The extract of *Elaeocarpus sylvestris* inhibits human cytomegalovirus immediate early gene expression and replication *in vitro*

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Abstract. Human cytomegalovirus (HCMV) is a major cause of morbidity and mortality in newborn infants, immunocompromised individuals with HIV/AIDS and organ transplant recipients. In order to identify a novel antiviral candidate for HCMV-related diseases, crude ethanol extracts from plants were screened for their potential inhibitory activity on HCMV replication *in vitro*. Ethanol (70%) extract of *Elaeocarpus sylvestris* leaves (ESE) markedly inhibited the replication of the HCMV Towne strain without exhibiting any significant adverse effects on the viability of human foreskin fibroblasts (HFF). In addition, ESE significantly downregulated HCMV immediate early (IE) gene expression. Taken together, this is the first study, to the best of our knowledge, demonstrating that ESE has a potent antiviral activity against HCMV by downregulating HCMV IE gene expression and replication.

Introduction

Human cytomegalovirus (HCMV) belongs to the β subgroup of herpes viruses, which exhibit a restricted host range and slow replication cycle (1,2). HCMV has a linear double-stranded DNA genome of ~230 kb, which encodes potentially >200 viral proteins (2). The viral DNA genome can be divided into two segments known as the unique long (UL), which is ~175 kb, and the unique short (US), which is ~38 kb. Flanking the UL and US regions are repeat sequences termed the terminal repeat long (TRL), the terminal repeat short (TRS), the internal repeat long (IRL) and the internal repeat short (IRS). The genes of the HCMV are classified according to location in the UL, US, TRS, TRL, IRS or IRL followed by the open reading frame number (2).

During lytic replication, HCMV gene expression occurs according to a cascade of three consecutive phases: immediate early (IE), early (E) and late (L) (2). The IE genes are transcribed immediately following infection and they do not require *de novo* viral protein synthesis. The IE proteins are important transactivators of viral early genes and cellular genes. The early genes encode proteins that are important for the replication of the viral DNA. Following the onset of viral DNA replication, late genes, which encode structural proteins for virion components, are transcribed.

HCMV is transmitted by bodily secretions and infects 50-90% of the human population worldwide (1). Following primary infection, HCMV establishes a lifelong latent infection in blood monocytes and myelomonocytic precursors within the bone marrow with periodic reactivation (3-8). In immunocompetent individuals, infection with HCMV is usually asymptomatic. In rare cases, HCMV mononucleosis syndrome develops with potential complications, including pneumonia, hepatitis and meningitis (1).

In immunosuppressed or immunocompromised individuals, primary infection with HCMV or reactivation of the latent virus causes severe diseases, including pneumonitis, encephalitis, retinitis, hepatitis and gastroenteritis (1). In addition, HCMV can be transmitted to infants from their mothers during a primary infection or reactivation either *in utero* or while passing through the cervix at birth, which can cause infant mortality, premature deliveries and birth defects, including blindness, mental retardation and hearing loss (1).

Currently, there are only a few drugs approved by the Food and Drug Administration (FDA) for the treatment of HCMV-related diseases (9,10). Ganciclovir, valganciclovir, cidofovir and fomivirsen are synthetic nucleotide analogs and foscamet is a pyrophosphate analog (9,10). These drugs inhibit HCMV DNA replication by targeting the viral DNA polymerase. However, there are several disadvantages associated with the use of these synthetic drugs, including toxicity and inactivation by resistant viruses (9,11). Thus, it is necessary to develop new anti-HCMV drugs with high efficacy and fewer side effects. Natural products from plants have been reported to be a valuable source for antiviral agents with fewer side effects

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Abbreviations: HCMV, human cytomegalovirus; IE, immediate early; ESE, ethanol extract of *Elaeocarpus sylvestris*; HFF, human foreskin fibroblasts

Key words: Elaeocarpus sylvestris, human cytomegalovirus, antiviral

than synthetic drugs (12). In the present study, the effects of 70% ethanol extract of *Elaeocarpus sylvestris* leaves (ESE) on HCMV infection and/or replication were investigated.

Materials and methods

Cells, viruses and plant material. The maintenance and propagation of primary human foreskin fibroblasts (HFFs) and HCMV have been described previously (13). The HCMV Towne strain and a recombinant HCMV Towne strain expressing green fluorescent protein (HCMV-Towne-GFP) were kindly provided by Dr Mark Stinski (University of Iowa, Iowa City, IA, USA). The plant materials (*Elaeocarpus sylvestris* var. *ellipticus*) and 70% ethanol extracts used in the present study were collected from the Jeju island in Korea through the Jeju Biodiversity Research Institute (Jeju, Korea; specimen no. JBR-083).

Fluorescence microscopy. The fluorescence was assessed and images were analyzed using an inverted Nikon TS100-F fluorescence microscope (Tokyo, Japan) equipped with a digital camera and Nikon NIS-Elements microscope imaging software.

Quantification of HCMV DNA and RNA. HCMV DNA was quantified by quantitative PCR (qPCR). Total DNA was isolated using an AccuPrep Genomic DNA Extraction kit (Bioneer, Daejon, Korea) and HCMV DNA was amplified and quantified in an MxPro3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) using HOT FIREPol® EvaGreen qPCR mix Plus (Solis BioDyne, Tartu, Estonia). The primers used were as follows: HCMV UL123, forward 5'-CTGCAAACATCCTCCCATCA-3' and reverse 5'-AATATACCCAGACGGAAGAGAAATTC-3'; β-actin, forward 5'-ATCATGTTTGAGACCTTCAAC-3' and reverse 5'-CAGGAAGGAAGGCTGGAAGAG-3'.

HCMV RNA was quantified by quantitative reverse transcription PCR (qRT-PCR). Total RNA was isolated using an RNeasy® kit and reverse transcribed into complementary DNA (cDNA) using a QuantiTect® reverse transcription kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNAs were amplified and quantified in an MxPro3000P QPCR System (Agilent Technologies) using HOT FIREPol® EvaGreen qPCR mix Plus (Solis BioDyne) and the following primers: UL122 (immediate early, IE), forward 5'-ACCATGCAGGTGAACAACAA-3' and reverse 5'-CATGAGGAAGGGAGTGGAGA-3'; UL44 (E), forward 5'-TTTTCTCACCGAGGAACCTTTC-3' and reverse 5'-CCGCTGTTCCCGACGTAAT-3'; UL83 (L), forward 5'-GCAGCCACGGGATCGTACT-3' and reverse 5'-GGCTTTTACCTCACACGAGACTT-3'; β-actin, forward 5'-ATCATGTTTGAGACCTTCAAC-3' and reverse 5'-CAGGAAGGAAGGCTGGAAGAG-3'.

Western blot analysis. Cells were collected, fractionated and transferred onto nitrocellulose membranes as described previously (14). Antibodies to IE-86 and tubulin were purchased from EMD Millipore (Billerica, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Enhanced chemiluminescence detection reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and secondary peroxidase-labeled anti-mouse immunoglobulin G antibody (Amersham Biosciences, Piscataway, NJ, USA) were used according to the manufacturer's instructions.

Cell viability assays. Cell viability was determined using a CellTiter-Glo luminescent cell viability assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Results

ESE inhibits HCMV replication. By using HCMV-Towne-GFP, 70% ethanol extracts of 662 plants were screened for their inhibitory effect on HCMV replication. HFF cells were infected with HCMV-Towne-GFP at a multiplicity of infection (MOI) of 0.1 (low) or 1 (high) and treated with either dimethyl sulfoxide (DMSO) or plant extracts at 10 μg/ml. The cells were retreated with either DMSO or plant extracts at 3 days following infection and GFP was visualized using a fluorescence microscope. Among the screened extracts, ESE was found to interfere with HCMV replication (Fig. 1A; compare lanes 2 and 3 with lanes 4 and 5). HCMV-Towne-GFP replication was completely inhibited by ESE at a low MOI (compare lane 2 with lane 4) and significantly downregulated at a high MOI (compare lane 3 with lane 5).

To quantify HCMV replication, the relative amount of viral DNA was measured by qPCR using primers specific for UL123. Notably, ESE almost completely inhibited HCMV replication at a high MOI (Fig. 1B). ESE treatment reduced HCMV replication in HFF cells by 99.6% compared with DMSO-treated cells (Fig. 1B; compare lane 3 with lane 2). Thus, ESE significantly inhibited HCMV-Towne replication.

ESE interferes with HCMV lytic gene expression. During lytic infection, HCMV gene expression occurs according to a cascade of three consecutive phases: IE, E and L. To determine the effect of ESE on HCMV gene expression, the levels of IE, E, or L transcripts were measured by using qRT-PCR. HFF cells were infected with HCMV Towne at a high MOI and treated with either DMSO or ESE at 10 μg/ml. At 24, 48 and 72 h following infection, the levels of UL122 (IE), UL44 (E) or UL83 (L) transcripts were determined by qRT-PCR (Fig. 2). In DMSO-treated cells, the expression of IE, E and L genes was induced at 24 h and then increased at 48 and 72 h following infection (Fig. 2; lane 2). However, in ESE-treated cells, the expression of IE, E and L genes was significantly downregulated (Fig. 2; compare lane 3 with lane 2). Therefore, ESE interferes with the expression of HCMV IE and subsequently, E and L genes.

ESE inhibits HCMV major immediate early (MIE) gene expression. To confirm the inhibitory effect of ESE on HCMV lytic gene expression, the expression of the HCMV MIE gene, the most abundant IE gene, was determined by western blot analysis. HFF cells were infected with HCMV Towne at a high MOI and treated with either DMSO or ESE at 10 μg/ml. The expression of the IE86 protein encoded by the MIE2 gene (UL122) was determined by western blot analysis with an anti-IE86 antibody. In DMSO-treated cells, the HCMV

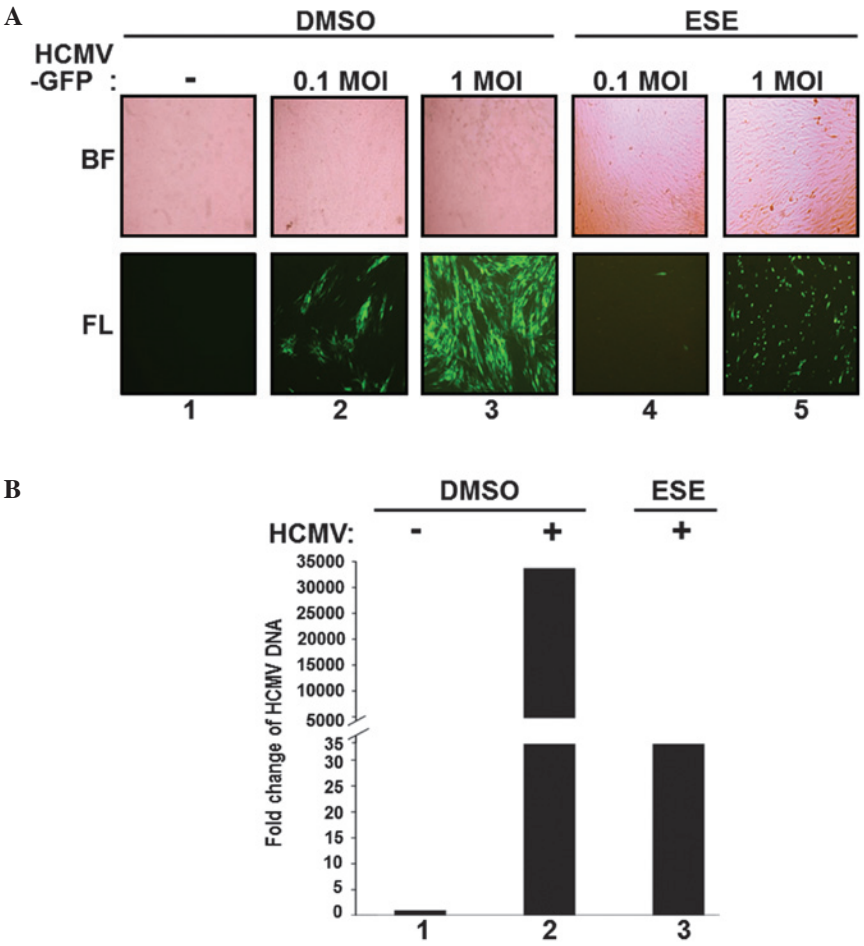

Figure 1. ESE inhibits HCMV replication. (A) HFF cells were mock- (lane 1) or HCMV-Towne-GFP infected at an MOI of 0.1 (low; lanes 2 and 4) or 1 (high; lanes 3 and 5) and treated with either DMSO (lanes 1-3) or ESE (lanes 4 and 5) at 10 μg/ml. HCMV- or mock-infected HFF cells were treated again with either DMSO or ESE at 3 days following infection. At 7 days following infection, GFP fluorescence was measured using a Nikon TS100-F inverted fluorescence microscope. (B) HFF cells were mock- (lane 1) or HCMV-Towne-infected at an MOI of 1 (high; lanes 2 and 3) and treated with either DMSO (lanes 1 and 2) or ESE (lane 3) as described above. At 7 days following infection, total DNA was harvested and the relative amount of viral DNA was measured by qPCR using primers specific for UL123 as described in Materials and methods. BF, bright field microscopic image; FL, fluorescence microscopic image; ESE, ethanol extract of *Elaeocarpus sylvestris* leaves; HCMV, human cytomegalovirus; HFF, human foreskin fibroblasts; HCMV-Towne-GFP, HCMV-Towne strain-expressing green fluorescent protein; MOI, multiplicity of infection; qPCR, quantitative PCR; DMSO, dimethyl sulfoxide; UL, unique long.

IE86 protein was expressed at 24 h following infection and the level of IE86 protein was markedly induced at 48 and 72 h following infection (Fig. 3; lane 2). Consistent with the qRT-PCR data, ESE significantly reduced the expression of the HCMV IE86 protein (Fig. 3; compare lane 3 with lane 2). These data indicated that ESE inhibits HCMV replication possibly by downregulating IE gene expression.

ESE has a minor effect on the viability of HFF cells. To determine whether the effect of ESE on HCMV gene expression and replication was associated with its cytotoxicity, the effect of ESE on the viability of HFF cells was further investigated. HFF cells were treated with 10 μg/ml of ESE and the cell viability was determined by the CellTiter-Glo assay, a luciferase-based assay measuring cellular ATP which represents the presence of metabolically active cells, at 24, 48 or 72 h following treatment (Fig. 4). Within the first 24 h, the viability of HFF cells treated with ESE was similar to those treated with DMSO (Fig. 4; 24 h). At 48 and 72 h following ESE treatment, HFF cell viability was reduced by 26 and 32%, respectively (Fig. 4; 48 and 72 h). Although the viability of HFF cells treated with ESE was slightly reduced at 48 and 72 h following treatment, ESE had little cytotoxic effect on HFF cells at 10 μg/ml.

Discussion

HCMV is a major health threat in newborn infants, immunocompromised individuals with HIV/AIDS and organ transplant recipients. Although certain synthetic drugs that may be used to treat HCMV-related diseases have been approved by the FDA, several problems with these drugs, including toxicity and inactivation by resistant viruses, have arisen. Natural products have been a major source for drug development. Particularly, anti-cancer or anti-infective agents isolated from natural sources have been extensively developed. Therefore, the aim of the present study was to identify plant extracts with anti-HCMV activities.

By screening 70% ethanol extracts of 662 plant extracts, ESE was identified to induce an inhibitory effect on HCMV replication. *Elaeocarpus* is the largest genus in Elaeocarpaceae (15) and *Elaeocarpus sylvestris* var. *ellipticus* is distributed in the

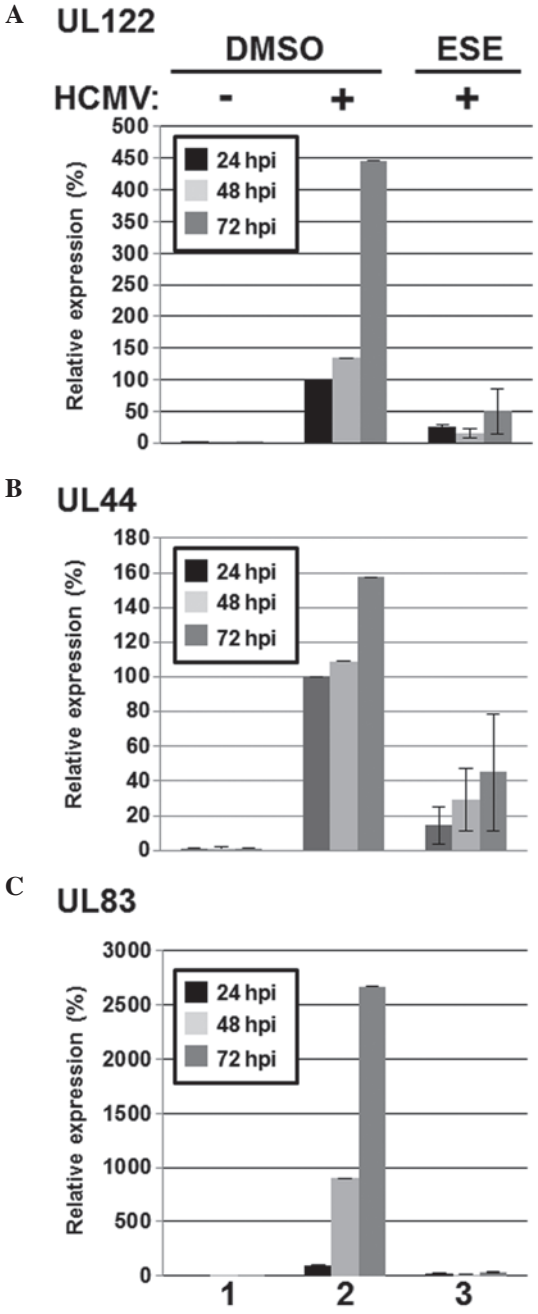

Figure 2. ESE interferes with HCMV lytic gene expression. HFF cells were mock- (lane 1) or HCMV-Towne infected at an MOI of 1 (lanes 2 and 3) and treated with DMSO (lanes 1 and 2) or ESE (lane 3) at 10 μg/ml. At 24, 48 or 72 h following infection, total RNA was harvested and reverse transcribed into cDNA. The relative amount of HCMV (A) UL122, (B) UL44 or (C) UL83 transcripts was measured by qRT-PCR as described in Materials and methods. To calculate the relative gene expression, the amount of transcripts in DMSO-treated HCMV-infected cells at 24 h was set at 100%. The qRT-PCR data are representative of three independent experiments. ESE, ethanol extract of *Elaeocarpus sylvestris* leaves; HCMV, human cytomegalovirus; HFF, human foreskin fibroblasts; MOI, multiplicity of infection; DMSO, dimethyl sulfoxide; cDNA, complementary DNA; UL, unique long; qRT-PCR, quantitative reverse transcription PCR.

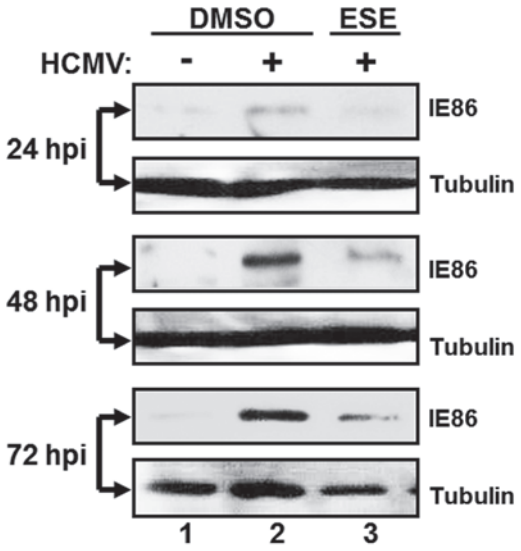

Figure 3. ESE downregulates HCMV MIE gene expression. HFF cells were mock- (lane 1) or HCMV-Towne-infected at an MOI of 1 (lanes 2 and 3) and treated with DMSO (lanes 1 and 2) or ESE (lane 3) at 10 μg/ml. At 24, 48 or 72 h following infection, equal amounts of cell extracts were subjected to western blot analysis with anti-IE86 or anti-tubulin antibody. ESE, ethanol extract of *Elaeocarpus sylvestris* leaves; HCMV, human cytomegalovirus; MIE, major immediate early; HFF, human foreskin fibroblasts; MOI, multiplicity of infection; DMSO, dimethyl sulfoxide.

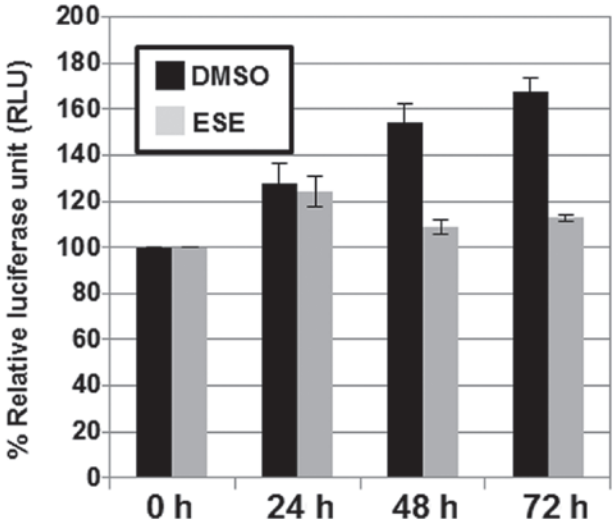

Figure 4. ESE has a minor effect on the cell viability of HFF cells. HFF were treated with either DMSO or ESE at 10 μg/ml and cell viability was determined at 0, 24, 48 or 72 h following treatment using a CellTiter-Glo® luminescent cell viability assay. Luciferase data shown are representative of three independent experiments. ESE, ethanol extract of *Elaeocarpus sylvestris* leaves; HFF, human foreskin fibroblasts; DMSO, dimethyl sulfoxide.

Jeju island in Korea, Southern China, Okinawa and Kyushu in Japan and Taiwan. The extract of *Elaeocarpus sylvestris* contains chemical compounds, including 2-hydroxybenzaldehyde, coniferyl alcohol, umbelliferone, scopoletin, β-sitosterol and daucosterol (16). Notably, the biological activities of ESE have not been extensively investigated. It was reported that ESE protects mice from radiation injury and possesses radioprotective activity by enhancing hematopoietic stem cell regeneration, although the detailed mechanisms remain to be elucidated (17).

Our data indicate that ESE markedly inhibits HCMV replication possibly by downregulating IE gene expression. The anti-HCMV activity of ESE is not associated with cytotoxicity as ESE has almost no adverse effect on the viability of HFF cells. The manner in which ESE inhibits HCMV IE

gene expression remains to be elucidated and may be the subject of future studies. ESE may directly inhibit functional activity of transcription factors for the HCMV IE promoter or indirectly interfere with a signal transduction pathway(s) to activate a transcription factor(s) that is critical for HCMV IE gene expression.

Taken together, this is the first study, to the best of our knowledge, demonstrating that ESE induces an inhibitory effect on HCMV replication without affecting the viability of HFF cells. ESE may be a good candidate for new drug discovery to treat HCMV-related diseases.

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