Antitumor activity of fucoidan against diffuse large B cell lymphoma \textit{in vitro} and \textit{in vivo}

Guang Yang\textsuperscript{1,\dagger}, Qianqiao Zhang\textsuperscript{1,\dagger}, Yuanyuan Kong\textsuperscript{1}, Bingqian Xie\textsuperscript{1}, Minjie Gao\textsuperscript{1}, Yi Tao\textsuperscript{1}, Hongwei Xu\textsuperscript{2}, Fenghuang Zhan\textsuperscript{2}, Bojie Dai\textsuperscript{3}, Jumei Shi\textsuperscript{1,*}, and Xiaosong Wu\textsuperscript{1,*}

\textsuperscript{1}Department of Hematology, Shanghai Tenth People’s Hospital, Tongji University School of Medicine, Shanghai 200072, China, \textsuperscript{2}Department of Internal Medicine, University of Iowa, Carver College of Medicine, Iowa City IA 52242, USA, and \textsuperscript{3}School of Life Science and Technology, Tongji University, Shanghai 200092, China

\dagger These authors contributed equally to this work.

*Correspondence address. Tel: +86-21-66306764; Fax: +86-21-66301362; E-mail: wux163@163.com (X.W.)/shijumei@tongji.edu.cn (J.S.)

Received 25 July 2015; Accepted 17 August 2015

Abstract

Fucoidan is one of the major sulfated polysaccharides isolated from brown seaweeds. In this study, we determined the anti-cancer activity of fucoidan on diffuse large B cell lymphoma (DLBCL) cells both \textit{in vitro} and \textit{in vivo}. Fucoidan inhibited the growth of DLBCL cells in a dose- and time-dependent manner, and fucoidan treatment provoked G\textsubscript{0}/G\textsubscript{1} cell cycle arrest, which was accompanied by p21 up-regulation and cyclin D1, Cdk4, and Cdk6 down-regulation. Fucoidan also induced caspase-dependent cell apoptosis in DLBCL cell lines and primary DLBCL cell. In addition, fucoidan treatment caused the loss of mitochondrial membrane potential and the release of cytochrome c and apoptosis-inducing factor from the mitochondria into the cytosol. Fucoidan also potentiated the activities of carfilzomib in killing DLBCL cells. Oral administration of fucoidan effectively inhibited tumor growth in xenograft mouse models. Our findings reveal the novel function of fucoidan as an anti-DLBCL agent, which can be used in the clinical treatment of DLBCL.

Key words: fucoidan, diffuse large B cell lymphoma, cell cycle, apoptosis

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most prevalent type of aggressive lymphoma in adults and accounts for 40\% of non-Hodgkin lymphomas (NHL) and 30\% of all lymphomas [1]. Based on gene expression profiling, DLBCLs have been classified into three groups: germinal center B cell (GCB)-like DLBCL, activated B cell (ABC)-like DLBCL, and primary mediastinal large B cell lymphoma or unclassified subtypes [2–4]. Although combined chemoimmunotherapy improves overall survival of DLBCL patients, over 30\% of these patients relapse or develop refractory disease [5–7].

Fucoidan is a natural sulfated polysaccharide extracted from the extracellular matrix of brown seaweed [8]. The structures and compositions of fucoidans from different brown seaweed vary from species to species [9]. Fucoidan from \textit{Fucus vesiculosus} is composed of 44.1\% fucose, 26.3\% sulfate, and 31.1\% ash, plus a small amount of amino-glucose [10]. Fucoidan is reported to have several biological activities, including anti-bacterial [11], anti-viral [12,13], anti-inflammatory [14], anti-angiogenic [15], and antioxidant [16] effects. In particular, the anti-cancer activity of fucoidan has recently attracted considerable attention. Fucoidan exerts antitumor effects in different types of cancer, both \textit{in vitro} and \textit{in vivo} [17–23]. Importantly, a combined Phase I and II clinical trial in humans showed the low toxicity of fucoidan [24].

In the present study, we demonstrated that fucoidan inhibited cell proliferation, caused cell cycle arrest, and induced apoptotic cell death in human DLBCL cell lines and primary cells. We also showed that oral administration of fucoidan effectively inhibited tumor growth...
in xenograft mouse models. Together, the results demonstrate the anti-cancer activity of fucoidan and suggest that fucoidan is a promising therapeutic agent for DLBCL.

Materials and Methods

Cell culture

SUDHL-4 and DB (both GCB subtype) cells were purchased from ATCC (Manassas, USA). OCI-LY8 (GCB subtype) and NU-DUL-1 (both ABC subtype) cells were provided by Professor Xiaoyan Zhou of the Department of Pathology, Fudan University Shanghai Cancer Center (Shanghai, China). TMD8 and U2932 (both ABC subtype) cells were obtained from Professor Dongsheng Xu of the Shanghai Tenth People’s Hospital, Tongji University School of Medicine (Shanghai, China). OCI-LY8 cells were maintained in Iscove’s Modified Dulbecco’s Medium (Gibco, Carlsbad, USA), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin streptomycin-glutamine (Gibco). U2932 cells were maintained in Dulbecco’s Modified Eagle’s Medium/Low Glucose (Gibco), supplemented with 10% FBS and 1% penicillin streptomycin-glutamine. Other cells were grown in suspension in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin streptomycin-glutamine. All cells were incubated at 37°C in 5% carbon dioxide air atmosphere. Primary cells were obtained from the bone marrow of three patients with DLBCL and extensive marrow infiltration. Normal CD34+ cells were isolated from the bone marrow of healthy donors using magnetic bead selection (Miltenyi Biotech, Auburn, USA). Primary cells and CD34+ cells were maintained in RPMI 1640 medium containing 10% FBS. Informed consent was obtained from each patient.

Reagents

Fucoidan of Fucus vesiculosus was purchased from Sigma (St Louis, USA). Carfilzomib (CFZ) was purchased from Onyx Pharmaceuticals (South San Francisco, USA). The primary antibodies against different proteins including cyclin D1, Cdk4, Cdk6, p21 Waf1/Cip1, E2F transcription factor 1 (E2F1), phospho-Retinoblastoma (Rb) (Ser780), cleaved caspase-3, cleaved caspase-8, caspase-9, poly (ADP-ribose) polymerase (PARP), cytochrome c, apoptosis-inducing factor (AIF), and β-actin were from Cell Signaling Technology (Beverly, USA). Horseradish peroxidase-conjugated secondary antibodies including anti-rabbit IgG and anti-mouse IgG were also from Cell Signaling Technology. Pan-caspase inhibitor Z-VDAD-FMK was obtained from Selleckchem (Houston, USA). JC-1 Mitochondrial Membrane Potential Detection Kit was purchased from Nanjing KeyGEN Biotech. Co. Ltd (Nanjing, China).

Western blot analysis

Cellular proteins were extracted using radio-immunoprecipitation assay (RIPA) buffer (Sigma). Equal amounts of protein (30 μg) were separated by 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% defatted milk and then probed with the indicated primary antibodies overnight. Phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) was used to wash the membranes three times before and after incubation with the secondary antibody (anti-rabbit or anti-mouse IgG) for 1 h at room temperature. The Odyssey two-color infrared laser imaging system (LICOR, Lincoln, USA) was used to detect the strength of the protein signals.

Cell cycle analysis

Cells were treated with fucoidan and collected at different times. Then, cells were fixed with ice cold 70% ethanol overnight. After washed once with PBS, cells were stained with propidium iodide (PI) and analyzed on a BD FACS Canto II flow cytometer (BD BioScience, San Jose, USA).

Cell proliferation and apoptosis assays

DLBCL cell lines (SUDHL-4, OCI-LY8, NU-DUL-1, TMD8, U2932, and DB) were treated with different concentrations of fucoidan for 48 h. CCK8 kit (Dojindo Molecular Technologies, Shanghai, China) was used to monitor the cell proliferation. The half maximal inhibitory concentration (IC50) values were calculated by using CalcuSyn software. To evaluate the drug combination and their synergy quantification, the method of Chou was used [25]. Data were analyzed, and the combination index (CI) was calculated by using CalcuSyn software (CI < 1: synergism, CI = 1: additive effect, and CI > 1: antagonism). For cytometric analyses of apoptosis, cells were treated with different concentrations of fucoidan and then stained with annexin V-FTIC and PI (BD) to detect apoptosis. Cells were considered to be apoptotic if they were either annexin V+/PI− (early apoptotic) or annexin V+/PI+ (late apoptotic).

Tumor xenograft models

OCI-LY8 human DLBCL cells (5 × 106) in 100 μl serum-free culture medium were subcutaneously injected into the upper flank region of 6-week-old male NOD/SCID mice (Shanghai Laboratory Animal Center, Shanghai, China). On the seventh day, 10 mice were randomly divided into two groups (control group and fucoidan group, 5 mice in each group). Mice were orally fed doubly distilled (dd) H2O (control) or 100 mg/kg (body weight) of fucoidan every day for 21 days. At the end of the experiment, mice were sacrificed and the tumors were weighed and imaged. Tumor size was monitored every 2 days. The mice were housed in a standard animal laboratory with free access to water and food. All procedures were approved by the Animal Care and Use Committee of Shanghai Tongji University.

Statistical analysis

Data are expressed as means ± standard deviations (SD). Differences among the experimental groups were determined with Student’s t-test. All statistical analyses were performed with SPSS v22.0 statistical analysis software. Significance was established at a P-value of ≤0.05.

Results

Fucoidan inhibits the growth of DLBCL cell lines

To investigate the effects of fucoidan on the growth and survival of DLBCL cells, different concentrations of fucoidan were used to treat six DLBCL cell lines (OCI-LY8, SUDHL-4, and DB, which are the GC subtype; TMD8, U2932, and NU-DUL-1, which are the ABC subtype) for 48 h. The CCK8 assay showed that fucoidan inhibited the growth of DLBCL cells in a dose-dependent manner (Fig. 1A). The IC50 values for these cell lines were 82.3 (OCI-LY8), 95.5 (DB), 80.0 (SUDHL-4), 97.5 (TMD8), 101.6 (U2932), and 93.7 μg/ml (NU-DUL-1). The action of fucoidan on DLBCL cells was also time dependent (Fig. 1B).
Fucoidan provokes cell cycle arrest in DLBCL cells

Regulation of cell cycle progression and cell death are important parameters of an antitumor drug. Fucoidan treatment induced a G_0/G_1 phase arrest in OCI-LY8 cells (Fig. 2A), and similar results were obtained in SUDHL-4 and NU-DUL-1 cell lines (data not shown). To further explore the mechanism of fucoidan-induced cell cycle arrest, the expression of cell cycle proteins was evaluated. Exposure of DLBCL cells to fucoidan significantly decreased the levels of cyclin D1, Cdk4, and Cdk6 (Fig. 2B). As a member of the Cip/Kip family of cell cycle regulators, p21 plays an important role in controlling the G_1/S transition [26]. Western blot analyses of DLBCL cells after fucoidan treatment showed that it increased the levels of p21Cip1. Rb is an important checkpoint of G_1 transition, so we further investigated the proteins in the dynamic complex of Rb and E2Fs. As shown in Fig. 2B, fucoidan treatment decreased the phosphorylation of Rb protein at Ser795 and decreased the level of E2F1.

Fucoidan induces caspase-dependent apoptosis of DLBCL cells

Using the cell cycle assay, fucoidan was shown to induce an increase in the sub-G_0/G_1 cells (from 2%–10%), which indicated that fucoidan caused cell death. We, therefore, performed an apoptosis assay by using the annexin V-FITC/PI kit to characterize the cytotoxic effect of fucoidan on DLBCL cells. It was found that fucoidan induced apoptosis of OCI-LY8 cells in a time- and dose-dependent manner (Fig. 3A). Fucoidan also induced apoptosis of primary DLBCL cells but had no effect on the viability of normal CD34+ cells (Fig. 3B). To further identify the mechanism of apoptosis in fucoidan-treated DLBCL cells, we detected caspase-3, caspase-8, and caspase-9 activities by western blot analysis. The results showed that fucoidan induced caspase-3, caspase-8, and caspase-9 cleavage, as well as PARP cleavage (Fig. 3C). Mitochondria have been shown to play a central role in the apoptotic process, so we investigated the effect of fucoidan treatment on mitochondrial membrane potential (ΔΨm). As shown in Fig. 3D, a decrease of ΔΨm was seen in fucoidan-treated DLBCL cells. The reduced depolarization effect of ΔΨm by fucoidan treatment was consistent with the release of cytochrome c and AIF from the mitochondria into the cytosol (Fig. 3E). We then explored whether fucoidan caused apoptosis of DLBCL cells through a caspase-dependent pathway. Our data showed that pretreatment with caspase inhibitor Z-VAD-FMK blocked fucoidan-induced cell death (Fig. 3F).

Synergy of fucoidan and CFZ in killing DLBCL cells

Combination chemotherapy is a widely accepted first-line regimen for the management of DLBCL. We were interested in whether fucoidan could enhance the action of these anti-DLBCL agents. DLBCL cells were treated with combinations of fucoidan and several chemotherapeutic agents commonly used to treat DLBCL, as well as novel drugs. Fucoidan did not enhance the efficacy of cyclophosphamide, doxorubicin, vincristine, prednisone, or vorinostat (data are not shown). However, fucoidan significantly increased the anti-DLBCL action of CFZ. As shown in Fig. 4A, low concentrations of CFZ killed a small percentage of DLBCL cells, but when combined with fucoidan, CFZ significantly increased its anti-DLBCL activity. Similar results were obtained when low concentrations of fucoidan were combined with CFZ (Fig. 4B). Median dose–effect analyses revealed that the CI value was <1.0, indicating a synergistic interaction of CFZ with fucoidan (Fig. 4C). Finally, combination of CFZ and fucoidan increased the cytotoxicity toward primary DLBCL cells but exhibited modest toxicity toward normal CD34+ cells (Fig. 4D). We failed to observe any significant difference when fucoidan was added prior to, together with, or after the addition of CFZ (data are not shown).

Antitumor activity of fucoidan in vivo

Our previous studies suggested that fucoidan inhibited cell growth and induced apoptosis in DLBCL cells. Based on the in vitro studies, we injected OCI-LY8 cells into NOD/SCID mice to determine the antitumor activity of fucoidan. At the end of the experiment, the mice were sacrificed and the tumors were weighed and imaged. Mice fed with fucoidan showed a significant reduction in tumor volume (Fig. 5A, B) and tumor weight (Fig. 5C). There was no difference in the mouse body weight between the fucoidan-treated and the control groups (Fig. 5D).

Discussion

Fucoidan is one of the major sulfated polysaccharides isolated from brown seaweeds. The anti-cancer activity of fucoidan has been studied both in vitro and in vivo in different types of cancers. In the present study, we demonstrated the anti-DLBCL activity of fucoidan both in vitro and in vivo, and found that fucoidan treatment inhibited the growth of three GC subtypes and three ABC subtypes of DLBCL cell lines. The growth inhibition caused by fucoidan occurred in a dose- and time-dependent manner. Our findings were in agreement with studies of other cancer types [19,23].
Previous studies showed that G0/G1 cell cycle arrest occurred in a non-small-cell bronchopulmonary carcinoma line and breast cancer MCF-7 cells [27,28]. In this study, fucoidan was found to cause an accumulation of cells in the G0/G1 phases, with a decrease in the percentage of cells in proliferative phases (S and G2-M) in DLBCL cells. Western blot analyses showed that fucoidan significantly downregulated the levels of cyclin D1, Cdk4, and Cdk6. Furthermore, fucoidan reduced the phosphorylation of Rb at Ser795 and decreased the levels of E2F1, suggesting the repression of the activity of the E2F complex. Western blot analyses showed that fucoidan treatment increased the levels of p21cip1, a cyclin-dependent kinase inhibitor (CDKI). Furthermore, increases of p21 have also been reported in prostate cancer cells and bladder carcinoma cells treated with fucoidan [20,29].

Besides the effects of fucoidan on the cell cycle, fucoidan was also found to induce cell apoptosis in both DLBCL cell lines and primary DLBCL cells. Further studies showed that fucoidan induced caspase-3, caspase-8, and caspase-9 cleavages, as well as PARP cleavage. Caspase-8 and caspase-9 are the two best characterized molecules of extrinsic and intrinsic pathways. Our results are in agreement with previous studies in breast cancer cells and colon cancer cells [30,31]. Pretreatment with caspase inhibitor Z-VAD-FMK rescued the cell death caused by fucoidan, indicating that fucoidan-induced apoptosis was caspase dependent. Flow cytometric analyses showed that fucoidan treatment decreased the level of ΔΨm. Loss of ΔΨm often reflects an increase in mitochondrial outer membrane permeability, which results in the release of proteins that trigger cell death [32]. Western blot analyses showed that cytochrome c and AIF were released from the mitochondria into the cytosol, suggesting that fucoidan performed its anti-DLBCL ability through this mitochondrial function.

Combination chemotherapy is a widely accepted first-line regimen for the management of DLBCL. The R (Rituximab)-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen has been demonstrated to increase the complete response rate and prolong the event-free and overall survival in elderly patients with DLBCL [33]. We, therefore, investigated whether the combination of fucoidan with these agents could enhance their anti-DLBCL activities. Unexpectedly, we did not detect any synergism in this combination. CFZ is a second-generation proteasome inhibitor. It has been shown that CFZ interacts synergistically with vorinostat in inducing the apoptosis of DLBCL cells [34]. In our study, CFZ was potentiated by fucoidan in DLBCL cell lines and primary DLBCL cells but exhibited little cytotoxicity toward normal CD34+ cells. The synergistic interactions between fucoidan and CFZ support the possible clinical use of fucoidan in treating DLBCL.

Fucoidan has been reported to inhibit the growth of tumor cells in several animal models upon oral, intraperitoneal, or intravenous administration [15,22,35,36]. We found that oral administration of fucoidan effectively inhibited the growth of implanted OCI-LY8 cells in xenograft mouse models. Hsu et al. [22] used 48, 96, or 144 mg of fucoidan per kg body weight to orally feed mice for 21 days, and no difference in body weight was found between the treated and the control groups. We also found that 100 mg/kg oral administration did not affect the body weight of the mice. The in vivo studies suggested the low toxicity of fucoidan and the potential use of fucoidan as an antitumor agent.

In summary, we have shown that fucoidan treatment induces G0/G1 cell cycle arrest and apoptosis in DLBCL cells. Oral administration of fucoidan inhibits the tumor growth in the xenograft mouse model. In addition, fucoidan interacts synergistically with CFZ in killing DLBCL cells. Our results demonstrate the anti-DLBCL activity of fucoidan both in vitro and in vivo, suggesting that fucoidan may be used as a clinical agent in DLBCL patients.
Figure 3. Fucoidan induces apoptosis in DLBCL cells

(A) OCI-LY8 cells were treated with different concentrations or durations of fucoidan, and the cell viability was examined by flow cytometry. (B) Primary DLBCL cells and normal CD34+ were treated with 75 or 150 μg/ml fucoidan for 24 h and analyzed by flow cytometry. (C) Western blot analysis was performed to determine the levels of proteins related to apoptotic pathways. (D) OCI-LY8 cells were treated with 100 μg/ml fucoidan, and the ΔΨm was examined by flow cytometry using JC-1 kit. (E) OCI-LY8 cells were treated with fucoidan for indicated durations. The protein levels of cytochrome c and AIF in mitochondria and in the cytosol were determined by western blot analysis. (F) OCI-LY8 cells were pretreated with or without 50 μM of pan-caspase inhibitor Z-VAD-FMK for 1 h and then exposed to 100 μg/ml fucoidan for 24 h. *P<0.05.

Anti-cancer activity of fucoidan on DLBCL
Figure 4. Fucoidan enhances the anti-DLBCL action of CFZ. (A) OCI-LY8 cells were treated with different concentrations of CFZ in combination with 50 µg/ml fucoidan. (B) OCI-LY8 cells were treated with different concentrations of fucoidan in combination with 1 nM CFZ. (C) OCI-LY8 cells were treated with the combination of CFZ and fucoidan at fixed ratio, and the fractional effect values were calculated using CalcuSyn software. (D) Primary DLBCL cells were treated with the combination of CFZ and fucoidan for 24 h, and cell death was determined by flow cytometry.

Figure 5. Fucoidan inhibits the growth of implanted OCI-LY8 cells in xenograft mouse model. OCI-LY8 human DLBCL cells (5 x 10^6) was subcutaneously injected into the upper flank region of 6-week-old male NOD/SCID mice. On the seventh day, mice were randomly assigned into two groups: one group was orally fed with dd H2O (control), and the other group was fed with 100 mg/kg (body weight) fucoidan every day for 21 days. (A) Tumor samples were collected and imaged using a high-definition digital camera, and (B) tumor sizes were measured. (C) Tumor weight and (D) mouse weight were recorded. *P < 0.05.

**Funding**

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81372391 and 31271496) and Shanghai Tenth People’s Hospital Funds (No. 040113015).

**References**