Table 2 | Primers used for ChIP

Primer	Sequence (5′-3′)
5′ inverted terminal region forward	CAATATGATAATGAGGGGGTGG
5′ inverted terminal region reverse	ACTACAACATCCGCCTAAAACC
E1A enhancer region forward	CGGTGTACACAGGAAGTGACAAT
E1A enhancer region reverse	AGTCTCCACGTAAACGGTCAAAGT
E1A promoter region forward	GGGTCAAAGTTGGCGTTTTA
E1A promoter region reverse	CAAAATGGCTAGGAGGTGGA
E1A coding region forward	GAGACATATTATCTGCCACGGAG
E1A coding region reverse	AGTGAGTAAGTCAATCCCTTCCTG
E1A 3′ region forward	CCTTCTAACACACCTCCTGAGATAC
E1A 3′ region reverse	ACACACGCAATCACAGGTTTAC
E1B promoter region forward	GTGTGTGGTTAACGCCTTTGT
E1B promoter region reverse	GAGGTACTGTTAGAGCTCTGTTCCA
IX promoter region forward	GGCTCTAGCGATGAAGATACAGAT
IX promoter region reverse	CATCACATTCTGACGCACCC
ML promoter region forward	AGGTGATTGGTTTGTAGGTGTAGG
ML promoter region reverse	CTCCTCGTTTTTGGAAACTGAC
VA I gene region forward	GTGCAAAAGGAGAGCCTGTAAG
VA I gene region reverse	AGGAAGCCAAAAGGAGCACT
Hexon ORF region forward	CGCAGTGGTCTTACATGCAC
Hexon ORF region reverse	CACACGGTTATCACCCACAG
E2B ORF region forward	AGAAGAACATGCCGCAAGAC
E2B ORF region reverse	TCGAAGGCGAGCTTAAGTGT
E2 late promoter region forward	ATTATCGGTACCTTTGAGCTGC
E2 late promoter region reverse	AGAATGTGGCCCTGGGTAAT
E3 promoter region forward	AAGTTCAGATGACTAACTCAGGGG
E3 promoter region reverse	AGAGTTAGGATTGCCTGACGAG
E4 ORF3 region forward	TGGCGTGGTCAAACTCTACA
E4 ORF3 region reverse	GATTTTTACAATGGCCGGACT
E4 promoter region forward	CCATAACAGTCAGCCTTACCAGT
E4 promoter region reverse	GTGACGATTTGAGGAAGTTGTG

Thermal Cycler Dice Real Time System (Takara) according to the manufacturers' protocol.

Table 1 indicates primer sequences for *GAPDH*, *CTCF*, E1A, E4, MLP, and E2A genes.

ChIP assays. ChIP assays were carried out according to the protocol from Chromatin Immunoprecipitation Assay Kit (Millipore) with minor modification, essentially as described previously[12,14]. Briefly, cells were fixed with 1% formaldehyde, followed by the addition of glycine at the final concentration of 125 mM for quenching. After centrifugation, cell pellets were lysed with SDS lysis buffer (50 mM Tris-HCl [pH 7.9], 10 mM EDTA, and 1% SDS), and lysates were subjected to sonication to shear the chromatin DNA to ~1 kbp in size. Sonicated samples were diluted 10 fold with ChIP dilution buffer (16.7 mM Tris-HCl [pH 7.9], 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, and 0.01% SDS) and then pre-cleared with Protein A Sepharose 4 Fast Flow (GE Healthcare). An antibody was added to the pre-cleared sample solution and incubated overnight at 4°C. Antibody-protein-DNA complexes were incubated with Protein A Sepharose at 4°C for 1 hr, and then the beads were washed with Low Salt Wash Buffer (20 mM Tris-HCl [pH 7.9], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), High Salt Wash Buffer (20 mM Tris-HCl [pH 7.9], 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, and 0.1% SDS), LiCl Wash Buffer (10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 0.25 M LiCl, 1% NP-40, and 1% deoxycholic acid), and TE (10 mM Tris-HCl [pH 7.9] and 1 mM EDTA) successively. Protein-DNA complexes were eluted from the beads with elution buffer (1% SDS, 100 mM $NaHCO_3$, and 10 mM DTT), and crosslinking was reversed by incubation at 65°C for 4 hr. After treatment with Proteinase K, DNAs were recovered by phenol/chloroform extraction and ethanol precipitation. Obtained DNA samples were subjected to qPCR as described above.

Table 2 shows primer sequences for 5′ inverted terminal region (5′ ITR), the E1A enhancer (E1A enh), the E1A promoter (E1A pro), the E1A coding region (E1A cds), the E1A 3′ region (E1A 3′), the E1B promoter (E1B pro), the IX promoter (IX pro), the ML promoter (MLP), the VA I gene (VA I), the Hexon ORF (Hexon), the E2B ORF (E2B orf), the E2 late promoter (E2late pro), the E3 promoter (E3 pro), the E4 ORF3 (E4 orf), and the E4 promoter regions (E4 pro).

siRNA-mediated knockdown, quantification of viral DNA, and western blot assays. These experiments were carried out essentially as described previously[12,14,19]. siRNA targeted for CTCF was commercially purchased (Stealth siRNA; Invitrogen). siRNAs were introduced into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Total DNAs were purified by treatment with Proteinase K, followed by phenol/chloroform extraction and ethanol precipitation, as described previously[19]. Quantitative determination of viral DNA was carried out by qPCR as described above. For western blot analyses, cell lysates were subjected to SDS-PAGE, and proteins were transferred to a PVDF membrane (Millipore). The membrane was incubated with primary antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (GE Healthcare). The blot was developed using Chemi-Lumi One (Nacalai tesque).

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Author contributions

T.K. and K.N. designed the study; T.K. and T.S. performed the experiments; T.K. and K.N. prepared the manuscript; All authors reviewed the manuscript.

Additional information

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ORIGINAL PAPER

Anti-influenza virus activity of *Ginkgo biloba* leaf extracts

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Abstract We examined the influence of *Ginkgo biloba* leaf extract (EGb) on the infectivity of influenza viruses in Madin–Darby canine kidney (MDCK) cells. Plaque assays demonstrated that multiplication of influenza viruses after adsorption to host cells was not affected in the agarose overlay containing EGb. However, when the viruses were treated with EGb before exposure to cells, their infectivity was markedly reduced. In contrast, the inhibitory effect was not observed when MDCK cells were treated with EGb before infection with influenza viruses. Hemagglutination inhibition assays revealed that EGb interferes with the interaction between influenza viruses and erythrocytes. The inhibitory effect of EGb was observed against influenza A (H1N1 and H3N2) and influenza B viruses. These results suggest that EGb contains an anti-influenza virus substance(s) that directly affects influenza virus particles and disrupts the function of hemagglutinin in adsorption to host cells. In addition to the finding of the anti-influenza virus activity of EGb, our results demonstrated interesting and important insights into the screening system for anti-influenza virus activity. In general, the plaque assay using drug-containing agarose overlays is one of the most reliable methods for detection of antiviral activity. However, our results showed that EGb had no effects either on the number of plaques or on their sizes in the plaque assay. These findings suggest the existence of inhibitory activities against the influenza virus that were overlooked in past studies.

Keywords Antiviral effect · *Ginkgo biloba* leaf extract · Hemagglutination · Influenza virus

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Introduction

Influenza viruses, members of the *Orthomyxoviridae* family, cause epidemics in the human population every year despite the availability of effective vaccines. In a severe pandemic year, millions of people die from the infection. Influenza viruses are classified on the basis of the antigenic properties of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1–H16) and nine NA subtypes (N1–N9) have so far been defined. Influenza virus infection is initiated by the interaction between HA and sialic acid moieties of glycoconjugates on host cells [16].

Several synthetic drugs such as amantadine and rimantadine (M2 ion channel inhibitors) and oseltamivir and zanamivir (NA inhibitors) have been available for decades, but all have side effects and thus somewhat limited usefulness [6, 11]. Therefore, novel substances and approaches are needed to control and prevent this viral disease. Various natural products have distinct anti-influenza virus activities [14]. We have demonstrated that a high-molecular-weight lignin-related fraction extracted from cones of *Pinus parviflora* Siebold et Zucc. suppresses the multiplication of influenza viruses by preventing viral RNA synthesis [9, 15]. We also reported that *Sanicula europaea* L. leaf extract contains an anti-influenza virus substance(s) that selectively inhibits influenza A viruses, but

not influenza B viruses [13]. Studies on the anti-influenza virus activity of natural products have dramatically increased over the past several years [14].

Ginkgo biloba leaf extract (EGb) is a potential phytomedicine with various pharmacologic effects: in particular, anticoagulant, vasodilator, and anti-inflammatory effects [17]. In many countries, EGb and similar products are prescribed as therapeutic agents for cerebral or peripheral vascular inefficiency and for cognitive impairments associated with aging [2, 3]. Unlike other herbal drugs, however, EGb has hardly been tested for its anti-influenza virus activity. In the present study, we examined the inhibitory effect of EGb on influenza viruses.

Materials and methods

Reagents

The powder of *Ginkgo biloba* leaf extract was prepared by Mitsubishi Paper Mills Co., Ltd., Japan. In brief, the dried *Ginkgo biloba* leaves were finely ground and then extracted with water containing alcohol. After removing the residue, the extracts were concentrated under reduced pressure. The concentrate was then filtered and treated with adsorption resin to eliminate the impurities. Finally, the extracts were concentrated under reduced pressure again and then dried to use as powder. The powder of *Ginkgo biloba* leaf extract was dissolved in DMSO at a concentration of 100 mg/ml and stored at −30 °C until use.

As main active ingredients, it is known that the extract contains not only flavonoids such as kaempferol, quercetin and isorhamnetin but also terpene lactones such as bilobalide, ginkgolide A, B, C and J as specific components derived from *Ginkgo biloba* leaves [1, 4, 5].

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were maintained in Eagle's minimum essential medium (MEM) at 37 °C, in a 5 % CO_2 atmosphere, supplemented with 10 % fetal bovine serum, 0.03 % L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses were grown at 35.5 °C for 48 h in allantoic sacs of 11-day-old embryonated eggs (Miyake Hatchery), and then the infected allantoic fluid was collected and stored at −80 °C until use.

Neutral red assay

The neutral red assay is based on incorporation of neutral red into lysosomes in living cells. To determine the effect of EGb on cell viability, MDCK cells (3.5×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37 °C overnight. After removal of the culture medium, 0.4 ml of MEM containing various concentrations of EGb or DMSO was added to each well of the plates. After incubation for 24 h at 37 °C, 0.2 ml of neutral red solution (0.15 mg/ml) was added to each well. After incubation at 37 °C for 3 h, wells were washed with 0.2 ml of a fixative (1 % formalin and 1 % $CaCl_2$). To extract the dye, 0.2 ml of 1 % acetic acid in 50 % ethanol was added to each well. After incubation at room temperature for 20 min, the amount of neutral red in each well was determined by measuring absorbance at 550 nm using a spectrometer. Results were represented as the cell number that was calculated from the standard curve of cell numbers. Furthermore, to determine the effect of EGb on the cell growth, MDCK cells (2.0×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37 °C overnight. After removal of the medium, 0.4 ml of MEM containing 0, 10 and 100 μg/ml of EGb were added to each well. As control groups, DMSO was added to each well at final concentrations of 0.01 or 0.1 %. After incubation at 37 °C for 0, 24, 48 and 72 h, viable cells were determined with the neutral red assay as described above.

Treatment of viruses and cells by EGb

For pre-treatment of viruses by EGb, influenza A/PR/8/34 virus (500 pfu/ml) was mixed with EGb at several concentrations, incubated at room temperature for 10 min, and then subjected to the plaque formation assay. For post-treatment by EGb, MDCK cells infected with influenza viruses were overlaid with 0.8 % agarose containing EGb at several concentrations in the plaque formation assay. To investigate the direct effect of EGb on host cells, MDCK cells were exposed to EGb at several concentrations and incubated at 37 °C for 1 h. After removing the medium containing EGb, MDCK cells were infected with influenza viruses followed by the plaque formation assay.

Plaque formation assay

A confluent monolayer culture of MDCK cells in a 6-well tissue culture plates was washed with serum-free MEM and then infected with 0.5 ml of influenza virus solution [500 pfu/ml = multiplicity of infection (MOI) of 2.5×10^{-4}] in serum-free MEM. After allowing 1 h at 37 °C for virus adsorption, the cells were washed with serum-free MEM and then overlaid with MEM containing 0.8 % agarose, 0.2 % BSA and 1 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). After incubation at 37 °C for 2–3 days, plaques were visualized by staining cells with 0.5 % amido black.

Results were represented as a ratio of the plaque number formed in the presence of EGb to that in the absence of EGb.

Hemagglutination assay

Influenza A/PR/8/34 virus (2×10^8 pfu/ml) was diluted nine times with phosphate buffered saline (PBS) (−) by twofold dilution each time, while 200 μg/ml of EGb was also diluted ten times with PBS (−) containing 0.2 % DMSO by twofold dilution each time. Fifty microliters of each diluted virus was mixed with 50 μl of each diluted EGb. These mixtures were then maintained at room temperature for 5 min. One hundred microliters of 0.5 % chicken erythrocyte suspension (Nippon Bio-Test Laboratories Inc., Japan) was added to each of these mixtures in 96-well round-bottom plates, and then the plate was incubated at room temperature for 30 min for hemagglutination. Results were represented as a plot where the *x*-axis and *y*-axis indicate concentrations of EGb and HA titer, respectively.

Statistical analysis

All of the data were represented as mean ± standard error of the mean (SEM). Comparisons for all pairs were performed by Student's *t* test. A *p* value >0.05 was considered to be not significant. The calculations of 50 % cytotoxicity concentration (CC_{50}) and inhibitory concentrations with 50 % plaque reduction (IC_{50}) were performed by nonlinear regression using GraphPad Prism's "log (inhibitor) versus response – variable slope" function (GraphPad Prism Version 5.01 for Windows, GraphPad Software Inc.).

Results

Effect of EGb on the viability and growth of MDCK cells

Before examining the anti-influenza virus activity of EGb, we investigated whether EGb affects the viability and growth of MDCK cells, which are routinely used as host cells for influenza viruses. We evaluated the cell viability and growth by counting the number of living cells as a function of time using the neutral red assay as described in "Materials and methods". Cytotoxic effects of EGb were not observed at concentrations of <10 μg/ml (CC_{50} = 180 μg/ml) (Fig. 1a). Neither the growth rate nor the final cell density was affected by the presence of 10 μg/ml of EGb, whereas a marked decrease in the cell growth rate was observed at 100 μg/ml (Fig. 1b). Thus, EGb at a concentration of <10 μg/ml could be considered essentially

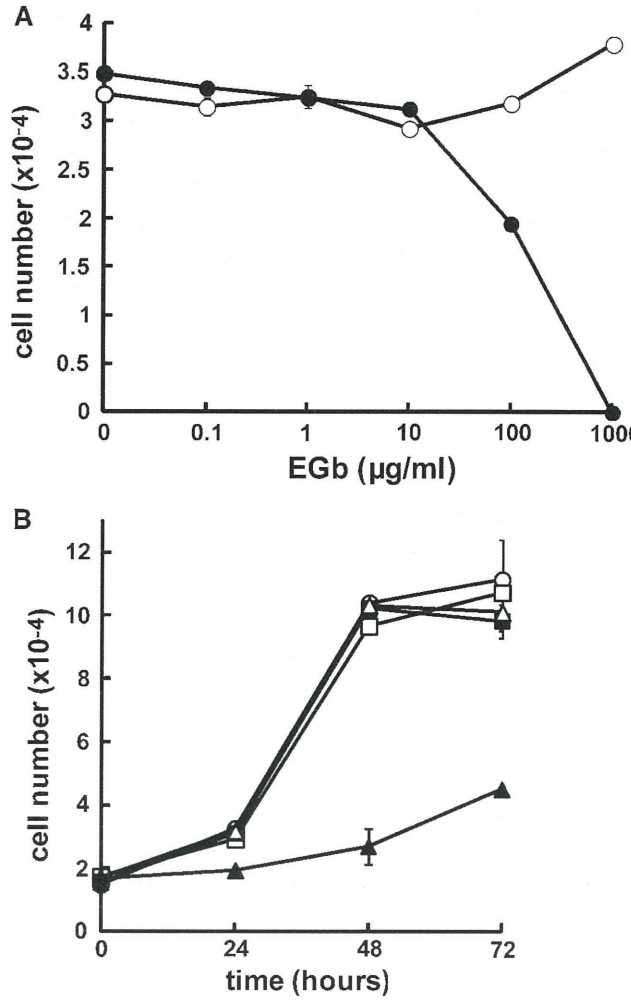

Fig. 1 Effect of EGb on the viability and the growth of MDCK cells. **a** MDCK cells (3.5×10^4) were seeded in 24-well tissue culture plates and incubated at 37 °C in the presence of various concentrations of EGb (*closed circles*) or solvent DMSO alone (*open circles*). After incubation for 24 h, the viable cell number was determined by the neutral red assay. **b** MDCK cells (2×10^4) were seeded in 24-well tissue culture plates and incubated at 37 °C in the absence (*open circles*) or presence of 10 μg/ml (*closed square*) and 100 μg/ml (*closed triangles*) of EGb, and 0.01 % (v/v) and 0.1 % (v/v) of DMSO alone (*open square* and *open triangle*, respectively). After incubation for the indicated periods, the viable cell number was determined by the neutral red assay

nontoxic to MDCK cells. We confirmed that the solvent DMSO had no effect on the viability and growth of MDCK cells in the range of concentrations used in this study (data not shown).

Inhibition of influenza virus infectivity by EGb

To examine whether EGb inhibits multiplication of influenza viruses, plaque assays were carried out as described in "Materials and methods". Cells were infected with influenza A/PR/8/34 virus at 37 °C for 1 h. The cells were

washed extensively with serum-free MEM and then overlaid with 0.8 % agarose in MEM containing EGb at various concentrations. The number of plaques and their sizes in the presence of EGb did not differ from those in the absence of EGb (Fig. 2a), indicating that EGb does not inhibit plaque formation by influenza virus infection. We further examined whether EGb is effective when mixed with viruses before exposure to cells. Influenza viruses were mixed with EGb at various concentrations at room temperature for 10 min and then exposed to MDCK cells. Under these conditions, EGb markedly inhibited viral infectivity in a dose-dependent manner (Fig. 2b). EGb at a concentration of 5 μg/ml almost completely inhibited the plaque-forming activity. These findings suggest that EGb inhibits the initial step of influenza virus infection before the virus enters the cytoplasm. Next, we examined whether the inhibitory effect of EGb against influenza virus was direct or indirect. Plaque assays were performed using MDCK cells treated with EGb at various concentrations for 1 h before infection with the influenza viruses. The number and sizes of the plaques of the tested groups in the presence of EGb did not differ significantly from those of the control group in the absence of EGb (Fig. 3), suggesting that EGb directly interacted with the influenza viruses and markedly reduced their infectivity.

Inhibition of hemagglutination by EGb

Influenza virus infection is initiated by the interaction of hemagglutinin (HA) on the virion with sialic acids on the host cell surface. To understand how EGb prevents virus adsorption to cells, we examined whether EGb competitively inhibits influenza virus-mediated hemagglutination. As shown in Fig. 4, EGb inhibited hemagglutination in a dose-dependent manner, suggesting that EGb interferes with the interaction between HA and sialic acids.

Susceptibility of other influenza virus strains to EGb

Our results suggest that EGb binds to HA and prevents virus adsorption to cells. We further examined whether the inhibitory effect of EGb is dependent on the type of influenza virus. EGb inhibited the infectivity of both influenza A/Udorn/72 (H3N2) and influenza B/Lee/40 viruses as well as of influenza A/PR/8/34 (H1N1) virus in an adsorption inhibition-dependent manner (compare Fig. 5a and b), albeit

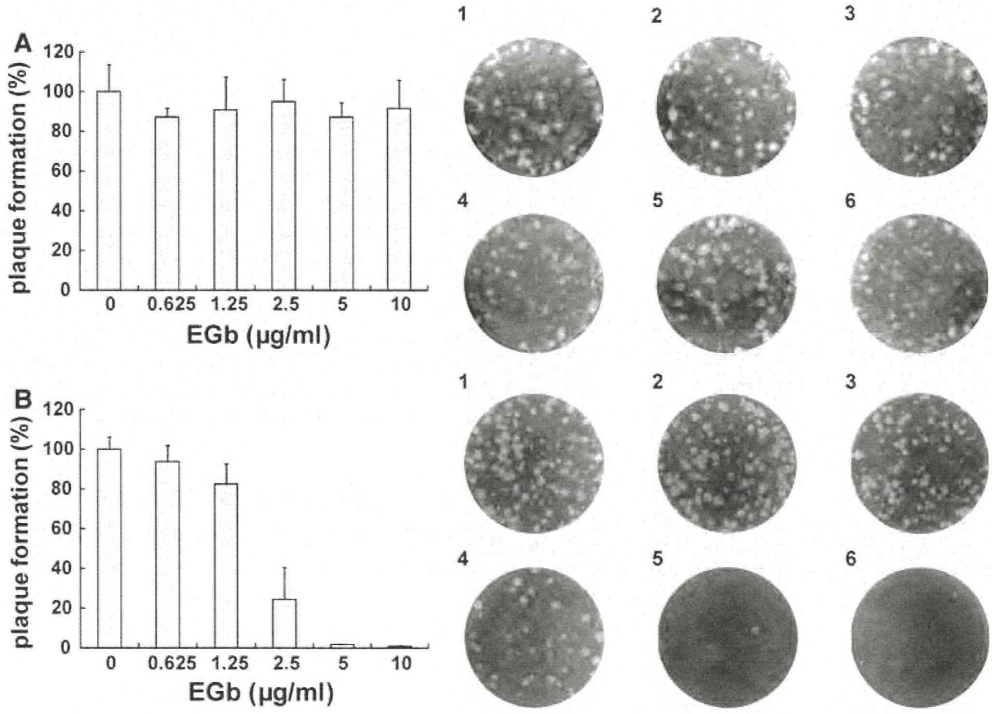

Fig. 2 Effect of EGb on plaque formation. Plaque assays were carried out as described in "Materials and methods". **a** MDCK cells were infected with virus suspension (500 pfu/ml) and then overlaid with the overlay medium containing various concentrations of EGb. The profile of plaques is shown in the *right panels*. *Panels 1*, *2*, *3*, *4*, *5* and *6* represent assays carried out in the presence of 0, 0.625, 1.25, 2.5, 5 and 10 μg/ml of EGb, respectively. **b** Influenza A virus (500 pfu/ml) was incubated with various concentrations of EGb prior to exposure to MDCK cells. The profile of plaques is shown in the *right panels*. *Panels 1*, *2*, *3*, *4*, *5* and *6* represent assays in the presence of 0, 0.625, 1.25, 2.5, 5 and 10 μg/ml of EGb, respectively. Results are represented as the percentage of the plaque number formed in the absence of EGb

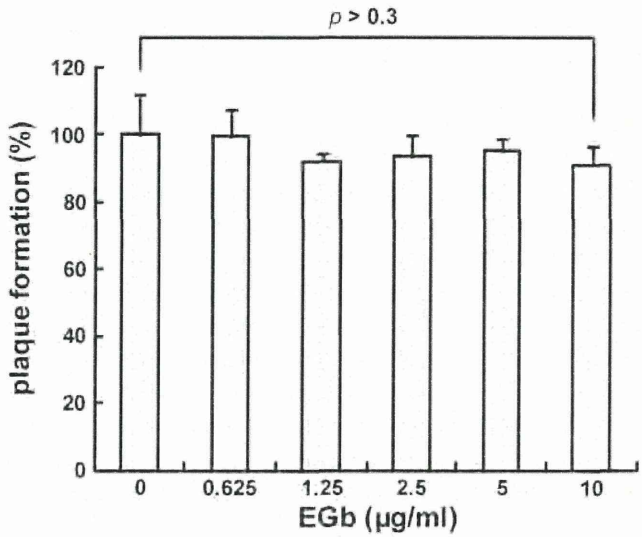

Fig. 3 Effect of pre-treatment of host cells with EGb on influenza virus infection. MDCK cells were exposed to EGb at various concentrations and incubated at 37 °C for 1 h prior to virus infections. After removing EGb, MDCK cells were inoculated with influenza A/PR/8/34 viruses (500 pfu/ml), and plaque formation assays were carried out as described in "Materials and methods". Results are represented as the percentage of the plaque number formed in the absence of EGb. All data are represented as mean ± SD, and the statistical analysis was performed using the *t* test to compare two groups

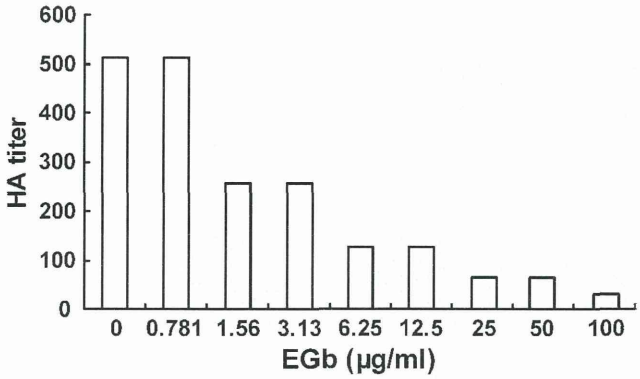

Fig. 4 HA titers of influenza A virus treated with various concentrations of EGb. Influenza A/PR/8/34 virus and EGb were diluted by twofold dilution each time and then mixed. After incubation at room temperature for 5 min, 0.5 % chicken erythrocyte suspension was added to each of these mixtures in a 96-well assay plate, and the plate was incubated at room temperature for 30 min for hemagglutination. Results are represented as a plot where the *x*-axis and *y*-axis indicate concentrations of EGb and HA titer, respectively. The result is representative of three independent experiments

with slightly different sensitivities. To confirm the difference in infectivity inhibition, the 50 % inhibitory concentration (IC_{50}) value of EGb was calculated for these three different strains of influenza viruses. Furthermore, the selectivity index (SI) was also calculated as the ratio of CC_{50} to IC_{50} (Table 1). The influenza A/PR/8/34 virus showed most

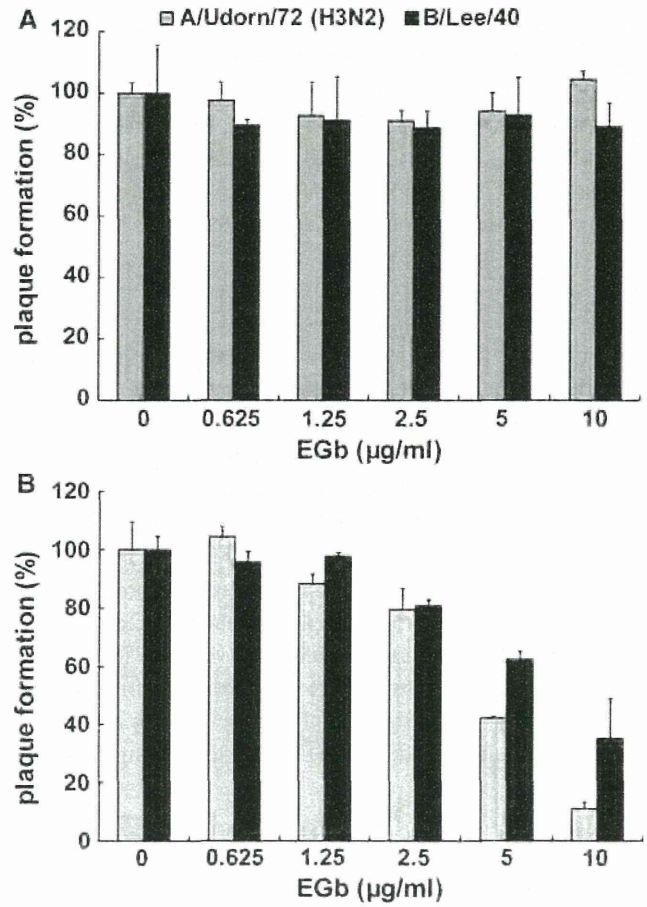

Fig. 5 Effect of EGb on plaque formation by two different subtypes of influenza virus. Plaque assays were carried out as described in "Materials and methods". **a** MDCK cells were infected with 0.5 ml of 500 pfu/ml of influenza A/Udorn/72 (H3N2) and B/Lee/40 viruses and then overlaid with the overlay medium containing various concentrations of EGb. **b** Each influenza virus strain was diluted to 500 pfu/ml and incubated with various concentrations of EGb prior to exposure to MDCK cells. One hour after virus inoculation, MDCK cells were washed with serum-free MEM and subsequently overlaid with the overlay medium without EGb. Results are represented as the percentage of the plaque number formed in the absence of EGb. **a**, **b** Results of A/Udorn/72 (H3N2) and B/Lee/40 are represented by *gray bars* and *black bars*, respectively

sensitivity to EGb (Table 1). These findings suggest that the antiviral activity of EGb is not dependent on the type of influenza virus.

Discussion

In this study, we revealed the anti-influenza virus activity of *Ginkgo biloba* leaf extract (EGb). EGb acted directly on the influenza viruses and prevented their adsorption to the host cell surface, suggesting that EGb interfered with the interaction between the HA on the influenza virion and sialic acids on the host cell surface, although we could not

Table 1 Selectivity indices of EGb in three different influenza virus strains

Virus strain	IC_{50} (μg/ml)[a]	SI[b]
A/PR/8/34 (H1N1)	1.86	96.8
A/Udorn/72 (H3N2)	4.41	40.8
B/Lee/40	6.79	26.5

[a] IC_{50}: 50 % inhibitory concentration of EGb was calculated from the results of the plaque formation assay performed as shown in Figs. 2b and 5b

[b] SI: selectivity index was evaluated as the ratio of CC_{50} to IC_{50}, i.e., $SI = CC_{50}/IC_{50}$

CC_{50}: 50 % cytotoxic concentration of EGb was calculated from the dose–response curve shown in Fig. 1a and its value (=180 μg/ml) was used for the calculation of each SI

All calculation was performed by using GraphPad Prism software as described in "Materials and methods"

exclude the possibility that EGb had a viricidal activity and directly inactivated the influenza virus.

The active constituents of EGb are standardized around the world; i.e., they contain 24 % flavonol glycosides (quercetin, kaempferol and isorhamnetin) and 6 % terpene lactones (ginkgolides and bilobalide). EGb also contains a class of condensed tannins, which are polymers composed primarily of flavan-3-ols (catechin and epicatechin) with a covalent bond between the individual flavonol units. Nakayama et al. [10] previously reported that two condensed tannins present in teas, (−)-epigallocatechin gallate (EGCG) and theaflavin digallate, bind to the HA of the influenza virus and inhibit its adsorption to MDCK cells. Furthermore, Song et al. [12] showed that catechin derivatives, including EGCG from green tea, inhibit not only the hemagglutination but also the NA activity of the influenza virus. The neuraminidase activity is thought to play a key role in the release of progeny virions from infected cells by cleavage of the sialic acid moieties of host cell receptors and in the prevention of self-aggregation of virions by cleavage of sialic acid still bound to the virus surface. These findings provide important insights into the molecular mechanisms of action of EGb.

Ginkgetin, a biflavone originally isolated from *Ginkgo biloba* leaves, has been found to inhibit the influenza virus sialidase [7]. However, our results showed that EGb prevented adsorption in the initial step of influenza virus infection. Therefore, in our study, a substance(s) in EGb other than ginkgetin may have been effective against influenza virus infection.

EGb was effective against the three different types of influenza viruses tested here, viz., the influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses, although the sensitivity towards EGb was slightly different among the three viruses. This finding suggests that EGb may be a potential wide-range inhibitor against influenza virus infection.

When plaque assays were performed with overlay medium containing EGb, neither the number of plaques nor their sizes were affected (Fig. 2a). Since our results suggest that EGb acts directly on the influenza virus and prevents the initial step in viral infection, we expected that the infectivity of the progenitor virions would be decreased owing to interaction with EGb present in the overlay medium and, consequently, that the size of individual plaques would be reduced in the plaque assay. This discrepancy between the predicted and the experimental results may be explained by our recent findings: we disclosed a novel transmission mode for influenza viruses, the so-called cell-to-cell transmission mode [8]. Influenza viruses have generally been believed to be capable of spreading via *cell-free* virions released from infected cells depending on the activity of NA. However, in cell-to-cell transmission, progeny virions are retained on the infected cell surface even after budding and transmitted from infected cells to adjacent uninfected cells without being released into the outer environment. The cell-to-cell transmission of the influenza virus is dependent on functional HA but independent of NA activity. The present study may demonstrate that EGb cannot inhibit the cell-to-cell transmission of influenza viruses but is highly effective in decreasing the infectivity of *cell-free* virions. This suggestion is in line with the findings of a previous study in which higher concentrations of anti-HA antibody were needed for inhibition of infection through cell-to-cell transmission than for that through *cell-free* viruses [8].

The plaque assay using drug-containing agarose gels is one of the most reliable methods for detecting anti-influenza virus activity and is frequently used as a screening method. However, our findings raise concerns that a particular anti-influenza virus activity, such as the inhibitory effect found here in EGb, may have been largely overlooked in past studies.

In conclusion, we have shown that EGb interacts directly with influenza viruses and markedly reduces the infectivity of the viruses by preventing their adsorption to host cells. Furthermore, the inhibitory effect of EGb seemed not to be restricted to a certain subtype of influenza virus. Taken together, these findings indicate the usefulness of EGb as an antiviral agent for influenza, although further studies are necessary to confirm its anti-influenza virus activity in vivo.

In addition to the finding of the anti-influenza virus activity of EGb, we demonstrated an interesting and important insight(s) into the screening system for anti-influenza virus activity. As was the case for the anti-influenza virus activity of EGb found in this study, some candidates for antiviral agents may have been overlooked

in past studies because of the existence of the cell-to-cell transmission mode of influenza viruses. Therefore, our results signal a need for caution on the part of investigators trying to find anti-influenza virus compounds.

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Insight into structural diversity of influenza virus haemagglutinin

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Influenza virus infects host cells through membrane fusion, a process mediated by the low pH-induced conformational change of the viral surface glycoprotein haemagglutinin (HA). We determined the structures and biochemical properties of the HA proteins from A/Korea/01/2009 (KR01), a 2009 pandemic strain, and A/Thailand/CU44/2006 (CU44), a seasonal strain. The crystal structure of KR01 HA revealed a V-shaped head-to-head arrangement, which is not seen in other HA proteins including CU44 HA. We isolated a broadly neutralizing H1-specific monoclonal antibody GC0757. The KR01 HA-Fab0757 complex structure also exhibited a head-to-head arrangement of HA. Both native and Fab complex structures reveal a different spatial orientation of HA1 relative to HA2, indicating that HA is flexible and dynamic at neutral pH. Further, the KR01 HA exhibited significantly lower protein stability and increased susceptibility to proteolytic cleavage compared with other HAs. Our structures provide important insights into the conformational flexibility of HA.

INTRODUCTION

The past three pandemic influenza viruses, viz. 1957 H2N2, 1968 H3N2 and 2009 H1N1 originated from reassortants between the genes of animal influenza viruses and the virus that had been circulating in humans since 1918. The haemagglutinin (HA) genes specifically originated from avian influenza viruses (Garten *et al.*, 2009; Salomon & Webster, 2009; Zimmer & Burke, 2009). The recent H1N1 2009 pandemic (2009pdm) influenza virus that had been in worldwide circulation was genetically homogeneous (99.5 % genome sequence identity) (Garten *et al.*, 2009). Although the current genetic variation has now accumulated in the circulating viruses, all 2009pdm isolates sequenced to date encode a glutamate at position 627 in viral PB2 instead of lysine, carry truncations of both PB1-F2 and NS1, and lack a multibasic proteolytic activation site in HA. They therefore lack previously identified molecular determinants for assessing pandemic potentials implicated in the pathogenicity and transmission of the 1918 H1N1 and H5N1 viruses (Conenello *et al.*, 2007; Garten *et al.*, 2009; Lazarowitz & Choppin, 1975; Steel *et al.*, 2009). However, they replicate more extensively in the respiratory tract of infected ferrets, mice and non-human primates than seasonal influenza viruses (Huang *et al.*, 2011; Itoh *et al.*, 2009; Maines *et al.*, 2009; Munster *et al.*, 2009). Clinical severity associated with the 2009pdm virus is akin to the 1957 H2N2 pandemic outbreak (Fraser *et al.*, 2009).

Three supplementary tables and supplementary methods are available with the online version of this paper.