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Immunomodulatory activity of *Salicornia herbacea L.* Components

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Abstract – *Salicornia herbacea* is an annual herb growing in salt marshes and on muddy seashores. *Salicornia herbacea* has been used as a fork medicine as well as a seasoned vegetable. In fork medicine, *Salicornia herbacea* has been used to treat a variety of diseases such as constipation, obesity, diabetes, asthma, arthritis and cancer. However, the biological mechanisms for these activities have not been characterized, nor the active components. The immunomodulatory activity of *Salicornia herbacea* components were studied in the present study. The components of *Salicornia herbacea* were prepared from the whole plant by passage through a fine screen, and then dialyzed against PBS overnight. Immunomodulatory activities of the *Salicornia herbacea* components were examined on a mouse macrophage cell line, RAW 264.7 cells. The *Salicornia herbacea* components were shown to stimulate cytokine production, nitric oxide release, and expression of surface molecules in a dose dependent manner. The *Salicornia herbacea* components also induced further differentiation of slightly adherent RAW 264.7 cell into strongly adherent macrophages. These results indicate that *Salicornia herbacea* contains immunomodulator(s) that induces activation of macrophages.
Keywords – *Salicornia herbacea*, Immunomodulator (s), Macrophage

Introduction

Salicornia herbacea has no leaves, but is formed of cylindrical, jointed branches of a light green color, smooth, very succulent and full of salt, bitterish juice. The whole plant is greedily devoured by cattle for its saltish taste. In South Korea, *Salicornia herbacea* grow naturally in the western coast area (Lee., 1997). *Salicornia herbacea* has been used as a fork medicine as well as a seasoned vegetable by some people living in coastal area. *Salicornia herbacea* contains very useful organic matters and many important minerals to human. Chemical composition of *Salicornia herbacea* reported by Korea National Fisheries Research and Development Institute is as follows : moisture content 90.9%, Fe 84.8 mg, Ca 650 mg, Na 1888.8 mg, Mg 50 mg, K 650 mg, Zn 29.6 mg and I 70 mg per 100 g of dry weight. Amino acids such as glutamic acid, aspartic acid, tyrosine, lysine, taurine and praline have been identified from *Salicornia herbacea* (Min, *et al.*, 2002). *Salicornia herbacea* also contains also betaine and choline (Shin, *et al.*, 2002). Betaine plays a critical role in the health of the cardiovascular system and liver. Betaine reduces potentially toxic levels of homocysteine, an amino acid found normally in the body. In recent years studies have suggested that a high level of homocystaine increases a person's chance of developing heart disease, stroke, liver disease, and peripheral vascular disease (Millian, *et al.*, 1998). Choline is a major component of phospholipid and acethylcholine (Zeisel, *et al.*, 1994).

Salicornia herbacea has long been used as a fork medicine, but the biological mechanisms of these activities and active components have not been clarified. In this study, we examined the effects of *Salicornia herbacea* on macrophage cell line, RAW 264.7 cells. LPS was used as a positive control for macrophage activation. Macrophage activation was expressed as increased cell size, cytoplasmic spreading, nitric oxide (NO) release, cytokine production, and expression of some adhesion molecules.

Material and Methods

Plant material and preparation of *Salicornia herbacea* components – *Salicornia herbacea* was collected in September, 2001 from the Boryeong, Chungnam in South Korea. The components of *Salicornia herbacea* were prepared from the whole plant by passage through a fine screen, and then dialyzed (MWCO, 3,500) against PBS overnight. To ensure that the effects on RAW 264.7 cell were not due to endotoxin contamination of *Salicornia herbacea*, all solution

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were pass through the Affi-Prep Polymyxin Matrix (*BIO-RAD*) column. Briefly, 1ml of Affi-Prep Polymyxin Matrix was packed with 1 ml of *Salicornia herbacea* components in a Bio-spin column (*BIO-RAD*) and incubated overnight at 4°C.

Cell culture – RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 streptomycin (Invitrogen), and 50 μM 2-mercptoethanol (Invitrogen) at 37°C, 5% CO_2 condition.

Cytokine production – RAW 264.7 cells were cultured in DMEM in 24-well-flat plates at a density of 5×10^5 cells/well. Cells were cultured in the presence of *Salicornia herbacea* components at different concentrations for 48 hours. Each dilution was tested in triplicated. Supernatant concentrations of IL-1β and TNF-α were measured using commercially available ELISA kit (R&D system). The absorbance at 450 nm was measured in ELISA plate reader (Dynatech MR 500).

Nitric Oxide production – RAW 264.7 cells were cultured in the presence of *Salicornia herbacea* components in a 24-well flat bottom microtiter plate (5×10^5 cells/well) in a total volume of 1ml for 48h. NO concentration of culture supernatant was determined by measuring the accumulation of the nitrate using the method described by Lalitha Rananoorthy *et al.* (1996).

Phenotypic analysis – RAW 264.7 cells were seeded in 6 well tissue culture plates at a density of 1×10^6 cells / well and incubated in the presence of *Salicornia herbacea* components for 48 hours. Cells were stained with monoclonal antibodies recognizing murine cell surface markers according to method of Lee *et al* (2001). The monoclonal antibodies, anti-CD40 (clone 3/23), anti-ICAM-1 (clone3E2), anti-I-Ab (clone AF6-120.1), anti-B7-1(clone 16-10A1), anti-B7-2 (clone GL1), and isotype-matched control antibodies were purchased from Pharmingen (San Diego, CA). Flow cytometric analysis was performed on FACS Caliver (Becton-Dickinson). Dead cells were gated out by their low forward angle light scatter intensity. In most analysis, 10,000 cells were scored.

Proliferation assay – RAW 264.7 cells were cultured in 96-well plates at a concentration of 2×10^4 cells/well with *Salicornia herbacea* components. DNA synthesis was measured by [^{3}H]thymidine (Du Pont) incorporation (0.5 μCi/well) for the final 6 h of the 2 day culture period. At the end of the incubation period, the cells and their medium were aspirated onto GF/C glass fiber filter paper using a cell harvester. The filters were washed, dried, and then a microbeta liquid scintillation counter (Wallac, USA) was used to detect the incorporation of [^{3}H]thymidine. The counts were expressed as count per minute (cpm).

Results

Inhibition of growth – RAW 264.7 cells were cultured in the presence of different concentrations of *Salicornia herbacea* components for 48 h, and the degree of cell proliferation was measured by a [^{3}H]thymidine incorporation assay. As shown in Fig. 1, *Salicornia herbacea* components inhibited the growth of RAW 264.7 cells in a dose dependent manner. The growth inhibitory activity of *Salicornia herbacea* components is not due to direct cytotoxicity on the cells, but appears to be due to induction of further differentiations of RAW 264.7 cells. Normal RAW 264.7 cells, when cultured in medium alone, tended to be round. None of them appeared to have spread in the surface. When the morphological changes were observed using a phase-contrast microscope, we found that most of the cells has spread cytoplasm when cultured with 10 μl/ml of *Salicornia herbacea* components. Furthermore, we also found that slightly adherent RAW 264.7 cells became strongly adherent cells with a typical morphology of mature macrophages (data not shown). Because mature macrophage are end-stage cells and thus do not proliferate, we believe that the growth inhibitory activity of *Salicornia herbacea* components is due to the induction of further different of RAW 264.7 cells

Induction of cytokine production – Because *Salicornia herbacea* components appeared to induce differentiation of RAW 264.7 cells, we examined the effect of *Salicornia herbacea* components on the production of cytokine production in RAW 264.7 cells. Culture supernatants were collected from RAW 264.7 cells that were stimulated with *Salicornia herbacea* components, and the amounts of IL-1β and TNF-α were measured by ELISAs. *Salicornia herbacea* induced the production IL-1β strongly (Fig. 2). *Salicornia herbacea*

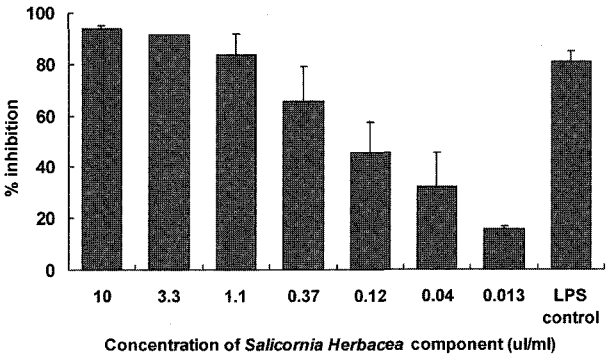

Fig. 1. Growth inhibition of RAW 264.7 cells in response to *Salicornia herbacea*. Raw 264.7 cells (1×10^5 cells/ml) were cultured with indicated amount of *Salicornia herbacea* components for 48 h. DNA synthesis was measured by ^{3}H-thymidine incorporation for the final 6 h of the culture period of 48 h. LPS was used at a concentration of 100 ng/ml. The illustrated data is a representative one from 3~4 independent experiments.

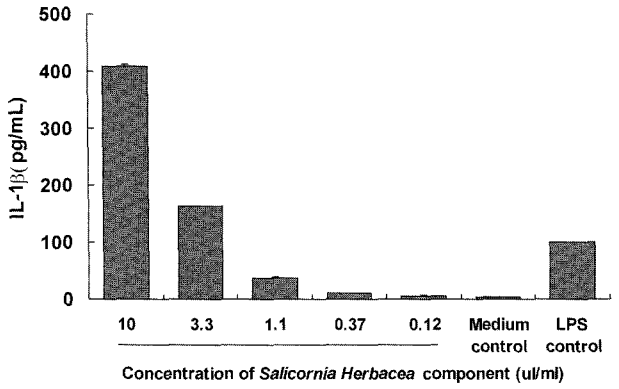

Fig. 2. IL-1β production in response to *Salicornia herbacea*. Raw 264.7 cells (5×10^5 cells/ml) were cultured with indicated amount of *Salicornia herbacea* components for 48 h. The culture supernatants were collected, and the amount of IL-1β was measured by an ELISA kit. Each dilution was tested in triplicated. LPS was used at a concentration of 100 ng/ml. The illustrated data is a representative one from 3~4 independent experiments.

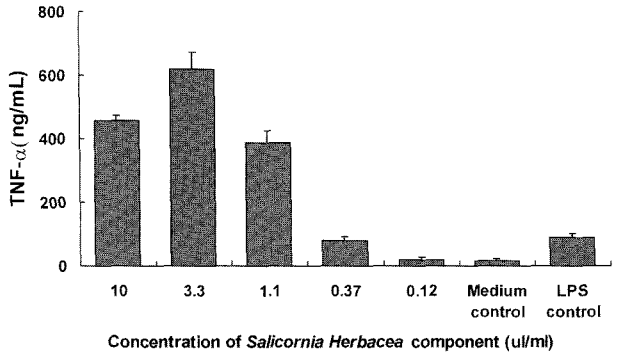

Fig. 3. TNF-α production in response to *Salicornia herbacea*. Raw 264.7 cells (5×10^5 cells/ml) were cultured with indicated amount of *Salicornia herbacea* components for 48 h. The culture supernatants were collected, and the amount of TNF-α was measured by an ELISA kit. Each dilution was tested in triplicated. LPS was used at a concentration of 100 ng/ml. The illustrated data is a representative one from 3~4 independent experiments.

components also induced the production of TNF-α (Fig. 3).

Induction of nitric oxide production – RAW 264.7 cells were stimulated with *Salicornia herbacea* components for 48 h after which accumulation of nitrate in the media was measured using Greiss reagent. As shown in Fig. 4, *Salicornia herbacea* components induced the production of NO in a dose dependent manner. *Salicornia herbacea* components induced maximum level of NO production even a low concentration (1.1 μl/ml).

Induction of surface molecule expression – As an another parameter of activation of RAW 264.7 cells, *Salicornia herbacea* components were added to cultures of RAW 264.7 cells, and then the cells were harvested and the level of expression of major co-stimulatory molecules were examined in a flow cytometry (Fig. 5). Expression of CD40 and B7-1

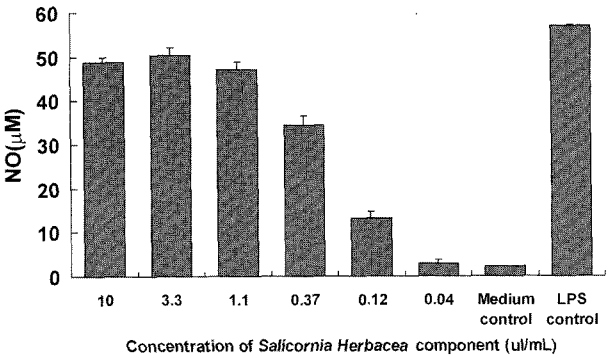

Fig. 4. Nitric oxide production in response to *Salicornia herbacea*. Raw 264.7 cells (5×10^5 cells/ml) were cultured with indicated amount of *Salicornia herbacea* components for 48 h. NO production was measured using Griess reagent. The amount of NO was determined using a standard curve generated with sodium nitrite. LPS was used at a concentration of 100 ng/ml. The illustrated data is a representative one from 3~4 independent experiments.

were increased by *Salicornia herbacea* components treatment. ICAM-1 molecules, which were constitutively expressed on resting cells, were not induced by *Salicornia herbacea* components. Ia and B7-2 molecules, which were expressed in a barely detectable level on resting cells, were not induced by *Salicornia herbacea* components.

Removal of endotoxin – To ensure that the activation and diggerentiation-inducing activity of *Salicornia herbacea* components is not due to endotoxin contamination, *Salicornia herbacea* components were treated with polymyxin B to remove possible contaminations of endotoxin. LPS incubated under similar condition in the absence of polymixin B were used as controls. As shown in Fig. 6, NO production in LPS treated cells was substantially blocked by polymyxin B treatment. In contrast, the amount of NO released from *Salicornia herbacea* components treated cells was not significantly decreased. These results suggested that the macrophage activation effects were not due to endotoxin contamination of *Salicornia herbacea*.

Discussion

The present study shows that *Salicornia herbacea* contains immunomodulator(s) that induces activation and further differentiation of macrophages. The *Salicornia herbacea* components were shown to stimulate cytokine production, nitric oxide release, and expression of surface molecules in a dose dependent manner. The immunomodulatory activity of *Salicornia herbacea* components on macrophage was not due to contamination of endotoxin.

Macrophages are activated by a wide variety of mechanisms (Adams, *et al.*, 1984), and are involved in at

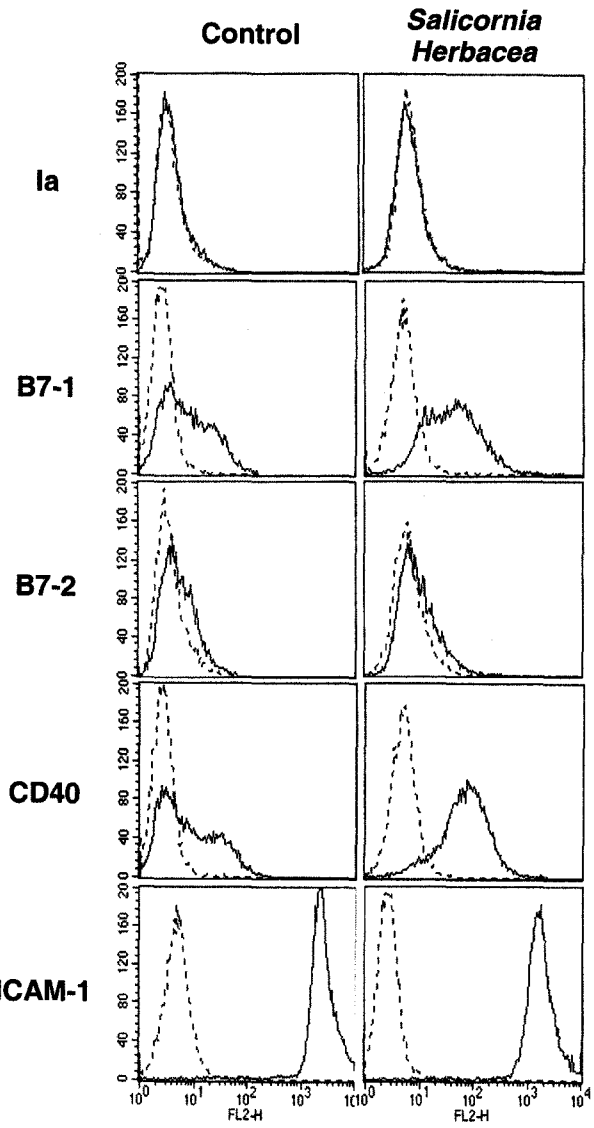

Fig. 5. Phenotypic analysis of RAW 264.7 cells stimulated with *Salicornia herbacea*. RAW 264.7 cells were stimulated with *Salicornia herbacea* components (10 μl/ml) for 48 h. Levels of expression (thin line) were illustrated in comparison to isotype control (dotted line). The illustrated data is a representative one from 3~4 independent experiments.

all stages of immune responses, particularly primary immune responses (Shamash, *et al.*, 2002). A number of different inflammatory stimuli, including IFN-γ, TNF-α and LPS, have been reported to induce NO production by macrophages (Kolls, *et al.*, 1994; Lowenstein, *et al.*, 1993; Xie, *et al.*, 1993). Reactive oxygen intermediates and NO are potent microbicidal agents that are produced within macrophages and able to kill ingested microbes. Activated macrophages stimulate acute inflammation through the secretion of cytokines, mainly TNF-α and IL-1β. IL-1β is a potent proinflammatory cytokine produced by a variety of cells

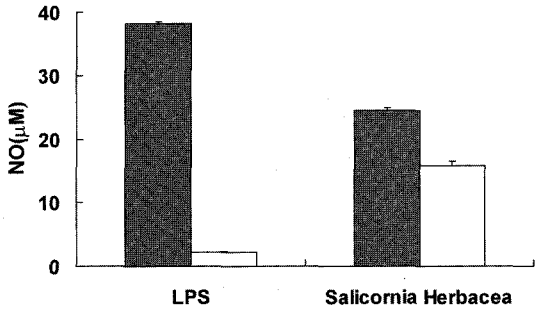

Fig. 6. Macrophage stimulatory activity of endotoxin-free *Salicornia herbacea* components. The samples, *Salicornia herbacea* components (10 μl/ml) and LPS (100 ng/ml), were treated with polymyxin B affinity column, respectively, and then examined for the NO stimulatory activity as described in Fig. 4. –■– : before polymyxin B treatment, – □ – : after polymyxin B treatment. The illustrated data is a representative one from 3~4 independent experiments.

including monocytes and macrophages. Among its functions are mediation of acute-phase response, chemotaxis and activation of inflammatory and antigen-presenting cells, upregulation of adhesion molecules, enhancement of neovascularization, and the ability to serve as a cofactor in lymphocyte activation. TNF-α and IL-1β share many biologic activities, in particular that of endogenous pyrogen and an ability to stimulate T cell proliferation (Shalaby, *et al.*, 1988; Yokota, *et al.*, 1988) and resistance to number of pathogens (Chen, *et al.*, 1992; Hoffman, *et al.*, 1993; Limper, *et al.*, 1997). Thus, activated macrophages become more efficient APCs because of increased levels of molecules involved in increased surface expression of class II MHC molecules and co-stimulators. These macrophage responses to pathogens enthance T cell activation, thus serving as amplication mechanisms for cell-mediated immunity.

The present study demonstrates that *Salicornia herbacea* components activate macrophages increasing NO release, cytokine production, expression of co-stimulatory molecules such as B7-1, CD40. In addition, *Salicornia herbacea* components induced further differentiation of RAW 264.7 cells, inhibiting their growth. Because LPS is a strong activator of macrophages, and is contaminated in many plant materials, we removed LPS from the *Salicornia herbacea* components by polymyxin B affinity column. The inability of polymyxin B to block the effect of *Salicornia herbacea* components suggests that the macrophage activating activity was not due to endotoxin contamination in the *Salicornia herbacea* components. *Salicornia herbacea* components were dialyzed (MWCO, 3,500) against PBS overnight. Thus, the immunomodulatory components of *Salicornia herbacea* must be high molecular weight substance. The growth inhibitory activity of *Salicornia herbacea* components was not due to direct cytotoxicity

on the cells, but appeared to be due to induction of further differentiations of RAW 264.7 cells. Taken together, these results show that *Salicornia herbacea* contains immunomodulator(s) that activates macrophages. Further studies are needed to clarify the active entity of *Salicornia herbacea* that activates macrophages.

Acknowledgments

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