

Immunomodulatory Activity of Pine Needle (*Pinus densiflora*) Extracts in Macrophages

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Abstract

Pinus densiflora belongs to the Pinaceae family which has been widely used for health promoting purposes as folk medicine or as a food. Various curative effects of different parts of the pine have been reported including as a remedy for carcinoma. We examined the effects of pine needle water extracts (PNE) on macrophage function using peritoneal macrophage, pre-osteoclast bone macrophage (Raw 264.7 cell) and brain macrophage (C6 microglia). When peritoneal macrophages were treated with various concentrations of PNE (1 ~ 100 µg/mL) for 24 hours, phagocytic activity was significantly increased, whereas it had no effect on tumoricidal activity and NO production. However, the treatment of Raw 264.7 with PNE resulted in the enhancement of NO production at high concentration (100 µg/mL). Furthermore, the treatment of C6 with PNE increased the production of NO in a concentration-dependent manner, whereas PNE suppressed NO production in LPS/IFN- γ -stimulated microglia. These results suggest that PNE has differential immunomodulatory effects on macrophages.

Key words: pine needle, *Pinus densiflora*, nitric oxide, macrophage, immunomodulation

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 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. During develop other stages, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (3,5).

Especially, there has been great interest in 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).

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. *Pinus densiflora* belongs to the Pinaceae family which have been widely used for health promoting purposes as folk medicine or as a food (9,10). Various curative effects of different parts of the pine have been reported indicating its physiological activities and therapeutic effects, including as a remedy for carcinoma (11). In addition, it has been shown that pine needle water extracts (PNE) displayed an antitumor effect in various cancer cell lines, but these effects were direct cytotoxic to cancer cells (12,13). However, immunomodulatory activity of PNE is still unknown. Here we examined the immunomodulatory effects of PNE on the production of NO and the activation of the macrophage families such as peritoneal macrophage, pre-osteoclast Raw 264.7 and C6 microglia.

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MATERIALS AND METHODS

Preparation of PNE

The dried materials (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 PNE was fractionated with D.W. Yields of water extracts were 16.5% (w/w) relative to starting material for pine needle.

Macrophage-mediated antitumor activity

The assay for macrophage cytotoxicity was based on an assay described by Verstovsek et al. (2). Briefly, macrophages (1×10^5 cells/well) from mice first incubated in either medium alone or in medium supplemented with various doses of PNE for 24 hr in 96-well plates. Macrophages were washed with RPMI-FBS to remove PNE 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:

$$\frac{[1 - \{OD \text{ of (target cells + macrophages)} - OD \text{ of macrophages}\} / OD \text{ of target cells}] \times 100}{}$$

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 viability

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay adapted from Mosmann (14). Briefly, the cells were seeded in 96-well plates at a suitable densities of cells per well with various concentrations of PNE. 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 nitro blue tetrazolium (NBT) reduction assay (15). Peritoneal macrophages were seeded in 96-well plates at a density 5×10^4 cells per well, treated with various concentration of PNE and cultured for 24 hr. The cultured media was then removed and 50 μ L of 5×10^6 particles/mL zymosan and 0.6 mg/mL NBT was added into each well. After an ad-

ditional 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 the test compounds and the accumulation of nitrite in culture supernatant was measured using the assay system described by Ding et al. (16). 100 μ L 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.

Statistical analysis

The data is represented as a mean \pm 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.

RESULTS AND DISCUSSION

Effects of PNE on macrophage-mediated tumoricidal activity

To examine whether PNE treatment alter the tumoricidal activities of macrophages against target tumor cells, inflammatory macrophages obtained from thioglycollate (TG)-injected mice. These peritoneal macrophages were co-cultured with B16 cells for 24 hr. B16 tumor cells were used as target since they are either TNF- α or NO sensitive (17). Recently data displayed PNE have a significant antitumor effect on various cancer cell lines including human gastric carcinoma (KATO III), human lung carcinoma (A549), human hepatocellular carcinoma (Hep3B) and human breast adenocarcinoma (MCF-7) (13), but these effects were direct cytotoxic to cancer cells (12,13). Since, immunomodulation activity of PNE is still unknown, we researched the effects of PNE on macrophage-mediated antitumor activity. As shown in Fig. 1, PNE-primed macrophage did not enhanced tumoricidal activities.

Effect of PNE on phagocytosis

Since the primary role of the macrophage is phagocytosis, phagocytosis stimulates a diverse range of antimicrobial/cytotoxic responses, including generation of

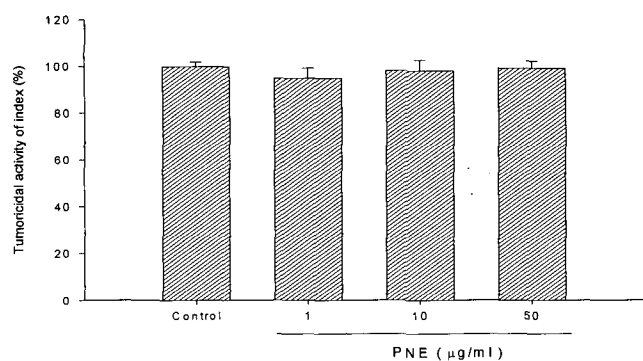


Fig. 1. The effects of PNE on macrophage-mediated tumoricidal activity. Peritoneal macrophages were isolated from TG-injected mice. Peritoneal macrophages were treated with various doses of PNE and co-cultured with the target at initial effector/target cell ratio of 10:1. Macrophage tumoricidal activity was determined as described in material and methods. The data represents the mean \pm SE of quadruplicate experiments. * $p < 0.05$; significantly different from the control (no treatment).

respiratory burst, secretion of inflammatory mediators and antigen presentation. In this study, we measured the effect of PNE on phagocytosis of peritoneal macrophages. Phagocytosis of PNE-treated macrophages was significantly increased in a dose dependent manner (Fig. 2). Enhancement of phagocytosis function in peritoneal macrophages by PNE provides more evidence for the modulatory effect of PNE on immune system. The data showed that PNE has no effect on macrophage-mediated antitumor activity, suggesting that even though these inflammatory peritoneal macrophages were not able to respond to PNE to become fully activated, the cells had an increased capacity to take up the microorganism or antimicrobial activity. Therefore, PNE maybe develop primed stage of macrophage activation.

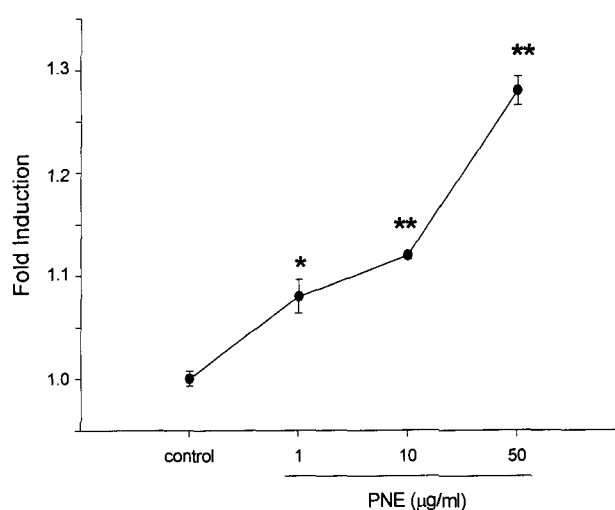


Fig. 2. The effects of PNE on phagocytosis in the peritoneal macrophages. Peritoneal macrophages isolated from TG-injected mice. Peritoneal macrophages (1×10^5 cells/well) were treated with various concentrations of PNE for 24 hr. The phagocytic activity of peritoneal macrophages was assessed by NBT reduction assay. The purple insoluble formazan was measured at 540 nm. The data represents the mean \pm SE of quadruplicate experiments. ** $p < 0.01$, * $p < 0.05$; significantly different from the control (no treatment).

Effect of PNE on nitric oxide production

Since macrophages activation play an important role in the host defense mechanism and NO is related to cytotoxic function of macrophages against a variety of tumors and microorganisms (7,8,18,19), we examined the effects of PNE on NO production in various macrophages. As shown in Fig. 3, the treatment of the cells with PNE induced an increase in the production of NO by pre-osteoclast cells, whereas NO production was decreased in peritoneal macrophages. Since peritoneal macrophages are inflammatory stage induced by TG-injection,

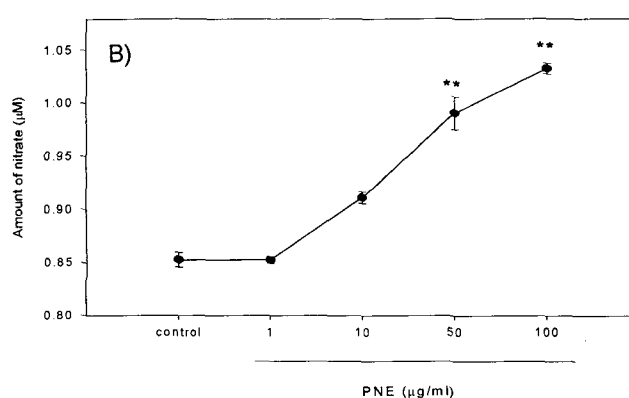
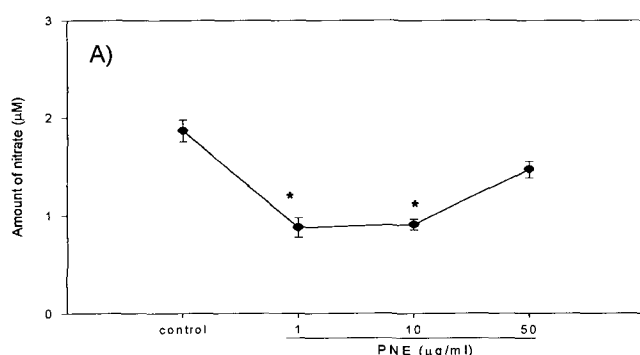


Fig. 3. The effects of PNE on production of nitrite by peritoneal macrophages (A) and Raw 264.7 cells (B). Peritoneal macrophage (1×10^5 cells/well) and Raw 264.7 (1×10^4 cells/well) were treated with various concentration of PNE for 24 hr. Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. As a positive control, IFN- γ combined with LPS was used. The data represents the mean \pm SE of quadruplicate experiments. * $p < 0.05$, ** $p < 0.01$; significantly different from the control (no treatment).

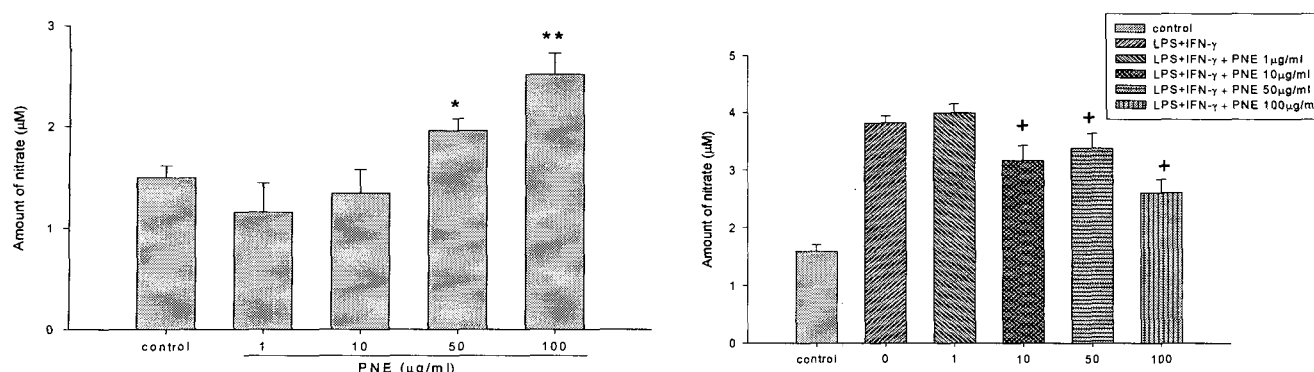


Fig. 4. The effects of PNE on production of nitrite by C6 cells. C6 (5×10^4 cells/well) were treated with various concentration of PNE for 24 hr in the absence (A) or presence (B) of LPS/IFN- γ . Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. The data represents the mean \pm SE of quadruplicate experiments. * $p < 0.05$, ** $p < 0.01$; significantly different from the control (no treatment). + $p < 0.05$; significantly different from the treatment of LPS/IFN- γ .

PNE could increase NO production in resident macrophages and suppress NO production induced by some stimuli in inflammatory macrophages.

Microglia are resident monocyte-lineaged cells in the brain. These cells defend the central nervous system against invading microorganisms and clear the debris from damaged cells. Recent biochemical and neurobiological studies have further indicated that they significantly affect the pathological state and/or regulate the regenerative state and remodeling of the brain by producing a variety of biologically active molecules including radicals such as superoxide and NO (20). The immunomodulating effects of PNE on NO production also are examined in microglia cells. As shown in Fig. 4, PNE induced the production of NO in microglia whereas PNE suppressed NO production in LPS/IFN- γ -stimulated microglia. At present time the mechanisms by which PNE has a biphasic response to microglia are not clear. And further study should be needed. In the present study, our data demonstrate that PNE has an immunomodulatory effects on various macrophages functions.

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