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Immunomodulatory Effects of *Cimicifugae Rhizoma* Extracts in Macrophages

Suhkneung Pyo[1], Byung-Oh Kim[2], Hye-Sook Choi[1] and Eunwha Son[3†]

[1]*College of Pharmacy, Sungkyunkwan University, Gyeonggi 440-746, Korea*
[2]*Department of Applied Biology, Sangju National University, Gyeongbuk 742-711, Korea*
[3]*Department of Pharmacognosy Material Development, Kangwon National University, Gangwon 245-711, Korea*

Abstract

***Cimicifugae Rhizoma* (CR) belongs to the Ranunculaceae family, which has been traditionally used to treat climacteric complaints, antipyretics and diaphoresis as an alternative medicine for estrogen hormone replacement therapy with estrogens. Recently, it has been reported that different extract fractions of CR have various effects such as anti-allergic, anti-inflammatory and anti-proliferative activities. The current study investigated the immunomodulatory effects of *Cimicifugae Rhizoma* water extracts (CRE) in the macrophage-like cell line, Raw 264.7. Our results showed that CRE (1～50 μg/mL) stimulated tumoricidal activity and NO production, whereas phagocytic activity was inhibited at the same concentrations. Additionally, iNOS mRNA expression was significantly increased in Raw 264.7 exposed to CRE as demonstrated by RT-PCR. These results indicate that the tumoricidal activity induced by CRE may be mediated by the production of NO and these activities may be useful for the treatment of diseases such as cancer.**

Key words: *Cimicifugae Rhizoma*, nitric oxide, macrophage, immunomodulation, tumoricidal activity

INTRODUCTION

Macrophage have been shown to be an important component of host defense against bacterial infection and cancer (1,2). Large pools of macrophages, thought to be of mononuclear phagocyte origin, are located throughout the body and historically have been identified by different names including peritoneum (peritoneal macrophages), brain (microglia), bone (osteoclast) and liver (Kupffer cells). This host wide system of macrophages undergoes developmental changes in response to various signals in which they acquire or increase some functions and lose or decrease others. In the murine peritoneal system, four general stages of activation have been defined and cells in these stages are called resident, inflammatory (or responsive), primed and fully activated macrophages (3,4). When exposed in vitro IFN-γ and LPS, inflammatory macrophages can develop the capacity to kill tumor cells, and these stages are called fully activated macrophages. While at other stages of development, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (3,5). There has been especially great interest in the reactive nitrogen intermediate, nitric oxide (NO) which is considered to be a central molecule in the regulation of the immune response to tumors because of its cytotoxic effects (6-8). It is known that inducible nitrogen synthase (iNOS) is expressed in many different cell types and increased iNOS enzymes produce high levels of NO.

Recently, the biological and pharmacological properties of Korea traditional and Chinese herbs have begun to receive more attention in the scientific community and have become a very important research focal point. *Cimicifugae Rhizoma* (CR) belongs to the Ranunculaceae family which has been used traditionally to treat climacteric complaints, antipyretics and diaphoresis as an alternative for hormone replacement therapy (HRT) with estrogens. Recently anti-allergic, anti-inflammatory and anti-proliferative effects of different extract fractions of CR have been reported (9,10). It has been shown that CRE has an antitumor effect on various cancer cell lines and these effects were directly cytotoxic to cancer cells (11,12). However, the immunomodulatory effects of *Cimicifugae Rhizoma* water extracts (CRE) on macrophages are still unknown. Here we examined the immunomodulatory effects of CRE on the effects of macrophage-mediated tumoricidal activity and phagocytic acti-

†Corresponding author. E-mail: ehson@kangwon.ac.kr
Phone: +82-33-570-6492, Fax: +82-33-570-6499

vitis related to antimicrobial functions. Additionally, we determined the production of the intermediate regulatory molecule, NO, and the expression of iNOS transcriptional levels in Raw 264.7.

MATERIALS AND METHODS

Preparation of CRE

Dried *Cimicifugae Rhizoma* herb (1 kg) was ground into a powder and subsequently extracted with MeOH (1:1) for 2 hr at room temperature. The MeOH extract was evaporated under vacuum and suspended with D.W (800 mL). The suspension was extracted with n-butanol and CRE was fractionated with D.W. Yields of water extracts were 16.5% (w/w) relative to starting material for CR.

Macrophage-mediated antitumor activity

Macrophage cytotoxicity was assayed as described by Verstovsek et al. (2). Briefly, macrophages (1×10^5 cells/well) from mice were first incubated in either medium alone or in medium supplemented with various doses of CRE for 24 hr in 96-well plates. Macrophages were washed with RPMI-FBS to remove CRE and then co-incubated with B16 melanoma cells (1×10^4 cells/well; effector:target cell ratio of 10:1). After 24 hr, plates were stained with crystal violet containing 10% formaldehyde for 15 min. Absorbance of each well at 540 nm was determined by using Molecular Devices microplate reader (Menlo Park, CA, USA). Cytotoxic activity is expressed as the percentage of tumor cytotoxicity by the following formula:

[1−{OD of (target cells+macrophages)−OD of macrophages}/OD of target cells]×100

OD of target cells is the optical density of B16 melanoma cells and OD of macrophages is the optical density of macrophages.

MTT assay for cell proliferation

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay adapted from Mosmann (13). Briefly, the cells were seeded in 96-well plates at suitable densities of cells per well with various concentrations of CRE. After an incubation period of 24 hr, the enzyme activity of viable cells were measured by addition MTT to each well. After 4 hr of additional incubation, the amount of formazan was determined by absorbance at 540 nm using a microplate reader.

NBT assay for phagocytosis

Phagocytosis was measured by the nitro blue tetrazolium (NBT) reduction assay (14). Peritoneal macrophages were seeded in 96-well plates at a density 5×10^4 cells per well, treated with various concentration of CRE and cultured for 24 hr. The cultured media was then removed and 50 μL of 5×10^6 particles/mL zymosan and 0.6 mg/mL NBT was added into each well. After an additional incubation for 1 hr, wells were washed with cold D-PBS 2 times and the optical density of reduction product of NBT, a purple insoluble formazan, was determined at 540 nm using a microplate reader. It was not required to solubilize the formazan before taking the measurement of absorbance.

Nitrite determination

The cells were treated with the control media for 24 hr in the presence or absence of various doses of CRE and the accumulation of nitrite in culture supernatants was measured using the assay system described by Ding et al. (15). 100 μL aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNAs were extracted from Raw 264.7 cells cultured in presence or absence of CRE for 4 hr in 6-well plates using Trizol reagent. The total harvested RNA was measured using UV/Vis spectrophotometer at 260 nm. One μg of total RNA was reverse transcribed to cDNA using SuperScriptTM II (Invitrogen). Polymerase chain reaction (PCR) then was carried out in a final volume of 20 μL containing 1 μL of template, 0.25 μL of Taq DNA polymerase (Takara, Korea) and 20 pM of each primer for 20 cycles for β-actin and iNOS in a minicycler. Each cycle was held at 94°C for 30 sec.

The PCR products were applied to a 1.2% agarose/ethidium bromide gel for electrophoresis. The resulting gel was photographed under UV illumination. Single stranded cDNA was then amplified by PCR with specific primers of iNOS and β-actin: iNOS sense; 5′-GACAA GCTGCATGTGACATC-3′, iNOS antisense; 5′-GCTG GTAGGTTCCTGTTGTT-3′, β-actin sense; 5′-TGGAA TCCTGTGGCATCCATGAAAC-3′, β-actin antisense; 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Statistical analysis

The data is presented as a mean±SEM. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with a Dunnett's *t*-test. A p value$<$0.05 was considered significant.

Fig. 1. The effect of CRE on the proliferation of Raw 264.7 cells. Raw 264.7 (1×10^4 cells/well) were treated with CRE of various concentration for 24 and 48 hr. The proliferation of cells was determined by MTT assay. Cell density was measured at 540 nm. The data represents the mean±SE of quadruplicate experiments. $^{*}p<0.05$, $^{**}p<0.01$; significantly different from the control (no treatment).

Fig. 2. The effects of CRE on macrophage-mediated tumoricidal activity. Raw 264.7 (1×10^5 cells/well) were treated with various doses of CRE and co-cultured with the target at initial effector/target cell ration of 10:1. Macrophage tumoricidal activity was determined as described in material and methods. The formazan formation of macrophages was determined by MTT assay. Cell density was measured at 540 nm. The data represents the mean±SE of quadruplicate experiments. $^{*}p<0.05$; significantly different from the control (no treatment).

RESULTS AND DISCUSSION

Determine of CRE treatment condition

To determine the optimal concentration and cultivation time for CRE stimulation of macrophages, we evaluated the proliferative effect of CRE on Raw 264.7 for 24 and 48 hr. As shown in Fig. 1, CRE has a proliferative effect for 24 hr, but no effect at 48 hr. In addition, when the cells were treated with CRE at 500 μg/mL for 48 hr, cell cytotoxicity appeared. Therefore, in all subsequent experiments CRE 1～50 μg/mL and cultivation time 24 hr were used.

Effects of CRE on macrophage-mediated tumoricidal activity

To examine whether CRE treatment stimulates the tumoricidal activities of macrophages against target tumor cells, we co-cultured Raw 264.7 with B16 cells for 24 hr. B16 tumor cells were used as targets since they are either TNF-α or NO sensitive. Recently data suggested that CRE has a significant inhibitory effect on human prostate cancer cell lines (LNCaP) (10), but these effects were directly cytotoxic to cancer cells. Because immunomodulatory activity of CRE is still unknown, we examined the effects of CRE on macrophage-mediated antitumor activity. As shown in Fig. 2, CRE enhanced tumoricidal activity of macrophage at 50 μg/mL. It has been reported that CR contains biologically active compounds such as the cinnamic acid ester fukinolic acid, cimifugin, and cimicifugoside (16). Hemmi (11,12,17) reported that cimicifugoside has cytotoxicity against murine leukemia L1210 and mouse lymphoma L-5178Y cells. He also demonstrated the immunosuppressive effects of cimicifugoside on B- and T-cell functions (11,12,17). Based on these findings, it is reasonable to suggest that cimicifugoside plays a central role in the tumoricidal activity of CRE against macrophages.

Effect of CRE on phagocytosis

Phagocytosis is one of the primary functions of macrophages and leads to the enhancement of a diverse range of antimicrobial/cytotoxic responses, including generation of the respiratory bust, secretion of inflammatory mediators and antigen presentation. In this study, we examined the effect of CRE on phagocytosis by macrophages. Unexpectedly, CRE inhibited phagocytosis at the same concentration (50 μg/mL) which enhanced the tumoricidal activity (Fig. 3). When stimulated macrophages reach the fully activated stage which can delete tumor cells, they decrease other functions such as phagocytic activity (4). Therefore, we can postulate that CRE stimulated macrophage into the fully activated stage, at which point the activated macrophage lose phagocytic activity.

Effect of CRE on nitric oxide production and iNOS mRNA expression

Since macrophages activation plays an important role in the host defense mechanism and NO is related to cytotoxic function of macrophages against a variety of tu-

(A)

Fig. 3. The effects of CRE on phagocytosis in macrophages. Raw 264.7 cells (1×10^4 cells/well) were treated with various concentrations of CRE for 24 hr. The phagocytic activity of peritoneal macrophages was assessed by NBT (nitro blue tetrazolium) reduction assay. The purple insoluble formazan was measured at 540 nm. The data represents the mean±SE of quadruplicate experiments. $^{*}p<0.05$; significantly different from the control (no treatment).

(B)

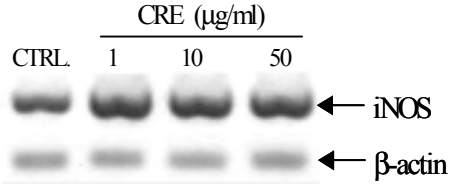

Fig. 4. The effects of CRE on production of nitrite (A) and expression of iNOS mRNA in Raw 264.7 cells (B). Raw 264.7 (1×10^4 cells/well) were treated with various concentration of CRE for 24 hr. Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. The data represents the mean±SE of quadruplicate experiments. $^{**}p<0.01$; significantly different from the control (no treatment). Raw 264.7 were treated with CRE for 6 hr. Total RNA was extracted and subjected to RT-PCR analysis for iNOS and β-actin mRNA.

mors and microorganisms (7,8,18,19), we examined the effects of CRE on NO production in macrophages. NO was produced in high amounts by iNOS in activated macrophages. It is known that excessive formation of NO mediates the bacterial and tumoricidal actions of macrophages. Thus, we also determined that iNOS mRNA expression in Raw 264.7 induced CRE. As shown in Fig. 4, the treatment of the cells with CRE increased the production of NO and iNOS transcriptional expressions. In the present study, our data demonstrates that CRE has immunomodulatory effects on macrophage functions, especially tumoricidal activity. Moreover, CRE increased NO production and iNOS mRNA expression. Therefore, the tumoricidal activity induced by CRE appeared to be mediated by the production of NO. Several recent publications demonstrate that CRE reduces the inflammatory reaction related to inflammatory mediators including histamine, bradykinin, and cyclooxygenase induction (20). However, there are no reported studies in the literature of either in vitro or animal studies showing CRE modulation of macrophage NO production. This study demonstrated that CRE augments the tumoricidal activity of macrophages that could be correlated with inducing the release of NO. Therefore, CRE might have therapeutic potential for cancer patients. However, it is possible that, under pathological conditions, high output of NO is associated with tissue damage observed in inflammatory conditions such as arthritis and nephritis (6-8). Moreover, NO plays important roles in the pathogenesis of gram-positive septic shock (21). Thus, the level of NO could be important in various physiological conditions. At the present time, the molecular mechanisms by which CRE facilitates tumoricidal activity and the signal pathway to iNOS gene expression by exposure to CRE remain to be elucidated. In the present study, our data suggest that CRE treatment may have immunotherapeutic usefulness in cancer therapy.

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