Glucosyl Hesperidin Prevents Influenza A Virus Replication in Vitro by Inhibition of Viral Sialidase

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Hesperidin, a flavonoid obtained from citrus fruits, is known to have multiple biological activities and antimicrobial activities for human viruses; however, hesperidin has very low solubility in water and the target molecule of hesperidin for influenza virus remains unknown. A water-soluble derivative of hesperidin, glucosyl hesperidin (GH), which was synthesized by regioselective transglycosylation with cyclodextrin glucanotransferase, has been reported to have biological activities that are as or stronger than those of hesperidin. To determine the inhibitory effect of GH on influenza A virus (IAV) infection, Madin–Darby canine kidney (MDCK) cells were treated with GH before, at the same time as, and after IAV inoculation. GH treatment before IAV inoculation had no effect on virus replication, whereas, treatment with GH at the same time as or after IAV inoculation induced distinct reduction in IAV replication. Inhibition analysis of GH against two surface glycoprotein spikes of IAV revealed that GH prevents IAV replication by inhibition of viral sialidase activity that is involved in the entry and release stages on IAV infection but not by receptor binding inhibition. GH had no cytotoxic effects on MDCK cells in a dose range of 0—25 mM. Our results provide useful information for the development of novel sialidase inhibitors for influenza prevention.

Key words glucosyl hesperidin; influenza virus; anti-influenza drug

Influenza is a common infectious disease in birds and mammals with high rates of morbidity and mortality. Four influenza pandemics occurred in the 20th century and caused more than 20—50 million of deaths. The World Health Organization is working to stockpile antiviral drugs for prevention of possible pandemics. Inhibitors of viral M2 or neuraminidase protein have been used as medicines for treatment of influenza-infected patients. Amantadine and rimantadine, which block the ion channel activity of the M2 protein, are not so effective due to the frequent emergence of drug-resistant viruses and the lack of inhibitory activity against the influenza B virus. The neuraminidase inhibitor oseltamivir is also compromised due to the emergence of drug-resistant viruses and the lack of inhibitory activity against the influenza B virus. The neuraminidase inhibitor oseltamivir is also compromised due to the emergence of drug-resistant viruses and the lack of inhibitory activity against the influenza B virus. The neuraminidase inhibitor oseltamivir is also compromised due to the emergence of drug-resistant viruses and the lack of inhibitory activity against the influenza B virus. Inhibitor (\{4S,5R,6R\}-5-acetamido-4-hydroxy-6-\{(1R,2R)-1,2,3-trihydroxypropyl\}-5,6-dihydro-4H-pyran-2-carboxylic acid) (DANA) were purchased from Sigma-Aldrich Co., Ltd., MI, U.S.A. Hybridoma-SFM complete DPM (HSFM) was purchased from Invitrogen Corporation, CA, U.S.A. The fluorogenic sialidase substrate 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) was purchased from Nacalai Tesque Inc., Tokyo, Japan. All chemicals were of the highest purity available.

Reagents Glucosyl hesperidin (GH) was a generous gift from Hayashibara Biochemical Laboratories (Okayama, Japan). Minimum essential medium (MEM) and sialidase inhibitor (\{4S,5R,6R\}-5-acetamido-4-hydroxy-6-\{(1R,2R)-1,2,3-trihydroxypropyl\}-5,6-dihydro-4H-pyran-2-carboxylic acid) (DANA) were purchased from Sigma-Aldrich Co., Ltd., MI, U.S.A. Hybridoma-SFM complete DPM (HSFM) was purchased from Invitrogen Corporation, CA, U.S.A. The fluorogenic sialidase substrate 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) was purchased from Nacalai Tesque Inc., Tokyo, Japan. All chemicals were of the highest purity available.

Cells Madin–Darby canine kidney (MDCK) cells were grown in Gibco minimal essential medium (MEM, Invitrogen Corporation, NY, U.S.A.) supplemented with 5% heat-inactivated (v/v) fetal bovine serum (FBS) and an antibiotic (50 µg/ml gentamicin).

Viruses Human and avian IAV strains, A/Puerto Rico (PR)/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/duck/HK/
Inhibition of IAV Infection by GH  Activity of GH against IAV infection was determined by three different protocols as shown in Fig. 2. In the first protocol (Protocol 1), MDCK cells were preincubated with HSFM containing various concentrations of GH (0.39–25 mM) for 12 h at 37 °C before inoculation of each IAV strain. After washing with HSFM, the cells were inoculated with each IAV strain (2.5 × 10^2 pfu per well) for 1 h at room temperature. After washing with HSFM, the cells were incubated for 12 h at 34 °C. In the second protocol (Protocol 2), each IAV strain suspension was preincubated with various concentrations of GH (0.39–25 mM) for 30 min at room temperature. Then MDCK cells were inoculated with the mixtures for 1 h at room temperature. After washing with HSFM, the cells were incubated for 12 h at 34 °C. In the third protocol (Protocol 3), MDCK cells were infected with each IAV strain at 2.5 × 10^2 pfu per well for 1 h at room temperature. After IAV inoculation, the cells were washed with HSFM and incubated with HSFM containing various concentrations of GH (0.39–25 mM) for 12 h at 34 °C. Progeny virus titers in the supernatant from infected cells were determined by a focus-forming assay. Cells initially infected with IAV were fixed with cold methanol for 30 s, and they were incubated with anti-nucleoprotein (NP) monoclonal antibody (Mab) for 30 min and then with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, U.S.A.) for 30 min at 4 °C. After washing with HSFM, the cells were incubated with mouse anti-NP Mab and HRP-conjugated goat anti-mouse IgG + M (Jackson Immuno Research, West Grove, PA, U.S.A.), and determined as previously described. Standard deviations were calculated from results of three independent experiments.

Sialidase Inhibition Assay  Sialidase activity was determined by a modified fluorometric assay previously described. Viral suspensions at 2 μl/well of a 96-well microtiter plate (black, flat-bottom, BD Falcon, U.S.A.) were preincubated for 30 min at 4 °C with 1 μl of GH solution diluted at various concentrations. The enzymatic reaction was initiated by addition of 1 μl of a substrate, 2′-[(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid. After incubation for 30 min at 37 °C, the reaction was terminated by addition of 100 μl of 100 mM carbonate buffer (pH 10.7). The fluorescence intensity resulting from substrate cleavage by the viral sialidase activity was measured at 355 nm (excitation) and 460 nm (emission) using a multilabel counter Wallac 1420 ARVO®x (PerkinElmer, MA, U.S.A.). Agent concentrations causing 50% inhibition (IC50) were calculated by plotting the percent inhibition of sialidase activity against the agent concentration. DANA was used as a positive control. Standard deviations were calculated from results of three independent experiments.

Hemagglutination Inhibition Assay  Hemagglutination inhibition (HAI) assay was carried out as described previously. Briefly, 25 μl of virus suspension (4 HA unit) was incubated for 1 h at 4 °C with 25 μl of inhibitors serially diluted two-fold with PBS (pH 7.2, 131 mM NaCl, 14 mM Na2HPO4, 1.5 mM KH2PO4, and 2.7 mM KCl) in 96-well microtiter plates. After adding 50 μl of 0.5% (v/v) guinea pig erythrocytes to each well, the plates were kept for 8 h at 4 °C. The maximum dilution of the agents showing complete inhibition of hemagglutination was defined as the HAI titer.
Bovine fetuin (10 mg/ml) was used as a positive control.

**Competitive Inhibition Assay with HRP-Conjugated Fetuin** Competitive inhibition assay with HRP-conjugated fetuin was performed in polystyrene microtiter wells (Nunc, Immuno plate Maxisorp, Denmark) with a slight modification as described previously.\(^{25}\) GH (300 mM) was dissolved in PBS containing 0.01% Tween 20 (T-PBS) and was diluted serially two-fold with T-PBS. Influenza virus A/Aichi/2/68 (H3N2)-PBS suspension (2\(^4\) HAU) was added to each well (50 \(\mu l\)/well) and incubated at 4\(^\circ\)C for 5 h. The virus suspension was removed and washed three times with T-PBS. After blocking with 250 \(\mu l\) of T-PBS and incubating at 4\(^\circ\)C for 16 h, each GH solution was added to the wells (50 \(\mu l\)/well) and incubated at 4\(^\circ\)C for 1 h. The solutions were removed from the wells and washed with T-PBS. HRP-conjugated fetuin was diluted 400 times with T-PBS and added to each well (50 \(\mu l\)/well). After incubation at 4\(^\circ\)C for 2 h, the wells were washed five times with T-PBS. HRP-conjugated fetuin that had bound to the viruses immobilized on the wells was detected with o-phenylenediamine (OPD) solution containing 4 mg of OPD and 0.01% \(\text{H}_2\text{O}_2\) in 100 mM phosphate-citric acid buffer, pH 5.0. The reactions were stopped with 4 N \(\text{H}_2\text{SO}_4\), and viral binding activities in the form of color development were determined at 492 nm, with a reference wavelength of 630 nm. As a positive control, bovine fetuin was used instead of GH.

**RESULTS AND DISCUSSION**

The antiviral effect of GH on human and avian IAVs was demonstrated by inhibition of viral infection in MDCK cells as shown in Fig. 2. The number of infected cells was scored as inhibition percentage of IAV-infected cells that were not treated with GH. Pretreatment of cells with GH (12 mM) for 12 h and washed with HSFM before IAV inoculation (Protocol 1) did not inhibit viral replication. However, GH treatment at the same time as IAV inoculation (Protocol 2) resulted in a clear decrease in the number of IAV-infected cells for all viruses tested, A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2) and A/duck/HK/313/4/78 (H5N3) (Fig. 3). The effect of GH treatment at the same time as IAV inoculation was further evaluated in a dose-dependent manner (Fig. 4). The IC\(_{50}\) values of GH treatment against the viruses A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2) and A/duck/HK/313/4/78 (H5N3), were 2.0 ± 0.03, 12.34 ± 1.32 and 2.8 ± 0.54 (mM ± S.D.), respectively. Inhibitory effects of GH on virus infection were about 2—70-times weaker than those of fetuin. The effect of GH treatment after IAV inoculation (Protocol 3) was also evaluated by a focus-forming assay for measuring the amounts of infectious progeny viruses that were produced from cells cultured for 12 h. GH treatment after IAV inoculation inhibited IAV replication (Table 1). The results suggest that GH works directly on the process of IAV replication.

The viral spike glycoprotein hemagglutinin (HA) is known to bind to sialic acid on the surface of host cells and mediate membrane fusion for the viral uncoating process.\(^{26,27}\) Hemagglutination of erythrocytes with IAV is mediated by the interaction of HA with a terminal sialic acid linked to sugar chains on the surfaces of erythrocytes. We examined the inhibitory effect of GH on IAV binding to sialic acid by HAI assay and a competitive inhibition assay with HRP-conjugated fetuin. However GH had no hemagglutination inhibition activity against the IAV strains tested (Table 2).

GH also showed no binding activity to A/Aichi/2/68...
(H3N2) in a dose range of 0—300 mM (Fig. 5) in the competitive inhibition assay.

Sialidase activity of the viral spike glycoprotein neuraminidase is indispensable for budding of IAV particles. The sialidase activity is also important for the initiation of IAV infection. We evaluated the effect of GH on the viral sialidase activity. GH exhibited an obvious inhibitory effect on sialidase activities of the three IAV strains tested (Fig. 6). IC_{50} values of GH against IAV strains A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/duck/HK/313/4/78 (H5N3) were 9.44±0.63, 17.08±0.25 and 19.77±0.27 (mM±S.D.), respectively. Inhibitory effects of GH on sialidase activities were about 100—2000 times weaker than those of DANA.

In this study, we demonstrated that GH inhibited both the initial viral infection and viral replication in MDCK cells. GH treatment at the same time as inoculation of IAV resulted in an obvious inhibition of viral replication. The sialidase activity of the spike glycoprotein neuraminidase (NA) plays a critical role in the budding of viral particles in the late stage of IAV infection. Matrosovich et al. reported that viral sialidase was important for the initiation of influenza virus infection. Recently, sialidase activity of influenza virus sialidase has been reported to function not only in the late stage but also in the initial stage of virus infection cycles, indicating that viral sialidase activity in late endosome/lysosome traffic on cell entry facilitates production of infectious progeny viruses from the infected cells. The results indicate that GH has effects not only in the late stage of IAV infection, such as the assembly and budding of progeny viruses, but also in the early stage, such as virus entry process via the endocytic pathway.

GH treatment at the same time as IAV inoculation appeared to have a stronger inhibitory effect than that after viral adsorption. This potential of GH treatment could preferentially target the early stage and late stage of virus infection as direct inhibitor of viral sialidase activity but not receptor binding activity. The inhibitory effects of GH on IAV replication were different from A/Aichi/2/68 (H3N2) and other strains in comparison with the effects of GH on their sialidase activities. Part of human IAV strains maintains avian-like low-pH stability of sialidase activity. The low-pH stability of IAV sialidase is functionally linked to virus entry via the endocytic pathway and contributes to the ability of virus replication. The difference of GH effects between A/Aichi/2/68 (H3N2) and other strains may depend on the low-pH stability of their sialidase activities.

The safety of high-dose and long-term administration of GH has been demonstrated in studies using mice and rats. Cytotoxic effects of GH on MDCK cells were examined by lactose dehydrogenase activity released from dead cells. Measurement of lactose dehydrogenase activity indicated that GH had no cytotoxic effects on the cells in a dose range of 0—25 mM (data not shown).

In conclusion, the present study showed that GH inhibits IAV infection in vitro and that GH is a potential additive and seed compound of easy-to-use commercial and clinical tools, due to its high water solubility, for prevention of influenza A

### Table 1. Inhibition of Progeny Virus Production from MDCK Cells by GH Treatment

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inhibition (%) (\pm S.D.)</th>
</tr>
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<tbody>
<tr>
<td>A/PR/8/34 (H1N1)</td>
<td>60.26±5.44</td>
</tr>
<tr>
<td>A/Aichi/2/68 (H3N2)</td>
<td>32.67±4.87</td>
</tr>
<tr>
<td>A/duck/HK/4/313/78 (H5N3)</td>
<td>21.07±5.30</td>
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</table>

MDCK cells were inoculated with each IAV strain. The IAV-infected cells were incubated with HSFM containing 12 mM GH. After incubation for 12 h at 34 °C, infectious progeny virus titers in the supernatants collected from the infected cells were determined by focus-forming assay. Inhibition of IAV replication was expressed as a percentage relative to the total number of foci per area without GH treatment.

### Table 2. Effect of GH on IAV Hemagglutination

<table>
<thead>
<tr>
<th>Virus</th>
<th>HAI titer (^a)</th>
<th>Fetuin</th>
<th>GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34 (H1N1)</td>
<td>4</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>A/Aichi/2/68 (H3N2)</td>
<td>8</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>A/duck/HK/4/313/78 (H5N3)</td>
<td>4</td>
<td>&lt;2</td>
<td></td>
</tr>
</tbody>
</table>

500 mM GH was serially two-fold diluted with 25 μL of PBS. HAI assay was performed as described in Materials and Methods. Bovine fetuin (10 mg/mL) was used as a positive control. Bars indicated standard deviations of three independent experiments.

### Fig. 5. Competitive Inhibition Assay for GH binding Affinity to IAV

The relative binding of GH (closed circle) to influenza virus A/Aichi/2/68 (H3N2) was determined by competitive inhibition assay with HRP-conjugated fetuin as described in Materials and Methods. Bovine fetuin (open circle) was used as a positive control. Bars indicated standard deviations of three independent experiments.

### Fig. 6. Inhibition of IAV Sialidase Activity by GH Treatment

Each IAV strain was incubated with GH solution (closed circle) serially diluted for 30 min at 4 °C. Sialidase activities were assayed by the fluorometric assay as described in Materials and Methods. Bovine fetuin (open circle) was used as a positive control. The experiments were carried out in triplicate. Bars indicate standard deviations.
virus infection.

REFERENCES