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Original Article

In Vitro Anti-Cytomegalovirus Activity of Kampo (Japanese Herbal) Medicine

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We examined the effect of three types of Kampo medicines on human cytomegalovirus (CMV) replication in the human embryonic fibroblast cell line, MRC-5. Treatment of cells with at least 0.01 μg/ml of Kampo medicines inhibited the cytopathic effects of CMV-infected MRC-5 cells. Moreover, Kampo medicine decreased the replication of CMV without affecting the inhibition of host cells, with a concomitant decrease in CMV DNA levels. However, Kampo medicine demonstrated no virocidal effect on cell-free CMV. Furthermore, western blotting analysis demonstrated that the Kampo medicine decreased the amount of 65 kDa late antigen expression in the infected cells. These results suggest that Kampo medicine may be sufficient to inhibit viral DNA replication and late protein synthesis, resulting in anti-CMV effects. Therefore, these three Kampo medicines have the potential of being a source of new powerful anti-CMV compounds.

Keywords: cytomegalovirus – Kampo medicine – late antigen

Introduction

Human cytomegalovirus (CMV) is a widespread human pathogen that has a minor clinical impact on healthy individuals, but causes various organ diseases in immunosuppressed patients and neural damage in fetuses infected *in utero* (1), and remains present as a lifelong latent infection. However, latently infected CMV is frequently activated in immunocompromised individuals such as patients with AIDS or organ transplants, thereby causing severe morbidity and eventual mortality (2–5). The mechanism of viral pathogenesis is still not well understood. Symptomatic CMV infection has been treated successfully with ganciclovir (GCV), but the appearance of GCV-resistant viruses is a current problem in the treatment of immunocompromised patients with CMV infection. Although foscarnet (PFA) and cidofovir (CDV) have been used for combined treatment with GCV and for the treatment of GCV-resistant CMV, these treatments are not always successful (6). New or alternative efficacious anti-CMV agents need to be developed (7–10).

Several Kampo (Japanese herbal) medicines are widely used in Japan and China as an effective medication against some disorders of the human body. Sho-seiryu-to (TJ-19; Xiao-Qing-Long-Tang in Chinese) showed potent anti-influenza virus activity in nasal and bronchoalveolar cavities (11) and showed potent antiviral activity against H1N1 and H3N2 subtypes of influenza A and influenza B virus *in vivo* (12). Hochu-ekki-to (TJ-41; Bu-Zhong-Yi-Qi-Tang in Chinese) has been reported to enhance killing activities of peritoneal macrophages against infected *Listeria monocytogenes* (13) and augment splenic natural killer (NK) cell cytotoxicity against Meth-A sarcoma (14). Furthermore, TJ-41 induced the accumulation and activation of NK cells and markedly reduced the viral load of murine CMV in the spleen in the early phase of infection (15). Juzen-taiho-to (TJ-48; Shi-Quan-Da-Bu-Tang in Chinese) is reported to have a stimulatory effect on the immune response (16,17) and phagocytosis (18). We studied these three types of Kampo medicines for anti-CMV activity *in vitro*. These three kampo medicines are shown to inhibit the

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cytopathic effects (CPE) on CMV-infected MRC-5 cells and CMV replication.

Materials and Methods

Cells and Specimens

The Towne strain of human CMV was used throughout the study, and has been described elsewhere (19). CMV was propagated in MRC-5 cells, and the clarified supernatant was stored in liquid nitrogen until use. Viral infectivity was titrated by plaque assay (20).

Medical plants used for preparation of Kampo medicines, TJ-19, TJ-41 and TJ-48, were kindly provided by Tsumura Co., Ltd (Tokyo, Japan). Mixtures of many types of medicinal herbs: for TJ-19, *Pinelliae tuber* (6 g), *Ephedrae herba* (3 g), *Schizandrae fructus* (3 g), *Cinnamonomi cortex* (3 g), *Paeoniae radix* (3 g), *Asari herba cum radice* (3 g), *Glycyrrhizae radix* (3 g) and *Zingiberis siccatum rhizoma* (3 g); for TJ-41, *Ginseng radix* (4 g), *Actractylodis rhizoma* (4 g), *Astragali radix* (4 g), *Angeliceae radix* (3 g), *Zizyphy fructus* (2 g), *Bupleuri radix* (2 g), *Glycyrrhizae radix* (1.5 g), *Zingiberis rhizoma* (0.5 g), *Cimicifuga rhizoma* (1 g) and *Aurantii nobilis pericarpium* (2 g); and for TJ-48, *Astragali radix* (3 g), *Cinnamonomi cortex* (3 g), *Relmanniae radix* (3 g), *Paeoniae radix* (3 g), *Cnidii rhizoma* (3 g), *Actractylodis lancease rhizoma* (3 g), *Angelicae radix* (3 g), *Ginseng radix* (3 g), *Poriacocos hoelen* (3 g) and *Glycyrrhizae radix* (1.5 g) were added with water and extracts at 100°C over a period of 1 h. The extracted solution was filtered and spray-dried to obtain a dry extract powder (Tsumura Report). The blended powder was dissolved in cell culture medium. The quality of these herbs was controlled by the Japanese Pharmacopeia. These extracts (TJ-19, TJ-41 and TJ-48) whose chemical patterns were obtained by three-dimensional HPLC analysis, have been reported previously (21–23).

Cell Culture

MRC-5 cells (24) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Nissui Pharmaceutical Co. Ltd, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS; Z.L. Bocknek Laboratory, Ontario, Canada), L-glutamine (0.3 mg/ml), gentamicin (50 mg/ml) and amphotericin B (2.5 mg/ml). All cell cultures were maintained in a humidified incubator at 37°C in 5% CO_2/95% air.

Viral Production

When MRC-5 cells in 24-well plates (IWAKI Microplate, IWAKI Glass Co., Funahashi, Japan) reached confluency, the cells were inoculated with CMV at a multiplicity of infection (m.o.i.) of 0.1. After adsorption for 1 h, the cells were added with 1 ml of DMEM containing 2% FCS in the presence or absence of various concentrations of Kampo medicine for the indicated time intervals. Production of infectious virus was titrated using a plaque assay (20).

Dot Blot Hybridization

The conditions used for dot blot hybridization were essentially those described previously (25,26), and signals were detected using ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection systems (Amersham Pharmacia Biotech, Inc., NY) according to the manufacturer's instructions. The amplified polymerase chan reaction (PCR) product from the UL54 DNA polymerase gene region as previously described was used as the hybridization probe (27).

Western Blot Analysis

The CMV-infected cell proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate (SDS)–-polyacrylamide gel. Proteins were then transferred to a PVDF membrane (Hybond-p, Amersham Pharmacia Biotech AB, Uppsala, Sweden) using 20 mM Tris, 150 mM glycine, pH 8.3 in 20% methanol. Membranes were incubated for 1 h at room temperature with the blocking reagent [5% skim milk, Tris-buffered saline–0.5% Tween-20 pH 7.6 (TBS-T)], and then incubated for 1 h at room temperature with primary antibody (mouse monoclonal antibody specific for a structural late antigen, 65 kDa, ViroStat, Portland, ME) diluted 1:2000 in TBS-T. The membranes were washed three times in TBS-T and incubated with peroxidase-conjugated second antibody diluted 1:10 000 in TBS-T for 1 h at room temperature. After washing three times in TBS-T, immune complexes were detected by the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using Student's *t*-test.

Results

Kampo Medicines Decreased CMV Replication

The effects of three types of Kampo medicines upon replication of CMV in MRC-5 cell cultures were analyzed. Figure 1 shows a typical experiment to determine the growth of CMV in either untreated or Kampo-treated cultures. MRC-5 cells infected with CMV produced infectious CMV 3 days post-infection (d.p.i.), with a titer increase of at least 6 d.p.i. We demonstrated a significant decrease in viral production in cells treated with all three types of Kampo medicines ($P < 0.05$). Next, to examine the dose-dependent effect of Kampo medicines on the replication of CMV, we determined the production of CMV in MRC-5 cells treated with various concentrations of Kampo medicines. An inhibitory effect on CMV production was observed in cells treated with at least 0.01 μg/ml of all three types of Kampo medicine (Fig. 1A, B and C). TJ-19 was the most effective herb for the inhibition of CMV production among the three kinds of Kampo medicines (Fig. 1A) ($P < 0.05$). The virocidal effect of TJ-19 was then examined after incubation of cell-free CMV with various concentrations of TJ-19. The infectivity of cell-free CMV was unchanged (Fig. 1D)

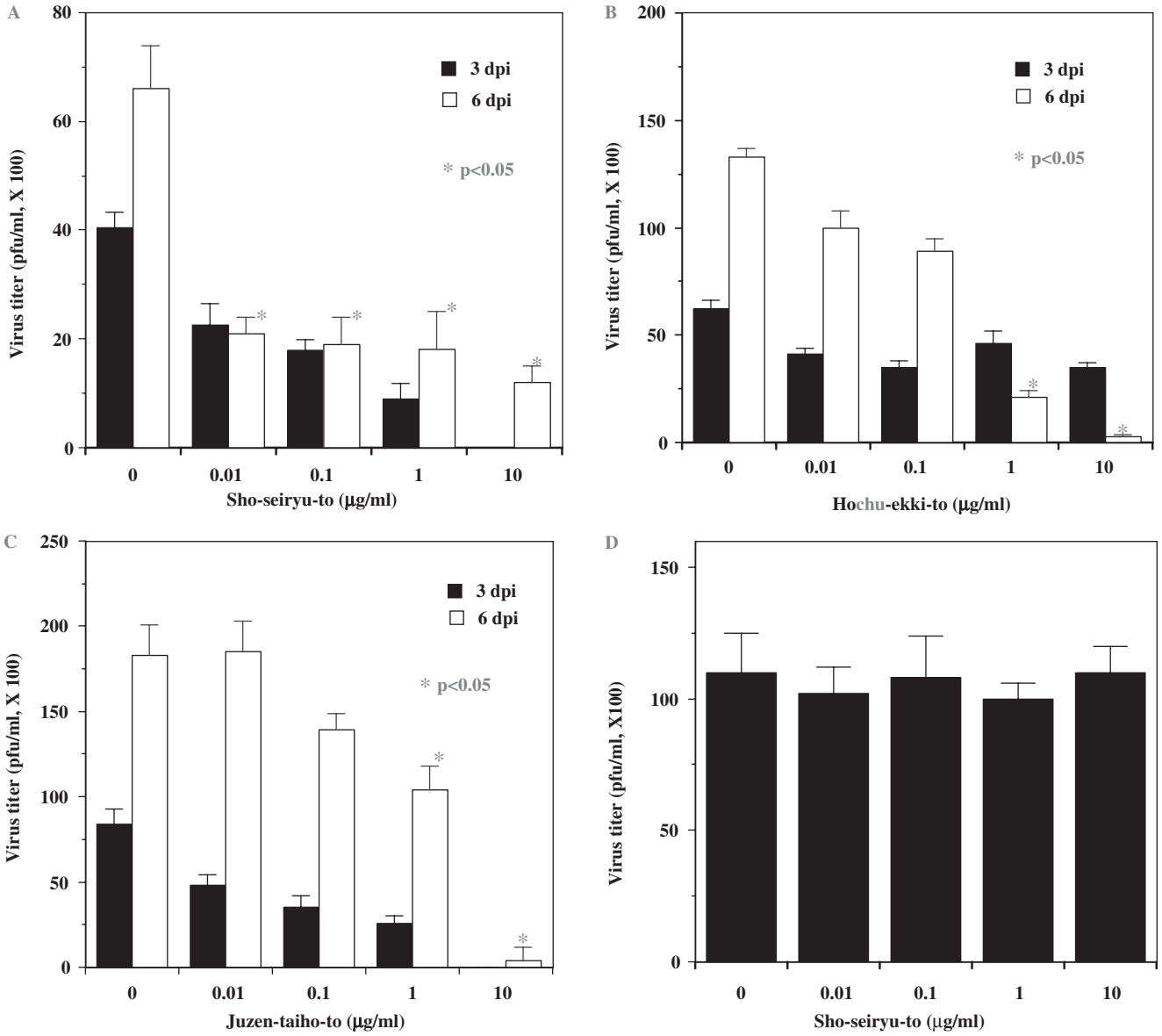

Figure 1. Infectious human CMV production in lung embryonic fibroblasts inhibited by three kinds of Kampo medicines. Sho-seiryu-to (**A**), Hochu-ekki-to (**B**) and Juzen-taiho-to (**C**) inhibited infectious CMV production in a dose-dependent manner. MRC-5 cells were incubated with the indicated concentration of each Kampo medicine after infection with CMV. The production of virus in culture supernatant was determined 3 and 6 days after the infection by using a plaque assay. Data represent means ± SE from three independent experiments. (**D**) Sho-seiryu-to at any concentration has no virocidal effect against cell-free CMV. Cell-free CMV was incubated with the indicated concentration of Sho-seiryu-to. Virus titer was determined 5 days after incubation using a plaque assay. Data represent means ± SE from three independent experiments. *$P < 0.05$, statistical significance compared with the untreated group 6 d.p.i. by the Student's *t*-test.

Reduction of Cytopathic Effects on CMV-Infected MRC-5 Cells with Kampo Medicine

CMV inoculated onto monolayered MRC-5 cells [m.o.i. of 0.1 plaque-forming unit (p.f.u.)/cell] was allowed to adsorb for 1 h at 37°C. The cultures were then washed twice and incubated with or without TJ-19 (0.1 or 1 μg/ml). At the indicated time intervals (Fig. 2), cells were observed through a phase difference microscope. We observed the reduction of CPE by incubation with TJ-19 (Fig. 2C and G, and D and H), which was characterized by the decrease of round cells. At 6 d.p.i., most of the cells showed rounding without TJ-19 (Fig. 2F). In contrast, only a small percentage of cells treated with TJ-19 showed rounding at 3 d.p.i., while at 6 d.p.i., ~20% of these cells showed rounding. There was very little rounding in most of the cells treated with only TJ-19 and in the cells not infected with CMV (Fig. 2A and E).

Inhibition of CMV DNA Replication with TJ-19

To evaluate the effect of TJ-19 on replication of CMV DNA, CMV-infected MRC-5 cells were cultured in the presence or absence of TJ-19 (1 or 0.01 μg/ml), and the extracted DNA was used to estimate the synthesis of CMV DNA by dot blot

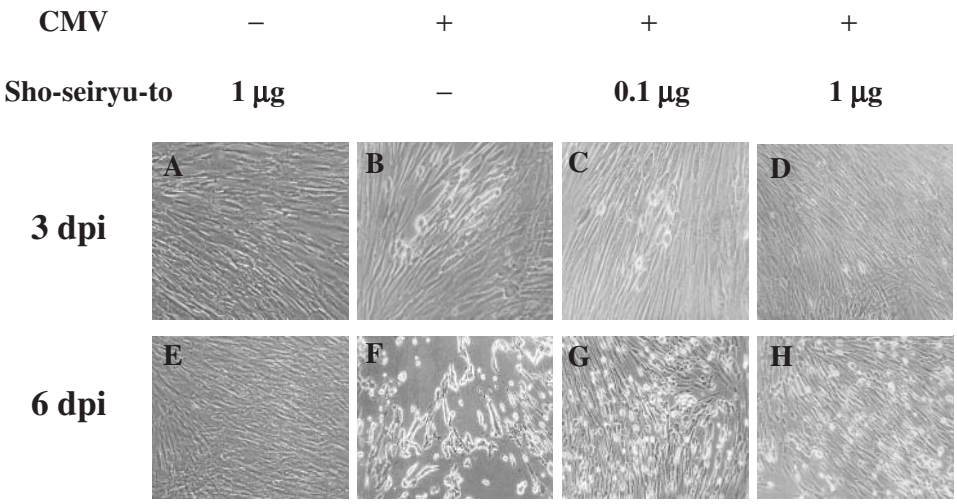

Figure 2. Sho-seiryu-to decreased the degree of cytopathic effects in CMV-infected MRC-5 cells. MRC-5 cells were incubated with either Sho-seiryu-to or medium alone after infection with CMV. The degree of cytopathic effects of CMV-infected (**B, C, D, F, G** and **H**) or uninfected cells (**A** and **E**) was observed 3 and 6 days after infection using a phase difference microscope. Photomicrographs are 200× original magnification.

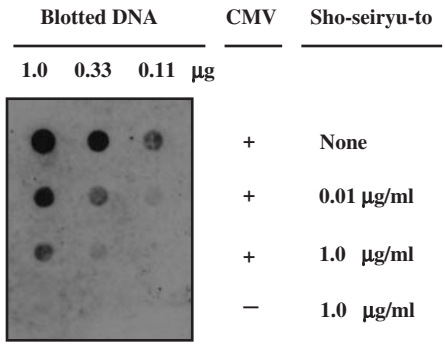

Figure 3. Sho-seiryu-to inhibited the replication of CMV DNA. The cells infected with CMV were incubated in the absence or presence of the indicated concentration of Sho-seiryu-to. DNA was extracted 5 days after infection. Dot blotting analysis was performed with serially 3-fold diluted DNA and detected using the ECL direct nucleic acid detection system.

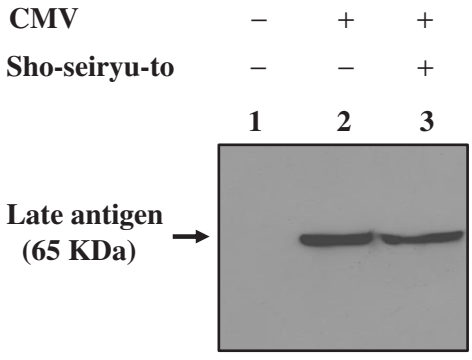

Figure 4. Sho-seiryu-to regulates protein expression of CMV-infected cells. MRC-5 cells were incubated with or without Sho-seiryu-to (0.01 μg/ml) after infection with CMV. Proteins were prepared 5 days after infection. Western blotting analysis was performed using antibody against late antigen (65 kDa). Proteins after electrophoresis on an SDS–polyacrylamide gel were transferred to a nylon membrane. Immunoblotting was detected by the ECL system.

hybridization. As shown in Fig. 3, TJ-19 inhibited CMV DNA replication in CMV-infected MRC-5 cells ~3-fold dose-dependently, compared with that in the untreated cells.

Contrasting Effect of TJ-19 on CMV Protein Synthesis

To determine the molecular nature of the late protein found in the TJ-19-treated cells, we examined the late protein synthesized in MRC-5 cells by western blot analysis. As shown in Fig. 4, the CMV 65 kDa late protein was synthesized in CMV-infected MRC-5 cells (lane 2). In contrast, the amount of late protein synthesis was reduced by treatment of the infected cells with TJ-19 (Fig. 4, lane 3).

Discussion

We demonstrated the effect of Kampo medicines on the replication of human CMV in MRC-5 cells. These three Kampo medicines were first studied for anti-human CMV activity in MRC-5 cells and an inhibitory effect was observed even at 0.01 μg/ml. These anti-CMV activities were demonstrated further through plaque and gene expression assays. Yukawa *et al*. (8) reported that hot water extracts of some traditional herbs have been shown to have anti-CMV activity at 1–5 μg/ml *in vitro*. Commonly, the clinical daily treatment concentration of these Kampo medicines is 7.5 g per adult human in Japan. The concentration of the effective element *Glycyrrhizae radix*, a major component herb of the three Kampo medicines, in plasma remained at ~0.1 μg/ml for at least 36 h after treatment (Tsumura Report). These low concentrations are sufficient to inhibit CMV replication *in vitro*. Although the inhibitory effect on the growth of the host cells might reduce the production of CMV virions, these three Kampo medicines did not inhibit the growth of MRC-5 cells, i.e. no decrease in the number of cells was observed in MRC-5 cells incubated for 5 days in the presence of Kampo medicine compared with those in the absence of Kampo medicine (data not shown). It is of interest that, in

spite of the massive inhibition of viral replication and degree of CPE, the percentage of viable cells in the presence of Kampo medicines was unaltered when compared with untreated control cultures.

Hossain *et al*. (15) reported that the oral administration of TJ-41 increased the splenic and hepatic cellularities in murine CMV-infected mice and induced the accumulation and activation of NK cells. This treatment markedly reduced the viral load in the spleen but not in the liver at the early phase of infection. These herbal medicines may thus play an important role in the treatment of HIV-infected AIDS patients where opportunistic human CMV infections are very common and also tend to be severe. Kampo medicine was found to be safe and has been used clinically in Japan and in many other countries. Kampo medicines may thus be valuable as a co-therapeutic agent against latent CMV infection.

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