Fucoidan induces Apoptosis in MDA-MB-231 Cells by Activating Caspase Cascade and Down-regulating XIAP

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Summary: *Previous studies have shown fucoidan could induce apoptosis in breast cancers, while the detailed mechanisms need to be investigated.Results of this study illustrated that number of viable MDA-MB-231 cells was decreased by fucoidan with a dose-dependent manner, while the fucoidan did not affect the viable epithelial cells number of normal human mammary (MCF10A).. After incubation with fucoidan, several apoptotic events such as chromatin condensation and increase of the population of G2/M phase were observed in MDA-MB-231 cells. RT-PCR and Western blot results showed that the activation of caspase cascade such caspase-3, caspase-8 and caspase-9, as well as the expression of XIAP (X-linked inhibitor of apoptosis protein), was involved in the apoptosis process. Besides that, reactive oxygen species(ROS) production was increased after fucoidan treatment. These results indicated that fucoidan could induce the apoptotic cell death through modulation of XIAP and caspases activation in MDA-MB-231 cells and could also induce the generation of ROS.*
Keywords: *Fucoidan, Apoptosis, MDA-MB-231 Cells, Caspase Cascade, XIAP, reactive oxygen species (ROS).*

I. Introduction

Natural dietary compounds have been widely and safely consumed over centuries, and preclinical studies suggest that most of them have potential applications in pharmacology and cancer therapy. Fucoidan, a class of fucose-enriched sulfated polysaccharides, is a naturally occurring polysaccharide compound in brown alga, such as Fucus vesiculosus, Cladosiphon okamuranus and Laminaria saccharina [1, 2]. It has recently been reported that fucoidan possesses a wide variety of biological activities, including anticoagulant, antiviral, anti-angiogenic, anticancer and immune-modulatory activities [3] . Studies in vitro have indicated that fucoidan exhibits protection effect against various cancers, including human lymphoma, promyelocytic leukemia, colon carcinoma, breast carcinoma, hepatoma and melanoma. Several investigations have demonstrated that fucoidan can effectively suppress proliferation and colony formation of cancer cells in vitro [4].

Apoptosis, or programmed cell death, is an important aspect of chemotherapy-induced tumor cell death and also the major mechanism of tumor cell death induced by many anticancer drugs and natural products [5]. Caspase-dependent apoptosis is characterized by activation of either the extrinsic pathway, initiated by activation of death receptors leading to the cleavage of caspase-3 and -8, or the intrinsic pathway, triggered by mitochondrial depolarization, release of cytochrome c and the subsequent activation of caspase-9 [6, 7]. Disruption to the factors regulating these apoptotic pathways contributes substantially to the transformation of a normal cell into a tumor cell, and the cells of some tumor types are relatively resistant to apoptosis [8, 9].

Significant effect of fucoidan has been shown to be associated with both decreased proliferation and increased apoptosis. Mechanisms associated with apoptosis have been described, including production of oxidative stress and activation/expression of modulation proteins, such as caspases-3, -8, -9, XIAP, CDK1 and cyclin B.

Caspases are aspartate-specific cysteine proteases that are instrumental in the apoptotic process. They exist in all nucleated cells as inactive zymogens and are activated by proteolytic cleavage. Caspases are categorized into initiators and effectors on the basis of their positions in apoptotic proteolytic cascades. Initiator caspases trigger and amplify apoptosis by activating effector caspases and pro-apoptotic factors. Effector caspases act at cytoplasmic sites, and eventually at other organelles, including the nucleus, to execute the proteolytic program that finalizes cell destruction.

Caspase-3 is a caspase protein that interacts with caspase-8 and -9. It is encoded by the CASP3 gene. The caspase-3 removes the IBM (The tethering site) of caspase-9 by proteolysis at Asp^{330}, thereby denying XIAP the ability to efficiently inhibit caspase-9. XIAP (X-linked inhibitor of apoptosis protein), is one from many members of the IAP (inhibitor of apoptosis protein) family capable of binding amino acid sequences

similar to that of the neo-N-terminus of caspase-9, most of which are characterized as having an alanine residue in the first position and often a proline residue in the third position.

ROS are considered to be an apoptotic death signal [6]. However, low physiological levels of ROS also serve as a signaling messenger to mediate various biological responses, including cell proliferation, angiogenesis, innate immunity, gene expression, apoptosis and senescence [7]. It has also been established that increased levels of these short-lived reactive molecules can exert harmful effects by inducing oxidative damage to biological macromolecules and disrupting the cellular reduction-oxidation (redox) balance. Such disturbances to ROS homeostasis are generally considered to be a risk factor for the initiation and progression of diseases such as atherosclerosis, neurodegeneration and cancer [8]. ROS induce depolarization of the mitochondrial membrane potential (MMP, $\Delta\psi m$) and the release of cytochrome c from the mitochondria into the cytosol, where cytochrome c triggers activation of caspase-9 and initiates the caspase cascade, which ultimately induces the cell to undergo apoptosis [9]. Tumor cells are more sensitive to fluctuations in the levels of ROS than normal cells; therefore, ROS are also considered as an important target in anticancer drug research [10], [11].

There are two major pathways involved in the process of the apoptosis mediation. These pathways are the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). Stimulation of death receptors in the extrinsic pathway such as fatty acid synthase (FAS), leads to clustering and formation of death-inducing signaling complex, which includes the adaptor protein Fas-associated death domain (FADD) and initiator caspases, such as caspase-3 and -8. The activated caspases-8 directly activates downstream effector caspases, such as caspase-3 [12]. This study illustrated that apoptosis in breast cancer MDAMB-231 cells is induced by fucoidan without affecting the viability of normal epithelial cells mediated by extrinsic pathway. In fact, caspase-3 and -8 are regarded as essential for fucoidan-induced apoptosis. Therefore we examined whether FAS could protect against apoptosis induced by agents that stimulate the extrinsic pathway [12, 13].

Breast cancer is one of the most common human malignancies and the second leading cause of cancer-related deaths in women, and its incidence in the developing world is on the rise [14]. Different treatment strategies have been employed to reduce the mortality rate, including surgery, radiotherapy, and adjuvant chemo or hormone-therapies [15]. Unfortunately, breast cancer is particularly challenging, because it is highly resistant to radiation and conventional chemotherapeutic agents, and such resistance is associated with a poor prognosis for this metastatic disease, particularly in hormone receptor-positive breast cancer [14], [16]. About 30-40% of women with this form of cancer will develop metastasis and eventually die from this disease [17]. Novel therapeutic agents are therefore needed to deal with the increasing incidence of human breast cancer. The MDA-MB-231 cell line is an estrogen receptor alpha (ERα) negative human breast cancer cell line, which was obtained from a patient in 1973 at M. D. Anderson Cancer Center. With epithelial-like morphology, the MDA-MB-231 cells appear phenotypically as spindle shaped cells as shown in **Fig 1**.

II. Materials and Methods

1.1 Chemicals

Fucoidan was purchased from Sigma (USA). Dulbecco's Modified Eagle's Medium (DMEM) medium was obtained from Gibco (USA). Annexin V-FITC/PI assay kit was purchased from Beijing Zoman Biotechnology Co., Ltd (China). Hoechst 33258 was purchased from Beyotime Institute of Biotechnology (China). Fetal bovine serums (FBS) and phosphate-buffered saline (PBS) were purchased from Invitrogen (USA). Dimethylsulfoxide (DMSO), Propidiumiodide (PI), TritonX-100, 3- (4, 5-dimethyl-thiazol-2-yl) -2, 5-diphenyltetrazoliumbromide (MTT) and ethylene diaminetetraacetic acid (EDTA) were purchased from Sigma (USA). Monoclonal antibodies against GAPDH, cyclin B1, CDK1, Bax, and Bcl-2 were purchased from Santa Cruz Biotechnology (USA). The secondary horseradish peroxidase (HRP) linked antibodies were obtained from Wuhan Boster Biological Engineering Co. Ltd., China. Alcohol is from Wuhan Anke Biological Technology Co. Ltd.

1.2 Cell Culture

MDA-MB-231 cell line and the control cell line MCF-A10, obtained from the China Center Type Culture Collection (China), were cultured in DMEM medium supplemented with FBS, 100 units/ml of penicillin and 100mg/ml of streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

1.3 MTT Assay

The effects on cell proliferation were measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded in 96-well microplates and incubated overnight. Then the cells were treated with different concentrations of the fucoidan (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) for 24 h. At the end of these periods, 20 μl of MTT stock solution (5 mg/ml, Sigma, St. Louis, MO) were added to each well and the plates were further incubated for 4 h at 37°C. The supernatant was removed and 100 μl of DMSO were added to each well to solubilize the water-insoluble purple formazan

crystals. The absorbency at a wavelength of 570 nm was measured with Multiscan MCC 340 microplate reader (Titertek, Huntsville, AL). All the measurements were performed in triplicate. Results are expressed as the % proliferation with respect to fucoidan-treated cells.

1.4 Cell cycle analysis

Cells were treated with fucoidan for 24 h at different concentrations. Then, the cells were collected by trypsinization and washed once with PBS. The cells were fixed with 80% ethanol for 2 h at -20 °C. Then the cells were collected by centrifugation at 15,000 rpm for 5 min and then resuspended in PBS containing 10μg/mL PI. After incubation at 37 °C for 30 min in dark,the DNA contents of the cells were analyzed using Cytomics TM FC 500 Flow Cytometer (Beckman, USA). The data were analyzed using the Modi FIT program.

1.5 AnnexinV-FITC/PI Assay

The annexinV-PI double-staining method was used. MDA-MB-231 cells were seeded into 6 well plates ($5x10^3$ cells/well) and cultured for 24 h. After treatment with or without fucoidan for varying concentrations, the cells were stained with the annexin V-FITC labeling solution (annexin V-fluorescein in a binding buffer containing PI). The plates were further incubated for 15 min in the dark, and then images of the cells were acquired using Cytomics TM FC 500 Flow Cytometer (Beckman, USA). The cells with apoptotic morphology of nuclei condensation, or annexin V-positive cells were analyzed using the flow Jo 7.6.1 software.

1.6 Hoechest Test

The induction of apoptosis by fucoidan in MDA-MB-231 cells was confirmed in fluorescence photomicrographs of cells stained with Hoechst 33258 at dose depending manner.

1.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from MDA-MB-231 cells using the RNA Extraction Kit (Beijing Zoman Biotechnology Co. Ltd.) and transcribed into cDNA. Reaction mixtures were incubated for examining it with primers we need in our pathway like caspas-3, caspase-8, caspase-9, XIAP, cyclin-B and Cdk-1. The expression level of each mRNA relative to that of GAPDH was calculated using a standard curve.

Primers	Sequence
Cyclin B	5-CAGTCAGCCAAAATACCTACCTGGGT-3, 5-ACACCAACCAGCTGCATCTTCTT-3
CDK1	5-GGATGTGCTTATGCAGGATT-3, 5-CAGCCAAAGAAAgACCAGAC-3
Caspase-9	5_-ATG GAC GAA GCG GAT CGG-3 5_-CCC TGG CCT TAT GAT GTT-3
XIAP	5-GGATTCTCCATGGCAGATTATGAAGC-3 5′-CTCTCGAGTTACCCCATGGATCCCAGATAGTTTTCAACT-3′
Caspase-3	5'-TTAATAAAGGTATCCATGGAGAACACT-3' 5'-TTAGTGATAAAAA TAGAGTTCTTTTGTGAG-3
Caspase-8	5_-CGGGATCCGCCACCATGGACTTCAGCAGAAATC-3 5_-TCCCCCGGGCACCATCAATCAGAAGGG-3
GAPDH	5_-GTG GAA GGA CTC ATG ACC ACA G-3 5_-CTG GTG CTC AGT GTA GCC CAG-3

1.8 Western Blot

MDA-MB231 cells were treated with fucoidan and then washed once with ice-cold phosphate-buffered saline containing 1 mM Na_2VO_4 and extracted with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na_2VO_4, 10 mg/mL of each aprotinin, leupeptin and pepstatin). The preparation of cytoplasmic was conducted using the NE-PER cytoplasmic extraction reagents (Pierce, Rockford, IL). Cell lysates were frozen and thawed three times and were further centrifuged at 14,000 g for 10 min at 4℃ to pellet insoluble material. The supernatant of cell extracts was analyzed for protein concentration by a DC protein assay kit based on the Lowry method (Bio-Rad, Hercules, and CA). Equal amounts of protein from each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (MSI, Westborough, MA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and then incubated with rabbit antibodies against caspase-3, caspase-8, caspase-9, XIAP and GAPDH, which was used as control for equal protein loading. The immunoblots were then washed three times with TBS-T buffer, incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgM, Santa Cruz Biotechnology), and developed using chemiluminescent substrate (Pierce).

1.9 Measurement of ROS

MDA-MB231 cells (2×10^6 cells per assay) were treated with fucoidan at various concentrations. Following treatment, the cells were wash once with PBS and incubated with 10 μ M carboxy-H 2 DCFDA, a fluorescent indicator of intracellular levels of ROS (Molecular Probe, Carlsbad, CA), for 30 minutes at 37°C in PBS. The cells were harvested and analyzed by flow cytometry using Flow Jo 7.6 software.

III.   Results

2.1 Effect of Fucoidan on MDA-MB-231 Cells Growth

The anti-proliferative effect of fucoidan on MDA-MB-231cells was examined by exposing them to different concentrations of fucoidan for 24 h. When MDA-MB-231 cells were exposed to fucoidan, cell growth was inhibited in a dose-dependent manner as illustrated in Fig 2. Compared to the control, 0.2 to 1.0 mg/ml of fucoidan significantly inhibited cell growth. After treatment with fucoidan for 24 h, a dose-dependent inhibition of cell viability was observed. Therefore, fucoidan induced growth inhibition of MDA-MB-231 cell line.

2.2 Effect of Fucoidan on MDA-MB-231Cell Cycle Distribution

The cell cycle distribution was also examined with flow cytometry at indicated time and various doses. As shown in Fig 3, fucoidan induced a significant cell population increase in G2/M phase following 24 h treatment with different concentrations (0-1.0 mg/ml). As shown in table 1, the percentage of cells at G1 phase decreases, while that at G2/M phase increases and apoptotic cells increases with increasing fucoidan concentration.

2.3 Fucoidan Induces Apoptosis in MDA-MB-231

Fucoidan treatment also caused cells to lose their phospholipid membrane asymmetry. The exposure of phosphatidylserine to the outside of the plasma membranes was detected by annexin V-FITC staining in MDA-MB-231 cells. The annexin V staining results as shown in Fig 4 indicate that fucoidan causes a significant increase in the number of apoptotic cells (annexin V positive) in a dose-dependent manner. The effect of fucoidan (1 mg/mL) on MDA-MB-231 cells was the most significant.

2.4 Cell Morphplogical Assessment (Hoechst Test)

The induction of apoptosis by fucoidan in MDA-MB231 cells was confirmed in fluorescence photomicrographs of cells stained with Hoechst 33258. **Fig 5** clearly demonstrates a shrunken nucleus and peripherally clumped and fragmented chromatin. These characteristics are typical of apoptotic cells.

2.5 Effect of fucoidan on expression of the caspases and XIAP at transcriptional level in MDA-MB 231 cells

Caspases are activated in a sequential cascade of cleavages from their inactive forms. Activation of caspase-8 leads to the cleavage of a number of caspases, one of which is caspase-3, while XIAP could inhibit activation of caspases-3.

In order to investigate the potential involvement of caspases in the cell death induced by fucoidan, we examined the expression of XIAP which is correlated with inhibition of apoptosis and further investigated expression of those critical caspases. Our results indicated the expression of caspase-8, caspase-3 and caspase-9 was increased gradually and inversely that of XIAP ,CDK1 and cyclin-B was decreased gradually after fucoidan incubation (Fig.6).

2.6 Effect of fucoidan on the caspases and XIAP protein expression in MDA-MB 231 cells

To assess the role of caspases in this apoptotic process, the expression of caspase-8, caspase -3, caspase -9 and XIAP was examined by Western blot analysis. As shown in Fig 7, the increase of caspases (-3, -8, and -9) and decrease of XIAP both indicate the effect of fucoidan on caspase cascade.

2.7 Effect of fucoidan on ROS generation in MDA-MB-23 cells

Fucoidan induced oxidative damage in human breast cells and increased intracellular reactive oxygen species. We have examined activation of members of the caspase family as well as   expression of mitochondrial-dependent apoptotic factors and ROS level. Our results indicate that fucoidan induces apoptosis via a caspase-8 activation pathway.

Increased levels of ROS have been demonstrated to induce depolarization of the mitochondrial membrane, which eventually produces an increase in the level of other pro-apoptotic molecules in cells[9]. With the increase of fucoidan concentration, increasing ROS level was observed in Fig.8 (from left to right side), which means that fucoidan induces apoptosis via promoting ROS accumulation.

Fucoidan dose-dependently increased the reactive oxygen species production. MDA-MB-231 cells were treated with or without fuciodan (a–e: 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) for 24 h and then were harvested. The amount of reactive oxygen species was measured using the fluorescent probe DCFH-DA by flow cytometry.

IV.   Discussion

Fucoidan is one of the major sulfated polysaccharides of brown seaweeds whose sulfate group plays a vital role in various biological activities[18, 19]. We used commercial available fucoidan purified from F.vesiculosus. A number of genes that are associated with apoptotic cell death were tested in order to clarify the mechanism by which fucoidan treatment induces apoptosis in MDA-MB-231 cells. According to some previously conducted studies, caspases act as key players of fucoidan-induced apoptosis [20-27]. These findings have guided the researcher to propose a model in which fucoidan promotes apoptosis in MDA-MB-231 cells through increasing caspases activity.

In this paper, MTT results domenstrated that fucoidan inhibited cell proliferation and significant cytotoxicity on MDA-MB-231cells in dose-dependent manner. Flow cytometry results further indicated that treatment of fuciodan induced G2/M phase arrest, which was due to the downregulation of the genes related to G2/M phase (cyclin B1, CDK1) in dose-dependent manner in MDA-MB231 cells. In the meanwhile this study also found the decrease of Bcl-2 level as well as the increase of Bax level in MDA-MB231 cells by fucoidan treatment (data not shown). This apoptotic effect is triggered via a pathway depending upon caspase-3 and -8 activation.

In fact, it is known that activation of caspase-3, -8 and -9 cleavages by caspase-3 are hallmarks of apoptosis in MDA-MB231 cells. Caspase-8 plays a vital role in apoptosis triggered by the interaction of ligand with death receptor as FAS and directly cleared by caspase-8 activation which after that activate caspase -3 (Fig. 9). Caspase-3 is active over a broad pH range that is slightly higher (more basic) than many of the other executioner caspases. This broad range indicates that caspase-3 will be fully active under normal and apoptotic cell conditions[28]. XIAP binds and inhibits initiator caspase-9, which is directly involved in the activation of executioner caspase-3 [29]. During the caspase cascade, however, caspase-3 functions to inhibit XIAP activity by cleaving caspase-9 at a specific site, preventing XIAP from being able to bind to inhibit caspase-9 activity[30]. Caspase-3 has been found to be necessary for typical role in apoptosis, where it is responsible for chromatin condensation and DNA fragmentation[31].

In addition, we observed that fucoidan treatment enhanced intracellular ROS levels in dose-dependent manner, suggesting that induction of oxidative stress was an important event in the cell death induced by fucoidan [32].

V.   Conclusion

Fucoidan inhibits the MDA-MB-231 cell proliferation by inducing cell apoptosis through activation of caspase cascade and induction of ROS, and the apoptosis is related to the activation of caspase-8, caspase-3, and caspase -9 and the inhibition of XIAP.

Acknowledgements

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