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Fucoidan Preferentially Induces the Mitochondrial Pathway-Mediated Cell Death in Hematopoietic Malignant Cells

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ABSTRACT

Fucoidan is a sulfated polysaccharide that occurs at high levels in some seaweed and in the eggs of sea urchin and bodies of sea cucumbers. Uptake of fucoidan-rich food is suggested to be one of the causes of longevity in Japan. Fucoidan shows anti-coagulant, anti-virus, anti-inflammatory, anti-tumor and other various favorable biological activities both *in vitro* and *in vivo*. In this study, the cell death induction activities of fucoidan were investigated using eight different human cancer as well as normal cell lines at the cellular and molecular levels. Two hematopoietic cell lines, Jurkat and K562, showed maximum sensitivities against fucoidan. Fucoidan at 100 μg/ml induced cell death of Jurkat cells. To further investigate the cell death induction mechanism of fucoidan against hematopoietic cell lines, we detected membrane turnover activities by annexin-V assay and detected active caspases by western blotting. An annexin-V assay indicated higher apoptosis induction activities in Jurkat cells. Higher inductions of executioner caspases, caspase-3, -6, and -7 were also observed in Jurkat cells. In particular, the activation of caspase-6 was relatively high and detected 12 hours after fucoidan treatment. Treatment with inhibitors against pan caspases and caspase-9 showed significant reduction effects on fucoidan-induced cell death. An apparent decrease of mitochondrial membrane potential was observed in both of the hematopoietic cell lines. These results strongly suggest the involvement of a mitochondrial apoptotic pathway by fucoidan in hemaotopoietic cells.

Keywords: annexin-V, apoptosis, caspase, caspase-9, F562, Jurkat
Abbreviations: 7-AAD, 7-aminoactinomycin D; **Bip**, immunoglobulin heavy-chain binding protein; **EDTA**, ethylenediaminetetraacetic acid; **EGTA**, ethylene glycol-bis (β-amino ethylether)-N,N,N',N'-tetraacetic acid; **ER**, endoplasmic reticulum; **FACS**, fluorescence activated cell sorter; **IFN**, interferon; **LDH**, lactate dehydrogenase; **PARP**, poly (ADP-ribose) polymerase; **PBS**, phosphate-buffered saline; **SDS**, sodium dodecyl sulfate; **TNF**, tumor necrosis factor; **TRAIL**, TNF-related apoptosis-inducing ligand

INTRODUCTION

Fucoidan is a sulfated polysaccharide mainly consisting of sulfated L-fucose. It was first isolated from brown marine algae, *Fucus vesiculosus* as well as *Laminaria digitata* by Killing and named fucoidin (Killing 1913). Later, fucoidin was re-named as fucoidan (McNeely 1959). The cell walls of brown marine algae contain fucoidan, as much as 40% of the dry weight, and their estimated role in algae is to protect the body of the seaweed against mechanical stress. The egg jelly coat of sea urchins and body walls of sea cucumbers also contains fucoidan (Alves *et al.* 1997). Interestingly, fucoidan is suggested to function as a species-specific marker between sperm and eggs of sea urchins. Fucoidan has both specific and non-specific protein-binding activities. Although the mechanism is mostly unrevealed, fucoidan shows various favorable biological activities, including anti-coagulant (Pereira *et al.* 1999), anti-virus (Damonte *et al.* 2004), anti-inflammatory, hypotensive and hypocholesterol actions. Anti-tumor activities of fucoidan have been shown against Sarcoma180, L-1210 and Ehrlich carcinoma maintained in mice (Yamamoto *et al.* 1974; Usui *et al.* 1980; Chida and Yamamoto 1987).

In this work, we examined cell death and apoptosis induction activities of fucoidan extracted from *Fucus vesiculosus* on different human cell lines using an apoptosis analysis assay system we have recently developed (Yamaguchi *et al.* 2007). On the basis of the results obtained, we propose that fucoidan obtained from *F. vesiculosus* induces both necrotic and apoptotic cell death in hematopoietic malignant cell lines.

MATERIALS AND METHODS

Reagents

Fucoidan from *Fucus vesiculosus* (Lot Number: 046K3785), staurosporine, Rhodamine 123 and 7-Aminoactinomycin D (7-AAD) were purchased from Sigma Chemical Co. (St. Louis, MO). Fucoidan was dissolved in PBS and sterilized by filtration through a 0.45 mm pore filter. Recombinant human TNF-related apoptosis-inducing ligand (TRAIL) and Interferon-γ (IFN-γ) were purchased from PeproTech Inc. (Rocky Hill, NJ). Caspase family inhibitors were products of BioVision (Lyon, France). All the other chemicals used were of analytical grade.

Cell culture

The human embryonic kidney 293, cervical carcinoma Hela, heaptocellular carcinoma Hep G2 and breast adenocarcinoma MCF-7 cells were cultured in E-MEM supplemented with 1% Non Essential Amino Acid (NEAA). The human myeloid cell line, K562 and T-cell lymphoblastoid cell line, Jurkat were grown in RPMI 1640 medium, and the human neuroblastoma SH-SY5Y cells were grown in D-MEM/ F-12 with 1% NEAA. The cells were maintained in the medium supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio Inc., Texas), and penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. Neonatal normal human fibroblasts (Lot Number: 5F0474) were purchased from Lonza (Walkersville, MD) and cultured according to the manufacture's instructions.

Received: 8 September, 2008. Accepted: 6 October, 2008.

Original Research Paper

Cell viability and cytotoxicity assay

The cells were seeded onto 96-well plates at the following density; HEK; 1.2 × 10^4 cells/cm^2, Hela; 0.8 × 10^4 cells/cm^2, HepG2; 2 × 10^4 cells/cm^2, Jurkat; 30 × 10^4 cells/ml, K562; 20 × 10^4 cells/ml, MCF-7; 4 × 10^4 cells/cm^2, SH-SY5Y; 2 × 10^4 cells/cm^2, normal human dermal fibroblast; 1 × 10^4 cells/cm^2. After a 24 hr incubation, the cells were exposed to the indicated concentrations of fucoidan for 48 hr. Half the volume of the supernatants was used to measure the cytotoxicity by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, WI). A WST-8 assay was performed in the residual medium containing the cells using Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan). The experimental results were calculated as described using the manufacture's instructions and expressed as means of triplicates of two or three separate experiments ± standard deviation (SD).

Immunofluorescence microscopy

Jurkat and K562 cells treated with fucoidan were fixed with 70% ethanol, followed by labeling with Hoechst 33258. The nuclear condensation and morphological changes were observed under a confocal laser scanning fluorescence microscope LSM 510 (Carl Zeiss, Jena, Germany).

FACS analysis

Apoptosis was determined by externalization of phosphatidylserine on the outer membrane and 7-AAD exclusion, and mitochondrial potential by rhodamine 123 staining. After treatment with fucoidan for the indicated time, 1 × 10^6 cells were washed with PBS and stained with Annexin-V-Fluos (Roche Diagnostics GmbH. Penzberg, Germany) and 7-AAD for apoptosis assay. The cells were incubated with 10 μM Rhodamine 123 for 30 min at 37°C for mitochondrial membrane potential assay. Then, the cells were subjected to FACS analysis using FACSCalibur (BD Biosciences, San Diego, CA). The data was analyzed using the CellQuest program.

DNA ladder detection

2 × 10^6 apoptotic cells were lysed in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EGTA and 0.5% Triton-X 100. After digestion with RNase A and Proteinase K, DNA fragments were extracted with isopropanol precipitation. The DNA obtained was electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Immunoblot analysis

Cells were lysed in homogenate buffer [50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 μg/ml leupeptin, 1 mM PMSF, 0.1% Triton-X 100, 1X Complete Mini (Roche)] by sonication. Protein content was measured by Protein Assay Lowry Kit (Nacalai Tesque, Inc., Kyoto, Japan). Fifty μg of total proteins were subjected to SDS-polyacrylamide gel electrophoresis and then immunoblotted using antibodies against active caspases, cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Inc., Beverly, MA), Bip (BD Biosciences), and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

RESULTS

Fucoidan preferentially induced cell death of hematopoietic cell lines

We first examined the sensitivity of cell lines derived from various human tissues after a 48-hr treatment of 100 μg/ml fucoidan applying the WST-8 cell viability assay (**Fig. 1A**). Among the cell lines tested, the Jurkat T-cell lymphoblastoid cell line was most sensitive, and the proliferative activity was inhibited by about 80%. In addition, the K562 myeloid cell line derived from different hematopoietic lineage was also sensitive, but to a lesser extent. In contrast, at 100 μg/ml concentration, fucoidan showed little effect, if any, on not only normal neonatal human dermal fibroblasts (NHDF) but also on the HEK, Hela, HepG2, MCF-7, and SH-SY5Y cell lines. This result demonstrated the cell type-selective anti-proliferative effect of fucoidan.

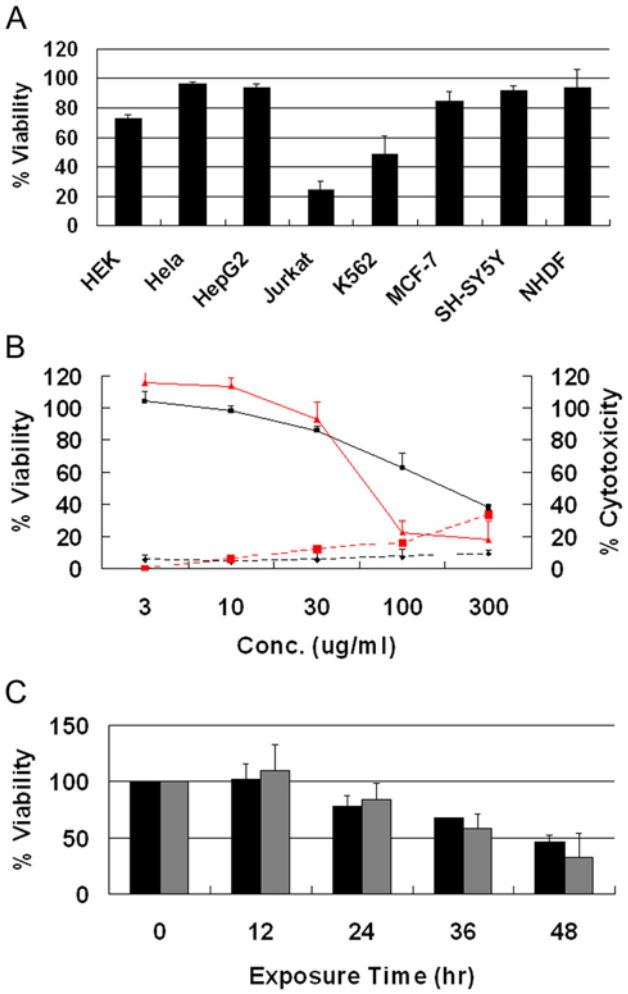

Fig. 1 Fucoidan preferentially induces cell death of hematopoietic cell lines. (**A**) Cell-type selective anti-proliferative effects of fucoidan. Human various cell lines and normal dermal fibroblasts were incubated with 100 μg/ml of fucoidan for 2 days, and then cell viability was determined by an WST-8 assay. (**B**) Dose-responses of Jurkat and K562 cells to fucoidan. Jurkat (red lines) and K562 cells (black lines) were treated with the indicated concentration of fucoidan for 2 days. Cell viability (solid lines) and cytotoxicity (dot lines) were measured as described in experimental procedure. (**C**) Time-course study of fucoidan's effect. Jurkat (gray bars) and K562 (black bars) cells were treated with 300 μg/ml of fucoidan for the indicated exposure time. Cell viability was calculated by counting viable cell number using a hematocytometer.

Next, we investigated the dose-dependent and exposure time-dependent responses of hematopoietic cells to fucoidan. As shown in **Fig. 1B**, the presence of fucoidan inhibited the growth of both the Jurkat and K562 cells in a dose-dependent manner after 2 days of treatment. To determine whether the inhibition was caused by growth arrest or cell death, the cytotoxicity of fucoidan was examined by measuring the activity of LDH released from lysed cells into the medium. Cytotoxicity showed clear inverse correlation with cell viability, although the maximum increase of cytotoxicity was very small. This implies that fucoidan inhibited the proliferative activity by killing the cell.

A time-course study was then performed by counting the number of viable Jurkat and K562 cells after treatment for the indicated time in **Fig. 1C**. As a result, 300 μg/ml of fucoidan induced cell death after 24 h of treatment and the death rates increased up to 48 hr. These results indicate that fucoidan induced cell death of hematopoietic cells in a dose- and time-dependent manner.

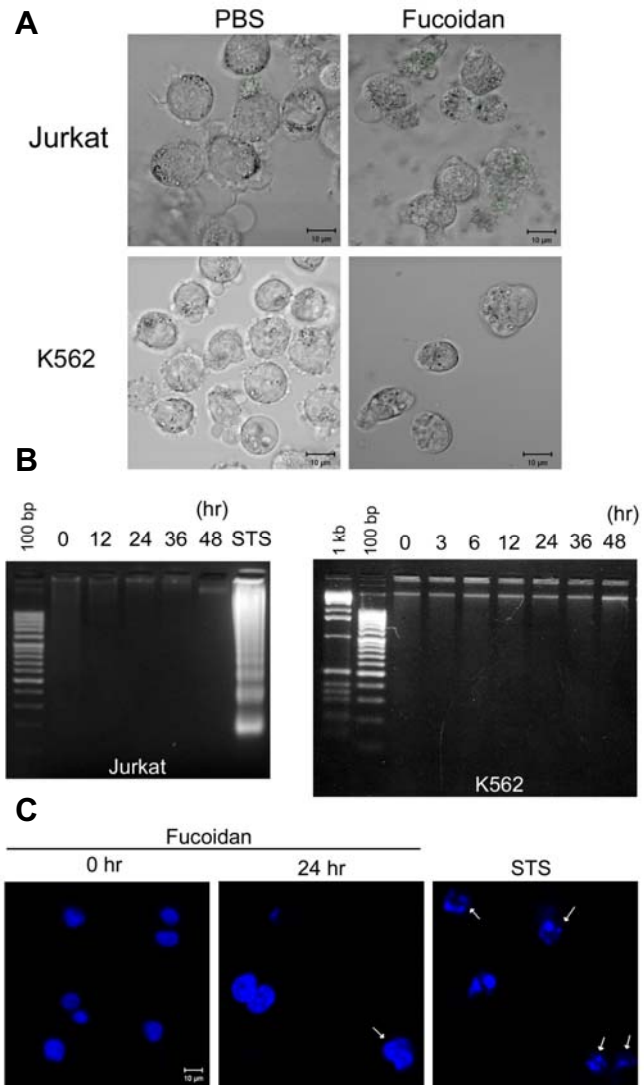

Fig. 2 Fucoidan induced cell shrinkage and nuclear condensation. (**A**) Optical microscopic images of fucoidan-treated Jurkat (upper panel) and K562 cells (lower panel). After a 48-hr exposure of control PBS (-) or 300 μg/ml fucoidan, Jurkat and K562 cells were observed under a light microscope. *Bars*, 10 μm. (**B**) DNA ladder/ fragmentation assay. (**C**) Nuclear condensation of Jurkat cells exposed to 300 μg/ml of fucoidan for 24 hr. 3 μM staurosporine (STS) treatment for 8 hr was applied for detection of the nuclear fragmentation in jurkat cells (as the arrow shows). *Bars*, 10 μm.

Fucoidan induced cell shrinkage and nuclear condensation, but not DNA fragmentation

Because the released LDH activity did not increase before the decrease of mitochondrial activity, as measured by the WST-8 assay, we considered that the fucoidan-induced death could have been caused by apoptosis. To verify this possibility, we observed the morphological changes of cell shape and nucleus in fucoidan-treated cells. Under the control conditions, Jurkat and K562 cells were round and bright with microvilli on the cell surface. After the 48 hr treatment with fucoidan, the microvilli disappeared and cell shrinkage was also observed (**Fig. 2A**). Then, the nucleosomal DNA ladder formation and fragmentation was investigated (**Fig. 2B**). For all the following apoptosis analysis, staurosporine, a powerful apoptosis inducer triggering the mitochondrial apoptotic pathway, was used. This also provided insight on susceptibility differences between the Jurkat and K562 cells. The treatment with saturosporine induced DNA fragmentation in the Jurkat cells (**Fig. 2B**), but not in K562 cells (data not shown). Furthermore, the nuclear condensation and fragmentation was observed after staurosporine treatment and Hoechst dye staining (**Fig. 2C**). On the other hand, fucoidan induced large fragmentation of the DNA but no DNA ladder formation was detected in both the Jurkat and K562 cell lines (**Fig. 2B**). The shrinking of the nucleus in dying cells were observed after the 24 hr treatment of 300 μg/ml of fucoidan (**Fig. 2C**). Thus, some of apoptotic hallmarks could be observed in fucoidan-exposed Jurkat and K562 cells.

Fucoidan induced early apoptotic membrane turnover and mitochondrial membrane disruption in hematopoietic cells

To further elucidate if the cell death was by apoptotic or necrotic, we performed FACS analysis using Annexin-V and 7-AAD (**Fig. 3A**). Population of apoptotic Jurkat cells with Annexin-V positive and 7-AAD negative signal levels increased from 12 h and reached to a maximum at 24 hr, followed by an increase of Annexin-V and 7-AAD double positive population (showing late apoptosis and cell death). Unlike the Jurkat cell line, an early apoptosis population was undetectable in fucoidan-treated K562 cells. Instead, its late apoptosis population was increased moderately after 48 hr. It should be noted that K562 cells could not be stained by Annexin-V in staurosporine treatment.

We then investigated the decrease of mitochondria membrane potential using Rhodamin 123 as a fluorescence marker (**Fig. 3B**). Nine period five percent of the untreated Jurkat cell population showed low Rhodamine signals and treatment with fucoidan increased the signal up to 39.9%. In K562 cells, a 2.9 to 24.6% increase of low Rhodamine signals was observed after fucoidan treatment. The increase of the low Rhodamine signals indicates a loss of mitochondrial integrity due to the treatment of fucoidan in both hemaopoietic cell lines.

Cell death induced by fucoidan is accompanied by caspase activation through the mitochondrial pathway

We also performed Western blotting using anti-active caspase antibodies to examine if active fragments of caspases were indeed produced upon fucoidan treatment (**Fig. 4A**). First, the cleavage of the caspase substrate PARP was assessed. Prior to the fucoidan treatment, full length PARP had already processed to its cleaved 89 kDa form in K562 cells, which might have resulted in the large fragmentation of the genomic DNA in control K562 cells (**Fig. 2B**). The cleaved fragments of PARP were increased after the 48 hr treatment in the Jurkat and K562 cells. These results indicate that fucoidan activated a caspase-dependent apoptosis program. Furthermore, active fragments of the apoptotic executioner caspases including caspase-3, -6 and -7 were detected in both hematopoietic cells after 48 hr, thereby confirming the caspase activation. Multiple pathways, including the mitochondrial apoptotic pathway triggered by external stimuli (staurosporine, ultraviolet, etc.), the death receptor-mediated pathway (TRAIL, TNF-a, etc.) and endoplasmic reticulum (ER) stress-induced pathway are involved in apoptosis. We further examined which pathway leading to apoptosis was activated by fucoidan. The markers used in this study were as follows; Bip is an ER stress-induced ER chaperone protein. Capase-8 and -9 are initiator caspases activated by death receptor and mitochondria pathways, respectively. Western blot analysis using these markers showed caspase-9 was activated much earlier than the executioner protease activation after the 6 hr treatment, and this effect was more prominent in K562 cells. In contrast, caspase-8 and Bip were not changed. These results suggested that fucoidan triggered the mitochondrial apoptotic pathway.

To elucidate the contribution of caspase activation in fucoidan-induced cell death, a pan-caspase inhibitor Z-VAD-FMK was used. Treatment with the pan-caspase inhibitor resulted in a significant ($p<0.01$) decrease of fucoidan-induced apoptosis both in the Jurkat cells and K562 cells (**Fig. 4B**). To further confirm which caspase is essential for apoptosis induction by fucoidan, specific caspase inhibitors were

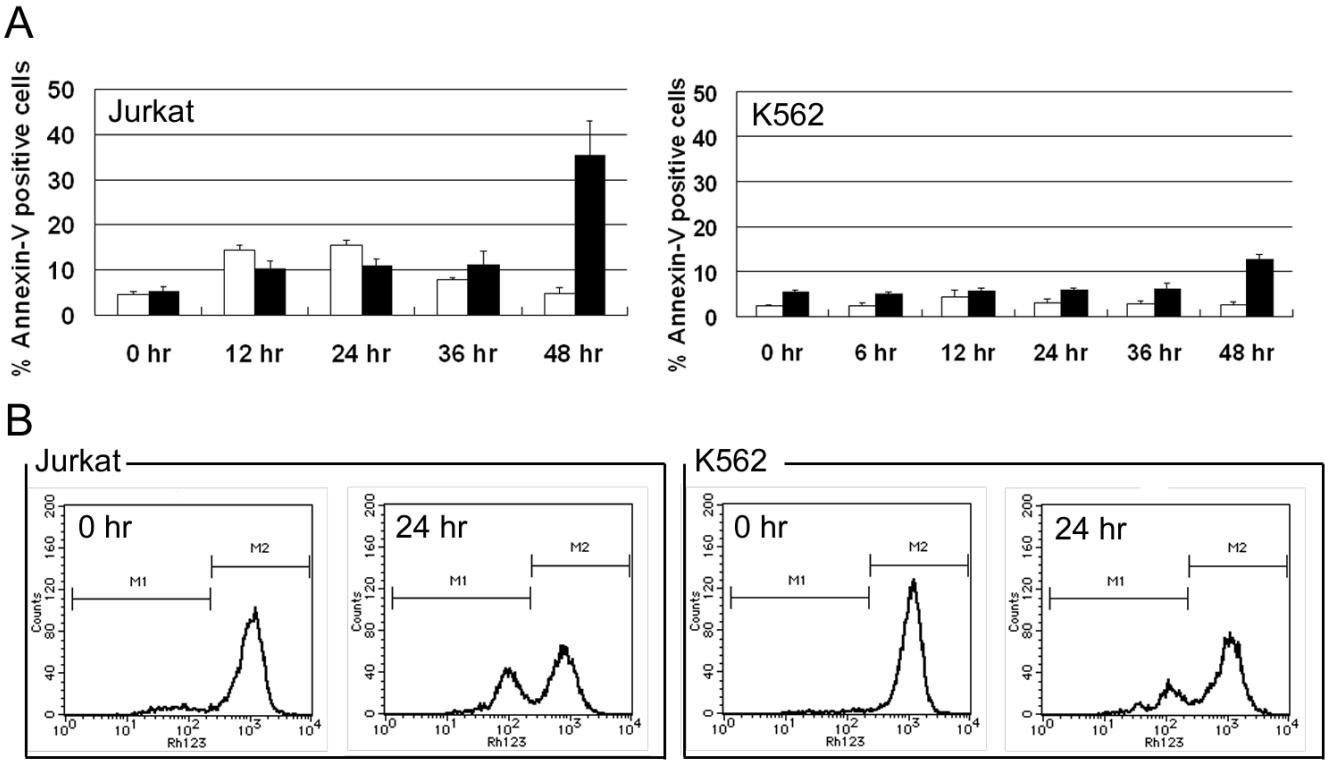

Fig. 3 Fucoidan induced membrane turnover and decrease of mitochondrial membrane potential. (**A**) Annexin-V and 7-AAD staining of fucoidan-treated Jurkat and K562 cells. Jurkat and K562 cells were treated with 300 μg/ml fucoidan for the indicated times, followed by FACS analysis using Annexin-V-FITC and 7-AAD. Early apoptosis population was represented by an Annexin-V positive and 7-AAD negative fraction (white bars), and late apoptosis and cell death population by an Annexin-V and 7-AAD double positive fraction (black bars). (**B**) Analysis of mitochondrial membrane potential in fucoidan-treated cells. M1 and M2 population represent the cell population with lower mitochondrial potential and that with the higher potential, respectively.

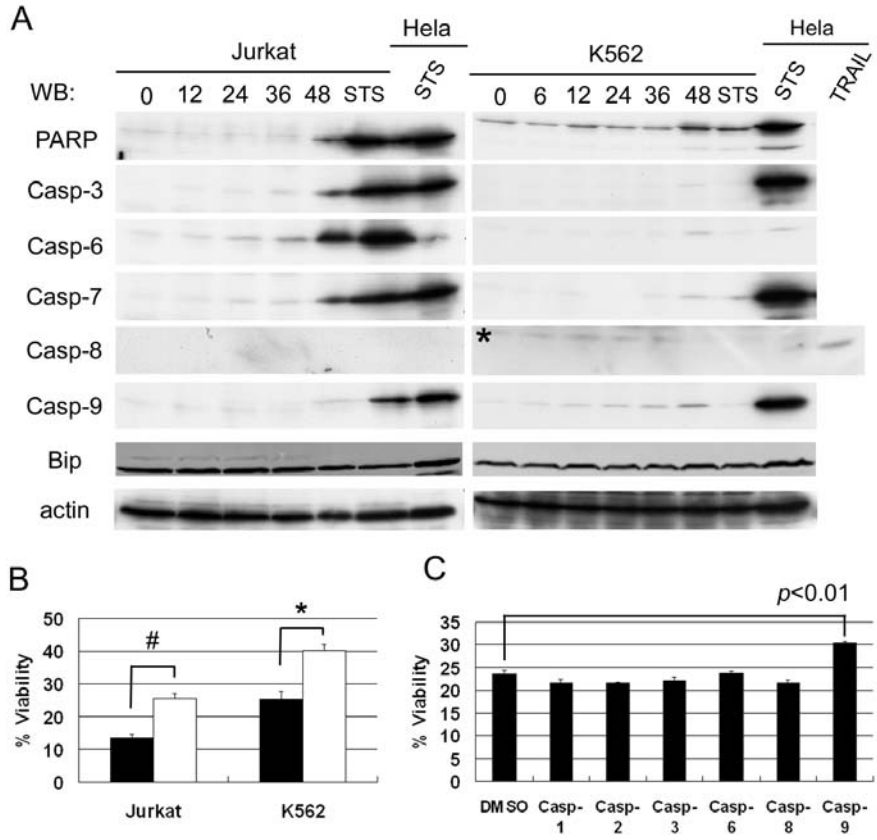

Fig. 4 Fucoidan-induced cell death accompanied by caspase activation. (**A**) Western blot analysis using antibodies against active caspases, PARP, Bip and actin. The asterisk shows non-specific band. (**B**) Effect of pan-caspase inhibitor, Z-VAD-FMK on fucoidan-induced cell death. Jurkat and K562 cells were pre-treated with the indicated concentration of Z-VAD-FMK for 1 hr, followed by exposure to 300 μg/ml of fucoidan for 48 hr in the presence of the inhibitor. #; $p<0.05$, *; $p<0.01$. (**C**) Effect of specific inhibitors on fucoidan-induced cell death. K562 cells were pre-treated with each inhibitor at a concentration of 10 μM, followed by exposure to 300 μg/ml of fucoidan for 48 hr in the presence of the inhibitor.

used with fucoidan in Jurkat cells. Among the specific caspase inhibitors tested in this experiment, only caspase-9 specific inhibitor, Z-LEHD-FMK, significantly inhibited fucoidan-induced death of K562 cells (**Fig. 4C**). In sum, fucoidan induced cell death via the mitochondrial apoptotic pathway, as demonstrated by active caspase-9 production prior to other caspases and the prevention of apoptosis induction with its specific inhibitor.

DISCUSSION

In Japan, the annual consumption of seaweed per person is more than 1 kg, and it is widely used as food in salads, soup, and in the food industry. In Okinawa, a group of isolated islands situated in the southern part of Japan, the population consumes large quantities of red algae, *Cladosiphon okamuranus* (Okinawa Mozuku) and *Laminaria japonica* (Kombu), and it is known for its longevity. The anti-tumor activity of fucoidan shown in mice could result from cell death/apoptosis induction, up-regulation of immune response against malignant neoplasm, and suppression of the development of blood vessels during cancer development. We focused on investigating the cell line specificity of the cell death/apoptosis induction activity of fucoidan. Among the cells tested, two cell lines, both hematopoietic in origin, showed the highest sensitivities to fucoidan. Jurkat is a T-lymphoid cell line and K562 is myeloid cell line. Both apoptotic and necrotic cell death were observed and Jurkat cells showed higher rates of apoptotic cells.

The major pathway of apoptosis is the mitochondrial pathway. The death receptor pathway and endoplasmic reticulum pathway are also known to cause apoptosis. From our studies using various caspase specific inhibitors, the mitochondrial pathway is suggested in K562 cell. Our observation of decreased mitochondrial membrane potential by fucoidan also suggests the importance of the mitochondrial pathway. Further analysis, for example the detection of ROS or NO production, may be required to elucidate the detailed mechanism of apoptosis induction by fucoidan.

In Jurkat cells treated with fucoidan, high levels of caspase-6 were apparently detected earlier at high levels. Caspase-6 is essential for the cleavage of lamin A in nuclei (Ruchaud *et al*. 2002) and generally known as an effector caspase activated downstream of caspase-3 (Srinivasula *et al*. 1998). An upstream role is also speculated in anti-cancer drug induced apoptosis (Bernhard *et al*. 2000; Loegering *et al*. 2006). In K562 cells, although the detection level of caspases is much less, similarly caspase-6 is the major caspase induced by fucoidan. Caspase-6 is generally recognized as the effector caspase, however, a few molecules are reported as direct activators of this casapase (Doostzadeh-Cizeron *et al*. 2000) The capase-3 processing activity of caspase-6 (Guerrero *et al*. 2008) suggests a possible initiator caspase function of caspase-6 in fucoidan-induced apoptosis. Also, the lack of caspase-8 activation indicates the death receptor pathway is not playing a major role in the apoptosis process of fucoidan-treated hematopoietic cells.

Biological activities of fucoidan extracted from nine different species of brown algae were compared (Cumashi *et al*. 2007). These fucoidan have distinct polysaccharide backbones, sulfate contents, and carbohydrate substituents. The structural difference causes different anti-inflammatory, anticoagulant, antiangiogenic and antiadhesive properties of the fucoidan. It is interesting to compare the apoptosis induction activities of structurally divergent fucoidan. Native fucoidan containing 13.5% of sulfate showed no apoptosis induction activities on the human leukemia cell line U937 at 100 µg/ml, but after chemically oversulfated (to 32.8% sulfate), apparent apoptosis induction was observed at the 20 µg/ml level (Teruya *et al*. 2007). A negative charge introduced by sulphate might be important for apoptosis induction.

In summary, we showed fucoidan preferentially induced the death of hematopoietic cells. Interestingly, fucoidan was less toxic to normal dermal fibroblasts. Furthermore, by use of staurosporine, susceptibility differences of responses to apoptosis inducers between different hematopoietic lineages were also demonstrated. These kinds of experiments should provide novel aspects in understanding cell type-specific mechanism of apoptosis as well as tumor treatments.

ACKNOWLEDGEMENTS

We thank members of Biosignal Research Center, Kobe University, for technical assistance, and Prof. Anthony Torbert for critically reading the manuscript. We also thank Professor Junichiro Yamamoto for his kind advice.

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