Fucoidan inhibits proliferation of the SKM-1 acute myeloid leukaemia cell line via the activation of apoptotic pathways and production of reactive oxygen species

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Abstract. Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid disorders characterized by peripheral blood cytopenias and a high risk of progression to acute myeloid leukaemia (AML). Fucoidan, a complex sulphated polysaccharide isolated from the cell wall of brown seaweeds, has recently attracted attention for its multiple biological activities and its potential as a novel candidate for cancer therapy. In the present study, the anti-cancer activity of fucoidan was investigated in the MDS/AML cell line SKM-1. Fucoidan inhibited proliferation, induced apoptosis and caused G1-phase arrest of the cell cycle in SKM-1 cells as determined by a cell counting kit 8 assay and flow cytometry. Furthermore, reverse transcription quantitative polymerase chain reaction and western blot analyses indicated that treatment with fucoidan (100 µg/ml for 48 h) activated Fas and caspase-8 in SKM-1 cells, which are critical for the extrinsic apoptotic pathway; furthermore, caspase-9 was activated via decreases in phosphoinositide-3 kinase/Akt signaling as indicated by reduced levels of phosphorylated Akt, suggesting the involvement of the intrinsic apoptotic pathway. In addition, fucoidan treatment of SKM-1 cells resulted in the generation of reactive oxygen species (ROS) as determined by staining with dichloro-dihydro-fluorescein diacetate. These results suggested that the mechanisms of the anti-cancer effects of fucoidan in SKM-1 are closely associated with cell cycle arrest and apoptotic cell death, which partly attributed to the activation of apoptotic pathways and accumulation of intracellular ROS. Our results demonstrated that Fucoidan inhibits proliferation and induces the apoptosis of SKM-1 cells, which provides substantial therapeutic potential for MDS treatment.

Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal and potentially malignant bone marrow disorders characterized by ineffective, inadequate haematopoiesis in one or more of the lineages of bone marrow (1). It has a variable propensity of transformation to acute myeloid leukaemia (AML) (2) and is described as a ‘pro-leukaemia state’ (3). MDS can arise de novo or as a consequence of previous chemotherapy or radiotherapy in cancer patients (4). It occurs most frequently in aged populations with a median age of 65-70 years at diagnosis (2). However, there is a paediatric population of MDS patients in which inherited bone marrow-failure syndromes are associated with high-risk factors. The median survival time of MDS patients following diagnosis is 0.5-6 years (5,6). Numerous types of therapy for MDS have been developed based on the molecular mechanisms of the diseases; for example, inhibitors of DNA methylation have been proven effective in the treatment of patients with MDS (7). Although available treatments have alleviated MDS-associated symptoms of certain patients, few treatments are able to transform the natural course of the disease (4). In addition, numerous chemotherapeutic treatment options induce undesirable side effects. The lack of safe and effective therapeutic options emphasizes the urgent requirement for the development of novel therapies. The ultimate goal is to identify an effective treatment that can extend the overall survival of patients with MDS.

Natural products have attracted considerable attention as anti-cancer agents over the past few years. Several of these compounds, including vincristine, paclitaxel and etoposide, have been tested and used in clinical treatment (8). Fucoidan, a complex sulphated polysaccharide natural product with a molecular weight of 5-627 kDa, was isolated from the cell wall matrix of brown seaweeds, which have been used in Traditional Chinese Medicine for nearly 2,000 years for the treatment of a wide variety of diseases, including thyroid disease, skin diseases, arteriosclerosis, hypertension and cancer (9-11). The anti-cancer effects of fucoidan are particularly promising (12). Previous studies reported that fucoidan effectively suppressed the proliferation and colony formation of cancer cells in vitro (13); furthermore, fucoidan inhibited metastasis and angiogenesis of Lewis lung adenocarcinoma
and B16 melanoma xenografts in vivo (14). Natural products to attenuate or prevent the progression of carcinogenesis via three major mechanisms: Selective promotion of apoptosis in cancer cells, interference with the cell cycle and inhibition of angiogenesis and metastasis (14). However, whether fucoidan affects the apoptosis of MDS/AML cells has remained elusive.

The present study therefore examined the anti-cancer effects of fucoidan as well as its underlying molecular mechanisms of action in the human MDS/AML cell line SKM-1. For this purpose, the effects of fucoidan on the proliferation, cell cycle, apoptosis, generation of reactive oxygen species (ROS) and expression of apoptosis-associated genes in SKM-1 cells were assessed. The present study suggested that fucoidan may be a candidate drug for the treatment of MDS.

Materials and methods

Drugs and cell culture. Fucoidan was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human MDS/AML cell line SKM-1 was provided by Professor Jianfeng Zhou (Department of Hematology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China). Cells were maintained in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco-BRL, Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) (15).

Cell counting kit (CCK-8) assay. The CCK-8 assay (cat. no. C0038; Beyotime Institute of Biotechnology, Shanghai, China) was performed to estimate the effects of fucoidan on the proliferation of SKM-1 cells. Cells were seeded (3x10^4 cells/ml) in a 96-well plate in 100 µl RPMI-1640 containing 10% FBS at 37°C in a 5% CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing various concentrations (50, 100, 200, 300, 400 and 500 µg/ml) of fucoidan, and the cells were incubated for an additional 24, 48 or 72 h at 37°C in the 5% CO_2 incubator. After incubation, the CCK-8 reagent (10 µl) was added to each well, and the cells were incubated for 2 h at 37°C and 5% CO_2. The optical density (OD) values were measured at 450 nm using a microtiter plate reader (SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA) (16). The results were expressed as the percentage of growth inhibition calculated according to the following formula: (OD\_Control - OD\_Experimental group)/OD\_Control x 100%.

Assessment of apoptosis. For the analysis of cell apoptosis, 2x10^5 SKM-1 cells were seeded into six-well plates in 1 ml RPMI-1640 medium containing 10% FBS and cultured overnight. Next, the medium was replaced with fresh RPMI-1640 or the same media containing 100 µg/ml fucoidan. After an additional incubation for 48 h, the medium was discarded and the SKM-1 cells were washed twice in phosphate-buffered saline (PBS). Apoptosis was evaluated using Annexin V and propidium iodide (PI) staining (Beyotime Institute of Biotechnology) followed by flow cytometric analysis (FACSCalibur®; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Cell cycle analysis. Cells were incubated with fucoidan (100 µg/ml) for 48 h, harvested and washed twice with PBS.

Table I. Primers used for polymerase chain reaction.

| Gene   | Primers                        |
|--------|--------------------------------|
| Caspase3 | F: 5'-ATGACATCTCGGTCTGGT-3'  |
|         | R: 5'−AGAAACATCAGCCTAA-3'    |
| Caspase8 | F: 5'-AAGGAAGCAGAAGACCCT-3' |
|         | R: 5'-TGACCTGTAGGCAAGAAA-3'  |
| Fas     | F: 5'-TCCCATCTCCTGACAC-3'    |
|         | R: 5'-TCGTAACGCCTTACCCT-3'   |
| Caspase9 | F: 5'-CCAAGCCCTTCCTTTACCCACC-3' |
|         | R: 5'−CATCGTTCTGCACTAC-3'    |
| Actin   | F: 5'-CCACAGAACTCACTCCTAACTA-3' |
|         | R: 5'-GTGATCTCTTCTTCATCTCTGT-3' |
| AKT     | F: 5'-GCAAGGTGATCCTGGTGAA-3' |
|         | R: 5'-TCGTGGGTCCTGAAAGAGTA-3' |

The medium was discarded, the SKM-1 cells were washed twice with PBS and fixed with 70% alcohol for 4 h. The cell cycle was determined by flow cytometry following PI staining of the nuclei.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using RNAiso Plus (Takara Biotechnology, Otsu, Japan) following incubation of the cells with fucoidan (100 µg/ml) for 48 h. Subsequently, cDNA was generated using a two-step RT-PCR kit (cat. no. RR037A; Takara Biotechnology). The quantitative PCR reaction was performed in a mixture with a total volume of 20 µl and contained SYBR Premix Ex Taq (10 µl), 1 µl of each primer (10 μmol/l), 2 µl cDNA template and double distilled H_2O (6 µl). A 7500 Real-Time PCR System (Applied Biosystems Life Technologies, Foster City, CA, USA) was used and the primers were designed using Primer 5 software (version 5; Jikai Co., Shanghai, China) and synthesized by Sangon Biotech (Shanghai, China; Table I). The thermocycling conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec, 65°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 10 min. The 2^−ΔΔCT method was used to quantify the PCR products.

Western blot analysis. SKM-1 cells were treated with fucoidan (100 µg/ml) for 48 h. Cells were harvested and washed twice with PBS, and the total protein was obtained after cell lysis using lysis buffer [components: 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and sodium orthovanadate sodium fluoride, EDTA and leupeptin (Beyotime Institute of Biotechnology)]. Total protein was quantified using a BCA protein assay kit (cat. no. P0012S; Beyotime Institute of Biotechnology) and 50 µg protein was loaded per lane and separated using 10% SDS-PAGE, then transferred onto a polyvinylidene fluoride membrane with glycine transfer buffer (composed of 3.05 g Tris, 14.4 g glycine, 200 ml methanol and 800 ml H_2O; Beyotime Institute of Biotechnology). After blocking with 5%...
nonfat milk, the membrane was incubated with the following primary antibodies: Rabbit polyclonal Fas (cat. no. YT1676), rabbit polyclonal caspase-8 (cat. no. YT0660), rabbit polyclonal caspase-9 (cat. no. YT0664), rabbit polyclonal caspase-3 (cat. no. YT5204; Immunoway Biotechnology Co., Newport, DE, USA) all at a dilution of 1:500, for 12 h at 4°C. Subsequently the membrane was incubated with the following secondary antibodies: Rabbit polyclonal anti-β-actin [dilution, 1:200 (cat. no. Ab19716); Abcam, Cambridge, UK], rabbit polyclonal phosphorylated-Akt [dilution, 1:100 (cat. no. Sc-135651); Santa Cruz Biotechnology, Inc., Dallas, TX, USA], rabbit polyclonal Akt [dilution, 1:100 (cat. no. Sc-8312); Santa Cruz Biotechnology, Inc.], all incubated at 4°C for 12 h, as well as horseradish peroxidase-conjugated goat polyclonal immunoglobulin G [dilution, 1:7,000 (cat. no. ZDR-5036); ZSGB-BIO, Beijing, China] at 37°C for 2 h. Protein bands were visualized using an enhanced chemiluminescence kit (cat. no. P0018; Beyotime Institute of Biotechnology) and analyzed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of intracellular ROS. The T-AOC detection assay kit (cat. no. S0119; Beyotime Institute of Biotechnology) was used to assess intracellular ROS production. SKM-1 cells were collected after incubation with fucoidan (100 µg/ml) for 48 h and labelled with 10 µM dichloro-dihydro-fluorescein diacetate at 37°C in the dark for 30 min. After washing the cells twice with RPMI-1640, the cellular fluorescence intensity was measured using a flow cytometer (excitation and emission wavelength, 488 and 525 nm, respectively).

Statistical analysis. Values are expressed as the mean ± standard deviation and one-way analysis of variance, Dunnett's test and independent samples t-test were performed to evaluate statistical significance. Statistical analyses were performed using SPSS version 20.0 (IBM SPSS, Armonk, NY, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

Fucoidan inhibits the proliferation of SKM-1 cells in a dose- and time-dependent manner. The present study first examined the effects of fucoidan on the proliferation of SKM-1 cells using a CCK-8 assay. SKM-1 cells were seeded in 96-well plates at 3x10^3 cells/well. After 24 h of attachment, the cells were treated with fucoidan (50, 100, 200, 300, 400 or 500 µg/ml) for 24, 48 or 72 h. The OD values were measured using the metabolic dye-based CCK-8 assay. Values are expressed as the percent inhibition calculated according to the following formula:

\[ \text{Inhibition rate} = \left(1 - \frac{OD_{\text{experimental group}}}{OD_{\text{control group}}} \right) \times 100\% \]

Figure 1. Inhibition rates of SKM-1 cells evaluated using the CCK-8 assay. SKM-1 cells were seeded in 96-well plates at 3x10^3 cells/well. After 24 h of attachment, the cells were treated with fucoidan (50, 100, 200, 300, 400 or 500 µg/ml) for 24, 48 or 72 h. The OD values were measured using the metabolic dye-based CCK-8 assay. Values are expressed as the mean ± standard deviation (n=3). P<0.05 between all concentrations of fucoidan at each time point. CCK-8, cell counting kit 8; OD, optical density.

Fucoidan induces apoptosis in SKM-1 cells via the extrinsic and intrinsic pathways. To gain further insight into the mechanism of fucoidan-induced apoptosis of SKM-1 cells, the expression of the apoptosis-associated molecules Fas, caspase-8, caspase-9 and caspase-3 was detected using the metabolic dye-based CCK-8 assay. Values are expressed as the mean ± standard deviation. The G1/G0-phase population in the fucoidan-treated group was 47.7±1.23% compared with 33.3±1.52% in the control group. However, the S-phase population decreased from 53.0±0.67 to 44.9±0.99% and the G2/M-phase population decreased from 13.6±1.69 to 7.3±1.35% following treatment with fucoidan compared with the control-group populations (Fig. 2B). These findings demonstrated that treatment with fucoidan increased the apoptotic rate and induced cell cycle arrest in G1/G0 phase.

Fucoidan blocks phosphoinositide-3 kinase (PI3K)/Akt signaling in SKM-1 cells. To investigate whether the PI3K/Akt signaling pathway is involved in fucoidan-induced apoptosis in SKM-1 cells, the mRNA levels of AKT were examined using RT-qPCR (Fig. 3A) and western blot analysis (Fig. 3B), respectively. The expression levels of the extrinsic pathway-associated molecules Fas and caspase-8 as well as the intrinsic pathway-associated molecule caspase-9 were gradually increased in response to fucoidan treatment (100 µg/ml for 48 h). In addition, the downstream effector caspase-3 was also activated.

Fucoidan induces apoptosis of SKM-1 cells and blocks the cell cycle in G1 phase. To determine whether the inhibitory effects of fucoidan on cell proliferation resulted from its induction of apoptotic cell death, SKM-1 cells treated with fucoidan (100 µg/ml) for 48 h were stained with Annexin V and PI, and the apoptotic rate was detected using flow cytometry. The apoptotic rate of SKM-1 cells following treatment with fucoidan (100 µg/ml) was 28.2±0.94% compared with 16.4±0.75% in the control (Fig. 2A). The underlying mechanism of the anti-proliferative effects of fucoidan was further investigated by determining its effect on the cell cycle of SKM-1 cells. Following incubation with fucoidan (100 µg/ml) for 48 h, cells were stained with PI and cell cycle analysis was performed using flow cytometry. The G1/G0-phase population in the fucoidan-treated group was 47.7±1.23% compared with 33.3±1.52% in the control group. However, the S-phase population decreased from 53.0±0.67 to 44.9±0.99% and the G2/M-phase population decreased from 13.6±1.69 to 7.3±1.35% following treatment with fucoidan compared with the control-group populations (Fig. 2B). These findings demonstrated that treatment with fucoidan increased the apoptotic rate and induced cell cycle arrest in G1/G0 phase.
group. Furthermore, the PI3K/Akt signaling pathway protein phospho-Akt and Akt were quantified using western blot analysis (Fig. 4B). The protein expression of phospho-Akt was decreased in SKM-1 cells treated with fucoidan (100 µg/ml) for 48 h. These results indicated that fucoidan inactivated the PI3K/Akt signaling pathway.

**Fucoidan increases intracellular ROS production.** The present study investigated the generation of intracellular ROS to determine whether changes in ROS levels have a role in fucoidan-induced apoptosis in SKM-1 cells. Cells were incubated with 100 µg/ml fucoidan for 48 h and ROS were measured using flow cytometry. The mean value of ROS production was 264.3x10³/mg protein in the fucoidan-treated group compared with 179.1x10³/mg in the control group (Fig. 5). As ROS production was markedly enhanced in SKM-1 cells following fucoidan treatment, the generation of ROS may, at least in part, be the underlying molecular mechanism of the induction of cancer-cell apoptosis by fucoidan.

**Discussion**

Fucoidan, a complex sulphated polysaccharide extracted from brown seaweeds, has been shown to exhibit anti-cancer
activity in a wide variety of tumour cell types and is therefore considered to be a promising novel candidate for cancer therapy with low toxicity to normal cells (17-20). In the present study, treatment with fucoidan inhibited the proliferation and induced apoptosis in the MDS/AML cell line SKM-1.

Previous studies have also indicated that fucoidan directly inhibited the proliferation of various cancer cell lines, including that of PC-3 cells at 10-200 µg/ml (21), MCF-7 cells at 82-820 µg/ml (22) and U937 cells at 20-100 µg/ml (23) where the incubation times were between 12 and 96 h. In the present study, fucoidan inhibited the proliferation of SKM-1 cells at all studied concentrations (50, 100, 200, 300, 400, 500 µg/ml) according to a CCK-8 assay. The inhibition rate of cells was 7.5±1.11% when the concentration of fucoidan was 50 µg/ml, which increased to 43.4±2.72% at a concentration of 500 µg/ml. Additionally, the inhibition rate increased by 9.2% when cells were exposed to 50 µg/ml fucoidan for 72 h. Thus, fucoidan treatment inhibited the proliferation of SKM-1 cells in a dose- and time-dependent manner. Furthermore, flow cytometric analysis was performed to determine whether the inhibitory effects of fucoidan on cell proliferation resulted from apoptotic cell death and cell cycle arrest. The apoptotic rate of SKM-1 cells treated with fucoidan (100 µg/ml) for 48 h was 28.2% compared to 16.4% in the control group. In addition, following treatment with fucoidan, the G1/G0 phase population of SKM-1 cells was markedly increased compared with that in the normal control group, while the S-phase and G2/M-phase populations were significantly decreased. Therefore, flow cytometric analysis confirmed that the inhibition of proliferation by fucoidan was based on the induction of cell cycle arrest and apoptosis.

It is well known that apoptosis, the process of programmed cell death, has an important role in the normal development and differentiation of multicellular organisms; it is characterized by distinct morphological features and energy-dependent biochemical mechanisms (24). Apoptosis also serves as a critical protective mechanism against carcinogenesis caused by genetic mutations of normal cells, which may occur spontaneously or which may be induced by various stimuli or carcinogens. Fucoidan has been previously demonstrated to induce apoptosis in leukemia cells via B-cell lymphoma 2 and mitogen-activated protein kinase signaling (23). In the present study, the observed fucoidan-induced cell cycle arrest in G1/G0-phase, which may have been a response to cellular damage by ROS, may have caused the activation of apoptotic pathways, which was further confirmed by the increased apoptotic rate. Furthermore, the extrinsic pathway (death receptor-mediated) and the intrinsic pathway (mitochondrial mediated) of apoptosis represent two major pathways implicated in the induction of apoptotic cell death (25). Activation of caspsases is a pivotal step in the extrinsic as well as the intrinsic apoptotic pathways and is triggered by signals from
death factors, mitochondrial alterations or DNA damage due to external and/or internal insults (26). Fas, one of these death receptor factors, results in the clustering and formation of a death-inducing signaling complex. The results of the present study showed that treatment of SKM-1 cells with fucoidan increased the expression of Fas, which activates the extrinsic pathway. Further downstream in the apoptotic signaling cascade, initiator caspases, including caspase-8, significantly amplify the complex death-inducing signaling (24). The results of the present study showed that incubation of SKM-1 cells with fucoidan decreased the mRNA expression of AKT as well as the levels of phosphorylated AKT protein, therefore inhibiting the PI3K/Akt signaling pathway. The PI3K/Akt signaling pathway is another regulator of cell survival, cell growth and apoptosis, and inactivation of the PI3K/Akt signaling pathway inhibits the proliferation and induces the apoptosis of cancer cells by activating apoptotic signals through caspase-9 (19,27). The present study further demonstrated that treatment of SKM-1 cells with fucoidan increased caspase-9 levels, which was likely to be mediated via decreases of PI3K/AKT signaling via the intrinsic pathway of apoptosis. In addition, activation of caspase-3 was observed, which is a downstream effector of caspase-8 and caspase-9. Upon its activation, the executioner caspase-3 disassembles the cytoskeleton, leading to cell-morphological changes associated with apoptosis. These results indicated that fucoidan induced apoptosis of SKM-1 cells via activation of extrinsic as well as intrinsic apoptotic pathways. Furthermore, ROS have a key role in oxidative stress and are generated as by-products of cellular metabolism, primarily in the mitochondria (28). The maintenance of an appropriate level of intracellular ROS is important for the maintenance of a redox balance and signaling associated with cellular proliferation (29). However, upon its overproduction, ROS can degrade cellular proteins, DNA and lipids, resulting in a state of oxidative stress (30). Previous studies have demonstrated that cancer cells can be effectively killed using natural products with the ability to increase intracellular ROS levels (29,31). In the present study, fucoidan treatment of SKM-1 cells for 48 h caused a rapid accumulation of intracellular ROS. High levels of ROS increase the vulnerability of tumor cells to apoptosis; furthermore, ROS may have initiated the cellular damage signaling cascade, leading to cell cycle arrest and apoptosis. The observations of the present study suggested that the generation of ROS is involved in fucoidan-induced apoptosis in SKM-1 cells.

In conclusion, fucoidan, a natural product from the cell wall of brown seaweeds, caused cell cycle arrest and induced apoptosis via the activation of apoptotic pathways. Furthermore, the induction of apoptosis by fucoidan was likely to be associated with enhanced production of ROS. The results of the present study suggested that fucoidan is a promising candidate drug for the treatment of MDS.

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