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Original Article

Caffeic acid, a coffee-related organic acid, inhibits infection by severe fever with thrombocytopenia syndrome virus *in vitro*☆

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) virus (SFTSV) causes tick-borne hemorrhagic fever in East Asia. The disease is characterized by high morbidity and mortality. Here, we evaluated the effects of caffeic acid (CA), a coffee-related organic acid with antiviral effects, against SFTSV infection. CA dose-dependently inhibited SFTSV infection in permissive human hepatoma Huh7.5.1–8 cells when SFTSV was added into the culture medium with CA. However, quinic acid (QA), another coffee-related organic acid, did not inhibit SFTSV infection. The 50% inhibitory concentration (IC_{50}) of CA against SFTSV was 0.048 mM, whereas its 50% cytotoxic concentration was 7.6 mM. The selectivity index (SI) was 158. Pre-incubation of SFTSV with CA for 4 h resulted in a greater inhibition of SFTSV infection (IC_{50} = 0.019 mM; SI = 400). The pre-incubation substantially decreased viral attachment to the cells. CA treatment of the SFTSV-infected cells also inhibited the infection, albeit less effectively. CA activity after cell infection with SFTSV was more pronounced at a low multiplicity of infection (MOI) of 0.01 per cell (IC_{50} = 0.18 mM) than at a high MOI of 1 per cell (IC_{50} > 1 mM). Thus, CA inhibited virus spread by acting directly on the virus rather than on the infected cells. In conclusion, CA acted on SFTSV and inhibited viral infection and spread, mainly by inhibiting the binding of SFTSV to the cells. We therefore demonstrated CA to be a potential anti-SFTSV drug for preventing and treating SFTS.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne hemorrhagic fever caused by the SFTS virus (SFTSV), a phlebovirus of the *Bunyaviridae* family. SFTSV is transmitted to humans by bites of various tick species such as *Haemaphysalis longicornis* and *Amblyomma testudinarium* [1].

SFTS was initially reported in China in 2010 [2]. From 2011 to 2014, more than 3500 cases of SFTS have been reported in approximately 20 provinces in China, with an average mortality rate of 7.8% [3]. In Japan, the first case of SFTS was reported in 2013 [4], after which approximately 300 SFTS cases have been reported, with a fatality rate of 20.7% [5]. The main clinical symptoms of SFTS are rapid onset of high fever, gastrointestinal tract symptoms, hemorrhagic tendency, and thrombocytopenia. Most cases occur between spring and autumn, corresponding to the tick's seasonal activity. Effective preventive and therapeutic methods are needed, but neither vaccines nor antiviral drugs are currently available.

Caffeic acid (CA), a coffee-related organic acid, is a degradation product of chlorogenic acid, abundant in coffee beans. It is responsible for the deep aroma, color, and bitterness of coffee. This polyphenol is also present in a wide variety of foods. CA exerts various beneficial biological effects, including the suppression of metastasis and proliferation of cancer cells [6,7] and antiviral effects [8,9].

In this study, the inhibitory effect and mechanism of action of CA on SFTSV infection was examined.

2. Materials and methods

2.1. Cells and viruses

A Japanese isolate of SFTSV, the YG1 strain, was propagated in the Huh7.5.1–8 cell line, a highly permissive derivative of human

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hepatoma Huh7 cells [10]. Briefly, the cells were cultured at 37 °C in Dulbecco's minimum essential medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich Japan Co. LLC, Tokyo, Japan) and antibiotics (DMEM-10FCS). The virus-infected cells were maintained in DMEM-10FCS. The infectious dose of SFTSV was determined as described previously [11].

2.2. Virus quantitation by quantitative reverse transcription PCR

The viral genome was quantified by quantitative reverse transcription PCR (qRT-PCR) using TaqMan-based chemistry after extracting total RNA from the medium and cells using a viral nucleic acid extraction kit (Favorgen Biotech Corporation, Changzhi, Taiwan) and the Blood/Cultured Cell Total RNA Mini kit (Favorgen Biotech Corporation). Virus-specific primers and a TaqMan probe suitable for the detection of strain YG1 were designed according to a previous report [12]: SFTS-S-F: 5′-GAGAGGATCCCTGAAAGAGTTGTAAA-3′; SFTS-S-R: 5′-TGCCTTCACCAAGACTATCAATGT-3′; SFTS-S-probe: [FAM]-5′-TCTGTCTTGCTGGCTCCGCGC-3′-[TAMRA]. qRT-PCR was performed using the THUNDERBIRD® Probe One-step qRT-PCR kit (Toyobo Co., Ltd., Osaka, Japan) in a LightCycler 96 (Roche Diagnostics, Basel, Switzerland).

2.3. Inhibitory effect of CA when SFTSV was incubated with CA in the culture medium

Huh7.5.1–8 cells (2×10^5 cells/well) were plated in a 12-well plate and incubated overnight to reach about 100% confluency. SFTSV was mixed with CA or quinic acid (QA) (Wako Pure Chemical Industries, Ltd.) (0–1 mM) to make the multiplicity of infection (MOI) of the virus solution 1 in DMEM-10FCS. Huh7.5.1–8 cells were inoculated with the mixture immediately. After 24 h, total RNA was extracted from the cells for viral genome quantitation by qRT-PCR. The 50% cytotoxic concentrations (CC_{50}) of CA and QA were evaluated using an XTT assay (Cell Proliferation Kit II; Roche Diagnostics).

2.4. Inhibitory effect of CA on SFTSV infection when SFTSV was incubated with CA before infection

SFTSV was diluted to the equivalent of an MOI of 400. Before inoculation, SFTSV was incubated with CA or QA (0–1 mM) for 4 h at 37 °C. The mixture was then diluted further with DMEM-10FCS to reach a MOI of 1 and was used to inoculate Huh7.5.1–8 cells. The dilution step was essential to minimize the direct inhibitory effect of CA on the cells. After 1 h incubation, the cells were washed and cultured in CA- or QA-free DMEM-10FCS. Twenty-four hours after inoculation, total RNA was extracted from the cells for viral genome quantitation by qRT-PCR. Furthermore, after SFTSV was incubated with CA (0–1 mM) for 4 h at 37 °C, the infectious dose of SFTSV in the mixture was also determined by the conventional virus titration assay using immunofluorescent staining as described previously [11].

2.5. Effect of CA when it was added after SFTSV inoculation

Huh7.5.1.–8 cells were infected with SFTSV at a low (MOI = 0.01) or high MOI (MOI = 1) for 1 h. The cells were then washed and cultured in DMEM-10FCS with 0–1 mM CA. After 72 h, total RNA was extracted from infected cells for viral genome quantitation by qRT-PCR.

2.6. Virus fractionation by sucrose density gradient ultracentrifugation

After incubating SFTSV solution with 1 mM CA for 4 h at 37 °C, the CA-treated virus solution was fractionated by sucrose density gradient ultracentrifugation as described previously [13], and 12 fractions were collected. Total RNA was extracted from each fraction to determine the virus copy number by qRT-PCR. Infectious virus in each fraction was determined by the conventional virus titration assay [11].

2.7. Virus-binding assay

After incubating SFTSV solution with 1 mM CA for 4 h at 37 °C, Huh7.5.1–8 cells were incubated with the CA-treated virus solution on ice. Two hours later, RNA was extracted from the cells and subjected to viral quantification by qRT-PCR. Statistical significance was evaluated by Welch's *t*-test, and $P < 0.01$ was considered significant.

3. Results

3.1. Inhibitory effect of CA when SFTSV was incubated with CA in the culture medium

We first examined the efficacy of CA when CA and SFTSV were mixed in the culture medium; the mixture was used to inoculate and culture Huh7.5.1–8 cells. Co-culture of the virus with CA inhibited virus infection in a dose-dependent manner (Fig. 1). We then evaluated the efficacy of another organic acid, QA (pKa 3.4), which has similar acidity to that of CA (pKa 3.6). However, QA was found to not inhibit SFTSV infection (Fig. 1). These results suggested that CA inhibited SFTSV infection specifically, and the mechanism of action did not depend on its acidity. The 50% inhibitory concentration (IC_{50}) of CA against SFTSV was 0.048 mM, whereas the CC_{50} of CA against Huh7.5.1–8 cells was 7.6 mM. The selectivity index (SI) was 158.

3.2. Inhibitory effect of CA on SFTSV infection when SFTSV was incubated with CA before infection

To elucidate the mechanism of the anti-SFTSV activity of CA, SFTSV was incubated with CA before inoculating the virus into

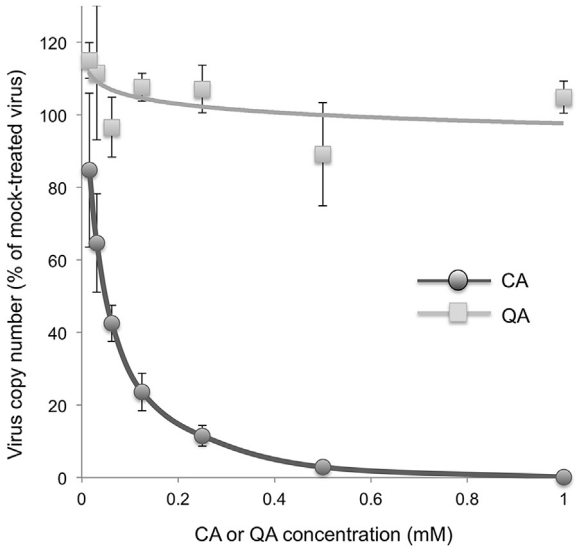

Fig. 1. Inhibitory effect of caffeic acid (CA) and quinic acid (QA) on SFTSV infection by culturing Huh7.5.1–8 cells inoculated with SFTSV in the presence of each compound. SFTSV (MOI = 1) and CA or QA at the designated concentrations were mixed in DMEM-10FCS. The inoculum was applied to Huh7.5.1–8 cells, and the cells were harvested at 24 h post infection for viral genome quantitation by qRT-PCR. Data are presented as percent virus copy number relative to that of mock-infected cells (virus treated with DMSO only). Results are presented as the mean ± SD (n = 4).

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