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Chrysanthemum morifolium inhibits inflammatory responses in IFN-γ and LPS-induced mouse peritoneal macrophages

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SUMMARY

Chrysanthemum morifolium (CM) is a herb widely used in medicine for the treatment of a variety of diseases. In this study, using mouse peritoneal macrophages, we have examined whether CM affects nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 induced interferon (IFN)-γ and lipopolysaccharide (LPS). CM inhibits IFN-γ and LPS-induced NO in dose dependent manner. We also found that CM inhibits pro-inflammatory cytokine, TNF-α and IL-6. The expression of cyclooxygenase-2 was reduced by CM. These finding means that CM can be used in controlling macrophages-mediated inflammatory disease.

Key words: *Chrysanthemum morifolium*; Nitric oxide; TNF-α; IL-6; COX-2

INTRODUCTION

The dried flower-heads of *Chrysanthemum morifolium* (CM) (Compositae) are an oriental drug, which has been used for the treatment of eye disease in Korea and China. They have also been used as an herbal tea in Chinease folklore and are known as "Ju Hua". They have been found to possess antibacterial, antifungal, antiviral, antispirochetal, and anti-inflammatory activities (Jiangsu New Medical College, 1977). Macrophages are a first line of defence against microbial invaders and malignancies by nature of their phagocytic, cytotoxic and intracellular killing capacities (Adams and Hamilton, 1984). Macrophage activation by lipopolysaccharide (LPS), the major component of gram-negative bacteria cell wall, results in the release of several inflammatory mediators such as nitric oxide (NO) and the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 (Schimmer and Parker, 2001). The physiologic or normal production of NO from phagocytes is beneficial for the host defense against microorganism, parasites, and tumor cells (Thiemermann and Vane, 1990). However, overproduction of NO can be harmful and result in septic shock, neurologic disorders, rheumatoid arthritis, and autoimmune diseases (Thiemermann and Vane, 1990; Evans, 1995; O'Shea *et al.*, 2002). Therefore, inhibition of NO production is a very important therapeutic target in the development of anti-inflammatory agents.

The pro-inflammatory cytokine, TNF-α regulates systemic responses to microbial infection or tissue injury (Evans, 1995). TNF-α induces other inflammatory cytokines such as IL-1, IL-2, IL-6, IL-18 and granulocyte-macrophage colony-stimulating factor. The involvement of TNF-α as a pathogenic factor has been documented in

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several immunoinflammatory diseases, including arthritic diseases, inflammatory bowel diseases, type 1 diabetes mellitus, multiple sclerosis, and Guillain-Barre syndrome (O'Shea *et al.*, 2002). In patients with rheumatoid arthritis, juvenile idiopathic arthritis and ankylosing spondylitis, for example, neutralizing anti-TNF antibodies and soluble TNF-α receptors are powerful means of controlling disease activity (Gorman *et al.*, 2002). IL-6 is macrophage-or monocyte-related cytokine (Sradnyk, 1997). It is essential for the inflammatory response to pathogenic germs or toxicants (Liew, 2003).

NO and pro-inflammatory cytokine are well known to be important mediators of acute and chronic inflammation (Park *et al.*, 2000)/and are synthesized by cyclooxygenase (COX) enzymes. There are two isoform of COX, constitutively expressed COX-1 and the inducible isoform COX-2 (Kanazawa, *et al.*, 1995). COX-2 is upregulated in response to inflammatory and pro-inflammatory mediators and their products can influence many aspects of inflammatory cascade.

In the present study, we show that CM significantly inhibited LPS and IFN-γ-induced NO production in a dose-dependent manner. Furthermore, CM inhibited TNF-α and IL-6 production. The expression of COX-2 protein was markedly decreased. Thus, CM may be useful in certain type of inflammation, allergy and infectious disorders.

MATERIALS AND METHODS

Reagents

Murine rIFN-γ (1×10^6 U/ml) was purchased from Pharmingen (Mnchen, Germany). LPS and sodium nitrite were purchased from Sigma (St. Louis, MO). Recombinant TNF-α, biotinylated TNF-α and anti-murine TNF-α were purchased from R & D system Inc, USA. Recombinant IL-6, biotinylated IL-6 and anti-murine IL-6 were purchased from Pharmingen. Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). 0.4 μm syringe filter and tissue culture plates of 96 wells, 4 wells and 100 mm diameter dishes were purchased from Nunc (Naperville, IL). DMEM containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY). Male C57BL/6 mice were purchased from Damul Science Co. (Daejeon, Republic of Korea).

Peritoneal macrophages culture

TG-elicited macrophages were harvested 3 4 days after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously (Chung *et al.*, 2002). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of CM

The plant sample was obtained from Gochang GukHwa festival Committee (Gochanggun, chonbuk, Republic of Korea). The dried samples (250 g) were extracted with 6.72 ml of 85% methanol for 2 h at room temperature with ultrasonic waves. This step was repeated with sediment. The methanol extracts (total of 6,250 ml) were concentrated into 6.72 g plant materials using a Rotary evaporator. An extract of CM was lyophilized and kept at 4°C. Dilutions were made in distilled water then filtered through 0.45 μm syringe filter.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations of CM. The cells were then stimulated with rIFN-γ (20 U/ml). After 6 h, the cells were finally treated with LPS (10 μg/ml). NO synthesis in cell cultures was measured

by a microplate assay method, as previously described (Chung *et al.*, 2002). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were pretreated with various concentrations CM. The cells were then incubated with for 6 h with rIFN-γ (20 U/ml). They were finally stimulated with LPS (10 μg/ml) for 24 h. Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in phosphate-buffered saline (PBS)-Tween-20 (Sigma) for 1 h at room temperature and then incubated with anti-COX-2 antibodies. After washing in with PBS containing 0.05% tween-20 three times, the blot was incubated with secondary antibody for 1 h and the antibody-specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Assay of cytokine release

Peritoneal macrophages (2.5×10^5 cells/well) were incubated with rIFN-γ (20 U/ml), CM, rIFN-γ plus LPS (10 mg/ml) and rIFN-γ plus various concentrations of CM for 24 h. Then the amount of TNF-α and IL-6 secreted by the cells were measured by a modified enzyme-linked immunosorbent assay (ELISA), as described previously (Jeong *et al.*, 2003). ELISA (TNF-α and IL-6) was carried out in duplicate in 96-well ELISA plates coated with each of 100 μl aliquots of anti-human TNF-α and IL-6 monoclonal antibodies at 1.0 μg/ml in PBS at pH 7.4 and was incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, sample or TNF-α and IL-6 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 mg/ml of biotinylated anti-mouse TNF-a and IL-6 was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37°C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α and IL-6 in serial dilutions.

Statistical analysis

Results were expressed as the mean ± S.E.M. of independent experiments, and statistical analysis was performed by one-way analysis of variance (ANOVA) to express the difference among the groups.

RESULTS

Effects of CM on cell viability

To determine the effects of CM on viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with CM (100 μg/ml), it had no effect on cell viability (Fig. 1).

Inhibitory effects of CM on NO production

To determine the effect of CM on the production of NO by mouse peritoneal macrophages, we pretreated the cells with various concentration CM (1, 10 and

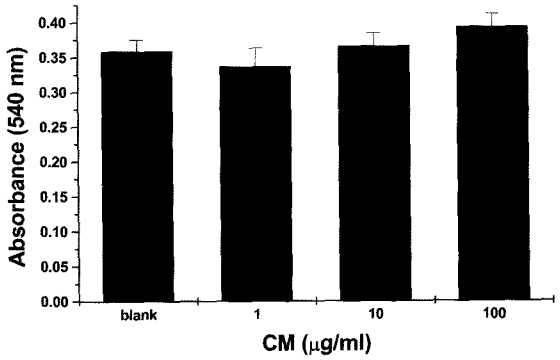

Fig. 1. Effect of CM on the cell viability. Cell viability was evaluated by MTT colorimetric assay 48 h after CM treatment in peritoneal macrophages. Values are the mean ± S.E.M. of three independent experiments duplicate in each run.

100 μg/ml). And then we stimulated them with rIFN-γ (20 U/ml) and LPS (10 μg/ml). The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. When mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with LPS, NO production was increased about 10 folds. CM had no effect on NO production in resting mouse peritoneal macrophages compared

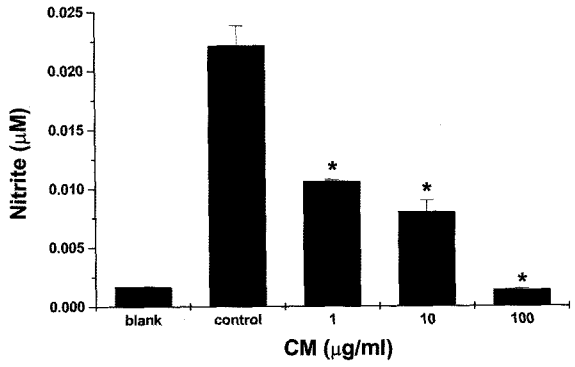

Fig. 2. Dose-dependent effects of CM on NO inhibition in rIFN-γ and LPS-treated peritoneal macrophages. Peritoenal macrophages (2.5×10^5 cells/well) were cultured with various concentration CM. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicate in each run. $^*P < 0.05$ compared to rIFN-γ + LPS.

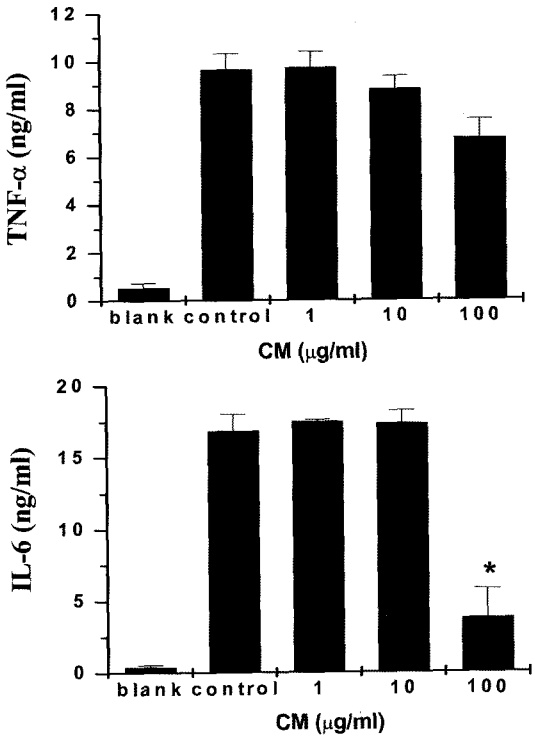

Fig. 3. Effects of CM on the rIFN-γ and LPS-induced TNF-α and IL-6 production in peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were stimulated with various concentrations of CM or rIFN-γ (20 U/ml) and LPS (10 μg/ml) plus CM. The amount of TNF-α and IL-6 secreted by peritoneal macrophages was measured by ELISA method after 24 h incubation. Values are the mean ± S.E.M. of three independent experiments duplicate in each run. $^*P < 0.05$ compared to rIFN-γ plus LPS.

to non-primed conditions. When CM was pretreated in primed cell, CM inhibits NO production dose dependently (Fig. 2).

Inhibitory effects of CM on TNF-α and IL-6 production

We examined the inhibitory effect of CM on LPS induced TNF-α and IL-6 production. Mouse peritoneal macrophages secreted low levels of TNF-α and IL-6 after 24 h incubation with medium alone. The basal level of TNF-α and IL-6 was little increased when incubated CM only. Upon IFN-γ (20 U/ml) plus LPS (10 mg/ml) treatment for 24 h,

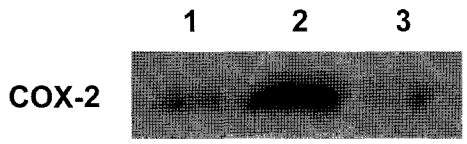

Fig. 4. Effects of CM on the expression of COX-2 by rIFN-γ plus LPS-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with CM and then stimulated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein extracts were prepared, and then samples were analyzed for COX-2 expression by Western blotting as described in the method. 1, blank; 2, rIFN-γ + LPS; 3, CM (100 μg/ml) + rIFN-γ + LPS.

TNF-α and IL-6 drastically increased in these cells, and pretreatment of cells with 100 mg/ml of CM for 24 h inhibited TNF-α induction in mouse peritoneal macrophages (Fig. 3).

Effects on expression of COX-2 protein

We investigate the effect of the CM (100 μg/ml) at translational level, As shown in Fig. 4, the expression of COX-2 protein were markedly increased after IFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 24 h. This increased expression of COX-2 protein was significantly reduced by CM (Fig. 4).

DISCUSSION

Murine macrophage exhibits a particularly vigorous response to endotoxin, which induces production of variety of inflammatory modulators such as NO, TNF-α, IL-6 and prostaglandins by inducible COX-2.

NO has been recognized to be an important mediator of celluar communication in several preparations such as macrophages, neutrophils, smooth muscle, autonomic nervous system, and central nervous system (Blackman *et al.*, 2000; Koyanagi *et al.*, 2000; Sharma *et al.*, 2000). In this study, exposure of macrophages to IFN-γ and LPS for 48 h was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This IFN-γ and LPS-induced NO production was inhibited by CM without notable cytotoxicity.

TNF-α is a key mediator in immunoinflammatory diseases. Administration of TNF-α induces shock, whereas treatment of mice with neutralizing monoclonal antibodies anti-TNF-α prevents the mortality caused by LPS challenge (Remick *et al.*, 1990). In this study, we showed that CM inhibit IFN-γ and LPS-induced TNF-α production. TNF-α also induces the secretion of cytokine such as IL-6 and activates T cells and other inflammatory cells (Vilcek and Lee, 1991). IL-6 plays an important role in the development of plasma cells and the induction of acute phase response, and it has also been demonstrated to induce neurite outgrowth in cell culture system. The secretion of IL-6 has been found to play a central role in the regulation of defense mechanism, and haematopoiesis. COX-2 plays a role in the pathophysiological processes including inflammation (Meade *et al.*, 1993).

We documented the increased production of COX-2 protein by macrophages exposed to IFN-γ and LPS. IFN-γ and LPS in combination with CM led to a significant reduction in COX-2 protein expression.

Here in our study, we have shown that CM exerts its anti-inflammatory effects probably by the suppression of COX-2 expression, and the final result is the inhibition of NO synthesis, TNF-α and IL-6 release. Based on our present results, it is possible that CM can offer a valuable means of therapy for the treatment of inflammatory diseases by attenuating IFN-γ and LPS-induced NO synthesis and pro-inflammatory cytokine and controlling of COX-2 expression.

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