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The Role of NK Cells in Antitumor Activity of Dietary Fucoidan from *Undaria pinnatifida* Sporophylls (Mekabu)

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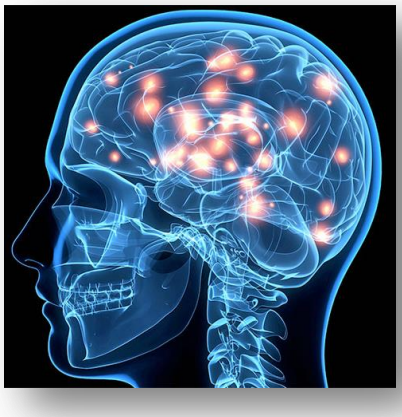

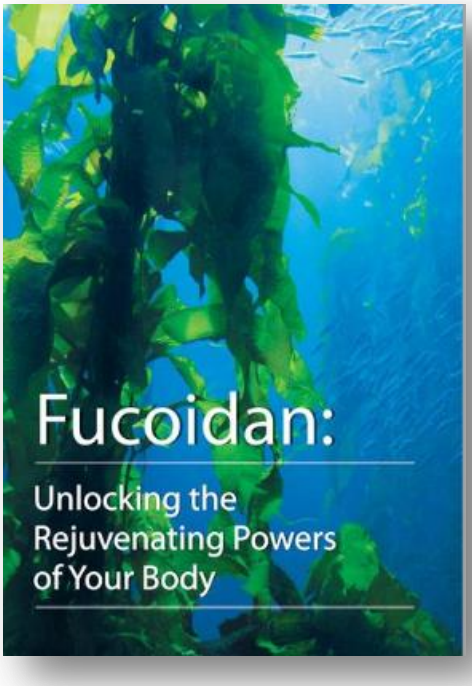

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Abstract

Fucoidan from Mekabu (sporophyll of *Undaria pinnatifida*), a dietary alga, exerts antitumor activity possibly through enhancing the immune response. The present report describes the effects of dietary Mekabu fucoidan on the tumor growth of mouse A20 leukemia cells and on T cell-mediated immune responses in T cell receptor transgenic (DO-11 - 10 - Tg) mice. The animals were fed with a diet containing 1 % Mekabu fucoidan (0.034 ± 0.003 g/mouse/day) for 10 days and subcutaneously (*s. c.*) inoculated with A20 leukemia cells. Thereafter, the mice were fed with the diet containing fucoidan for 40 days. Mekabu fucoidan inhibited tumors by 65.4 %. We studied how the killer activities of T cell-mediated and natural killer (NK) cells are augmented in DO-11 - 10 mice fed with Mekabu fucoidan. The cytolytic activities of ovalbumin (OVA), which is specific against OVA-transfected A20 (OVA-A20) B lymphoma cells, and NK cells against YAC-1 were significantly enhanced in the mice fed with fucoidan compared with a basic diet. Thus, these findings suggested that Mekabu fucoidan mediates tumor destruction through Th1 cell and NK cell responses.

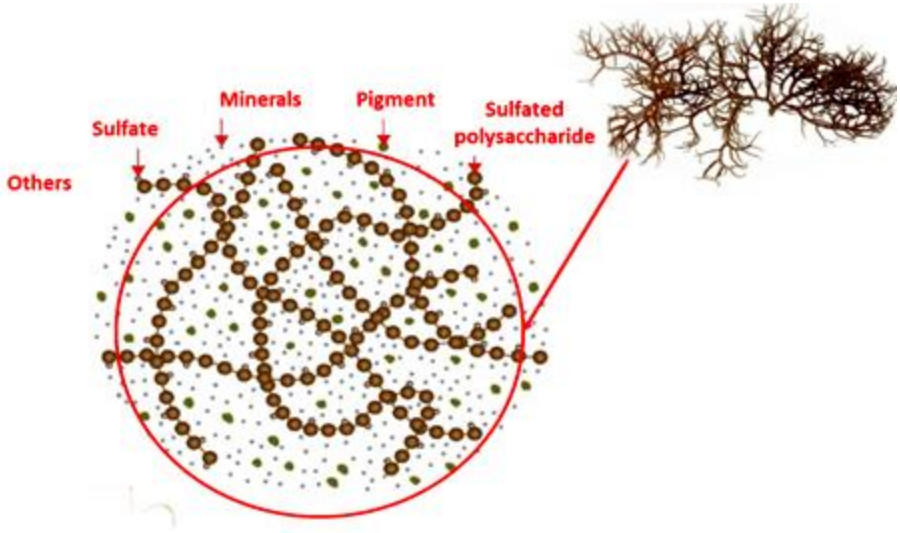
Studies from our and other laboratories have shown that intraperitoneally (*i. p.*) injected fucoidans from some brown alga have antitumor activity in mice [1], [2], [3]. We showed that injected Mekabu fucoidan inhibits the growth of P-388 leukemia cells inoculated *i. p.* into mice. The activation of NK cells is associated with IFN-γ and/or IL-12 [4], and activated cells might contribute to T cell activation [5], [6], [7], [8]. Natural killer activity might be an important factor in the anticancer activity [9], [10], [11]. We reported that the mechanism of antitumor activity by Mekabu fucoidan is through the enhancement of

natural killer (NK) cell activity augmented by γ-interferon (IFN-γ). The cytotoxicity level of the positive control, mitomycin C (MMC), was significantly higher than that of Mekabu fucoidan. The viability of the tumor cells was 48 % at a final Mekabu fucoidan concentration of 100 μg/mL and 43 % at 0.6 μg/mL of MMC. Therefore, Mekabu fucoidan acts as an immunopotentiator in tumor-bearing mice, leading to antitumor effectiveness [4]. Moreover, we demonstrated that Mekabu fucoidan suppressed the levels of the Th2 cytokines IL-4, IL-13 and of anti-OVA-specific IgE antibody in mice treated with OVA [12]. This approach was successful in mice injected *i. p.* with soluble Mekabu fucoidan.

On the other hand, we reported that a diet containing 2 % powdered fronds of *Undaria pinnatifida* (*U. pinnatifida*) inhibits 1,2-dimethylhydrazine (DMH)-induced intestinal tumorigenesis [13]. However, the same diet was ineffective against mammary tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in rats [14]. Others have found that water extracts of Mekabu significantly delay DMBA-induced mammary carcinogenesis in rats [15]. The powdered fronds and water extracts of *U. pinnatifida* appear to have antitumor activity during the post-initiation phase of carcinogen-induced mammary cancer. However, the most potent agents and the mechanisms of action remain obscure.

We initially examined whether dietary Mekabu fucoidan has antitumor activity against A20 lymphoma cells and whether it modulates Th1 cell and NK cell responses. Food intake by the mice in each group as measured weekly throughout the experimental period showed no significant difference between the groups fed with fucoidan (3.4 ± 0.3 g) and with a basic diet (3.7 ± 0.3 g). Therefore, the total daily intake of fucoidan per mouse was 0.034 g. A diet including Mekabu fucoidan for 10 days before and 40 days after inoculation with lymphoma A-20 cells significantly decreased the size and weight of the tumor [tumor inhibition ratio (TIR, 65.4 %) compared to controls (Figs. [1] A and B)]. However, the results were similar in control mice and in mice fed with the fucoidan diet only for 40 days after inoculation with A-20 cells (TIR, 22.4 %; Fig. [1] C). The difference between the mice that were given fucoidan or not for 10 days before tumor cell inoculation might be partly due to immune response activation. Accordingly, we examined the immune responses of ovalbumin (OVA)-specific TCR transgenic mice (Do-11 - 10 mice) fed with Mekabu fucoidan. Dietary fucoidan significantly increased NK activity compared with that in mice given a basic diet (Figs. [2] A and B). Dietary fucoidan also increased NK cell responses against YAC-1 lymphoma cells (NK sensitive) and the ovalbumin (OVA)-specific cytolytic activities against OVA-A20 cells whereas leukocyte activated killer (LAK) activity in mice fed with a basic diet was not altered (data not shown). The NK activity of splenic cells towards 50 mg/kg body fucoidan injected *i. p.* was 2.3-fold the normal level [4]. When the mice were injected *i. p.* with polyinosinic-cytidylic acid (poly I.C)(6 μg/kg body), that mimics a common product of viral infections rapidly up-regulating NK cell ability *in vivo*, the NK activity of poly I.C (71.7 %) was about 7-fold the normal level (10.5 %) (data not shown). The NK activity of mice fed the isoflavone genistein (20 mg/kg body weight 4.7.4′-trihydroxyisoflavone) was about 2.7-fold the normal level [16]. The NK activity of mice fed with fucoidan (18.9 %) was about 1.5-fold that of the control mice (12.7 %). These data indicate that fucoidan enhances NK activity.

Our data suggest that Mekabu fucoidan augments Th1 cell and NK cell activities in Do-11 - 10 mice. We examined whether the production of Th1 type cytokines (IFN-γ and IL-12) in T cells purified from the spleen is enhanced in Do-11 - 10 mice fed with Mekabu fucoidan. The IFN-γ production of Mekabu fucoidan fed mice was significantly increased than that of the control mice (Fig. [3] A). The amount of IL-12 in the T cell culture supernatants were essentially similar in the presence or absence of dietary Mekabu fucoidan



(Fig. [3] B). Nonetheless, a diet containing 1 % Mekabu fucoidan augments Th1 cell and NK activities.

Our observations indicate that fucoidan, a polysaccharide derived from *U. pinnatifida*, is a potent antitumor agent and that its mode of action is associated with the immune response. We postulate that *U. pinnatifida* extracts can activate NK cells during the post-initiation phase of cancer. Further study should elucidate the relationship between NK and dendritic cells in the antitumor activities of Mekabu fucoidan.

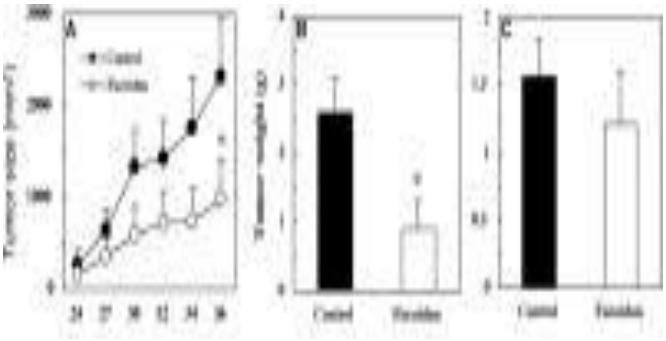


Fig.* 1- *Effect of Mekabu fucoidan on growth of A20 murine tumors.* A *Ten days after feeding with a fucoidan diet ora basic diet, BALB/c mice were inoculated s. c. with A20 leukemia cells (10^7 cells/mouse). Tumor volumes were measured at various times thereafter, using the formula, width × length × thickness. Data are shown as means ± SEM of ten mice.* B *Tumors were weighed 40 days after tumor cell inoculation.* C *Tumor weight in mice given Mekabu fucoidan diet from the time of tumor inoculation for 40 days. *, compared with control mice; p < 0.05 (Student's t-test).

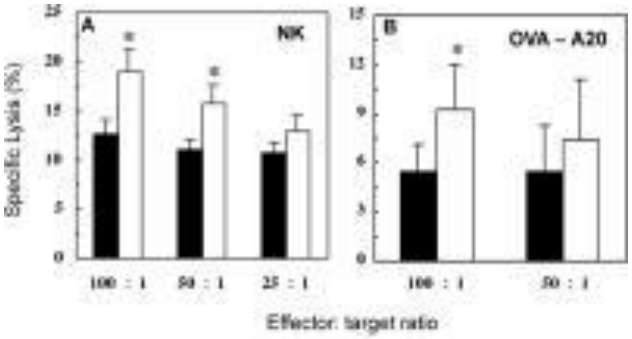


Fig.* 2 - *Evaluation of cellular immune response in Do-11 - 10 mice. Mice were fed with a diet containing 1 % Mekabu fucoidan for the entire experimental period. At 30 days after starting experiments, all mice were inoculated i. p. with 100 µg of OVA per day for 7 successive days. Twenty-four hours after the last injection, NK- (A), and OVA-specific cytotoxicity (B) was assayed in spleen cells. Data are shown as means ± SEM of nineteen mice. *, compared with controls; p < 0.05 (Student's t-test).

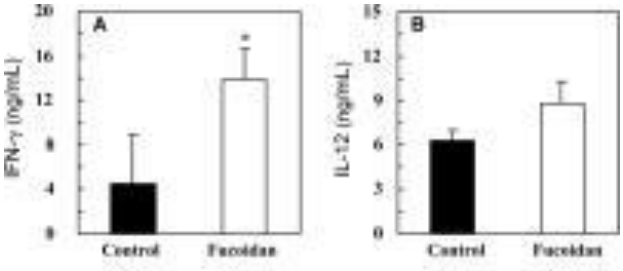


Fig.* 3 - *IFN-γ and IL-12 production measured in T cells from spleens from the same sets of mice as shown in Figure 2. T cells were stimulated with antiCD3 antibody at indicated antigen concentrations for 48 h. Cytokine levels in supernatants were then analyzed using ELISA. (A) IFN-γ, (B) IL-12. Data are shown as means ± SEM. *, compared with controls: p < 0.05 (Student's t-test).

Materials and Methods

Mekabu fucoidan: **Milled Mekabu (Jeollanam-do; Seoul, Korea), the sporophyll of *U. pinnatifida*; 1 kg was washed with 15 L of cold water (10 °C) for 2 min and extracted with 7 L of 10 µM HCl (pH 1) at room temperature for 15 h. The extract was centrifuged at 5,000 *g* for 10 min and the supernatant was passed through filter paper (No. 2; Whatman Japan K.K.; Tokyo, Japan) using Celite as filter aid. The filtrate was**

neutralized with 3 N NaOH and then ultrafiltered through ACP-1013 (MW: 6,000; Asahi Kasei Co. Ltd.; Tokyo, Japan) to obtain 93 g of lyophilized Mekabu fucoidan. The composition of Mekabu fucoidan was 15.6 % L-fucose, 20.7 % galactose, 8.2 % uronic acid, 23.0 % ester sulfate, and 32.5 % ash as a percentage of dry weight. The experimental diet containing 1 % Mekabu fucoidan was prepared at Oriental Yeast Co. Ltd. in the form of pellets by incorporating the fucoidan into the basic diet (MF: Oriental Yeast Co., Ltd.; Tokyo, Japan).

Experiment 1: **Male BALB/c mice (n = 10; Clea Japan Inc.; Tokyo, Japan) were housed under specific pathogen-free conditions and used at 6 weeks of age. We incorporated 1 % Mekabu fucoidan into a basic pelleted diet (MF; Oriental Yeast Co., Ltd.; Tokyo, Japan). Experimental mice were fed with either the basic diet or with the same diet containing Mekabu fucoidan for 10 days before inoculation *s. c.* with a suspension of 1×10^7 A20 lymphoma cells/mouse, and then both groups received the fucoidan diet ad libitum for 40 days. Control mice were similarly inoculated but received the basic diet throughout the experimental period. The tumor was measured and weighed. The tumor inhibition ratio (TIR) (%) was calculated as (C - T)/C × 100, where T is the mean tumor weight (g) of the treated group and C is that of the control group.**

Experiment 2: **Pairs of breeding Do-11 - 10 - Tg mice, which are transgenic for the TCR recognizing the ovalbumin peptide 323 - 339 (pOVA323 - 339) on a BALB/c background, were obtained from Dr. Ken Murphy (Washington University; St Louis, MO, USA). The mice were maintained under specific pathogen-free conditions at the Animal Facility of School of Allied Health Sciences, Kitasato University. Male mice (6 weeks of age, n = 19) were fed with the diet containing Mekabu fucoidan throughout the experimental period, and control mice received the basic diet for the entire experimental period. At 30 days from the start of the experiment, all mice were treated *i. p.* daily for 7 days with ovalbumin (OVA; Sigma Chemical Co.; St. Louis, MO, USA; 100 μg/mouse). Twenty-four hours later, the treated mice were anesthetized with ether and splenocytes separated from spleens were assayed for NK-, LAK-, and OVA-specific cytotoxicity.**

Spleen cells at a density of 1×10^6, 0.5×10^6, and 0.25×10^6 per mL were seeded in 0.1 mL of complete RPMI-1640 medium (Sigma) in 96-well microtiter plates. Target cells (YAC-1, RDM4 and OVA-A20 cells) (Dr. Habu, Department of Immunology, Tokai University, School of Medicine, Kanagawa, Japan) for NK-, LAK- and OVA-specific cytotoxicity assays, respectively, were labeled for 45 min with ^{51}Cr (sodium chromate, 37 MBq/mL; Dupont New Research Products; Boston, MA, USA), washed and adjusted to a density of 1×10^4/mL. Then, 0.1 mL aliquots were added to the wells. The amount of radioactivity in the supernatant was measured using a γ-counter (Gamma 5500B; Beckman Instruments Inc.; Fullerton, CA, USA) and the % specific lysis was calculated as described previously [4].

For IFN-γ and IL-12 analyses, splenocytes were passed through a column containing sterilized nylon fiber (Wako Chemical Industries, Ltd; Osaka, Japan) (0.7 g/10 mL syringe) for 1 h at 37 °C in a 5 % CO_2 atmosphere. Purified T cells at a density of 5×10^5 per mL in 0.2 mL of complete medium were seeded in each well of 96-well microtiter plates that had been coated overnight with anti-CD3 antibody (PharMingen; San Diego, CA, USA). The plates were incubated at 37 °C under a 5 % CO_2 atmosphere for 48 h. Levels of IFN-γ and IL-12 in the culture supernatants were measured in triplicate using ELISA kits (ENDOGEN, Inc.; Woburn, MA, USA) according to the manufacturer's instructions. The color reaction was stopped and absorbance was measured at 450 nm (Micro Plate Reader MOR A4I; Tosoh; Tokyo, Japan).

All experiments described herein adhered to the National Institutes of Health guidelines for the use of experimental animals. The Animal Use Committee of the Kitasato University School of Allied Health Sciences also approved the experimental protocol before starting the study.

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