

Fucoidan Induces Apoptosis of Human HS-Sultan Cells Accompanied by Activation of Caspase-3 and Down-Regulation of ERK Pathways

Yoshinobu Aisa,¹ Yoshitaka Miyakawa,^{1*} Tomonori Nakazato,¹ Hideyuki Shibata,² Kaori Saito,¹ Yasuo Ikeda,¹ and Masahiro Kizaki¹

¹Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

²Yakult Central Institute for Microbiological Research, Tokyo, Japan

Fucoidan, a sulfated polysaccharide in brown seaweed, was found to inhibit proliferation and induce apoptosis in human lymphoma HS-Sultan cell lines. Fucoidan-induced apoptosis was accompanied by the activation of caspase-3 and was partially prevented by pretreatment with a pan-caspase inhibitor, z-VAD-FMK. The mitochondrial potential in HS-Sultan cells was decreased 24 hr after treatment with fucoidan, indicating that fucoidan induced apoptosis through a mitochondrial pathway. When HS-Sultan was treated with 100 µg/mL fucoidan for 24 hr, phosphorylation of ERK and GSK markedly decreased. In contrast, phosphorylation of p38 and Akt was not altered by treatment with fucoidan. L-Selectin and P-selectin are known to be receptors of fucoidan; however, as HS-Sultan does not express either of these selectins, it is unlikely that fucoidan induced apoptosis through them in HS-Sultan. The neutralizing antibody, Dreg56, against human L-selectin did not prevent the inhibitory effect of fucoidan on the proliferation of IM9 and MOLT4 cells, both of which express L-selectin; thus it is possible fucoidan induced apoptosis through different receptors. These results demonstrate that fucoidan has direct anti-cancer effects on human HS-Sultan cells through caspase and ERK pathways. *Am. J. Hematol.* 78:7–14, 2005. © 2004 Wiley-Liss, Inc.

Key words: fucoidan; apoptosis; multiple myeloma; ERK; caspase-3

INTRODUCTION

The structure of fucoidan, a sulfated polysaccharide present in brown seaweed, has been reported to be a heparin-like molecule [1,2]. Fucoidan has anti-coagulant activities [1,3] and inhibitory effects on tube formation of human endothelial cells [4] as well as anti-cancer activities through the modulation of host immune systems [5–8]. It is reported that fucoidan can mobilize hematopoietic stem and progenitor cells from bone marrow into peripheral circulation, since the L-selectin on these cells are receptors for fucoidan [9,10]. Recently we reported that fucoidan prevents the attachment of *Helicobacter pylori* to mucin of gastric tract [11]. It has also been reported that fucoidan inhibits the growth of Ehrlich ascites carcinoma and lung cancer in vivo through inhibiting tumor angiogenesis [6,7]. In this study, we demonstrate that fucoidan directly induces apoptosis of

human HS-Sultan cells through caspase and ERK pathways.

MATERIALS AND METHODS

Cell Lines

The human lymphoma and myeloma cell lines, HS-Sultan and IM9, were cultured in RPMI1640 medium

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*Corresponding author: Yoshitaka Miyakawa M.D., Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: yoshi@sc.itc.keio.ac.jp

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(Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) in a humidified atmosphere with 5% CO₂. MOLT4 is a human T-cell lymphoma cell line. The cell lines were obtained from the Japan Cancer Research Resources Bank (JCRB, Tokyo, Japan).

Reagents

Fucoidan (from *Fucus vesiculosus*) and lithium chloride were purchased from Sigma (St. Louis, MO). Fucoidan was dissolved in PBS (Sigma) and sterilized by a 0.45- μ m syringe filter (Millipore, Carrigtwohill, Ireland). Pan-caspase inhibitor, z-VAD-FMK, was purchased from Calbiochem (San Diego, CA). A neutralizing antibody against human L-selectin (clone: Dreg 56) was obtained from BD Biosciences (San Jose, CA). Phospho-specific antibodies against ERK, p38, Akt, and GSK were purchased from Cell Signaling Technologies (Beverly, MA). Monoclonal antibody against GSK was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against total ERK, p38, and Akt were from Cell Signaling Technology, Inc. (Beverly, MA).

FACS Analysis of the Expression of CD62L and CD62P

The expression of L-selectin (CD62L) and P-selectin (CD62P) was analyzed using FITC-conjugated CD62L and PE-conjugated CD62P antibodies (BD Biosciences), respectively. FITC- or PE-conjugated isotype-matched IgG (BD Biosciences) were used as negative controls for FACS.

Cell Proliferation Assay

Cells were suspended at a final concentration of 1×10^5 cells/mL in RPMI 1640 with 10% FBS in a 96-well plate in triplicate. Fucoidan was added to each well at various concentrations and incubated for 2 days. MTT assays were performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). In some experiments, IM9 and MOLT4 cells were preincubated with 50 μ g/mL anti-L-selectin antibody (clone: Dreg 56) for 1 hr followed by the incubation with or without 1,000 μ g/mL fucoidan for 48 hr.

Analysis of Cell Cycle and Apoptosis

Cell cycle and apoptosis were analyzed using FACS as previously described [12]. In some experiments, HS-Sultan was pretreated with 50 μ M z-VAD-FMK for 1 hr followed by a 48-hr incubation with 100 μ g/mL fucoidan before analysis of apoptosis. Apoptotic

bodies were observed by Giemsa staining, after the cells were cytospun onto glass slides. Mitochondrial potential was determined by Rhodamine 123 staining using FACS Calibur (BD Biosciences), as previously reported [13]. Briefly, the cells were washed twice with PBS and incubated with 1 μ g/mL rhodamine 123 (Sigma) at 37°C for 30 min to assess the mitochondrial potential by FACS. A higher intensity of rhodamine 123 indicated a higher mitochondrial potential [13].

Caspase-3 Assays

The activation of caspase-3 was analyzed using a caspase-3 assay kit from BD Biosciences. Briefly, the FITC-conjugated antibody against the active form of caspase-3 provided in the kit was used for FACS analysis according to the manufacturer's instructions [12]. HS-Sultan was cultured with 100 μ g/mL fucoidan for the indicated times and subjected to caspase-3 assay to estimate the percentage of the cells with the active form of caspase-3.

Western Blotting

HS-Sultan was cultured with 100 μ g/mL fucoidan for 24 hr. The cells were washed twice with ice-cold PBS and lysed in a buffer composed of 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1 mM NaF, 1% Triton X-100 accompanied by 0.1 mM sodium orthovanadate and protease inhibitors (1 mM PMSF, 20 U/mL aprotinin) [14]. Protein concentration was measured by a Protein DC assay kit (Bio-Rad, Hercules, CA). Western blot was performed with specific antibodies and ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ), as previously reported [14].

RESULTS

Growth Inhibition by Fucoidan

We studied the inhibitory effect of fucoidan on the proliferation of HS-Sultan cells cultured with 0, 10, and 100 μ g/mL fucoidan for 48 hr followed by MTT assay (Fig. 1A). Fucoidan inhibited proliferation of HS-Sultan in a dose-dependent manner. The cell cycle analysis of HS-Sultan was analyzed using FACS (Fig. 1B). The percentage of sub-G1 fraction was increased in a time-dependent manner (0 hr, 1.1%; 24 hr, 4.0%; 36 hr, 28.7%; 48 hr, 89.0%) after stimulation with 100 μ g/mL fucoidan (Fig. 1B). However, cell cycle arrest in G1 or G2/M phases was not observed by fucoidan in HS-Sultan cells (Fig. 1B).

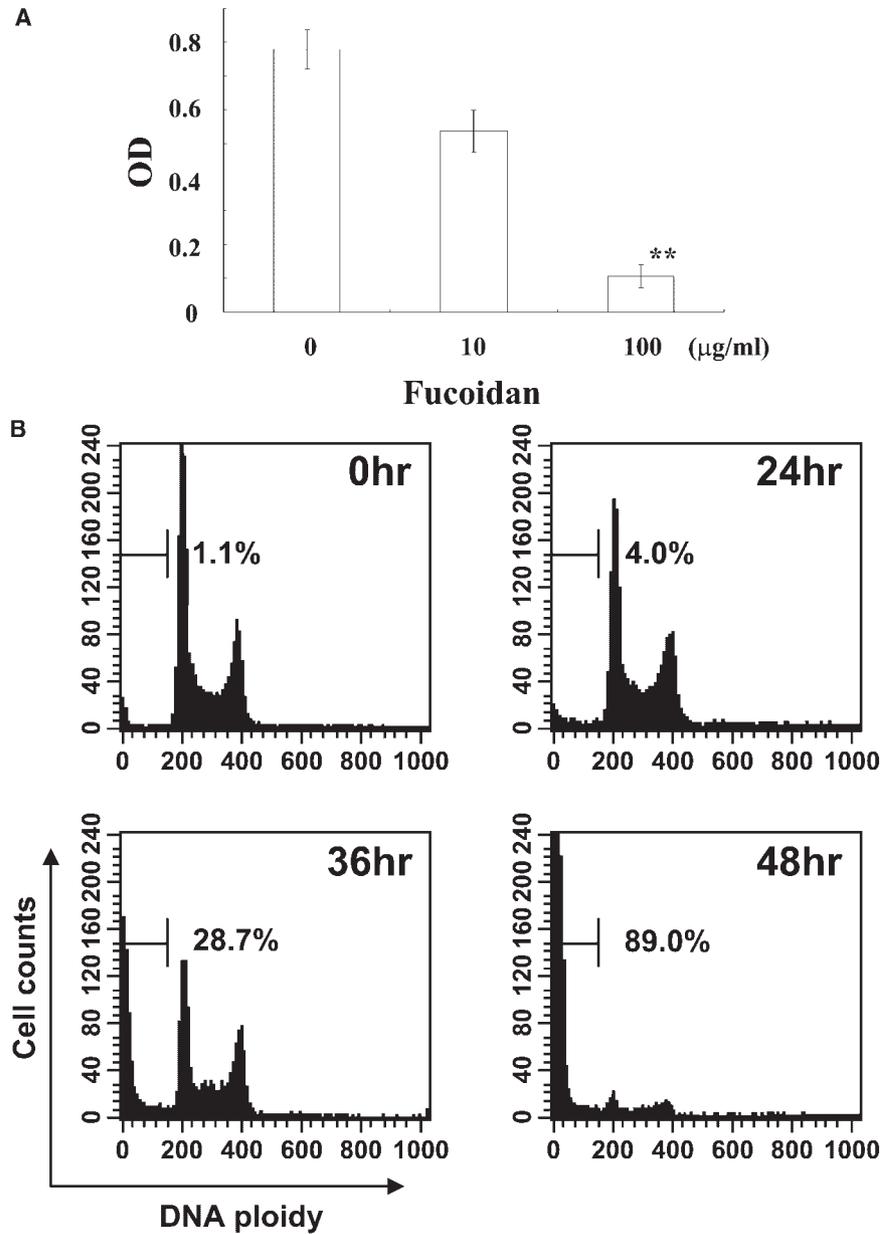


Fig. 1. Proliferation assay and cell cycle analysis of HS-Sultan cells. (A) HS-Sultan cells were cultured with 0, 10, and 100 µg/mL fucoïdan for 48 hr followed by MTT proliferation assay. Similar results were obtained in three independent experiments. Error bars indicate standard deviations of the triplicate experiments (** $P < 0.01$). (B) The cell cycle of HS-Sultan was analyzed by FACS. HS-Sultan was treated with 100 µg/mL fucoïdan for the indicated times, followed by propidium iodide staining. The number in each inset indicates the percentage of the cells in the sub-G1 fraction.

Analyses of Fucoïdan-Induced Apoptosis

Twenty-four hours after incubation with 100 µg/mL fucoïdan, HS-Sultan was cytopspun onto glass slides for morphological study. Apoptotic bodies were easily found by Giemsa staining in fucoïdan-treated cells but not in control cells (without fucoïdan) (Fig. 2A, and data not shown). To quantify the percentage of apoptosis caused by fucoïdan, HS-Sultan cells were subjected to annexin-V assay. The percentage of

annexin-V-positive cells was increased nearly 13-fold from 6.3% in control cells to 79.9% in cells treated for 48 hr with fucoïdan (Fig. 2B). Pretreatment of HS-Sultan with pan-caspase inhibitor, z-VAD-FMK, modestly inhibited the fucoïdan-induced apoptosis to 25.0% (Fig. 2B), indicating the involvement of caspase. To clarify the involvement of the activation of caspase-3 and the mitochondrial pathway, the percentage of the cells expressing the active form of caspase-3 and

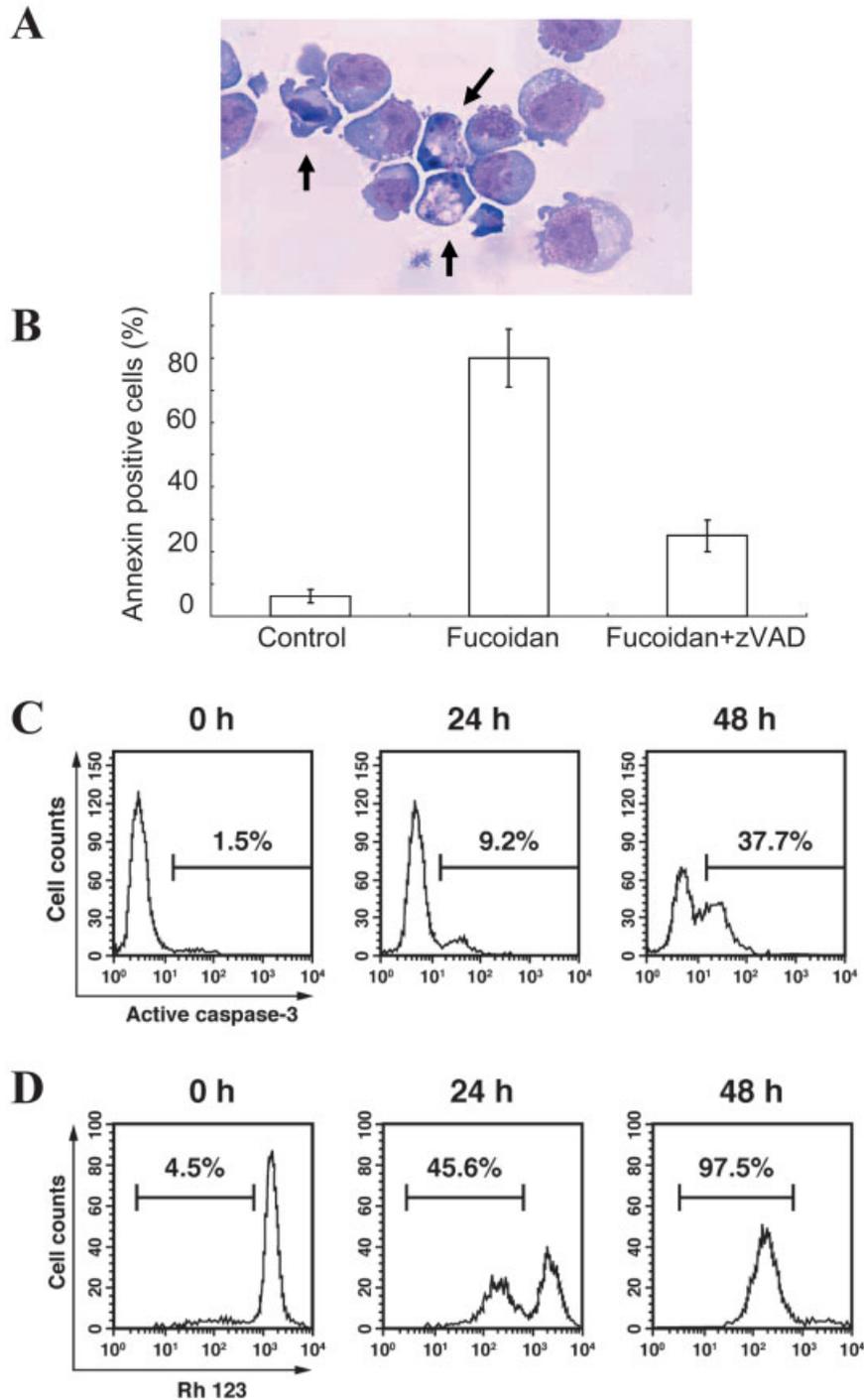


Fig. 2. Fucoidan-induced apoptosis in HS-Sultan cells. (A) Giemsa staining of HS-Sultan cells 24 hr after treatment with 100 µg/mL fucoidan. Arrows indicate the apoptotic bodies and nuclear condensation (original magnification 400×). (B) Detection of apoptosis by annexin-V staining. HS-Sultan was cultured with 100 µg/mL fucoidan for 48 hr, followed by annexin-V staining. In some experiments, HS-Sultan was pre-incubated with 50 µM z-VAD (pan-caspase inhibitor) for 1 hr followed by treatment with fucoidan. (C) Detection of the active form of caspase-3 by FACS. HS-Sultan was treated with 100 µg/mL fucoidan for the indicated time. The percentage of the cells expressing the active form of caspase-3 (the numbers in Fig. 2C) was analyzed using the specific antibody by FACS. (D) Analyses of mitochondrial electric potential. HS-Sultan was treated with 100 µg/mL fucoidan for 24 hr, followed by staining with Rh123. The numbers in the graphs are the percentages of cells with lower mitochondrial electric potential. Similar results were obtained in two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mitochondrial electric potential was analyzed by FACS. After incubation with 100 $\mu\text{g}/\text{mL}$ fucoïdan for 24 and 48 hr, the percentages of HS-Sultan cells expressing the active form of caspase-3 were 9.2% and 37.7%, respectively (Fig. 2C). The percentages of HS-Sultan cells with lower expressions of Rh123 increased in a time-dependent manner (4.5% at 0 hr, 45.6% at 24 hr, 97.5% at 48 hr), indicating that the mitochondrial electric potential was decreased by fucoïdan treatment (Fig. 2D). These results demonstrated that fucoïdan induced apoptosis in HS-Sultan cells through mitochondrial and caspase-3 pathways.

Intracellular Signaling of Fucoïdan-Induced Apoptosis

We observed that fucoïdan induced caspase-dependent apoptosis in HS-Sultan cells. To investigate fucoïdan-induced intracellular signaling, we analyzed the phosphorylation of ERK and Akt pathways by Western blot. Twenty-four hours after treatment with 100 $\mu\text{g}/\text{mL}$ fucoïdan, phosphorylation of ERK was clearly down-regulated (Fig. 3). It has been reported that p38, one of the ERK pathways, is involved in apoptosis and cellular differentiation [15–17]. Phosphorylation of p38, or of Akt kinase, was not altered by fucoïdan treatment in HS-Sultan (Fig. 3). In contrast, phosphorylation of GSK was decreased by fucoïdan in HS-Sultan (Fig. 3). Since dephosphorylation of GSK and ERK was reported to be involved in the induction of apoptosis [18–20], it is likely that this down-regulation of GSK and ERK by fucoïdan contributed to the fucoïdan-induced apoptosis in HS-Sultan cells.

Inhibition of GSK by Lithium Chloride

Since dephosphorylation of GSK was observed in HS-Sultan cells after stimulation with fucoïdan,

we investigated the role of GSK in apoptosis and caspase-3 activation using a GSK-specific inhibitor, lithium chloride. HS-Sultan cells were pretreated with 10 mM lithium chloride followed by stimulation with 100 $\mu\text{g}/\text{mL}$ fucoïdan for 48 hr and subjected to the analysis by annexin-V and caspase-3 assays (Fig. 4). Lithium chloride failed to inhibit fucoïdan-induced apoptosis and caspase-3 activation, indicating the activation of GSK is not essential for apoptosis by fucoïdan in HS-Sultan.

Expression of CD62L and CD62P on Human Myeloma and Leukemia Cell Lines

Since CD62L (L-selectin) and CD62P (P-selectin) are reported to be receptors of fucoïdan *in vitro* and *in vivo*, respectively [9,21,22], we analyzed their expression on human myeloma and leukemia cell lines by FACS (Fig. 5). HS-Sultan cells did not express CD62L or CD62P, indicating that these selectins were not involved in fucoïdan-induced apoptosis in these cell lines. In contrast, the IM9 myeloma cell line and the human T-cell lymphoma cell line, MOLT4, modestly expressed CD62L but not CD62P (Fig. 5).

Neutralization of CD62L

Because IM9 and MOLT-4 cells expressed CD62L, we studied whether CD62L is involved in the fucoïdan-induced inhibition of cellular proliferation using a neutralizing antibody against CD62L (clone: Dreg 56) (Fig. 6). Fucoïdan at 1,000 $\mu\text{g}/\text{mL}$ inhibited the proliferation of IM9 and MOLT4 cells (Fig. 6A, B). Dreg 56 antibody did not prevent the inhibitory effect of fucoïdan on IM9 and MOLT4 cells, indicating that CD62L is not involved in the effects of fucoïdan that inhibit cellular proliferation.

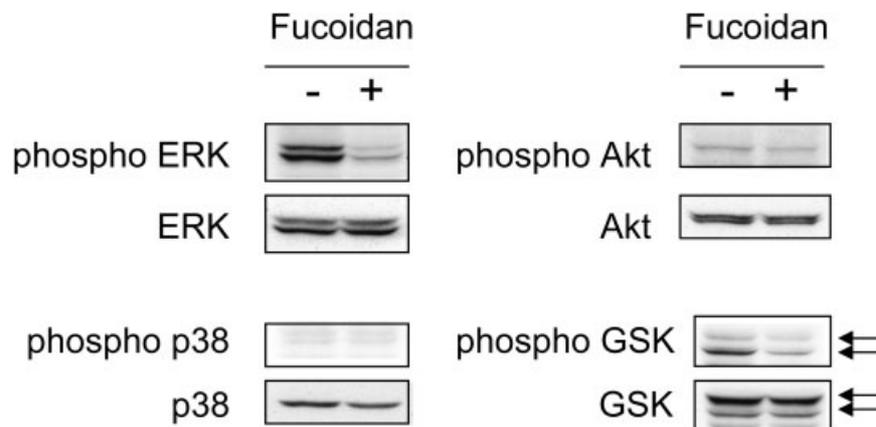


Fig. 3. Analyses of the intracellular signaling of fucoïdan by Western blot. HS-Sultan cells were treated with 100 $\mu\text{g}/\text{mL}$ fucoïdan for 24 hr. Phosphorylation of ERK, p38, Akt, and GSK was analyzed using phospho-specific antibodies. Each membrane was re-probed with anti-ERK, p38, Akt, and GSK antibodies to confirm equal protein loading.

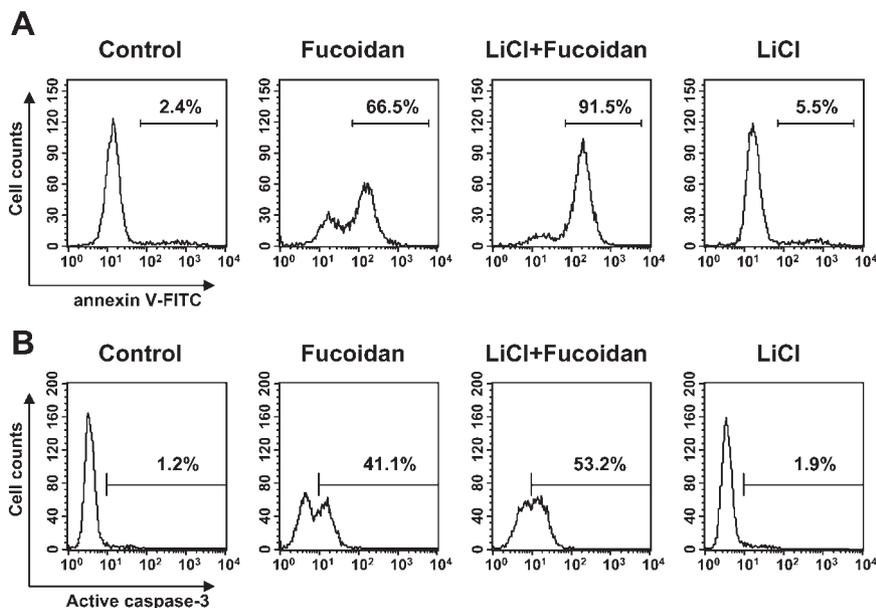


Fig. 4. Inhibition of GSK by lithium chloride. HS-Sultan cells were pretreated with or without 10 mM GSK-specific inhibitor, lithium chloride (LiCl), for 1 hr followed by the incubation with 100 μ g/mL fucoidan for 48 hr. Detection of apoptosis by annexin-V staining (A) and active caspase-3 (B) was performed as described for Fig. 2. Similar results were obtained in three independent experiments.

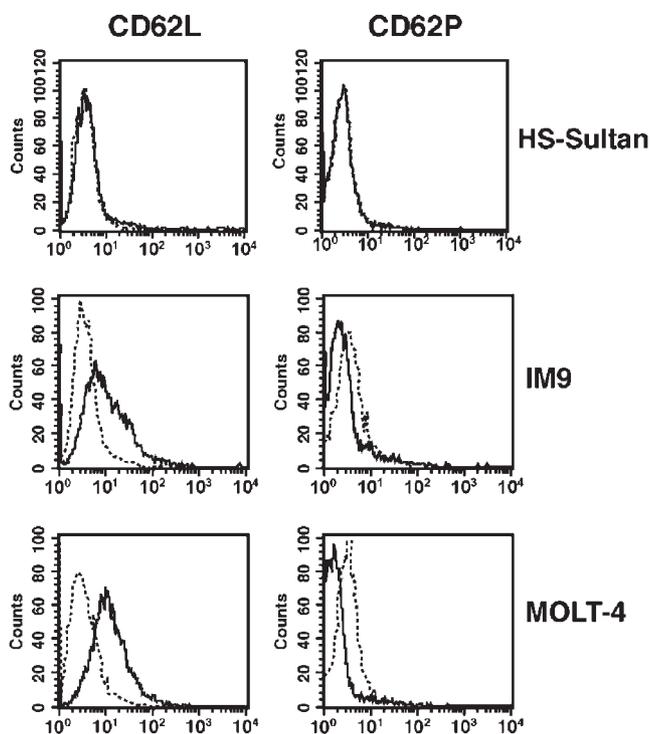


Fig. 5. Expression of CD62L and CD62P on human myeloma and lymphoma cell lines. Expression of CD62L (I-selectin) and CD62P (P-selectin) was analyzed using specific antibodies by FACS. Solid lines indicate the cells stained with anti-CD62L or CD62P antibodies, respectively. The dotted lines indicate cells stained with an isotype-matched IgG as a negative control.

DISCUSSION

Fucoidan is a sulfated polysaccharide from the brown alga, *Fucus vesiculosus*. It has been reported that fucoidan has several biological effects such as heparin-like anti-coagulant activity [1,3], interference with primary adhesion events during lymphocyte recirculation [9,10], inhibition of endothelial cell spreading [4], and an influence on the attachment of *H. pylori* to the gastric wall [11]. These different biological activities of fucoidan come from its different branched structures and sugar moieties. In this study, we used a commercial available fucoidan (Sigma) purified from *F. vesiculosus*. We demonstrated, to our knowledge for the first time, that fucoidan induced apoptosis in human myeloma and leukemia cells. We found that fucoidan induced the activation of caspase-3 and reduced the mitochondrial electric potential in HS-Sultan. Apoptotic bodies were observed in HS-Sultan 24 hr after treatment with fucoidan. These results indicated that fucoidan induced apoptosis through caspase and mitochondrial pathways. The pan-caspase inhibitor, zVAD, prevented the fucoidan-induced apoptosis by 69%, but not 100%, suggesting that caspase-independent pro-apoptotic pathways are also involved (Fig. 2B).

Because MAPK pathways are involved in cellular proliferation, differentiation, and apoptosis [17, 23–25], we studied phosphorylation of ERK and p38

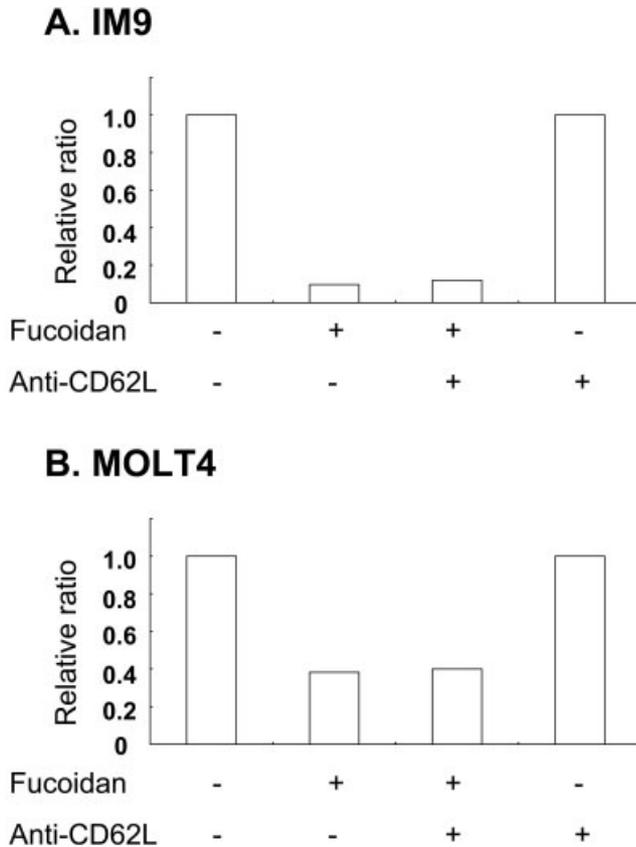


Fig. 6. Neutralizing antibody against CD62L failed to prevent fucoïdan-induced growth suppression of human myeloma and lymphoma cell lines. The human myeloma cell line, IM9, and the human T-cell lymphoma cell line, MOLT4, were incubated with 1,000 $\mu\text{g}/\text{mL}$ fucoïdan for 48 hr followed by MTT proliferation assay. In some experiments, the cells were pretreated with 50 $\mu\text{g}/\text{mL}$ of a neutralizing antibody (clone: Dreg 56) against human CD62L. Similar results were obtained in two independent experiments. The Y axis demonstrates the ratio to controls.

by Western blotting. Fucoïdan clearly decreased the phosphorylation of ERK but not p38. Phosphorylation of Akt was not altered by fucoïdan in HS-Sultan. The serine/threonine kinase, GSK, is part of Wnt signaling and is involved in cell cycle regulation and apoptosis [26,27]. Fucoïdan induced the dephosphorylation of GSK in HS-Sultan, suggesting that GSK is involved in fucoïdan-induced apoptosis. However, the GSK-specific inhibitor, lithium chloride, failed to prevent fucoïdan-induced apoptosis and activation of caspase-3 (Fig. 4), suggesting that GSK is not essential in fucoïdan-induced apoptosis in HS-Sultan. Religa et al. reported fucoïdan inhibits smooth muscle cell proliferation and reduces ERK activity [28], which is compatible with our observations in this study. The PI3K/Akt pathway is the most frequent upstream mediator of GSK phosphorylation, but phosphoryla-

tion of Akt was not altered by fucoïdan in HS-Sultan (Fig. 3). As p70 ribosomal S6 kinases (p70S6K), p90RSK (also known as MAPKAP-K1), protein kinase A (PKA), and PKC are known to phosphorylate GSK [29], it will be important to investigate the role of these GSK mediators in future studies. We also analyzed protein expressions of other intracellular signaling molecules that regulate apoptosis, but the expression levels of bcl-2 or bcl-X_L protein were not altered by fucoïdan in HS-Sultan (data not shown). Our data regarding cellular signaling by fucoïdan is limited in HS-Sultan; thus, it will be necessary to study other cell lines in the future.

CD62L and CD62P work as fucoïdan receptors [9,10]. Although HS-Sultan is sensitive for fucoïdan, it does not express CD62L or CD62P. These results suggested that other receptors might exist on HS-Sultan. The neutralizing antibody against human CD62L, Dreg 56, did not prevent fucoïdan-induced inhibition of IM9 and MOLT4 cells. Although integrin induced outside-in and inside-out signaling are involved in many physiological responses, such as activation of platelets and white blood cells [30,31], there are few reports about CD62L-induced signaling. Downey et al. reported that the activation of CD62L on neutrophils by the activation of antibodies induced elevation of intracellular calcium concentrations accompanied by the phosphorylation of intracellular proteins [21]. As fucoïdan is a complex sulfated polysaccharide with a high molecular weight, we cultured HS-Sultan cells with D-fucose (up to 1,000 $\mu\text{g}/\text{mL}$) to exclude the possibility that fucoïdan killed the cells by changing the osmotic pressure of the culture condition. D-Fucose at 1,000 $\mu\text{g}/\text{mL}$ did not demonstrate any inhibitory effects on the proliferation of HS-Sultan by MTT assay (data not shown).

In this study, we used fucoïdan extracted from *F. vesiculosus*. Other polysaccharides, extracted from the brown seaweeds *Sargassum thunbergii* and *Sargassum kjellmanianum*, revealed antitumor effects for Ehrlich carcinoma-bearing mice and L1210 leukemia-bearing mice, respectively [7,32]. In these in vivo experimental models, it was suggested that fucoïdan-induced enhancement of immune responses are involved in the anti-cancer effects of fucoïdan. In contrast, Riou et al. reported that fucoïdan extract obtained from the brown seaweed *Ascophyllum nodosum* directly inhibited the proliferation of a human non-small cell lung cancer cell line (NSCLC-N6) [5]. We acknowledge our observations are limited as they were obtained from in vitro experiments; therefore, we hope in future studies to investigate the anti-tumor effects of fucoïdan using a tumor-bearing murine model.

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