

Immunomodulatory Effects of Fucoidan on NK Cells in Ovariectomized Rats

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Abstract - In this study we investigated the effects of supplementation with fucoidan from brown alga on the function of natural-killer (NK) cells to evaluate the possibility as an immunomodulator in ovariectomized (OVX) rats. A total of 18 female Wistar rats (six weeks) were used this study and 12 rats were OVX, and the rest of rats were sham-operated. The sham and one OVX group were fed standard diet, and the remaining OVX group received fucoidan (0.05% supplemented diet). After 12 weeks of supplementation, rats were sacrificed to assess the tumoricidal activity of the NK cells and the NO-iNOS regulation from splenocytes. The mass of body and the immune organs such as spleen and thymus were also studied. In OVX rats, body and thymus weights increased, however fucoidan supplementation did not change the body mass and organs weight compared to OVX group. Fucoidan supplementation increased NK cell activity and reduced NO-iNOS production in OVX rats. *Ex vivo* treatment of fucoidan increased NK cell activity in splenocytes from sham and OVX rats. *Ex vivo*, we confirmed that fucoidan partially reduced the NK cell activity in the presence of iNOS inhibitors in OVX-splenocytes. These results indicate fucoidan supplementation has a NK cell tumoricidal activity, which are regulated by the iNOS production in OVX rats. This suggests that fucoidan is useful for potential therapeutic strategies as a nutrient in regulating the NK cells in postmenopausal osteoporosis patients.

Key words - Antitumor effect, *In vivo*, iNOS, Osteoporosis

Introduction

Aging plays an important role in many biological functions within a body, and it is the main predictor for osteoporosis. With advancing aging and particularly with post-menopausal conditions, the activity of osteoclasts, cells that break down bone through bone resorption, is greater than that of osteoblasts, cells responsible for bone formation, in women. This ultimately gives rise to osteoporosis (Yamaguchi, 2006). Aging is also associated with a decline in immune function, known as immunosenescence, that contributes to the increased susceptibility to infection, cancer, and autoimmune diseases observed in humans (Mocchegiani and Malavolta, 2004; Sohal and Allen, 1990). Aging-related alterations of the cellular components of innate immunity might therefore be involved in the impairment of adaptive immunity observed in the elderly (Mocchegiani and Malavolta, 2004; Jang *et al.*, 2010).

Ovariectomy induced bone loss in rats and postmenopausal bone loss in humans share many similar characteristics, and similar skeletal response to therapy with 17 β -estradiol (Kalu, 1991; Kalu *et al.*, 1991). These similarities are strong evidence that the OVX rat bone loss model is suitable for studying the prevention and treatment of postmenopausal bone loss.

NK cells are an important component of the innate immune system and mediate cytolytic activities against tumors and virus-infected targets. Recently increasing evidences indicate that NK cells may play a pivotal role in some female predominant diseases or in normal physiological conditions. Some studies suggest that estrogen may be involved in the regulation of immunocompetence in immune system and it modulates activities of NK cells (Nakaya *et al.*, 2006). Baral *et al.* also found that estrogen reduced NK cell cytotoxicity against tumor targets but this effect was dependent on duration of exposure to estrogen (Baral *et al.*, 1995).

The brown seaweeds, which have been consumed as a food or relish to provide a dietary fiber source, are the most important

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economic seaweeds cultured in Asia. Fucoidan, which is found in extracts from brown seaweeds (e.g., *Ascophyllum nodosum* and *Fucus vesiculosus*), is a polyanionic macromolecule composed predominantly of sulfated fucose moieties. In recent years, fucoidan has been extensively studied due to its numerous biological activities including its anticoagulant, antithrombotic, antitumor, antiviral, anti-complement and anti-inflammatory effects (Patel *et al.*, 2002; O'Leary *et al.*, 2004; Mahony *et al.*, 1991; Baba *et al.*, 1988).

Although these favorable effects of fucoidan have been reported and evaluated, the effects of fucoidan on activity of NK cells in osteoporosis have not been evaluated. The estrogen-deficient ovariectomized (OVX) osteoporosis model is useful for evaluation of osteoporotic drugs because several bone-related parameters are clearly decreased by ovariectomy within 10 to 14 weeks after the operation. In this model, we examined the effects of fucoidan on the NK cell function in OVX rats and identify the possible involvement of iNOS that may mediate the regulatory effect of NK cell.

Materials and Methods

Experimental animals and diets

Female Wistar rats (six weeks) were used for the experiment. The animals were kept for 5 days before the onset of the experiment to acclimatize to our laboratory conditions (the room temperature was 25 °C with a 12 h/12 h light/dark cycle), then 12 rats were OVX and 6 rats were sham-operated under anesthesia with intraperitoneal (IP) injection of sodium pentobarbital at a dose of 30 mg/kg body weight. After operation, rats were kept in separate cages and fed standard diet before being sacrificed. To prevent hyperphagia associated with ovariectomy, the OVX rats were pair-fed to the mean intake of those in the sham group (Wronski *et al.*, 1987). All rats were allowed free access to drinking ion-exchanged distilled water for the duration of the whole experiment. The sham and one OVX group were fed standard diet, and the remaining OVX group received fucoidan (0.05% fucoidan modified AIN-93 M diet) supplemented diet. We confirmed that the ovariectomy significantly decreased (*t*-test, $p < 0.05$) bone mineral density at 10 weeks after surgery. The animals were sacrificed after ether anesthesia at 12 weeks. Spleen samples were

collected aseptically for the NK cell assays. The rats were observed daily for clinical signs. None of the rats showed any abnormal growth and behavior and no sign of illness. Animal care and all experimental protocols were performed following the Institute for Laboratory Animal Research (ILAR) guideline.

Preparation of fucoidan and Reagents

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO). Fucoidan extracted from *Fucus vesiculosus* was also purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in distilled water. The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). The XTT {2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt} cell viability assay kit was purchased from WelGENE (Daegu, South Korea). All the tissue culture reagents and fucoidan were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate, Sigma) for in vitro assay, and the endotoxin levels were found to be < 10 pg/ml.

NK cell-mediated tumoricidal activity

A modification of the method reported by Mosmann *et al.* was used (Mosmann, 1983; Namkoong *et al.*, 2011). The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^5 cells/ml. The culture medium was RPMI 1640 containing 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Spleen cells from the sham and OVX rats were tested as effector cells and YAC-1 mouse lymphoma cells (ATCC, Rockville, MD) were used as the target cells. The NK cell assays were carried out in 96-well v-bottom plates at effector/target (E:T) cells ratios 50:1 with 1×10^4 of the target cells in a final well volume of 200 µl at 37 °C for 4 h in a 5% CO₂ humidified incubator. After incubation of cells for 4 h, 20 µl of phenazine methosulphate (PMS); electron-coupling reagent and 25 µl of XTT were added to each well. The cells were further incubated for 3h to allow XTT formazan production. The absorbance were determined with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Western blot analysis

The amount of iNOS was measured by Western blot analysis. Rats were immediately sacrificed and isolate spleen. After homogenized of spleen tissue and lysed in RIPA buffer. Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of each sample was electrophoresed on 10% sodium dodecylsulfate polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with 5% skim milk in TBS-T for 1 h. The membranes were incubated with primary antibody against iNOS for 24 h. They were then washed with TBS-T once for 15 min and three times for 5 min and incubated with secondary HRP-conjugated anti-rabbit antibody for 1 h. The membranes were washed again as described above. Auto-radiography was carried out using an enhanced chemiluminescence kit.

Statistical analysis

Each experiment was repeated at least two times, and the results of one representative experiment are shown. The results were expressed as means \pm S.E.M. and analyzed *via* Student's t test and ANOVA. The significant values are represented by an asterisk (*, # $p < 0.05$ and **, ## $p < 0.01$).

Results and Discussion

The most characteristic function of NK cells is non-MHC restricted killing of target cells, such as tumor, and thus NK cells are important for maintaining health in body (Borrego *et al.*, 1999). It has been reported that aging attributed to various factors of NK cells. For example, NK cells exhibited decreased responsiveness to its positive modulator, IL-2, in aged group (Miller, 1991) and this explains the high frequency of cancer and infectious diseases with aging. Osteoporosis is one of the aging-related diseases (Yamaguchi, 2006). With advancing aging and particularly after menopause in women, the activity of osteoclasts, cells that break down bone, is greater than that of osteoblasts, cells that build bone. In this study, we determined the tumoricidal activity of the NK cells in OVX and the regulatory effects of the supplementation with edible seaweed fucoidan. We showed that the increasing effects on NO-iNOS

production and the suppressive effect of NK cells tumoricidal activity in OVX rats compared to sham group. Furthermore, fucoidan supplementation in OVX group recovered all these effects such as NO-iNOS production and NK cells tumoricidal activity (Fig. 1-3).

Recently it has been reported that NK cell tumoricidal activity could be regulated by sex hormones. Yovel *et al.* found that there was an inhibition of specific lysis of Yac-1

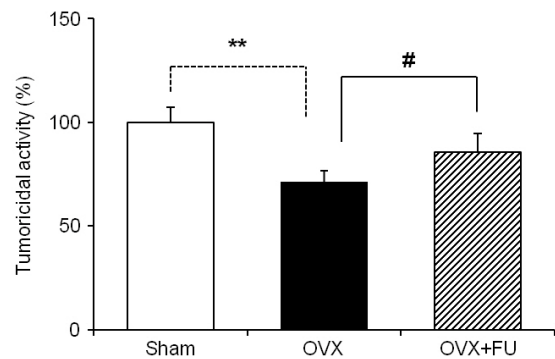


Fig. 1. Effect of the fucoidan (FU) supplementation on the NK cells tumoricidal activity from experimental rats. Splenocytes were cultured with Yac-1 tumor cells for 4 h. The NK cells tumoricidal activity examined at effector/target cells ratio of 50:1 by XTT assay. The data is expressed as a means \pm S.E.M of quadruplicates of a representative experiment. ** $P < 0.01$: significantly different from the sham group. # $P < 0.05$: significantly different from the OVX group.

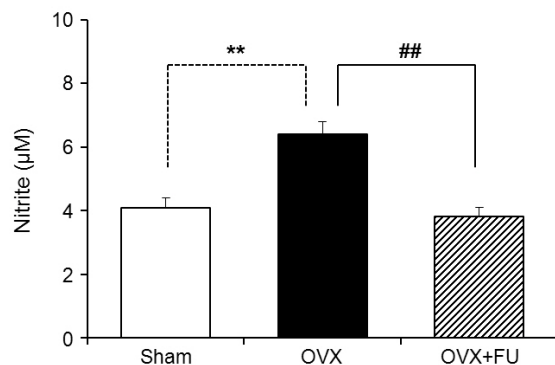


Fig. 2. The effects of the fucoidan (FU) supplementation on the production of NO in splenocytes from experimental rats. These results are means \pm S.E.M of quintuplicates from a representative experiment. ** $P < 0.01$: significantly different from the sham group. ## $P < 0.01$: significantly different from the OVX group.

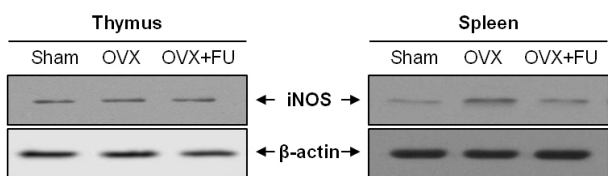


Fig. 3. The effects of the fucoidan (FU) supplementation on the expression iNOS in splenocytes and thymocytes from experimental rats. Spleen and thymus tissue lysate were determined by Western Blot assay.

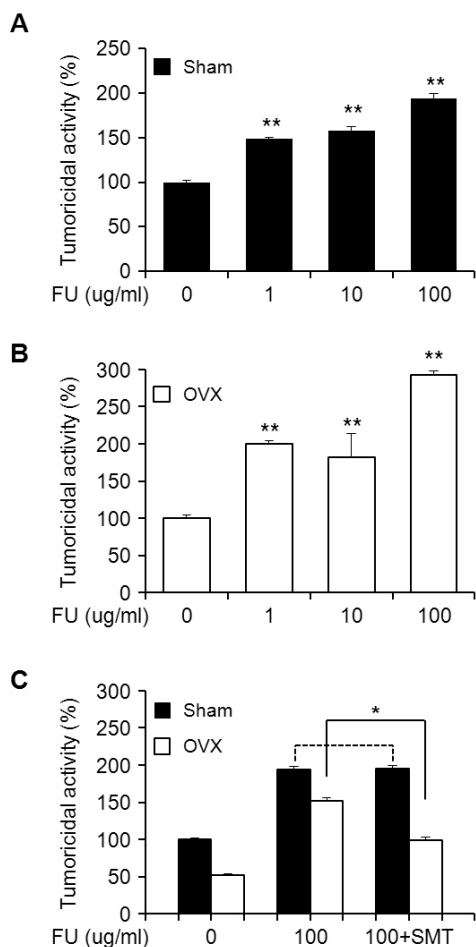


Fig. 4. The effects of fucoidan (FU) on the NK cell tumoricidal activity in the splenocytes from sham (A), OVX group (B) and the NK cell tumoricidal activity of FU in the absence or presences of s-methylisothiourea (SMT; 100 μ M), iNOS inhibitors (C). Splenocytes (5×10^4 cells/well) were treated with various FU (1-100 μ g/ml or 100 μ g/ml) or in the presence of SMT 4 h. The tumoricidal activity was examined at E:T ratio of 50:1. The data represents the means \pm S.E.M. of quadruplicate experiments. ** $p < 0.01$, * $p < 0.05$: significantly different from the control or FU alone treated cells.

target after a long-term exposure of the NK cells to 17β -estradiol (Yovel *et al.*, 2001). In addition, several data in animal model studies also provided direct evidence that chronic administration of high doses of 17β -estradiol to mice resulted in a decreased activity of NK cells (Yovel *et al.*, 2001). Thus, estrogen imbalance level caused change of NK cell cytotoxicity and the low NK activity induced by IL-2 was found to be a predictor of morbidity for various infection and cancer in osteoporosis (Levy *et al.*, 1991; Kim *et al.*, 2009).

To examine whether the effect of fucoidan on NK cell tumoricidal activity is mediated by NO production which is involved in iNOS expression, the splenocytes from sham and OVX rats were treated with fucoidan (100 μ g/ml) in the absence or presence of s-methylisothiourea, preferential inhibitor of iNOS. As shown in Fig. 4, the treatment of fucoidan resulted in a significant enhancement of NK cell activity in splenocyte from sham and OVX rats in dose dependant manners. Moreover it appears that iNOS expression was involved in the regulation of NK cell activity in fucoidan-treated OVX splenocytes, whereas inhibition of iNOS has no effects of the NK cells activity in fucoidan-treated sham cells. These data imply that the estrogen-deficient stress activates innate immune cells such as NK cells and changed intracellular NO levels. Thus it is speculated that fucoidan and cytokines produced by these cells could exert combinatory effects on NK cell activities in OVX state.

In previous study, red ginseng acidic polysaccharide (RGAP) alone had no effect on the function of macrophages for killing tumor cells. However, a combination of RGAP and recombinant IFN- γ (rIFN- γ) enhanced their killing of tumor cells (Choi *et al.*, 2008). There was a report that IFN- γ was a major product of murine NK cells during pregnancy and its production was enhanced by a long-term exposure of cells to estradiol. Therefore, we could postulate that hormonal imbalance such as estrogen-deficiency changed the secretion of various cytokines of NK cells. Moreover, the fucoidan exerted combinatory influence on such cytokines regulation for NK cell activities, and it was regulated by secondary signals such as NO-iNOS production.

In our experimental systems using OVX rat model, as shown in Table 1, ovariectomy significantly increased the body mass and thymus weight index but not spleen compared

Table 1. Effects of fucoidan supplement on the body mass and index of immune organs in ovariectomized (OVX) rats

Organ / Body weight	Sham	OVX	OVX+FU
Thymus [†]	0.15±0.005	0.24±0.009 ^a	0.24±0.011
Spleen [†]	0.28±0.012	0.27±0.008	0.28±0.007
Body weight (g)	268.5 ±3.7	330.25±6.5 ^a	325.75±8.3

[†]Index = (organ mass (g)/body mass (g)) × 100, FU: fucoidan, ^aP<0.01 statistically significant differences in comparison with the sham control rats.

to the sham rats ($p < 0.01$). However, fucoidan supplementation did not affect the changes of the body weight and thymus/spleen weight index of the OVX rat.

Collectively, we could suggest that fucoidan supplementation have a possibility as a potential immunotherapeutic agent for osteoporotic patients. Though we do not know whether iNOS expression is involved in NK cell activities in details, our data demonstrate that the effect of fucoidan on NK cell-mediated tumoricidal activity is regulated by iNOS expression and fucoidan provides secondary signals for the regulatory induction of NK cell activities in OVX states. The precise mechanism of estrogen in modulating NK cells and the exact role of NK cells in the osteoporosis remain unclear. In addition, the exact explanation for modulation of NK activity by fucoidan in OVX rats is not known yet. Therefore we need to examine further the effects of fucoidan on the regulation of NO-iNOS and the level of cytokines such as IL-2 and IFN- γ , which are may necessary for activation and maturation of NK cells.

In summary, our data imply that the estrogen-deficient stress activates innate immune cells such as NK cells and changed the secretion of NO and iNOS expression, and these were regulated by fucoidan supplementation. This suggests that fucoidan is useful for potential therapeutic strategies as a nutrient in regulating the NK cells in postmenopausal osteoporosis patients.

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