RESEARCH ARTICLE

Steroidal Saponins from *Paris polyphylla* Induce Apoptotic Cell Death and Autophagy in A549 Human Lung Cancer Cells

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Abstract

Background: *Paris polyphylla* (Chinese name: Chonglou) had been traditionally used for a long time and shown anti-cancer action. Based on the previous study that paris polyphylla steroidal saponins (PPSS) induced cytotoxic effect in human lung cancer A549 cells, this study was designed to further illustrate the mechanisms underlying. Materials and Methods: The mechanisms involved in PPSS-induced A549 cell death were investigated by phase contrast microscopy and fluorescence microscopy, flow cytometry and western blot analysis, respectively. Results: PPSS decreased the proportion of viable A549 cells, and exposure of A549 cells to PPSS led to both apoptosis and autophagy. Apoptosis was due to activations of caspase-8, caspase-3, as well as cleavage of PARP, and autophagy was confirmed by up-regulation of Beclin 1 and the conversion from LC3 I to LC3 II. Conclusions: PPSS was able to induce lung cancer A549 cell apoptosis and autophagy *in vitro*, the results underlining the possibility that PPSS would be a potential candidate for intervention against lung cancer.

Keywords: Paris polyphylla - steroidal saponins - lung cancer - apoptosis - autophagy

Asian Pac J Cancer Prev, **16 (3)**, 1169-1173

Introduction

Lung cancer is one of the most common types of cancer in the world, both in terms of incidence and mortality, causing millions of deaths annually and resulting in an enormous global health problem. The majority of the cases now occur in the developing countries (55%), a large increase since the estimates in 1980 (Siegel et al., 2014). The primary symptoms of lung cancer include chest pain, shortness of breath, cough and coughing up blood. Lung cancer may metastasis, and when spreading to the brain can cause headache, vomiting, psychosis, visual impairment and unilateral limb paresthesia. The diagnosis of overall survival at five years is only 15%, and more than 75% lung cancer patients at the time of diagnosis is already presenting with advanced stage and therapeutic options are very limited (Patz et al., 2007).

Cancer chemotherapy drugs clinically used are partly derived from natural products which are still the hotspots for discovery novel leads (Newman and Cragg, 2012). Paris polyphylla (Chinese name: Chonglou) is a well-known traditional Chinese medicine with heat-clearing and detoxicating (qing re jie du) functions. It has been traditionally used for the treatment of inflammation and cancer, especially lung cancer for thousands of years (Committee, 2010; Li et al., 2013). Many studies showed that the main active ingredients of paris polyphylla are steroidal saponins (Wang et al., 2007; Yan et al., 2009).

Programmed cell death (PCD) is a critical mechanism for maintaining cell homeostasis in multicellular organisms, mainly including two classical forms: apoptosis and autophagy (Savill and Fadok, 2000). Apoptosis prevents abnormal cell proliferation and removes damaged cells. This is a crucial process for eliminating cancer cells. Induction of apoptosis is a useful approach in the management and prevention of cancer (Pandurangan and Esa, 2013). Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and the activation of specific cysteine proteases known as caspases. The term caspases is derived from cysteine dependent aspartate-specific proteases. Caspases take major and a central role in apoptotic mechanism. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspase-3 may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 (Sankari et al., 2012).

Autophagy is a dynamic process by which autophagosomes fuse with lysosomes for subsequent degradation and recycling of impaired organelles by lysosomal enzymes. Microtubule-associated protein light chain 3 (LC3) protein is localized in autophagosomes and autolysosomes, the amount of LC3 II cleaved product is correlated with the extent of autophagosome formation, providing the marker for the detection of autophagic activity (Kabeya et al., 2000; Pandey and Chandravati,

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2012). Class III PI3K is the homologue of Beclin 1, in this regulatory frame, Beclin 1 undertakes a central role because of its necessary function in the formation of autophagic vacuoles, and results in the occurrence of autophagy (Chen and Klionsky, 2011).

The present study is designed to evaluate the *in vitro* anti-tumor activities of paris polyphylla steroidal saponins (PPSS), and the mechanisms of PPSS-induced cell death in human lung cancer A549 cells.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. (Zhejiang, People's Republic of China); RPMI 1640 was obtained from HyClone (Logan, UT, USA). Propidium iodide (PI), monodansylcadaverine (MDC), acridine orange (AO) were purchased from Sigma (St. Louis, MO, USA). Polyclonal antibodies against caspase-3, caspase-8, poly-ADP-ribose polymerase (PARP), LC3, Beclin 1, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) reagent was from Thermo Scientific (Rockford, IL, USA).

Plant material

Steroidal saponins were prepared from Paris polyphylla Smith var. chinensis (Franch.) Hara. by our lab. The dried root and rhizome of paris polyphylla were extracted with 70% ethanol for three times. Then the solvent was removed under reduced pressure, and the ethanol extract was suspended in H2O, subjected to macroporous resin (D101, Sunresin New Materials Co. Ltd., Xi' an, China) column chromatography and eluted with increasing amounts of ethanol (i.e. 0 % ethanol, 20 % ethanol, 40 % ethanol, 60 % ethanol, 80 % ethanol, and 95 % ethanol). The 60 %-ethanol fraction was collected and the solvent was removed under reduced pressure, finally yield paris polyphylla steroidal saponins (PPSS) after freezing drying. PPSS was dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI 1640 medium before the experiments. The DMSO concentration was kept below 0.05 % in all cell cultures used and did not exert any detectable effect on cell growth or cell death.

Cell culture

Human lung cancer A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5 % CO2 at 37°C. All cells in the exponential phase of growth were used in the experiments.

Observation of morphological changes

A549 cells (2×10^4 cells/well) were inoculated into 24-well culture plates (Corning, NY, USA) with or without PPSS for 24 h. The cellular morphology was observed by using a phase contrast microscope (Olympus, Tokyo, Japan).

Fluorescent Microscopy of Apoptotic (with AO) and Autophagy (with MDC) Staining

A549 cells (2×10^4 cells/well) were inoculated in 24-well culture plates and cultured for 24 h. Then, the cells were treated with or without PPSS for another 24 h. After this, the cells were stained with 20 μg/mL AO in a dark place for 15 min or stained with 0.05 mM MDC (a marker for autophagic vacuoles) at 37°C for 1 h. Fluorescent changes were observed by an Olympus IX71 reverse fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometric analysis of apoptosis and autophagy

A549 cells were dispensed in a 25 mL culture bottle at a density of 4×10^5 per bottle and then incubated for 24 h. Then, the cells were treated with or without PPSS for 12, 24, and 48 h. The cells were harvested and rinsed with PBS. For measuring apoptosis, the collected cells were fixed with 500 μL of PBS and 10 mL of 70% ethanol at 4°C for 18 h; then after washing twice with PBS, they were suspended with 1 mL of PI solution (PI 50 μg/mL and

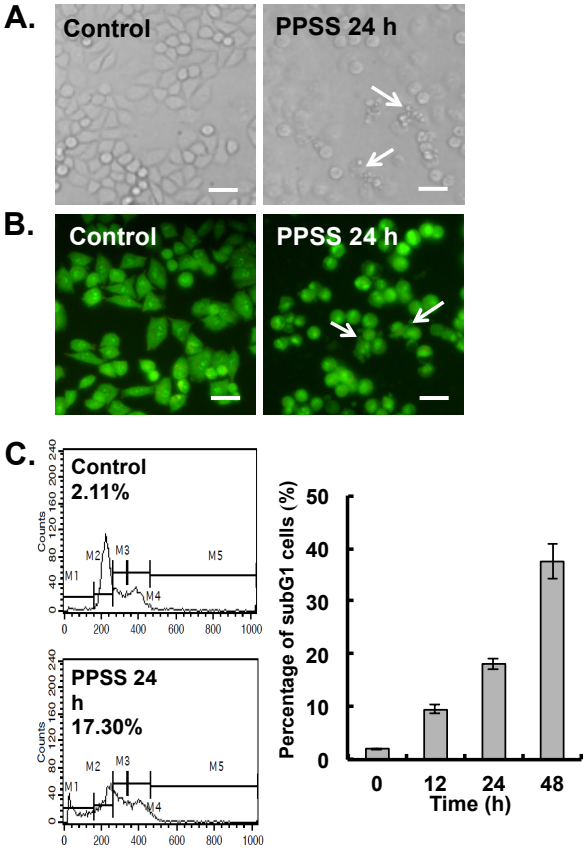

Figure 1. PPSS Induces Apoptosis in A549 Cells. A, B) The cells were incubated with 50 mg/L PPSS for 24 h, and the cellular morphological changes were observed by phase contrast microscopy (A, 200×magnification, bar=20 μm) or by fluorescence microscopy with AO staining (B, 200×magnification, bar=20 μm). Arrows indicate apoptotic bodies. **C)** Cells were cultured in the presence of PPSS for 0, 12, 24, and 48 h, and the apoptotic cells stained with PI (sub-G1 fraction) were measured by flow cytometric analysis (Data were represented as means±S.D. of three independent experiments)

RNase A 1 mg/mL). For measuring autophagy, collected cells were suspended with 0.05 mM MDC at 37°C for 1 h. Then the samples were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis

A549 cells were treated with PPSS for 0, 12, 24, and 48 h, then were harvested and lysed in RIPA lysis buffer at 4°C for 60 min. Lysates were centrifuged at 12,000×g for 15 min, and the protein content of the supernatant was determined by the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were separated by 12 % SDS-polyacrylamide gel electrophoresis and electrophoretically (SDS-PAGE) transferred to Immobilon®-PTransfer Membrne (Millipore Corporation, Billerica, MA, USA). The membranes were soaked in blocking buffer (5 % skimmed milk). Proteins were detected with the primary antibodies against capase-3, caspase-8, PARP, LC3, Beclin 1, and GAPDH followed by horseradish perosidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized by using ECL as the HRP substrate.

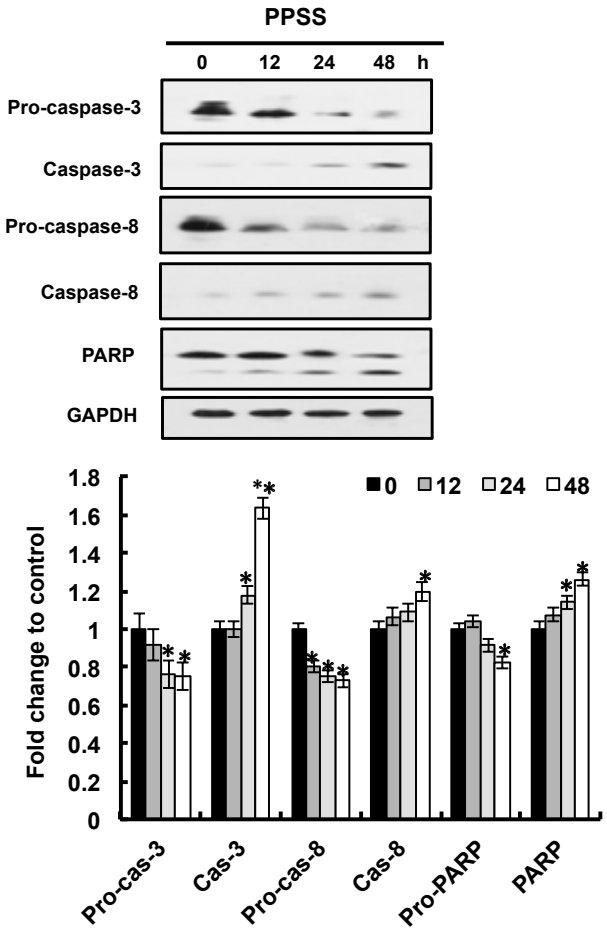

Figure 2. The Levels of Apoptosis-Associated Proteins in PPSS Treated A549 Cells. The cells were lysed after treatment with 50 mg/L PPSS for 0, 12, 24, and 48 h, and the protein levels of caspase-3, caspase-8, and PARP were determined by western blot analysis. It showed that PPSS cleaved pro-caspase-8, pro-caspase-3 and PARP in a concentration-dependent manner. GAPDH was used as a loading reference. Band density of the specific protein was analyzed with Quantity One image software and the results were expressed as average density to GAPDH. (n=3, means±S.D.; *p<0.05, **p<0.01 vs control group)

Statistical analysis

All the presented data and results were confirmed in at least three independent experiments and were expressed as mean±S.D. Statistical comparisons were made by Student's t-test and One-way ANOVA followed by Tukey's post hoc test. p<0.05 was considered to represent a statistically significant difference.

Results

PPSS time-dependently induced apoptotic death in A549 cells

Our previous study showed that PPSS exhibited inhibitory effects against A549 human lung cancer cells with IC_{50} at 24 h were 49.96 mg/L. To clarify the features of the decreased cell viability induced by PPSS, the morphologic changes of A549 cells were examined. Compared with the control group, PPSS treatment caused remarkable morphologic changes including membrane blebbing and granular apoptotic bodies (Figure 1A-B). Flow cytometric analysis was also carried out and showed a significant increase in the percentage of subG1 cells after treatment with PPSS (Figure 1C).

PPSS activated apoptosis-associated proteins in A549 cells

The levels of apoptosis-associated proteins in PPSS

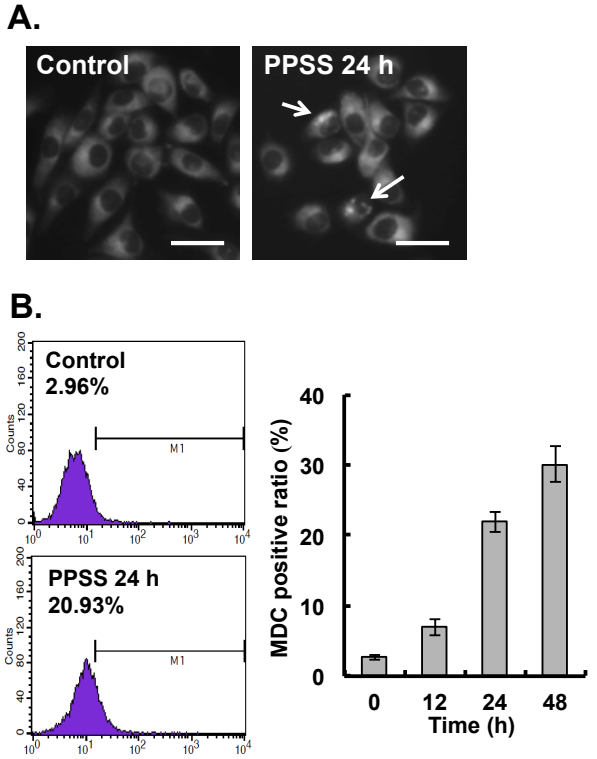

Figure 3. PPSS Induces Autophagy in A549 Cells. **A)** Cells were cultured with 50 mg/L PPSS for 24 h and then observed by fluorescence microscopy with MDC staining (400 × magnification). Arrows indicate cells containing autophagolysosomes. **B)** The cells were treated with 50 mg/L PPSS for 0, 12, 24, and 48 h, quantitative analysis detected a positive ratio of MDC staining by flow cytometric analysis, n =3, means±S.D

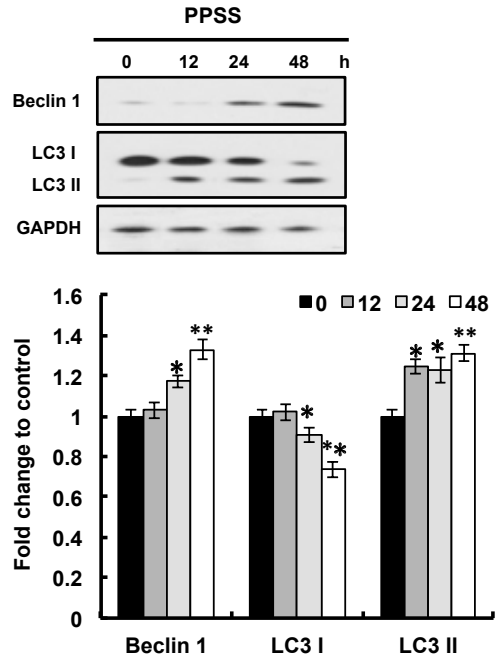

Figure 4. The Protein Levels of Beclin 1 and LC3 in PPSS Treated A549 Cells. The cells were lysed after treatment with 50 mg/L PPSS for 0, 12, 24, and 48 h, the protein levels of Beclin 1 and LC3 were detected by western blot analysis. GAPDH was used as an equal loading control. Band density of the specific protein was analyzed with Quantity One image software, and the results are expressed as average density to GAPDH (Mean±S.D. of three separate experiments; *p<0.05, **p<0.01 vs. control group)

treated A549 cells were measured. Western blot analysis showed that PPSS treatment induced significant changes of apoptosis-related proteins, being initiated by the activation of cleaved procaspase-8, procaspase-3 and PARP, leading to DNA fragmentation in nuclei (Figure 2). It is concluded that PPSS induced apoptotic death in A549 cells.

PPSS time-dependently induced autophagy in A549 cells

The morphologic changes were observed in the MDC stained A549 cells by fluorescence microscopy. PPSS treatment caused a marked increase in the number of MDC-labeled autophagolysosomes compared with the control group (Figure 3A). The quantitative analysis of PPSS-induced autophagy by flow cytometric analysis demonstrated that the MDC-positive cells increased in a time-dependent manner (Figure 3B). The changes at protein levels were also examined. Western blot analysis revealed that the up-regulation of Beclin 1 and the conversion from LC3 I to LC3 II were detected in PPSS-treated cells in a time-dependent manner (Figure 4). All the results showed that PPSS induced autophagy in A375-S2 cells.

Discussion

Lung cancer continues to be a significant worldwide public health problem, more than 75% of lung cancer patients present with advanced stage of disease when therapeutic options are limited (Mountain, 1997). Therefore, researchers continue to search for anti-cancer drugs that place extra emphasis on lung cancer.

Traditional Chinese medicine has been used as therapeutic agents for thousands of years. Compared to the purely synthetic drugs, some compounds and extracts from natural plants exhibited higher activity and lower toxicity (He et al., 2013; Li et al., 2014). Traditional Chinese medicine has its features and advantages in the treatment of cancer, such as reduce the toxicity of radiation and chemotherapy, improve general conditions of patient, release cancer-related symptoms, and prolonging the survival of patient. However, traditional Chinese medicine also has its boundedness that was due to active ingredients being unclear in cancer treatment. Natural products have been proven to be a rich resource for anticancer drug discovery, with the development of separation and analysis technology, nature extracts provide leads for the potential anti-cancer agents (Lee, 1999). This provides guide to identifying new anti-cancer compounds and serve as a source of alternative cancer therapy, and have received increased scientific attentions.

Saponins are major active components for the traditional Chinese medicine Paris polyphylla Smith var. chinensis (Franch.) Hara., which has shown antitumor activities. In this study, we have found that paris polyphylla steroidal saponins (PPSS) induced both apoptosis and autophagy in A549 human lung cancer cells. The mechanism of apoptosis was due to activations of caspase-8, caspase-3, and autophagy was confirmed by up-regulation of Beclin 1 and the conversion from LC3 I to LC3 II. The activation of caspase-3 was demonstrated in reduction of procaspase-3 as well as cleavage of PARP, one of the caspase-3 substrates, which is required for repairing DNA damage.

In mammalian cells, the amount of LC3 II is a marker for the formation of autophagosomes. LC3 is modified via an ubiquitylation-like system (Tanida et al., 2004). The carboxyl terminal region of LC3 is cleaved, generating a soluble form known as LC3 I and exposing a carboxyl terminal glycine essential for further reactions. Then, LC3 I conjugated to phosphatidylethanolamine and modified to a membrane-bound form, LC3 II, which localized to autophagosomes and autolysosomes. The amount of LC3 II correlated with the extent of autophagosome formation and involved in the autophagic pathway. Conversion from LC3 I to LC3 II is an essential step in autophagosome formation. Therefore, the cellular level of LC3 II has been regarded that reflects the activation of autophagy (Tanida et al., 2005). Beclin 1 is part of a Class III PI3K complex that is involved in the signaling pathway activating autophagy and also participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane. In particular, LC3 II is a specific marker of the autophagic process since it directly correlates with the number of autophagosomes. Additionally, Beclin 1 is an essential modifier of the autophagic process. Thus, Beclin 1 and the conversion of LC3 are all involved in the occurrence of autophagy, and can be used to monitor autophagy (Kassiotis et al., 2009; Xing et al., 2012).

In conclusion, these results indicated that PPSS was able to induce lung cancer cell apoptosis and autophagy,

the former was attributed to the cleavage of caspase-3 and PARP, the latter was demonstrated by up-regulation of Beclin 1 and the conversion from LC3 I to LC3 II, respectively. With the undermining of the mechanism, PPSS would be a potential candidate for intervention against lung cancer.

Acknowledgements

This work was supported by the Fund of the Educational Department of Shaanxi Province, P.R. China (14JK1621).

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