

RESEARCH COMMUNICATION

Apoptosis of Human Ovarian Cancer Cells Induced by *Paris Chinensis* Dioscin via a Ca²⁺-Mediated Mitochondrion Pathway

Lin-Lin Gao^{1*}, Fu-Rong Li², Peng Jiao³, Shu-Tong Yao¹, Hui Sang¹, Yan-Hong Si¹

Abstract

Background: Study of the mechanisms of apoptosis in tumor cells is an important field of tumor therapy and cancer molecular biology. Apoptosis triggered by activation of the mitochondrial-dependent caspase pathway represents the main programmed cell death mechanism. The mitochondrial-dependent apoptosis pathway is activated by various intracellular stresses that induce permeabilization of the mitochondrial membrane, leading to cytochrome C release. This study was to investigate the anti-tumor effects of Dioscin from traditional Chinese anti-snake venom medicine *Paris chinensis* (PCD) and correlated mechanisms regarding apoptosis in human ovarian cancer SKOV3 cells. **Methods:** Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cell apoptosis was evaluated by flow cytometry and Laser Scanning Confocal Microscope (LSCM) using Annexin-V/PI staining. Intracellular calcium ions were detected using fluorescence microscopy. The expression of apoptosis-related proteins cytochrome C and caspase-3 was measured by immunohistochemical staining. **Results:** PCD had an anti-proliferation effect on human ovarian cancer SKOV3 cells in a dose- and time-dependent manner. After treatment with PCD, the apoptotic rate significantly increased, and accompanied with the increased levels of caspase-3 and cytochrome C protein in SKOV3 cells. Morphological changes typical of apoptosis were also observed with LSCM by Annexin V/PI staining. Moreover, intracellular calcium accumulation occurred in PCD-treated cells. **Conclusions:** The molecular determinants of inhibition of cell proliferation as well as apoptosis of PCD may be associated with the activation of Ca²⁺-related mitochondrion pathway in SKOV3 cells.

Keywords: *Paris chinensis* dioscin - ovarian cancer cells - apoptosis, Ca²⁺ - cytochrome C

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Introduction

Epithelial ovarian cancer (EOC) is one of the most frequent gynecologic cancers and the leading cause of death among gynecological malignancies (Jemal et al., 2010). Recent evidence suggests that apoptosis of cells is closely related to the occurrence, progress and metastasis of tumors (Hung et al., 2008). Study of the mechanisms of apoptosis in tumor cells is an important field of tumor therapy and molecular cancer biology (Ye et al., 2007; Yang, et al 2009). Apoptosis is regarded as the preferred mechanism for managing cancer cells (Fesik, 2005).

Paris chinensis (Liliaceae) is distributed in many regions of the world, such as India, China, Vietnam, and Germany. As a traditional Chinese medicine, it grows wildly throughout South China and has been used mainly as a folk remedy for treatment of abscesses, throat swelling and pain, thanatophidia bites, contused wounds and convulsions for centuries. It is also the major component medicine of the famous Chinese patent medicine yunnan baiyao powder and snake-bite therapeutics. It also has been used to treat liver cancer in China for many

decades (Lee, et al., 2005; Shoemaker, et al., 2005). The active components of *Paris chinensis* are the saponin steroids polyphyllin D, dioscin, and balanitin 7. Among its three chemical constituents, polyphyllin D has been previously reported (Deng, et al., 1999; Cheung, et al., 2005) to circumvent drug resistance and elicit apoptosis in HepG2 and R-HepG2 cells via mitochondrial damage. However, as there has been no documentation of the use of the other important steroid saponin dioscin in the treatment of cancer, its mechanisms in human ovarian cancer cells remain unknown. We investigated the effects of PCD on cell growth and apoptosis in ovarian cancer cells, particularly focusing on releasing mitochondrial cytochrome c into the cytosol, increasing intracellular calcium ion and activating caspase-3, and the signaling pathways involved in PCD-induced apoptosis.

Materials and Methods

Chemicals and reagents

Paris chinensis dioscin (PCD) was purchased from Yuancheng Scientific and Technical Corporation (Wuhan,

¹Department of Pathology and Pathophysiology, ²School of Pharmaceutical Science, ³Institute of Basic Medical Sciences, Taian Medical University, Taian, China *For correspondence: ytg98@yahoo.cn

China) at 99% purity. RPMI-1640 medium, HEPES (4-hydroxyethyl piperazine ethanesulfonic acid), fetal calf serum and trypsin were purchased from GIBCO (Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, and trypsin were purchased from Amresco Chemical Co. Ltd. (USA). Propidium iodide (PI) was purchased from Sigma (USA). The fluorescent probe Fluo-3/AM was product of Molecular Probes Incorporated (USA). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (USA). The primary antibodies for caspase-3, cytochrome C and β -actin and the secondary antibody were acquired from Santa Cruz Biotechnology (USA), and all chemicals were of analytical grade and were obtained from Tianjin Chemical Reagents Co. Ltd. (Tianjin, China).

Cell culture

SKOV3 cells were obtained from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China), cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂; the medium was changed every other day. When the cultures were 80 to 90% confluent, the SKOV3 cells were washed with phosphate-buffered saline (PBS, pH 7.4), detached with 0.25% trypsin, centrifuged and re-plated onto 96- or 24-well plates at an appropriate density according to each experimental scale.

Cell viability and cytotoxicity

The cultured cells at the exponential growth phase were harvested from the culture flasks by trypsin and then resuspended in fresh medium. The cell suspensions were dispensed into a 96-well microplate at 100 μ l/well and incubated in an incubator with 5% CO₂ at 37°C. After 24 hours, 200 μ l of various concentrations (0 to 500 μ g/ml) of PCD were added and incubated for 12, 24, 36, 48, 60 and 72 hours to evaluate their anti-proliferation effects on SKOV3. The cell proliferation in the microplate was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) assay after incubation (Chang et al., 2008). Twenty microliters of PBS solution containing 5 mg/ml MTT was added to each well. After incubation for 4 hours, the cells from each well were solubilized with 100 μ l DMSO for optical density determination at 570 nm. Cell proliferation activity was expressed as the percentage of MTT counts of treated cells relative to those of the control (% of control). The IC50 was taken as the concentration that caused 50% inhibition of cell viabilities and was calculated by the Logit method.

Observation of morphological changes

The SKOV3 cells were seeded in six-well plates (2.0 \times 10⁵ cells/well) and incubated in RPMI1640 at 37°C in an atmosphere of 5% CO₂ for 24 h. The cells were treated with several concentrations of PCD. After incubation for 24 h, cellular morphology was observed using a phase contrast microscope (Nikon, Japan). The photographs

were taken at a magnification of 40 \times .

Flow cytometry and LSCM analysis of cell apoptosis

SKOV3 cells were cultivated in RPMI1640 with 10% fetal bovine serum. Before the cell density was modulated to 1 \times 10⁵ cells, cell synchronization was conducted to force the cells to the G0 phase via a serum-free culture for 12 h, and the cells were washed twice with PBS before being suspended in a binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 140 mM NaOH, and 2.5 mM CaCl₂). Five microliters of fluorescein isothiocyanate (FITC)-labeled Annexin V was mixed with 100 μ l of cell suspensions containing 1 \times 10⁵ cells, and the cells were incubated at room temperature for 5 min. Thereafter, 50 μ l of propidium iodide (PI) solution (10 μ g/ml) was added to the cells, followed by an additional 5-min incubation period. The scatter parameters of the cells (20,000 cells per experiment) were analyzed using a FAC Scan flow cytometer and Cell Quest analysis software (Becton–Dickinson, CA, USA). Four cell populations were identified according to the following interpretations: viable population in the lower-left quadrant (low-PI and FITC signals), early apoptotic population in the lower-right quadrant (low-PI and high-FITC signals), necrotic population in the upper-left quadrant (high-PI and low-FITC signals), and late apoptotic or necrotic population in the upper-right quadrant (high-PI and high-FITC signals). At this point, cells treated in the manner described above were examined on a glass slide using a laser-scanning confocal microscope (LSCM, Bio-Rad Radiance 2100, U.S.A) with 488-nm excitation and 525-nm emission wavelengths. Bright green fluorescence was manifested in cell membranes of cells undergoing prophase apoptosis because of Annexin V-FITC staining, while nuclear cardinal red fluorescence was associated with advanced stage apoptosis because of PI staining.

Measurement of intracellular calcium

The intracellular calcium ion ([Ca²⁺]_i) was measured as previously described (Li et al., 1996). After confluence, SKOV3 cells on a coverslip were loaded by the [Ca²⁺]_i indicator Fluo-3/AM in HEPES solution at 37°C in the dark for 30 min. HEPES solution contains (concentration in mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH. The final concentration of Fluo-3/AM was 5 μ M. After loading with Fluo-3/AM, a fluorescence image of [Ca²⁺]_i was taken using a LSCM at 600 \times , and qualitative changes of [Ca²⁺]_i were inferred from the fluorescence intensity using Simple PCI Imaging Systems (Simple PCI, Compix Inc., USA).

Western blot analysis

The 20 μ g of protein in each 20- μ l sample was electrophoresed through 10% SDS-PAGE gels as previously described (Rasmussen et al., 2008). Separated proteins were incubated with primary antibodies overnight at 4°C, transferred to nitrocellulose membranes, and blocked with a 5% skim milk solution. They were incubated with secondary antibodies for 1 h at 37°C. Each antigen-antibody complex was visualized by enhanced chemiluminescence (ECL) western blotting detection

kits (Amersham Pharmacia Biotech, Piscataway, NJ), and band densities were determined using Chemi Doc Software (BioRad); β -Actin was used as a loading for normalization.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are given as the mean \pm SE. Statistical analysis was performed using the statistical software package SPSS 13.0 (SPSS). A p-value of 0.05 (two-sided) was considered statistically significant.

Results

Cytotoxic activity of PCD on SKOV3 cells

As shown in Figure 1A, untreated SKOV3 cells grew well with clear skeletons, whereas cells treated with PCD exhibited cytoplasmic shrinkage and either detached from each other, floated in the medium, or became distorted and blurry under a phase contrast microscope. The number of sloughed cells increased with increasing drug concentrations. The MTT assay showed that PCD significantly inhibited the viability of SKOV3 cells (Figure 1B). The cells were incubated in the absence or presence of various concentrations of PCD for specified time periods, and the IC50 values were 14.6 ± 0.11 , 7.64 ± 0.40 , and 5.81 ± 0.33 mg/ml for 24, 48, and 72 h, respectively. The MTT assay showed that PCD decreased the viability

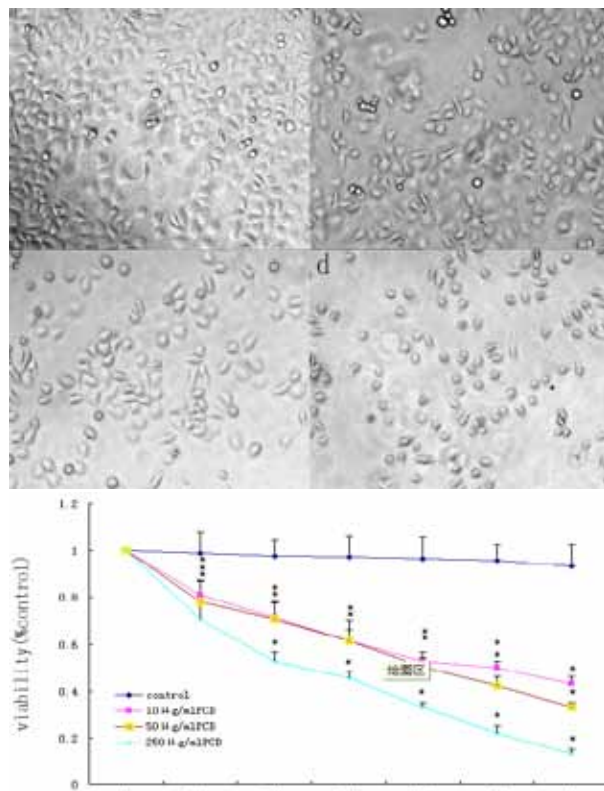


Figure 1. PCD inhibited the viability of SKOV3 cells. (A) Morphological changes of SKOV3 cells exposed to PCD for 24 h imaged with a phase contrast microscope at 40 \times . (B) Effect of PCD on SKOV3 cells viability. SKOV3 cells were treated with PCD at the indicated concentrations for 0-72 h. Cell viability was then determined by MTT assay and expressed as the mean \pm SD, n = 3. The OD value at 570 nm is proportional to the number of cells with PCD. *P<0.01 vs.control.

of SKOV3 cells in a concentration-dependent and time-dependent manner (P<0.05 and P<0.01, respectively).

Effect of PCD on apoptosis in SKOV3 cells

To identify whether PCD induces apoptosis, the treated cells were also stained with Annexin V-FITC/PI, and the population of apoptotic cells was analyzed by flow cytometry. As seen in Figure 2A, the drug treatment significantly increased the proportion of apoptotic cells. In the vehicle treated samples, $10.2 \pm 1.51\%$ of cells stained

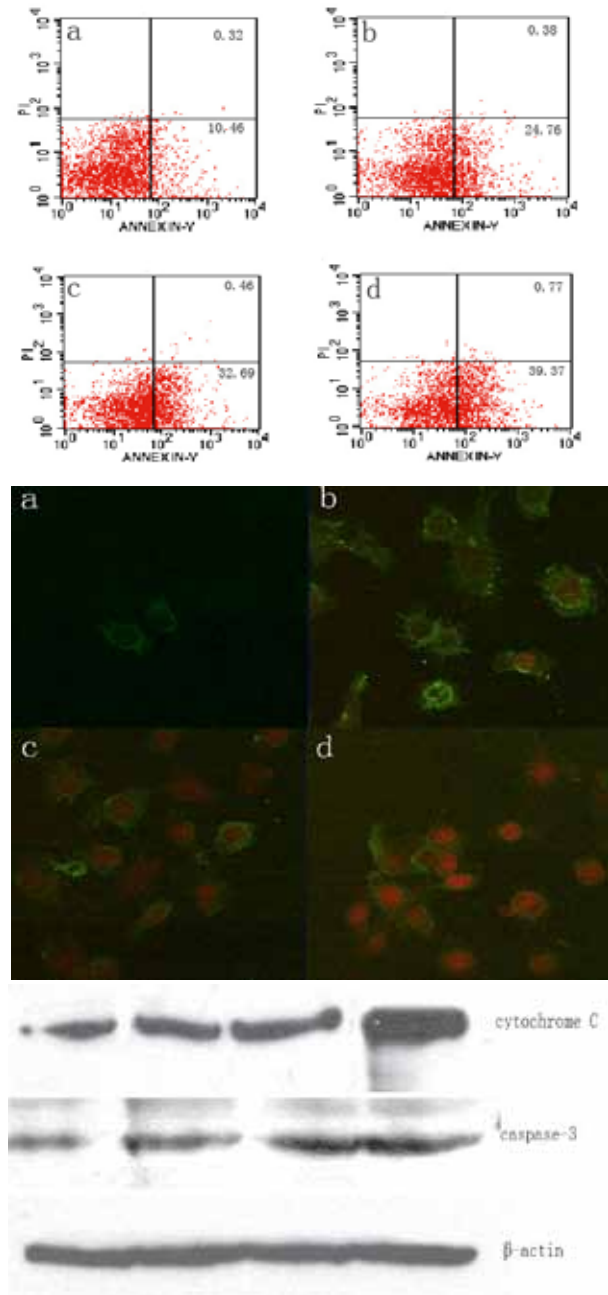


Figure 2. PCD induced apoptosis in SKOV3 cells after 24h following the treatment. (A) Apoptotic cells determined with Annexin V/PI staining by flow cytometry assay. a: vehicle treated; b: 10 μ g/ml, c: 50 μ g/ml, d: 250 μ g/ml. (B) Morphological changes of SKOV3 cells as determined with a LSCM at 600 \times followed by flow cytometry with labeled with Annexin V/PI. a: vehicle treated; b: 10 μ g/ml, c: 50 μ g/ml, d: 250 μ g/ml. (C) Western blot analysis of the expressions of the level of caspase-3, cytochrome C and β -actin (internal control) protein in vehicle treated and PCD-treated SKOV3 cells

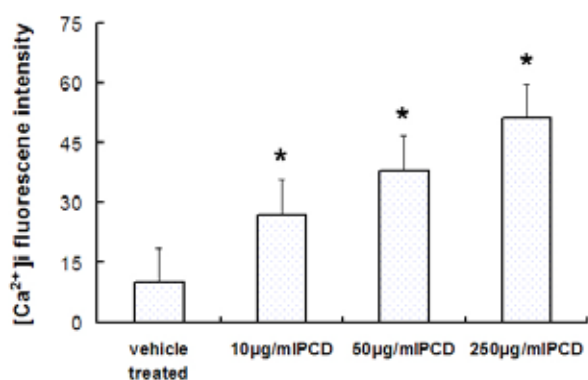
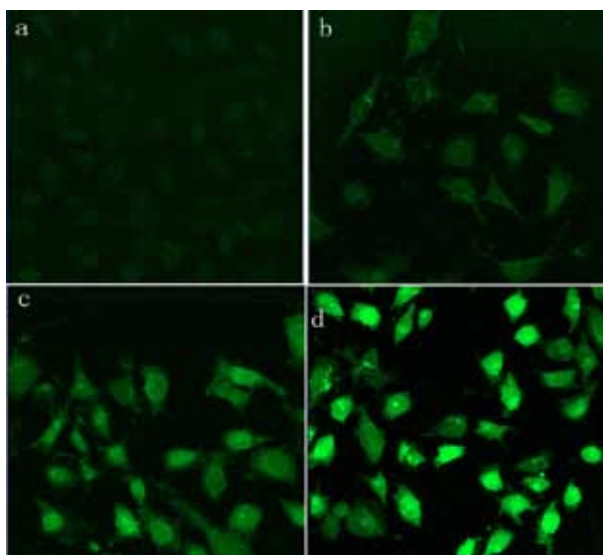


Figure 3. Effects of PCD Treatment for 24 h on intracellular [Ca²⁺]_i expression in human ovarian cancer SKOV3 cells. (A) Fluorescence image of [Ca²⁺]_i by LSCM at 600×, a: vehicle treated; b: 10 µg/ml; c: 50 µg/ml; d: 250 µg/ml. (B) Qualitative changes of [Ca²⁺]_i were inferred from the fluorescence intensity after PCD treatment for 24 h, using Simple PCI Imaging Systems. Data are presented as mean ± SD (error bar). * P<0.01 vs. vehicle treated

positive for Annexin V-FITC, while PCD treatment showed increases of 24.6±2.78%, 32.6±2.17%, and 39.34±3.22% in early stage apoptosis (P<0.01), but no significant difference at late stage apoptosis (P>0.05). These results demonstrate the ability of PCD to induce apoptosis mainly early stage apoptosis in SKOV3 cells. The morphologic changes of cells treated in the manner described above were also observed with LSCM by Annexin V/PI staining. As shown in Figure 2B, typical morphological changes, such as the formation of apoptotic bodies, appeared after the cells were treated for 24 h with 250 µg/ml PCD, whereas the vehicle treated cells did not show the evident apoptotic morphological changes.

To determine whether apoptosis induced by PCD was due to a mitochondrial-dependent caspase pathway, we further tested whether cytochrome C could be released from the mitochondria into the cytoplasm. We next investigated the levels of cytochrome C and caspase-3, which was the core protein in the caspase cascade in the soluble cytosolic fractions of SKOV3 cells after PCD treatment for 24 h. Figure 2C shows that PCD increased the level of cytochrome C to be released into the cytosol, and the expression of caspase-3 was increased after PCD

treatment for 24 h compared to the vehicle treated, which indicated that PCD increased the caspase-3 level in SKOV3 cells.

Effects of PCD on [Ca²⁺]_i in SKOV3 cells

To explore whether PCD-induced apoptosis involved [Ca²⁺]_i, we used the [Ca²⁺]_i indicator Fluo-3/AM to detect [Ca²⁺]_i changes after PCD treatment with various densities. As shown in Figure 3, [Ca²⁺]_i fluorescence intensity in the group treated with 250 µg/ml PCD was brighter than were the vehicle treated and lower concentration groups (P<0.01), and PCD treatment with 10, 50 and 250 µg/ml induced an increase by 45±4.13%, 53±5.04%, and 75±7.17% vs. the vehicle treated (23.4±2.36%) (P < 0.01) in Fluo-3/AM fluorescence intensity after 24 h of treatment, respectively. These results suggest that the PCD can induce a dose-dependent [Ca²⁺]_i influx and might induce apoptosis or necrosis that follows via calcium ion overload.

Discussion

Natural products with anticancer properties could be valuable substances in cancer treatment. In this study, we assessed the inhibitory effects and molecular mechanisms of PCD using human ovarian cancer SKOV3 cells. MTT showed that PCD inhibited the growth of SKOV3 cells in both time-dependent and concentration-dependent manners. To determine whether the cytotoxic activity of PCD was due to apoptosis, SKOV3 cells were treated for 0-72 h with indicated concentrations of PCD. Not only were morphological changes such as cytoplasmic shrinkage, detachment from each other, floating in the medium, distortion and some blurring under a fluorescence microscope observed, but marked chromatin condensation and apoptotic body formation in PCD-treated cells were also observed in cells stained with Annexin V-FITC/PI using an LSCM.

Membrane blistering and apoptotic bodies can be seen under the phase contrast microscope, consistent with previous studies (Sgonc et al., 1994). A major disadvantage in morphological detection of apoptosis is that it is only qualitative. The quantitative analysis of apoptosis mainly relies on flow cytometry with fluorescent markers. Annexin V-FITC/PI double staining, which can detect cell apoptosis, is one of the most sensitive and ideal indicators (Dai et al., 2009).

As shown in Figure 2A and 2B, Annexin V-FITC/PI staining assay revealed that early and total apoptotic rates of PCD treatment (10, 50 and 250 µg/ml) were significantly higher than those of vehicle treated. This may be because of the fact that early apoptosis was mainly revealed by Annexin V-FITC/PI double staining assay. Moreover, Annexin V-FITC/PI staining assay and typical morphological changes with LSCM afterwards, such as the formation of apoptotic bodies, appeared after the cells treated by PCD, confirming that PCD induced apoptosis in SKOV3 cells.

The apoptotic process is preceded by the collapse of the mitochondrial potential, the opening of a multi-protein structure named the permeability transition pore

(PTP), which could be triggered by multiple stimuli such as changes in Ca²⁺, oxygen radicals, PH, swelling of the matrix and rupture of the outer membrane with ensuing changes in the permeability of the outer mitochondrial membrane, and release of apoptogenic factors including cytochrome C from mitochondria (Petronilli, et al., 1994; Bernardi et al., 1998).

The accumulated data suggest that the mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to those stimuli. In the mitochondria-initiated death pathway, mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome C or apoptosis-inducing factor from the mitochondrial inter membrane space into the cytosol. Released cytochrome C can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3.

Over the last few years, several studies have shown that increases of cytosolic Ca²⁺ concentration ([Ca²⁺]_c) occur, both at early and late stages of the apoptotic pathway (Martikainen et al., 1991). More specifically, it has been suggested that both Ca²⁺ release from the endoplasmic reticulum (ER) and capacitive Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ channels are apoptogenic (Wertz et al., 2000). There are also data suggesting that very high intracellular Ca²⁺ levels can promote cell death through necrosis, whereas lower intracellular Ca²⁺ increases induced by milder insults promote cell death through apoptosis (Choi, 1995; Skulachev, 1996; Sara et al., 2005). Corbiere (Corbiere et al., 2004) reported that diosgenin-induced apoptosis in different human cancer cells is caspase-3-dependent and is concomitant with a fall in the mitochondrial membrane potential.

In this study, western blot showed cytochrome C increased in cytoplasm accompanying caspase-3 upregulation after PCD treatment, suggesting that changes in cytochrome C and caspase-3 might contribute to the apoptosis promotion activity of PCD. At meantime, the [Ca²⁺]_i fluorescence intensity of cells loaded with Fluo-3/AM under a fluorescence microscope in the group treated with 250µg/ml PCD was obviously brighter than were the vehicled treated and lower concentration groups, Ca²⁺ overload has even been suggested to be the final common pathway for all types of cell death (Lynch et al., 2000). Ca²⁺ plays a critical role in this process, and intracellular Ca²⁺ overload appears to mediate the lethal effects of receptor overactivation (Pinton et al., 2001). These occurrences of mitochondrial apoptotic events were correlated with the modulation of PCD, not only indicating that the mitochondrial apoptotic pathway played a pivotal role in PCD-induced early stage apoptosis of SKOV3 cells, but also confirming that PCD induced apoptosis is associated with regulation of intracellular Ca²⁺ increase.

In conclusion, the findings indicate that PCD could significantly inhibit the growth and induce apoptosis via the Ca²⁺ related mitochondria-mediated pathway of human ovarian cancer cell line SKOV3 in vitro. The promotion of the specific apoptosis pathway, key molecular targets that specifically initiate apoptosis of ovarian cancer cells and quality standards of PCD should be further explored. Thus, PCD would be worth investigating as a novel therapeutic

agent originating from a natural source, and the induction of apoptosis by PCD in other cancer cell lines is the subject of on-going investigations.

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