Diosgenin induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma cells

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Abstract. Diosgenin is a major compound of Dioscoreaceae plants such as yam, which is used as a drug in Traditional Chinese Medicine, and a common vegetable worldwide. The anticancer effect of diosgenin has been reported in various tumor cells, including leukemia, gastric, colorectal, and breast cancer. However, the activity of diosgenin on hepatocellular carcinoma (HCC) and the underlying mechanism have not been completely investigated. Therefore, we investigated the efficacy and associated mechanisms of diosgenin in HCC cells. Flow cytometric analysis was performed to determine the presence of cell cycle arrest and apoptotic cells. Diosgenin significantly inhibited the growth of Bel-7402, SMMC-7721 and HepG2 HCC cells in a concentration-dependent manner. Diosgenin treatment for 24 h induced G2/M cell cycle arrest and apoptosis of hepatoma cells. Diosgenin inhibited Akt phosphorylation and upregulated p21 and p27 expression, but did not alter the expression of p53, suggesting diosgenin-induced upregulation of p21 and p57 is p53-independent in HCC cells. Diosgenin treatment for 24 h induced G2/M cell cycle arrest and apoptosis of hepatoma cells. Diosgenin inhibited Akt phosphorylation and upregulated p21 and p27 expression, but did not alter the expression of p53, suggesting diosgenin-induced upregulation of p21 and p57 is p53-independent in HCC cells. Diosgenin induced HCC cell apoptosis by activating caspase cascades -3, -8 and -9. However, diosgenin did not affect Bcl-2 and Bax levels. In conclusion, results of the present study suggest that diosgenin may be an active anti-HCC agent obtained from natural plants and provide new insights in understanding the mechanisms of diosgenin.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China (1). Although surgical resection remains the optimum option for HCC, many patients are unresectable due to the tumor size, metastasis, hepatic functional reserve and/or portal hypertension. Trans-arterial embolization (TAE), percutaneous injection of ethanol and radiofrequency ablation have also been used in the treatment of unresectable patients. However, these methods only target the local tumors and the high recurrence and metastatic rate limit the outcome. In addition, HCC is not sensitive to most chemotherapeutic drugs, such as paclitaxel, doxorubicin, fluorouracil, cisplatin and mitomycin. Thus, new agents that are safe and effective are to be identified for the treatment of HCC.

Findings of recent studies have demonstrated that many compounds from Traditional Chinese Medicine (TCM) are effective in the treatment of malignant tumors, including HCC (2-4). In a previous study, we found that Ganzaoning granule, a Traditional Chinese Medicine formula, is able to inhibit diethylnitrosamine-induced hepatocarcinogenesis in rats (5,6). However, the active ingredients of Ganzaoning exerting an anti-HCC effect have not been clarified. Diosgenin, a steroidal saponin, is abundant in a variety of plants, such as yam (Dioscorea villosa) which is a main drug in Ganzaoning granule. Results of recent studies have shown that diosgenin exerts anticancer effects against a wide variety of tumor cells, including leukemia, gastric, colorectal, and breast cancer (7-11). However, the anticancer effect of diosgenin on HCC and the mechanisms has not been completely elucidated.

Therefore, we investigated the inhibitory effect of diosgenin on HCC, and the molecular mechanism of the antitumor effect in this study. Our results showed that diosgenin reduced the proliferation of Bel-7402, SMMC-7721 and HepG2 cells in a dose-dependent manner. In addition, diosgenin exerted an anti-proliferative effect in the three HCC cells by inducing G2/M cell cycle arrest and apoptosis. Furthermore, diosgenin upregulated p27 and p21 expression and activated the caspase cascade. Diosgenin-induced p27 and p21 upregulation was independent of p53. The results suggest that diosgenin
potentially exerts chemopreventive effects on the relevant cell cycle regulation and death receptor apoptotic pathways.

Materials and methods

Cell culture and drug treatment. SMMC-7721, Bel-7402, HepG2 HCC cell lines were cultured in DMEM medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France). The cells were cultured at 37°C with 5% CO₂. Diosgenin, with a purity of >98% was purchased from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China) and dissolved in ethanol.

MTT assay. The cells were seeded in 96-well plates and treated with diosgenin at concentrations of 0-40 µM for the indicated time-points. After the exposure period, the media were removed. Cell viability was measured using the MTT method as previously described (12). The experiment was performed in triplicate. The inhibitory rate was calculated as a percentage using the formula: \( \frac{1 - OD_{\text{diosgenin}}/OD_{\text{control}}}{100\%} \).

Cell morphology was observed under an inverted microscope and the images were obtained at magnification of x200.

Cell cycle and apoptosis assay. Flow cytometric analysis was performed to determine the presence of cell cycle arrest and apoptotic cells. After treatment with diosgenin for 24 h, the cells were collected by trypsinisation and washed twice with PBS, fixed in ice-cold 80% ethanol, and stored overnight at 4°C. For analysis, the cells were washed with PBS twice, and suspended in 1 ml of cold propidium iodide (PI) solution. The cells were then incubated on ice for 30 min in the dark and then analyzed using flow cytometry.

FITC-labeled Annexin V/PI staining was performed according to the manufacturer’s instructions (Keygen, Nanjing, China). Briefly, 1x10⁶ cells/well were suspended in buffer containing FITC-conjugated Annexin V/PI at appropriate concentrations. The samples were analyzed by flow cytometry and 20,000 events from each sample were obtained to ensure adequate data.

Quantitative RT-PCR. Total RNA was extracted from SMMC-7721 cells after diosgenin treatment with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA (5 µg) was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (FSK-100) (Toyobo, Osaka, Japan). Amplification of the cDNA was achieved in triplicate using a commercially available SYBR-Green PCR Master mix (Toyobo). cDNA was amplified under the following conditions: 95°C for 5 min for denaturation and subjected to 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 25 sec. The relative expression level of mRNA in each sample was normalized to its β-actin content. The relative expression levels of mRNA were calculated as \( 2^{-ΔΔCt} \).

Western blotting. SMMC-7721 cells were seeded in 6-well plates at a density of 1x10⁶ cells/well with 2 ml completed DMEM medium. Following diosgenin treatment for the indicated times, total protein was extracted as previously described (12,13). The protein concentration was determined using the BCA method. Equal quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting onto a nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) overnight at 4°C. The membrane was incubated with specific primary antibodies for 2 h and a secondary antibody for 1 h. The signal was visualized with an enhanced chemiluminescence kit (ECL) (Thermo Scientific, San Jose, CA, USA).

Statistical analysis. Data are presented as means ± SD. Statistical significance was determined using SPSS 17.0 for Windows. The Student’s t-test was used to compare means for two groups and one-way ANOVA was performed for multiple comparisons followed by the Newman-Keuls test for multiple comparisons. Differences were considered significant when \( P<0.05 \).

Results

Diosgenin inhibits HCC cell proliferation. Diosgenin treatment for 24 and 48 h induced changes in SMMC-7721 cell morphology, including cell shrinkage, disappearance of tentacles and round-up shapes, indicating cell damage (Fig. 1A). The effect of diosgenin was concentration- and time-dependent. We examined the inhibitory rate of diosgenin in Bel-7721, SMMC-7721 and HepG2 HCC cells. The results showed that diosgenin treatment significantly inhibited Bel-7721, SMMC-7721 and HepG2 cell proliferation in a concentration-dependent manner (Fig. 1B).

Diosgenin induced G2/M cell cycle arrest and apoptosis of HCC cells. To investigate the mechanisms of diosgenin-induced growth repression, the flow cytometric analysis was performed. Diosgenin treatment caused a concentration-dependent increase of G2/M phase cell population in Bel-7721, SMMC-7721 and HepG2 HCC cells, indicating diosgenin was able to arrest the cell cycle in G2/M phase (Fig. 2). The proportion of G2/M phase cells increased with concentration of diosgenin.

We also determined whether diosgenin-induced HCC cell proliferation and inhibition involved apoptosis. Flow cytometry using Annexin V-PI staining was performed. After treatment with different concentrations of diosgenin for 24 h, the proportions of apoptotic cells were markedly increased in Bel-7721, SMMC-7721 and HepG2 HCC cells (Fig. 3), suggesting that diosgenin was able to induce the HCC cell apoptosis. The effect of diosgenin on HCC cell apoptosis was concentration-dependent.

Effect of diosgenin on cell cycle-related proteins. To investigate the mechanisms of diosgenin-induced cell cycle arrest, we examined the expression of cell cycle-related proteins by quantitative RT-PCR and western blotting. Diosgenin (40 µM) treatment for 24 h significantly upregulated the p21 and p27 mRNA levels in SMMC-7721 cells (Fig. 4A). Following treatment with diosgenin for 24 h, Akt phosphorylation was significantly inhibited in SMMC-7721 cells (Fig. 4A). Following treatment with diosgenin for 24 h, Akt phosphorylation was significantly upregulated after diosgenin treatment in a
concentration-dependent manner. However, diosgenin treatment did not alter the expression of p53 in the mRNA and protein levels (Fig. 4A and B).

**Effects of diosgenin on cell apoptosis-related proteins.** To determine the signaling pathway responsible for diosgenin-induced apoptosis in HCC cells, the expression levels of apoptosis-related proteins were examined subsequently. We first examined whether diosgenin could alter the balance between pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 in SMMC-7721 cells. The results showed that diosgenin treatment did not alter Bax and Bcl-2 levels (Fig. 5).
The expression levels of caspase-3, -8 and -9 were also detected in diosgenin-treated SMMC-7721 cells. After various concentrations of diosgenin treatment for 24 h, the expression of caspase-3, -8 and -9 was markedly reduced in a concentration-dependent manner (Fig. 5), whereas the cleaved caspase-3, -8 and -9 were obviously increased.

Discussion

In the present study, we investigated the anti-HCC effect of diosgenin in three HCC cell lines. The results demonstrated that diosgenin exerted a strong growth inhibitory activity against Bel-7402, SMMC-7721 and HepG2 human liver cancer cells. Diosgenin induced G2/M cell cycle arrest and apoptosis in these cells. Further study showed that the upregulation of cell cycle-related proteins, p21 and p27, and activation of caspase cascade may be involved in diosgenin-induced cell cycle arrest and apoptosis.

Although the anticancer property of diosgenin has been widely reported in many cancer cells, the anti-HCC effect and the mechanisms involved in diosgenin have not been extensively investigated. In the present study, the results showed that...
Diosgenin inhibited the proliferation of Bel-7402, SMMC-7721 and HepG2 HCC cells. We also studied the mechanisms of diosgenin at cellular and molecular levels. It is well recognized that dysregulation of the cell cycle is a hallmark of tumor cells. Numerous anticancer drugs play a therapeutic role by inducing cell cycle arrest. In the present study, we showed that treatment with diosgenin induced G2/M cell cycle arrest in Bel-7402, SMMC-7721 and HepG2 cells in a concentration-dependent manner. Although previous studies showed that diosgenin treatment caused cell cycle arrest in G1 phase in osteosarcoma cells and C3A hepatoma cells (14,15), diosgenin also induced G2/M cell cycle arrest in erythroleukemia HEL cells and human leukemia K562 cells (9,16). Those results suggest that the action of diosgenin on cell cycle and its mechanism may be determined by the cell type.

The potent cyclin-dependent kinase inhibitors (CK1) (17), p21 and p27, negatively regulate multiple phases of the cell cycle progression (18). After binding to the Cyclin-CDk complexes, p21 and p27 inhibit their kinase activities and prevent cell cycle progression (19). Since the upregulation of p21 and p27 suppresses the proliferation of many cancer cells by inducing cell cycle arrests, they are recognized as important tumor suppressors (20,21). Diosgenin treatment induced a significant increase of p21 and p27 in SMMC-7721 cells, suggesting that the regulation of p21 and p27 may be involved in diosgenin-induced cell cycle arrest. However, p53 expression, which is conventionally considered as a regulator of p21, was not altered by diosgenin, suggesting the diosgenin-induced upregulation of p21 and p27 is not p53-dependent. Phosphatidylinositol 3 kinase (PI3K)/Akt pathway is a vital regulator of cell survival, proliferation and migration (22). Deregulation of the PI3K/Akt signaling pathway is important in cancer development and has been suggested as a therapeutic target for cancer (22-24). The inhibition of PI3K/Akt pathway by its specific inhibitor LY294002 is able to upregulate the expression of p21 and p27 (25) and lead to cell cycle arrest (26). Diosgenin is known to suppress Akt activation in various cell types (27-29). Our data also showed that diosgenin treatment inhibited Akt phosphorylation. These results suggest that the inactivation of the PI3K/Akt pathway accompanied by an increased expression of p21 and p27 is involved in diosgenin-induced cell cycle arrest in HCC cells.

Cell growth is also regulated by apoptosis. Diosgenin-induced G2/M cell cycle arrest provides an opportunity for HCC cells to undergo apoptotic progression. In this study, flow cytometry showed that treatment with diosgenin caused concentration-dependent cell apoptosis. Since activation of the caspase cascade and its downstream molecules turns on cell apoptotic death progression, we first assessed the effect of diosgenin on the caspase cascade, which mediates the death receptor pathway. Western blotting showed that diosgenin decreased the total caspase-3, -8 and -9 levels, but increased the cleaved caspase-3, -8 and -9 levels in a concentration-dependent manner. The Bcl-2 family proteins are crucial for mitochondrial pathway-induced apoptosis (30). Diosgenin treatment for 24 h did not alter the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. These results suggest that diosgenin-induced apoptosis of HCC cells may be mediated by the death receptor pathway, but not the mitochondrial pathway. Although Kim et al (31) reported that diosgenin induced apoptosis in HepG2 cells through the generation of reactive oxygen species and mitochondrial pathway, our results may indicate a diverse mechanism of diosgenin.

In conclusion, our study demonstrated that diosgenin inhibited HCC cell proliferation by inducing G2/M cell cycle arrest and apoptosis. The inactivation of Akt, upregulation of p21 and p27 expression and activation of the caspase cascades were involved in the anti-HCC effect of diosgenin. Therefore, our study may provide evidence for the anti-HCC effect of diosgenin and elucidate the underlying mechanisms of diosgenin.

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References


