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Effects of Fucoidan on NO Production and Phagocytosis of Macrophages and the Proliferation of Neuron Cells

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Abstract

Fucoidans, high-molecular-weight sulfated polysaccharides extracted from brown seaweeds, have various biological activities. Here we examined whether fucoidan could increase the immunomodulation and proliferation capacity of cells in vitro. When peritoneal macrophages were treated with various concentrations of fucoidan (1~100 μg/mL) for 24 hours, NO production was significantly increased. In addition, exposure of macrophages to 10 μg/mL of fucoidan induced a phagocytic activity. Treatment of neuroblastoma cells (SK-N-SH) with fucoidan enhanced cell proliferation and NO production in a concentration-dependent manner. These results indicate that fucoidan has both immunomodulatory and cell proliferative properties, and may thus be a a candidate for development as an immunomodulating agent.

Key words: fucoidan, nitric oxide, phagocytosis, immunomodulation

INTRODUCTION

Fucoidan, collective algal sulfated polysaccharides extracted components of edible brown seaweeds, are considered the most economically important seaweeds in China and are also widely distributed in Korea and Japan. Cell walls from brown seaweeds contain abundant sulfated polysaccharides and alginate. Fucoidan was first isolated by Kylin almost one century ago, containing substantial percentages of L-fucose and sulfate ester groups (1). In recent years, fucoidan has been extensively studied due to its numerous biological activities including anticoagulant, antithrombotic, antitumor, antiviral, anti-complement and anti-inflammatory activities (2-6).

The nonspecific immunomodulators may represent the ideal strategy for the controlling invading microorganisms. Numerous nonspecific immunomodulators have been identified, including substances isolated from fungi or microorganisms and from plants. Among plant-origin substances, polysaccharides seem to exert considerable immuno-potentiating activities like enhancement of macrophage function. Macrophages play an important role in host protection against a wide range of tumors and microorganisms. Macrophages also present antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase their phagocytic activity which is essential in host defense against microbial pathogens and in the clearance of apoptotic cells. Macrophages express many phagocytic receptors which stimulate ingestion of the pathogens they recognize. The interaction of these soluble receptors with pathogens leads in turn to binding of the receptor-pathogen complex by macrophage, either through direct interaction with the pathogen-binding receptors, or through receptors for complement, thus promoting phagocytosis and killing of the bound pathogen and the induction of other cellular responses. In addition to triggering phagocytosis, binding of pathogens by macrophages can also trigger the release of various materials such as cytokines and reactive intermediates and then carry out nonspecific immune responses (7,8). The reactive nitrogen intermediate, NO (nitric oxide), has now been implicated in many antibacterial and antitumor activities. The clinical effects of fucoidan, such as antiviral, antitumor activites, might be due to the stimulation of macrophage function mediated by the generation of NO (9,10).

This study was designed to investigate the effects of fucoidan on NO production and phagocytosis of macrophages. Additionally, fucoidan has been shown to exhibit

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the inhibitory effects on the pathogenesis of Alzheimer disease (AD) (11,12), a progressive neurodegenerative disease characterized by memory impairment and cognitive dysfunction that is linked to the loss of cholinergic neurons in the basal forebrain (13). Even though NO produced in neural cells appears to be both neuroprotective and neurotoxic, the possibility of NO involvement in the neuroprotective effect of fucoidan was investigated in neuroblastoma cells.

MATERIALS AND METHODS

Mouse and chemicals

C57BL/6 mice (5 wk) were obtained from Charles River Breeding Laboratories (Japan). Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). Thioglycollate (TG) broth was purchased from Difco Laboratories (Detroit, MI).

Cell culture and treatment

Raw 264.7 (murine macrophage cells) and SK-N-SH (neuroblastoma cells) were obtained from ATCC. Peritoneal macrophages were collected from the peritoneal cavities of C57BL/6 mice that had been i.p. injected with 4% TG 3 days previously. Cells were harvested and centrifuged at 1000 rpm. After seeding 2 h, non adherent cells were removed by washing with medium three times. More than 98% of the adherent cell populations were macrophages according to morphology. All three cell types were cultured at 37°C with 5% CO_2 in RPMI 1640 medium containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin.

MTT assay for proliferation

Cell proliferation 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 suitable densities of cells per well with various concentrations of fucoidan. After an incubation period of 24 h, the enzyme activities of viable cells were measured by addition of MTT to each well. After 4 h of additional incubation, the amount of formazan was determined by absorbance at 540 nm using a microplate reader (Menlo Park, CA).

Nitrite determination

The cells were treated with the control media for 24 h in the presence and 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. (15). 100 μL 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.

NBT assay for phagocytosis

Phagocytosis was measured using the nitro blue tetrazolium (NBT) reduction assay (16). Peritoneal macrophages were seeded in 96-well plates at a density 5×10^4 cells per well, treated with various concentration of fucoidan and cultured for 24 h. The cultured media was then removed and 50 μL of 5×10^6 particles/mL zymosan and 0.6 mg/mL NBT was added into each well. After additional incubation for 1 h, wells were washed twice with cold D-PBS 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.

Statistical analysis

The data is represented as a mean±SE. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with Dunnett's *t*-test. A p value<0.05 was considered significant.

RESULTS AND DISCUSSION

Effect of fucoidan on nitric oxide production

Nitric oxide, one of the smallest biological molecular mediators, plays important roles in many different aspects of mammalian biology, such as vascular relaxation, neurotransmission, platelet aggregation and immune regulation (17-19). To investigate the effect of fucoidan on NO production, we measured the accumulation of nitrite, the stable end product of NO, in peritoneal macrophages, Raw 264.7 and SK-N-SH cells. As shown in Fig. 1B, the treatment of the cells with fucoidan (1, 5, 10, 50 μg/mL) caused an increase in the production of nitrite by Raw 264.7. This increase was dose-dependent and reached the highest value at 50 μg/mL. Fucoidan also significantly induced NO production in peritonel macrophages at 100 μg/mL, but not at lower concentrations (0.1, 1, 10, 50 μg/mL) (Fig. 1A). Since macrophage activation plays an important role in the host defense mechanism and NO is related to the cytolytic function of macrophages against a variety of tumors and microorganisms (7-10), the increased synthesis of NO by fucoidan in Raw 264.7 and peritoneal macrophages implied that fucoidan might have an activating effect on macrophages.

In some recent studies, estrogen is reported to have many effects on the nervous system, including regulation

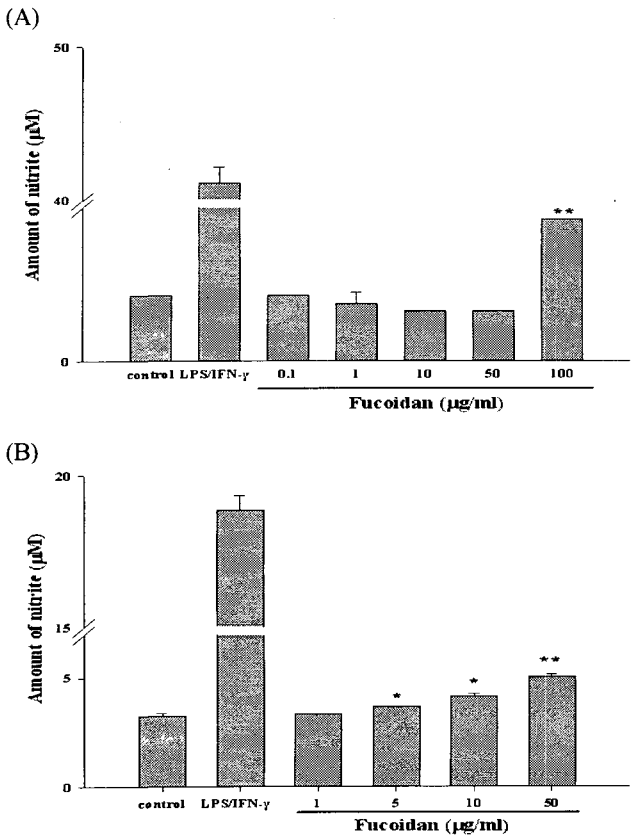

Fig. 1. The effects of fucoidan on nitrite production in peritoneal macrophages (A) and Raw 264.7 cells (B). Peritoneal macrophage (1×10^5 cells/well) and Raw 264.7 (1×10^4 cells/well) were treated with fucoidan of various concentration for 24 h. Culture supernatants were collected and the levels of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

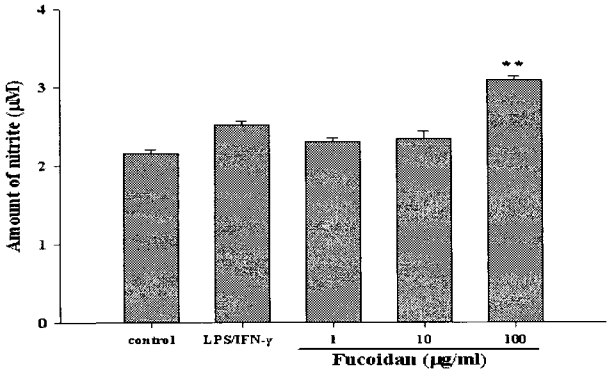

Fig. 2. The effect of fucoidan on production of nitrite by SK-N-SH cells. SK-N-SH (5×10^4 cells/well) were treated with fucoidan of various concentration for 24 h. Culture supernatants were collected and the levels of nitrite were measured as described in materials and methods. As a positive control, IFN-γ combined with LPS was used. The data represents the mean ±SE of quadruplicate experiments. **p<0.01; significantly different from the control (no treatment).

of neuroendocrine activities, modulation of autonomic functions, neuroprotective effects, and improvement on cognitive tasks (20,21). Many of these effects are mediated or influenced by nitric oxide (NO). Our data showed that fucoidan at 100 μg/mL increased the production of nitrite in SK-N-SH neuroblastoma cells (Fig. 2). Since this effect of fucoidan was similar to that of estrogen, we expect that fucoidan might have neuroprotective effects.

Unlike the large increase in NO release induced by tissue injury and inflammation, fucoidan-induced increases in NO levels were relatively small. NO is a highly reactive molecule that can be toxic to tumor cells. However, fucoidan cannot upstimulate mononuclear cells into fully activated macrophages which are cytotoxic to tumor cells. However, the slight enhancement of NO production by fucoidan showed health benefits.

Effect of fucoidan on phagocytosis

Phagocytosis stimulates a diverse range of antimicrobial/cytotoxic responses, including generation of a respiratory bust, secretion of inflammatory mediators and antigen presentation. It has been demonstrated that fucoidan acts as an activator of the reticular-endothelial system and an enhancer of phagocytosis (22). In this study, we measured the effect of fucoidan on phagocytosis function of peritoneal macrophages. Phagocytosis of fucoidan (10 and 100 μg/mL)-treated macrophages was significantly increased compared to that of control at 24 h (Fig. 3). Enhancement of phagocytic function in peritoneal macrophages by fucoidan in our study provides further evidence for the stimulatory effect of fucoidan in the immune system.

Effect of fucoidan on cell proliferation

In addition to immune stimulation, fucoidan has also been shown to have an effect on cell proliferation. Giraux et al. (23) reported that fucoidan enhanced the proliferation of endothelial progenitor cells (EPC). Proliferation was enhanced in a concentration-dependent manner, starting at 1 μg/mL, but it had no effect on human umbilical vein endothelial cells (HUVEC) at concentrations below 100 μg/mL. Regardless of the fucoidan concentration, EPC always proliferated significantly more strongly than HUVEC (23). On the contrary, Religa et al. (24) demonstrated an inhibitory effect of fucoidan on rat aortic smooth muscle cell (SMC) proliferation at 100 μg/mL. These studies suggest that the effect of fucoidan on cell proliferation is variable among cell types. Within the context of AD, fucoidan has been

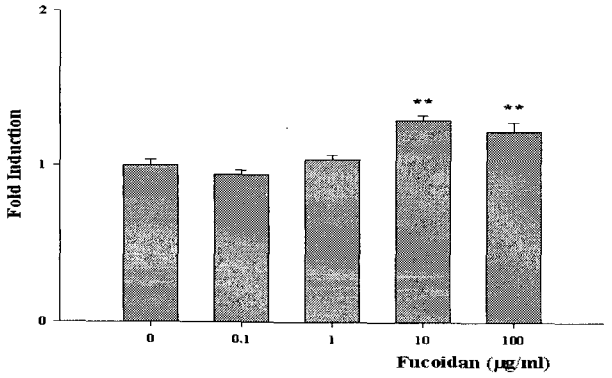

Fig. 3. The effect of fucoidan on phagocytosis in peritoneal macrophages. Peritoneal macrophages (1×10^5 cells/well) were treated with fucoidan of various concentration for 24 h. The phagocytic activity of peritoneal macrophages was assessed by the NBT reduction assay. The purple insoluble formazan was measured at 540 nm. The data is expressed as the mean±SE of quadruplicate experiments. Increase in phagocytosis upon stimulation of macrophages with fucoidan was calculated in relation to the basal phagocytosis to nonstimulated macrophages that was set to 1. **p<0.01; significantly different from the control (no treatment).

shown to have neuroprotective effects against β-amyloid-induced neurotoxicity. However, the effect of fucoidan on proliferation of neuroblastoma cells was not reported. Data in this study showed that fucoidan enhanced SK-N-SH proliferation in a concentration dependent manner, from 1 μg/mL to 100 μg/mL (Fig. 4A). Since AD is a progressive neurodegenerative disease characterized by memory impairment and cognitive dysfunction that is linked to the loss of cholinergic neurons in basal forebrain, the enhancement of fucoidan on the proliferation of SK-N-SH suggests a possible role for fucoidan in the prevention or treatment of AD. We also checked the effect of fucoidan on the proliferation of Raw 264.7 cells and the results showed that fucoidan did not enhance their proliferation (Fig. 4B). Our results lead to the same conclusion as previous studies showed that the effect of fucoidan on cell proliferation is different depending on cell types. Therefore, to apply fucoidan for clinical use, a suitable concentration of fucoidan must be defined to produce the desired effects only on target cells. Moreover, the present data suggest that fucoidan has effects on macrophage functions including phagocytic activity and NO production but can not upstimulate them into fully activated macrophages with cytotoxicity against tumor cells. We reconfirmed that fucoidan had no effect on tumorcidal acivity (data not shown). This means fucoidan might be more useful for adjuvants in cancer immunotherapy.

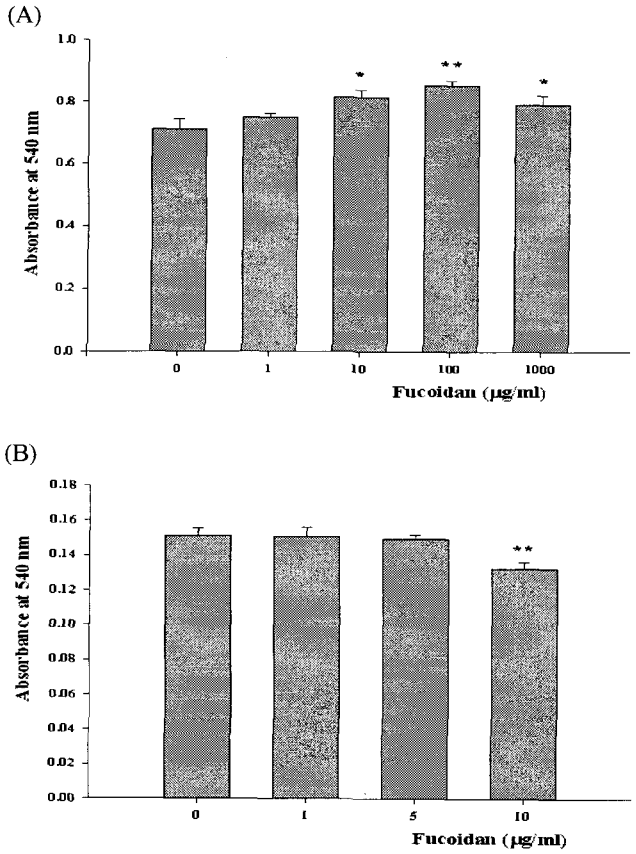

Fig. 4. The effect of fucoidan on proliferation of the SK-N-SH (A) and Raw 264.7 cells (B). SK-N-SH (5×10^4 cells/well) and Raw 264.7 (1×10^4 cells/well) were treated with fucoidan of various concentration for 24 h. The proliferation of cells was determined by the MTT assay. Cell density was measured at 540 nm. The data is expressed as the mean±SE of quadruplicate experiments. **p<0.01; significantly different from the control (no treatment).

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