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Antiviral effect of catechins in green tea on influenza virus

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Abstract

Polyphenolic compound catechins ((−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin (EGC)) from green tea were evaluated for their ability to inhibit influenza virus replication in cell culture and for potentially direct virucidal effect. Among the test compounds, the EGCG and ECG were found to be potent inhibitors of influenza virus replication in MDCK cell culture and this effect was observed in all influenza virus subtypes tested, including A/H1N1, A/H3N2 and B virus. The 50% effective inhibition concentration (EC_{50}) of EGCG, ECG, and EGC for influenza A virus were 22–28, 22–40 and 309–318 μM, respectively. EGCG and ECG exhibited hemagglutination inhibition activity, EGCG being more effective. However, the sensitivity in hemagglutination inhibition was widely different among three different subtypes of influenza viruses tested. Quantitative RT-PCR analysis revealed that, at high concentration, EGCG and ECG also suppressed viral RNA synthesis in MDCK cells whereas EGC failed to show similar effect. Similarly, EGCG and ECG inhibited the neuraminidase activity more effectively than the EGC. The results show that the 3-galloyl group of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5′-OH at the trihydroxy benzyl moiety at 2-position plays a minor role. The results, along with the HA type-specific effect, suggest that the antiviral effect of catechins on influenza virus is mediated not only by specific interaction with HA, but altering the physical properties of viral membrane.
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Keywords: Influenza virus; Catechins; Green tea; EGCG; ECG; Hemagglutination

1. Introduction

Green tea is produced from the leaves of the evergreen plant *Camellia sinensis*. The major active ingredients of green tea are polyphenolic compounds, known as catechins. Catechins of green tea leaves are account for about 10% of dry weight, including (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) (Fig. 1) and (−)-epicatechin (EC), where EGCG accounts for approximately 50% of the total amounts of catechins in green tea (Balentine et al., 1997).

Various biological and pharmacological activities have been reported for EGCG, including antioxidative (Valcic et al., 1999), antibacterial (Toda et al., 1992), antitumor and antiviral activity. The antitumor effects of EGCG have been investigated in detail and the compound demonstrated inhibitory action against carcinogenesis on several different organs in animal models (Gensler et al., 1996; Katiyar and Mukhtar, 1996; Mimoto et al., 2000; Yamane et al., 1995; Yang et al., 2002). Studies have shown that antitumor effect correlates with inactivation of tumor-related proteases (Jankun et al., 1997), nitric oxide synthase (NOS) (Lin and Lin, 1997), and is mediated by PI3-to Akt kinase in the NF-kB pathway (Pianetti et al., 2002). Recently, laminin receptor has been identified as a receptor for EGCG mediating the anti cancer activity (Tachibana et al., 2004).

With regard to the antiviral activities, EGCG acts as a strong inhibitor of HIV replication in cultured peripheral blood cells (Fassina et al., 2002), and EGCG and ECG were found more effective than EGC or EC in the inhibition of the HIV-1 reverse transcriptase in vitro (Nakane and Ono, 1990). EGCG also binds directly to CD4 molecule with consequent inhibition of gp120 binding (Kawai et al., 2003). EGCG also induced inactivation of virus in vitro by deformation of phospholipids (Yamaguchi et al., 2002). Distinct antiviral activities of EGCG were reported for Epstein–Barr virus inhibition of expression of viral proteins (Chang et al., 2003)

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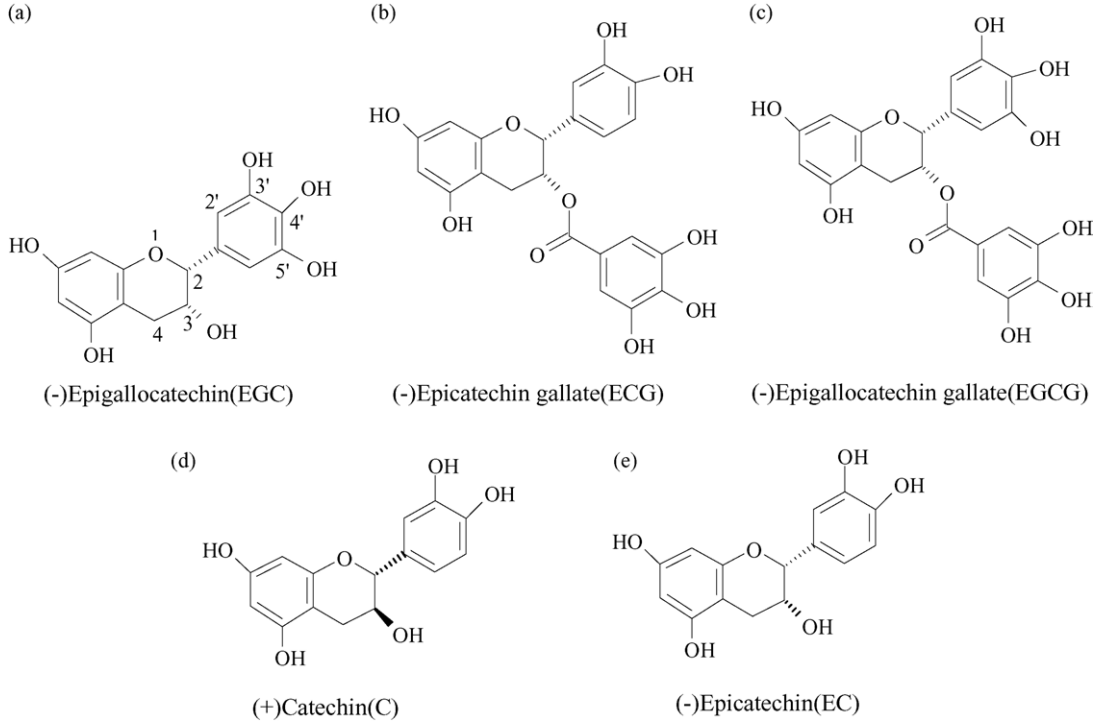

Fig. 1. Structures of (−)-epigallocatechin (EGC) (a), (−)-epicatechin gallate (ECG) (b), (−)-epigallocatechin gallate (EGCG) (C), (+)-catechin (C) and (−)-epicatechin (EC).

and inhibition of host factors (Weber et al., 2003). Antiviral effects of EGCG have also been reported for influenza virus. EGCG affected the infectivity of influenza virus in cell culture, and it was shown to agglutinate the viruses, preventing the viruses from absorbing to MDCK cells (Nakayama et al., 1993). It was also shown that green tea extract exerted an inhibitory effect on the acidification of intracellular compartments such as endosomes and lysosomes, resulting in inhibition of growth of influenza virus in cell culture (Imanishi et al., 2002). So far, however, biochemical studies on the anti-influenza effects of green tea polyphenols have focused on EGCG. With a view to investigate the structure-activity relationships of the green tea polyphenolic compounds, we investigated in this report the antiviral effects of various catechin compounds, EGCG, ECG and EGC, on influenza virus. The studies were further extended to all three currently circulating virus subtypes, including two different H3N2 and H1N1 influenza A types and one influenza B type. Here, we found that ECG and EGCG were much more effective than EGC, and besides the known inhibition of hemagglutination, the compounds also exerted inhibitory effect on neuraminidase and affects viral RNA synthesis at high concentration.

2. Materials and methods

2.1. Preparation of catechins

Green tea extract was prepared by infusing the leaves (Sulloc Cha, Amore-Pacific Co. Ltd., Korea) with 75 °C distilled water in the ratio of 1:7 (w/w). After 20 min of infusion, the tea extract was quickly separated from the tea leaves by filtration and the tea extract was freeze-dried for further tests. To isolate catechins, green tea leaves were extracted with five times of 90 °C distilled water for 5 h, and the tea extract was washed with chloroform and extracted with ethyl acetate three times. The ethyl acetate layers were combined and vacuum evaporated. The powder remained was re-dissolved in 95% ethanol, and loaded to Sephadex LH-20 HPLC column for further purification. The catechin fractions collected were freeze-dried and stored in the freezer (−20 °C) before use. Both the green tea extract and isolated catechins were analyzed for their composition by C18 reverse phase column chromatography (elution with 22% THF at the flow of 1 ml/min). Each purified catechins were dissolved in DMSO (dimethylsulfoxide) and stored in the freezer (−20 °C). (−)-Epicatechin and (+)-catechin (C) were purchased from Sigma-Aldrich.

2.2. Viruses and cells

Influenza A/Chile/1/83 (H1N1), A/Sydney/5/97 (H3N2) and B/Yamagata/16/88 viruses were propagated in 11-day chick embryos. Allantoic fluids were harvested and stored at the freezer (−80 °C). Madin–Darby canine kidney (MDCK) cells were cultured as monolayers in the MEM medium (JBI, Korea) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, USA).

2.3. Plaque inhibition assay

For plaque inhibition assays, confluent monolayer MDCK cells cultured in a 6-well tissue culture plate (1×10^5 cells/cm^2) were infected with a mixture of approximately 500 PFU/ml of virus. After 60 min for virus adsorption, the solution was removed and the cells were washed twice with pre-warmed MEM medium, and replaced with overlay medium (DMEM containing 10 μg/ml trypsin, 1% low melting agarose, without serum), containing catechins at different concentration. After incubating cultures for 2–3 days at 37 °C with 5% CO_2, monolayers were fixed with 4% formaldehyde solution for 30 min and the agarose was then removed by flowing water and stained with 1% (w/v) crystal violet solution. The plaques were counted by visual examination and percentage of plaque inhibition was calculated as relative to the control without catechins. A required concentration to reduce the 50% plaque number (EC_{50}), was calculated by regression analysis of the dose–response curves generated from these data.

2.4. Virus growth inhibition assay

Confluent monolayers of MDCK cells in 12-well plates were washed once with phosphate buffered saline (PBS) and then infected with influenza A/Chile/1/83 (H1N1) virus at 0.1 multiplicity of infection (MOI). The plates were regularly inverted on a shaker for 45 min at room temperature in compounds-free conditions for virus adsorption. The solution was removed and replaced with MEM medium containing catechins of various concentrations. Viruses were harvested at 8, 24, 36 h post-infection, and the viral yield was estimated by plaque assay on MDCK cells. As a control, the infected cells incubated in catechin-free medium were included throughout the experiment.

2.5. Hemagglutination inhibition assay

Hemagglutination inhibition assay was employed to test the effect of catechins in virus adsorption to target cells. Catechin solutions (25 μl) with two-fold serial dilution with PBS were mixed with equal volume of influenza virus solution (200 HAU/25 μl). After incubation for 30 min at room temperature, 50 μl of the solution was mixed with equal volume of 1% chicken erythrocyte suspension and incubated for 30 min at room temperature.

2.6. Quantitative RT-PCR analysis

MDCK cells were grown at about 90% confluence and infected with influenza A/Chile/1/83 virus at 0.1 MOI and cultured in the presence of catechins at various concentrations. At 16 h post-infection, cells were scraped off and collected by centrifugation (500 g for 5 min). Cell pellets were washed with PBS twice. Total cellular and viral RNAs were isolated from pellets using the RNeasy mini kit (QIAGEN) following the manufacture's protocol. First-strand cDNA was synthesized from 1 μg of total RNA with Omniscript RT kit (QIAGEN) using specific primers. PCR reactions were performed with 50 μl of reaction buffer [5 μl of cDNA template, 50 pmols of primers, 0.1 mM dNTPs, and 0.5 U of EX-Taq polymerase (Takara)]. The amplification conditions were as follows: 94 °C for 5 min (1 cycle), 94 °C for 1 min, 55 °C 40 s and 72 °C 1 min 40 s (18 cycles, respectively). NP RNA was chosen for detection and the primer sequences used for the detection of viral RNA were 5′-TGC TGG ATT CTC GTT CGG TC (sense) and 5′-CCT TTA TGA CAA AGA AGA AAT AAG GCG (antisense). The β-actin was used as internal control of cellular RNAs, with primer sequences of 5′-TCA CCC GAG TCC ATC ACG AT (sense) and 5′-GAA GTA CCC CAT TGA GCA CGG (antisense). The reverse transcription and PCR products were resolved on 1.0% agarose gels and stained with ethidium bromide.

2.7. Neuraminidase inhibition assay

Neuraminidase inhibition assay was employed to test the effect of catechins in neuraminidase activity in virus. Catechin solutions (25 μl) with two-fold serial dilutions with PBS were mixed with equal volume of influenza A/Chile/1/83 virus solution. Equal volume (50 μl) of the substrate solution (4-MU-NANA; 2′-(4-methylumbelliferyl)-A-D-*N*-acetylneuraminic acid sodium, Sigma) was added and the mixture was further incubated at 37 °C for 2 h, protected from light. Optical density is then measured by fluorescence of 4-methylumbelliferone with fluorescence spectrophotometer (excitation 365 nm, emission 460 nm).

Relative activities were calculated by the following formula:

$$\text{Relative activities (\%)} = \frac{\text{NA activites with catechins}}{\text{NA activites without catechins}} \times 100$$

2.8. Cytotoxicity test by MTT assay

MDCK cells were grown (8800 cells/well) in 96-well plate for 24 h. The medium was replaced with that containing serially diluted catechins and the cells were further incubated for 48 h. The culture medium was removed and 25 μl MTT, 3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide (Sigma) solution was added to each well and incubated at 37 °C for 4 h. After removal of supernatant, 50 μl of DMSO was added for solubilization of formazan crystals and incubated for 30 min. The optical density was measured at 540 nm in ELISA reader.

2.9. Statistical analysis

The results were expressed as mean ± S.E.M. for three independent experiments. Student's unpaired *t*-test was used to evaluate the difference between the test sample and

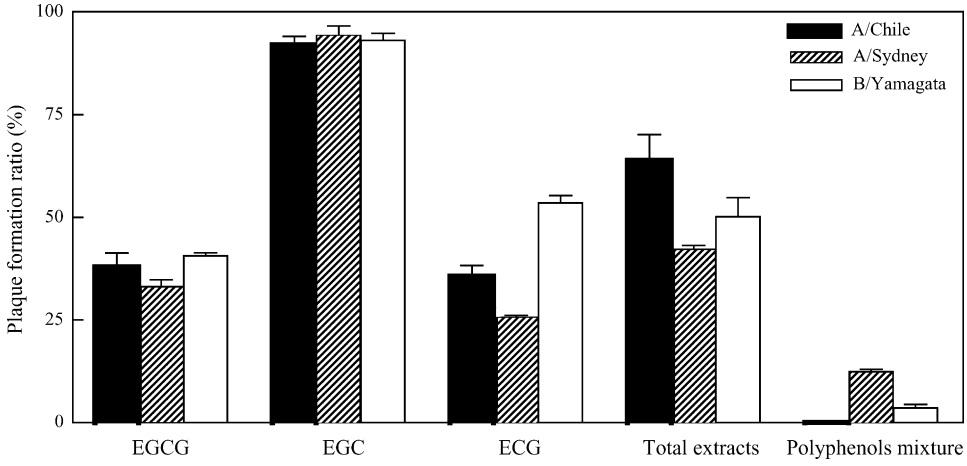

Fig. 2. Inhibitory effects of catechins on plaque formation. Six-well plates containing monolayers of MDCK cells were washed and infected with equal volume of virus suspension (500 PFU/ml). The overlay medium, mixed with 2% low melting agarose and DMEM, containing each catechin on 50 μM or 50 μg/ml with total extracts and polyphenols mixture were added to each plate. The plates were incubated at 37 °C for 48 h. Plaques were stained with crystal violet and counted. The percentage of plaque formation ratio relative to the control (no catechin) was determined for each catechin.

untreated control. A P value of <0.05 was considered statistically significant. And also one-way ANOVA was used to evaluate the difference between the test samples. We used GraphPad PRISM software for statistical analysis.

3. Results

3.1. Inhibitory effects of catechins on plaque formation by influenza A and B viruses in MDCK cells

For initial screening of the antiviral activity, each compound was tested by plaque inhibition assay in MDCK cells at a fixed concentration. As shown in Fig. 2, EGCG and ECG at 50 μM concentration inhibited more than 50% of the plaque forming activity of influenza A and B viruses whereas EGC exhibited little inhibition. Notably, polyphenols mixture is more efficient than any other single compounds in plaque inhibition. For a given compound, similar inhibitory effect was observed for all influenza viruses tested, including A/H1N1, A/H3N2 and B virus.

Each catechin compounds were then tested at various concentrations. The mean 50% inhibitory concentration (EC_{50}) of catechins, represented as the mean of at least three independent experiments, are summarized in Table 1. In summary, EGCG and ECG was about 10-fold more effective than EGC. This finding was confirmed for all different influenza viruses, including two different subtype of A viruses (A/Chile/1/83 (H1N1) and A/Sydney/5/97 (H3N2)) and B virus (B/Yamagata/16/88).

On the contrary (−)-epicatechin, the back bone compound of green tea polyphenols, had no antiviral effect even at 1 mM concentration and (+)-catechin, the geometric isomer of EC, exerted little inhibition on plaque formation. (+)-Catechin showed plaque inhibition of only about 20% at 600 μM, and at higher concentration (summarized in Table 1).

3.2. Inhibitory effects of catechins on influenza virus replication in MDCK cells

Next, we analyzed the effect of catechins on virus yield at various times post-infection of MDCK cells. The cells were infected with 0.1 MOI influenza A virus and various concentrations of catechins were added in culture medium. At 8, 24 and 36 h post-infection, virus yields were determined by plaque assay. As shown in Fig. 3, the virus yields in MDCK cell were reduced by 2–6 log units depending on the concentration of the compounds. Virtually complete reduction in virus yields was observed at highest concentration tested (120, 1200 and 120 μM for EGCG, EGC and

Table 1
Inhibitory effects of catechin compounds on plaque formation on MDCK cells

Virus	EC_{50}[a]					
	EC and C (μM)	EGCG (μM)	EGC (μM)	ECG (μM)	Total extracts (μg/ml)	Polyphenols mixture (μg/ml)
A/Chile/1/83	>600	28.4 ± 1.4	318 ± 3.0	26.4 ± 2.0	70.5 ± 1.2	13.1 ± 1.4
A/Sydney/5/97	>600	22.8 ± 1.1	309 ± 1.2	22.2 ± 1.1	63.8 ± 1.2	15.2 ± 1.2
B/Yamagata/16/88	>600	26.1 ± 1.4	311.1 ± 2.4	40.4 ± 1.7	68.2 ± 2.1	14.1 ± 1.6

Values represent the mean ± S.E.M. for three independent experiments.

[a] EC_{50} represents the concentration of catechin necessary to reduce the plaque number by 50% relative to control wells without test compound, calculated from dose–response data of plaque inhibition.

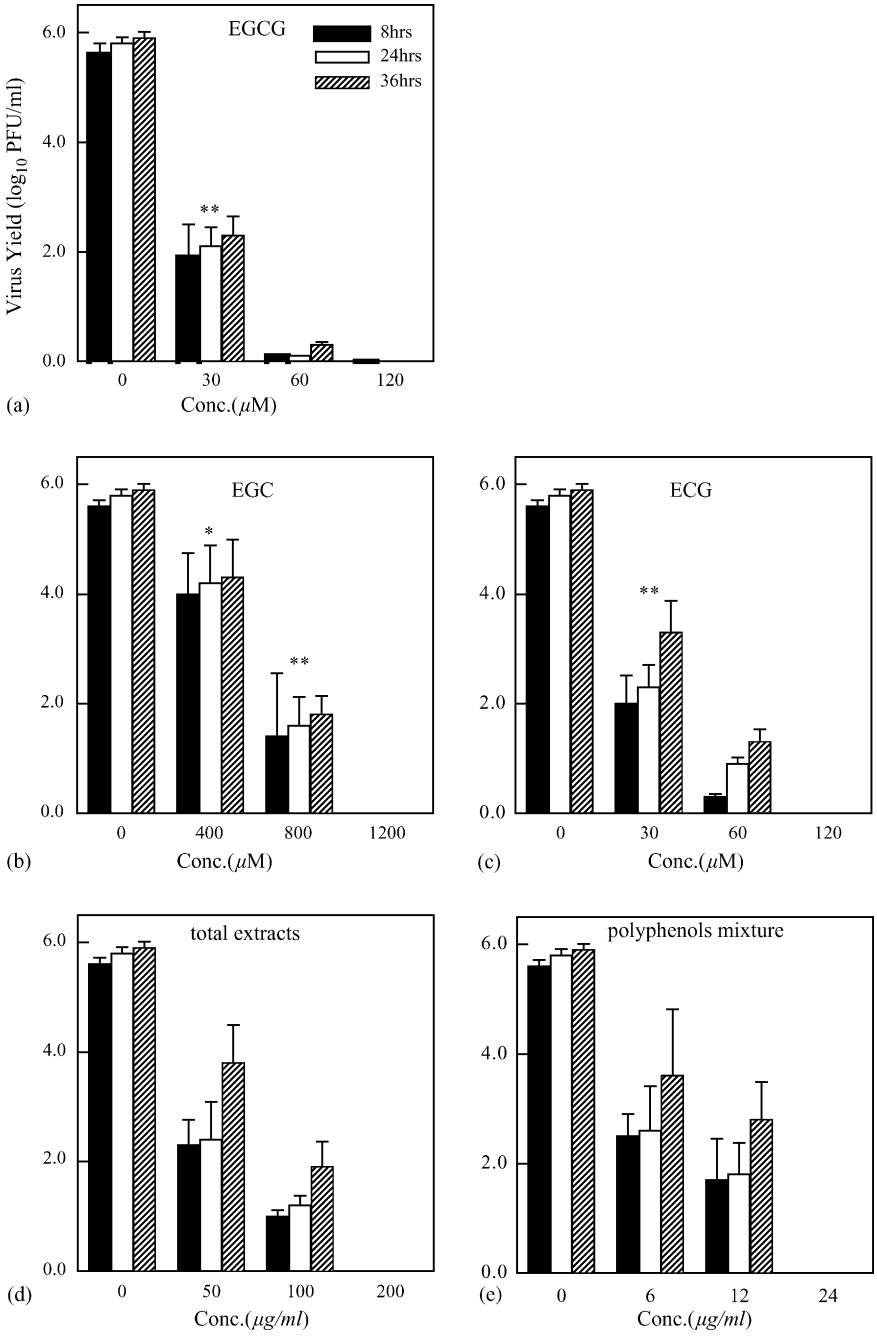

Fig. 3. Inhibitory effects of catechins on influenza virus yield in MDCK cells. Confluent monolayers of MDCK cells in 12-well plates were infected with A/Chile/1/83 (H1N1) virus at 0.1 MOI. After 45 min it was replaced with MEM medium containing each catechin in various concentration without serum. Culture medium was harvested at 8, 24 and 36 h post-infection. The viral yield was estimated by plaque assay on MDCK cells. The asterisk indicates a significant difference between test sample and control, $^{*}P < 0.05$, $^{**}p < 0.001$.

ECG, respectively, and 200 and 24 μg/ml for total extracts and polyphenols mixture, respectively). For example, EGCG reduced about 4 log units of virus yields at 30 μM, and this significant reduction ($P < 0.001$) was observed at all phases of infection (8, 24 and 36 h post-infection). Complete inhibition was observed at 120 μM, where we failed to observe any release of viruses. In this assay, EGCG was by far the most effective, and EGC was least effective among three isolated compounds tested. Again, we observed that polyphenols mixture was very effective (failure to detect any viral yield at 24 μg/ml concentration). The results further extend previous data on plaque inhibition (Fig. 2).

Overall, the inhibitory effect is observed in concentration dependent manner, and throughout the virus infection cycle after initial infection. The results suggest that the antiviral effect is exerted not only on the initially infecting viruses but newly propagated viruses as well.

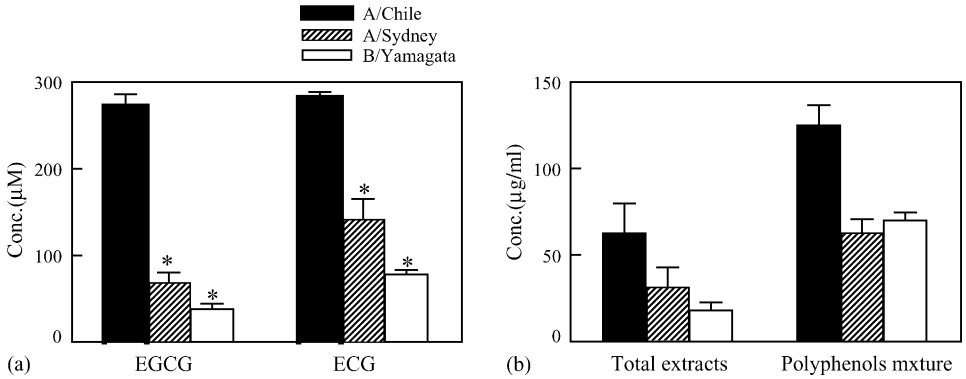

Fig. 4. Inhibitory effects of catechins on virus adsorption to chicken red blood cells. Twenty-five micro moles of influenza virus suspension (200 HAU/25 μl) were mixed with an equal volume of different concentrations of catechins and incubated for 30 min at room temperature. The mixture was further incubated with 50 μl of 1% chicken red blood cells suspension for 30 min at room temperature for hamagglutination. We determined the minimum concentration of catechins that completely inhibited the adsorption of virus. The asterisk indicates a significant difference between test samples, $^{*}P < 0.05$.

3.3. Inhibitory effects of catechins on the virus adsorption with chicken red blood cells

Influenza A virus has an ability to adsorb to chicken red blood cells resulting in hemagglutination. We investigated if cathechins could interfere with the viral adsorption to RBC resulting in hemagglutiniation inhibition, and analyzed the minimum concentration of catechins that completely inhibited the adsorption ability of virus. As shown in Fig. 4, EGCG, ECG and polyphenols mixture exhibited, to some degree, inhibition of viral adsorption of RBC, but the effect was surprisingly different among type of viruses and catechins ($P < 0.05$). In case of EGCG, for examples, influenza A/Sydney/5/97 virus was three- to four-fold more sensitive and the B/Yamagata virus was seven-fold more sensitive than A/Chile/1/83, respectively. Albeit less pronounced, clearly different sensitivity is also observed with ECG. In another set of experiment, the sensitivity towards EGCG was up to 15-fold among the viruses tested (data not shown). The result is in clear contrast to the results of plaque inhibition assay where difference in EC_{50} between the two influenza A strains was within 40%. Moreover, contrary to previous results on plaque inhibition assay and viral growth on cell culture, total extract was more effective than the polyphenols mixture. However, in case of EGC, we failed to observe inhibitory effect even at the highest concentration tested in this experiment (3.5 mM).

3.4. Measurement of the viral mRNA expression in infected cell with quantitative RT-PCR

We then tested the effect of catechins on transcription of viral genes in infected cells by quantitative RT-PCR of influenza virus specific mRNA. MDCK cells were infected with A/Chile/1/83 virus and were incubated 16 h in the presence of various concentrations of catechins. Total RNAs were isolated from infected MDCK cells and RT-PCR analysis was performed using primers specific for viral NP RNA.

The results showed significant differences among various catechin compounds (Fig. 5). Down regulation of viral RNA synthesis was evident especially at high concentration (over 600 μM EGCG, 1200 μM ECG, respectively). The inhibitory effect was most pronounced with polyphenols mixture (about 80% inhibition at 500 μM). However, we failed to observe inhibitory effect with EGC even at the highest concentration tested in this experiment (3.2 mM). As an internal control, the transcription of cellular β-actin mRNA was not affected in all catechin concentrations tested. Therefore, the inhibitory effect on viral replication observed at high concentration was not due to a general cytotoxic effect on the cell. The results suggest that, in addition to direct virucidal effect by binding to virus particles, the catechins may have additional inhibitory effect on viral RNA synthesis, especially at high concentration.

3.5. Inhibitory effects of catechins on the viral neuraminidase activity

Neuraminidase is thought to play a key role in the release of newly made virus particle from infected cells by cleavage of target cell receptor sialic acid moieties. Additionally, the enzyme activity is responsible for preventing self-aggregation of virus particles by cleavage of sialic acids still bound to the virus surface. We therefore tested the potential effect of catechins on the viral neuraminidase activity (see Section 2). The activity of neuraminidase decreased significantly by EGCG and ECG, but not by EGC (Fig. 6). Reduction of half enzymatic activity was shown at relatively high concentration (about 350 and 550 μM for EGCG, ECG, respectively). The results suggest that the catechins may have additional inhibitory effect on virus release step through inhibition of neuraminidase, especially at high concentration.

3.6. Cellular toxicity of catechins

We evaluated the cytotoxicity of catechins by the MTT assay. Half-confluent MDCK monolayers were incubated with media in the absence or presence of two-fold diluted

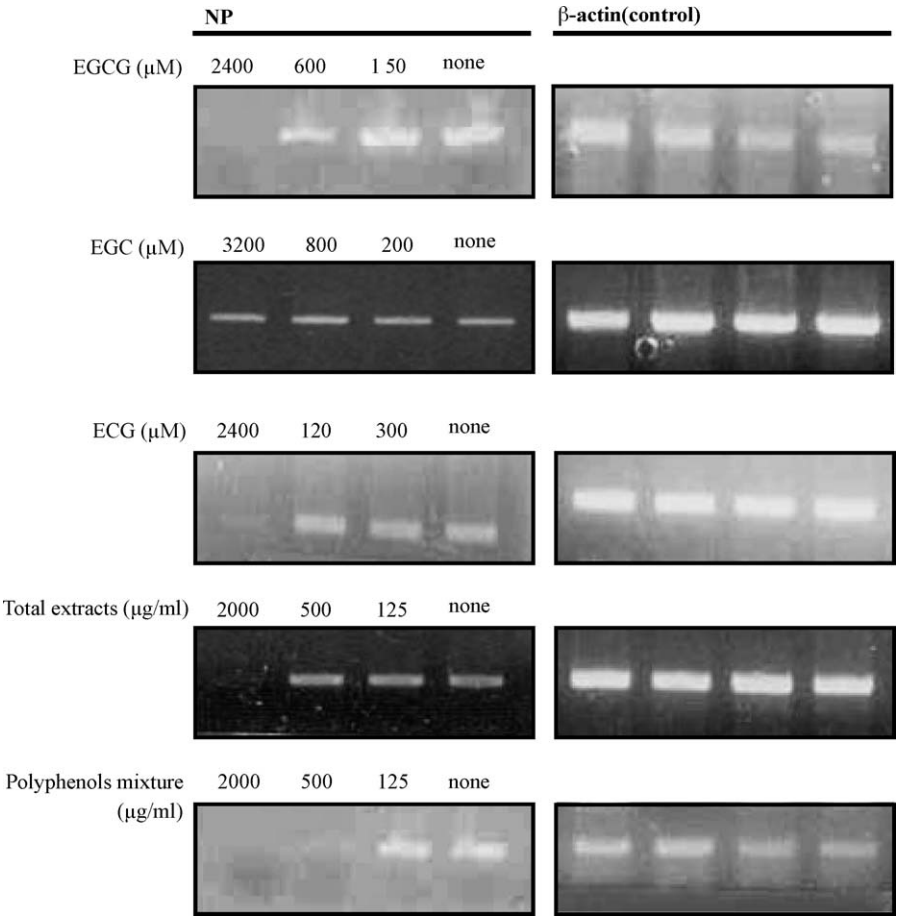

Fig. 5. Effect of catechins on influenza viral RNA synthesis in infected cell as analyzed by quantitative RT-PCR. Ninety percent confluent cells were infected with virus at 0.1 MOI and cultured with MEM medium containing different concentrations of each catechin and 1% serum. Cells were lysed 16 h after infection, and total cellular and viral RNA isolated from cell pellets. Quantitative RT-PCR was performed using specific primers for viral RNA (NP) and cellular RNA (actin). Similar results were obtained in three other experiments.

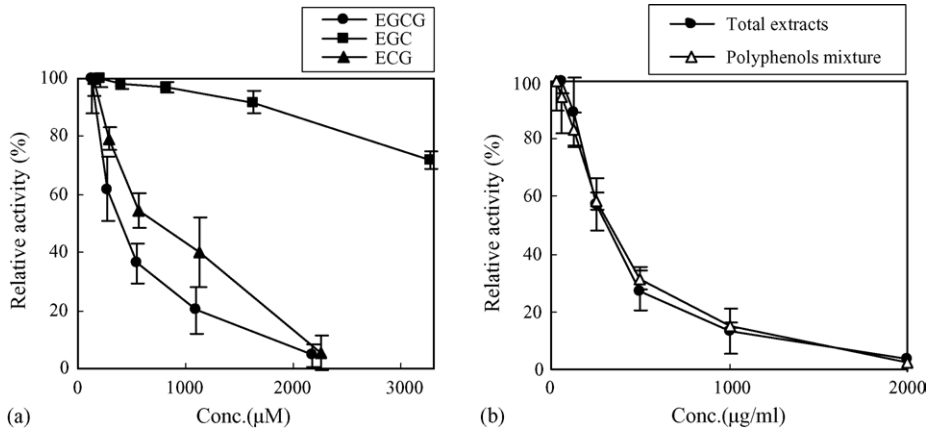

Fig. 6. Inhibitory effects of catechins on the viral neuraminidase activity. Catechin solutions (25 μl) with two-fold serial dilution with PBS were mixed with equal volume of influenza virus solution. Fifty microliters of the substrate solution (4-MU-NANA) was mixed with equal volume catechins/virus mixture and incubated for 2 h at 37 °C. Neuraminidase activity was monitored by fluorescence of 4-methylumbelliferone with fluorescence spectrophotometer. Each point represents the mean ± S.E.M. for three independent experiments.

Table 2
Cellular toxicity and selectivity index of catechins on MDCK cells

	EGCG (μM)	EGC (μM)	ECG (μM)	Total extracts (μg/ml)	Polyphenols mixture (μg/ml)
CC_{50}[a]	275.4 ± 22.8	1233.1 ± 44.9	525.9 ± 30.7	353.3 ± 11.2	330.0 ± 57.9
Selectivity index[b] (SI)	12.1	4.0	23.7	5.5	21.7

[a] CC_{50}, 50% cell toxicity concentration determined by MTT assay. The values of CC_{50} are mean ± S.E.M.
[b] SI is the ratio of CC_{50} to EC_{50}.

catechins and total extracts (0–30 mM or 20 mg/ml) for 48 h. MTT reagents were then added to the monolayer of MDCK cells. After incubation at 37 °C for 4 h, absorbance (540 nm) was measured by ELISA reader. The estimated doses that reduced cell viability about 50% are described in Table 2. The results showed that EGC has lowest toxicity among three isolated compounds and ECG was about two-fold less toxic than EGCG. The viabilities of the all test sets were at least 20% at the highest dose tested (data not shown).

4. Discussion

Green tea contains various useful chemical compounds such as catechins, caffeine and vitamins, most notable components being catechins including EGCG, ECG, ECG and EC. As the most abundant component, EGCG has been most extensively studied for various biological activities. Previous reports have demonstrated that EGCG inhibits influenza virus infection when they contact with influenza virus directly (Nakayama et al., 1993), but indirect effect on host cell that might interfere with the virus-cell membrane fusion was also suggested (Imanishi et al., 2002).

As an approach to structure-function relationship of anti-influenza activities, we have tested three isolated catechin compounds in in vitro culture of influenza viruses in MDCK cells. Among the test compounds, the EGCG and ECG were found to be potent inhibitors of influenza virus growth, and this effect was observed in all virus subtypes tested, including A/H1N1, A/H3N2 and B virus that currently afflicts human population (Fig. 2, Table 1). The EC_{50} values of EGCG, EGC and ECG for influenza A virus were 22–28, 309–318 and 22–40 μM, respectively, EGCG being most effective. In all experiments two different controls were included; polyphenols mixture (major constituents EGCG, ECG, EGC) and total green tea extracts. Considering the molecular weights of each catechins and the composition of polyphenols mixture, 1 μg/ml concentration of polyphenols mixture would be equivalent to 3 μM of EGC or 2 μM of EGCG and ECG. Considering this, the inhibitory activity in both plaque formation and the viral growth of polyphenols mixture would roughly correlate to that of EGCG and ECG.

Strong inhibitory effect ($P < 0.001$) was observed regardless of the contact time of the compounds throughout the growth. This raised possibility that the antiviral effect is exerted not only on the initially infecting viruses but other steps of infectious cycle. Although it is difficult to dissect the effect at each steps involved, we are analyzed the antiviral effects at various steps in influenza virus infection. Inhibition of influenza virus adsorption to MDCK cells and chicken red blood cells by EGCG was previously reported (Nakayama et al., 1993). Further extending the report, here we observed that EGCG is most effective among three isolated compounds in hemagglutination inhibition activity. The sensitivity towards catechin compounds was widely different among three different subtypes of influenza viruses tested. It should also be mentioned that total tea extract was much more effective than any other isolated or mixture of polyphenols. This strongly suggests that, although dietary uptake of tea would be beneficial for direct intervention of influenza virus infection, components other than catechins are more responsible especially for the hemagglutination inhibition. It should also be noted that, despite differential sensitivity in HA inhibition, the growth of all subtypes of influenza viruses were effectively inhibited by EGCG, suggesting that catechins may affect others steps of infectious cycle as well. This may involve interference of viral membrane fusion by inhibition of acidification of endosome (Imanishi et al., 2002). Here, by quantitative RT-PCR analysis of influenza-specific RNA in infected cells showed that, at high concentration, ECG and EGCG also suppressed viral RNA synthesis (Fig. 5). Similarly, EGCG and ECG inhibited the neuraminidase activity at relatively high concentration more effectively than the EGC (Fig. 6). The ability to raise a resistant mutant against these catechins may provide an avenue for identification of primary target for catechin compounds.

However, the physiological relevance of the present in vitro data should be interpreted with caution especially considering the pharmacokinetic properties of catechins, such as cellular uptake, chemical modification and distribution (Kroon et al., 2004; Vaidyanathan and Walle, 2003; Williamson, 2002). For example, the cellular uptake and efflux of tea polyphenols in human intestinal cell line Caco2 is dependent on the transport mechanism (Vaidyanathan and Walle, 2003). Moreover, many polyphenolic compounds are metabolized into various conjugated form—sulfates, sulfoglucuronide, etc., and the degree of conjugation is widely different among catechin compounds (Kroon et al., 2004) and the accumulation of metabolized compounds is widely different among various tissues (Williamson, 2002).

With potential exception of different subtypes of influenza virus, the observed inhibitory effects of various catechin derivatives were consistent, in the order of EGCG > ECG > EGC. The results show that the galloyl group at the 3-hydroxyl of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5′-OH of the

trihydroxybenzyl moiety at 2-position plays a minor role (see Fig. 1). The result is consistent and further extends previous finding that galloyl group is important for the anti HIV reverse transcriptase activity (Chang et al., 1994; Yamaguchi et al., 2002).

The results also suggest that, besides the known inhibitory activity on viral attachment of host cells, the antiviral activities of polyphenols are associated with various steps in the influenza virus life cycle. The differential antiviral effect among different catechins, which was consistently observed at various steps of influenza virus infection cycle albeit at different concentrations, strongly suggests that primary target for catechins is likely to be membrane. The effect would be altering physical integrity of virus particles or host membrane. The interpretation is in good agreement with the inhibitory effect of catechins on acidification of intracellular endosome compartments required for fusion of viral and cellular membranes (Imanishi et al., 2002). In addition, as suggested by the subtype-specific effect on hemagglutination inhibition (Fig. 4), catechin is expected to affect the conformation of HA or its interaction with influenza virus. The HA type specific effect of catechins, hitherto unknown before, remains to be explored. The structure-activity relationships of anti-influenza properties merit further investigation and could be further extended by chemical modification of catechin derivatives.

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