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Platycodin D triggers autophagy through activation of extracellular signal-regulated kinase in hepatocellular carcinoma HepG2 cells

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ABSTRACT

Platycodin D (PD), isolated from the Chinese medicinal herb named Platycodonis Radix, is a triterpenoid saponin with well-known anti-tumor effects. In this study, we provided reliable evidence that PD triggered autophagy in a number of cell lines *in vitro*. PD-triggered autophagy was identified by observation of cytoplasmic vacuole, up-regulation of microtubule-associated protein 1 light chain 3 II (LC3-II), and accumulation of autophagosomes. The Akt/mammalian target of rapamycin (mTOR) pathway may be not involved in PD-triggered autophagy, as evidenced by the increased phosphorylation of Akt (Thr308), mTOR (Ser2448), ribosomal protein S6 kinase (Ser371), and ULK1 (Ser757). However, the extracellular signal-regulated kinase (ERK) was activated after PD treatment. The decreased ERK phosphorylation caused by pretreatment with U0126, an inhibitor of MEK, suppressed the expression of LC3-II compared with PD treatment alone, suggesting that ERK pathway may have a critical function in PD-triggered autophagy. In addition, the PD-induced proliferative inhibition and apoptosis were enhanced when pretreatment with autophagy inhibitor chloroquine (CQ) or bafilomycin A1 (BAF), indicating that PD may trigger a protective autophagy in HepG2 cells. To the best of our knowledge, this paper is the first to report that PD triggers autophagy in a series of cell lines and ERK activation is important for PD-triggered autophagy in hepatocellular carcinoma HepG2 cells. The combined treatment with PD and CQ or BAF may be a promising regimen for hepatocellular carcinoma treatment.

1. Introduction

Autophagy is an evolutionary highly conserved process that mediates the degradation of misfolded proteins and damaged organelles (Lockshin and Zakeri, 2004). During this process, cytoplasmic materials isolated from the rest of the cell are targeted for double-membrane cytosolic vesicles to form autophagosomes, which are then fused with lysosomes and degraded (Periyasamy-Thandavan et al., 2009; Xu, 2012; Yang and Klionsky, 2010). In the context of cancer, the function of autophagy is complicated and controversial. Autophagy is frequently activated during nutrient deprivation, hypoxia, and a wide range of anti-cancer therapy, which may be regarded as a pro-survival mechanism (Shi et al., 2013). On the contrary, persistent and excessive autophagy contributes to tumor cell death and functions as a tumor-suppression mechanism (Pardo et al., 2010).

Platycodin D (PD), the marker for quality control of Chinese medicinal herb Platycodonis Radix (Pharmacopoeia Commission of the Ministry of Health of the P.R.China, 2010), is a triterpenoid saponin that exerts various pharmacological activities (Choi et al., 2004; Chun and Kim, 2013; Hwang et al., 2013; Kim et al., 2012; Lee et al., 2010; Wu et al., 2012; Xie et al., 2009). PD has been increasingly recognized as a promising anti-cancer agent because of its potent anti-proliferative activities against a broad spectrum of cancer cell lines (Kim et al., 2008a, 2008b; Lee et al., 2008). Our previous study demonstrated that PD inhibits HepG2 cell proliferation and induces apoptosis, as well as effectively suppresses cell adhesion, migration, and invasion (Li et al., 2014). Moreover, doxorubicin combined with PD exerts stronger anti-proliferation

Abbreviations: PD, Platycodin D; CQ, chloroquine; DMSO, dimethylsulfoxide; MTT, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide; MDC, monodansylcadaverin; ERK, extracellular signal-regulated kinase; BAF, bafilomycin A1; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; p70S6K, ribosomal protein S6 kinase; PARP, poly-ADP-ribose polymerase; PBS, phosphate buffer saline; PVDF, polyvinylidene Fluoride; PMSF, phenylmethanesulfonyl fluoride; FBS, fetal bovine serum; ANOVA, one-way analysis of variance.

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effect on human breast cancer MCF-7 and MDA-MB-231 cells than doxorubicin or PD alone (Tang et al., 2014). However, understanding on the mechanisms of PD remains incomplete.

In our preliminary experiments, we found that PD induced cytoplasmic vacuoles in HepG2 cells and speculated that it may probably induce autophagy in cancer cells. We further demonstrated that PD triggers autophagy in various cell lines. Activation of extracellular signal-regulated kinase (ERK) may be the key mechanism for its triggered autophagy in HepG2 cells. Inhibition of autophagy by chloroquine (CQ) and bafilomycin A1 (BAF) enhance PD-induced cytotoxicity and apoptosis.

2. Material and methods

2.1. Reagents

PD was purchased from Best-Reagent (Chengdu, Sichuan, China). PD was diluted using dimethylsulfoxide (DMSO); the stock concentration was 20 mM. 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT), DMSO, CQ, BAF, phenylmethanesulfonyl fluoride (PMSF), Cocktails and monodansylcadaverin (MDC) were obtained from Sigma (Saint Louis, MO, USA). RIPA and U0126 were purchased from Beyotime (Nantong, Jiangsu, China). The primary antibodies against microtubule-associated protein 1 light chain 3 (LC3), ERK, p-ERK (Thr202/Tyr204), p-mTOR (Ser2448), mTOR, p-ULK1 (Ser757), ULK, p-p70S6K (Ser371), p70S6K, Akt, p-Akt (Thr308), and GAPDH, and secondary antibody anti-rabbit IgG with HRP-linked antibody were all purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

Human hepatocellular carcinoma HepG2 and Hep3B cells, human breast cancer MCF-7 and MDA-MB-231 cells, and human lung cancer A549 cells were obtained from ATCC (Rockville, MD, USA). Lung cancer 95D cell line was purchased from China Center for Type Culture Collection (CCTCC, Shanghai, China). HepG2, Hep3B, and MCF-7 were cultured in DMEM (Gibico, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA), whereas MDA-MB-231, A549, and 95D were cultured in RPMI 1640 medium (Gibico, Grand Island, NY, USA) that contains 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified incubator in an atmosphere containing 5% CO_2. Exponentially growing cells were used in the experiments.

2.3. MTT assay

Cells were plated in 96-well plates at a density of 8000 cells per well for 24 h. The cells were pretreated with 10 μM CQ or 50 nM BAF for 1 h, followed by different concentrations of PD diluted in a medium containing 0.5% FBS for another 24 h. The supernatant was then removed and loaded with 100 μl per well of MTT solution (1 mg/ml). The cells were maintained in a humidified environment for 4 h. Cell proliferation was determined by addition of 100 μl DMSO and shaking for 10 min in the dark to solubilize formazan. The absorbance at 570 nm was recorded using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was shown as percentage of control viability.

2.4. Observation of morphologic changes

Cells were transferred to 96-well plates at a density of 8000 cells per well. After treatment with PD diluted in a medium containing 0.5% FBS for 24 h, the cellular morphology was observed with an Axiovert 200 inverted microscope (Zeiss, Oberkochen, Germany).

2.5. Western blot analysis

Cells were lysed in the lysis buffer, which contains RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, sodium orthovanadate, and sodium fluoride), PMSF, and the protease inhibitor, Cocktails. After incubation for 20 min on ice, the lysis buffer containing cellular contents were centrifuged at 4 °C, 12,500 rpm for another 20 min. And then the cell supernatants containing the aimed protein were obtained and quantified using a BCA™ protein assay kit (Pierce, Rockford, IL, USA). Approximately 50 μg of total proteins were subjected to SDS-PAGE, transferred onto polyvinylidene fluoride 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 continuous rocking. The membranes were probed with specific primary antibodies overnight at 4 °C. The membranes were then washed with TBST three times for 15 min each and incubated with anti-rabbit IgG with HRP-linked secondary antibody in TBST at room temperature for 1 h. Specific protein bands were visualized using an ECL advanced Western blot detection kit (GE Healthcare, Uppsala, Sweden).

2.6. MDC staining

Cells were seeded into a 96-well black culture plate and maintained in the incubator. After adhesion, the cells were treated with different concentrations of PD diluted in the medium that contains 0.5% FBS. At indicated periods, the cells were labeled with MDC (0.05 mM) for 10 min at 37 °C and washed three times with phosphate buffer saline (PBS). The cells were then imaged and analyzed by an In Cell Analyzer 2000 System (GE Healthcare, Uppsala, Sweden).

2.7. Transmission electron microscopy analysis

PD-treated or untreated cells were harvested and washed with PBS, and then fixed in ice-cold 2.5% glutaraldehyde for 1 h. The cells were then washed with PBS three times for 15 min, post-fixed in 1% OsO_4 for 1 h, and stained with 2% uranyl acetate for 30 min at room temperature. Afterward, the cells were dehydrated through a graded series of ethanol (50%, 70% and 90%) for 15 min each, ethanol 100% for 20 min and 100% acetone for 20 min, and then embedded in Epon812. Ultrathin sections (120 nm) were obtained before staining with 2% uranyl acetate for 20 min and lead citrate for 5 min, and examined using a TECNAI 10 transmission electron microscope (Phillips, Eindhoven, The Netherlands) at high voltage of 80 kV.

2.8. Statistical analysis

Data were expressed as mean ± S.D. Statistical significance was analyzed using one-way analysis of variance test by Graph Pad Prism (Demo, Version 5) with $^{*}P$-value < 0.05, $^{**}P$-value < 0.01, $^{***}P$-value < 0.001.

3. Results

3.1. PD induced formation of cytoplasmic vacuoles in various cell lines

In our previous study, we reported that PD inhibits the proliferation of hepatocellular carcinoma HepG2 and Hep3B cells in a

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