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Expression of antiviral cytokines in Crandell-Reese feline kidney cells pretreated with Korean red ginseng extract or ginsenosides

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ABSTRACT

The antiviral activity and protective mechanism of Korean red ginseng (KRG) is not well understood. The aim of this study was to investigate the protective mechanism of KRG extract and ginsenosides against feline calicivirus (FCV), a human norovirus surrogate. CRFK cells that were pretreated for 48 h with 10 μg/mL of KRG extract or purified ginsenoside Rb1 or Rg1, were inoculated with FCV. RNA extracted from each treated group was examined for the expression of antiviral cytokines, including interferon-α (IFN-α), interferon-β (IFN-β), interferon-ω (IFN-ω), Mx, and zinc finger antiviral protein shorter isoform (ZAPS), by relative real-time reverse transcription-polymerase chain reaction. mRNA expression of IFN-α, IFN-β, IFN-ω, Mx, and ZAPS was significantly induced in the FCV-challenged group pretreated with the KRG extract or ginsenosides, and it was higher than the group treated with FCV alone. Mx protein expression was confirmed by western blotting of CRFK cells pretreated with the ginsenoside Rb1 or with Rg1. Induction of antiviral cytokines contributes to the reduction of the viral titer in CRFK cells pretreated with the KRG extract and purified ginsenosides. In future studies, the antiviral protective mechanism of KRG should be demonstrated using other viruses such as human norovirus.

1. Introduction

Human norovirus (HuNoV) is one of the most common food-borne pathogens worldwide. From 2009 to 2010, a total of 491 food-borne outbreaks due to HuNoV resulted in 9737 cases of illness and 109 hospitalizations in the United States. Norovirus outbreaks constitute 42% of the total outbreaks related to food-borne diseases (CDC, 2013). The Foodborne Viruses in Europe network reported 7647 HuNoV outbreaks from July 1, 2001 to June 30, 2006 in European countries, including England and Wales, Finland, France, Germany, Hungary, the Netherlands, Norway, Sweden, and Slovenia (Kroneman et al., 2008). In Japan, the Ministry of Health, Labour, and Welfare reported that 41 and 8 people died in the winters of 2006 and 2012, respectively. In 2012, the Korean Centers for Disease Control and Prevention reported that outbreaks of HuNoV increased by 88.5% from 2011.

HuNoV, a member of the family *Caliciviridae*, is a 7.6-kb positive-sense, single-stranded RNA virus, 28–32 nm in diameter, has no lipid envelope, and its genome consists of 3 open reading frames (Hansman et al., 2010). HuNoV is presently classified into 6 genogroups (GI to GVI) (Martella et al., 2011). Only GI, GII, and GIV cause nausea, vomiting, abdominal pain, and diarrhea in humans (Atmar and Estes, 2001). They are transmitted through the fecal-oral route, by ingestion of contaminated food or water, and by person-to-person contact (Heijne et al., 2009).

Although several research groups have reported HuNoV culture systems *in vitro* or *in vivo*, a standard cell culture system for HuNoV has not been established. Due to the lack of a cell culture system for HuNoV, viability and titration of HuNoV cannot be measured, and the development of vaccines or antiviral drugs has been limited (Bok et al., 2011; Duizer et al., 2004). Thus, many scientists use norovirus surrogate models such as feline calicivirus (FCV), murine norovirus (MNV), porcine norovirus, bovine norovirus, poliovirus, and bacteriophage MS2 (Cannon et al., 2006; Richards, 2012). FCV is a representative human norovirus surrogate that is cultivable in Crandell-Reese feline kidney (CRFK) cells. FCV was first used as a HuNoV surrogate in the late 1990s (Hansman

Abbreviations: KRG, Korean red ginseng; HuNoV, human norovirus; FCV, feline calicivirus; MNV, murine norovirus; ZAPS, zinc finger antiviral protein shorter isoform; PARP, poly (ADP-ribose) polymerase.

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et al., 2010; Richards, 2012). FCV is a member of the *Caliciviridae* family, like human norovirus, and it has similar structural characteristics and genomic structure (Bentley et al., 2012). It is easily propagated in CRFK cells, and the reverse genetics system has been studied (Hansman et al., 2010).

When humans or animals are infected with viruses, various types of cytokines are expressed and activated to block virus replication. Among them, antiviral activities of some cytokines, including interferon (IFN), Mx, and zinc finger CCCH type antiviral protein 1 (ZAP), are known. IFN was first discovered in 1957 by Isaacs and Lindenmann (Isaacs and Lindenmann, 1957), and it can be classified into type I IFN and type II IFN (Ho and Armstrong, 1975). Type I IFNs include IFN-α, IFN-β, and IFN-ω, and they work synchronously to block the virus infection (Samuel, 2001). Type II IFNs include IFN-γ, and they are related to the immune system (Samuel, 2001). Mx is an IFN-inducible gene product that has antiviral activity. Mx is induced by IFN-α, and IFN-β has the ability to block viral replication (Samuel, 2001). ZAP is known as the full-length poly (ADP-ribose) polymerase-13 (PARP-13), which interacts with viral RNA and degrades mRNA (Gao et al., 2002). ZAPS, the shorter isoform of PARP-13, can induce type I IFN genes and inhibit viral replication (Hayakawa et al., 2011).

Panax ginseng C.A. Meyer has many beneficial functions, including anti-cancer, anti-oxidant, anti-aging, and immunostimulatory properties. While Korean red ginseng (KRG) is made by repeated steaming and drying of *Panax ginseng*, the same components undergo chemical transformation and fortification (Lee et al., 2011a). The anti-stress (Kim et al., 2013a,2013b), anti-oxidant (Kim et al., 2011), anti-microbial (Lee et al., 2008), and anti-virus properties (Lee et al., 2011b; Yoo et al., 2012) of KRG have been established in previous studies. Red ginseng acidic polysaccharide and ginsenosides, which are ingredients of KRG, are known to improve immune system functioning (Byeon et al., 2012). Although it was reported that treatment with KRG inhibited FCV and MNV, the antiviral protective mechanisms of KRG have not been investigated (Lee et al., 2011b). Therefore, the aim of this study was to investigate the antiviral cytokine induction of KRG and the ginsenosides Rb1 and Rg1 against feline calicivirus, as a HuNoV surrogate.

2. Materials and methods

2.1. Viruses and cell lines

Crandell-Reese Feline Kidney (CRFK) cells and a feline calicivirus (FCV)-F9 strain were purchased from ATCC (Manassas, VA, USA). CRFK cells were maintained in Eagle's minimal essential medium (EMEM; Cat. No. 30-2003, ATCC) containing 10% heat-inactivated bovine viral diarrhea virus (BVDV)-free fetal bovine serum (Cat. No. 16000, Gibco-Invitrogen, Grand Island, NY, USA), and 1% antibiotic–antimycotic (Gibco-Invitrogen) at 37 °C under 5% CO_2 conditions. CRFK cells were infected with FCV and cultured for 1 day for virus propagation. The cultured virus was then frozen and thawed 3 times and centrifuged at 2500*g* for 15 min. The supernatant virus was used as stock virus. The titration of stock virus was determined by the protocol of a previous study (Lee et al., 2011b).

2.2. KRG extract and ginsenosides

KRG (*Panax ginseng* C.A. Meyer) extract was prepared and provided by the Korea Ginseng Corporation (Daejeon, Korea). The moisture content of KRG extract was 36.68%, and the amount of red ginseng content in the KRG extract was 85.58 mg/g. The total content of ginsenoside Rb1 and ginsenoside Rg1 was 4.547 mg/g, as determined by high-performance liquid chromatography analysis of the KRG extract; it was then diluted to the proper concentration for treatment. Purified ginsenoside Rb1 and ginsenoside Rg1 were purchased from Wako (Osaka, Japan), resuspended in sterile distilled water, and diluted with EMEM.

2.3. Cytotoxicity test

CRFK cells were treated with the KRG extract and purified ginsenosides Rb1 and Rg1. The stock concentrations of KRG extract or ginsenosides were adjusted to 40 μg/mL of total saponin. CRFK cells were treated with 2-fold serially diluted KRG extract and purified ginsenosides in 96-well plates and incubated at 37 °C with 5% CO_2 for 24 h. In order to evaluate cytotoxicity, a colorimetric MTT (tetrazolium) assay was performed as previously described (Mosmann, 1983). Briefly, MTT (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in PBS at 5 mg/mL and sterilized using a 0.2-μm syringe filter. Stock MTT solution was added to each well of a 96-well cell culture plate (10 μL of stock MTT solution/100 μL medium), and the plates were incubated at 37 °C for 4 h. Acid-isopropanol containing 0.04 N HCl was added to all the wells and mixed thoroughly to dissolve the dark blue crystals. The absorbance value was read at a wavelength of 570 nm on an Epoch spectrophotometer (Biotek, Winooski, VT, USA) within 1 h after adding isopropanol. Results are expressed as cell viability (%), which was calculated using the following formula: Cell viability (%) = $(Abs_{treatment}/Abs_{control}) \times 100$.

2.4. Experimental design

To examine the expression of antiviral cytokines by KRG or ginsenosides, CRFK cells were seeded in 60-mm cell culture dishes. In order to set the basal expression level, the CRFK cells were cultured for 24 h to confluency. The concentration of KRG extract and purified ginsenosides Rb1 and Rg1 used to treat CRFK cells was 10 μg/mL of total saponin. The experimental groups were divided into 8 groups (Table 1): NC, negative control; K, 10 μg/mL of total saponin containing KRG extract treated for 48 h; Rb, 10 μg/mL of purified ginsenoside Rb1 treated for 48 h; R, 10 μg/mL of purified ginsenoside Rg1 treated for 48 h, V, 2 $\log_{10}$ PFU/mL of FCV infected for 24 h; KV, 10 μg/mL of total saponin containing KRG extract treated for 48 h and then FCV infected; RbV, 10 μg/mL of Rb1 treated for 48 h and then FCV infected; and RgV, 10 μg/mL of Rg1 treated for 48 h and then FCV infected. For preparation of a positive control for the expression of cytokines, CRFK cells were treated with 10 ng/mL of purified IFN-α (Cat. No. H6041, Sigma) for 24 h. The cells were harvested from the 8 experimental groups and the positive control, and then RNA extraction was performed.

2.5. Expression of antiviral cytokines

2.5.1. RNA extraction and DNase treatment

RNA extraction was performed on each sample using the TRIzol® method. Briefly, 500 μL of TRIzol® reagent (Invitrogen, CA, USA) was added to the cells. The sample mixture was moved to an Eppendorf tube and incubated for 10 min at room temperature. Then, 300 μL of chloroform was added, and the samples were maintained at room temperature. Ten minutes later, the mixtures were centrifuged at 7500 rpm for 15 min. Then, 500 μL of supernatant was transferred to a fresh Eppendorf tube, 500 μL of isopropyl alcohol (Sigma) was added, and the tube was stored at −20 °C overnight for RNA precipitation. The next day, the samples were centrifuged at 14,000 rpm for 30 min. To clean the RNA precipitate, the top liquid layer was removed, and 500 μL of 75% ethanol was added to the removed liquid before vortexing gently. RNA was centrifuged at 14,000 rpm for 30 min, and the supernatant was carefully removed. Finally, the precipitate was air-dried for 30 min and dissolved in 30 μL of RNase-free water.

For elimination of contaminating DNA, RNA was treated with deoxyribonuclease I (DNase I) of amplification grade (Invitrogen, CA, USA). Briefly, a mixture of 1 μg total RNA, 1 μL 10 × DNase I reaction buffer, 1 U DNase I, and DEPC-treated distilled water (up to 10 μL) was incubated for 15 min at room temperature. Then, 1 μL of 25 mM EDTA solution was added to the reaction mixture and heat-inactivated for 10 min at 65 °C. The RNA concentration was measured using an Epoch spectrophotometer (Biotek, Winooski, VT, USA) at a test wavelength of 280 nm.

2.5.2. Relative quantification of mRNA

For reverse transcription (RT), a mixture was prepared with 1 × PCR buffer, 10 mM dNTP, 25 μM oligo d(T), 10 U RNase inhibitor, 25 U of MuLV reverse transcriptase, and 1 μL of total RNA. The mixture was incubated at 42 °C for 15 min, and the enzymes were inactivated at 99 °C for 5 min.

To evaluate the relative quantification of antiviral cytokines, real-time RT-polymerase chain reaction (real-time RT-PCR) was performed, and the results were read by TaqMan probe or SYBR green. Expression of *IFN-α*, *-β*, *-ω*, *Mx*, and *ZAPS* were examined at the mRNA level, and the housekeeping gene β-glucuronidase (*GUBS*) was used as a control (Kessler et al., 2009). For expression of *IFN-α*, *IFN-β*, *IFN-ω*, and *Mx*, the reactions contained 1 × Premix Ex TaqTM (TAKARA, Shiga, Japan), 1 pM forward and reverse primers, 1 pM probe (Table 2), 100 ng cDNA, and distilled water to a final volume of 25 μL. Real-time PCR amplification was performed in a thermocycler (TAKARA) as follows: initial denaturation for 30 s at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 45 s. All the probes were labeled with the reporter dye FAM (6-carboxyfluorescein) at the 5′ end and the quencher dye TAMRA (6-carboxytetramethylrhodamine) at the 3′ end for TaqMan probe amplification. For the expression of *ZAPS* mRNA, the reactions contained 1 × SYBR® Premix Ex Taq (TAKARA), 1 pM forward and reverse primer, 100 ng cDNA, and distilled water to a final volume of 25 μL. SYBR green amplification was performed as follows: initial denaturation for 30 s at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 45 s, followed by an additional cycle to generate the dissociation curve at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.