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Cytokine-regulatory activity and therapeutic efficacy of cinnamyl derivatives in endotoxin shock

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Abstract

We characterized the regulatory activity of cinnamyl derivatives and related compounds on pro-inflammatory cytokine production in vitro and in vivo. Among the 51 compounds examined, 7-amino-4-methylcoumarin (AMC) suppressed the production of interleukin-1α, interleukin-6 and tumor necrosis factor (TNF)-α, and their lipopolysaccharide-induced mRNAs in P388D1 cells. AMC suppressed pro-inflammatory cytokine transcription by reducing the DNA-binding amounts of nuclear factor-κB (NF-κB) and activator protein 1. Further, oral administration of AMC (30 mg/kg) as well as anti-TNF-α and anti-interleukin-1α antibodies significantly prevented death from endotoxin shock in mice without body weight loss and toxicity. AMC did not affect basal cytokine levels in control mice but suppressed the rise of systemic pro-inflammatory cytokine level, especially TNF-α. Thus, AMC might contribute to the recovery of endotoxin shock mainly by suppressing pro-inflammatory cytokine transcription. AMC may be useful in understanding the regulation and role of cytokine production in the pathogenesis of cytokine-mediated diseases.

Keywords: Cinnamyl derivative; Endotoxin shock; Cytokine

1. Introduction

Cytokines play important roles in the pathogenesis of inflammatory, infectious and autoimmune diseases. Therefore, the modification of cytokine production in cytokine-mediated diseases may contribute to the understanding of their pathogenesis and provide evidences to improve the treatment of cytokine-mediated diseases. We have been studying the roles of medicinal herbs in the modification of the course of viral infection by assessing both their antiviral activities and influence upon cytokine levels (Nagasaka et al., 1995; Kurokawa et al., 1995, 1996a,b, 1998a,b, 1999, 2001, 2002; Tsurita et al., 2001). In an influenza virus-intranasal infection model in mice, a medicinal herb, Kakkon-to, was effective in reducing the severity of the pneumonia and prolonging survival times by enhancing interleukin-12 production in the respiratory tract. However, it did not exhibit direct anti-influenza virus activity in vitro nor reduced virus yield in the respiratory tract (Kurokawa et al., 1996b, 2002). In addition, the augmentation of interleukin-12 production by the oral administration of Kakkon-to in the infected mice has been shown to play an important role in the alleviation of influenza infection (Tsurita et al., 2001; Kurokawa et al., 2002). Such efficacies were due to the modification of cytokine productions by the medicinal herbs. Further, cinnamyl derivatives and related compounds (Kurokawa et al., 1998b) derived from the medicinal herb were demonstrated to possess antipyretic activity by suppressing the rise of interleukin-1α production subsequent to interferon production induced by influenza infection in mice (Kurokawa et al., 1996a, 1998b). Interestingly, cinnamyl derivatives and related compounds preserved the basal level of interleukin-1α production but suppressed its rise in influenza infection (Kurokawa et al., 1996a, 1998b). The compounds were suggested to contribute to the modification of cytokine production and the alleviation of infection without affecting the basal cytokine levels. One aspect of anti-inflammatory activity has been evaluated in a murine endotoxin shock model using lipopolysaccharide injection (Tracey et al., 1987; Parrillo, 1993; Marsh and Wewers, 1996). In this model, the elevation of circulating pro-inflammatory cytokine levels, such as tumor necrosis factor (TNF)-α and interleukin-1α, is a critical

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factor in the pathogenesis of endotoxin shock (Tracey et al., 1987). Thus, this murine model is very useful for evaluating the cytokine-regulatory activity and therapeutic efficacy of substances on endotoxin shock.

In this study, we have examined the cytokine-regulatory activity of the 51 compounds and characterized 7-amino-4-methylcoumarin (AMC) among them for its cytokine-regulatory activity in vitro and in vivo. AMC suppressed all of the interleukin-1α, interleukin-6 and TNF-α gene expression in lipopolysaccharide-exposed macrophage-like P388D1 cells as well as their secretion from the cells. AMC was suggested to suppress pro-inflammatory cytokine transcription by reducing the DNA-binding amounts of nuclear factor-κB (NF-κB) and activator protein 1 (AP1). Further, we have assessed the cytokine-regulatory activity of AMC and its efficacy in an endotoxin shock model in mice. AMC suppressed the rise of systemic pro-inflammatory cytokine levels, especially TNF-α, without affecting the basal cytokine levels and prevented death from endotoxin shock without toxicity. It was confirmed that AMC suppresses the production of pro-inflammatory cytokines in vivo. Thus AMC may be a cytokine-regulator specific for inducible pro-inflammatory cytokines in cytokine-mediated diseases.

2. Materials and methods

2.1. Cells

Macrophage-like P388D1 cells (American Type Culture Collection, Rockville, MA), originated from DBA/2 mice, were used for in vitro screening of the cytokine-regulatory activity of cinnamyl derivatives and related compounds. The cells were grown and maintained in Rosewell Park Memorial Institute (RPMI)-1640 medium supplemented with 5% and 2% heat-inactivated fetal bovine serum, respectively.

2.2. Compounds

Fifty-one of cinnamyl derivatives and related compounds (Kurokawa et al., 1998b) were supplied from Tsumura (Tokyo, Japan) or purchased from Sigma (St. Louis, MO), Wako (Osaka, Japan) or Nakarai Chem. (Tokyo, Japan). The compounds were dissolved in dimethylsulfoxide (DMSO) at 100 mg/ml and used for in vitro screening as described below. For administration to mice, AMC was suspended in 5% arabic gum and orally administered to mice. As a control, 5% arabic gum alone was used.

2.3. Assay of cytokines secreted from P388D1 cells

The effects of the cinnamyl derivatives and related compounds on the secretion of pro-inflammatory cytokines from P388D1 cells were examined. The P388D1 cells were seeded at 5×10^5 cells/well in 24-well plates and grown at 37 °C overnight. The culture medium was replaced by fresh maintenance medium (1 ml) containing 30 μg/ml of lipopolysaccharide (W *E. coli* O127:B8, Difco, Detroit, MI) and various concentrations of cinnamyl derivatives and related compounds. As a negative control, fresh maintenance medium containing 0.1% of DMSO was used. At various times after lipopolysaccharide exposure, the culture medium was centrifuged and the supernatants were stored at −30 °C until enzyme-linked immunosorbent assay (ELISA) analysis for cytokines was performed. The concentrations of interleukin-1α, interleukin-6 and TNF-α in the supernatants were determined by ELISA (Amersham Pharmacia Biotech, Buckinghamshire, England, or BioSource, Camarillo, CA) according to the manufacturers' instructions. The 50% inhibitory concentrations (IC_{50}) of the compounds for cytokine production were determined from a curve relating the concentrations of each cytokine to the concentration of each compound.

2.4. Cytotoxicity of compounds

The cytotoxicity of the cinnamyl derivatives and related compounds was examined by a growth inhibition assay. The P388D1 cells were seeded at a concentration of 5×10^4 cells/well in 24-well plates and grown at 37 °C for 2 days. The culture medium was replaced by fresh medium containing the compounds at various concentrations, and the cells were further grown for 2 days. The cells in triplicate wells for each concentration of the compounds were treated with trypsin and the number of viable cells was determined by the trypan blue exclusion test (Kurokawa et al., 1995). The 50% cytotoxic concentrations (CC_{50}) of the compounds were determined graphically.

2.5. Analysis of cytokine production in P388D1 cells

In order to examine the regulatory activity of AMC on cytokine production in P388D1 cells, the cells were seeded and treated with lipopolysaccharide and AMC in 24-well plates as described above. After the removal of the culture medium, the cells were lysed in 100 μl of a sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 2 or 8 h after lipopolysaccharide exposure. The lysates were applied to SDS-PAGE and the migrated proteins in gels were blotted to a polyvinylidene difluoride filter (Millipore, Bedford, MA). The immunoblots were probed with rabbit anti-mouse interleukin-1α antibody (Genzyme, Cambridge, MA), rabbit anti-mouse interleukin-6 antibody (Pepro Tech, Rocky Hill, NJ), rabbit anti-mouse TNF-α antibody (Endogen, Woburn, MA) and rabbit anti-mouse β-actin antibody (Sigma), and developed with the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

2.6. Analysis of cytokine mRNA in P388D1 cells

The regulatory activity of AMC on the expression of cytokine genes was examined in P388D1 cells. P388D1

cells were seeded at 7.5×10^6 cells/60-mm dish and treated with lipopolysaccharide and AMC as described above. After 2 or 8 h lipopolysaccharide exposure, the cells were lysed and total RNA was prepared from the lysates using the SV Total RNA Isolation System (Promega, Madison, WI). The total RNA (4–7 μg/lane) was electrophoresed in 1.2% agarose formaldehyde gels and blotted to nylon filters (GeneScreen Plus, NEN, Boston, MA) as described previously (Kurokawa et al., 1990). The filters were prehybridized and then hybridized with denatured digoxigenin-labeled probes for the detection of mouse interleukin-1α, interleukin-6, TNF-α and β-actin mRNAs at 62 °C overnight. RNA specific for each murine cytokine was detected by using immunochemical methods, the DIG Luminescent Detection Kit (Boehringer, Mannheim, Germany) and Gene Images CDP-Star Detection Model (Amersham Pharmacia Biotech) according to the procedures recommended by the manufacturers. The digoxigenin-labeled probes were prepared by polymerase chain reaction (PCR) amplification of each murine cytokine cDNA and labeled with digoxigenin-11-dUTP by PCR. The cDNA was synthesized from total RNA of lipopolysaccharide-stimulated P388D1 cells using cDNA Synthesis Kit (Qiagen, Hilden, Germany). Amplification with PCR primers was performed with Taq polymerase (Gibco BRL, Gaithersburg, MD) under the following conditions: denaturation at 96 °C, 1 min; annealing at temperatures as described below, 1 min; polymerization at 72 °C, 1 min; 35 cycles. The sequences of the PCR primers used (Bristulf et al., 1994; Tingsborg et al., 1996; Chai et al., 1996) were as follows (the annealing temperatures and product sizes are given between brackets):

interleukin-1α: forward primer, 5′GCCAGTTGAGTAGGATAAAGG3′; reverse primer, 5′GGAGTTCTTCCTCTGTCTGAC3′ (53 °C, 156 bp).
interleukin-6: forward primer, 5′AAGAAAGACAAAGCCAGAGTC3′; reverse primer, 5′CACAAACTGATATGCTTAGGC3′ (51 °C, 265 bp).
TNF-α: forward primer, 5′TCAGCCTCTTCTCATTCCTGC3′; reverse primer, 5′TTGGTGGTTTGCTACGACGTG3′ (56 °C, 203 bp).
β-actin: forward primer, 5′AGGGAAATCGTGCGTGACAT3′; reverse primer, 5′CATCTGCTGGAAGGTGGACA3′ (53 °C, 452 bp).

In order to examine the effect of AMC on the stability of pro-inflammatory cytokine mRNAs synthesized in lipopolysaccharide-exposed P388D1 cells, the cells were treated with or without lipopolysaccharide at 30 μg/ml in the presence of cycloheximide (Wako) at 150 μg/ml. After 2 h incubation, actinomycin D (Wako) at 5 μg/ml and AMC at 100 μg/ml, or actinomycin D at 5 μg/ml alone was added to the culture medium and the cells were further incubated for 0, 0.5, 1, 2 and 3 h. As a control, 0.1% DMSO was added instead of AMC. Total RNA (10 μg) was prepared from the cells and then cytokine and β-actin mRNAs were analyzed as described above.

2.7. Preparation of nuclear protein extracts

Nuclear protein extracts were prepared from lipopolysaccharide-exposed P388D1 cells treated with AMC for gel shift assay as described by Zhou et al. (1990). P388D1 cells were seeded at 5×10^7 cells/75-cm^2 flask, grown at 37 °C overnight and then treated with lipopolysaccharide at 30 μg/ml and AMC at 0, 30 and 100 μg/ml as described above. After 2 or 8 h lipopolysaccharide exposure, the cells were collected and washed with cold PBS. The collected cells (1.5×10^7 cells) were suspended in 1 ml of ice-cold lysis buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride supplemented with a protease inhibitor mixture tablet (one tablet per 10 ml; Roche, Mannheim, Germany)] and homogenized on ice with a Dounce homogenizer. The homogenates were kept on ice for 15 min and then 25 μl of 10% Nonidet P-40 was added. They were further incubated on ice for 20 min after a brief vortexing and centrifuged at 12,500 rpm for 30 s at 4 °C. The pellets of nuclei were resuspended in 200 μl of extraction buffer [20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride supplemented with a protease inhibitor mixture tablet (one tablet per 10 ml; Roche)] and kept on ice for 30 min. The nuclear suspension was centrifuged at 12,500 rpm for 15 min at 4 °C to collect the supernatants containing nuclear proteins. The concentrations of nuclear proteins in the resultant supernatants were determined with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Aliquots of the nuclear extracts were stored at −80 °C for the gel shift assay.

2.8. Gel shift assay

Effects of AMC on DNA-binding abilities of transcription factors, NF-κB and AP1, in the nuclear extracts of lipopolysaccharide-exposed P388D1 cells were examined using gel shift assay system (Promega). To assay DNA-transcription factors binding, an aliquot of the nuclear extracts (10 μg) was incubated in a reaction buffer [10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 50 μg/ml poly(dI-dC)]. To identify the DNA binding of NF-κB, an excess amount of unlabeled consensus NF-κB or AP2 oligonucleotide (Promega) or anti-NF-κBp65 antibody (1 μg) (Santa Cruz Biotechnology) was added as a competitor. To identify the DNA binding to AP1, an excess amount of unlabeled consensus AP1 or SP1 oligonucleotide (Promega) was added. After 10 min incubation of the mixtures at room temperature, ^{32}P-end-labeled consensus NF-κB oligonucleotide, AP1 oligonucleotide or mutant NF-κB oligonucleotide (Santa Cruz Biotechnology) ($0.5–3 \times 10^5$

cpm) was added to the reaction mixture, which was then incubated for an additional 20 min at room temperature. For analysis of DNA-binding proteins, the reaction mixture was electrophoresed on a 4% nondenaturing polyacrylamide gel. The electrophoresed gels were dried under vacuum and the dried gels were exposed to an X-ray film at −80 °C.

2.9. Mice

Female DBA/2 CrSlc mice (6 weeks old, 17–19 g, Sankyo Labo Service, Tokyo, Japan) were used for the endotoxin shock model. Mice were housed five per cage in a temperature-controlled room, with feed (CE-2, Clea Japan, Tokyo) and pyrogen-free water (Otsuka Distilled Water, Otsuka Pharmaceutical, Tokyo) ad libitum and under a 12:12-h light/dark diurnal cycle (light at 7.00 a.m.). The temperature in the room was kept at 23 ± 2 °C. The mice were acclimated for at least 3–4 days before starting the experimental procedures. The animal experimentation guidelines of Toyama Medical and Pharmaceutical University were followed in the animal studies.

2.10. Endotoxin shock model in mice

The efficacy and cytokine-regulatory activity of AMC were examined in an endotoxin shock model in mice. Ten DBA/2 mice were used for each group. Lipopolysaccharide (LD_{50}, 35.7–36.2 mg/kg for intraperitoneal injection to mice, W *E. coli* O127:B8, Difco) was dissolved in phosphate-buffered saline (PBS) and injected intraperitoneally in mice at 15 mg/kg. In this model, most of mice died 2 days post-lipopolysaccharide injection. Therefore, AMC at 10 and 30 mg/kg was orally administered once at 2 h prior to lipopolysaccharide exposure and five times daily on days 0 and 1 after lipopolysaccharide exposure, and then on days 2–5, it was administered three times daily. Mice were monitored twice daily and the number of survivors was determined at 7 days after lipopolysaccharide exposure. In the different experiments, sera were prepared from four to five mice in each group at various times after lipopolysaccharide exposure. The concentrations of cytokines in the sera were determined by ELISA as described above.

The efficacy of AMC in endotoxin shock was compared with those of anti-interleukin-1α antibody, anti-TNF-α antibody and dexamethasone in the murine lipopolysaccharide-stimulated model to confirm the cytokine-regulatory activity of AMC. In these experiments, 0.1 ml of fourfold diluted rabbit anti-mouse interleukin-1α serum (Genzyme) or monoclonal rat anti-mouse TNF-α antibody at 50 μg/0.1 ml (Upstate Biotechnology, Lake Placid, NY) was intravenously administered to a mouse once at 2 h before lipopolysaccharide exposure. Dexamethasone (Wako) was intraperitoneally administered once daily for 7 days after lipopolysaccharide exposure. Mice were monitored continuously and the number of survivors and their body weights were determined for 7 days after lipopolysaccharide exposure.

2.11. Statistical analyses

One-way analysis of variance (ANOVA) followed by Dunn's procedure as a multiple comparison procedure was used to evaluate the significance of the differences in mean cytokine levels on the days examined. The Student's *t*-test was used to evaluate the significance of the differences in mean TNF-α levels at 1.5 h after lipopolysaccharide exposure. The Kaplan–Meier method with log-rank test was used to evaluate the significance of the difference in survival rates of lipopolysaccharide-injected mice. A *P* value of less than 0.05 was statistically defined as significant.

3. Results

3.1. Effects of cinnamyl derivatives and related compounds on cytokine secretion in vitro

Fifty-one cinnamyl derivatives and related compounds were examined for their cytokine-regulatory activity on the secretion of interleukin-1α, interleukin-6 and TNF-α from lipopolysaccharide-exposed P388D1 cells. Among the compounds, 22 showed regulatory activity against at least one of three pro-inflammatory cytokines examined (Table 1). Of the 22, AMC (7 in Table 1) and 2-methoxycinnamaldehyde (2 in Table 1) suppressed the secretion of all pro-inflammatory cytokines examined. Although the IC_{50} values of 2-methoxycinnamaldehyde were similar to its CC_{50} value, the IC_{50} values of AMC towards the three cytokines were at least 9.8-fold lower than its CC_{50} value, indicating that the suppressive activity of AMC was not due to cytotoxicity. The suppressive activity of AMC (IC_{50} values, 11.7–30.7μg/ml) was stronger than the other coumarin derivatives (4, 8, 9, 10 and 12 in Table 1). Six compounds (6, 8, 10, 12, 20 and 22 in Table 1) showed suppressive activity against two of three cytokines at concentrations less than 100 μg/ml. The IC_{50} values of compounds 8, 10 and 12 were lower than their CC_{50} values, but IC_{50} values of the other compounds (6, 20 and 22 in Table 1) corresponded to their CC_{50} values. Eleven compounds (1, 3, 4, 5, 9, 13, 14, 15, 18, 19 and 22 in Table 1) showed suppressive activity against one of three cytokines at concentrations less than 100 μg/ml. Thus, only AMC was effective in suppressing the secretion of all three pro-inflammatory cytokines examined.

On the other hand, eight cinnamyl derivatives and related compounds (1, 9, 11, 12, 14, 17, 20 and 21 in Table 1) augmented the secretion of at least one of the pro-inflammatory cytokines examined at concentrations less than 100 μg/ml. Among the eight compounds, compound 22 showed both an enhanced and suppressive activity at their different concentrations against TNF-α. Five of the eight compounds

Table 1
Cytokine regulatory activity of cinnamyl derivatives and related compounds

Compound	CC_{50} (μg/ml)	IC_{50} (μg/ml)		
		Interleukin-1α	Interleukin-6	TNF-α
(1) 2-Hydroxy cinnamic acid	97.0	>100[a]	>100[a]	99.0[b]
(2) 2-Methoxycinnamaldehyde	20.8	21.0[b]	20.6[b]	7.9[b]
(3) 3,4,5-Trimethoxycinnamic acid	–	–	–	−48.3[b]
(4) 3-Hydroxycoumarin	–	>100	<50[b]	>100
(5) 4-Formylcinnamic acid	41.2	–	<100[b]	>100
(6) 4-Hydroxy, 3-methoxycinnamaldehyde	26.3	<50[b]	<50[b]	>100
(7) 7-Amino-4-methylcoumarin	>300	30.7[b]	23.3[b]	11.7[b]
(8) 7-Hydroxycoumarin	234	30.0[b]	34.0[b]	>100
(9) 7-Hydroxycoumarin 3-carboxylic acid	45.9	<100[b]	>100[a]	>100[a]
(10) 7-Methoxycoumarin (Herniarin)	175.5	78.6[b]	<100[b]	>100
(11) Benzylalcohol	26.4	>100	>100[a]	>100[a]
(12) Coumarin	>300	97.2[b]	78.7[b]	>100[a]
(13) Cuminaldehyde	–	<50[b]	>100	>100
(14) Eugenol	120.9	>100	>100[a]	72.2[b]
(15) Furfural	–	<50[b]	>100	>100
(16) Geraniol	–	<100	>100	>100
(17) Guaiacol	23.1	>100[a]	>100[a]	>100[a]
(18) Methyleugenol	32.4	>60	>100	40.1[b]
(19) Phenylalanine	>300	>100	>100	38.1[b]
(20) Salycilaldehyde	7.84	<100[b]	<50[b]	>100[a]
(21) α-Cianocinnamic acid	–	<100[b]	>100	>100
(22) α-Methylcinnamaldehyde	48.7	>100	<100[b]	34.5[a,b]

Interleukin-1α, interleukin-6 and TNF-α concentrations in culture medium were determined at 48, 48 and 4 h, respectively, after lipopolysaccharide exposure as described in text.

[a] Compounds showing augmentative activity.

[b] Compounds showing suppressive activity.

(1, 9, 12, 14 and 20 in Table 1) exhibited augmentative and suppressive activity to different cytokines and two compounds (11 and 17 in Table 1) exhibited only augmentative activity. The cinnamyl derivatives and related compounds showed the various kinds of regulatory activity towards the secretion of pro-inflammatory cytokines. AMC was chosen as a substance for further characterization of suppressive activity towards interleukin-1α, interleukin-6 and TNF-α.

3.2. Effects of AMC on cytokine production in P388D1 cells

The suppressive activity of AMC on the production of interleukin-1α, interleukin-6 and TNF-α was examined in P388D1 cells exposed to lipopolysaccharide. In our time-course experiments of the cytokine production in P388D1 cells, the production of TNF-α and interleukin-1α and -6 reached to their maximum levels at 2 and 8 h after lipopolysaccharide exposure, respectively. Thus, the three cytokines were detected under various concentrations of AMC at 2 or 8 h after lipopolysaccharide exposure by Western blot analysis. As shown in Fig. 1, DMSO at 0.1%, which was used to dissolve AMC into culture medium, did not affect the production of cytokines examined in comparison with lipopolysaccharide-unexposed controls. AMC did not affect the production of β-actin at concentrations used. However, AMC suppressed the production of TNF-α, interleukin-1α and interleukin-6 in P388D1 cells in a dose-dependent manner. As shown in Fig. 2, when the mRNA levels of TNF-α, interleukin-1α and interleukin-6 were examined by Northern blot analysis at 2 or 8 h after lipopolysaccharide exposure, the mRNA

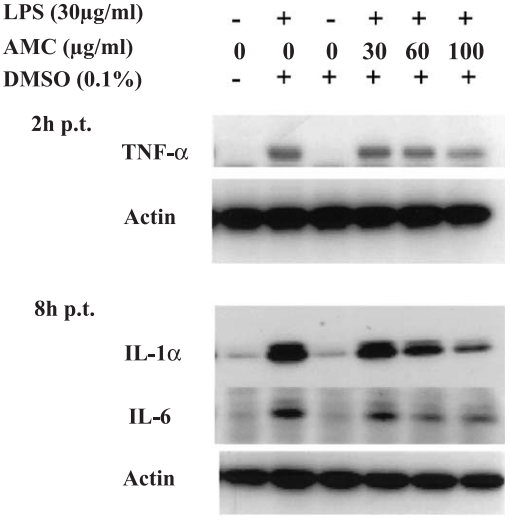

Fig. 1. Cytokine production in lipopolysaccharide-exposed P388D1 cells treated with AMC. P388D1 cells were incubated in culture medium with or without lipopolysaccharide at 30 μg/ml in the presence of AMC (0, 30, 60 and 100 μg/ml) for 2 or 8 h after lipopolysaccharide exposure. Interleukin-1α, interleukin-6, TNF-α and β-actin were detected by Western blot analysis followed by chemiluminescence detection with anti-mouse interleukin-1α, interleukin-6, TNF-α and β-actin antibodies using the ECL system.

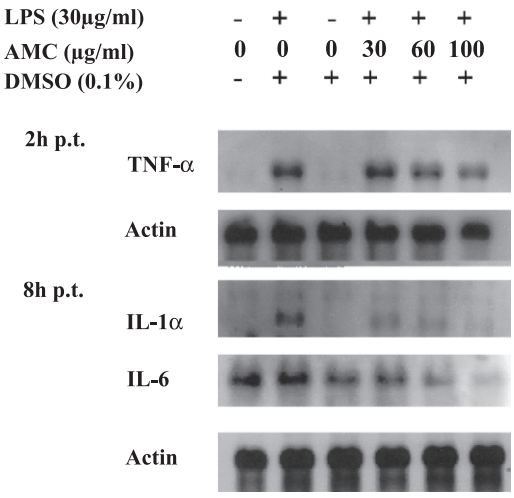

Fig. 2. Cytokine production in lipopolysaccharide-exposed P388D1 cells treated with AMC. The P388D1 cells were incubated in culture medium with or without lipopolysaccharide at 30 μg/ml in the presence of AMC (0, 30, 60 and 100 μg/ml) for 2 or 8 h after lipopolysaccharide exposure. interleukin-1α, interleukin-6, TNF-α and β-actin mRNAs were detected using digoxigenin-labeled probes with immunochemical methods.

levels of the three cytokines were suppressed by AMC in a dose-dependent manner. Neither DMSO at 0.1% activated the transcription of the three cytokines nor AMC affected the expression of β-actin. AMC reduced the production of TNF-α, interleukin-1α and interleukin-6 and their mRNA expression.

3.3. Effects of AMC on stability of cytokine mRNAs

In order to evaluate the effect of AMC on the stability of pro-inflammatory cytokine mRNAs synthesized in lipopolysaccharide-exposed P388D1 cells, the amounts of synthesized mRNAs were compared after the addition of actinomycin D in the presence or absence of AMC at 100 μg/ml (Fig. 3). At 0.5, 1, 2 and 3 h after the addition of actinomycin D, the amounts of TNF-α mRNA decreased in a time-dependent manner, but AMC had no effect on its decrease. In the cases of interleukin-1α and interleukin-6 mRNAs as well as β-actin mRNA, AMC had no detectable effect on their stability although there was no obvious degradation for 3 h. In the lipopolysaccharide-unexposed cells, TNF-α and interleukin-1α mRNAs were not detected although interleukin-6 mRNA as well as β-actin mRNA was observed. AMC did not alter the stability of lipopolysaccharide-induced cytokine mRNAs in P388D1 cells.

3.4. Effects of AMC on transcription factors-DNA binding

Transcription factors, NF-κB and AP1, are important in mediating transcriptional control of pro-inflammatory cytokines (Baldwin, 1996; Drouet et al., 1991; Stain et al., 1993; Zhang et al., 1995). We examined the effects of AMC on the DNA-binding activity of NF-κB and AP1 in the nuclear extracts of lipopolysaccharide-exposed P388D1 cells. In a gel shift assay using ^{32}P-labeled NF-κB oligonucleotide, several bands as DNA-binding complexes were detected in lipopolysaccharide-exposed nuclear extract in the absence of AMC (Fig. 4A, lane 6) but some of the bands were less in their density in lipopolysaccharide-unexposed nuclear extract (Fig. 4A, lane 5). Such less observed bands were also competed away by the excess of the unlabeled consensus NF-κB oligonucleotide (Fig. 4A, lane 4) but were not completely by the excess of the unlabeled AP2 oligonucleotide. These indicated that the bands are specific NF-κB complex bands. When using ^{32}P-labeled mutant NF-κB oligonucleotide instead of ^{32}P-labeled NF-κB oligonucleotide, no specific NF-κB complex bands were observed (Fig. 4A, lane 2). They were also competed away by the anti-NF-κBp65 antibody added to the extract before the addition of ^{32}P-labeled NF-κB oligonucleotide. In the lipopolysaccharide-exposed cells for 2 h, the binding levels of DNA to the specific NF-κB complexes obviously increased as compared with the basal binding level in the lipopolysaccharide-untreated cells and AMC suppressed the binding level in a dose-dependent manner. In the lipopolysaccharide-exposed cells for 8 h, the levels of NF-κB-DNA binding were slightly higher than the basal level detected in the lipopolysaccharide-unexposed cells and retained at a low level even in the presence of AMC at 30 and 100 μg/ml for 8 h.

In the case of ^{32}P-labeled AP1 oligonucleotide, the identity of the AP1 complex was confirmed using an excess unlabeled AP1 and SP1 oligonucleotides, which decreased

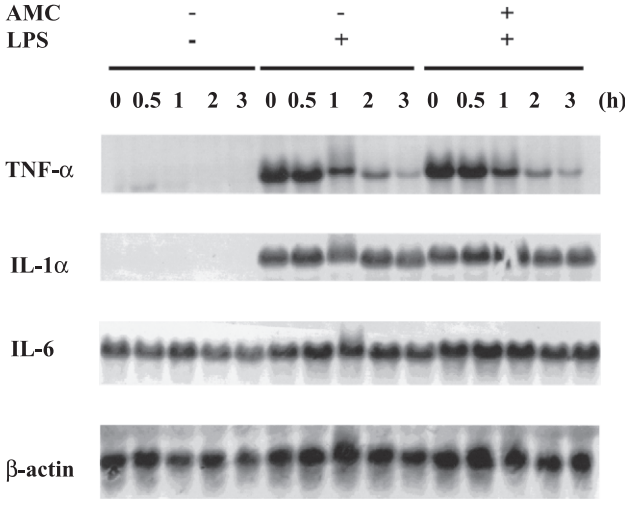

Fig. 3. Effect of AMC on the stability of pro-inflammatory cytokine mRNAs. P388D1 cells were treated with lipopolysaccharide (LPS+) or without lipopolysaccharide (LPS−) at 30 μg/ml in the presence of cycloheximide at 150 μg/ml for 2 h, and then were treated with AMC at 100 μg/ml and actinomycin D at 5 μg/ml (AMC+), or actinomycin D at 5 μg/ml alone (AMC−) for 0, 0.5, 1, 2 and 3 h. As a control, 0.1% DMSO was added instead of AMC. Total RNA (10 μg) was prepared from the cells, and then cytokine mRNAs (TNF-α, interleukin-1 α and interleukin-6) and β-actin mRNAs were detected by Northern blot analysis as described in text. Similar results were obtained in three independent experiments.

and did not affect, respectively, AP1-DNA binding (Fig. 4B). In the nuclear extracts of lipopolysaccharide-exposed cells for 2 h, AMC reduced lipopolysaccharide-induced AP1-DNA binding in a dose-dependent manner. In the lipopolysaccharide-exposed cells for 8 h, the AP1-DNA binding level was retained at the basal level even in the presence of AMC at 30 and 100 μg/ml. Thus, AMC dose-dependently suppressed the DNA-binding amounts of NF-κB and AP1 in P388D1 cells in the early phase of lipopolysaccharide exposure, but the longer exposure of AMC had less influence on the basal levels of DNA binding of the transcription factors.

3.5. Efficacy of AMC in an endotoxin shock model

We evaluated the efficacy of AMC in an endotoxin shock model in DBA/2 mice. Since P388D1 cells used in vitro are derived from DBA/2 mice, this mouse strain was used for an endotoxin shock model. In this model, most of the untreated mice died 2 days post-lipopolysaccharide injection; however, dexamethasone at 3 mg/kg significantly reduced the mortality ($P<0.01$ by Fischer's exact test in Fig. 5A). Administration of AMC at 30 mg/kg significantly protected the mice from death ($P<0.01$ by Kaplan–Meier method)

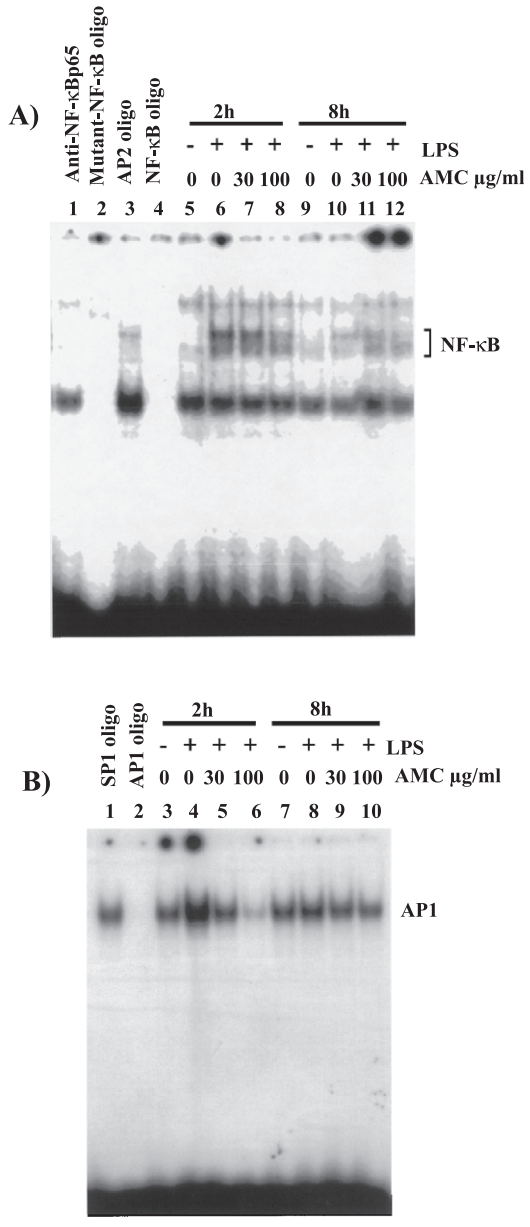

Fig. 4. Effect of AMC on transcription factors-DNA binding. (A) Effect of AMC on NF-κB-DNA binding. P388D1 cells were treated with or without lipopolysaccharide at 30 μg/ml in the presence of AMC at 0, 30 and 100 μg/ml for 2 and 8 h, and then nuclear protein extracts were prepared from the cells. The extracts were examined for the activity of NF-κB-DNA binding by a gel shift assay as described in text. Antibody and competition analyses were performed on nuclear extract from cells treated with lipopolysaccharide at 30 μg/ml for 2 h in the absence of AMC, which was used in lane 6. Anti-NF-κBp65 (lane 1): antibody to NF-κBp65; Mutant NF-κB oligo (lane 2): mutant NF-κB oligonucleotide; AP2 oligo (lane 3): excess amount of unlabeled AP2 consensus oligonucleotide; NF-κB oligo (lane 4): excess amount of unlabeled NF-κB consensus oligonucleotide. Similar results were obtained in three independent experiments. (B) Effect of AMC on AP1-DNA binding. P388D1 cells were treated with or without lipopolysaccharide at 30 μg/ml in the presence of AMC at 0 (lanes 3, 4, 7 and 8), 30 (lanes 5 and 9) and 100 (lanes 6 and 10) μg/ml for 2 and 8 h, and then nuclear protein extracts were prepared from the cells. The extracts were examined for the activity of AP1-DNA binding by a gel shift assay as described in text. Competition analysis was performed on nuclear extract from cells treated with lipopolysaccharide at 30 μg/ml for 2 h in the absence of AMC, which was used in lane 4. SP1 oligo (lane 1): excess amount of unlabeled SP1 consensus oligonucleotide; AP1 oligo (lane 2): excess amount of unlabeled AP1 consensus oligonucleotide. Similar results were obtained in two independent experiments.

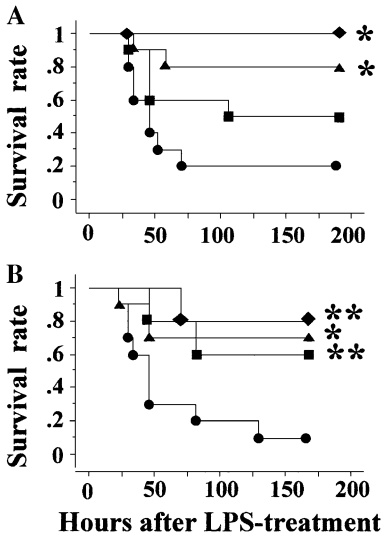

Fig. 5. Protection against endotoxin shock by AMC. (A) AMC at 0 (●) 10 (■) or 30 mg/kg (▲) was orally administered to the lipopolysaccharide-injected DBA/2 mice ($n=10$ per group). Dexamethasone at 3 mg/kg (◆) was intraperitoneally administered once daily for 7 days after lipopolysaccharide exposure. *$P<0.01$ compared with AMC at 0 mg/kg by the Kaplan–Meier method. (B) The efficacy of AMC at 0 (●) or 30 mg/kg (■) was compared with those of anti-interleukin-1α antibody (▲) and anti-TNF-α antibody (◆) in lipopolysaccharide-injected mice ($n=10$ per group). *$P<0.01$ and **$P<0.05$ compared with AMC at 0 mg/kg by the Kaplan–Meier method.

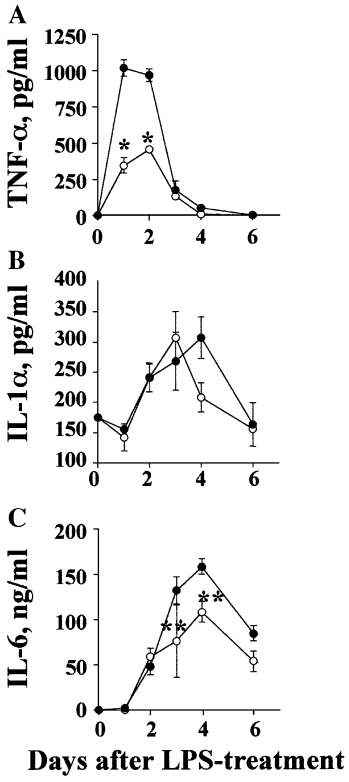

Fig. 6. Suppression of cytokine production by AMC in lipopolysaccharide-injected mice. AMC at 0 (●) or 30 mg/kg (○) was orally administered to the lipopolysaccharide-injected mice and sera were prepared from four to five mice in each group. The concentrations of TNF-α (A), interleukin-1α (B) and interleukin-6 (C) in sera are expressed as the mean ± S.E.M. *$P<0.001$ and **$P<0.05$ compared with AMC at 0 mg/kg by one-way ANOVA followed by the Dunn's procedure as a multiple comparison procedure.

although at 10 mg/kg this was not significant (Fig. 5A). Neither lethal toxicity nor body weight loss was observed in lipopolysaccharide-non-injected mice treated with AMC at 30 mg/kg for 7 days (data not shown).

Circulating concentrations of TNF-α and interleukin-1α are elevated in patients with sepsis after lipopolysaccharide administration in humans and laboratory animals (Cannon et al., 1990) and these cytokines mimic the effects of lipopolysaccharide (Tracey et al., 1986a,b; Mathison et al., 1988). Thus, the efficacy of AMC at 30 mg/kg was compared with that of anti-TNF-α and interleukin-1 α antibody. As shown in Fig. 5B, the efficacy of AMC at 30 mg/kg against endotoxin shock was confirmed in the repeated experiments.

Table 2
Comparison of TNF-α levels in the sera of lipopolysaccharide-exposed mice administered with and without AMC

Treatment	TNF-α (pg/ml)
Control	4893.9 ± 563.1
AMC at 30 mg/ml	3103.9 ± 489.1[a]

Sera were prepared from the mice ($n=6$) at 1.5 h after lipopolysaccharide exposure, and cytokine levels were determined as described in text.

[a] $P<0.05$ vs. control by the Student's t-test.

Table 3
Effect of AMC administration on cytokine levels in the serum of uninfected mice

Treatment	Interleukin-1α pg/ml on day 4	Interleukin-6 pg/ml on day 4	TNF-α pg/ml on day 2
Control	105.1 ± 31.0	631.0 ± 290.3	2.3 ± 0.9
AMC at 30 mg/ml	115.0 ± 16.7	504.8 ± 166.0	1.3 ± 0.4

Sera were prepared from AMC-administered mice ($n=3$ or 4) on days 2 and 4 after mock lipopolysaccharide exposure, and cytokine levels were determined as described in text.

Anti-TNF-α and anti- interleukin-1α antibodies significantly increased the survival rate similar to oral AMC-administration at 30 mg/kg ($P<0.01$ and $P<0.05$ by Kaplan–Meier method, Fig. 5B). Thus, AMC at 30 mg/kg, as well as anti-TNF-α and anti-interleukin-1α antibodies, exhibited significant efficacy against endotoxin shock in mice.

3.6. Effect of ACM on cytokine level in endotoxin shock model

The effect of AMC at 30 mg/kg on systemic TNF-α, interleukin-1α and interleukin-6 levels was examined in lipopolysaccharide-injected mice at 1, 2, 3, 4 and 6 days (Fig. 6). The level of TNF-α production was higher at 1–2 days than at 3–6 days (Fig. 6A) and the levels of interleukin-1α and interleukin-6 production were higher at 3–4 days than at 1, 2 and 6 days (Fig. 6B and C). AMC significantly reduced TNF-α and interleukin-6 levels on days 1 and 2 ($P<0.001$, Fig. 6A) and on days 3 and 4 ($P<0.05$, Fig. 6C), respectively. Interleukin-1α level was also reduced by AMC on day 4, although the reduction was not statistically significant (Fig. 6B). The production of TNF-α has been shown to occur at 1–2 h after lipopolysaccharide exposure in mice (Rees et al., 1998; Zuckerman and Bendele, 1989; Nicoletti et al., 1997). We examined the effect of AMC on TNF-α level at 1.5 h after lipopolysaccharide exposure as shown in Table 2 and confirmed that AMC was significantly effective to reduce the level of TNF-α production in the early stage of lipopolysaccharide exposure. When AMC was administered to lipopolysaccharide-unexposed mice, AMC administration did not affect the basal cytokine levels (Table 3). Thus, AMC suppressed the rise of systemic pro-inflammatory cytokine levels, especially in the early phase of endotoxin shock.

4. Discussion

Cinnamyl derivatives and related compounds are common metabolites in plants and components in many herbal medicines, although their contents vary. We have shown that herbal medicines exhibit cytokine-regulatory activity in influenza virus-infected mice and that the regulatory activity is associated with alleviation (Kurokawa et al., 1996b, 1998b,

2002). In this study, we have demonstrated that cinnamyl derivatives and related compounds have a variety of cytokine-regulatory activity in P388D1 cells and that one of them, AMC, possesses cytokine-suppressive activity in vitro and in vivo. Cinnamyl derivatives and related compounds have been suggested to be possible components that contribute the cytokine-regulatory activity of herbal medicines.

AMC among the cinnamyl derivatives and related compounds examined suppressed the secretion of interleukin-1α, interleukin-6 and TNF-α from murine P388D1 cells (Table 1). AMC also suppressed their production in the cells and diminished lipopolysaccharide-induced pro-inflammatory mRNAs in a dose-dependent manner, but β-actin production was not affected by AMC (Figs. 1 and 2). We used higher concentrations of AMC (30 and 100 μg/ml) than its IC_{50} concentrations (11.7–30.7 μg/ml) to evaluate the suppression of lipopolysaccharide-induced pro-inflammatory cytokine production and their mRNAs. However, trypan blue exclusion tests confirmed that the concentrations used were more than 9.8-fold lower than the CC_{50} concentration (>300 μg/ml, Table 1) of AMC and did not exhibit cytotoxicity. It is probable that AMC selectively suppressed the production of pro-inflammatory cytokines.

Activation of transcription factors, such as NF-κB and AP1, is key events in the signal transduction pathways mediating pro-inflammatory cytokine production after lipopolysaccharide exposure (Baldwin, 1996; Drouet et al., 1991; Stain et al., 1993; Zhang et al., 1995). The activated NF-κB and AP1 bind to the promoter regions of pro-inflammatory cytokines and induce their transcription. NF-κB and AP1 have been reported to form synergistic complexes that enhance transcription (Stain et al., 1993). Our gel shift assay (Fig. 4) demonstrated that AMC reduced the DNA-binding amounts of NF-κB and AP1 in a dose-dependent manner at 2 h after lipopolysaccharide exposure. However, AMC was not so active in suppressing the DNA binding of NF-κB and AP1 at 8 h after lipopolysaccharide exposure as compared with the results at 2 h after lipopolysaccharide exposure and their DNA-binding capacity was retained at lower levels (Fig. 4). The production of TNF-α reached at the maximal levels at 2 h after lipopolysaccharide exposure (Table 1) and the stability of pro-inflammatory cytokines mRNAs was not affected by AMC (Fig. 3). Thus, in the early phase of lipopolysaccharide exposure, AMC might be effective in suppressing TNF-α transcription by reducing the DNA-binding capability of NF-κB and AP1 through lipopolysaccharide-induced activation. TNF-α released by lipopolysaccharide exposure, in turn, contributes to the triggering of an inflammatory cascade involving the induction of various kinds of immune-mediators, including interleukin-1 and interleukin-6 (Marsh and Wewers, 1996; Parrillo, 1993; Dinarello, 1996). Some of lipopolysaccharide-triggered mediators, such as TNF-α activated through NF-κB, also activate NF-κB, resulting in promoting their own secretion and augmenting the cytokine cascade and the inflammatory response (Baeuerle and Henkel, 1994; Baldwin, 1996; May and Ghosh, 1998). Thus, it is possible that the suppression of TNF-α by AMC in the early phase of lipopolysaccharide exposure led to the suppression of interleukin-1α and interleukin-6 production in P388D1 cells. AMC might contribute mainly to the suppression of TNF-α transcription.

We used an endotoxin shock model in DBA/2 mice to evaluate the efficacy of AMC in one of the inflammatory diseases in relation to its cytokine-regulatory activity observed in macrophage-like P388D1 cells derived from DBA/2 mice. In the murine model, AMC was significantly effective in suppressing inducible pro-inflammatory cytokines (Fig. 6). Especially, the systemic production of TNF-α was suppressed by AMC in the early phase of lipopolysaccharide stimulation. This result was consistent with the result in vitro that AMC mainly suppressed TNF-α production in the early phase of lipopolysaccharide exposure. The cytokine-suppressive activity of AMC was confirmed in both in vitro and in vivo systems based on the same genetic background. Thus, mode of suppressive production of pro-inflammatory cytokines by AMC observed in P388D1 cells may act on suppressing inducible pro-inflammatory cytokines by AMC in the murine model.

The protective efficacy of AMC at 30 mg/kg against endotoxin shock was similar to those of anti-TNF-α and anti-interleukin-1α antibodies (Fig. 5). This suggested that the pro-inflammatory cytokines play an important role in the pathogenesis of endotoxin shock. AMC might exert its anti-TNF-α and anti-interleukin-1α-like effects in lipopolysaccharide-stimulated mice. In the sera of lipopolysaccharide-injected mice, the marked suppression of systemic TNF-α level by AMC was observed before the suppression of interleukin-1α and interleukin-6 levels after lipopolysaccharide stimulation (Fig. 6). The suppression of TNF-α level followed by that of interleukin-1α and interleukin-6 levels was consistent with a result that TNF-α is a major inducer of them (Shalaby et al., 1989; Roth et al., 1993; Ng et al., 1994). It is possible that the suppression of TNF-α and subsequent interleukin-1α and interleukin-6 production by AMC contributed the protection of mice from endotoxin shock. Our results are in agreement with previous findings where TNF-α is the key mediator in the cytokine cascade and influences the severity of endotoxin shock (Grau et al., 1987; Tracey et al., 1986a,b; Camenisch et al., 1999; Tracey, 1991). We confirmed that AMC was effective in reducing the production of pro-inflammatory cytokines in vivo.

In this study, we demonstrated that AMC exhibited anti-endotoxin shock activity in vivo. The results presented here strongly support that the cytokine-suppressive activity of AMC is responsible for its efficacy. In control DBA/2 mice, AMC had no effect on the systemic basal level of the three pro-inflammatory cytokines (Table 3). AMC is a cytokine-regulator specific for inducible cytokine, especially TNF-α, in cytokine-mediated diseases, and would be useful to the understanding of the pathogenesis of cytokine-mediated diseases.

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References

Baeuerle, P.A., Henkel, T., 1994. Function and activation of NK-κB in the immune system. Annu. Rev. Immunol. 12, 141–179.

Baldwin Jr., A.S., 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. 14, 649–683.

Bristulf, J., Gatti, S., Malinowsky, D., Bjork, L., Sundgren, A.K., Bartfai, T., 1994. Interleukin-1 stimulates the expression of type I and type II interleukin-1 receptors in the rat insulinoma cell line Rinm5F; sequencing a rat type II interleukin-1 receptor cDNA. Eur. Cytokine Netw. 5, 319–330.

Camenisch, T.D., Koller, B.H., Earp, H.S., Matsushima, G.K., 1999. A novel receptor tyrosine kinase, Mer, inhibits TNF-alpha production and lipopolysaccaride-induced endotoxic shock. J. Immunol. 162, 3498–3503.

Cannon, J.G., Tompkins, R.G., Gelfand, J.A., Michie, H.R., Stanford, G.G., van der Meer, J.W., Endres, S., Lonnemann, G., Corsetti, J., Chernow, B., 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. J. Infect. Dis. 161, 79–84.

Chai, Z., Alheim, K., Lundkvist, J., Gatti, S., Bartfai, T., 1996. Subchronic glucocorticoid pretreatment reversibly attenuates IL-1β induced fever in rats; IL-6 mRNA is elevated while IL-1α and IL-1β mRNAs are suppressed, in the CNS. Cytokine 8, 227–237.

Dinarello, C., 1996. Cytokines as mediators in the pathogenesis of septic shock. Curr. Top. Microbiol. Immunol. 216, 133–165.

Drouet, C., Shakhov, A.N., Jongeneel, C.V., 1991. Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-α promoter in primary macrophages. J. Immunol. 147, 1694–1700.

Grau, G.E., Fajardo, L.F., Piguet, P.F., Allet, B., Lambert, P.H., Vassalli, P., 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science 237, 1210–1212.

Kurokawa, M., Ochiai, H., Nakajima, K., Niwayama, S., 1990. Inhibitory effect of protein kinase C inhibitor on the replication of influenza type A virus. J. Gen. Virol. 71, 2149–2155.

Kurokawa, M., Nagasaka, K., Hirabayashi, T., Uyama, S., Sato, H., Kageyama, T., Kadota, S., Ohyama, H., Hozumi, T., Namba, T., Shiraki, K., 1995. Efficacy of traditional herb medicines in combination with acyclovir against herpes simplex virus type 1 infection in vitro and in vivo. Antivir. Res. 27, 19–37.

Kurokawa, M., Imakita, M., Kumeda, C.A., Shiraki, K., 1996a. Cascade of fever production in mice infected with influenza virus. J. Med. Virol. 50, 152–158.

Kurokawa, M., Imakita, M., Kumeda, C.A., Yukawa, T.A., Shiraki, K., 1996b. Kakkon-to suppressed interleukin-1α production responsive to interferon and alleviated influenza infection in mice. J. Trad. Med. 13, 201–209.

Kurokawa, M., Hozumi, T., Basnet, P., Nakano, M., Kadota, S., Namba, T., Kawana, T., Shiraki, K., 1998a. Purification and characterization of eugeniin as an anti-herpes virus compound from *Geum japonicum* and *Syzygium aromaticum*. J. Pharmacol. Exp. Ther. 284, 728–735.

Kurokawa, M., Kumeda, C.A., Yamamura, J., Kamiyama, T., Shiraki, K., 1998b. Antipyretic activity of cinnamyl and related compounds in Kakkon-to, a traditional medicine, in influenza virus-infected mice. Eur. J. Pharmacol. 348, 45–51.

Kurokawa, M., Basnet, P., Ohsugi, M., Hozumi, T., Kadota, S., Namba, T., Kawana, T., Shiraki, K., 1999. Anti-herpes simplex virus activity of moronic acid purified from *Rhus javanica* in vitro and in vivo. J. Pharmacol. Exp. Ther. 289, 72–78.

Kurokawa, M., Hozumi, T., Tsurita, M., Kadota, S., Namba, T., Shiraki, K., 2001. Biological characterization of eugeniin as an anti-herpes simplex virus type 1 compound in vitro and in vivo. J. Pharmacol. Exp. Ther. 297, 372–379.

Kurokawa, M., Tsurita, M., Brown, J., Fukuda, Y., Shiraki, K., 2002. Effect of interleukin-12 level augmented by Kakkon-to, a herbal medicine, on the early stage of influenza infection in mice. Antivir. Res. 56, 183–188.

Marsh, C.B., Wewers, M.D., 1996. The pathogenesis of sepsis: factors that modulate the response to Gram-negative bacterial infection. Clin. Chest Med. 17, 183–197.

Mathison, J.C., Wolfson, E., Ulevitch, R.J., 1988. Participation to tumor necrosis factor in the mediation of Gram negative bacterial lipopolysaccaride-induced injury in rabbits. J. Clin. Invest. 81, 1925–1937.

May, M.J., Ghosh, S., 1998. Signal transduction through NK-κB. Immunol. Today 19, 80–88.

Nagasaka, K., Kurokawa, M., Imakita, M., Terasawa, K., Shiraki, K., 1995. Efficacy of Kakkon-to, a traditional herb medicine, in herpes simplex virus type 1 infection in mice. J. Med. Virol. 46, 28–34.

Nicoletti, F., Mancuso, G., Cusumano, V., Di Marco, R., Zaccone, P., Bendtzen, K., Teti, G., 1997. Prevention of endotoxin-induced lethality in neonatal mice by interleukin-13. Eur. J. Immunol. 27, 1580–1583.

Ng, S.B., Tan, Y.H., Guy, G.R., 1994. Differential induction of the IL-6 gene by tumor necrosis factor and interleukin-1. J. Biol. Chem. 269, 19021–19027.

Parrillo, J.E., 1993. Pathogenic mechanisms of septic shock. N. Engl. J. Med. 328, 1471–1477.

Rees, D.D., Monkhouse, J.E., Camgridge, D., Moncada, S., 1998. Nitric oxide and the haemodynamic profile of endotoxin shock in the conscious mouse. Br. J. Pharmacol. 124, 540–546.

Roth, J., Conn, C.A., Kluger, M.J., Zeisberger, E., 1993. Kinetics of systemic and intrahypothalamic IL-6 and tumor necrosis factor during endotoxin fever in guinea pigs. Am. J. Physiol. 265, 653–658.

Shalaby, M.R., Waage, A., Aarden, L., Espevik, T., 1989. Endotoxin, tumor necrosis factor α and interleukin 1 induce interleukin 6 production in vivo. Clin. Immunol. Immunopathol. 53, 488–498.

Stain, B., Baldwin Jr., A.S., Ballard, D.W., Greene, W.C., Angel, P., Herrlich, P., 1993. Cross-coupling of the NF-kappa B p65 and fos/jun transcription factors potentiated biological function. EMBO J. 12, 3879–3891.

Tingsborg, S., Zetterstrom, M., Alheim, K., Hasanvan, H., Schultzberg, M., Bartfai, T., 1996. Regionally specific induction of ICE mRNA and enzyme activity in the rat brain and adrenal gland by LPS. Brain Res. 712, 153–158.

Tracey, K.J., 1991. Tumor necrosis factor (cachectin) in the biology of septic shock symdrome. Circ. Shock 35, 123–128.

Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W., Hariri, R.J., Fahey III, T.J., Zentella, A., Albert, J.D., 1986a. Shock and tissue injury induced by recombinant human cachetin. Science 234, 470–474.

Tracey, K.J., Lowry, S.F., Beutler, B., Cerami, A., Albert, J.D., Shires, G.T., 1986b. Cachetin/tumor necrosis factor mediates changes of skeletal muscle plasma membrane potential. J. Exp. Med. 164, 1368–1373.

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., Cerami, A., 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330, 662–664.

Tsurita, M., Kurokawa, M., Imakita, M., Fukuda, Y., Watanabe, Y., Shiraki, K., 2001. Early augmentation of interleukin (IL)-12 level in the airway of mice administered orally with clarithromycin or intranasally with IL-12 results in alleviation of influenza infection. J. Pharmacol. Exp. Ther. 298, 362–368.

Zhang, Y., Broser, M., Rom, W., 1995. Activation of the interleukin 6

gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF IL6 and NF-κB. Proc. Natl. Acad. Sci. U. S. A. 92, 3632.

Zhou, D., Brown, S.A., Yu, T., Chen, G., Barve, S., Kang, B.C., Thompson, J.S., 1990. A high dose of ionizing radiation induces tissue-specific activation of nuclear factor-κB in vivo. Radiat. Res. 151, 703–709.

Zuckerman, S.H., Bendele, A., 1989. Regulation of serum tumor necrosis factor in glucocorticoid-sensitive and resistant rodent endotoxin shock models. Infect. Immun. 57, 3009–3013.