Inhibition of ganciclovir-resistant human cytomegalovirus replication by Kampo (Japanese herbal medicine)

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We examined the effect of Kampo on the replication of ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) in the human embryonic fibroblast cell line MRC-5. Treatment of HCMV-infected cells with Sho-seiryu-to (SST; Xiao-Qing-Long-Tang in Chinese) resulted in the inhibition of viral replication without affecting the cell growth. SST treatment decreased the synthesis of viral DNA, but had no virucidal effect on cell-free HCMV. However, the inhibitory effect of SST on HCMV replication was ablated by anti-interferon-β (IFN-β) antibody suggesting that SST inhibits the replication of GCV-resistant HCMV through the induction of IFN-β. These results suggest that SST is a novel compound with potential as an anti-HCMV.

Keywords: cytomegalovirus, ganciclovir, Kampo medicine, interferon-β

Introduction

Human cytomegalovirus (HCMV) is one of the eight herpesviruses known to infect humans. It 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 (Britt et al., 1996) and HCMV remains present as a lifelong infection. However, HCMV is frequently activated in immunocompromised individuals such as AIDS patients or organ transplants, thereby causing severe morbidity and eventual mortality (Ho, 1977; Meyers, 1986; Sissons & Carmichael, 2002; Zaia, 1993). Symptomatic HCMV infection has been treated successfully with ganciclovir (GCV); however, the emergence of GCV-resistant viruses is a current problem in the treatment of immunocompromised patients with apparent HCMV infection. Foscarnet (PFA) and cidofovir (CDV) have been used for treatment of GCV-resistant HCMV. This alternative treatment is not always successful (Freitas et al., 1989). Therefore, new or alternative anti-HCMV agents need to be developed (Buerger et al., 2001; McSharry et al., 2001; Shigeta et al., 1991; Yukawa et al., 1996).

Several Kampo (Japanese herbal medicines) are widely used in Japan and many Asian countries as an effective medication against some human disorders. We have previously examined the anti-HCMV activities of three types of Kampo medicine: SST, Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang in Chinese), and Juzen-taiho-to (Shi-Quan-Da-Bu-Tang in Chinese) (Harada et al., 1995; Hossain et al., 1999; Komatsu Y, 1986; Li et al., 1992; Maruyama H, 1988; Nagai et al., 1996; Nagai & Yamada, 1998; Ohnishi et al., 1990). Of these, SST was the most active against HCMV replication (Murayama et al., 2004). In this study, we have explored the inhibitory effect of SST on the replication of drug-resistant HCMV in vitro and found that SST strongly suppresses the replication of GCV-resistant HCMV through the induction of IFN-β.

Materials and methods

Cells and viruses

The human embryonic lung fibroblast cell line MRC-5 (Jacobs et al., 1970) and human embryonic lung fibroblasts were grown in Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; Z.L. Bocknek Lab., Ontario, Canada), gentamicin (50 mg/ml), and amphotericin B (2.5 mg/ml). All cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO₂.
The laboratory-adapted HCMV strain Towne was used as a standard strain throughout the experiment (Furukawa et al., 1973). Both GCV-resistant and -sensitive HCMV strains were isolated from a patient with HCMV retinitis/encephalitis (Sasaki et al., 1997). They were plaque-purified twice and designated 93-1R and 91-7S, respectively (Harada et al., 1997). These HCMVs were propagated in human embryonic lung fibroblasts. The viral infectivity was determined by a plaque assay (Wentworth & French, 1970).

Reagents
A mouse monoclonal antibody (mAb) specific for IFN-β, MCA B-02 (Kawade, 1980), was purchased from Yamasa Shouyu Co., Ltd. (Chiba, Japan). The medical plants used for the preparation of SST (code No. TJ-19) were kindly provided by Tsumura Co., Ltd., (Tokyo, Japan). A mixture of 8 kinds of medicinal herbs, Pinelliae tuber (6.0 g), Ephedrae herba (3.0 g), Schizandrae fructus (3.0 g), Cinnamonomi cortex (3.0 g), Paeoniae radix (3.0 g), Asari herba cum radice (3.0 g), Glycyrrhizae radix (3.0 g), and Zingiberis siccatum rhizoma (3.0 g), was added to water and extracted at 100°C for 1 hour. The extracted solution was filtered and dried to obtain the powder extract. The quality of these herbs was controlled by the Japanese Pharmacopoeia. The chemical patterns of TJ-19 extract obtained by three-dimensional HPLC analysis has been reported previously (Amagaya et al., 2001).

Figure 1. Effects of SST on HCMV replication

MRC-5 cells were infected with (A) Towne strain, (B) GCV-sensitive clinical isolate (91-7S) or (C) GCV-resistant strain of clinical isolate (93-1R), incubated with the indicated concentrations of Sho-seiryu-to (SST) or ganciclovir (GCV). The virus titres in the culture supernatants were determined on day 3 and 6 (A) or day 5 (B and C) after infection by using a plaque assay. Data represent means ± standard error (SE) for three independent experiments. Statistical significance was compared with the untreated group by the Student’s t-test (*P < 0.05). Cell-free HCMV (Towne strain) was incubated with the indicated concentration of SST (D). Virus titre was determined on day 5 after incubation by a plaque assay. Data represent means ± SE for three independent experiments.
Dot blot hybridization
The conditions used for dot blot hybridization were essentially identical to those described previously (Furukawa et al., 1994; Murayama et al., 1994). The hybridization probe was a 641-bp length PCR product from the UL54 DNA polymerase gene of HCMV, as previously described (Harada et al., 1997). The hybridization signal was detected using an ECL direct nucleic acid labelling and detection system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Bucks, UK), according to the manufacturer’s instructions.

Western blot analysis
The infected cell lysate was subjected to electrophoresis on a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE).

IFN-β assay
The amount of IFN-β was measured using a commercially available enzyme-linked immunoabsorbance assay (ELISA) kit for human IFN-β (PBL Biomedical Laboratories, Piscataway, NJ, USA), according to the manufacturer’s instructions. The detection limit of this assay was 100 pg/ml.

Statistical analysis
Data were analysed by Student’s t-test.

Results
Effect of SST on HCMV replication
We recently demonstrated that SST was the most effective herb against HCMV production among the selection of Kampo medicines tested (Murayama et al., 2004). To confirm that SST inhibits HCMV replication, a monolayer culture of MRC-5 cells was inoculated with HCMV at a multiplicity of infection of 0.1. After adsorption for 1 h, the cells were added with 1 ml of culture medium (DMEM medium containing 5% fetal calf serum) in the absence or presence of various concentrations of SST at the indicated intervals. In the MRC-5 cells infected with Towne strain, the virus titre increased until 6 days after infection in the absence of SST. However, in the presence of SST, virus production was significantly inhibited in a dose-dependent manner (Figure 1A). This anti-HCMV activity of SST was compared with that of GCV against GCV-sensitive (91-7S) and GCV-resistant (93-1R) strains. After adsorption for 1 h, the cells were incubated in the presence of various concentrations of either SST or GCV and cultured for 5 days. SST and GCV inhibited the replication of the GCV-sensitive strain at concentrations ranging from 0.0001 to 0.01 mg/ml and 0.1 to 1 µM, respectively (Figure 1B). Although GCV could not inhibit the GCV-resistant strain, SST equally inhibited GCV-resistant and -sensitive strains (Figure 1C), suggesting that SST inhibits HCMV replication through a mechanism different from that of GCV. In the next experiment, the virucidal effect of SST was examined. Cell-free HCMV (Towne strain) was incubated with DMEM medium containing 5% FCS and various concentrations of SST for 5 days. However, there was no virucidal effect even at the highest concentration of SST (Figure 1D). We also observed the decreased cytopathicity in the cells infected with the 93-1R strain, when incubated with SST (data not shown).

The effect of SST on viral DNA synthesis
To evaluate the effect of SST on HCMV DNA synthesis, the MRC-5 cells infected with 93-1R strain were cultured in the presence or absence of SST (0.1 or 0.001 µg/ml). On day 5 after infection, DNA was extracted from the infected cells by Hirt’s method (Hirt, 1967), and viral DNA synthesis was determined by dot blot hybridization. Inhibition of HCMV DNA synthesis by SST was approximately threefold higher in the infected cells compared with untreated cells (Figure 2).

Induction of IFN-β by SST
As viral DNA synthesis was inhibited in SST-treated cells, we examined whether SST induced IFN-β production. Mock-infected MRC-5 cells were cultured in the absence or presence of SST (0.0001 to 1 mg/ml), and IFN-β in the culture supernatants was measured by ELISA. MRC-5 cells secreted IFN-β protein within 1 h after SST-treatment, and the level of IFN-β peaked at 3 h. SST-treatment induced IFN-β production from MRC-5 cells in a dose-dependent manner.
manner (Figure 3A). The induction of IFN-β was not observed in HCMV-infected cell (data not shown).

We examined the effect of a neutralizing anti-IFN-β mAb on the inhibitory effect of SST on HCMV replication. When the diluted (1/30) mAb was added to the SST-treated cell culture, the virus titre in the culture supernatant recovered and reached 83% of the control level (Figure 3B, columns 1 and 3). Such recovery was not observed for the culture supernatant of the cells treated with an isotype-matched control mAb (Figure 3B, column 5). Furthermore, the anti-IFN-β mAb itself did not affect HCMV replication (Figure 3B, column 6). These results indicate that the mechanism of HCMV inhibition by SST can be in part attributed to the induction of IFN-β.

Discussion

We demonstrated the effect of Kampo medicines on the replication of GCV-resistant HCMV in MRC-5 cells. HCMV infection has been treated with GCV, PFA, CDV, immunoglobulin, etc., but the treatment is not always successful in immunocompromised hosts. SST was first examined for its inhibitory effect on drug-resistant HCMV in MRC-5 cells, and the effect was observed even at a concentration of 0.0001 mg/ml. Its anti-HCMV activity was further demonstrated by plaque formation and gene expression assays (Murayama et al., 2004). Yukawa et al. (1996) reported that hot water extracts of some traditional herbs had anti-HCMV activity at 1-5 µg/ml in vitro and in vivo. Many Kampo medicines have been used for the treatment of chronic diseases in Japan and many Asian countries. The information of their efficacy and adverse effects for long-term use has been accumulated for practical use. In fact, the clinical dose of Kampo medicines for daily treatment is generally 7.5 g per adult in Japan. The concentration of Glycyrrhizae radix, a major and effective component herb of this Kampo medicine, in plasma reaches approximately 0.1 µg/ml at least 36 h after treatment (Tsumura Report). Such concentrations are sufficient to inhibit HCMV replication in vitro. Although it is possible that decreased growth of the host cells reduces the production of HCMV virions, SST did not inhibit the growth of MRC-5 cells at effective concentrations (Murayama et al., 2004). In spite of the profound inhibition of viral replication, the percentage of viable cells treated with SST was unaltered compared with that of the untreated control.

We found that SST induced IFN-β at 1-3 h after treatment of human embryonic lung fibroblast cells (Figure 3A), and that the effect of SST on HCMV replication was partially annihilated by an anti-IFN-β antibody in vitro (Figure 3B), suggesting that the induction of IFN-β is a mechanism of SST against HCMV replication. Orange and Biron (1996) claimed that IFN-α/β is necessary for natural killer (NK) cell blastogenesis and cytotoxicity, but not for IFN-β production. The IFN-β induced by SST-treatment may also stimulate NK cell activity against HCMV-infected cells in vivo. It was reported that the
in vivo neutralization of IFN-α/β resulted in higher levels of murine cytomegalovirus replication and inhibition of murine-cytomegalovirus-induced activation of NK cell cytotoxicities (Grundy et al., 1982). The activated NK cells by Kampo medicine markedly reduced the viral load in the spleen, but not in the liver at the early phase of infection (Hossain et al., 1999).

It is assumed that herbal medicines are useful in the treatment of HIV-1-infected patients, in which opportunistic HCMV infections are very common and also tend to be severe. In fact, traditional Kampo medicines have been used for the improvement of many chronic diseases in the Asian countries (Yukawa et al., 1996). Moreover, they were found to be safe. Therefore, Kampo medicines might be applicable as co-therapeutic and/or prophylactic agents against HCMV diseases in immunocompromised hosts.

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