

WE investigated the effect of ferulic acid (FA) and isoferulic acid (IFA), which are the main active components of the rhizoma of *Cimicifuga heracleifolia* (CH), an anti-inflammatory drug used frequently in Japanese traditional medicine, on the production of macrophage inflammatory protein-2 (MIP-2) in a murine macrophage cell line, RAW264.7, in response to respiratory syncytial virus (RSV) infection. Following the exposure of cells to RSV for 20 h, the MIP-2 level in condition medium was increased to about 20 ng/ml, although this level in mock-infected cells was negligible. In the presence of either FA or IFA, RSV-infected cells reduced MIP-2 production in a dose-dependent manner. These data suggest that FA and IFA might be responsible, at least in part, for the anti-inflammatory drug effect of CH extract through the inhibition of MIP-2 production.

Key words: Respiratory syncytial virus, Murine macrophage inflammatory protein-2, Ferulic and isoferulic acids, RAW264.7 cells

Inhibitory effect of ferulic acid and isoferulic acid on the production of macrophage inflammatory protein-2 in response to respiratory syncytial virus infection in RAW264.7 cells

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Introduction

Respiratory syncytial virus (RSV) is the major cause of acute lower respiratory tract illnesses such as bronchiolitis and pneumonia in infants and young children, and can cause severe, even fatal, infections in the elderly.¹ Several studies have indicated that RSV is released initially from upper respiratory tract infections and then reaches the bronchoalveolar region, where viruses induce inflammation of the airway epithelium accompanied by peribronchiolar infiltrations of neutrophils.^{1,2} It is well known that neutrophil accumulation is an important characteristic of inflammation and modulates various inflammatory reactions.³ Since the initial discovery of interleukin-8 as a novel neutrophil chemotactic cytokine,^{4–6} it has since been named a chemokine and been found in the conditioned medium (CM) of various cells in response to stimulation with lipopolysaccharide (LPS) as well as several virus infections. We previously reported that RSV infection strongly induces the production of macrophage inflammatory protein-2 (MIP-2), a murine counterpart of chemokine family,⁷ in RAW 264.7 cells.^{8,9}

On the other hand, the rhizoma of *Cimicifuga* sp., such as *Cimicifuga heracleifolia* (CH) Komarov and *Cimicifuga dahurica* Maxim are used frequently as antipyretic, analgesic and anti-inflammatory drugs in Japanese traditional medicine. Ferulic acid (FA),

3-(4-hydroxy-3-methoxyphenyl)-2-propenic acid and its isomer isoferulic acid (IFA), 3-(3-hydroxy-4-methoxyphenyl)-2-propenic, have been recently recognized as the main active components of CH extract in the inflammation model in rats.¹⁰

In the light of these findings, we examined whether FA and IFA show inhibitory effects on MIP-2 production in response to RSV infection.

Materials and methods

Preparation of drugs

FA and IFA, purchased from Carl Roth GmbH (Karlsruhe, Germany), were freshly prepared in serum-free Dulbecco's modified Eagle's minimal essential medium (DMEM) at a concentration of 5 mM. The dissolved drugs were sterilized through a Millipore filter before use.

Cell

A murine macrophage cell line, RAW264.7, was obtained from American Type Culture Collection and maintained in culture with DMEM supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere containing 5% CO₂ at 37°C. Hep-2 cells were cultured in MEM supplemented with 10% of FBS in the same manner as above.

Virus

RSV A2 strain, which was kindly supplied by Dr Watanabe (Rational Drug Design Laboratories, Fukushima, Japan), was propagated in a confluent monolayer of Hep-2 cells in a maintenance medium (MEM supplemented with 2% FBS).¹ When the cytopathic effect reached 80%, the cultures were processed with three cycles of freezing and thawing. After centrifugation at 2000×g for 10 min, the culture supernatant was collected and stored in a small portion at -84°C as a virus stock solution. Virus titers were determined as plaque-forming units (PFU) on Hep-2 cells as described previously.¹

RSV infection

RAW 264.7 cells were inoculated into a 96-well microplate at a density of 1×10^5 cells/well and cultured overnight at 37°C. Thereafter, the cells were washed once with serum-free media and exposed for 3 h at 37°C to 20 µl of virus solution at a multiplicity of infection (MOI) of 3 PFU/cell. After virus adsorption, 200 µl of serum-free medium with or without drugs were added (0 h post-infection) and further cultured for additional time periods as indicated.

MIP-2 assay

MIP-2 levels in the CM were examined by antibody-sandwich enzyme-linked immunosorbent assay (ELISA) in which rabbit unlabeled and biotinylated anti-murine MIP-2 antibodies were used as the capture and second-layer antibodies, respectively, as described previously.¹¹ Purified MIP-2 was used for standardization of MIP-2 levels, and MIP-2 levels in experimental groups were expressed as percent of control culture, in which infected cells were cultured in the absence of drugs. For statistical analysis, three wells were used for each experimental point.

Statistical analysis

The results were expressed as mean±standard deviation (SD). The differences were examined by Bonferroni's least-significant difference test.

Results and discussion

In the control culture, MIP-2 levels in the CM increased to the level of 20.4 ± 0.8 ng/ml until 20 h after infection in a time-dependent manner. In the uninfected cells, no significant production (less than 0.5 ng/ml) was observed throughout the experiments (data not shown). When the infected cells were cultured in the presence of FA and IFA, these

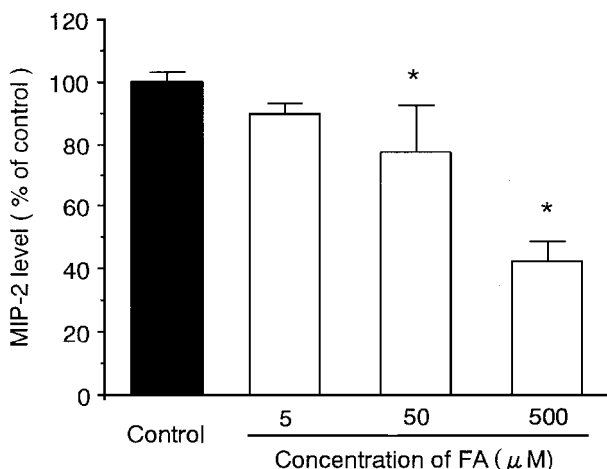


FIG. 1. Inhibitory effect of FA on MIP-2 production in RAW264.7 cells in response to RSV infection. RAW264.7 cells were infected with RSV at an MOI of 3 PFU/cell. After absorption for 1 h, the cells were cultured in the presence of FA at the indicated concentrations (open bar). The MIP-2 levels in 20-h conditioned media were determined and expressed as MIP-2 level by percent of control cultured in the absence of drugs (closed bar). Asterisks indicate a significant difference from control with a *P* value of <0.05. Data are shown as the mean (thick bar)–SD (thin bar) of three determinations.

drugs showed inhibitory effects on MIP-2 production in a dose-dependent manner. At 20 h, FA reduced MIP-2 levels to 74.4 and 42.8% of control at doses of 50 and 500 µM, respectively (Fig. 1). In addition, IFA also reduced MIP-2 levels to 61.8, 58.0 and 35.6% of control at doses of 5, 50 and 500 µM, respectively (Fig. 2). These values were significantly

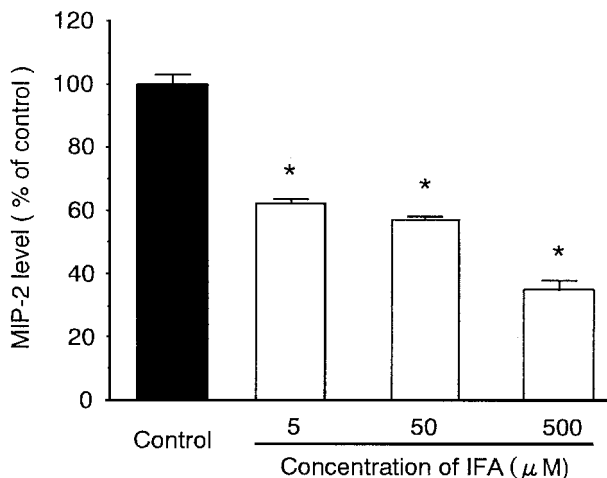


FIG. 2. Inhibitory effect of IFA on MIP-2 production in RAW264.7 cells in response to RSV infection. RAW264.7 cells were infected with RSV by the same manner as described in the legend for Fig. 1. After absorption for 1 h, the cells were cultured in the presence of IFA at the indicated concentrations (open bar). The MIP-2 levels in 20-h conditioned media were determined and expressed as MIP-2 level by percent of control cultured in the absence of drugs (closed bar). Asterisks indicate a significant difference from control with a *P* value of <0.05. Data are shown as the mean (thick bar)–SD (thin bar) of three determinations.

lower than that of the control. At the highest dose (500 μ M), both FA and IFA reduced MIP-2 levels to 42.8 and 35.6% of the control, respectively. In contrast to FA, IFA exhibited a significant inhibitory effect even at the lowest dose (5 μ M). The cytotoxic effect of FA or IFA treatment at 500 μ M for 24 h could be ruled out by Trypan blue exclusion test, that is, FA- or IFA-treated cells were viable at more than 95% which was comparable to drug-untreated cells.

In Japanese oriental medicines, the rhizoma of CH are used frequently as antipyretic, analgesic and anti-inflammatory drugs, and FA and IFA have been recently recognized as the main active components of CH extract in the inflammation model in rats.¹⁰ However, the mechanisms of anti-inflammatory effect of FA and IFA are not understood completely. In addition to a previous report indicating that FA inhibits MIP-2 production in response to LPS stimulation in RAW264.7 cells,¹² this study confirmed that both FA and IFA inhibit MIP-2 production even in response to RSV infection in RAW264.7 cells in a dose-dependent manner. Since peribronchiolar infiltrations of neutrophils are important pathogenesis in RSV infections,^{13,14} MIP-2-inducing activity of RSV should play a key role. Taking into account the fact that neutrophils can cause tissue injuries, such as lung damage in adult respiratory distress syndrome and other inflammatory diseases, through the production of superoxide or some enzymes, in addition to killing ingested or invading microbes,¹⁵⁻¹⁷ these data suggest that FA and IFA might contribute at least in part to the anti-inflammatory drug effect of CH extract through the reduction of MIP-2 levels, and thereby the reduction of neutrophil infiltration into the inflammatory sites. In addition, the use of FA and IFA or CH extract seems to be a novel and attractive strategy for control of pneumonia induced by certain virus infections, including RSV infection.

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Received 26 March 1999
accepted 6 April 1999