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Inhibition of Human Parainfluenza Virus Type 2 Growth in Vitro by Catechin is caused by the Inhibition of Genome and mRNA Syntheses and by the Disruption of Cytoskeleton, and that by Tannic Acid is Mainly Caused by Genome Synthesis Inhibition and the Disruption of Cytoskeleton

Jun Uematsu[1], Sahoko Kihira[2], Mika Uchida[2], Yurika Sakakura[2], Sayuri Deguchi[2], Kae Sakai[2], Hidetaka Yamamoto[3], Mitsuo Kawano[4], Masato Tsurudome[4], Myles O'Brien[5], Hiroshi Komada[1]

[1]Microbiology and Immunology Section, Department of Clinical Nutrition, Graduate School of Suzuka University of Medical Science, 1001-1 Kishioka, Suzuka, Mie 510-0293, Japan

[2] Department of Clinical Nutrition, Faculty of Health Science, Suzuka University of Medical Science, 1001-1 Kishioka, Suzuka, Mie 510-0293, Japan

[3] Faculty of Pharmaceutical Science, Suzuka University of Medical Science, 3500-3 Minamitamagaki, Suzuka, Mie 513-8670, Japan

[4]Department of Microbiology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan

[5]Graduate School of Mie Prefectural College of Nursing, 1-1-1 Yumegaoka, Tsu, Mie 514-0116, Japan

Abstract: The antiviral activities of catechin mixture (catechin) and tannic acid against human parainfluenza virus type 2 (hPIV-2) were investigated *in vitro*. Catechin and tannic acid both inhibited cell fusion induced by hPIV-2 in $LLCMK_2$ cells. However, high concentrations of them caused cell toxicity. Both catechin and tannic acid reduced the number of viruses released from the cells. Real time PCR showed that catechin almost completely inhibited virus genome RNA synthesis, and tannic acid largely inhibited it. Virus nucleoprotein (NP), fusion (F) and hemaggulutinin-neuraminidase (HN) gene syntheses were largely inhibited by catechin, and mRNA syntheses of these proteins were partly inhibited by catechin. However, tannic acid did not cause inhibition. An indirect immunofluorescence study showed that catechin partly inhibited virus NP, F and HN protein syntheses, but tannic acid did not inhibit either virus NP, F or HN protein syntheses. Using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP), it was found that virus entry into the cells was not inhibited by catechin or tannic acid, and that spreading of virus to the adjacent cells was not blocked by them. Catechin and tannic acid disrupted both actin microfilaments and microtubules. These results indicated that the inhibitory effect of catechin was caused by the inhibitions of both viral genome RNA and mRNA syntheses, and by the disruption of actin microfilaments and microtubules. However, inhibition by tannic acid was mainly caused by vial genome synthesis inhibition and the disruption of cytoskeleton.

Keywords: human parainfluenza virus type 2, catechin mixture, tannic acid, a recombinant green fluorescence protein expressing hPIV-2 without matrix protein

Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (1). hPIV-2 has 7 structural proteins, NP, V, phospho (P), matrix (M), F, HN and large (L) proteins. The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group (2-7).

Hiroshi Komada (Correspondence)
komada@suzuka-u.ac.jp
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Monoclonal antibodies (mAbs) against hPIV-2 were made, and antigenic diversity of clinical isolates was investigated, by Tsurudome (8). The infectious hPIV-2 from cDNA clone was constructed by Kawano, and it was shown that its growth property was the same as that of natural hPIV-2 (9).

Catechins are major biologically active components of green tea, and are a mixture of epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. Steinmann *et al*. reviewed anti-infective properties of epigallocatechin-3-gallate on many viruses (10) such as human immunodeficiency virus-1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), Epstein-Barr virus (EBV), Adenovirus (AdV), influenza virus and enterovirus. Some mechanisms have been elucidated. For instance, catechins inhibit HIV-1 by the inhibition of integrase and reverse transcriptase, cause reduction of HBV antigen expression, inhibit HCV entry by interference with virus binding to target cells, and inhibit influenza virus entry by binding to hemaggulutinin (HA).

Tannic acid is also derived from plants, and the roll of tannic acid is reported to be resistance to the infection of microorganisms. Ueda *et al.* (11) reported the inhibitory effect of tannic acid against a broad range of viruses, including Sendai virus (SeV) and Newcastle disease virus (NDV) (both belong to the family *Paramyxoviridae*). Tannic acid-treated and diluted solutions were inoculated to susceptible cells and assayed by immunofluorescent infectious focus assay. They showed that persimmon extract and green tea extract decreased the infectivity of SeV and NDV.

In the present investigation, catechin and tannic acid were tested for hPIV-2 growth, and it was found that the both had inhibitory effects on hPIV-2 growth. The numbers of viruses released from infected cells cultured with catechin and tannic acid were determined. To investigate the effect of catechin and tannic acid on viral RNA synthesis, viral RNA was prepared and amplified by polymerase chain reaction (PCR), and viral genome synthesis was quantified by real time PCR. Viral mRNA was also detected. Viral protein expression was observed by indirect immunofluorescence study using mAbs against NP, F and HN proteins of hPIV-2 (8). The inhibitory effects of catechin and tannic acid on hPIV-2 entry into the cells, and cell-to-cell spreading were analyzed using rhPIV-2ΔMGFP (9,12). Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication (13). Tubulin also acts as a positive transcription factor for *in vitro* RNA synthesis by SeV (14). The effects of catechin and tannic acid on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin α mAb, respectively.

MATERALS AND METHODS

Catechin mixture and tannic acid

Catechin mixture and tannic acid were purchased from Wako Chemicals (Osaka, Japan). They were dissolved at 100 mg/mL in 10 mM phosphate buffered saline, pH 7.2 (PBS), pH was adjusted to 7.0 by 1 M NaOH, and they were sterilized by filtration. They were diluted to 1 mg/mL with sterilized PBS, and kept at -20^{o}C before use. Catechin mixture from green tea (Wako) was a mixture of epicatechin, epigarocatechin, epicatechin gallate and epigarocatechin gallate.

Virus and recombinant virus

The virus and recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science.

hPIV-2 (Toshiba strain) was used.

rhPIV-2ΔMGFP was constructed according to the method described previously (9,12), and it was shown that it did not produce infectious virus particles without addition of M protein gene *in trans* (data not shown). The full-length cDNA was constructed from 5 overlapping RT-PCR fragments (9,15). To make rhPIV2ΔM construct, Not I-Sac I region and Sac I-Kpn I region, from pPIV-2 that contains a full anti-genomic cDNA copy, were sub-cloned into pBluescriptIISK(-), and PCR was carried out using appropriate primers (Fig.1A). The EGFP gene open reading frame was engineered to be flanked by hPIV-2 specific gene end of NP gene (R2), intergenic sequence (IG), and gene start (R1) transcriptional signal of V/P gene. It was inserted into a cloned cDNA of rhPIV-2ΔM anti-genome at a Not I site that had been engineered to be at 5'-noncoding region of NP gene. The construct of rhPIV-2ΔMGFP is shown in Fig.1B. The virus was recovered by co-transfection of the plasmid and plasmids expressing the NP, P and L, which were cloned in a mammalian gene expression vector (pCAGGS) (16), into BSRT7/5 cells (17) expressing T7 RNA polymerase. The cells were harvested and co-cultured with fresh Vero cells every 48 hr. About 90% of the cells showed syncytia formation in the 10th generation of co-cultured cells. For virus propagation, Cos7 cells were transfected with the plasmid expressing M gene, and co-cultured with the above mentioned 10th generation of cells for 72 hr. The virus titer was determined using Vero cells. The virus yield was about 1x10^{5} $TCID_{50}$/mL.

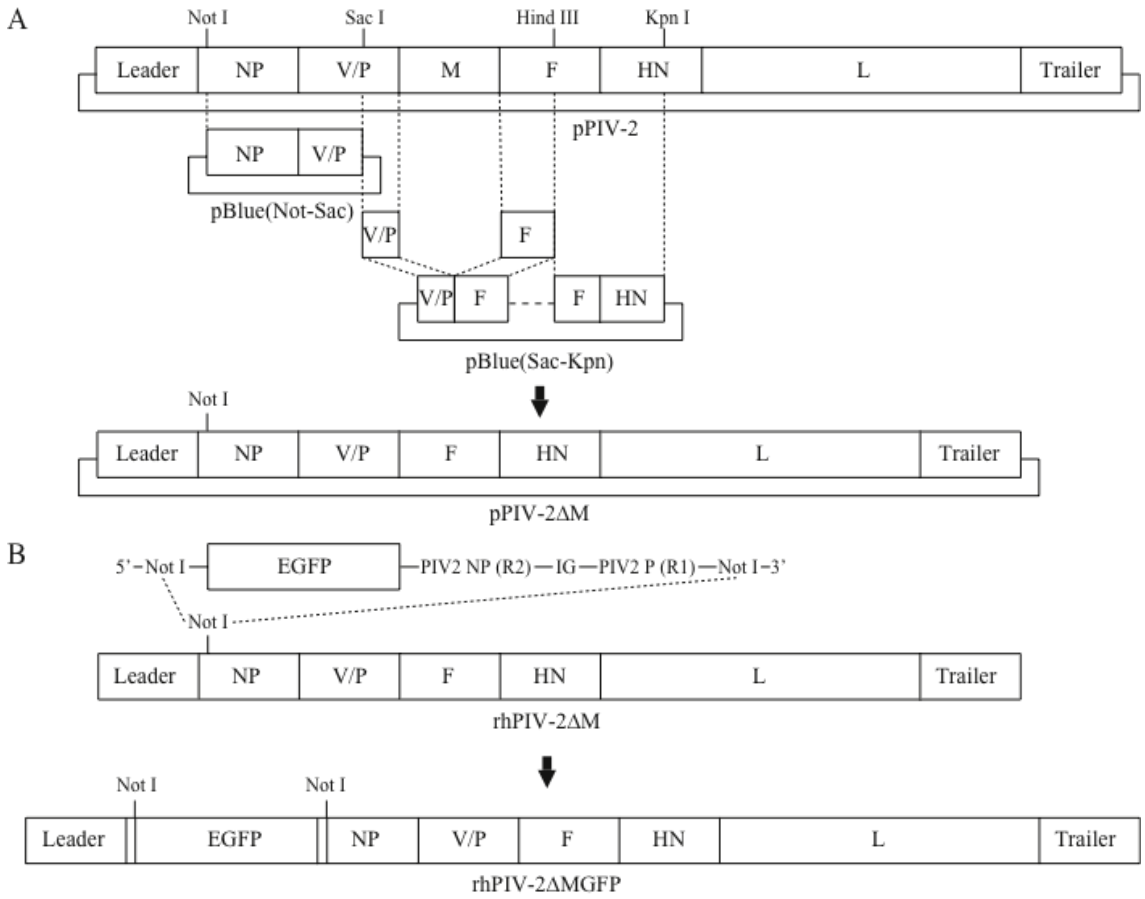

Figure 1. Schematic diagram for constructing pPIV-2ΔM (A), and the construction of rhPIV-2ΔMGFP (B). hPIV-2ΔM was constructed from pPIV-2 (9). The EGFP gene open reading frame was inserted into a cloned cDNA of hPIV-2ΔM anti-genome at a Not I site that had been engineered to be 5'-noncoding region of NP gene.

Cell line and cultivation of cells

$LLCMK_2$ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium α (MEMα: Wako, Osaka, Japan), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin, was used. The cells were cultured at 37ºC in a humidified atmosphere with 5% CO_2. After 3 days, when the cells became confluent ($5x10^5$ cells), the medium was changed to MEMα with 0.5% FCS and 0.1 mg/mL kanamycin. The reagents were added to the cells, and the cells were infected with hPIV-2 ($3x10^2$ $TCID_{50}$).

Cytopathogenic assay

Cell fusion was observed under a cell culture light microscope at 4 days post infedtion.

RNA preparation, cDNA synthesis and real time PCR

RNA was extracted from the cells ($2x10^6$ cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method. cDNA was synthesized with 1 µg RNA using Reverse Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and NP gene specific primer (nucleotide number 1661-1679: 5'-CAACATTCAATGAATCAGT-3'). Real time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Life Technologies, Tokyo, Japan) using TaqMan Probe (1932-1956: 5'-FAM-AAGCACCGGATTTCTAACCCGTCCG-TAMRA-3'), forward primer (1851-1875: 5'-ACACACTCATCCAGACAAATCAAAC-3'), and reverse primer (1958-1980: 5'-TGTGGAGGTTATCTGATCACGAA-3').

Detection of virus RNA

cDNA was synthesized with 1 µg RNA using forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489: 5'-CCTCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681:

5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara, Shiga, Japan).

Detection of messenger RNA (mRNA)

cDNA was synthesized with 1 µg RNA using oligo dT primer (5'-TTTTTTTTTTTTTTTTTTTT-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489), F (6,661-6,681) and HN (8,481-8,500:) genes and Ex Taq (Takara).

Immunofluorescence study

To detect virus proteins in the infected cells, the cells were fixed with 3.7% formaldehyde solution in PBS at room temperature for 10 min. After washing with PBS, the cells were further incubated with 0.1% TritonX-100 in PBS at room temperature for 3 min, washed with PBS, and incubated with mouse mAbs against NP, F and HN proteins of hPIV-2 at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody to mouse IgGs (Invitrogen) at room temperature for 30 min, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Actin was detected using rhodamine phalloidin (Invitrogen) and microtubules were observed using anti-tubulin α mAb against sea urchin tubulin α (clone B-5-1-2, Sigma-Aldrich, St Louis, MO, USA) at 20 hr of cultivation. The cells were fixed with 3.7% formaldehyde solution in PBS at 37°C for 10 min, washed with PBS, and the cells were further incubated with 0.1% TritonX-100 in PBS at 37°C for 3 min. All procedures were carried out at 37°C except for the microscopic observation.

Entry and cell-to-cell spreading of hPIV-2

The reagents were added to the cells, and immediately after the addition, the cells were infected with rhPIV-2ΔMGFP ($1x10^4$ $TCID_{50}$), and cultured for 4 days. They were then fixed with 1.2% formaldehyde solution in PBS at room temperature for 10 min and observed under a fluorescence microscope.

RESULTS

Titration of virus released from the infected cells

The titers of virus released from cells cultured with and without catechin or tannic acid for 4 days were determined. Fig. 2 shows the dose dependent curves. The virus titer was reduced dependent on the concentrations. Without the reagents, the virus titer was about $5x10^5$ $TCID_{50}$/mL, and with catechin, it reduced to $3x10^2$ $TCID_{50}$/mL (Fig.2A). However catechin showed slight cell damage at 50 µg/mL (data not shown), and at more than 50 µg/mL considerable cell damage was observed (data not shown). At 25 µg/mL, tannic acid reduced the released virus to $1x10^4$ $TCID_{50}$/mL (Fig.2B), and also slight cell damage was observed (data not shown). At more than 25 µg/mL, considerable cell damage was also observed (data not shown). We used 50 µg/mL of catechin and 25 µg/mL of tannic acid in the following experiments.

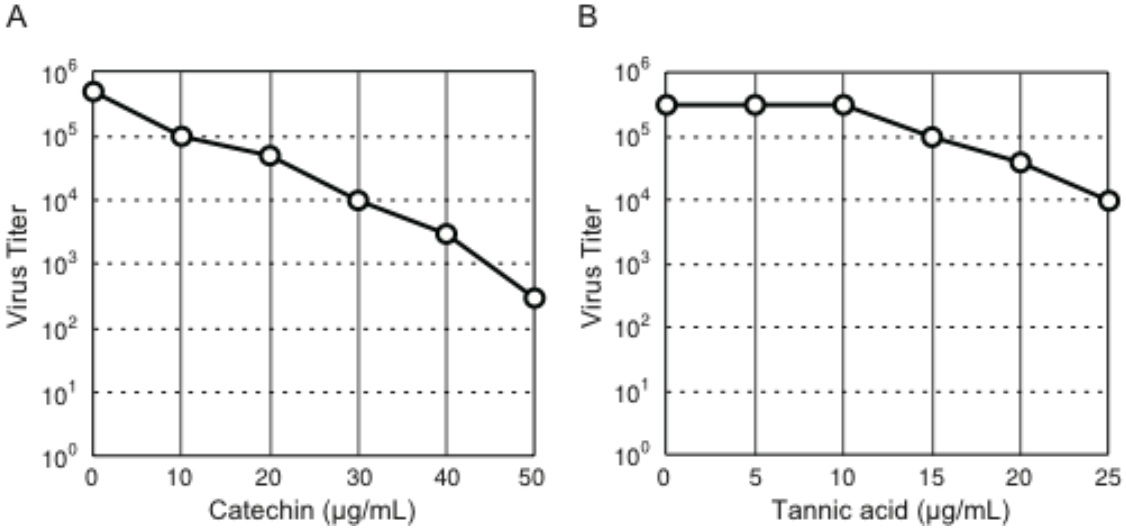

Figure 2. The effects of catechin (A) and tannic acid (B) on the number of the virus released from the cells. Catechin and tannic acid inhibited the virus release in a dose dependent manner.

Viral genome RNA and mRNA syntheses

RNA was prepared from the infected cells at 4 days post infection, and the viral genome RNA was analyzed by real time PCR. Table 1 shows that catechin almost completely inhibited viral genome RNA synthesis and tannic acid largely inhibited it.

Table 1. **Catechin and tannic acid inhibited hPIV2 genome RNA synthesis.**

	Virus	Catechin + Virus	Tannic acid + Virus
No. of copies	3764,844	2,961	227,185

The numbers of viral genome copies were detected by real time PCR.

Virus-synthesized RNAs of NP, F and HN proteins were analyzed using hPIV-2 specific primers for NP, F and HN genes by PCR. The number of base pairs between forward and reverse primers of NP, F and HN genes was about 400, 860 and 760, respectively. Fig. 3 shows that NP (lane 4), F (lane 5) and HN (lane 6) genes were detected in the virus-infected cells, and NP (lane 7), F (lane 8) and HN (lane 9) gene syntheses were largely inhibited in the catechin-treated infected cells. In the tannic acid-treated infected cells, NP (lane 10), F (lane 11) and HN (lane 12) genes were synthesized.

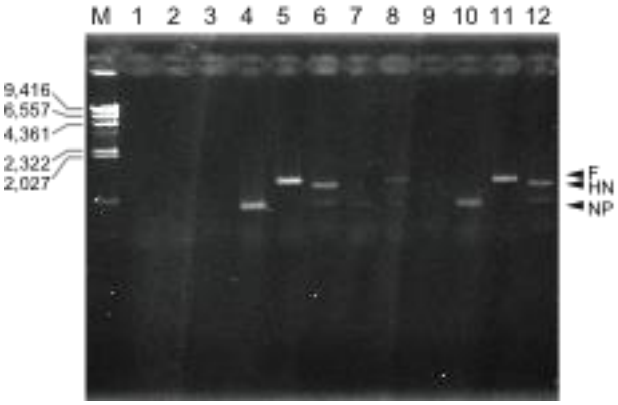

Figure 3. The effect of catechin and tannic acid on viral RNA synthesis. Lane M: marker (base pair), lanes 1, 2 and 3: non-infected cells, lanes 4, 5 and 6: hPIV-2 infected cells, lanes 7, 8 and 9: hPIV-2 infected cells cultured with catechin, lanes 10, 11 and 12: hPIV-2 infected cells cultured with tannic acid. Lanes 1, 4, 7 and 10: NP gene, lanes 2, 5, 8 and 11: F gene, lanes 3, 6, 9 and 12: HN gene. Catechin largely inhibited RNA syntheses, but tannic acid did not inhibit.

In the following experiment, mRNA synthesis was analyzed. cDNA was synthesized using oligo dT primer, and PCR was carried out using hPIV-2 specific primers for NP, F and HN genes. Fig. 4 shows that NP (lane 4), F (lane 5) and HN (lane 6) mRNAs were clearly detected in the virus-infected cells, and mRNA syntheses were partly inhibited in the catechin-treated infected cells (NP: lane 7, F: lane 8 and HN: lane 9). However, in the tannic acid-treated infected cells, mRNAs of NP (lane 10), F (lane 11) and HN (lane 12) proteins were detected.

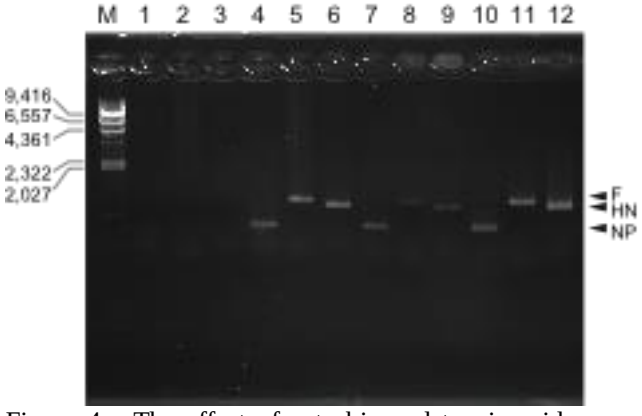

Figure 4. The effect of catechin and tannic acid on viral mRNA synthesis. Lane M: marker (base pair), lanes 1, 2 and 3: non-infected cells, lanes 4, 5 and 6: hPIV-2 infected cells, lanes 7, 8 and 9: hPIV-2 infected cells cultured with catechin, lanes 10, 11 and 12: hPIV-2 infected cells cultured with tannic acid. Lanes 1, 4, 7 and 10: NP gene, lanes 2, 5, 8 and 11: F gene, lanes 3, 6, 9 and 12: HN gene. Catechin partly inhibited mRNA syntheses, but tannic acid did not inhibit.

Immunofluorescence study

Indirect immunofluorescence study was performed to investigate the effects of catechin and tannic acid on hPIV-2 protein expression. Catechin or tannic acid was added to the cells and they were infected with hPIV-2. At 4 days post infection, the cells were fixed and stained with the mAbs against NP, F and HN proteins of hPIV-2. Fig. 5A, D and G show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm, F and HN proteins were in small dots in the cytoplasm and on the cell surface. Catechin partly inhibited the syntheses of NP (Fig. 5B), F (Fig. 5E) and HN (Fig. 5H) proteins, due to the inhibition of transcription. However, tannic acid did not inhibit the syntheses of viral proteins (NP: Fig. 5C, F: Fig. 5F, HN: Fig. 5I).

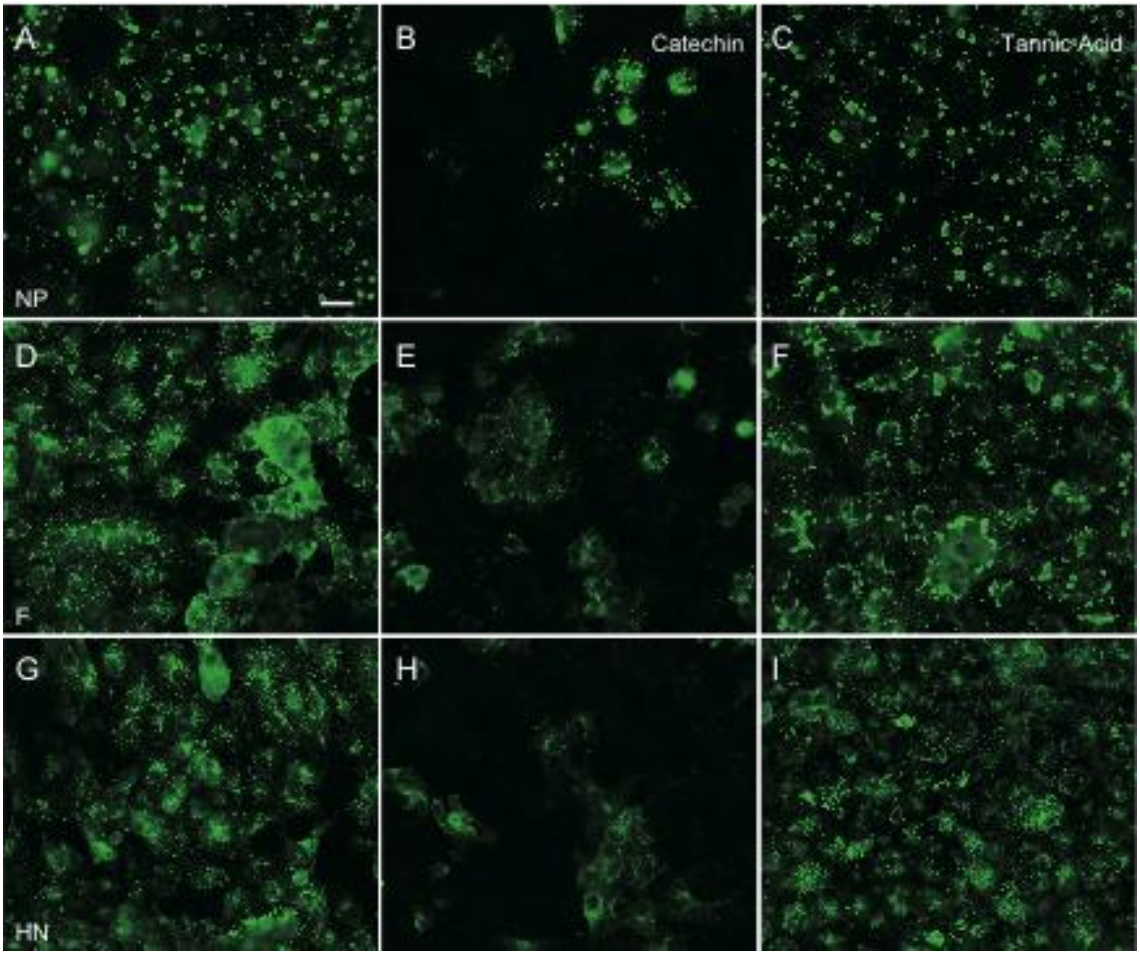

Figure 5. Effects of catechin and tannic acid on viral protein syntheses. The expression of NP (A), F (D) and HN (G) proteins of hPIV-2 infected cells. Catechin partly inhibited the expression of NP (B), F (E) and HN (H) proteins. Tannic acid did not inhibit the expression of NP (C), F (F) and HN (I) proteins. (bar: 50 μm)

Entry and cell-to-cell spreading of hPIV-2

In the following experiments, we determined the effect of both catechin and tannic acid on the entry and the cell-to-cell spreading of virus among the cells using rhPIV-2ΔMGFP (Fig. 6). The reagents were added to the cells, and immediately after the addition, the cells were infected with rhPIV-2ΔMGFP ($1x10^4$ $TCID_{50}$), and cultured for 4 days. The cells were then fixed and observed under a fluorescence microscope. Fig. 6B is a positive control: there are multinucleated giant cells with strong fluorescence. Fig. 6C and D show the infected cells cultured with catechin and tannic acid, respectively. Fig. 6C and D show that there are multinucleated giant cells, indicating that the two did not inhibit either virus entry or cell-to-cell spreading of the virus.

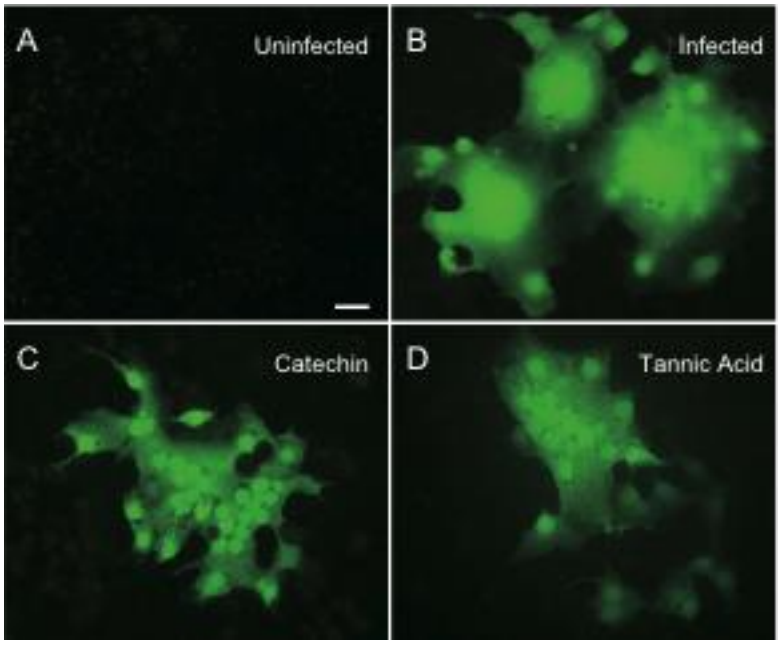

Figure 6. Effects of catechin and tannic acids on hPIV-2 entry and cell-to-cell spreading. Uninfected cells (A), cells infected with rhPIV-2ΔMGFP (B), rhPIV-2ΔMGFP infected cells cultured with catechin (C) and tannic acid (D). Both in Fig. 6C and D, there were multinucleated giant cells with strong fluorescence, indicating that both catechin and tannic acid did not inhibit either virus entry or cell-to-cell spreading of the virus. (bar: 50 μm)

The effect of the reagents on cytoskeleton

Catechin or tannic acid was added to the cells, and cytoskeleton was observed under a fluorescence microscope at 20 hr of cultivation. Fig. 7A and D show actin microfilaments and microtubules, respectively, in $LLCMK_2$. As shown in Fig. 7, catechin disrupted both actin microfilaments (Fig. 7B) and microtubules (Fig. 7E), and tannic acid also disrupted both actin microfilaments (Fig. 7C) and microtubules (Fig. 7F), indicating that one of the causes of the inhibitory effects of catechin and tannic acid was the disruption of cytoskeleton.

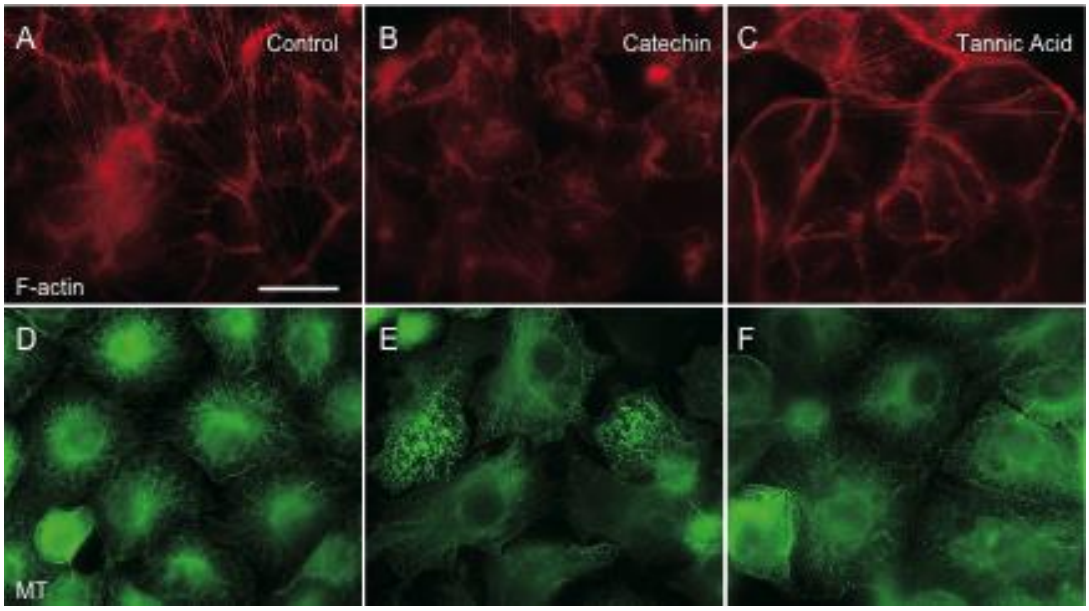

Figure 7. Catechin and tannic acid disrupted both actin microfilaments and microtubules. The cells were cultured with the reagents for 20 hr, and actin microfilaments and microtubules were stained with rhodamine phalloidin and anti-tubulin α mAb, respectively. Fig. 7 shows actin microfilaments of the control cells (A), the cells cultured with catechin (B) and tannic acid (C), and also exhibits microtubules of the control cells (D), the cells cultured with catechin (E) and tannic acid (F). (bar: 50 μm)

DISCUSSION

The present study showed that both catechin and tannic acid inhibited hPIV-2 replication in $LLCMK_2$ cells. Viral genome RNA synthesis was inhibited both in the catechin-treated infected cells and in the tannic acid-treated infected cells. Viral mRNA synthesis was largely inhibited in the catechin-treated infected cells, but not inhibited in the tannic acid-treated infected cells. The viral protein syntheses were partly inhibited in the catechin-treated infected cells, but not inhibited in the tannic acid-treated infected cells. Using rhPIV-2ΔMGFP, it was shown that both catechin and tannic acid did not inhibit either the entry of hPIV-2 into the cells or the cell-to-cell spreading of the virus among the cells. Catechin and tannic acid disrupted both actin microfilaments and microtubules, indicating that one of the causes of the inhibitory effects of both catechin and tannic acids was the disruption of the cytoskeleton.

Green tea has been a popular and healthy beverage in Japan, China and other Asian countries for many years. Catechins are the major and important active components of green tea. They have been shown to have anti-cancer, anti-oxidant, anti-bacteria, anti-fungi and anti-virus activities *in vivo* and *in vitro*. Major components of catechins are epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate. Epigallocatechin gallate has a broad range of anti-viral activity against many viruses including AdV, EBV, influenza virus, HBV, HCV, HIV-1, HSV and enterovirus. Some mechanisms have been reported. AdV (18) inhibition was by inactivation of virus particles, inhibition of intracellular virus growth and viral protease. Inhibition of transcription of immediate-early genes was reported for EBV (19). For influenza virus, inhibition of virus entry by binding to HA (20) and inhibition of endnuclease activity was reported (21). Reduction of HBV antigen expression was also reported (22). Mechanisms of HCV inhibition were as follows: the inhibition of viral entry with binding to target cells (23) and suppression of the gene expression of proinflammatory enzymes and cytokines (24). For HIV, inhibition of integrase (25), inhibition of reverse transcriptase (26), destruction of virions by binding to envelope (27), and by binding to CD4 and interference with gp120 binding (28). Inhibition of HSV (29) was caused by damage to and inactivation of virions. The suppression of viral replication via modulation of cellular redox milieu was reported on enterovirus (30). The present study reported that the mechanisms of inhibitory effect of catechin on hPIV-2 were different from those of catechin on other viruses. Catechin did not inhibit hPIV-2 entry into cells, but it had transcription inhibition activity, leading to the inhibition of protein synthesis in $LLCMK_2$ cells. In addition, it inhibited viral genome synthesis, and disrupted actin filaments and microtubules at 20 hr of culture.

Tannins are also plant polyphenols and also have inhibitory effect on a broad rage of viruses. Norovirus was inhibited by tannic acid from binding to histo-blood group antigen receptors (31). Ueda *et al.* (11) reported that persimmon extract (PE), wattle extract, etc inhibited influenza virus, HSV, VSV, SeV, NDV, poliovirus, Coxsachie virus, AdV, rotavirus, feline calicivirus and mouse norovirus replications when each virus was incubated with tannin solution for 3 min, and the remaining infectivity was measured. They showed that PE was effective within 30 seconds and inhibited attachment of influenza virus to cells. They also showed that PE did not cause cytotoxicity to many cell lines including $LLCMK_2$ cells at 24 hr of culture by MTT assay. However we showed that tannic acid caused disruption of actin microfilaments and microtubules, resulting to the inhibition of virus progeny. The difference may come from the nature of tannic acid. Ueda *et al.* (11) added the extracts from plants, while we used reagent from a company. They showed that PE works by inhibiting SeV attachment to cells. Our results showed that tannic acid did not inhibit virus entry or cell-to-cell spreading of hPIV-2. In addition, tannic acid did not inhibit either viral mRNA or protein synthesis. However, viral genome synthesis was largely inhibited by tannic acid.

In summary, the present investigation reported that the inhibitory effect of catechin was due to viral genome synthesis inhibition, transcription inhibition and the disruption of the cytoskeleton, but that of tannic acid was mainly due to viral genome inhibition and the disruption of cytoskeleton.

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DISCLOSURE

The authors declare no conflict of interests and agree to publication.

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