Platycodin D Induces Apoptosis, and Inhibits Adhesion, Migration and Invasion in HepG2 Hepatocellular Carcinoma Cells

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Abstract

**Background:** Platycodin D (PD), a triterpenoid saponin isolated from the Chinese medicinal herb *Platycodonis radix*, possesses anti-cancer effects in several cancer cell lines. The aim of this study was to evaluate its anti-cancer activities in hepatocellular carcinoma cells. **Materials and Methods:** MTT and colony formation assays were performed to evaluate cell proliferation, along with flow cytometry and Western blotting for apoptosis. Cell adhesion was tested by observing cellular morphology under a microscope, while the transwell assay was employed to investigate the cell migration and invasion. **Results:** PD concentration-dependently inhibited cell proliferation in both HepG2 and Hep3B cells, and significantly suppressed colony formation and induced apoptosis in HepG2 cells. The protein levels of cleaved poly ADP-ribose polymerase (PARP) and Bax were up-regulated while that of survivin was down-regulated after treatment with PD. Moreover, PD not only obviously suppressed the adhesion of HepG2 cells to Matrigel, but also remarkably depressed their migration and invasion induced by 12-O-tetradecanoylphorbol 13-acetate (TPA). **Conclusions:** PD presents anti-cancer potential in hepatocellular carcinoma cells via inducing apoptosis, and inhibiting cell adhesion, migration and invasion, indicating promising features as a lead compound for anti-cancer agent development.

Keywords: Platycodin D - HepG2 - apoptosis - metastasis - cancer

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**Introduction**

Hepatocellular carcinoma is one of the most common and most aggressive cancers worldwide and a leading cause of cancer deaths in China (Chen et al., 2013). Although surgical resection and transplantation have remarkably improved the survival in patients with no metastasis, the prognosis of hepatocellular carcinoma for late stage diseases remains quite poor (El-Serag, 2002). Besides, because of the high rates of recurrence, most patients are not eligible for surgery. What’s worse, there was lack of first line chemotherapy drugs for this type of cancer in a long period of time. Recently, sorafenib might be the limited choice for patients with advanced hepatocellular carcinoma (Llovet et al., 2008). However, sorafenib only prolongs patients’ median survival time by less than 3 months and many patients have to reduce their dosage or discontinue the drug therapy due to its side effects (Llovet et al., 2008; Cheng et al., 2009). Thus, efficient drugs for treatment of hepatocellular carcinoma are still urgently needed especially for those who are unable to process surgical resection or transplantation.

Natural products, a rich source of compounds with enormous structural diversity, have been extensively explored in the field of drug discovery. A large number of different sources of natural compounds present anti-cancer effects both in vitro and in vivo (Chen et al., 2012; Huang et al., 2012; Lu et al., 2012a; Lu et al., 2012b; Lu et al., 2013). Therefore, it is vital to screen efficient natural compounds for hepatocellular carcinoma treatment. Platycodin D (PD) is a triterpenoid saponin isolated from the Chinese medicinal herb *Platycodonis Radix*. It possesses important biological activities, including immune stimulation (Xie et al., 2008), anti-inflammation (Kim et al., 2001; Ahn et al., 2005), anti-angiogenesis (Choi et al., 2002), anti-obesity (Han et al., 2002; Lee et al., 2010), anti-atherosclerosis (Wu et al., 2012) and anti-cancer (Kim et al., 2005; Kim et al., 2008a; Kim et al., 2008b; Shin et al., 2009; Choi et al., 2010; Yu and Kim, 2010; Chun et al., 2012) etc. Due to the various pharmacological activities, PD has been listed as the marker for the quality control of *Platycodonis Radix* in the Pharmacopoeia of the People’s Republic of China (Pharmacopoeia Commission of the Ministry of Health of the P.R.China, 2010).

In this study, the anti-proliferative effects and apoptotic induction of PD as well as the anti-adhesion, anti-migration, and anti-invasion potentials of PD were determined which indicates that PD might be a promising lead compound for treatment of hepatocellular carcinoma.
Materials and Methods

Materials

PD was purchased from Best-Reagent (Chengdu, China). It was diluted using DMSO and the stock concentration was 20 mM. 3-[4, 5-Dimethyl-2-thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT) and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma (Saint Louis, Missouri, USA) and Beyotime (Nantong, China), respectively. Propidium iodide (PI) was purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

The hepatocellular carcinoma cell lines HepG2 and Hep3B (ATCC, Rockville, MD, USA) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin, and were grown in an incubator with 5% CO₂ at 37°C.

MTT assay

Exponentially growing HepG2 and Hep3B cells were plated into 96-well plates, and then treated with serial concentrations of PD for 48 h after adhesion. Cell proliferation was determined by addition of 1 mg/mL MTT-containing medium for 4 h, addition of 100 μL DMSO to solubilize the formazan, and shaking for 10 min in the dark. The absorbance at 570 nm was recorded using the SpectraMax M5 Microplate Reader (Molecular Devices).

Colony formation assay

HepG2 cells were treated with serial concentrations of PD for 24 h and then suspended and re-seeded into 6-well plates at a density of 2x10⁵ cells per well after treatment. Cells were subsequently fixed using 4% paraformaldehyde and stained with crystal violet staining solution (Beyotime, Nantong, China) after 10 days-incubation. Typical images were captured using an ordinary NIKON camera.

Flow cytometry analysis of DNA content

HepG2 cells seeded into 6-well plates were treated with series concentrations of PD for 24 or 48 h. Cells were harvested and fixed in 70% ethanol and then stored at 4°C overnight. Cells were stained in PBS containing 5 μg/mL RNase and 20 μg/mL PI in the dark at room temperature for 30 min and analyzed using a flow cytometry (Becton Dickinson FACS Canto™, Franklin Lakes, NJ). At least 10,000 events were counted for each sample.

Cell adhesion assay

Matrigel was diluted in sterile conditions and applied to 96-well plates overnight at 4°C. Non-specific binding sites were blocked with 1% bovine serum albumin for 1 h at 37°C. Cells were harvested after serum starved for 45 min and then plated in triplicate onto wells in low-serum medium (0.5% FBS) with series concentrations of PD. Following 1 h-incubation, the cellular morphology was photographed using a CCD camera (AxioCam HC, Carl Zeiss, Thornwood, NY) attached to a microscope. Non-adherent cells were washed away and adherent cells were fixed with 4% paraformaldehyde and stained with crystal violet. After extensive washing with PBS, the dye was extracted with 10% acetic acid and the absorbance at 595 nm was recorded using the SpectraMax M5 Microplate Reader (Molecular Devices).

Cell migration assay

A cell suspension containing 0.5% FBS DMEM medium and different concentrations of PD was deposited into the upper chamber of each well. The lower chambers were loaded with 10% FBS DMEM medium containing 100 nM TPA as well as different concentrations of PD. After 24-h incubation, the non-invasive cells were removed via gentle scraping. The invasive cells attached to the lower surface of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with crystal violet for 10 min. Three randomly selected views of the upper chamber were photographed using a CCD camera (AxioCam HC, Carl Zeiss, Thornwood, NY) attached to a microscope.

Cell invasion assay

Cell invasion assay was performed as described in migration with a slight modification in that both sides of the insert were pre-coated with Matrigel.

Western blot analysis

Cells were lysed in the lysis buffer, and the proteins of the lysates were quantified using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). About 50 μg of total proteins was subjected to SDS-PAGE, transferred onto PVDF membranes, and then blocked with 5% nonfat milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20) at room temperature for 2 h with rocking. The membranes were probed with specific primary antibodies against poly ADP-ribose polymerase (PARP), Bax, and survivin (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. After washing with TBST three times for 15 min each, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) in TBST at room temperature for 1 h, and specific protein bands were visualized using an ECL advanced western blot detection kit. Equal protein loading was verified through re-hybridization of the membranes and re-probing with anti-β-actin antibody.

Results

PD inhibited cell proliferation and colony formation in hepatocellular carcinoma cells

Herein, the hepatocellular carcinoma cell lines HepG2 and Hep3B were used to detect the anti-proliferative potential of PD. Cells were treated with various concentrations of PD for 48 h and the inhibition rate was tested by MTT assay. As shown in Figure 1A, B, PD dose-dependently inhibited the cell proliferation in both HepG2 and Hep3B hepatocellular carcinoma cell lines. To further investigate the anti-cancer effect of PD, the colony formation assay was used. The number of colony foci was obviously decreased after treatment with 10 μM
Figure 1. PD Inhibited Proliferation and Colony Formation in Hepatocellular Carcinoma Cells. (A, B) HepG2 (A) and Hep3B (B) cells were treated with indicated concentrations of PD for 48 h and the cell proliferative inhibition was tested by MTT assay. (C) HepG2 cells were treated with various concentrations of PD for 24 h. After treatment, cells were suspended and re-seeded into 6-well plates at a density of 2×10^4 cells per well and then fixed and stained with 4% paraformaldehyde and crystal violet after 10 days. The representative images of colony formation assay were obtained.

Figure 2. PD Induced Apoptosis in HepG2 Cells. (A-D) Cells were treated with indicated concentrations of PD for 24 h (A, B) or 48 h (C, D), and cells in the sub-G1 phase were counted using flow cytometry. Upon treatment with PD for 24 h, PD induced sub-G1 peak (Figure 2A, B) in a dose-dependent manner and the apoptotic cells appeared more obvious accompanied with the increased incubation time (Figure 2C, D). We further detected the levels of several proteins which are related with apoptosis. After PD treatment, cleavage fragment (CF) of PARP and pro-apoptotic protein Bax were obviously up-regulated while the anti-apoptotic protein survivin was remarkably down-regulated (Figure 2E), indicating PD indeed induces apoptosis in HepG2 cells.

PD induced apoptosis in HepG2 cells
As induction of apoptosis is one of the major contents that mediate cell proliferative inhibition, we tested cells in sub-G1 phase, which is a characteristic of apoptosis, after PD treatment using flow cytometry. Upon treatment with PD for 24 h, PD induced sub-G1 peak (Figure 2A, B) in a dose-dependent manner and the apoptotic cells appeared more obvious accompanied with the increased incubation time (Figure 2C, D). We further detected the levels of several proteins which are related with apoptosis. After PD treatment, a cleavage fragment (CF) of PARP and pro-apoptotic protein Bax were obviously up-regulated while the anti-apoptotic protein survivin was remarkably down-regulated (Figure 2E), indicating PD indeed induces apoptosis in HepG2 cells.

PD inhibited adhesion in HepG2 cells
In order to systematically evaluate the anti-hepatocellular carcinoma activities of PD, the potential of cancer cells adhering to Matrigel was tested. Cells were harvested and plated into wells with series concentrations of PD for 1 h and the cellular morphology was photographed. As shown in Figure 3 A, the cells without PD treatment showed spreading state while those treated with PD presented much more round phenotype in a concentration-dependent manner. Data obtained from the microplate reader showed the similar results as phenotype observation (Figure 3B), indicating PD effectively suppresses HepG2 cells adhering to Matrigel.
PD inhibited migration and invasion induced by TPA in HepG2 cells

We further investigated the anti-migration and anti-invasion abilities of PD in HepG2 cells. TPA is a well-known tumor promoter, and it may act as a potential inducer of tumor migration and invasion in cancer cells (Shimao et al., 1999). The results of the transwell assay (without Matrigel pre-coated) showed that after 24 h treatment, the amounts of cells translocated into the lower surface of the membrane were significantly increased in the group with 100 nM TPA treatment (Figure 4A). PD concentration-dependently inhibited TPA-induced migration in HepG2 cells (Figure 4A). Similarly, the amounts of cells degraded the Matrigel and invaded into the lower surface of the membrane were obviously increased after induced by TPA and PD also significantly reduced the invasive cells in the invasive model (Figure 4B). Figure 4C showed the semi-quantitative results obtained from (A) and (B).

Discussion

Herein, we found that PD obviously inhibited hepatocellular carcinoma cells proliferation and induced apoptosis in a concentration-dependent manner (Figure 1 and 2). PARP cleaved by caspases is a hallmark of apoptosis (Boulares et al., 1999) and Bax is a pro-apoptotic member of the Bcl-2 family which regulate apoptosis (Brady and Gil-Gomez, 1998). Survivin has multiple functions including cytoprotection and inhibition of cell death among others which favor cancer survival (Yamamoto et al., 2008). Together with the data obtained from flow cytometry with PI staining and western blot assay, PD indeed induced apoptosis in hepatocellular carcinoma cells and the up-regulation of Bax and down-regulation of survivin, at least in part, may contribute to the initial of apoptosis.

To reduce the metastasis could be a promising stratagem for treatment of hepatocellular carcinoma, as the poor prognosis of hepatocellular carcinoma is largely due to a high rate of metastasis (Genda et al., 1999; Ye et al., 2003). Metastasis of cancer cells includes several steps while change of cell-cell and cell–extracellular matrix (ECM) adhesion capacity is the initial step in this process. Matrigel mainly contains the structural proteins such as laminin and enactin (Hughes et al., 2010) which has been widely used to mimic the ECM in cancer. Herein, we found that PD significantly inhibited HepG2 cell adhesion to Matrigel in a concentration-dependent manner only after one hour treatment (Figure 3), indicating the potential anti-adhesion activity of PD. We further detected the anti-migration and anti-invasion potentials of PD employing transwell assays and the data showed that PD remarkably reduced TPA-induced HepG2 cell migration and invasion (Figure 4). It is noteworthy that combinational treatment with 5 µM PD and 100 nM TPA reduced the cell proliferation about 30% in HepG2 cells (data not shown), indicating the proliferative inhibition could not be excluded when the concentration of PD is high. Because cancer cells need to degrade and traverse the Matrigel-coated membrane to successfully penetrate the transwell chamber, PD might also affect the basement membrane hydrolysis which deserves further study.

In conclusion, PD inhibited HepG2 cell proliferation, and induced apoptosis by regulating the protein expression of Bax and survivin. Moreover, PD effectively inhibited HepG2 cell adhesion, migration and invasion in hepatocellular carcinoma cells. Our results support PD might be a novel candidate chemotherapeutic agent for treatment of hepatocellular carcinoma.

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