac ratios (%) of human IAV sialidases were 20.4–52.9 (median, 34.7). Substrate specificities of equine IAV sialidases to Neu5Gc were significantly lower than those of human IAV sialidases ($p<0.01$ using the Mann–Whitney $U$-test) (Fig. 2A). To determine whether this property is dependent on NA protein only, we obtained NA genes from some of the virus strains used in an experiment for which results are shown in Fig. 2A and measured sialidase activities of 293T cells transfected with eight equine and four human IAV NA genes using 4MU-Neu5Ac and 4MU-Neu5Gc. Neu5Gc/Neu5Ac ratios (%) of equine IAV sialidases were 4.4–12.4 (median, 5.8). On the other hand, Neu5Gc/Neu5Ac ratios (%) of human IAV sialidases were 23.9–56.6 (median, 44.2). As was found for virus sialidases, substrate specificities of equine IAV NAs to

Fig. 2. Comparison of Substrate Specificities of Equine and Human IAV Sialidases between Neu5Ac and Neu5Gc
A: Substrate specificities of equine (filled column) and human IAV sialidases (open column) for Neu5Gc against Neu5Ac. B: Substrate specificities of NA-expressed cell sialidases for Neu5Gc against Neu5Ac. Sialidase activities for 4MU-Neu5Gc (Neu5Gc/Neu5Ac ratio) are shown as a percentage of those for 4MU-Neu5Ac. Strain names are arranged in order of isolated year.
Neu5Gc were significantly lower than those of human IAV NAs \((p<0.01\) using the Mann–Whitney U-test) (Fig. 2B), indicating that substrate specificity to Neu5Gc was dependent on NA protein only. To search for an amino acid residue in NA responsible for the substrate specificities, amino acid sequences were compared among NA genes used in cell expression. Amino acid homology among NAs was 90–99\% for the same subtype and host and 43–44\% for different subtype and host. We focused on important amino acid residues for the enzymatic activity, residues of the active site and neighboring the active site. There was no significant difference in these residues between equine IAV NA and human IAV NA.

We calculated enzymatic parameters of four equine and four human IAV sialidases using 4MU-Neu5Ac and 4MU-Neu5Gc. \(K_m\) values, reciprocally related to affinity of enzymes to substrates, of equine IAV sialidases for 4MU-Neu5Ac and 4MU-Neu5Gc were significantly higher than those of human IAV sialidases \((p<0.05\) using the Mann–Whitney U-test) (Table 1). Sialidase activity of equine IAV showed significantly lower affinity for both 4MU-Neu5Ac and 4MU-Neu5Gc than that of human IAV. Median in 4MU-Neu5Ac/median in 4MU-Neu5Gc ratios from \(K_m\) values were 10.8 (2337.5/216.9) for equine IAV and 4.7 (385.8/82.7) for human IAV. Equine IAV sialidases showed low affinity, especially for 4MU-Neu5Gc, compared to human IAV sialidases. In NA subtypes of equine IAV, median in 4MU-Neu5Gc/median in 4MU-Neu5Ac ratios from \(K_m\) values were 13.4 (2337.5/173.9) for N7 and 15.4 (5106.1/330.5) for N8. In NA subtypes of human IAV, median in 4MU-Neu5Gc/median in 4MU-Neu5Ac ratios from \(K_m\) values were 6.5 (289.5/44.2) for N1 and 4.1 (496.1/121.9) for N2. The substrate specificities for Neu5Gc in N1 and N2 from enzymatic parameters were higher than those in N7 and N8. The specificity (affinity) for Neu5Gc was the highest in N2 of tested NAs.

Neu5Ac and Neu5Gc analysis of RBC by HPLC demonstrated that 100\% human RBC contained of 183.9 \(\mu\)M Neu5Ac (100\%) and 0 \(\mu\)M Neu5Gc (0\%) and that 100\% equine RBC contained of 54.3 \(\mu\)M Neu5Ac (14.9\%) and 310.7 \(\mu\)M Neu5Gc (85.1\%). To test substrate specificities of equine and human IAV sialidases to Neu5Ac and Neu5Gc from natural material, we measured sialidase activities of equine IAV [A/equine/Miami/1963 (H3N8)] and human IAV [A/Memphis/1/1971 (H3N2)] using equine RBC as a Neu5Gc substrate and human RBC as a Neu5Ac substrate. For both IAVs, Neu5Ac released from human RBC showed a dependency on the concentration of RBC. Neu5Gc released from equine RBC showed a dependency on the concentration of RBC. Neu5Gc released from equine RBC showed a dependency on the concentration of RBC. Neu5Gc released from equine RBC showed a dependency on the concentration of RBC. Neu5Gc released from equine RBC showed a dependency on the concentration of RBC.
dependency on the concentration of RBC for human IAV but not for equine IAV (Fig. 3). In the most efficient sialidase reaction using 45% RBCs, Neu5Gc/Neu5Ac ratios (%) were 24.8 for human IAV and 1.68 for equine IAV. The result is an experimental example using a natural substrate. It is possible that equine IAV sialidase also shows less substrate specificity than human IAV sialidase to Neu5Gc in natural substrates as in artificial substrates.

**DISCUSSION AND CONCLUSION**

We found that equine IAV sialidase showed less substrate specificity than human IAV sialidase to Neu5Gc conjugates in both artificial and natural substrates. This report is the first report showing substrate specificity of equine IAV sialidase to a molecular species of sialic acid. Our results showing more affinity to Neu5Gc for human IAV sialidase are in agreement with results for substrate specificity of human IAV sialidase to Neu5Gc reported by Kobasa et al. 10) Kobasa et al. also investigated substrate specificities of three avian and eight swine IAV strains for Neu5Gc. Avian IAVs had low specificity (5–20%) in percentages of Neu5Gc/Neu5Ac, like equine IAVs (3–14% in percentages of Neu5Gc/Neu5Ac) in the present study. Swine IAVs had the higher specificity (35–70% in percentages of Neu5Gc/Neu5Ac) than that of avian IAVs, like human IAVs (20–53% in percentages of Neu5Gc/Neu5Ac) in the present study. We also measured the substrate specificities of six avian and two swine IAV strains in the present study. Avian IAVs [A/duck/Hong Kong/86/1/1976 (H9N2), A/duck/Hong Kong/47/5/1976 (H7N2), A/hog/Hong Kong/92/1/1976 (H9N2), A/hog/Hong Kong/273/8/1978 (H2N2), A/duck/Hong Kong/313/4/1978 (H5N3), and A/duck/Hokkaido/8/1985 (H3N8)] had low specificity (8–25% in percentages of Neu5Gc/Neu5Ac). Swine IAVs [A/swine/Wisconsin/15/1930 (H1N1) and A/swine/Iowa/15/1930 (H1N1)] had slightly higher specificity (22–48% in percentages of Neu5Gc/Neu5Ac) than that of avian and equine IAVs (data not shown). Taken together, the results indicate that human and swine IAVs have higher substrate specificity for Neu5Gc than equine and avian IAVs. IAV infection is fundamentally limited to specific hosts. Substrate specificity may be involved in restriction of host specificity for IAV infection. A new subtype of human IAV emerged from transmission of swine IAV to humans. 11) It is possible that the higher substrate specificity of human IAV for Neu5Gc is derived from swine IAV.

Human cells are not able to synthesize Neu5Gc because the human Neu5Gc synthesis enzyme, CMP-Neu5Ac hydrolase, is not functional at the genome level. 1) Therefore, how more Neu5Gc recognition of human IAV sialidase affects human infection is not known. A pandemic in 1968 was caused by a new subtype of IAV, that emerged in pigs infected simultaneou sly with human IAV and swine IAV. 11) Neu5Gc is expressed in the pig tracheal epithelium. 8) Some human IAVs bind to Neu5Gc-linked sugar chains. 12) In infection of human IAV to pigs, Neu5Gc substrate specificity of human IAV sialidase may help replication in the pig trachea by promoting the virus release process from removal of Neu5Gc or by an unknown mechanism.

Sialidase activity of virus NA is thought to facilitate release of progeny virus from the surface of infected cells through removal of sialic acid to which virus HA binds. Neu5Gc has been reported to be the preferential binding receptor of most equine IAVs. However, lower specificity of equine IAV NA to Neu5Gc-conjugated substrates reported in this paper is in disagreement with this traditional function of virus NA. As an alternative function of NA, it has been reported that sialidase activity of IAV NA is important for efficient infection and virus replication via functions at the early infection stage (for example, maintenance of the activity through an endocytic pathway in the cell entry process). 13–15) For efficient infection and replication of equine IAV, high substrate specificity of NA for Neu5Ac may have an advantage at the early infection stage rather than at the time of the progeny virus release process from the surface of infected cells.

There is no report about a relationship between viral pathogenicity in an animal infection model and substrate specificity of viral sialidase activity for sialic acid molecular species. For human IAVs, A/Hong Kong/1/1968 (H3N2) and A/Shizouka/833/2009 (H1N1pdm) caused pandemics worldwide in 1968 and 2009, respectively. Substrate specificities of sialidase activity in these pandemic viruses were similar to those of other seasonal IAVs. It is thought that substrate specificity of human IAV for Neu5Gc is not associated with pandemic occurrence. A/Puerto Rico/8/1934 (H1N1) has been commonly used in a mouse infection model of IAV because the virus strain has high pathogenicity in mice. 16) Most human IAVs and equine IAVs show little or no pathogenicity in mice. It is thought that substrate specificity of IAV for Neu5Gc is not involved in its pathogenicity in mice.

HA of most equine IAVs has preferential binding to terminal Neu5Gc in sugar chains rather than to terminal Neu5Ac. Therefore, rich Neu5Gc-conjugated sugar chains in horses have been believed to be functional receptors of equine IAV. According to the hypothesis about a balance between the functions of HA and NA, 6) NA of most equine IAVs should have greater substrate specificity for terminal Neu5Gc-galactose linkage. However, the hypothesis is not supported by our results showing that equine IAV had less substrate specificity for Neu5Gc than that of human IAV preferring Neu5Ac binding (with human IAV A/Memphis/1/1971 showing no Neu5Gc binding). Our results also suggest that substrate specificity of equine IAV NA for Neu5Ac, rather than for Neu5Gc, is important for an advantage at the early infection stage and the process of progeny virus release from the surface of infected cells.

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