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Modulation of cytokine production by 7-hydroxycoumarin in vitro and its efficacy against influenza infection in mice

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ABSTRACT

We previously demonstrated that 7-hydroxycoumarin (7HC) was effective in reducing proinflammatory cytokine production in lipopolysaccharide-exposed macrophage-like P388D1 cells and fever production by suppressing the increase in interleukin (IL)-1α production in an influenza virus-intranasal infection model in mice. In this study, we assessed the effects of modulation of cytokine production by 7HC on influenza virus infection in relation to its efficacy in influenza virus-infected mice. 7HC was confirmed to suppress proinflammatory cytokine levels in P388D1 cells due to influenza virus infection. In the murine infection model, oral administration of 7HC (30 mg/kg) was significantly effective in reducing the weight loss of infected mice and virus titers in the bronchoalveolar lavage fluid (BALF) of lungs and in prolonging survival times without toxicity. The rise of proinflammatory and Th1 cytokine (IL-12 and interferon-γ) production in the BALF from infected mice was significantly suppressed by 7HC at two and four days post-infection, respectively. This suppression correlated with the reduction of virus titers and diminution of lung consolidation. Because 7HC did not exhibit direct anti-influenza virus activity in vitro, 7HC was suggested to suppress pneumonia in influenza virus-infected mice through suppression of the cytokine production induced by infection.

1. Introduction

Cytokines are produced locally and systemically in animals during influenza infection (Conn et al., 1995; Hennet et al., 1992; Monteiro et al., 1998; Peper and Van Campen, 1995; Swiergiel and Dunn, 1999; Van Reeth, 2000). Recently, proinflammatory cytokines have been reported to be markedly elevated in human cells and mice during highly pathogenic H5N1 influenza virus infection (Cheung et al., 2002; Kash et al., 2006; Szretter et al., 2007; Xu et al., 2006). The occurrence of the 'cytokine storm' has been proposed to contribute to the increased severity of the diseases caused by the highly pathogenic virus (de Jong et al., 2006; Peiris et al., 2004; Yuen et al., 1998). Intraperitoneal administration of tumor necrosis factor (TNF)-α antibodies to influenza virus-infected mice was reported to reduce lung lesion severity and prolong survival (Peper and Van Campen, 1995). Antagonism of TNF-α, interleukin (IL)-1α, and IL-6 partly prevented the decrease in feeding and loss of body weight of mice induced by influenza virus infection (Swiergiel and Dunn, 1999). Aldridge et al. (2009) demonstrated that the recruitment of TNF and inducible NOS-producing dendritic cells correlated with the severity of inflammatory responses induced by influenza virus. Proinflammatory cytokines were suggested to play an important role in influenza virus-induced pathogenesis. IL-12 was shown to contribute to the development and activation of innate immune response and the inhibition of virus replication in the early phase of influenza infection in mice (Monteiro et al., 1998). The importance of the augmentation of IL-12 and subsequently induced interferon (IFN)-γ levels in the early phase of influenza infection in alleviation of the infection has been verified (Kurokawa et al., 2002; Tsurita et al., 2001). In addition to the direct cytopathic effect, the involvement of host immune responses, including cytokine production, has been suggested to play an important role in the pathogenesis of influenza virus infection.

Traditional medicines have been used in the treatment of influenza infection in Asia. If they have beneficial effects, it is important to understand their mechanism of action. We have documented the efficacies of traditional medicines in animal models and clarified the mechanisms of action in herpes simplex virus infection and influenza virus infection (Kurokawa et al., 1996a,b; Nagasaka et al., 1995). Further, the active compounds in traditional medicines have been identified and the efficacies of herbal medicines reproduced in animal infection models (Kurokawa et al., 1998a,b, 1999, 2001). In a series of studies, we found that some cinnamyl derivatives and related compounds show cytokine-

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modulatory activity in lipopolysaccharide (LPS)-exposed murine macrophage-like P388D1 cells (Kurokawa et al., 2003). Among the compounds, 7-hydroxycoumarin (7HC), a coumarin derivative, was found to be effective in reducing fever production by suppressing the rise of IL-1α production in a murine influenza virus-infection model (Kurokawa et al., 1998b), suggesting that 7HC was capable of modulating the cytokine production induced by influenza infection in mice.

In this study, to evaluate the cytokine-modulatory activity of 7HC in influenza virus infection, we examined the effects of 7HC on proinflammatory cytokine production in influenza virus-infected P388D1 cells, and assessed the protective effects of 7HC as a potential antiviral and/or immunomodulatory compound in a murine model of influenza virus infection. The cytokine-modulatory activity of 7HC is discussed in relation to its contribution to the alleviation of influenza infection.

2. Materials and methods

2.1. Cells and viruses

P388D1 cells, originating from DBA/2 mice, were purchased from American Type Culture Collection (Manassas, VA). The cells have been characterized both morphologically and functionally as being a macrophage-like cell line (Koren et al., 1975; Snyderman et al., 1977) and reported to express CD11b/CD18, CD14, and Fc receptor (Barbour et al., 1998; Ochiai et al., 1988). We used the cells to examine the cytokine-modulatory activity of 7HC in vitro. The cells were grown and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2% or 5% heat-inactivated fetal bovine serum. Madin-Darby canine kidney (MDCK) cells were provided by Dr. H. Ochiai, Toyama University, Japan (Kurokawa et al., 1990). The cells were grown and maintained in Eagle's minimum essential medium supplemented with 2% or 5% heat-inactivated calf serum. Influenza A/PR/8/34(H1N1) virus was provided by Dr. H. Ochiai, Toyama University, Japan (Kurokawa et al., 1990). The mouse-adapted influenza virus was propagated in the lungs of mice by intranasal infection (Kurokawa et al., 1996a). The lungs of infected mice were removed and homogenized in phosphate-buffered saline. The homogenate was centrifuged at 3000 rpm for 15 min and then the supernatant was stored at −80 °C.

2.2. Compounds

7HC (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) at 100 mg/ml and used for the in vitro-assay as described below. For administration to mice, 7HC was suspended in 5% gum arabic and administered orally to mice (Kurokawa et al., 2003). As a control, 5% gum arabic alone was used.

2.3. Assay of cytokines secreted from P388D1 cells

The effect of 7HC on the secretion of IL-1α from P388D1 cells was examined. P388D1 cells were seeded at 1×10^6 cells/well in 24-well plates and grown at 37 °C overnight. As influenza virus has been shown to replicate in P388D1 cells (Ochiai et al., 1988), the cells were infected with influenza virus or the heat-inactivated virus at two plaque-forming units (PFUs)/cell for 1 h at room temperature, rinsed twice with RPMI-1640 medium, and then incubated in fresh maintenance medium (1 ml) containing 7HC at 0, 10, 30, or 100 μg/ml. As a negative control, fresh maintenance medium containing 0.1% of DMSO was used. At various times after infection, the culture medium was centrifuged, and the supernatants were stored at −30 °C until assayed by an enzyme-linked immunosorbent assay (ELISA) for IL-1α. Its concentrations in triplicate wells of supernatants were determined by ELISA kits using 96-well plates (Amersham Pharmacia Biotech, Buckinghamshire, England or BioSource, San Jose, CA) according to the manufacturer's instructions. The 50% inhibitory concentrations (IC_{50}) of 7HC for IL-1α secretion were estimated from a curve relating the concentration of IL-1α to the concentrations of 7HC.

For LPS exposure, P388D1 cells were seeded as described above. The culture medium was replaced by fresh maintenance medium (1 ml) containing 30 μg/ml of LPS (W *E. coli* 0127:B8, Difco, Detroit, MI) as described previously (Kurokawa et al., 2003). At various times after infection, the culture medium was collected for ELISA as described above.

2.4. Analysis of cytokine production in P388D1 cells

In order to examine the modulation of proinflammatory cytokine production in P388D1 cells by 7HC, the cells were seeded and infected with influenza virus as described above. The cells were lysed in 100 μl of a sample buffer at 15 h after infection. The lysates were applied to gel electrophoresis and blotted onto a filter (Kurokawa et al., 2003). The immunoblots were probed with rabbit anti-mouse IL-1α antibody (Genzyme, Waltham, MA), rabbit anti-mouse IL-6 antibody (Pepro Tech, Rocky Hill, NJ), rabbit anti-mouse TNF-α antibody (Endogen, Woburn, MA), or rabbit anti-mouse β-actin antibody (Sigma), and developed with the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech). Bands on the exposed films were scanned and quantitatively analyzed by NIH Image. The IC_{50} values of 7HC for cytokine production were estimated from a curve relating the amount of each cytokine to the concentrations of 7HC.

2.5. Plaque reduction assay and cytotoxicity assay

The anti-influenza virus activity of 7HC was examined by a plaque reduction assay to estimate the possible anti-influenza activity in vivo. Duplicate cultures of MDCK cells in 60 mm plastic dishes were infected with 100 PFU/0.2 ml of the PR8 strain for 1 h at room temperature. Cells were overlaid with 5 ml of nutrient agarose (0.8%) medium containing various concentrations of 7HC, and then cultured at 37 °C for 2–3 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a dissecting microscope (Kurokawa et al., 1990). The concentrations that reduced the number of plaques by 50% (EC_{50}) were determined from a curve relating the plaque number to the concentrations of 7HC.

The cytotoxicity of 7HC was examined by the growth inhibition of MDCK cells as described previously (Tsurita et al., 2001). Briefly, MDCK cells seeded at a concentration of 2.5×10^4 cells/well in 24-well plates were grown at 37 °C for 2 days. The culture medium was replaced by fresh medium containing 7HC at various concentrations. The cells were further grown for 2 days and the number of viable cells was determined by a trypan blue exclusion test (Shimizu et al., 2008). The concentration of 7HC reducing cell viability by 50% (CC_{50}) was determined from a curve relating percent cell viability to the concentrations of 7HC.

2.6. Mice

Female DBA/2 CrSlc mice (6-week-old, 17–19 g, Sankyo Labo Service, Japan) were housed five per cage in a temperature-controlled room with food (CE-2, Clea Japan, Inc., Tokyo, Japan) and pyrogen-free water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) ad libitum and under a 12 h light/12 h dark diurnal cycle (light on at 7.00 a.m.). The temperature in the room was kept at 23 ± 2 °C. The mice were acclimated for at least 5 days before starting the experimental procedures. The animal experimentation guidelines

of the University of Toyama and Kyushu University of Health and Welfare were followed in the animal studies.

2.7. Murine influenza virus infection model

7HC was examined for its efficacy on influenza infection in mice. Mice were infected intranasally or mock-infected with 700 PFU/20 μl of influenza virus as described previously (Kurokawa et al., 2002; Shimizu et al., 2008). 7HC (30 mg/kg/0.25 ml) or water (0.25 ml) was applied orally by gavage to the mice three times daily (approximately 8 h intervals) for 6 days starting one day before infection. We used the dose of 30 mg/kg as the maximum dosage to evaluate the immunomodulatory activity because a dosage of 14.1 mg/kg of 7HC was not toxic for mice, effectively inhibited IL-1α production, and reduced fever in infected mice in our previous studies (Kurokawa et al., 1998b). The body weights of 10 mice in each group were measured every morning after infection. The changes of body weight were calculated based on the body weight of each mouse on day 0. To determine mortality, the infected mice were fed and observed for at least half a month after infection.

The development of consolidation of the lungs was observed and scored according to the method of Ginsberg and Horsfall (1952) with modifications. The following scores were used: 0, no consolidation in a lobe; 1, consolidation of less than 50% of the surface area of a lobe; and 2, consolidation of more than 50% of the surface area of a lobe. The scores of each lobe were added, and the total was defined as the consolidation score of 5 lobes.

In order to assess the effect of 7HC administration on the coagulation of blood of mice, 7HC at 30 mg/kg or water was administered to the infected and mock-infected mice as described above. On day 4 after infection, blood was collected from 5 mice in each group and immediately thereafter the blood of 450 μl was mixed with 3.2% sodium citrate of 50 μl. The mixtures were centrifuged for 15 min at 1500 × *g* and the plasma was prepared and kept on ice. The coagulation times of plasma were measured using coagulation kits (Coagpia APTT-S, Sekisui Medical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

2.8. Determination of cytokine levels and virus titer in bronchoalveolar lavage fluid

Cytokine levels in the bronchoalveolar lavage fluid (BALF) of lungs were examined by ELISA. BALF was prepared on days 1, 2, 3, and 4 after infection as described previously (Shimizu et al., 2008; Tsurita et al., 2001). Cytokine levels in the BALF were determined using ELISA kits for mouse TNF-α, IL-6, IL-12, and IFN-γ (Amersham International or BioSource International) according to the manufacturer's instructions.

Virus titers in the BALF of influenza virus-infected mice were examined. Virus titers of the stored supernatant of BALF were determined by the plaque assay using MDCK cells as described previously (Kurokawa et al., 1990; Shimizu et al., 2008).

2.9. Statistical analyses

Student's *t*-test was used to evaluate the statistical significance of differences between two groups in the consolidation scores, mean days of death, coagulation times, virus titers, and concentrations of cytokines (IL-12 and IFN-γ). The one-way analysis of variance (one-way ANOVA) followed by Dunnett's test was used to evaluate the statistical significance of differences in the concentrations of cytokines (TNF-α and IL-6). The repeated measures ANOVA with Dunn's procedure as a multiple comparison procedure was used to analyze the interaction between 7HC and the control in the changes of net body weights of infected mice. Statistical differences in the mortality of mice were evaluated using the Kaplan–Meier method with a log-rank test. *P*-values of less than 0.05 were defined as statistically significant.

3. Results

3.1. Effects of 7HC on cytokine secretion in vitro

7HC was examined for its cytokine-modulatory effects on the secretion of IL-1α, TNF-α, and IL-6 as proinflammatory cytokines by macrophage-like P388D1 cells infected with influenza virus. In our preliminary experiments, IL-1β was not abundantly produced in influenza virus-infected P388D1 cells as compared to LPS-exposed cells and the level of IL-1β produced by live influenza virus was similar to that of IL-1α produced by the heat-inactivated virus (data not shown). Therefore, we focused on the secretion of IL-1α as a proinflammatory cytokine produced by live influenza virus. As shown in Fig. 1A, IL-1α production was induced in P388D1 cells by influenza virus infection as well as by LPS exposure but not by the heat-inactivated virus. In the presence of 7HC after infection (Fig. 1B), IL-1α secretion was suppressed in a concentration-dependent manner (IC_{50}, 66.2 μg/ml). When the

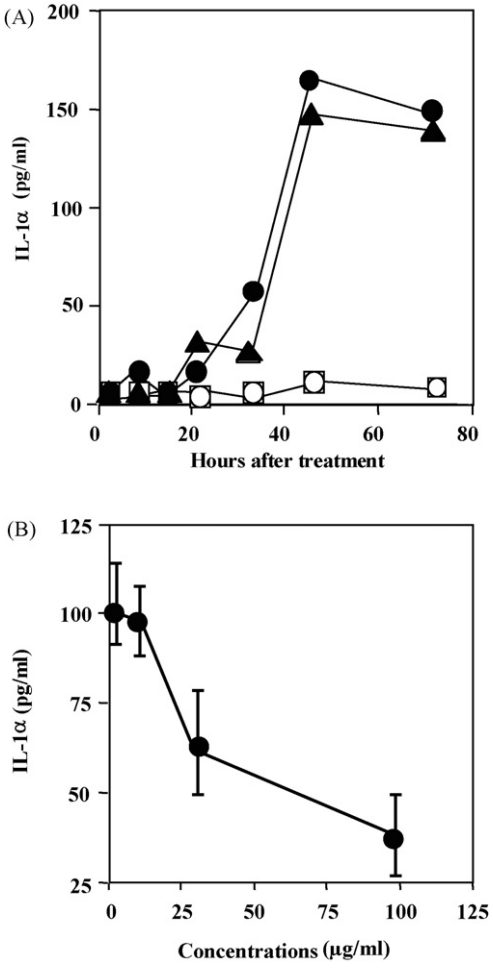

Fig. 1. IL-1α secretion from P388D1 cells and its suppression by 7HC. (A) P388D1 cells were mock-infected (□), infected with influenza virus (●), or heat-inactivated virus (○), and also exposed to LPS at 30 μg/ml (▲) as described in text. At various times after infection or exposure, IL-1α concentrations in the culture supernatants were determined by ELISA. Each value represents the mean of triplicate wells. (B) The infected P388D1 cells were incubated in the presence of various concentrations of 7HC for 48 h after infection, and then IL-1α concentrations in the culture supernatants were determined by ELISA. Each value represents the mean of three independent experiments. Bars indicate statistical errors.

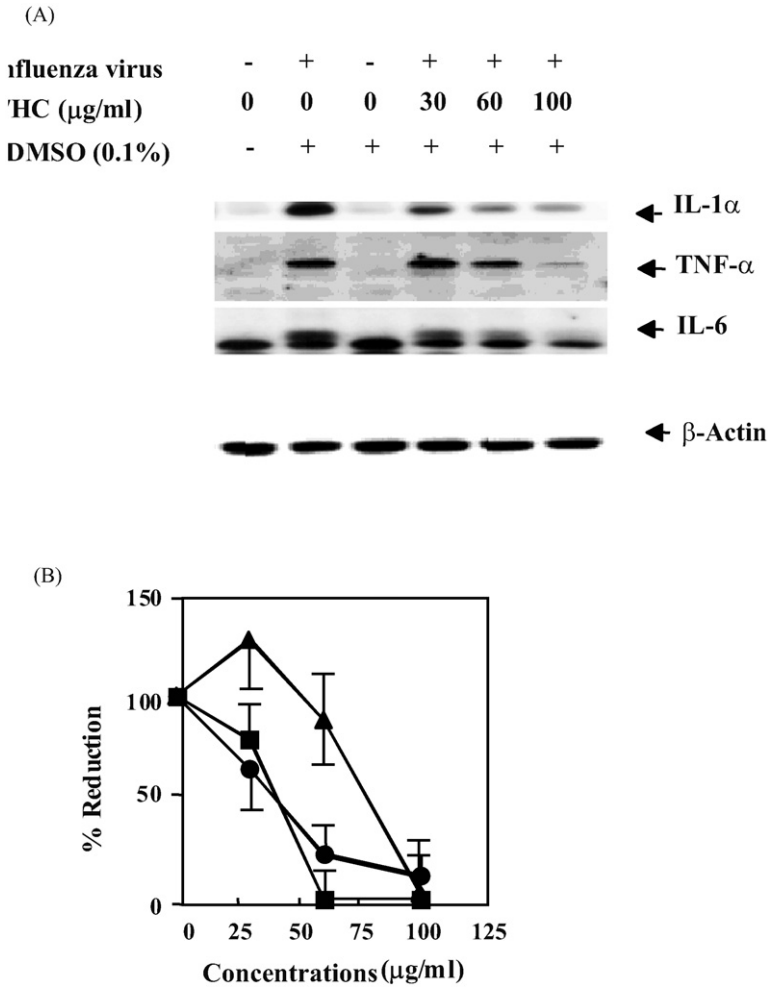

Fig. 2. Cytokine production in influenza virus-infected P388D1 cells treated with 7HC. (A) The infected P388D1 cells were incubated in the presence of 7HC (0, 30, 60, or 100 μg/ml) for 8 h after infection. IL-1α, IL-6, TNF-α, and β-actin in the cells were detected by Western blotting, followed by chemiluminescence detection with anti-mouse IL-1α IL-6,TNF-α, and β-actin antibodies using the ECL system. (B) Proinflammatory cytokine bands IL-1α (●), IL-6 (▲), and TNF-α (■) detected on the exposed films by the ECL system were scanned and quantified by NIH Image. Each value represents the mean of three independent experiments, which were normalized to the amounts of β-actin at the same concentration. Bars indicate statistical errors.

suppressive activity of 7HC on proinflammatory cytokine production in P388D1 cells was examined (Fig. 2A), 7HC suppressed the production of all proinflammatory cytokines examined, IL-1α, IL-6, and TNF-α, in P388D1 cells in a concentration-dependent manner and the IC_{50} values of 7HC for these cytokines were 40.8, 77.9, and 39.6 μg/ml, respectively (Fig. 2B). However, 7HC did not affect the amount of β-actin at the concentrations used. DMSO at 0.1%, which was used to dissolve 7HC in the culture medium, did not affect the production of the proinflammatory cytokines in comparison with the influenza virus-uninfected control. Thus, we confirmed the suppressive activity of 7HC on proinflammatory cytokine production induced by influenza virus infection in P388D1 cells.

3.2. *Efficacy of 7HC on influenza infection in mice*

The efficacy of the cytokine-modulatory activity of 7HC in vivo was examined in an intranasal influenza virus infection model in mice to assess its potential activity as an experimental immunomodulator. In our murine influenza virus infection model, oral administration of oseltamivir at 1 mg/kg twice daily for days 0–4 after infection was significantly effective in reducing the body weight loss of and virus titers in the BALF from infected mice but oseltamivir administration at 0.1 mg/kg was not (Shimizu et al., 2008). The net increases of body weights of mock-infected mice treated with and without 7HC at 30 mg/kg three times daily for 6 days were 0.28 ± 0.33 g and 0.62 ± 0.28 g, respectively, on day 6 after infection and there was no significant difference between the treated and untreated groups. When mice were infected with influenza virus, the body weight was significantly decreased at ≥4 days after infection (Fig. 3A) as reported previously (Kurokawa et al., 2002; Shimizu et al., 2008), and the consolidation of the lungs was obviously observed on days 3 and 4 post-infection (Fig. 3B). However, the mean body weights of mice receiving 7HC at 30 mg/kg were significantly higher compared to water-administered mice (Fig. 3A, $P < 0.05$). The reductions in the mean scores of consolidation in 7HC-administered mice were 56.0% and 40.7% at 3 and 4 days, respectively, after infection, as compared with mice not administered 7HC. The reduction due to 7HC administration was statistically significant on day 4 after infection ($P < 0.05$), indicating that the development of consolidation was also retarded by 7HC administration (Fig. 3B). As shown in Fig. 3C, 7HC prolonged the survival times of infected mice and reduced mortality ($P < 0.05$). The mean day of death for 7HC-treated mice (11.6 ± 0.7 days) was significantly longer than that for mice given water (9.1 ± 0.8 days) at day 13 after infection ($P < 0.05$) and three and one mice in the 7HC- and water-administered groups, respectively, were alive at day 20 after infection. The reduction of body-weight loss, diminution of

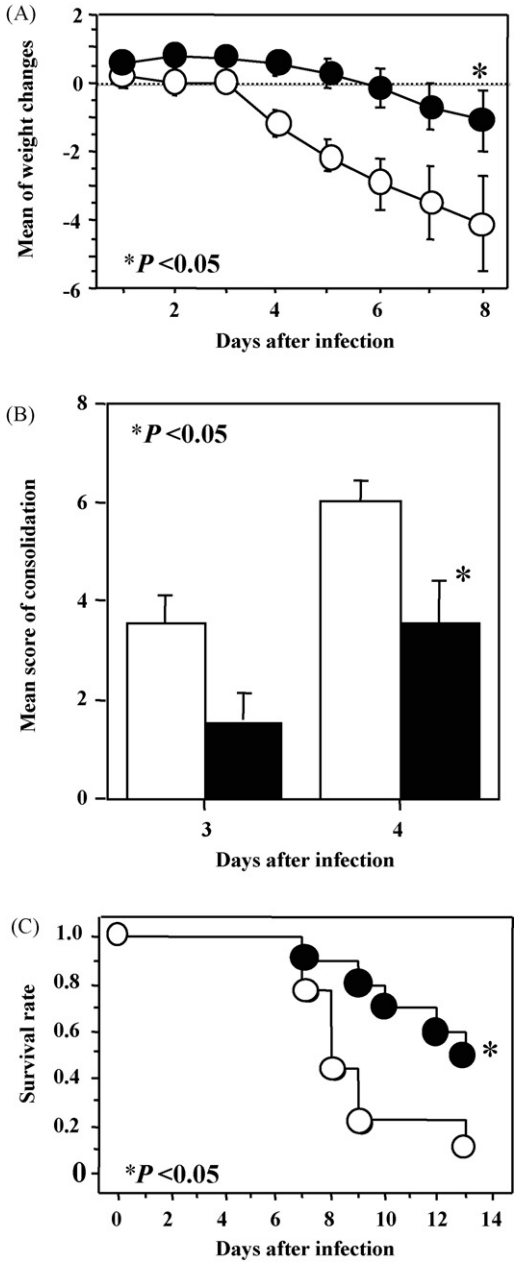

Fig. 3. Effects of 7HC on influenza infection in mice. (A) Changes in the mean body weights of influenza virus-infected mice. 7HC (●) and water (○) were administered to the infected mice (10 mice per group), and the body weights were measured daily. Bars indicate standard errors. The asterisk indicates the weight change in 7HC-administered group for 1–8 days and the statistical significance versus infected mice administered water for days 1–8 after infection by repeated measures ANOVA. (B) Effect of 7HC on the development of consolidation in the lungs of influenza virus-infected mice. Mice were intranasally infected with influenza virus. 7HC (closed columns) and water (open columns) were administered orally, and consolidation of the lungs was observed on days 3 and 4 after infection, as described in the text. Five mice were used in each group. Bars indicate standard errors. The asterisk indicates statistical significance versus the water-administered group by Student's *t*-test. (C) Effect of 7HC on the survival times of influenza virus-infected mice. Mice were infected intranasally with influenza virus, and 7HC (●) or water (○) was administered orally as described in the text. The asterisk indicates the 7HC-administered group for 0–11 days after infection and a significant difference from the water-administered group by the Kaplan–Meier method for 11 days after infection.

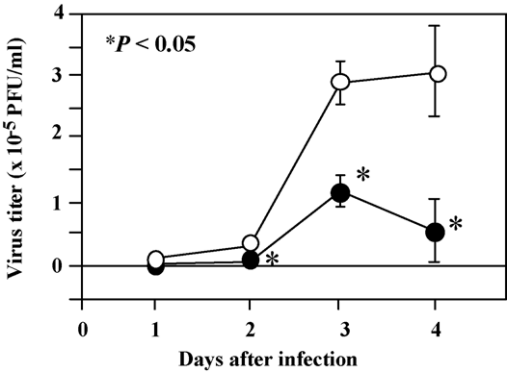

Fig. 4. Effect of 7HC on virus titers in BALF of infected mice. Mice were intranasally infected with influenza virus, and 7HC (●) or water (○) was administered orally as described in text. The virus titers in the BALF of 4 mice in each group were determined on days 1–4 after infection. Bars indicate standard errors. Asterisks indicate the virus titers in 7HC-administered mice and statistical significance versus water-administered mice on each day by Student's *t*-test.

consolidation, and reduction of mortality by 7HC administration at 30 mg/kg were confirmed by a repeated experiment.

It is well known that coumarin derivatives such as warfarin have anti-coagulation activity (Ansell and Bergqvist, 2004). However, in this study, there were no significant differences between the coagulation times of blood prepared from 7HC- and water-administered groups of infected mice (28.8 ± 1.6 s and 22.2 ± 2.4 s, respectively) and mock-infected mice (29.8 ± 0.4 s and 32.0 ± 1.9 s, respectively). Oral administration of 7HC in our influenza virus infection model did not affect the blood coagulation time.

3.3. Effect of 7HC on viral growth

The effect of 7HC on virus titers in BALF was examined in the early phase of influenza infection, as shown in Fig. 4. 7HC significantly reduced virus titers in the BALF of infected mice after infection, especially at 2–4 days post-infection, as compared with untreated mice ($P < 0.05$). The reductions of virus titers were 82.0%, 59.2%, and 82.0% at 2, 3, and 4 days, respectively, after infection. 7HC suppressed virus growth in the respiratory tract and exhibited anti-influenza virus activity in mice.

When the anti-influenza virus activity of 7HC was examined in a plaque reduction assay using MDCK cells, there was no significant difference between the EC_{50} (255.1 ± 10.8 μg/ml) and CC_{50} (252.1 ± 6.2 μg/ml) values. Thus, suppression of the virus titer in BALF by 7HC did not result from a direct anti-influenza virus activity of 7HC in mice.

3.4. Effect of 7HC on cytokine production in BALF of infected mice

To evaluate the potential activity of 7HC as a cytokine modulator in influenza infection, the effects of 7HC on cytokine levels produced by influenza infection were examined in influenza virus-infected mice. We measured the levels of proinflammatory cytokines (TNF-α and IL-6) and Th1 cytokines (IL-12 and IFN-γ) in BALF. On day 2 after infection, the levels of the proinflammatory cytokines TNF-α and IL-6 were significantly higher in infected mice administered water than those in mock-infected mice (Fig. 5A and B, $P < 0.05$). The levels of TNF-α and IL-6 in infected mice administered 7HC were significantly lower (42.8% and 35.3% of control, respectively) than those in infected mice administered water (Fig. 5A and B, $P < 0.05$), but were not significantly different from those in mock-infected mice. On day 4 after infection, the TNF-α level was higher in infected mice than in mock-infected mice, although the differences were not significant, and the IL-6 levels in

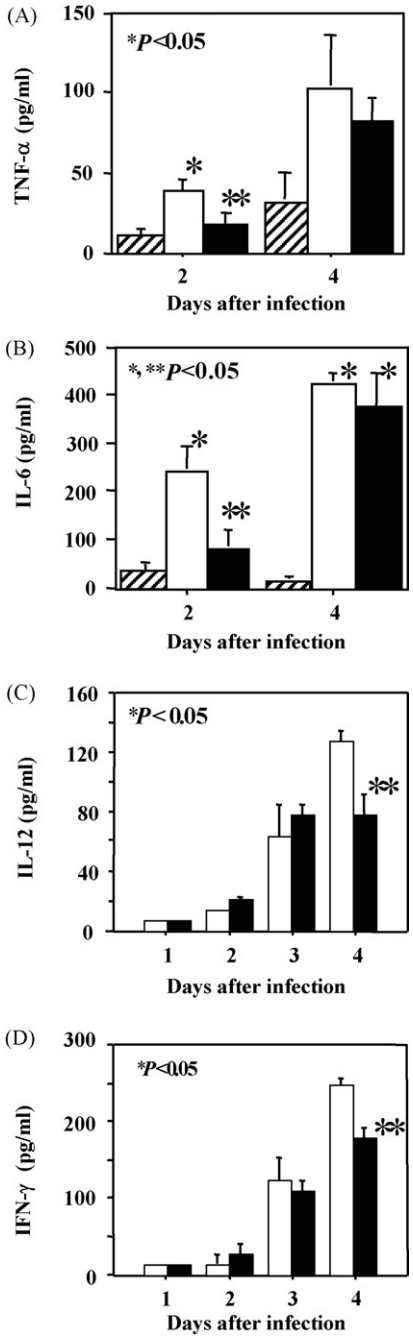

Fig. 5. Changes of TNF-α (A), IL-6 (B), IL-12 (C), and IFN-γ (D) levels in the BALF of influenza virus-infected mice administered with 7HC. Water (open columns) and 7HC (closed columns) were administered to the infected mice and BALF was prepared from the mice ($n = 4$) on days 1, 2, 3, or 4 after infection. For TNF-α and IL-6, mock-infected mice (hatched columns) were administered water, and BALF was prepared from the mice ($n = 4$) on days 2 and 4 after infection. The levels of TNF-α, IL-6, IL-12, and IFN-γ in the BALF were determined by ELISA. Bars indicate standard errors. *Statistical significance versus mock-infected mice with water administration on each day by one-way ANOVA followed by Dunnett's test in (A) and (B). **Statistical significance versus infected mice with water administration on each day by one-way ANOVA followed by Dunnett's test in (A) and (B) and by Student's *t*-test in (C) and (D).

infected mice was significantly higher than that in mock-infected mice. The proinflammatory cytokines were suppressed to their basal levels by 7HC on day 2 after infection. In the cases of Th1 cytokines, the levels of IL-12 and IFN-γ in infected mice increased from day 2 to day 4 (Fig. 5C and D). However, their levels were significantly reduced by 7HC administration only on day 4 after infection. Thus, 7HC was effective in suppressing the production of proinflammatory cytokines (TNF-α and IL-6) on day 2 after infection and Th1 cytokines (IL-12 and IFN-γ) on day after infection in mice infected with influenza virus.

4. Discussion

We previously showed that herbal medicines exhibit cytokine-modulatory activity in influenza virus-infected mice and that the modulatory activity is associated with alleviation of symptoms (Kurokawa et al., 1996b, 1998b, 2002). In this study, we demonstrated that 7HC has cytokine-modulatory activity in P388D1 cells and mice infected with influenza virus and that the modulation of cytokine production by 7HC correlated with the alleviation of influenza infection. Cinnamyl derivatives and related compounds including 7HC are common metabolites in plants and components of many herbal medicines, although their concentrations vary among herbs. Thus, 7HC was suggested to be a possible contributor to the cytokine-modulatory activity of herbal medicines.

IL-1α was secreted from macrophage-like P388D1 cells when they were infected with live influenza viruses but not with the heat-inactivated viruses (Fig. 1A). The time course of the secretion pattern was similar to that due to LPS exposure. These results suggested that IL-1α secretion required viral replication in the cells and that the mechanism of the secretion due to influenza infection was similar to that due to LPS stimulation. 7HC acted on the production of not only IL-1α but also IL-6 and TNF-α as proinflammatory cytokines in a concentration-dependent manner (Fig. 2). Previously, 7HC was shown to suppress the secretion of proinflammatory cytokines from LPS-exposed P388D1 cells (Kurokawa et al., 2003). Thus, the suppressive activity of 7HC on proinflammatory cytokine production induced by influenza virus infection was also confirmed in vitro. The concentrations of 7HC used in the present studies (up to 100 μg/ml) were lower than its 50% cytotoxic concentration on P388D1 cells (234 μg/ml) (Kurokawa et al., 2003) and did not affect the production of β-actin (Fig. 2). It is probable that 7HC selectively suppressed the production of proinflammatory cytokines in P388D1 cells and it is possible that 7HC suppresses the secretion of proinflammatory cytokines from macrophage-like cells infected with influenza virus in vivo.

Oral administration of 7HC at 30 mg/kg did not affect blood coagulation and was not toxic for mice. 7HC was found to show significant efficacy in influenza virus-infected mice (Fig. 3). The efficacy of 7HC was associated with a significant reduction of virus titers in the BALF of infected mice (Fig. 4). However, 7HC showed no direct anti-influenza virus activity in vitro. Thus, the reduction of the virus titer in the BALF of 7HC-treated mice is probably due to the modification of host immune responses against influenza infection rather than the direct anti-influenza virus activity of 7HC. The efficacy of 7HC on influenza infection in mice may result from the protective immune responses activated by 7HC. This may be part of the biological activity of 7HC as an immunomodulator in hosts.

It is known that the symptoms of influenza virus infection in mice are caused by overproduction of cytokines in the host rather than the direct effects of viral replication (Oda et al., 1989). The symptoms of influenza in humans have been revealed to correlate with elevated levels of local and systemic proinflammatory cytokines, including TNF-α and IL-6 (Kaiser et al., 2001). In mice, intranasal infection with influenza virus caused deadly pneumonia with an elevated production of proinflammatory cytokines (Conn et al., 1995; Tsurita et al., 2001) and infiltration of inflammatory cells (Deliyannis et al., 2002; Maeda and Akaike, 1991; Tsurita et al., 2001). In this study, 7HC significantly reduced the rise of proinflammatory cytokine (TNF-α and IL-6) production to the basal levels in the BALF of infected mice on day 2 after infection (but not on day 4) (Fig. 5A and B). The reduction in TNF-α and IL-6 by 7HC was most

prominent at the early time of infection, suggesting that the beneficial effect of 7HC was due to the suppression of inflammation early after infection. In addition, 7HC diminished the consolidation of lungs on days 3 and 4 after infection (Fig. 3B). Thus, the suppression of proinflammatory cytokine production by 7HC was associated with weakening inflammation in the lungs of infected mice and reducing the infiltrated cell population quantitatively and qualitatively, resulting in suppression of the overreaction of host immune responses causing pneumonia and lung consolidation.

Macrophages and monocytes are the major producers of IL-12 and IFN-γ (Fritz et al., 1999). IL-12 is a strong inducer of IFN-γ, IL-12 is important in the development of Th1 cells, which is followed by generation of cell-mediated immunity (Arulanandam et al., 1999; Hsieh et al., 1993; Manetti et al., 1994; Monteiro et al., 1998; Paul and Seder, 1994). IL-12 also induces cytotoxicity of activated T cells and natural killer cells that can induce IFN-γ (Kos and Engleman, 1996; Robertson et al., 1992). In this study, the increase in IL-12 and IFN-γ levels from day 3 (Fig. 5C and D) suggested that macrophages and cytotoxic T lymphocytes had infiltrated the lungs of infected mice. However, it has been reported that the reduction of IFN-γ production contributes to the alleviation of influenza infection by reducing the number of infiltrated cells in the lungs and the levels of NO metabolites (Sato et al., 1998). 7HC actually reduced proinflammatory cytokine production in the BALF of lungs on day 2 after infection (Fig. 5A and B). Therefore, the reduction of IL-12 and IFN-γ levels on day 4 was suggested to result from the reduction of excess inflammation and the decrease in the number of infiltrated cells in the early phase of infection under 7HC treatment rather than the suppression of their production from the infiltrates in the lungs. 7HC was characterized as a compound capable of alleviating influenza infection by reducing the levels of proinflammatory cytokines before the onset of pneumonia. The outcome of an influenza virus infection depends on a delicate balance between protective and harmful immunological factors. 7HC was probably effective in reducing the harmful factors such as the excessive production of proinflammatory cytokines causing overreaction of early host immune responses in influenza infection.

In this study, we demonstrated that 7HC exhibited cytokine-modulatory activity in influenza virus-infected mice, resulting in the alleviation of influenza infection. 7HC might be useful in understanding the modulation and role of cytokine production in the pathogenesis of influenza infection.

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