RESEARCH ARTICLE

Up-regulating of RASD1 and Apoptosis of DU-145 Human Prostate Cancer Cells Induced by Formononetin *in Vitro*

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Abstract

Prostate cancer is one of the most prevalent malignant cancers in men. The isoflavone formononetin is a main active component of red clover plants. In the present study, we assessed the effect of formononetin on human prostate cancer DU-145 cells in vitro, and elucidated posssible mechanisms. DU-145 cells were treated with different concentrations of formononetin and cell proliferation was assessed by MTT assay, cell apoptosis by Hoechst 33258 and flow cytometry, and protein levels of RASD1, Bcl-2 and Bax by Western blotting. The results showed that formononetin inhibited the proliferation of DU-145 cells in a dose-dependent manner. DU-145 cells treated with different concentrations of formononetin displayed obvious morphological changes of apoptosis under fluorescence microscopy. In addition, formononetin increased the proportion of early apoptotic DU-145 cells, down-regulated the protein levels of Bcl-2 and up-regulated those of RASD1 and Bax. The level of RASD1 reached its maximum at 48h post-treatment, and rapidly decreased thereafter. Together, we present evidence that formononetin triggered cell apoptosis through the mitochondrial apoptotic pathway by up-regulating RASD1.

Keywords: Formononetin - prostate cancer cells - apoptosis - RASD1

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Introduction

Prostate cancer is one of the most commonly diagnosed malignant cancers and is the second leading cause of cancer-related deaths in men after lung cancer in Western countries (kiJemal et al., 2007). Several observations reported that the incidence of prostate cancer in Asian was lower than that in Western (Nakamura et al., 2011).

However, the recent epidemiology studies also showed that, the incidence rates of prostate cancer increased in Asian countries in the last few years (Center et al., 2012) due to the change in diet and life style. A prospective study by Severson et al. (1989) showed the increasing consumption of phytoestrogens in men of Japanese ancestry living in Hawaii was associated with the decreasing risk of prostate cancer. Phytoestrogens, sometimes called dietary estrogens, are a diverse group of naturally occurring nonsteroidal plant compounds. Phytoestrogens have estrogenic or/and antiestrogenic effects for their structures, are similar to estrogen. Phytoestrogens have been shown to inhibit growth of both androgen-dependent and -independent prostate cancer cells *in vitro* (Zhao et al., 2009).

The isoflavone formononetin (C16H12O4) is a phytoestrogen and a main active component of red clover plants, a kind of traditional Chinese medicinal herbal which has been wildly used for centuries. The effects of isoflavones formononetin include regulating steroid receptor (AR and ER) expression (Fritz et al., 2002), inhibition of cell proliferation and DNA topoisomerase II (Constantinou et al., 1995), promotion of cancer cell apoptosis (Shapiro et al.,1999).

Our previous study have demonstrated that formononetin induced cell cycle arrest of human breast cancer at the G0/G1 phase by inactivating IGF1/PI3K/AKT pathways *in vitro* and *in vivo* (Chen et al., 2012). One of our other researches showed that formononetin caused apoptosis of human prostate cancer cells LNCaP through ERK1/2Mitogen-Activated Protein Kinase inactivation (Ye et al., 2012). Mitogen-Activated Protein Kinase (MAPK) which include the ERK1/2, Jun N-terminal of stress-activated protein kinases1/2 (JNK1/2), and p38 MAPK subgroups, are suggested to play important roles in cells proliferation and apoptosis (Fan et al., 2007). Our data confirmed that formononetin modulated both the extent and duration of ERK1/2 inactivation in LNCaP cells, which coincided with the finding that formononetin inhibited cell proliferation and induced cell apoptosis. However, the upstream of MAPK is not clear. Our preliminary experiment show that AGS1 (Activator of G-protein signaling1) / RASD1 (RAS, dexamethasone-induced1) which belongs to the RAS superfamily of small GTPases, has a close relationship with MAPK.

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Materials and Methods

Drugs and reagents

Formononetin (purity>98%) was purchased from Sigma (St. Louis, MO,USA). Formononetin was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock solution and stored at 4°C for further use. The chemical3-[4, 5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide (MTT) was from Sigma (St. Louis, MO, USA). Hoechst 33258 Staining kit was from Beyotime (Beyotime, China). Annexin-V-FITC was obtained from BD Pharmingen (San Diego, CA, USA). The antibodies against RASD1, Bax, Bcl-2 and β-actin were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from Zhongshan Golden Bridge Biotechnology (Zhongshan Golden Bridge, Beijing, China).

Cell culture

The human prostate carcinoma cell line DU-145 was obtained from the American Type Culture Collection. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media (Gibco-BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, USA), 100 u/ml penicillin and 100 ug/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Cell survival assay by MTT

The effect of formononetin on the viability of DU-145 cells was measured by MTT assay. Cells were plated at a density of 3×10^3 cells/well into 96-well plates. After 24 hours' incubation, various concentrations (6.25, 12.5, 25, 50, 100, 150, 200μM) of formononetin and blank control were added and incubated. After 48 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added into each well and then incubated for another 4 h. After that, the medium was removed, and 150 μl DMSO was added to each well to dissolve the sediment. The mixtures were shaken for 15 min. The optical density (OD) for each well was measured by a microplate reader (Bio-Tek, USA) at a wavelength of 490 nm.

Cell apoptosis analysis by hoechst 33258

DU-145 cells were seeded at a density of 5×10^4 cells/well in a 6-well plate for 24h, and then treated with formononetin. After 24h, the cellular morphology was observed by light microscope. DU-145 cells were then washed twice with PBS (phosphate-buffered saline, pH 7.4), fixed in 4% formaldehyde, stained with Hoechst 33258 (Beyotime, China Hoechst Staining kit) for 5 min, and observed under fluorescence microscope. Cells showing condensed and fragmented nuclei were defined as apoptosis.

Cell apoptosis analysis by flow cytometry

DU-145 cells were seeded in a 6-well plate with 5×10^4 cells each well. After 24 hours, DU-145 cells were incubated with formononetin. Cells were then harvested after 48 hours and washed twice with ice-cold PBS. Cells were then stained with 3ul Annexin V-FITC and 3ul propidium iodide (PI) for 60 min in dark at room temperature in 300 μl binding buffer. The cell apoptosis in DU-145 cells were detected by Flow Cytometry (FACSCalibur, USA). Nonstained cells were viable, and cells stained with Annexin V were at early stage apoptosis. Additional exposure to PI made it possible to differentiate the early apoptotic cells (Annexin V+/PI-) from the late apoptotic cells (Annexin V+/PI+).

Western blot analysis

DU-145 cells were incubated with various concentrations (0, 25, 50, 100uM) of formononetin for 48 h, the proteins were extracted from cellular lysates, and then protein concentrations were determined by Bio-Rad assay kit. Equal amounts of protein (40μg/lane) were separated by SDS-PAGE and then transferred into 0.22 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad laboratories, USA). The membranes were blocked in TBST (Tris-buffered solution, pH 7.6, 0.05% Tween 20) containing 5% nonfat dried milk for 2 hours. Membrane were further incubated with primary antibodies to different proteins, including, RASD1 (1:1000; Santa Cruz Biotechnology, USA), Bax (1:1000; Santa Cruz Biotechnology, USA), Bcl-2(1:1000; Santa Cruz Biotechnology, USA), β-action (1:1000; Santa Cruz Biotechnology, USA) at 4°C overnight. After 3 washes with Tris-Buffered Saline Tween-20 (TBST), membranes were subsequently incubated with appropriate secondary antibody(1:4000; Zhongshan Golden bridge Biotechnology Co, Beijing, China) conjugated to horseradish peroxidase at room temperature for 1h and then developed in electrochemiluminescence (ECL) western blot detection reagents (Beyotime, China). Expressions of the proteins were compared to the control, β-actin, base on the relative intensities of the bands. The bands were analyzed using the Kodak 1D image system (Kodak,USA).

Statistical analysis

Data were expressed as mean±standard deviation. The Statistical Package for Social Sciences (SPSS) 13.0 software (Chicago, IL, USA) was used for statistical analyses including one-way ANOVA and Student's t- test. A *p*-value of <0.05 was considered statistically significant.

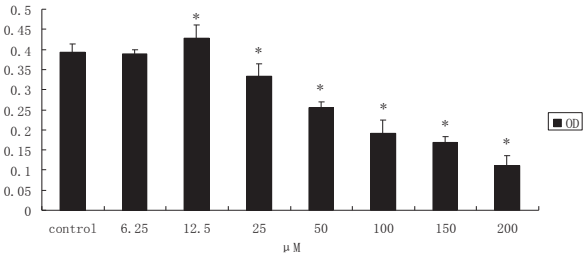

Figure 1. Effects of formononetin on cell proliferation of DU-145 cells. The cells were treated with formononetin for 48h. Final concentration of the compounds are shown. Cell viability was determined by MTT assay. 7 independent experiments were repeated.**p<0.05 *vs* control

Results

Effects of formononetin on proliferation of DU-145 cells

DU-145 cells were treated with different concentrations of formononetin for 48 h. The optical densities are 0.3757, 0.4214, 0.3437, 0.2894, 0.2127, 0.1734 and 0.1087 respectively. The MTT assay results are shown in Figure 1, compared with the vehicle control, formononetin dramatically inhibited the proliferation of DU-145 cells ($p<0.05$) depending on the dosage.

Effects of formononetin on the cell apoptosis

To understand whether the inhibitory effect of formononetin was related to apoptosis, we conducted Hoechst 33258 assay and flow cytometry. Compared with the blank control, characteristic condensed and fragmented nuclei were observed in DU-145 cells treated with formononetin in Hoechst 33258-staining assays (Figure 2a). Additionally, we found that only 0.31% of DU145 cells in the control group were positively stained by Annexin V. In contrast, 4.36%, 10.21%, 19.82% of the cells treated with 25, 50, 100μM formononetin for 48 h were positively stained by Annexin V (Figure 2b). It was indicated that formononetin could induce early apoptosis on DU145 cells, which was in accord with results of the MTT assay.

Effects of formononetin on RASD1, Bax, and Bcl-2 protein expression

The above experiments conducted showed that formononetin decreased the cell number and induced early apoptosis of DU-145. To obtain the direct evidence that the inhibitory effects of formononetin on DU-145 cells were mediated by RASD1 pathway, we determined whether different concentrations of formononetin altered RASD1, Bax and Bcl-2 protein levels in DU145 cells. As

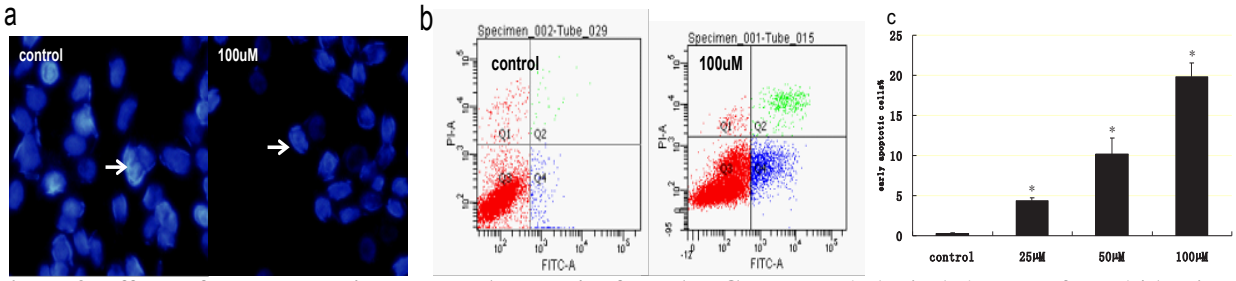

Figure 2. Effects of Formononetin on Early Apoptosis of DU-145 Cells. Morphological changes of DU-145 stained with Hoechst 33258 were observed by fluorescence microscope a(×200). Early apoptosis rate of DU145 stained with Annexin V-FITC and PI was tested by flow cytometry b

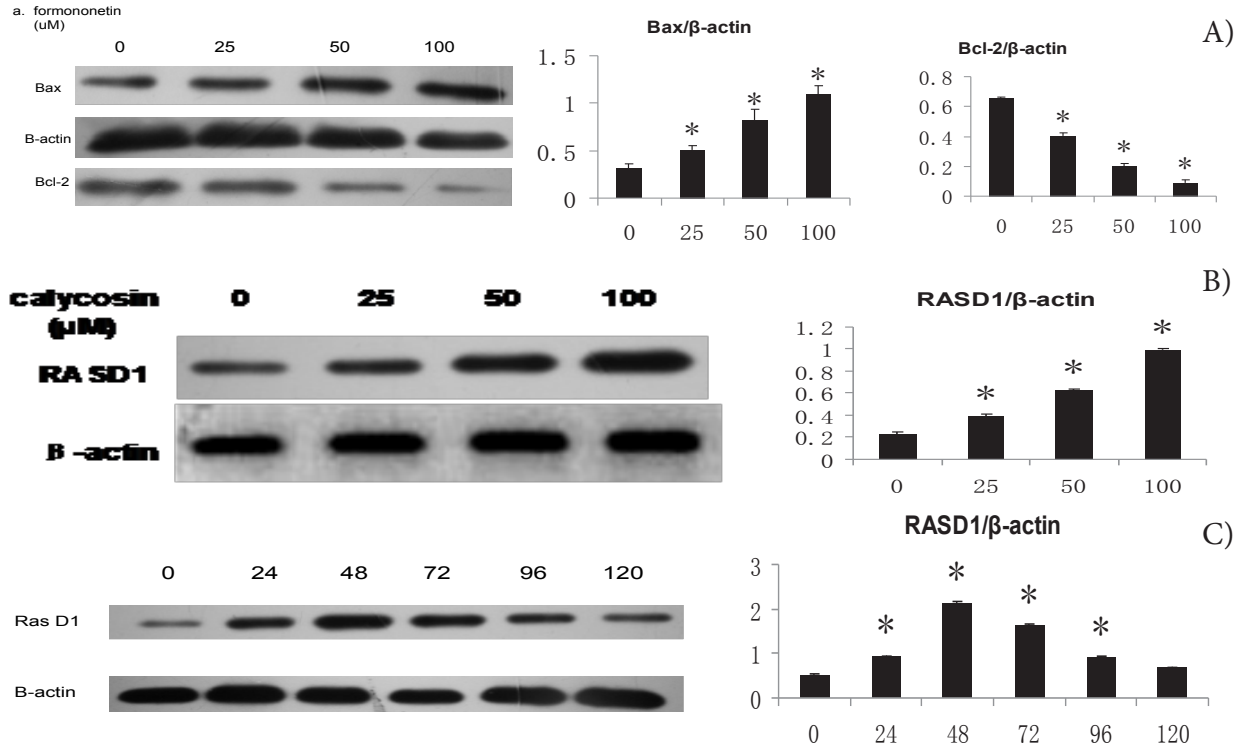

Figure 3. Effects of Formononetin on the Expression of Apoptosis-related Gene and Protein in DU-145 Cells. a Dose-dependent effects of formononetin on level of Bax and Bcl-2 protein. β-Actin (42 kDa) was used as a loading control. Results are representative of 3 independent experiments; b Dose-dependent effects of formononetin on level of RASD1 protein. Cells were incubated with various concentrations of formononetin for 48 h and RASD1 was determined. Results are representative of 3 separate experiments; c Time-dependent effects of formononetin on level of RASD1 protein. Results are representative of 3 separate experiments

shown in Figure 3, the levels of RASD1 and Bax protein in DU145 cells were increased after incubating at different concentrations (0, 25, 50, 100uM) of formononetin. However, the levels of Bcl-2 protein decreased in a dose-dependent manner. The level of RASD1 reached its maximum at 48h post-treatment, and decreased thereafter. These data showed the formononetin induced the activation of RASD1 and Bax and inhibited the levels of Bcl-2 protein in a dose-dependent manner.

Discussion

The sex-hormone receptor status of the prostate is complex (Smith et al., 2008). Both of the estrogen and estrogen receptors (ER) are expressed and seem to play an important role in prostate carcinogenesis (Hara et al., 2008). The estrogen stimulation of proliferation in prostate cancer cells and conversely, its inhibition by phytoestrogen, appeared to be related to the ER. After estrogen was bound to ER, the effects of estrogen that are attributable to transcriptional activation following ligand receptor binding can be supplemented by cytoplasmicsignaling pathways (Driggers et al., 2002), such as the MAPK pathway. The MAPK pathway is normally activated by estrogen or estrogen agonists by the membrane-associated receptor tyrosine kinases (Di et al., 2008; Lau et al., 2009) , which in turn activate Ras, followed by the activation of the protein kinase Raf. The activated Raf then mediates signal transduction to mitogen-activated protein kinase (MAPK) and downstream ERK pathway or p38 pathway (Bapat et al., 2001).

Formononetin is a phytoestrogen and is one of the main active components of red clover plants. Our previous studies demonstrated that formononetin could also induce the apoptosis by activation of Ras/p38 Mitogen-activated Protein Kinase, indicating the use of formononetin in the prevention of breast cancer carcinogenesis (Chen et al., 2012). We further found formononetin induced apoptosis of human prostate cancer cells through ERK1/2 Mitogen-activated Protein Kinase inactivation. In this study, higher concentrations formononetin (25, 50, 100 μM) exhibited inhibitory effects on hormone-independent prostate cancer cell lines (DU-145 cells), depending on the dosage.Our data confirmed that formononetin activated RASD1 (a member of Ras family) in a dose-dependent manner, which suggested that the inhibitory effects of formononetin on proliferation of human prostate cancer cells were related to Ras-Raf-MAPK pathway.

RASD1 or AGS1 (Activator of G-protein signaling 1) is a 30 kDa G-protein that belongs to the RAS superfamily of small GTPases (Takai et al., 2001). It was first discovered as a dexamethasone-inducible gene in the AtT-20 pituitary cell line (Kemppainen and Behrend et al., 1998). RASD1 mRNA and/or protein is generally widely expressed in normal human tissues including brain, heart, liver, kidney and bone marrow, but is not detected in various human cancer cell lines (HL-60, K-562, SW-480, A549, G-361, Raji and MCF-7) (Kemppainen et al., 2003).

As a Ras family member, RASD1 may regulate various aspects of cell growth, differentiation and mutations in many different types of tumors (Der et al., 1989). Transfection of several different cancer cell lines with a RASD1 expression vector inhibited their growth and survival, suggesting that RASD1 may be important in preventing aberrant cell growth. In clonogenic assays with NIH-3T3 murine fibroblast cells, the MCF-7 human breast cancer cell line and the human lung adenocarcinoma cell line A549, RASD1 transfection markedly diminished the number of G418-resistant colonies. A549 cell infection with adenovirus engineered to express AGS1/RASD1 (Ad.AGS1) inhibited log phase growth in vitro and increased the percentage of cells undergoing apoptosis. The anti-growth action was also observed in vivo as the expression of RASD1 inhibited the subcutaneous tumor growth of A549 cells in athymic nude mice. The mice injected with AGS1/RASD1-expressing cells (Ad.AGS1) remained tumor free even at day 60 following injection. So it showed that Ad.AGS1 completely prevented the formation of tumors in mice injected with A549 cells expressing AGS1/RASD1G (Vaidyanathan et al., 2004). These data indicate that RASD1, a member of the Ras superfamily of small G-proteins that often promotes cell growth and tumor expansion, plays an active role in preventing aberrant cell growth.

The B-cell CLL/lymphoma 2 (Bcl-2) family proteins include antiapoptotic members such as Bcl-2 and Bcl-xL and pro-apoptotic members such as Bax, Bad, and Bak. As one of the pro-apoptotic members of the Bcl-2 family, Bax protein exerts proapoptotic activity by translocation from the cytosol to the mitochondria, where it induces cytochrome c release (Li et al., 2011). Previous reports suggested that death signals induced a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome c release (Weidinger et al., 2010). On the other hand, anti-apoptotic proteins such as Bcl-2 are located on the outer membrane of the mitochondria and inhibit the release of cytochrome c. In the present study, activated Bax was dose-dependently increased by formononetin, while expression level of Bcl-2 protein was dose-dependently decreased by formononetin.

Activation of MAPKs followed by activation of the proapoptotic protein Bax seems to play an important role in the apoptosis of DU-145 cells induced by formononetin. During switching the balance from antiapoptotic to proapoptotic signals (Bcl-2, Bax), the mitochondrial membrane is permeabilized and cytochrome c is released. It was suggested that changes in the ratio of proapoptotic to anti-apoptotic Bcl-2 family proteins might contribute to the apoptosis-promotion activity of formononetin.

Taken together, our results indicated that formononetin inhibited cell proliferation and induced apoptpsis in DU-145 cells throughout the RASD1/MAPK/Bax pathway. Our study may provide experimental foundations for the future clinical use of formononetin in prostate cancer.

Acknowledgements

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