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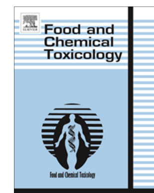

Differential effects of fucoidans with low and high molecular weight on the viability and function of spleen cells

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Fucoidan is an edible sulfated polysaccharide purified from brown algae that has multiple biological activities. However, the effects of fucoidans of different molecular weights on immune cells have not been determined. Thus, we treated spleen cells with low- and high-molecular-weight fucoidans (LMF and HMF, respectively). Viability assays demonstrated that HMF enhanced the viability and prevented the death of spleen cells. Furthermore, functional analysis revealed that HMF significantly increased the production of interferon-γ and nitric oxide. In contrast, LMF had low activity and was relatively toxic to spleen cells. Taken together, these results indicate that HMF makes the greatest contribution to the immunostimulatory activity of fucoidan mixtures. Additionally, fucoidans with different molecular weights may have different effects on the viability and function of immune cells. This study increases our understanding of fucoidans, and may broaden their use in the basic research and clinical fields.

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1. Introduction

Fucoidan is an edible algae-derived sulfated polysaccharide (Li et al., 2008) known for its biological activities (Fitton, 2011), including hematopoietic mobilization (Frenette and Weiss, 2000) and immunomodulation. The effects of fucoidan have been demonstrated on a variety of immune cell types, including B lymphocytes (Oomizu et al., 2006) and macrophages (Yang et al., 2006). In previous studies, we demonstrated that fucoidan has a strong immunostimulatory activity on bone marrow-derived dendritic cells, the most potent antigen-presenting cells in the immune system (Kim and Joo, 2008). In addition, fucoidan showed radioprotective effects on bone marrow cells and enhanced the survival rate in a mouse endotoxemia model (Byon et al., 2008; Ko and Joo, 2011).

At this time, the effect of fucoidans with different molecular weights (especially on immune cells) remains unknown. Recent studies demonstrated that high- and low-molecular-weight fucoidans have different effects in a collagen-induced arthritis mouse model (Park et al., 2010). Two other studies showed that low-molecular-weight fucoidan inhibits neointimal hyperplasia in aortic allograft and transplant coronaropathy in cardiac allograft (Alkhatib et al., 2006; Hlawaty et al., 2011).

In the present study, we investigated the effects of fucoidans of different molecular weights on immune cells. Spleen cells were cultured in the presence of low- and high-molecular-weight fucoidans (LMF and HMF, respectively). Specifically, we quantified viability/death and measured the function of treated cells, including cytokine production.

2. Materials and methods

2.1. Animals and reagents

Balb/c or C57BL/6 mice were purchased from ORIENT BIO (Seongnam, Republic of Korea) and maintained in our animal facility. 7- to 12-week-old mice were used in this study. All experiments using animal were performed based on the institutional guideline of Jeju National University for laboratory animal use and care. LMF and HMF were obtained from HAERIM FUCOIDAN Co. (Wando, Republic of Korea). Fucoidans were purified from *Undaria pinnatifida sporophylls* and separated by Gel permeation chromatography. According to the manufacturer's information, the average molecular weight of LMF and HMF are 30 kDa and 130 kDa, respectively. LMF and HMF similarly contain 21 ± 3% fucose, 20 ± 5% galactose, 2 ± 2% mannose, 30 ± 3% sulfate. And, the fucoidan originated from *Fucus vesiculosus*, was obtained from Sigma (St. Louis, MO, USA) and used as the positive control (PC). The molecular weight of Sigma fucoidan is 20–200 kDa. All fucoidans were tested for the endotoxin level using Endosafe® (Charles River, Charleston, SC, USA). The levels were less than 0.1 EU/ml at the highest concentration (50 μg/ml).

2.2. Preparation of spleen cells

Spleen cells were prepared from the spleen of mice (Joo et al., 2001). Briefly, spleens were mechanically disrupted and treated with hypotonic lysis buffer to remove red blood cells. After incubation on T75 culture flask for 30 min, the cells were

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passed through 40 μm cell strainer to get single cells. The adherent spleen cells, such as macrophages, dendritic cells, and some B lymphocytes, attach on the bottom of T75 flask during the incubation time. Thus, the single cells include mainly lymphocytes and natural killer cells. For IFN-γ and NO experiments, we used spleen cells containing adherent cells. Spleen cells were cultured in a lymphocyte culture medium, RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol.

2.3. Assessment of cellular viability

The viability of spleen cells was measured after treatments. Briefly, cells were seeded at a concentration of 2×10^6 cells/ml in 96-well plates and treated with fucoidans. After 3 days culture, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was treated at a concentration of 0.5 mg/ml for 4 h. The viable cells generate insoluble violet crystal, 100 μl/well 10% SDS solution directly solved it for 2 h. The optical density of samples was measured at 570 nm using a microplate reader (Multiskan FC, Thermo Scientific, Waltham, MA, USA).

2.4. Flow cytometry analysis

The spleen cells were cultured at a concentration of 2×10^6 cells/ml in 6-well culture plates in the absence or presence of 50 μg/ml PC, LMF, and HMF for 2 days for flow cytometry analysis. The treated cells were stained as established (Kim et al., 2007). The cells were stained with biotin-labeled anti-CD4, CD8a, CD19, or CD25 antibody, then streptavidin-phycoerythrin (PE), and PE-labeled anti CD69 antibody (all from BD Biosciences, San Jose, CA, USA). For analyzing the cell death, both apoptosis and necrosis, the cells were stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Life Technologies) according to the manufacturer' instruction. Annexin V specifically binds to phosphatidylserine on apoptotic cells (Martin et al., 1995). To check the membrane potential of mitochondria, the cells were incubated with 10 μg/ml rhodamine 123 (Sigma) for 30 min at room temperature. The stained cells were analyzed with FACSCaliber flow cytometer and CellQuest software (both from BD biosciences).

2.5. Nuclear staining analysis of apoptotic cells

To confirm the effects of different fucoidans on the viability of spleen cells, nuclear chromatin of cells were stained with 2.5 μg/ml Hoechst 33342 fluorochrome and 2.5 μg/ml propidium iodide, and followed by examination on a fluorescence microscope (Olympus Optical, Tokyo, Japan). Color changes in nuclear chromatin of stained cells were revealed. Intact blue, white, and pink nuclei were considered as viable, apoptotic, and necrotic cells, respectively.

2.6. Determination of interferon-gamma (IFN-γ) production

Fucoidans (50 μg/ml) was added on 4×10^6 cells/ml of spleen cells including adherent cells in 96-well culture plates. After 4 days, the supernatants were collected and used for the determination of IFN-γ, a critical cytokine for innate and adaptive immunity. The cytokine concentration in supernatants was determined by enzyme-linked immunosorbent assay (ELISA) using CytoSet™ antibody pairs (Life Technologies) based on the manufacturer's manual.

2.7. Determination of Nitric oxide (NO) production

The concentration of NO in the supernatants of treated cells was measured using Griess reagent (Sigma). Spleen cells were cultured at a concentration of 4×10^6 cells/ml in the absence or presence of 50 μg/ml PC, LMF, HMF for 4 days. The supernatants were collected and used for NO assay. The sample was reacted with the equal volume of Griess reagent in room temperature for 10 min. Standard curve was obtained using sodium nitrite solution. The optical density of samples was measured at 570 nm by using a microplate reader.

2.8. Statistical analysis

Data were presented as mean ± standard deviation (mean ± SD) and statistically analyzed by one-way ANOVA and Dunnett multiple comparisons test using InStat (GraphPad Software Inc., La Jolla, CA, USA). A p value of <0.05 was considered as significant. *, **, *** indicate $p < 0.05$, 0.01, 0.001 compared to control.

3. Results

3.1. Effect of HMF on spleen cell activity

MTT assays were performed to measure spleen cell activity. All fucoidans increased cell viability in a concentration-dependent manner (Fig. 1). Specifically, HMF induced the greatest increase in viability compared to the control and other treatments. This suggested that HMF might protect spleen cells from spontaneous death and enhance their proliferation.

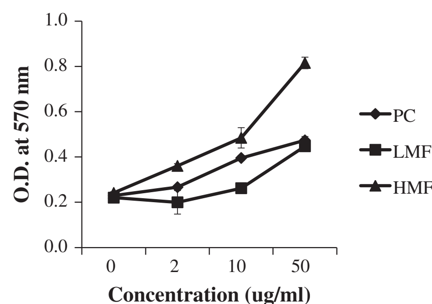

Fig. 1. The activity of spleen cells was enhanced by fucoidans. Spleen cells were seeded at a concentration of 2×10^6 cells/well in 96-well culture plates and treated with fucoidans for 3 days. MTT assay was performed as described in Section 2. Data are expressed as means ± SD.

3.2. Effect of HMF on CD19 and lymphocyte activation marker expression

To determine whether fucoidans induce proportional changes in spleen cells, we measured the levels of CD4, CD8, and CD19 expression by spleen cells after treatment. Flow cytometry analysis revealed that HMF increased the expression of CD19 on spleen cells (Fig. 2A). In addition, HMF significantly increased expression of the lymphocyte activation markers, CD25 and CD69 (Fig. 2B). LMF and PC also increased the expression of activation markers, but to a lesser degree than did HMF. Thus, HMF is a potent mitogen for B lymphocytes.

3.3. Effects of fucoidans on spleen cell viability

By chromatin analysis using fluorochromes, the LMF and HMF fucoidans showed different effects on spleen cell viability (Fig. 3). In the control, a proportion of spleen cells contained pink nuclei, indicative of dead or necrotic cells. PC- and LMF-treated spleen cells showed a decrease proportion of pink nuclei compared to the control. HMF-treated spleen cells showed a high proportion of blue nuclei, indicative of viable cells.

3.4. Effects of fucoidans on death of spleen cells

Harvested spleen cells gradually enter a spontaneous cell death process due to the lack of growth-related cytokines that provide survival and proliferation signals *in vivo* (Fleischer et al., 2007; Letai, 2006). Quantitative Annexin V-FITC/PI staining was used to investigate the protective effects of fucoidans against such death (Fig. 4A). Fucoidans consistently decreased the number of necrotic cells (AnnexinV−/PI+) and increased the number of viable cells (Annexin V−/PI−) compared to the control. Spleen cells treated with PC showed increased late apoptosis, whereas those treated with LMF showed increased necrosis. Treatment with HMF resulted in the highest proportion of viable cells. Thus, the protective effects of fucoidans differ, and HMF has the greatest protective effect. Mitochondria are known to play a critical role in apoptosis. Thus, cells were stained with rhodamine 123 since the action potential of the mitochondrial membrane of apoptotic or necrotic cells is lower than that of viable cells (Sugrue and Tatton, 2001). Treatment with fucoidans increased the action potentials of the mitochondrial membrane of spleen cells (Fig. 4B). HMF-treated cells showed the greatest increase in action potential, whereas LMF-treated cells showed a lesser increase.

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