

RESEARCH ARTICLE

Effect of Sesamin on Apoptosis and Cell Cycle Arrest in Human Breast Cancer MCF-7 Cells

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Abstract

Dietary prevention has been known to reduce breast cancer risk. Sesamin is one of the major components in sesame seeds and has been widely studied and proven to have anti-proliferation and anti-angiogenic effects on cancer cells. In this study, the influence of sesamin was tested in the human breast cancer MCF-7 cell line for cell viability (MTT assay) and cell cycling (flow cytometry). Results showed that sesamin dose-dependently (1, 10 and 50 μ M) reduced the cell viability and increased LDH release and apoptosis (TUNEL assay). In addition, there was a significant increase of sub-G1 phase arrest in the cell cycle after sesamin treatment. Furthermore, sesamin increased the expression of apoptotic markers of Bax, caspase-3, and cell cycle control proteins, p53 and checkpoint kinase 2. Taken together, these results suggested that sesamin might be used as a dietary supplement for prevention of breast cancer by modulating apoptotic signal pathways and inhibiting tumor cell growth.

Keywords: Sesamin - breast cancer - apoptosis - cell cycle

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Introduction

Breast cancer is one of the most common cancers among women, and is the leading cause of death globally (Jemal et al., 2011). Many women still die of this disease in spite of cancer therapy, therefore new treatment strategies are needed. Natural dietary substances have been used for reducing the incidence of cancer (Gullett et al., 2010) and natural compounds can downregulate anti-apoptotic proteins (Kong et al., 2014). Cancer cells are also known to lose the ability to negatively regulate the cell cycle leading to their continuous proliferation. The cell cycle is controlled by numerous mechanisms ensuring correct cell division including regulation of cyclin-dependent kinases (CDK) by cyclins, CDK inhibitors and phosphorylating events (Caldon et al., 2010). Cancer cells activate the apical kinase ATM (Ataxia Telangiectasia Mutated) and its target and effector checkpoint kinase Chk2 after DNA damage by genotoxic substance (Ciccia and Elledge, 2010). Following DNA damage, ATM phosphorylates Chk2 on Thr68, leading to its activation and phosphorylation of several substrates, including p53, HDMX, PML, TRF2 (Ouchi and Ouchi, 2014). A wide range of phytochemicals derived from medicinal plants have been reported to exhibit anticancer activity by targeting the cell cycle regulation and apoptosis. Sesamin, a major lignin from sesame seeds has been reported to prevent hypertension, protect brain and liver injuries (Noguchi et al., 2001; Hsieh et al., 2011; Chiang et al., 2014) and modulate the cell cycle in human cancer cells (Yokota et al., 2007).

We report here the effect of sesamin on the cell cycle and apoptosis of MCF-7 breast cancer cells

Materials and Methods

Reagents

Fetal bovine serum (FBS) was obtained from Gibco Invitrogen (Grand Island, NY, USA). Dulbecco's Modified Eagle's medium (DMEM) were purchased from GIBCO (Grand Island, NY, USA). Sesamin was kindly provided from Joben Bio-Medical Co. (Kaohsiung, Taiwan). Antibodies were obtained from various sources: β -Actin (mouse, 1:5000; Novus, Littleton, CO, USA), caspase-3 (rabbit, 1:1000; Cell Signaling, Danvers, MA, USA), p21WAF1 (mouse, 1:5000; Cell Signaling), p53 (mouse, 1:2000, Calbiochem, Darmstadt, Germany), human phospho-Chk2 ELISA kit (R and D, Minneapolis, MN, USA), Bax (mouse, 1:5000; Cell Signaling), and anti-rabbit or anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA).

Cell lines and culture

Human breast cancer MCF-7 cell line was purchased from ATCC. Cells were cultured in a humidified incubator at 37°C and 5% CO₂ atmosphere in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% FBS and 1% penicillin-streptomycin.

Cell viability assay

Cell viability was measured using blue formazan

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that was metabolized from colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are active only in live cells. MCF-7 cells were preincubated in 24-well plates at a density of 5×10^5 cells per well for 8 h and then treated with various concentrations of sesamin (1, 10, and 50 μM) for 24 h. The cells were washed by 1 \times PBS solution, and then incubated in 0.5 mg/ml MTT at 37°C for 1 h. A solubilization solution, 200 μl of DMSO (dimethyl sulfoxide), was added to each well and absorption values read at 540 nm on microtiter plate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA, USA). Data were expressed as the mean percent of viable cells vs. control.

LDH assay

Cytotoxicity was assessed by meaning the release of the cytosolic enzyme lactic dehydrogenase (LDH) from damaged cells. LDH released from the 24 h MCF-7 cell culture was using an LDH diagnostic kit (Boehringer Mannheim, Mannheim, Germany). Absorbance values were recorded at 490 and 630 nm with the aid of a SpectraMAX340 reader.

Cell cycle analysis

The effect of sesamin on cell cycle distribution was assessed by flow cytometry after staining the MCF-7 cells with propidium iodide (PI). Briefly, the cells ($1 \times 10^5/\text{ml}$) were treated with different sesamin concentrations (1, 10 and 50 $\mu\text{g}/\text{ml}$) for 48 h. The treated cells were harvested, washed with PBS and fixed with 70% ethanol on ice. Then cells washed with cold PBS, suspended in 200 μl 1X Propidium Iodide + RNase staining solution and incubate at 37°C in the dark for 30 min. Propidium Iodide Flow Cytometry Kit was used for cell cycle analysis. DNA content of the cells was measured by C6 flow cytometer and the population of each phase was determined using CFlow Plus analysis software (BD Accuri Cytometers, Ann Arbor, MI, USA).

TUNEL assay

The TUNEL assay uses an enzyme (terminal deoxynucleotidyl transferase) to add biotinylated nucleotides to the strand breaks found in the DNA of apoptotic cells. TUNEL assay was performed by the manufacture's protocol. Briefly, sesamin treated, or non-treated MCF-7 cells were cultured in 6-well plates for 24 h. After the incubation period, the culture medium was aspirated, and the cell layers were trypsinized. The trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained according to the TUNEL system protocol (BioVision, Milpitas, CA, USA). The stained cells were observed using a fluorescence microscope with excitation at 450-500 nm and detection wavelength at 515-565 nm. To determine the percentage of cells undergoing apoptosis, 100 cells were counted in each experiment.

CHK2 assay

Cell lysate samples were centrifuged (2000 \times g for 5 min) to pellet cells and cell debris. The supernatants were analyzed using an ELISA for Chk2 phosphorylated at

threonine 68 by human phospho-Chk2 (T68) kit (R and D) and measured at 450 nm with an ELISA reader according to the manufacturer's instructions.

Western blotting

Protein samples from sesamin-treated or untreated cells, containing 40 μg of protein each, were separated on 11.5 % (w/v) sodium dodecyl sulfate polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with rabbit anti-mouse beta-actin (Rockland, Gilbertsville, PA, USA), caspase-3, or anti-phospho-MAPKs (Cell Signaling, Danvers, MA, USA). The membranes were subsequently incubated with conjugated goat anti-rabbit IgG (PerkinElmer, Boston, MA, USA). p53, p21, Bax, and caspase-3 proteins were detected by chemiluminescence detection system (Western Lightning[®] Plus-ECL, PerkinElmer, Waltham, MA, USA) and quantified with a densitometric scanner (PDI, Huntington Station, NY, USA).

Statistics

All data are expressed as mean \pm S.D. For single variable comparisons, Student's t-test was used. For multiple variable comparisons, data were analyzed by one-way ANOVA using by Dunnett's test. P values of less than 0.05 or 0.01 were considered significant.

Results

Cytotoxic effect of sesamin on the viability of MCF-7 cells

Sesamin was tested for cytotoxic effect on the MCF-7 cells. Sesamin (10 and 50 μM) decreased the cell viability by 18 and 30% and increased LDH release, 22 to 50%,

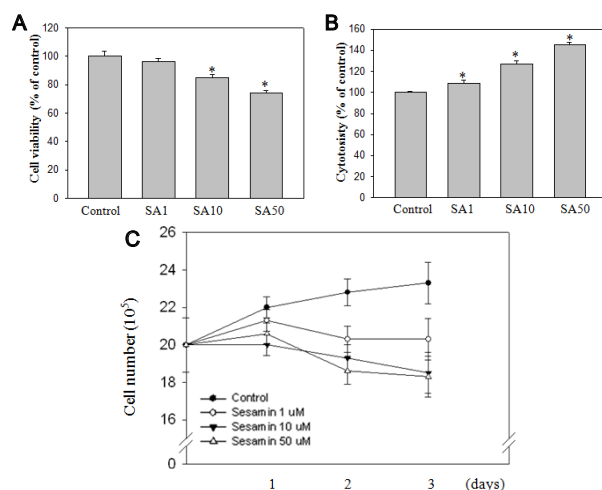


Figure 1. The Effect of Sesamin on the Viability and Cytotoxicity on MCF-7 Cells. The cells were treated with sesamin (0, 1, 5, 10 μM) for 24 h. The cell viability **A**) and cytotoxicity **B**) was determined by MTT and LDH assay, respectively. Data are expressed as the mean \pm SD of three separate experiments. * $P < 0.01$ as compared to the control. **C**) Proliferation of MCF-7 cells. The cells grown on 20 cm^2 culture dish were treated with sesamin (0, 1, 5, 10 μM) for 24 to 72 h and separated by trypsin EDTA. Viable cells were enumerated by trypan blue exclusion with an aid of hemocytometer. * $P < 0.01$ as compared to the control

respectively (Figure 1a, b). This effect was consistent with the number of viable cells from three days culture (Figure 1c). Sub-G1 phase arrest was increased 19, 27, and 41%, respectively by sesamin treatment (1, 10, and 50 μM) as compared to 16% of the control (Figure 2). Cell apoptosis was also increased by sesamin dose-dependently from TUNEL assay ($P < 0.01$, Figure 3).

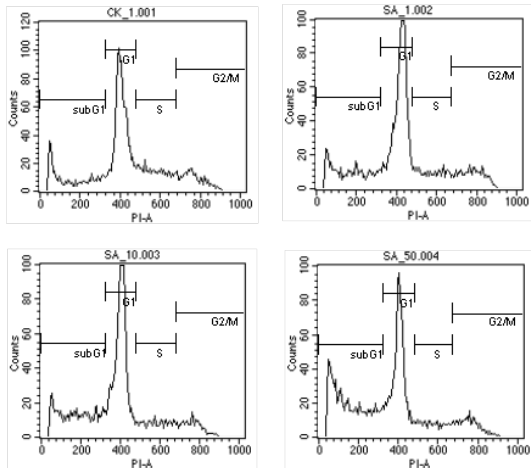


Figure 2. The Effect of Sesamin on Cell Cycle of MCF-7 Cells. The cells grown on 20 cm^2 culture dish were exposed to sesamin (0, 1, 5, 10 μM) for 24 h. The cells were fixed with 70% ethyl alcohol and then stained with propidium iodide. The cell cycle was analyzed by flow cytometer

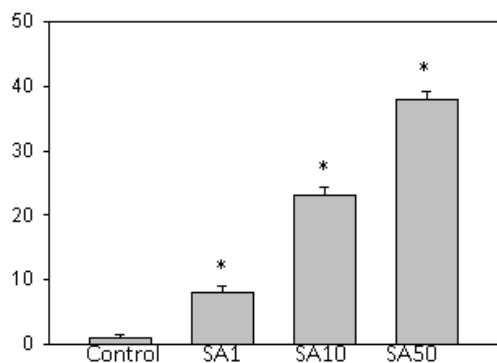
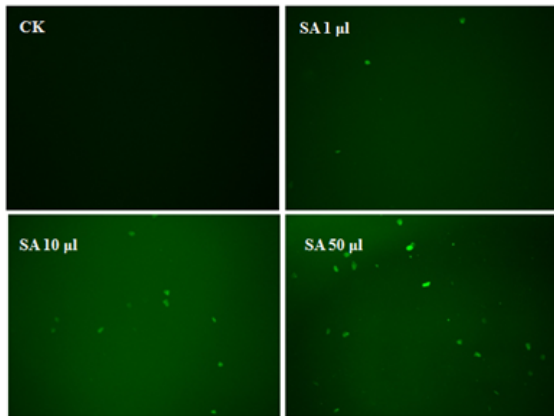


Figure 3. The Effect of Sesamin on the Apoptosis of MCF-7 Cells. Cell apoptosis was determined with TUNEL assay after 24 h of exposure to sesamin (0, 1, 5, 10 μM). Values represent the mean from three independent experiments. * $p < 0.05$ vs. the control

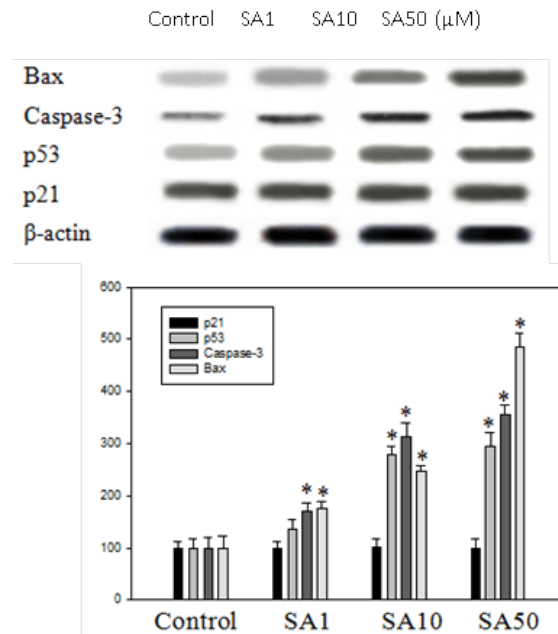


Figure 4. Effect of Sesamin Treatment on BAX, Caspase-3, p53 and p21 expression of MCF-7 Cells. Western blots results of scanning densitometer analysis are presented as value of proteins/beta-Actin. Values represent the mean from three independent experiments (lower panel). * $P < 0.05$, vs. non-treated control

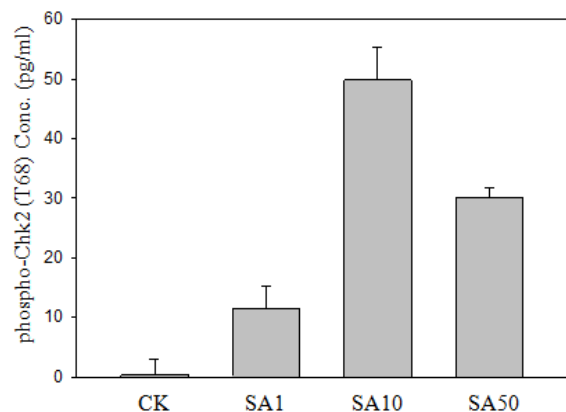


Figure 5. Effect of sesamin treatment on Chk2 expression of MCF-7 cells. Phosphorylated Chk2 in MCF-7 cells was increased significantly by sesamin as compared to that of the control ($P < 0.01$)

Cell cycle and apoptotic signaling pathways

Key cell cycle control proteins p53 and p21 and apoptotic proteins caspase-3 and Bax were examined by western blots. The results showed that MCF-7 cells treated with sesamin (1-50 μM) for 10 min increased Bax (75~310%), caspase-3 (70~187%), and p53 (35~194%) but not p21 protein expression (0%, Figure 4). Cell cycle checkpoints for damaged or abnormally structured DNA can slow or arrest the progression of cell-cycle (Ouchi and Ouchi, 2014). The result showed an increased phosphorylation of Chk2 in MCF-7 cells by sesamin as compared to that of the control ($p < 0.01$, Figure 5).

Discussion

In the present results, sesamin reduced the cell viability by inducing both necrosis and apoptosis in MCF-7 cells, as shown by LDH release and TUNEL assay, respectively. This result agreed with a study in the nude mice supplemented with 1 g/kg sesame seed reduced MCF-7 tumor size by 23% compared to control (Truan et al., 2012).

Several components of the apoptotic pathways were examined for the reduced cell viability by sesamin. The proteins of the Bcl-2 family play an essential role in apoptosis that BAX increases during apoptosis (Hasan et al., 2011). Our result showed that an increase of BAX expression in MCF-7 cells by sesamin. Furthermore, activated caspase-3 that played a crucial role in the final step of apoptosis (Vegran et al., 2011) was increased by sesamin. These data were consistent with results of natural compounds through p53/caspase-3 induced apoptosis of MCF-7 cells (Esmaili-Mahani et al., 2014). Our result also demonstrated a sub-G1 arrest in the cell cycle by sesamin. The cell cycle arrest was related with the increased expression of tumor suppressor p53. p53 plays an important role in response to DNA damage or other genomic instability. Functional p53 protein is crucial in p53-dependent pathway leading to cell cycle arrest or apoptosis (Lee et al., 2014). Several stresses lead to the activation of p53 and results in the arrest of cell cycle by increasing p21 or by inhibiting cyclins and CDKs directly (Yadav et al., 2012; Kim et al., 2015). The involvement of p53 and its downstream CDK inhibitor p21 in sesamin-treated MCF-7 cells was then investigated. However, present result showed that p53 but not p21 protein level was increased significantly with increasing dose of sesamin. These results indicate that a p53 was partly involved sub-G1 arrest by sesamin in the cell-cycle of MCF-7 cells.

We further examined the effect of sesamin on phosphorylation of checkpoint Chk2 and found that phosphorylation of Chk2 was increased by sesamin and suggested that would allow the accumulation of sub-G1 phase in the cell cycle. This is different from a study that reports an arrest at the G1 phase by sesamin with down-regulated cyclin D1 expression (Yokota et al., 2007). However, treatment to lung NSCLC cancer cells with obtusilactone A and (-)-sesamin from *Cinnamomum kotoense* induce sub-G1 arrest from the DNA damage responses, including G1/S checkpoint activation and apoptosis by phosphorylation of checkpoint proteins (H2AX, Nbs1, and Chk2), caspase-3 cleavage (Wang et al., 2010). Ellagic acid from pomegranate, muscadine grapes, walnuts and strawberries, has been shown to induce apoptosis and arresting cell cycle in the G0/G1 phase (Chen et al., 2015). In addition, rapamycin, a cytotoxic drug, shows characteristic features of apoptosis and an arrest in the G0/G1 phase to MCF-7 cells (Tengku Din et al., 2014). Therefore, increased phosphorylated Chk-2 by sesamin could lead to the Go/G1 arrest.

In conclusion, we demonstrate that anticancer activity of sesamin to MCF-7 cells. It inhibits cell proliferation by inducing cell cycle arrest and apoptosis. This induction of

cell cycle arrest is through the increasing of p53 and Chk2 and apoptosis is through the activation of the Bax and caspase-3 pathways. Future work will focus on isolation and characterization of the tentatively identified major compound and will explore the prospective *in vitro* and *in vivo* anticancer capabilities.

Acknowledgements

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