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Augmentation of macrophage functions by an aqueous extract isolated from *Platycodon grandiflorum*

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Abstract

Platycodon grandiflorum has been claimed to have a wide range of health benefits, which include immunostimulation and antitumor activity. The associated biological mechanisms are unclear; however, of the wide diversity of effects, it is believed that their activities may be exerted through several potent effector cells such as macrophages. Therefore, the effects of an aqueous extract from the root of *P. grandiflorum* (Changkil: CK) on mouse peritoneal macrophage function were investigated. It was found that CK stimulated macrophage proliferation, spreading ability, phagocytosis, cytostatic activity, and nitric oxide production in a dose-dependent manner, and that the production of cytokines such as TNF-α, IL-1β and IL-6 were similarly increased. CK significantly affected secretion at concentrations greater than 10 μg/ml; its maximal effects were at the concentration of 100 μg/ml. Reverse transcription-polymerase chain reaction showed that CK increased the appropriate cytokine mRNAs. These results suggest that CK is a potent enhancer of macrophage function.

Keywords: *Platycodon grandiflorum*; Macrophages; Activation; Cytokine

1. Introduction

Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), commonly known as Doraji (Chinese drug, 'Jiegeng', and Japanese name, 'Kikyo') has been used as a traditional oriental medicine [1]. Extracts from the roots of *P. grandiflorum* have been reported to have wide ranging health benefits. In Korea, the root of *P. grandiflorum* (4 years old) is used as a food, and employed as a folk remedy for adult diseases [1,2]. Some studies on its chemical [3,4] and immunopharmacological effects [1,5–7] have been performed but little is known about its immunostimulating effects and its associated mechanisms. Recently, it has been observed that the root of *P. grandiflorum* (22 years old) is beneficial to obese patients with adult-onset diabetes mellitus and lung cancer (unpublished data) and that dietary *P. grandiflorum* helps prevent hypercholesterolemia and hyperlipidemia [2].

In a previous study, we demonstrated that Changkil (CK) aqueous extract from the root of *P. grandiflorum*

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cultivated for more than 20 years (S.H. Lee (1991) Patent on the method of cultivating the perennial balloonflower, patent No. 045971, Korea) inhibits the growth of various transplantable tumors in experimental animals and increases the survival rate [8]. Thus, we believe that this preparation may well have important antitumor and immune stimulating properties. CK is considered to exert its antitumor activity through the potentiation of the host animal's defense system, rather than by directly inhibiting tumor cell growth [8]. It has also been reported that the methanolic extract from *P. grandiflorum* promoted the phagocytic activity of the mouse reticuloendothelial system and peritoneal macrophage [9].

Macrophages play a significant role in the immune system as part of the host's defense mechanism. Macrophages are the first cells to recognize invading foreign bodies and are central to cell-mediated and humoral immunity. When activated they enhance proliferation, cell morphologic changes such as spreading ability, phagocytosis, and nitric oxide (NO) and cytokine production and they inhibit the growth of a wide variety of tumor cells and microorganisms [10]. NO is identified as the major effector molecule in the destruction of tumor cells by activated macrophages [11]. The involvement of NO during non-specific host defense, macrophage-mediated killing, or the inhibition of the proliferation of microorganisms and tumor cells has been previously demonstrated [11]. Furthermore, several studies have demonstrated that activated macrophages produce NO, and suggested that the reactive nitrogen intermediates play a significant role in tumoricidal and microbicidal activity [12]. TNF-α and IL-1β produced by activated macrophages also have been recognized and well characterized as important host defense molecules, which affect tumor cells [13,14]. Cytokines like IL-1β and TNF-α are also involved in the generation of reactive nitrogen intermediates [15].

Although CK is believed to augment the immune response by modulation of macrophage function, the precise mechanism involved in the augmentation of cell-mediated immunity remains to be elucidated. At the present time, there is no clear understanding of the molecular or cellular basis of immunostimulation by CK, because cytokines are mediators in all aspects of immunoregulation [10]. The capacity to induce or enhance cytokine production could be a major mechanism which enables CK to exert its immunomodulating effect. We have previously reported upon the beneficial effects of CK on antitumor activity [8]. In this study, we examined the hypothesis that CK may act by enhancing macrophage function. As far as we know, there is no evidence that CK augments macrophage functions, and we now report that CK enhanced some of the functions of macrophages, such as proliferation, spreading ability, phagocytosis, cytostatic activity, NO secretion, and the gene expression of TNF-α, IL-1β, and IL-6.

2. Materials and methods

2.1. Plant material

CK is the aqueous extract from the roots of *P. grandiflorum* (22 years old), which were supplied by Jang Saeng Doraji Co., Jinju, South Korea. CK was prepared as follows. Distilled water at 90°C was added to powered root (5 ml/g) and the temperature was maintained for 10 h. The mixture was allowed to cool to room temperature, filtered, and lyophilized. The yield of lyophilized residue corresponded to 33.5% of the original dry root weight. The pale-yellow extract powder was dissolved directly in distilled water.

2.2. Chemicals

Chemicals and cell culture materials were obtained from the following sources: oligo(dT) 18 primers, Moloney leukemia virus (M-MLV) reverse transcriptase (RT), RPMI 1640, fetal bovine serum (FBS), and penicillin-streptomycin solution (Life Technologies, Inc.); *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) and Polymyxin B sulfate (Sigma); AmpliTaq® DNA polymerase (Perkin Elmer); dNTP solutions and ribonuclease (RNAse) inhibitor (Takara Technologies, Inc.); goat monoclonal antibody against murine TNF-α, biotinylated goat polyclonal antibody against murine TNF-α, recombinant murine TNF-α, horseradish peroxidase streptavidin, 3,3′,5,5′-tetramethylbenzidine, and H_2O_2 (R&D Systems). Polymerase chain reaction (PCR) oligonucleotide primer pairs were custom synthesized by Bioneer Co. (Korea).

2.3. Animals

Specific pathogen-free BALB/C mice (female, 5–7 weeks old) were obtained from KRIBB (South Korea). The mice were housed under normal laboratory conditions, i.e. at 21–24°C and 40–60% relative humidity, with a 12 h light/dark cycle with free access to standard rodent food and water.

2.4. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from mice that had been injected intraperitoneally with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated on a flat-bottom culture plate and then incubated for 2 h at 37°C in a 5% CO_2 humidified incubator. After removing the non-adherent cells, the mono-layered macrophages were treated with CK. Sarcoma 180 tumor cells were maintained in a RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and used as target cells in a cytostatic assay of macrophages.

2.5. Endotoxin assay

The E-Toxate (Limulus Amebocyte Lysate; Sigma) test was used, following the manufacturer's instructions, to test all solutions for the presence of the gram-negative bacterial endotoxin, LPS.

2.6. Assay for proliferative activity of macrophages

Proliferative activity was measured according to the MTT method previously described by Tada et al. [16] with slight modifications. Peritoneal macrophages on a 96-well culture plate (2×10^5/well) were incubated with CK for 48 h at 37°C in 5% CO_2, and MTT solution (50 μg/well) was added. After incubation for an additional 6 h, the formazan crystals were dissolved with acidified isopropanol. Plates were read on a FL600 (Bio-Tek) microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Mean background values were obtained by scanning blank wells. Proliferative activity is expressed as the ratio of the test absorbance to the control absorbance at 570 nm.

2.7. Measurement of the spreading ability of macrophages

Peritoneal macrophages on 96-well culture plates (1×10^5/well) were incubated with CK for 48 h at 37°C in 5% CO_2. After incubation, spreading macrophages exhibiting pseudopods were counted, and the spreading ability was expressed as a percentage of spreading macrophages in a total of 300 macrophages.

2.8. Assay for macrophage phagocytosis

Peritoneal macrophages on 48-well culture plates (1×10^6/well) were incubated with CK for 48 h and then treated with FITC-latex microbeads (ϕ 0.75 μm; Polysciences) and CK, and then incubated for 90 min at 37°C. After incubation, the cells were washed with phosphate-buffered saline containing 2% FBS, and phagocytic capability was measured with a fluorescence spectrophotometer (FL600, Bio-Tek) in intact cells grown in 48-well plates as previously described [17]. LPS was used as a positive control.

2.9. Assay for macrophage cytostaticity

Peritoneal macrophages on 96-well culture plates (2×10^5/well) were incubated with CK for 48 h at 37°C in 5% CO_2. After washing, cell cytostaticity on tumor cells was assayed by a co-culture contacted with 10^4 viable sarcoma 180. The cultures were incubated for 18 h, and MTT solution (50 μg/well) was added to each well. Macrophage cytostaticity was measured according to the MTT method previously described by Tada et al. [16] with slight modifications, as mentioned in Section 2.6. Peritoneal macrophages without CK treatment were cultured as controls. The percentage of cytostaticity was calculated by the following formula: cytostaticity (%) = [1 − (absorbance at 570 nm of experiment/absorbance at 570 nm of control)] × 100.

2.10. Nitrite assay

Peritoneal macrophages (2×10^5 cells/ml) prepared as above were cultured in 48-well plates. After 24 h of incubation, nitrite in culture supernatants, the stable

reaction product of NO with molecular oxygen, was determined by using Griess reagent.

2.11. Measurement of cytokine production

For cytokine immunoassay, peritoneal macrophages were cultured for 6 h at a density of 2×10^6 cells/ml in 24-well plates. Supernatants were removed at the allotted times and IL-1β, IL-6, and TNF-α production were quantified by sandwich immunoassays using a protocol supplied by R&D Systems.

2.12. RNA extraction

For RNA extraction, cells (1×10^6/ml) were cultured with CK. After 6 h incubation, the cells were lysed in 4 M of guanidinium thiocyanate solution. Total RNA was extracted according to the single-step method previously described by Chomczynski and Sacchi [18]. Before use, the integrity and purity of the RNA samples were checked by electrophoresis. Visualization of the 28S and 18S rRNA species as discrete bands, and the absence of high molecular weight species were taken to indicate that the samples were largely intact and free of genomic DNA contamination.

2.13. Reverse transcription-polymerase chain reaction

The reverse transcription reactions (20 μl total volume) were carried out in 5 mM $MgCl_2$, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) using 1 mM dNTPs, 1.75 units/μl RNAse inhibitor, 2.5 units/μl M-MLV RT, 25 μg/μl oligo(dT) primers and 25 ng of total RNA. Tubes were incubated at room temperature for 10 min before reverse transcription in a thermal cycler at 42°C for 60 min, and 94°C for 3 min. After the generation of the first strand cDNAs, the tubes were placed on ice for 5 min prior to either being stored at −20°C or used in the polymerase chain amplification step. The PCR reactions (100 μl total volume) were carried out in 2 mM $MgCl_2$, PCR buffer using 2.5 units of AmpliTaq® DNA polymerase, 5 μg/ml of sense and antisense primer pair and 20 μl of reverse transcription volume. The sense and antisense primer sequences used in this study are described in Table 1. These primer sets yielded PCR products of 754, 692 and 153 bp for iNOS, TNF-α and β-actin, respectively. PCR (35 cycles for iNOS and TNF-α cDNAs and 25 cycles for β-actin cDNA) was performed using the GeneAmp PCR System 2400 (Perkin Elmer). The initial PCR cycle was 94°C for 5 min, 60°C for 5 min and 72°C for 90 s, with the following temperature profile: denaturation at 94°C for 45 s, primer annealing at 60°C for 45 s and primer extension at 72°C for 90 s, with a final cycle of 94°C for 45 s, 60°C for 45 s and 72°C for 10 min.

Once the PCR reactions were complete 0.05 or 0.2 volumes of the reaction mix, depending upon the expected amount of product, were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation.

2.14. Statistical analysis

All experiments were repeated at least three times. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.05 was considered significant.

Table 1
Oligonucleotide primers used in the reverse transcription-polymerase chain reactions

Gene	Sense/antisense	Primer sequence	Expected size of PCR products (bp)
IL-1β	Sense	5′-ATGGCAACTGTTCCTGAACTCAAC-3′	563
	Antisense	5′-CAGGACAGGTATAGATTCTTTCCTTT-3′	
IL-6	Sense	5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′	638
	Antisense	5′-CACTAGGTTTGCCGAGTAGATCTC-3′	
TNF-α	Sense	5′-ATGAGCACAGAAAGCATGATCCGC-3′	692
	Antisense	5′-CCAAAGTAGACCTGCCCGGACTC-3′	
β-Actin	Sense	5′-CCTCTATGCCAACACAGT-3′	153
	Antisense	5′-AGCCACCAATCCACACAG-3′	

3. Results

3.1. Effects of CK on macrophage functions

Table 2 shows the effect of CK on some macrophage functions. The macrophage proliferative activity of CK was measured by MTT assay. CK significantly increased the proliferation of macrophages. The spreading ability of macrophages was measured by counting the number of spreading macrophages that exhibited pseudopods. CK enhanced the spreading ability of macrophages, and significantly enhanced some macrophage functions such as proliferative response in a dose-dependent manner in the concentration range 10–100 μg/ml (Table 2).

3.2. Effect of CK on macrophage phagocytosis

The stimulation effect of CK on macrophage phagocytosis was measured by internalization of the FITC-latex microbeads using a fluorescence spectrometer and estimated by the mean fluorescence intensity. CK-treated macrophages were efficient in the internalization of the microbeads, as when macrophages were stimulated with CK, they internalized microbeads more so than the controls (Table 3). These results demonstrate that CK has the ability to enhance macrophage phagocytosis.

Table 2
Effect of CK on macrophage functions

Treatment (μg/ml)[a]	Proliferative activity[b]	Spreading ability[c]
Control	1.00 ± 0.12[d]	1.00 ± 0.11
CK (1)	1.12 ± 0.13	1.16 ± 0.14
CK (10)	1.87 ± 0.19*[e]	2.25 ± 0.24*
CK (50)	2.57 ± 0.26*	3.02 ± 0.31*
CK (100)	3.71 ± 0.39*	5.22 ± 0.54*
LPS (0.5)	3.83 ± 0.40*	5.71 ± 0.59*

[a] Macrophages were incubated with CK for 48 h, and their functionality was assessed.
[b] Proliferative activity is expressed as the ratio of the test absorbance to the control absorbance at 570 nm.
[c] Spreading ability was expressed as a percentage of spreading macrophages in a total of 300 macrophages.
[d] Results are expressed as the mean ± SD of four independent experiments performed in triplicate.
[e] $*P < 0.01$, significantly different from the control.

Table 3
Effect of CK on macrophage phagocytosis and NO production

Treatment (μg/ml)	Macrophage phagocytosis[a]	Nitrite[b]
Control	236.4 ± 24.1[c]	4.61 ± 0.45
CK (1)	248.6 ± 25.3	5.41 ± 0.53
CK (10)	374.5 ± 38.4*[d]	12.68 ± 1.36*
CK (50)	513.2 ± 52.5*	31.45 ± 3.28*
CK (100)	645.7 ± 63.1*	47.63 ± 0.59*
LPS (0.5)	655.1 ± 65.9*	61.11 ± 6.34*

[a] Macrophages were incubated for 48 h, and then cells were washed twice and further incubated with FITC-latex microbeads for 90 min. The phagocytic capability of macrophages was measured using a fluorescence spectrophotometer. Macrophage phagocytosis is expressed as fluorescence intensity.
[b] Macrophages were incubated for 24 h and NO production was determined by measuring nitrite accumulation in the incubation medium.
[c] Results are expressed as the mean ± SD of four independent experiments performed in triplicate.
[d] $*P < 0.01$, significantly different from the control.

3.3. Effect of CK on NO production

Macrophages were incubated with CK for 24 h, and NO concentrations in the culture supernatants were assessed by the Griess reaction. CK showed a significant effect on NO production from a dose of 10 μg/ml. The effect of CK gradually increased between concentrations of 10 and 100 μg/ml, and reached a plateau at 100 μg/ml (Table 3). Upon CK stimulation, NO synthesis by peritoneal macrophages increased in a dose-dependent manner. The potent macrophage activator LPS (0.5 μg/ml) also increased nitrite synthesis compared to the control.

3.4. Effect of CK on cytostatic activity of macrophages

In order to assess the other functional activities stimulated by CK, the tumor cytostaticity of CK-stimulated macrophages was tested at various concentrations against sarcoma 180 (Fig. 1). The macrophages showed strong cytostatic activity against the sarcoma 180 tumor cells when stimulated at a concentration of 10–100 μg/ml. On the other hand, CK showed no direct killing potential against sarcoma 180 tumor cells at a concentration of 100 μg/ml. These findings demonstrate that CK has an ability to augment macrophage cytostaticity against tumor cells.

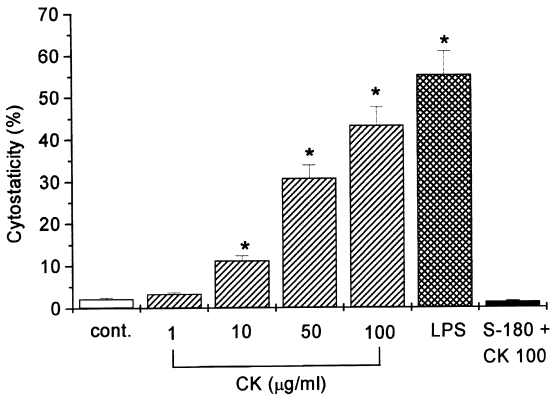

Fig. 1. Cytostatic activity of macrophages stimulated with CK to tumor cells. Macrophages were incubated with CK or medium alone for 48 h. Cells were then washed twice and further co-cultured with sarcoma 180 (S-180). The cultures were incubated for 18 h, and macrophage cytostaticity was measured according to the MTT method. Cytostaticity (%) = [1 − (absorbance at 570 nm of experiment/absorbance at 570 nm of control)] × 100. Each bar shows the mean ± SD of four independent experiments performed in triplicate. *$P < 0.01$, significantly different from the control.

3.5. Analysis of LPS contamination in CK

Macrophages can be induced to produce NO, and to ensure that the observed ability of CK to induce NO was due to LPS contamination, CK was tested for the presence of contaminating LPS by the *Limulus* amoebocyte lysate test. CK was found to have a LPS level below the lowest level of assay sensitivity, which was typically below 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used as an LPS inhibitor in macrophage cultures [19]. Although CK contained no detectable activity in the *Limulus* amoebocyte lysate assay, in order to further check for contaminating LPS in CK, CK and LPS were separately pre-treated with polymyxin B for 30 min at 37°C, and then examined for effectiveness in terms of macrophage activation, as judged by NO production. CK or LPS incubated under similar conditions in the absence of polymyxin B were used as controls. As shown in Table 4, nitrite production in LPS-treated macrophage was substantially blocked by the polymyxin B treatment. In contrast, the amount of nitrite released from CK-treated cells did not markedly decrease. These results suggest that the observed macrophage activation effects were not due to LPS.

Table 4
Effects of polymyxin B on NO production by CK and LPS

Treatment (μg/ml)[a]	Nitrite (μM)[b]
Control	2.47 ± 0.43*,**[c]
Polymyxin B (10)	2.27 ± 0.41*,**
CK (100)	48.47 ± 4.79
CK + polymyxin B	42.17 ± 4.32
LPS (0.5)	62.21 ± 6.38
LPS + polymyxin B	8.39 ± 0.85**

[a] Macrophages were cultured with CK or LPS in the presence or absence of polymyxin B.
[b] Supernatants were harvested 24 h later and assayed for NO. Values are the mean ± SD of three individual experiments performed in triplicate.
[c] *$P < 0.01$, significantly different from CK; **$P < 0.01$, significantly different from LPS.

3.6. Effects of CK on macrophage-related cytokines production

To assess the effects of CK on TNF-α, IL-1β and IL-6 production by activated macrophages, peritoneal macrophages were incubated in the presence of increasing concentrations of CK, and the quantities of these cytokines secreted into the culture supernatants were monitored by ELISA. As was the case for NO production, CK increased TNF-α, IL-1β, and IL-6 secretion in the supernatant in a dose-dependent manner (Table 5). The secretion of these cytokines might be due to the activation of macrophages via

Table 5
Effects of CK on macrophage-related cytokine production

Treatment (μg/ml)[a]	TNF-α (ng/ml)	IL-1β (ng/ml)	IL-6 (ng/ml)
Control	0.66 ± 0.07[b]	0.36 ± 0.04	0.22 ± 0.03
CK (10)	3.03 ± 0.29*[c]	1.81 ± 0.19*	0.81 ± 0.08*
CK (50)	6.52 ± 0.63*	2.44 ± 0.25*	1.27 ± 0.12*
CK (100)	8.73 ± 0.81*	3.52 ± 0.34*	2.15 ± 0.19*
LPS (0.5)	14.23 ± 1.51*	4.25 ± 0.43*	3.93 ± 0.41*

[a] Macrophages were cultured for 6 h (TNF-α and IL-6) or 12 h (IL-1β) in the presence of media alone with CK or LPS. The amount of TNF-α, IL-1β, and IL-6 released to culture medium was measured by immunoassay.
[b] Results are expressed as the mean ± SD of four independent experiments performed in triplicate.
[c] *$P < 0.01$, significantly different from control.

the autocrine or paracrine action of the secreted TNF-α, IL-1β or IL-6.

3.7. Effects of CK on the gene expression of macrophage-related cytokines

As described above, CK induced macrophage secretion of TNF-α, IL-1β and IL-6. In order to determine whether CK regulates these cytokine secretions at the mRNA level, a reverse transcription-polymerase chain reaction assay was conducted. LPS (0.5 μg/ml), a known immunostimulating agent, was used as a positive control. Consistent with the results obtained from the cytokine secretion immunoassays, TNF-α, IL-1β, and IL-6 mRNA levels were markedly increased by CK treatment (Fig. 2), which indicates that CK upregulated TNF-α, IL-1β, and IL-6 accumulation in macrophages in a dose-dependent manner. Control β-actin was constitutively expressed and was unaffected by CK treatment. Therefore, the increase of TNF-α, IL-1β, and IL-6 by CK is believed to be regulated by transcriptional activation.

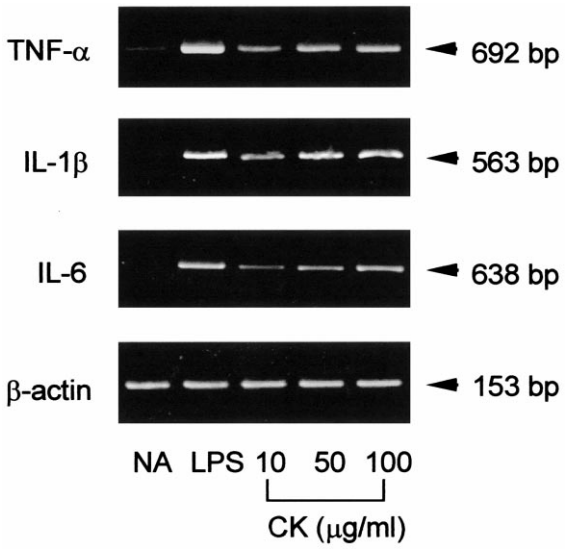

Fig. 2. Effects of CK on the gene expression of macrophage-related cytokines. Macrophages were cultured for 6 h in the presence of media alone, and with CK or LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for reverse transcription-polymerase chain reaction analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed on each sample. PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide as described in Section 2. One of three representative experiments is shown.

4. Discussion

Platycodon grandiflorum is claimed to have a wide range of health benefits, including immunostimulation and antitumor effects [1,8]. However, the biological mechanisms of these activities are unclear. Given this claimed wide diversity of biological activity it has been suggested that macrophage activation might present a common mechanism. In other to understand the beneficial effects of CK, which is an aqueous extract from the root of *P. grandiflorum*, we attempted to elucidate its immunostimulatory effects, especially its enhancement of macrophage activation, such as phagocytosis, cytostaticity, and cytokine production.

Macrophages are important regulatory cells that are central to cell-mediated and humoral immunity as antigen-presenting, and tumoricidal and microbicidal cells [10]. Many of these activities are mediated by the release of different cytokines, and therefore cytokine production can be taken as indicative of the degree of macrophage activation. The present results showed that CK enhances macrophage phagocytosis. The enhancement of macrophage phagocytosis by *P. grandiflorum* has been reported by Kubo et al. [9]. However, there is still no evidence as to whether it was attributable to the activation of macrophages by *P. grandiflorum*. In our previous study, we demonstrated that CK is capable of potently inhibiting the growth of sarcoma 180 in mice as revealed by a drastic reduction in tumor weight and prolongation of survival after treatment. Because CK did not affect the in vitro growth of cancer cells, the antitumor activity was assumed to be mediated by immunostimulation [8]. Therefore, the antitumor activity of CK is likely to be mediated by a potentiation of the host's defense system, such as by macrophage activation in animals, rather than by the direct inhibition of tumor cell growth. These observations support the view that CK has an ability to induce mononuclear phagocytes. In this report, we also showed that CK enhances the proliferation and cytostatic activity of macrophages. The present study evidences the induction of macrophage cytostaticity by CK. Therefore, it is possible to conclude that its antitumor effects are closely asso-

ciated with macrophage activation. This suggests that macrophages stimulated by CK may participate in the further activation of lymphocyte functions.

Humoral defense via antibody response is mediated by B cells and other immune cells involved in antigen processing and immunization. In these processes of humoral response, CK might accelerate or potentiate each step by macrophage activation. Cell-mediated immune defense is mediated specifically by T cells, including cytotoxic T cells, and non-specifically by macrophages and natural killer cells. Because CK activates macrophages, it may potentiate cell-mediated defense. This hypothesis is supported by our previous observation that CK lengthened the survival time of tumor-bearing mice without showing direct cytotoxicity to tumor cells [8].

Cytotoxicity against tumor cells is dependent on the activation of macrophages, and the expression of these mechanisms strongly correlates with the pattern of production of several cytokines. Of these factors, NO, TNF-α, IL-1β, and IL-6 were investigated in the current study to confirm the possibility that CK might be an immunostimulator. The production of NO, TNF-α, IL-1β, and IL-6 was found to be augmented in CK-treated murine peritoneal macrophages. CK had a significant effect on NO, TNF-α, IL-1β, and IL-6 secretion at levels above 10 μg/ml. These results suggest that the secretion of NO, TNF-α, IL-1β, and IL-6 is regulated by the same mechanism, or that the TNF-α, which the first cytokine produced, induces NO, IL-1β, and IL-6 secretion via an autocrine or paracrine system. TNF-α is known to be the first compound in the NO, TNF-α, IL-1β, and IL-6 series to be secreted by macrophages [20]. Thus, TNF-α is involved in the early phase of the cytokine cascade and induces NO, IL-1β, and IL-6 production. The expression levels of NO, TNF-α, IL-1β, and IL-6 genes were found to be augmented in CK-treated macrophages. In this study, the results suggest that CK may induce TNF-α, IL-1β, and IL-6 both at the transcriptional and protein levels. Recently, we have shown that iNOS gene expression is increased in macrophages (unpublished data). The above results show the possible roles of macrophages in the anti-tumor activity of CK. Since NO is related to the cytolytic function of macrophages against a variety of tumors [21], the increased synthesis of NO might interfere with tumor growth. The indirect actions of macrophages were also activated by CK; for example, augmented processing of tumor antigen and lymphokine-mediated helper T cell activation by macrophages also possibly helped in the retardation of tumor growth.

Macrophages stimulated by CK produced TNF-α and IL-1β, which suggests that CK can induce the production of cytokines in TNF-α and IL-1β resulting in the enhancement of TNF-α cytostaticity. IL-6 is a cytokine produced mainly by antigen-presenting cells, and it has multiple biological activities in various cell types. In the development of cell-mediated immune responses, IL-6 synergizes with IL-1β and promotes T cell proliferation, the differentiation of T helper cells and the development of T cell-mediated cytotoxicity by $CD8^+$ cells [22,23]. In summary, CK stimulates macrophage-derived NO, TNF-α, IL-1β, and IL-6 production and is able to upregulate the expression of these cytokines in macrophages, which may explain the antitumorigenic properties of *P. grandiflorum*. These results also raise the possibility that CK may induce Th immune responses.

In conclusion, the results reported here suggest that CK augmented several macrophage functions, increased the secretion of several cytokines by macrophage, and thus potentially modulated the host's immune response. It is likely that CK will be useful clinically as a biological response modifier to activate macrophages. Although we showed that CK activates peritoneal macrophages to induce cytokines, we cannot exclude the possibility that CK activates normal B and T cells. This should be further studied. In order to investigate the overall antitumor effect of CK, a study on the in vivo induction of gene expression and the production of immunomodulatory cytokines in mice is in process (unpublished data). The in vitro approaches employed here should be useful in any future mechanistic characterization of the immune properties of CK and in any study into enhancing its health benefits.

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References

[1] E.B. Lee, Pharmacological studies on Platycodon grandiflorum A. DC. IV. A comparison of experimental pharmacological effects of crude platycodin with clinical indications of platycodi radix, Yakugaku Zasshi J. Pharm. Soc. Jpn. 93 (1973) 1188–1194.

[2] K.S. Kim, O. Ezaki, S. Ikemoto, H. Itakura, Effects of Platycodon grandiflorum feeding on serum and liver lipid concentrations in rats with diet-induced hyperlipidemia, J. Nutr. Sci. Vitaminol. 41 (1995) 485–491.

[3] A. Tada, Y. Kaneiwa, J. Shoji, S. Shibata, Studies on the saponins of the root of *Platycodon grandiflornm.* A. DE CANDOLLE. I. Isolation and the structure of Platycodin-D, Chem. Pharm. Bull. 23 (1975) 2965–2972.

[4] T. Saeki, K. Koike, T. Nikaido, A comparative study on commercial, botanical gardens and wild samples of the roots of Platycodon grandiflorum by HPLC analysis, Planta Medica 65 (1999) 428–431.

[5] K. Takagi, E.B. Lee, Pharmacological studies on *Platycodon grandiflorum* A. DC. Activities of crude platycodin on respiratory and circulatory systems and its other pharmacological activities, Yakugaku Zasshi J. Pharm. Soc. Jpn. 92 (1972) 969–973.

[6] M. Kiibo, T. Nagao, H. Matsuda, K. Namba, Immune pharmacological studies on platicodi radix. I. Effect on the phagocytosis in the mouse, Shoyakugaku Zasshi J. Pharm. Soc. Jpn. 40 (1986) 367–374.

[7] T. Nagao, H. Matsuda, K. Namba, M. Kubo, Immune pharmacological studies on platicodi radix (II). Antitumor activity of inulin from platicodi radix, Shoyakugaku Zasshi J. Pharm. Soc. Jpn. 40 (1986) 375–380.

[8] Y.S. Kim, B.E. Lee, K.J. Kim, Y.T. Lee, K.B. Gho, Y.C. Chung, Antitumor and immunomodulatory activities of the *Platycodon grandiflorum* cultivated for more than 20 years, Yakhak Hoeji J. Pharm. Soc. Korea 42 (1998) 382–387.

[9] M. Kubo, T. Nagao, H. Matsuda, K. Namba, Immune pharmacological studies on platycodi radix (I). Effect on the phagocytosis in mouse, Shoyakugaku Zasshi J. Pharm. Soc. Jpn. 40 (1986) 367–374.

[10] J.M. Cavaillon, Cytokines and macrophages, Biomed. Pharmacother. 48 (1994) 445–453.

[11] R. Farias-Eisner, M.P. Sherman, E. Aerberhard, G. Chaudhuri, Nitric oxide is an important mediator of tumoricidal activity in vivo, Proc. Natl. Acad. Sci. USA 91 (1994) 9407–9411.

[12] R.B. Lorsbach, W.J. Murphy, C.J. Lowenstein, S.H. Snyder, S.W. Russell, Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing, J. Biol. Chem. 268 (1993) 1908–1913.

[13] F.R. Balkwill, M.S. Maylor, S. Malik, Tumor necrosis factor as an anticancer agent, Eur. J. Immunol. 26 (1990) 641–644.

[14] L. Gorelik, Y. Bar-Dagan, M.B. Mokyr, Insight into the mechanism(s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearer spleenic cells, J. Immunol. 156 (1996) 4298–4308.

[15] A. Khar, B.V.V. Pardhasaradhi, C.H. Varalakshmi, A.M. Ali, A.L. Kumari, Natural killer cell as the effector which mediates in vivo apoptosis in AK-5 tumor cells, Cell. Immunol. 177 (1997) 86–92.

[16] H. Tada, O. Shiho, K. Kuroshima, M. Koyama, K. Tsukamoto, An improved colorimetric assay for interleukin 2, J. Immunol. Methods 93 (1986) 157–165.

[17] I. Suzuki, H. Tanaka, Y. Adachi, T. Yadomae, Rapid measurement of phagocytosis by macrophages, Chem. Pharm. Bull. 36 (1988) 4871–4875.

[18] P. Chomczynski, N. Sacchi, Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 16 (1987) 156–159.

[19] C.L. Manthey, M.E. Brandes, P.U. Perera, S.N. Vogel, Taxol increases steady-state levels of lipopolysaccharide-inducible gene and protein-tyrosine phosphorylation in murine macrophages, J. Immunol. 149 (1992) 2459–2465.

[20] J.H. Martin, S.W. Edwards, Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. Reactive oxygen intermediates are involved in monocyte-mediated cytotoxicity, whereas reactive nitrogen intermediates are employed by macrophages in tumor cell killing, J. Immunol. 150 (1993) 3478–3486.

[21] J.B. Hibbs, R.R. Taintor, Z. Vavrin, Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite, Science 235 (1987) 473–476.

[22] M. Okada, M. Kitahara, S. Kishimoto, T. Matsuda, T. Hirano, T. Kishimoto, IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells, J. Immunol. 141 (1988) 1543–1549.

[23] F. Houssiau, J. Van Snick, IL-6 and the T-cell response, Res. Immunol. 143 (1992) 740–743.