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Sarsasapogenin induces apoptosis via the reactive oxygen species-mediated mitochondrial pathway and ER stress pathway in HeLa cells

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ABSTRACT

Sarsasapogenin is a sapogenin from the Chinese medical herb *Anemarrhena asphodeloides Bunge*. In the present study, we revealed that sarsasapogenin exhibited antitumor activity by inducing apoptosis in vitro as determined by Hoechst staining analysis and double staining of Annexin V-FITC/PI. In addition, cell cycle arrest in G2/M phase was observed in sarsasapogenin-treated HeLa cells. Moreover, the results revealed that perturbations in the mitochondrial membrane were associated with the deregulation of the Bax/Bcl-2 ratio which led to the upregulation of cytochrome c, followed by activation of caspases. Meanwhile, treatment of sarsasapogenin also activated Unfolded Protein Response (UPR) signaling pathways and these changes were accompanied by increased expression of CHOP. Salubrinal (Sal), a selective inhibitor of endoplasmic reticulum (ER) stress, partially abrogated the sarsasapogenin-related cell death. Furthermore, sarsasapogenin provoked the generation of reactive oxygen species, while the antioxidant N-acetyl cysteine (NAC) effectively blocked the activation of ER stress and apoptosis, suggesting that sarsasapogenin-induced reactive oxygen species is an early event that triggers ER stress mitochondrial apoptotic pathways. Taken together, the results demonstrate that sarsasapogenin exerts its antitumor activity through both reactive oxygen species (ROS)-mediate mitochondrial dysfunction and ER stress cell death.

1. Introduction

Cervical cancer is the second most common cancer among women worldwide and remains a critical public health problem, although mortality rates have declined over time due to early detection and screening programs. Cervical cancer is a leading cause of death from cancer among women in developing countries, killing approximately a quarter of a million women per year [1]. Platinum-based chemotherapy drugs have been proven to be effective among the chemotherapy drugs available; however, the serious side effects of such treatments cannot be ignored. To reduce the mortality rate of cervical cancer, the development of effective drugs to treat cervical cancer with minimal side effects is of great importance. Natural products are considered to be a suitable replacement or supplement for platinum drugs to cure cervical cancer.

To date, attention has been focused on finding safe agents with biological activity and isolating active compounds from natural sources. Chemopreventive phytochemicals including berberine [2], resveratrol [3], curcumin [4] and trichosanthin [5] have been shown to have great potential as cervical cancer drugs. These compounds exert their effects through various mechanisms, such as promoting apoptosis and inhibiting DNA methyltransferase.

Apoptosis, also known as type I programmed cell death, is the most common mechanism exploited by targeted chemotherapies (cytotoxic agents and radiation therapy) that induce cancer cell death or sensitize cells [6,7]. This process can be triggered either at the plasma membrane (extrinsic pathways) and/or within cells (intrinsic pathways) [8]. Recent studies have suggested that the intrinsic pathways are initiated by the biochemical events, which affect the organelles inside the cells causing intracellular stresses. Among these organelles, mitochondria and endoplasmic reticulum (ER) play important roles in the intrinsic pathways to execute apoptosis [9].

Sarsasapogenin (Fig. 1A), a sapogenin from the Chinese medical herb *Anemarrhena asphodeloides Bunge*, has been widely used in Chinese and Japanese folk medicine. It exhibits antidiabetic activity [10], antiplatelet aggregation activity [11] and diuretic activity [12]. Despite evidence indicating the benefits of sarsasapogenin treatment for cancer resistance, there is a lack of data describing the anti-tumor activity of sarsasapogenin on cervical cancer. We therefore investigate the roles and explore the underlying mechanisms of sarsasapogenin in HeLa cells in the present study.

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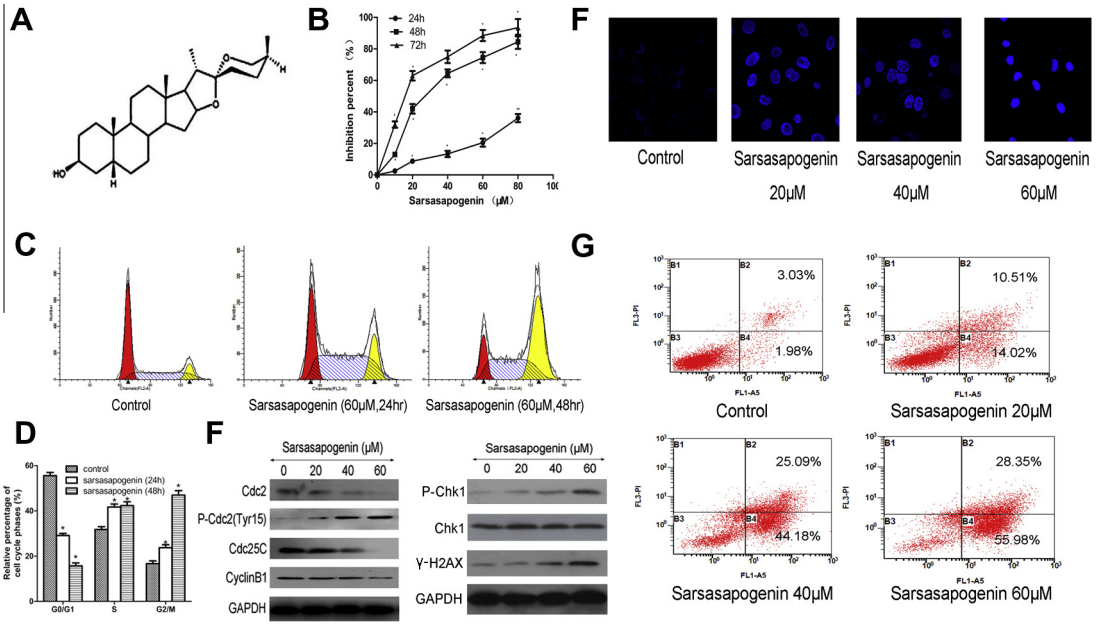

Fig. 1. Effects of sarsasapogenin on the cell cycle and apoptosis induction in HeLa cells. (A) Chemical structure of sarsasapogenin. (B) HeLa cells were treated with increasing doses of sarsasapogenin for various times. Results are expressed as means ± SD of data obtained in three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control). (C) The HeLa cells were treated with 60 μM sarsasapogenin for 24 or 48 h and then cell cycle analyzed by flow cytometry. (D) The proportions of cells in G1, S and G2 phases were calculated using Cell Quest software. Bar graphs are shown by mean ± SD from three independent experiments ($^{*}p < 0.05$ vs. control). (E) HeLa cells were treated with sarsasapogenin (0, 20, 40, 60 μM) for 48 h. Proteins (30 μg per lane) were probed with antibodies against Cyclin B1, Cdc2, Cdc25C, p-Cdc2 (Tyr15), p-Chk1 (Ser345), Chk1 and γ-H2AX as indicated. Each blot is representative of three similar experiments. (F) HeLa cells were incubated with sarsasapogenin (0–60 μM) for 48 h. Hoechst 33342-stained nuclei were visualized using confocal laser scanning microscopy (63×). The data are representative of three parallel experiments. (G) HeLa cells were treated with sarsasapogenin (0–60 μM) for 48 h, stained with Annexin V/PI and then analyzed by flow cytometry. The data represent similar results from three independent experiments.

2. Materials and methods

2.1. Reagents and antibodies

Primary antibodies were purchased from these companies: Santa Cruz (ATF4, ATF6, XBP-1s, CHOP, GAPDH, GRP78, GRP94, PERK, eIF2S1), Cell Signaling (cleaved caspase-3, cleaved caspase-9, phospho-PERK, phospho-eIF2S1, Cyclin B1, Bcl-2, Bax, Bak, Cdc2, Cdc25C, p-Cdc2 (Tyr15), cytochrome c (cyt c), p-Chk1 (Ser345) and Chk1), Upstate Biotechnology (γ-H2AX). The fluorescent probes dihydrorhodamine 123 (DHR123), Rh123 and naphthalene-2,3-dicarboxaldehyde (NDA) were from Molecular Probes (Eugene, OR). The synthesized sarsasapogenin was obtained from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals, except otherwise noted, were purchased from Sigma–Aldrich chemical.

Sarsasapogenin was dissolved in ethanol at 10 mM and diluted with fresh medium to achieve the desired concentration. The final ethanol concentration in cultures was <1% (v/v), which did not influence cell growth when compared with the vehicle-free controls. HeLa cells were cultured in DMEM containing 10% fetal bovine serum in humidified air at 37 °C with 5% CO_2.

2.2. Analysis of cell viability

The effect of sarsasapogenin on cell viability was measured by the MTT assay method. After exposure to sarsasapogenin in the absence or presence of Z-VAD-FMK pretreatment for the indicated time, cells in 96-well plates were incubated with MTT. The formazan precipitate was dissolved in 200 μl of dimethyl sulfoxide, and the absorbance at 570 nm was measured using a Benchmark microplate reader (Bio-Rad, CA, USA) [13].

2.3. Measurement of the cell cycle

HeLa cells were cultured in the presence or absence of 60 μM sarsasapogenin for 24 or 48 h. The cells were then treated with 20 μg/ml RNaseA, followed by 25 μg/ml propidium iodide (PI). The population of cells at each stage of the cell cycle was determined by examining the intensity of PI fluorescence with a flow cytometer using an argon laser and a 570 nm bandpass filter (FACSort, Becton Dickinson).

2.4. Morphological analysis after Hoechst 33342 staining

HeLa cells (1×10^5) were seeded in 6-well plates for 24 h. After sarsasapogenin treatment for another 48 h, the cells were stained with Hoechst 33342 solutions containing 5 μg/ml Hoechst 33342 in 0.1% sodium citrate. Then, the nuclear morphology of the cells was examined using confocal laser scanning microscopy (Zeiss, LSM 510, Germany).

2.5. Annexin V/propidium iodide flow cytometric analysis

Different stages of apoptosis were distinguished using an Annexin V-FITC/propidium iodide apoptosis kit (BioSource). Briefly, cells were cultured with sarsasapogenin at various concentrations for 48 h, and then 1×10^6 cells were harvested, washed twice with ice-cold PBS. Apoptotic (Annexin V^+/PI^-) or necrotic cells (Annexin V^+/PI^+) were evaluated by double staining with Annexin V-FITC and PI in binding buffer using by flow cytometry.

2.6. Measurement of mitochondrial transmembrane potential (MMP)

MMP was measured by flow cytometer using the cationic lipophilic green fluorochrome Rh123. Cells were harvested, washed twice with PBS, incubated with 1 μM Rh123 at 37 °C for 30 min,