Immunomodulatory activity of phytoprotein isol *Acanthopanax senticosus*

: Regulation of CTL responses and activation of macrop

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We previously reported that the phytoprotein extracted from Acanthopanax senticosus (PA) had anti-carcinogenic anti-metastatic activity via increase of inhibition of gap junctional intercellular communication. In the present study investigated the immunomodulatory mechanism of phytoprotein isolated from the stem bark of Acanthopanax sentic (PA). PA was found to significantly stimulate macrophages producing TNF-α and IL-1β in vitro. It also showed tumori activity indicating that PA had the ability to stimulate macrophage directly. Moreover, PA induced the $CD8^+$ CTL cy responses to recognize antigen on the B16-BL6 melanoma cells. Treatment of PA with B16-BL6 melanoma cells increased the proliferation of splenocytes compared with untreated control. These results demonstrate that PA immunomodulatory activity suggesting a useful anti-tumor agent applicable to treatment and prevention of cancer.

Key words : Acanthopanax senticosus, phytoprotein, macrophage, Cytotoxic T lymphocyte, cytokines, TNF-α, IL-1β

Introduction

Approaches to immunotherapy to cancer have advanced with identification of tumor antigens, cytokines and antigen presenting and interacting mechanisms[1,2]. The most important antigen presenting cells are dendritic cells and macrophages[3,4]. Macrophages recognize cellular debris, damaged cells and foreign invaders. Also, macrophages are continuously involved in the controlled metabolism of lipids and iron and in host response to injury such as inflammation. Macrophages not only provide the first line of defense against microbial infections and parasitic infestations, but also participate in the surveillance against foreign invaders and cancer. There are two major physiological pathways for the activiation of macrophages to become cytotoxic against microorganisms, parasites, or cancer. Macrophages are readily activated to a microbicidal state subsequent to interaction with microorganisms or their products. The other category of activating agents with the potential for therapeutic use is the cytokines[5-7].

Cytotoxic T lymphocytes (CTLs) recognize short peptides presented on MHC class I molecules. CTL responses are a major branch of the immune system which can remove infected cells and tumor cells and can inhibit viral replication by non-lytic means[8,9]. Because CTLs represent a major arm of the immune response against cancer, the elicitation of a specific CTL response against tumor antigens is one of the main aims of current immunotherapy trials[10].

Traditional Oriental medicine always pays attention to the strengthening of the patients general resistance against illness, and there are many Oriental herbal medicines such as Astragalus mongholicus and Panax ginseng used for thousands of years are considered as tonics, immunomodulating agents, which stimulated macrophages, promoted antibody formation, activated complement and increased T lymphocyte proliferation[11,12]. Acanthopanax senticosus has been used for the treatment arthritis and immunological ailments in Oriental and foreign medicines[11,13,14]. Acanthopanax senticosus was also used for treatment of skin diseases, variant tumors, inflammation, apoplexy and pain condition[15,16]. We previously

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reported that the phytoprotein extracted from Acanthopanax senticosus (PA) has anti-carcinogenesis and anti-metastatic activity via increase of inhibition of gap junctional intercellular communication induced H_2O_2 (unpublished data). However, its anti-cancer action and mechanism, specially immunomodula -ting property, were not fully investigated. Therefore, here we investigated the immunomodulating and tumoricidal properties of PA in vivo and in vitro. This report provides that PA may useful anticancer potential via activation of CTLs and macrophages.

Materials and methods

1. Extraction of Acanthopanax senticosus

An aqueous extract of Acanthopanax senticosus (Rupr. et Max.) Harms was prepared as previously reported[17]. Briefly, the chopped trunks of Acanthopanax senticosus were placed in approximately 10 volumes of distilled water, and then stirred at 4 ℃ overnight. The supernatant was filtered, evaporated and lyophilized to produce powder. An appropriate amount of the powder was dissolved in PBS, filtered through 0.2 (m filters and then stored at 4 ℃ until use. For extraction of phytoprotein, an aqueous extract of Acanthopanax senticosus was saturated with 50% ammonium sulfate at 4 ℃ for 3 hrs and centrifuged at 10,000 g for 20 min. After removal of supernatant, precipitate was resuspended, dialyzed and concentrated by removing ammonium sulfate using desalting column (Bio Rad Co. CA, USA).

2. Animals

Specific pathogen-free male C57BL/6 mice were purchased from Daehan Biolink Co., Ltd. (Korea). They were provided with standard laboratory mouse food and water ad libitum and were kept at 20 ± 1 ℃, 60% humidity in a 12-hr light and dark cycle. All mice were used at 6 ~ 12 week-old for the experiments.

3. Tumor Cells

B16-BL6 melanoma and S-180 fibrosarcoma cell lines were obtained from Korean Cell Line Bank (KCLB; Korea). B16-BL6 cells were maintained as monolayer cultures in Minimal Essential Medium (MEM; GibcoBRL, NY, USA) supplemented with 7.5% fetal bovine serum (FBS), non-essential amino acid, Antibiotic-Antimycotic and L-glutamine. S-180 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL) supplemented with 10% FBS, Antibiotic-Antimycotic and L-glutamine. All cultures were maintained in a 37 ℃ humidified atmosphere of a 5% CO_2/95% O_2 air.

4. Preparation of mouse splenocytes and peritoneal exudate macrophages (PEMs)

Splenocytes were obtained by passing pieces of spleen through a stainless mesh, treated with a hypotonic solution to lyse erythorocytes, and washed three times with PBS. The viability of the splenocytes was more than 95%, as assessed by the trypan blue dye exclusion method. Whole splenocytes were suspended in RPMI 1640 medium supplemented with 10% FBS and then used for experiments. Peritoneal exudate cells (PECs) were collected by peritoneal lavage 4 days after i.p. injection of 1 ml of 3% thioglycollate broth, and resuspended in serum free RPMI 1640 medium. The macrophage population was prepared by the plastic adherence procedure. PECs (1×10^6 cell/well) were incubated on 24-well culture plates for 2 hr at 37 ℃, and non-adherent cells were removed by vigorous washing with PBS. The remaining adherent cells were used as peritoneal exudates macrophages (PEMs).

5. Induction of cytokine production

TNF-α and IL-1 β levels in the macrophage culture supernatants were evaluated using specific ELISA kits (Endogen, IL, USA) according to the manufacturer's instructions. Cell-free supernatants was prepared as follows. Macrophages (1×10^6 cell/well) were prepared as described above and then cultured in the 24-well culture plates in the presence or absence of PA for 24 hrs. Lipopolysaccharide (LPS), a macrophage mitogen, was used as a positive control. The cell-free supernatants were collected from each well, centrifuged at 6,000 rpm for 1 min and then stored at -80 ℃ until ELISA assay.

6. Assay for cytostasis

Cytostatic activity was determined by using MTT assay with some modifications[18]. Briefly, BALB/c mice were injected i.v. with 500 μg of PA. After 36 hrs, peritoneal exudate macrophages (PEMs) were collected by peritoneal lavage from mice. S-180 target cells were incubated with PEM monolayers (2×10^5/well) in 96-well culture plates at PEM : target cell ratiso of 2.5, 5 and 10 : 1. After 20 hrs incubation, cytostatic activity against the tumor cells was assessed by the MTT assay. The cytostatic activity caused by PEM was calculated as follows:

$$\text{Cytostatic activity (\%)} = [1 - (\text{OD of target cells with PEM} - \text{OD of PEM}) / \text{OD of target cells}] \times 100$$

7. Assay of allogenic CTL activity

Allogenic CTL activity was evaluated by lactic acid

dehydrogenase (LDH) releasing method two days after the boosted immunization as described previously with some modifications[19]. Briefly, BALB/c mice were subcutaneously injected with inactivated B16-BL6 melanoma cells (UV irradiation for 2 hrs; 1 (10^4/mouse) with or without PA (500 mg/mouse) 2 times at intervals of 2 weeks. 4 days after the last injection, splenocytes (effecter cells) were harvested from mice. Harvested splenocytes (2 × 10^5/well) were incubated with live B16-Bl6 melanoma cells (target cells) in 96-well culture plates at E/T ratios of 12.5, 25, 50 and 100 :1. After 6 hrs incubation, viable cells were counted by LDH Kit. (promega, WI, USA). LDH activity was determined with a standard curve and specific lysis for each E/T ratio is expressed as follows:

CTL activity (%) = [(experimental release - spontaneous release) / (target maximum - target spontaneous release)] × 100

8. Restimulation assay of splenocytes administrated with PA

BALB/c mice were subcutaneously injected with inactivated B16-BL6 melanoma cells (UV irradiation for 2 hrs; 1 × 10^4/mouse) with or without PA (500 mg/mouse) 2 times at intervals of 2 weeks. 4 days after the last injection, splenocytes were harvested and the harvested splenocytes (2 × 10^5/well) were added to the 96-well culture plates containing inactived B16-BL6 melanoma cells (UV irradiation for 2 hrs; 3 (10^5 ~ 3.75 10^4/well). Viable cells were examined by XTT assay. Finally, the products were evaluated using an ELISA microplate reader (Molecular Devices, CA, USA) at 450-650 nm.

9. Statistical analysis

Representative data from each experiments are presented as mean values ± SD. The statistical differences between the groups were determined by applying the Student's two tailed t-test. Statistical significance was defined as a P value < 0.05.

Results

1. Induction of cytokines from macrophages stimulated with PA

The production of TNF-α, and IL-1α and β molecules has been shown to be associated with the proliferation and activation of cells of the monocyte/macrophage series[20]. Therefore, we first examined whether PA can induce the production of cytokines such as TNF-α and IL-1α by macrophages for the investigation of immunomodulating activities of PA,. Macrophages were collected by peritoneal lavage and treated with PA by the concentrations of 20, 100 and 500 μg/ml, respectively. As shown in Table 1, PA-treated macrophages increased the release of the IL-1α and TNF-α in a concentration-dependent manner. Specially, PA was shown similar tendency to LPS, a macrophage mitogen. Therefore, PA not only can induce cytokine production from macrophages, but also has the mitogenic ability to induce cytokine.

Table 1. Induction of cytokines from macrophages stimulated with PA

Groups	Concentration (μg/ml)	Production of cytokines (pg/ml)	
		IL-1β	TNF-α
Normal	(	2.2	0
Acanthopanax phytoprotein	20	12	261
	100	43	445
	500	36	700
LPS	1	127	851

LPS : macrophage mitogen, positive control

2. Cytostatic effect of macrophges administered with PA on tumor cells

We next examined the cytostatic activity of PEM obtained from BALB/c mice administered PA (500 μg/mouse). As shown in Fig. 1, the i.v. injection of PA activated PEM to become cytostatic against the S-180 sarcoma cells. And this cytostatic activity was E/T ratio dependent.

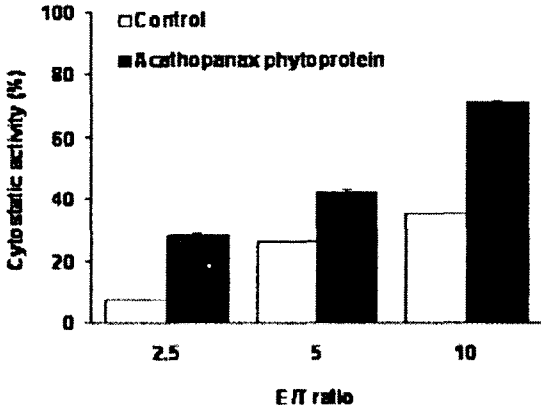

Fig. 1. Cytostatic activity of macrophages administered with PA on tumor cells (S-180) from mice. BALB/c mice were injected i.v. with 500 μg of PA. After 36 hrs, PEMs were collected by peritoneal lavage from mice and S-180 target cells were incubated with PEM monolayers (2 (10^5/well) in 96-well culture plates at a PEM : target cell ratio of 2.5, 5 and 10 : 1. After a 20-hrs incubation, cytostatic activity against the tumor cells was assessed by the MTT assay.

3. Effect of PA on enhancement of CTL activity against allogenic tumor cells.

The activity of CTLs treated with PA against tumor cells was investigated. As shown in Fig. 2, CTL activity was increased in B16-BL6-treated group comparing to control group. Moreover, CTL activity was higher in both B16-BL6 and PA-treated group than in only B16-BL6-treated group. An increase of E/T ratio also results in an increase of CTL activity.

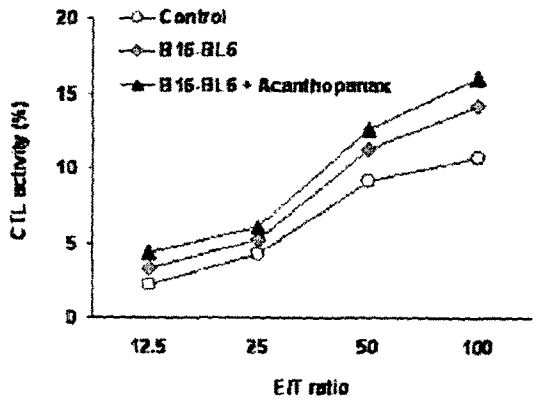

Fig. 2. Effect of PA on enhancement of CTL activity against allogenic tumor cells. BALB/c mice were injected subcutaneously inactivated B16-BL6 melanoma cells (UV irradiation for 2 hrs; 1 (10^6/mouse) with or without PA (500 mg/mouse) 2 times at intervals of 2 weeks. 4 days after the last injection, splenocytes (effecter cells) were harvested from mice and splenocytes (2 × 10^5/well) were incubated with live B16-Bl6 melanoma cells (target cells) in 96-well culture plates at E/T ratios of 12.5, 25, 50 and 100 :1. After 6-hrs incubation, viable cells were counted by LDH releasing method.

4. Effect of PA on the proliferation of splenocytes

The effect of PA on the proliferation of splenocytes was examined. The proliferation of splenocytes significantly increased when B16-BL6 alone or B16-BL6 and PA were treated (Fig. 3). These results were similar to the results of CTL activity. Therefore, these data supported the results of CTL activity by PA.

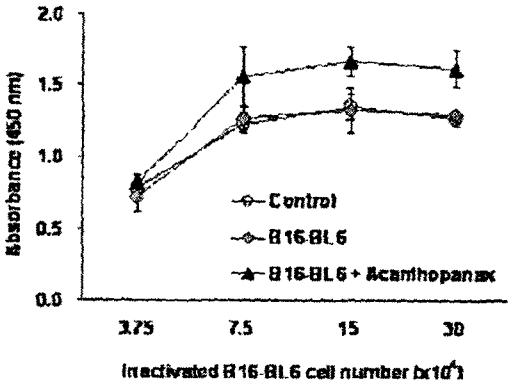

Fig. 3. Effect of PA on the proliferation of splenocytes. BALB/c mice were injected subcutaneously inactivated B16-BL6 melanoma cells (UV irradiation for 2 hrs; 1 (10^6/mouse) with or without PA (500 mg/mouse) 2 times at intervals of 2 weeks. 4 days after the last injection, splenocytes were harvested and the harvested and splenocytes (2 × 10^5/well) were added to the 96-well culture plates containing inactived B16-BL6 melanoma cells (UV irradiation for 2 hrs; 3 (10^6 ~ 3.75 10^4/well). Viable cells were examined by XTT assay.

Discussion

Individual tumor cells were originated by abnormalities of genes but the increase of tumor cells to cancer is due to decline of immune system. Therefore, it has long been recognized that modulation of the immune system by various agents may have potential for the management of certain infectious and neoplastic diseases. Natural products as well as chemically synthesized compounds have been investigated for immunotherapeutic potential[2,21]. Over the years, the clinical efficacy of those agents has been investigated[2,11,12,15,17,18]. Acanthopanax senticosus has been used for the treatment arthritis and immunological ailments in oriental medicines[11] and water crude extract of stem bark of Acanthopanax senticosus has been reported to have immunopharmacological activity[13,14] and anti-cancer effect[16]. We previously isolated the phytoprotein of Acanthopanax senticosus (PA) and investigated its anti-carcinogenesis and anti-metastatic activity (unpublished data). In the present study, we focused on the immunomodulating activity of PA.

Pro-inflammatory cytokines, such as IL-1β, and TNF-α, are playing integral roles in the inflammatory pathway of systemic inflammatory response syndrome (SIRS) [22] and the level of these cytokines is important to some kinds of cancers [22, 23]. IL-1β is produced by monocytes, macrophages and B cells and it activats helper T cells. TNF-α, in combination with interferon (IFN)-γ can also activate macrophages to destroy intracellular parasites and cancer cells[24]. PA significantly enhanced TNF-α and IL-1β production by macrophages in vitro, following activation of macrophage (Table. 1). These data suggest that the treatment of PA is corelated with the activation of macrophages and B lymphocytes[25]. We also found that the injection of PA caused PEM to become cytostatic against S-180 tumor cells in vitro (Fig. 1).

CTLs, a population of $CD8^+$ T lymphocytes, destroy target cells after a prior exposure to specific antigen. The induction of CTLs require the interaction of various cells and is therefore a prime target for evaluating potential immunomodulation[10,26]. Therefore, these CTL responses must not only be initiated, but must also be vigorous and sustained so as to achieve successful tumor regression. In the present study, the injection of PA resulted in a significant induction of CTLs at the concentration of 500 mg/mouse (Fig. 2) indicating that PA had antitumor activity by stimulating macrophage directly[27]. Also, the proliferation of splenocytes significantly increased when B16-BL6 alone or B16-BL6 and PA were treated (Fig. 3) supporting the results of CTL activity by PA (Fig. 2). It has been reported that the activity of several cytokines enhanced various aspects of the CTL responses. For example, IFN-γ and the combination of IL-1 and IL-6 induced cytolytic

responses and acted directly on $CD8^+$ cells[28-30]. Also, anti-tumor effects mediated by the administration of recombinant cytokines, including IL-1[31], IL-2[32], IL-12[33], IFN-α[34], IFN-γ[35] and TNF-α[36], have been shown in tumor bearing mice.

In conclusion, this study reported that PA mediated anti-cancer effects through its immunomodulating activity such as activation of macrophages and CTLs, and cytokine production. This report provides the evidence that PA can activate CTLs mediated by macrophage activation and following cytokine production. Thus, PA would be useful and might be contribute to the clinical application in cancer treatment. However, we just showed the tumoricidal activity of PA in this study and for the detailed mechanism of PA activity, further studies will be required.

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