Daucosterol induces autophagic-dependent apoptosis in prostate cancer via JNK activation

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Summary

Plant sterols (phytosterols) have been widely accepted as a natural anti-cancer agent in multiple malignant tumors. This study was designed to investigate the functions of daucosterol in prostate cancer progression and its possible molecular mechanisms. Our results showed that daucosterol inhibited cell proliferation and induced cell cycle arrest. Moreover, daucosterol treatment obviously promoted apoptosis and autophagy. An autophagy inhibitor, 3-methyladenine (3-MA) was proved to counteract daucosterol-triggered autophagy, growth inhibition, and apoptosis, indicating that daucosterol-induced apoptotic response was dependent on autophagy. Additionally, treatment with daucosterol resulted in increased phosphorylation of c-Jun N-terminal kinase (JNK). Furthermore, pre-treatment with a JNK-specific inhibitor SP600125 abated daucosterol-elicited autophagy and apoptotic cell death. Taken together, our findings demonstrated that daucosterol blocked prostate cancer growth at least partly through inducing autophagic-dependent apoptosis via activating JNK signaling, providing a promising candidate for the development of antitumor drugs in prostate cancer treatment.

Keywords: Prostate cancer, daucosterol, cell cycle, apoptosis, autophagy, JNK

1. Introduction

Prostate cancer, the most common non-cutaneous malignancy in male, ranks second in cancer-related death among men in the United States in 2016 (1). Chemoprevention has been generally used as a main therapy for prostate cancer patients (2). However, high cytotoxicity and drug resistance greatly limited its application (3). Hence, it is necessary to identify more effective therapeutic targets for prostate cancer treatment.

Natural compounds, multi-targeted and less toxic, have been found to be involved in the prevention and therapy of multiple cancers (4,5). Plant sterols (phytosterols), with similar structural and biological functions to cholesterol, are able to exert anti-cancer effects in different tumors through modulation of cell growth, invasion, metastasis, cell cycle arrest and apoptosis (6,7). Daucosterol, a β-sitosterol glycoside, is one of the major phytosterols in higher plants and extracted from an endemic medicinal plant of Iran, Salvia sahendica (8). Daucosterol was previously reported to display anti-inflammatory and immunomodulatory activities (9–11). Additionally, daucosterol showed neuroprotective action in \textit{in vitro} model of ischemia by activating IGF1 signaling (12). Moreover, daucosterol could promote neural stem cells proliferation through regulating multiple genes, especially increasing insulin-like growth factor I (IGF1) expression (13).

In recent years, daucosterol is also proved as...
important regulators in the occurrence and progression of cancers. A previous report delineated that daucosterol induced apoptosis in human breast cancer through regulating PTEN/PI3K/Akt pathway (14). Zeng et al. disclosed that daucosterol repressed proliferation, migration and invasion in hepatocellular carcinoma via Wnt/β-catenin signaling pathway (15). Zhao et al. discovered that daucosterol suppressed cancer cell proliferation by triggering autophagy through reactive oxygen species (ROS)-dependent manner (16). However, the exact roles and molecular mechanisms of daucosterol in prostate cancer deserve to be further investigated.

Apoptosis, type-I programmed cell death (PCD), is a common mechanism of cancer cell death for various anticancer drugs (17). Autophagy has a controversial role in cancer development. It could relieve tumor cell from nutrient and oxidative stress during the rapid expansion of cancer, while excessive and sustained autophagy may result in cell death and tumor shrinkage (18). Many researchers have highlighted the importance of autophagy in tumorigenesis and cancer therapy (19,20). Natural compounds and extracts have been documented as potential inducers of autophagy through diverse cellular mechanisms and pathways, thereby leading to cell senescence, apoptosis-independent death, and apoptotic death (21,22). Jun N-terminal kinase (JNK), a member of mitogen-activated protein kinase (MAPK) family, plays a vital part in many cellular events, including apoptosis and autophagy (23). JNK signaling has been found to be implicated in the process of natural product therapy in a variety of neoplasms (24,25).

In this current study, we elucidated that daucosterol suppressed cell proliferation, induced cell cycle arrest and enhanced apoptosis in prostate cancer. Furthermore, the anti-cancer effect of daucosterol was mediated by autophagy via JNK signaling.

2. Materials and Methods

2.1. Cell culture

Human prostate cancer cell lines (PC3 and LNCap) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA) at 37°C with an atmosphere of 5% CO2.

2.2. Reagents

Daucosterol was obtained from extractsupplier Biotechnology (Xi'an, China) and dissolved in DMSO at 10 mM as stock. Autophagy inhibitor 3-methyladenine (3-MA) was purchased from Sigma and JNK-specific inhibitor (SP600125) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Cell Counting Kit-8 (CCK-8) assay

PC3 and LNCap cells were seeded in 6-well plates (1 × 104 cells/well) and exposed to different concentrations of daucosterol (0, 5, 10, 20, 40, and 80 µM) for 48 h. Then, 10 µl of CCK-8 solution was added and incubated for another 2 h at 37°C. The absorbance at 450 nm was determined, and cell viability was normalized as the percentage of control.

2.4. Cell cycle and apoptosis assay

PC3 and LNCap cells were treated with various concentrations of daucosterol (0, 5, 10, 20, 40, and 80 µM) for 48 h. For cell cycle analysis, cells were collected, fixed in 75% ethanol, incubated with 50 µg/mL propidium iodide (PI) containing 40 µg/mL RNase for 30 min. Subsequently, cell cycle distribution was detected by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

After treatment, apoptotic rate was measured using the annexin-V-FITC/PI apoptosis kit (BD Biosciences) by a flow cytometry (FACScan; BD Bioscience).

2.5. Western blot analysis

Following daucosterol treatment, cell lysates were separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were sequentially probed with primary antibodies and peroxidase-conjugated secondary antibody. Finally, the protein bands were detected by ECL Advance Detection System (Amersham Biosciences, Piscataway, NJ, USA). The primary antibodies against cleaved caspase 3, cleaved caspase 9, Bax, Bel-2, LC3, Beclin 1, p62, were purchased from Cell Signaling Technology (Danvers, MA, USA), and antibodies against p-JNK, JNK and β-actin were obtained from Santa Cruz Biotechnology.

2.6. Statistical analysis

All experiments were performed at least 3 times and all data were displayed as mean ± SD. Student's t test or one-way ANOVA was used to evaluate statistical difference by GraphPad Prism (GraphPad Software Inc.). P < 0.05 is considered as statistically significant.

3. Results

3.1. Daucosterol suppressed proliferation in prostate cancer cells

To explore the effects of daucosterol on prostate
cancer, PC3 and LNCap cells were treated with various concentrations of daucosterol for 48 h. CCK-8 assay manifested that daucosterol inhibited PC3 and LNCap cell growth in a dose-dependent manner (Figure 1A and 1B). Moreover, flow cytometry analysis demonstrated that daucosterol treatment resulted in an obvious cell cycle arrest at G1 phase in both PC3 (Figure 1C) and LNCap (Figure 1D) cells. These data suggested that daucosterol impaired cell proliferation in prostate cancer.

3.2. Daucosterol promoted apoptosis in prostate cancer cells

To further investigate whether the growth inhibition elicited by daucosterol was associated with apoptosis, we evaluated the apoptotic rate of PC3 and LNCap cells after treatment with different doses of daucosterol for 48 h. Flow cytometry assay displayed that the apoptotic rate was greatly enhanced in both PC3 and LNCap cells following daucosterol treatment (Figure 2A and 2B). Moreover, western blot analysis exhibited a dramatic increase of cleaved caspase 3, cleaved caspase 9 and Bax protein expressions, while a prominent decrease of Bcl-2 expression in PC3 and LNCap cells after treatment with daucosterol (Figure 2C and 2D). Together, these results indicated that daucosterol exerted anticancer activity at least partly by facilitating apoptosis in prostate cancer.

3.3. Daucosterol induced autophagy in prostate cancer cells

Autophagy, type-II PCD, is demonstrated to participate...
in the regulation of natural products in cancers (26). Thus, we determined the protein levels of autophagy markers (LC3, Beclin 1 and p62) in PC3 and LNCap cells after treatment with different doses of daucosterol for 48 h. Western blot analysis revealed that daucosterol treatment resulted in an increase of LC3II/LC3I ratio and Beclin 1 expression, while a decline of p62 expression in PC3 and LNCap cells (Figure 3A and 3B), supporting the induction of autophagy by daucosterol in prostate cancer cells.

3.4. Suppression of autophagy weakened daucosterol-elicited growth inhibition and apoptosis in prostate cancer cells

To further analyze whether apoptosis and autophagy were related or independent events in response to daucosterol, we examined the effects of daucosterol on cell proliferation and apoptosis in the presence or absence of autophagy inhibitor 3-MA. As expected, 3-MA pre-treatment effectively attenuated daucosterol-
induced autophagy in PC3 cells, presented by reduced LC3II and Beclin 1 expressions, while enhanced p62 expression (Figure 4A). Moreover, the growth inhibition triggered by daucosterol was greatly reversed in PC3 cells following pre-treatment with 3-MA (Figure 4B and 4C). Further, treatment of PC3 cells with 3-MA abated daucosterol-induced apoptosis (Figure 4D). Collectively, the pro-apoptotic effect of daucosterol was modulated by autophagy.

3.5. Daucosterol induced autophagy in prostate cancer cells

JNK activation has been found to be involved in autophagy and apoptosis induced by natural compound (27). In order to explore the potential molecular basis of daucosterol in prostate cancer, we firstly determined the effects of daucosterol on JNK pathway in PC3 cells. As presented in Figure 5A, daucosterol treatment significantly increased the level of phosphorylated JNK. To further confirm whether daucosterol-induced apoptosis and autophagy require JNK activation, PC3 cells were pre-incubated with or without JNK-specific inhibitor (SP600125) prior to daucosterol treatment. The results demonstrated that SP600125 suppressed the conversion of LC3I to LC3II and Beclin 1 expression, while enhanced p62 expression in PC3 cells under daucosterol treatment (Figure 5B), suggesting the inhibitory effect of SP600125 on daucosterol-induced autophagy. Moreover, daucosterol-elicited growth inhibition (Figure 5C), cell cycle arrest (Figure 5D) and apoptosis (Figure 5E) were alleviated following SP600125 pre-treatment. From these data, we draw a conclusion that daucosterol induced autophagic-dependent apoptosis in prostate cancer by activating JNK signaling.

![Figure 5](https://www.biosciencetrends.com)

**Figure 5.** Daucosterol-triggered autophagic-dependent apoptosis by JNK activation in prostate cancer cells. (A) Western blot analysis of JNK and p-JNK expression in PC3 cells treated with daucosterol at indicated concentrations for 48 h; (B) PC3 cells were pre-incubated with SP600125 (10 μM) for 1 h prior to treatment with 40 µM daucosterol for 48 h, then (B) western blot was applied to evaluate the protein levels of LC3II, Beclin 1 and p62; (C) CCK-8 assay was performed to monitor cell viability; (D and E) flow cytometry analysis was conducted to analyze cell cycle distribution and apoptosis. *p < 0.05, **p < 0.01.
4. Discussion

Natural products have been discovered as a rich source for the discovery and development of cancer preventive and anticancer drugs (28,29). Daucosterol, a β-sitosterol glycoside, is one of the major phytosterols in higher plants (30). In this study, we found that daucosterol exerted tumor-suppressive function in prostate cancer by inducing autophagic-dependent apoptosis via activation of JNK.

Daucosterol are reported as an anti-cancer plant sterol in breast cancer (14), hepatic carcinoma (15) and gastric cancer (16) by various mechanisms and pathways. Rajavel et al. also revealed that the presence of phytosterols (β-sitosterol and daucosterol) significantly blocked the growth of lung cancer cells both alone and in combination (31). Wang et al. disclosed that daucosterol hindered proliferation, enhanced apoptosis, and inhibited migration and invasion in colon cancer via regulating caspase signaling (32). In this study, we firstly found that daucosterol suppressed prostate cancer cell viability in a dose-dependent manner. Deregulation of cell cycle progression has been regarded to be associated with cancer occurrence and development (33). Moreover, controlling or perturbing the cycle of tumor cells is an effective method for natural products to destroying malignant cell proliferation (34). Here, our results showed that daucosterol treatment resulted in a dramatic cell cycle arrest.

Apoptosis has been considered as a major mechanism to exert anti-cancer roles for chemotherapeutic agents (35). It has been proved that numerous plant-derived chemotherapy drugs kill cancer cells via inducing apoptotic cell death (36,37). In the present study, we demonstrated that daucosterol repressed cell apoptosis by flow cytometry. Moreover, daucosterol activated the mitochondria-dependent apoptotic signaling pathway, leading to increased expression of pro-apoptotic protein (Cleaved caspase 3, Cleaved caspase 9, Bax) and decrease expression of anti-apoptotic protein Bcl-2. These data suggested that daucosterol was cytotoxic to cells in prostate cancer through facilitating apoptosis.

Autophagy is a homeostatic cellular degradation process for eliminating damaged or unnecessary intracellular organelles and proteins in lysome (38). Autophagy is able to suppress early cancer development while contribute to advanced tumor progression (39). Numerous natural compounds have been discovered to induce cell apoptosis through modulating autophagy in cancers. For example, natural product peiminine repressed cell growth in colorectal cancer by enhancing autophagic cell death (40). Marchantin M promoted apoptosis in prostate cancer through induction of autophagy (41). Our results manifested that daucosterol treatment effectively enhanced autophagy in prostate cancer cells, indicated by up-regulated LC3II and Beclin 1 expression, while down-regulated p62 expression. Moreover, autophagy inhibitor 3-MA attenuated daucosterol-triggered growth inhibition and apoptosis in prostate cancer cells. These data confirmed that daucosterol impaired cell growth by contributing to autophagic-dependent cell death.

It is well known that JNKs are master protein kinases that modulate a variety of physiological events, such as inflammatory responses, morphogenesis, cell proliferation, differentiation, survival and death (42). Aberrant activation of JNK signaling has been reported to be associated with tumor progression (43). Our data exhibited that daucosterol increased the phosphorylation level of JNK in prostate cancer cells. Moreover, JNK-specific inhibitor (SP600125) inhibited daucosterol-induced autophagy. Furthermore, daucosterol-elicted growth inhibition, cell cycle arrest and apoptosis were abated by pre-treatment with SP600125. Together, daucosterol exerted anti-proliferation effect by inducing autophagic-dependent apoptosis via activation of JNK signaling. Consistent with our finding, JNK activation has been reported to be participate in apoptosis and autophagy mediated by multiple natural products (44,45).

In conclusion, our present study demonstrated that daucosterol inhibited cell proliferation, induced cell cycle arrest, and promoted autophagic-dependent apoptosis in prostate cancer. Moreover, the tumor-suppressive effect of daucosterol was at least partly mediated by JNK activation. Our findings indicated that daucosterol may be developed as a potential anti-cancer agent.

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