Original Research Article

Paris polyphylla extract inhibits proliferation and promotes apoptosis in A549 lung cancer cells

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Abstract

Purpose: To investigate the effect of Paris polyphylla extract (PPE) on proliferation and apoptosis in A549 human lung cancer cells.

Methods: Morphological changes were examined by microscopy in A549 cells after exposure to PPE. Trypan blue staining of living cells was used to aid the construction of the cell growth curve after treatment with different concentrations of PPE. The influence of PPE on cell proliferation, apoptosis and cell cycle were determined by MTT assay. Protein expressions of key apoptosis-related enzymes were determined by immuno-cytochemical method.

Results: PPE inhibited the growth of A549 lung cancer cells at a concentration range of 12.5 – 200.0 µg/mL. Flow cytometry revealed that PPE promoted apoptosis in A549 cells. The proportion of cells in G0/G1-phase increased significantly ($p < 0.01$), while the proportion of cells in S- and G2/M-phases decreased correspondingly, indicating that the cells were in G0/G1-phase arrest. Cell cycle arrest and apoptosis-inducing effect gradually increased with increase in PPE concentration. With increasing concentration of PPE, there was significant increase in the expressions of caspase-8, caspase-3 and caspase-9, but significant decrease in Ki-67, p21ras protein ($p < 0.01$).

Conclusion: PPE exerts pronounced inhibitory activity on the proliferation of A549 lung cancer cells. It also induces apoptosis in A549 cells, most probably by a mechanism related to Ki-67 and p21 ras protein expression, and arrest of cell cycle in G0/G1-phase.

Keywords: Paris polyphylla, Antitumor activity, Lung cancer, A549 cells, p21 ras protein expression, Caspase, Cell cycle arrest, Apoptosis

INTRODUCTION

The morbidity and mortality of lung cancer account for 13 and 19.4 %, respectively, worldwide [1]. Lung cancer is divided into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC accounts for most (about 80 %) lung tumors. At present, early-stage NSCLC is treated by surgical intervention, while mid-late NSCLC is treated with radiotherapy and chemotherapy. Although

effective for some patients in the short run, radiotherapy and chemotherapy are associated with adverse effects, toxicity, high recurrence of tumor and poor prognosis [2]. Therefore, it is necessary to reduce the side effects of chemotherapeutic drugs by searching for more effective anticancer drugs with lower toxicity. This has been a major challenge to medical science.

Paris polyphylla is a very valuable plant in

Guangxi, China. Studies have shown that *Paris polyphylla* has anti-aging, blood pressure-lowering, hypoglycemic and immunity-enhancing properties [3]. It also has antioxidant activity [4], and exerts cytotoxic effects on various malignant tumors [5,6]. The present study was carried out to study the effect of *Paris polyphylla* extract on apoptosis and proliferation in A549 human lung cancer cells.

EXPERIMENTAL

Materials

Human lung cancer cell line (A549) was obtained from Chinese Academy of Medical Sciences. *Paris polyphylla* was obtained from Guangxi, China. RPMI1640 culture medium was product of GIBCO Company, USA). Calf serum and phosphate buffer solution (PBS, American hyclone); dimethylthiozol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypsin were obtained from Sigma(Steinheim, Germany). Caspase-8, caspase-3, caspase-9, Ki-67, bcl-2 p21ras monoclonal antibodies, and SP generic kits were products of American NeoMarkers (Fremont, CA).

Samples of *Paris polyphylla* were collected from Shiyan City, Hubei Province, China in May 2016. Authentication of the plant material was carried out by Professor Yue Hu from Chongqing Medical University in China. A voucher specimen (no. PPE 20160508) was deposited in the herbarium of College of Pharmacy, Chongqing Medical University.

Preparation of PPE

Dry leaves of *Paris polyphylla* (500 g) were ground and extracted three times by refluxing with 95 % ethanol, each time for 2 h. The extracts were pooled and concentrated under reduced pressure to obtain PPE.

Cell culture

The A549 cells were cultured in RPMI-1640 medium, which contains 10 % fetal bovine serum (v/v) and 1 % penicillin-streptomycin at 38 °C in a humidified atmosphere containing 5 % CO_2, with 0.25 % trypsin digestion batches. The logarithmic phase of the cells were used for the study.

Drug sensitivity test

Cells at the logarithmic growth phase in RPMI1640 medium at a density of 1.0×10^5 cells/mL of cell suspension, were seeded in 48-well plates and cultured at 37°C for 24 h, until the cells became adherent. PPE at different final concentrations (25, 50, 100, 200 µg/mL) were added to different wells, and the wells were incubated in an incubator for different periods (24, 48 and 72 h) at 37 °C. A well containing 20 µg/mL cisplatin served as positive control, while another well containing saline in place of PPE served as negative control.

Cell morphology assay

A suspension of A549 cells at the logarithmic growth phase in RPMI 1640 medium was adjusted to a density of 1.0×10^5 cells/mL in a 48-well culture plate, and cultured for 24 h at 37 ℃ to achieve adherence. The wells were grouped into two. One group (experimental group) received different concentrations of PPE in the culture medium, while the other group (control group) received 0.04 % DMSO in place of PPE. The cells were observed under inverted microscope after 24, 48 and 72 h for changes in cell size, cell membrane and nucleus.

Growth curve and doubling time (TD) ofA549 lung cancer cells

A549 cells at logarithmic growth phase at a density of 1×10^4/ mL were seeded in 24-well plates. One (1) mL was taken from each of three wells at the same time point daily, and digested. The cell number well was counted, and the mean number of cells was determined. Counting was done for 8 days. A growth curve was plotted with mean cell count as vertical axis, and time as horizontal axis. TD for the human lung cancer A549 cells was calculated using Eq 1.

$$TD = t\{\log2/(\log N_t - \log N_o)\} \dots\dots\dots\dots (1)$$

where *t* is time interval, while N_o, and N_t are the initial mean count and mean count after time *t*, respectively.

MTT assay

A suspension of the A549 cells (0.2 mL) at logarithmic phase was seeded in a 96-well plate with each well containing 5×10^3 cells, and cultured overnight for cell adhesion. In the experimental group, the original culture medium was drained, and replaced with different concentrations of freshly-prepared PPE (one concentration in 24 wells, and 6 wells per group). The wells of the control group medium contained 0.04 % DMSO, while wells without cells served as blank control. All wells were incubated for 24, 48, 72 and 96 hat 37 °C in a humid atmosphere containing 5% CO_2. The supernatant was

discarded, and 20 μL of MTT solution (5 g/L) was added to each well and incubated for 4h at 37 °C for 4 h. Then, the supernatant was carefully removed by suction, and 150 μL DMSO was added to each well to dissolve the crystals. The absorbance of each solution was read at 490 nm against blank control, and the results were used for calculating the growth inhibition rate.

Flow cytometry

A suspension of 549 human lung cancer cells (5.0×10^4 cells/mL) at logarithmic growth phase was seeded in 24-well plates (100 μL in each well), and cultured in RPMI 1640 medium for 24 h for adherence. Thereafter, PPE was added to a final concentration of 40 μg/mL. Wells in the control group contained an equivalent volume of normal saline. The plates were cultured at 37 °C in an atmosphere of 5 % CO_2. At 24 and 48 h, the cells in each group were collected (cell number > 1×10^6), washed twice in cold PBS (pH 7. 2 ~ 7. 4), and centrifuged at 1000 rpm for 5 min. Then, 1 mL of cold precipitation PBS (pH 7. 2 ~ 7. 4) was added to the cell suspension, followed by 3 mL of pre-cooled 70 % ethanol. The cell suspension was kept overnight at 4 °C, and then centrifugal at 1 000 rpm for 5 min. The supernatant was discarded; and the cells were washed twice in 1 ml of cold PBS (pH 7. 2 ~ 7. 4), and centrifuged at 1 000 rpm for 5 min. The cell count was adjusted to 1×10^6/mL, and 200 uL NaseA was added, followed by incubation in a 37 °C water bath for 30 min. Iodide c ingot (PI) staining fluid 800 mu L was added, mixed and kept at 4 °C in the dark for 30 min. DNA single parameter analysis was carried out to determine cell distribution and the proportion of cells in G0/D1, S, and G2/M phases, as a function of time. The results were analyzed using Modifit LT software.

Immunocytochemistry staining

The A549 cells at logarithmic phase were digested with 0.25 % trypsin, and 2×10^5 cells were seeded into a 6-well plate and cultured overnight for cell adhesion. The culture medium was removed, and 3mL of PPE was added to different cells to final concentrations of 20, 40, 80 and 160 μg/mL. Each PPE concentration was replicated in six parallel wells. In the control wells, PPE was replaced with an equivalent volume of 0.04 % DMSO. The plates were incubated for 24 and 48 h; the glass slides were taken out, fixed with 4 % paraformaldehyde for 10 min at 4 °C according to instructions contained in the operation manual of S-P kit. The plates were observed for appearance of clear brown granules which is indicative of positive

cells. The presence of Bcl-2 and p21 ras in cytoplasm is indicative of positive expression; Ki67 tan is indicative of positive expression in the nucleus, caspase-9 is an index of positive expression in the cytoplasm and the nucleus; while caspase-3 and caspase-8 indicate positive expression in the cytoplasm or cytoplasm and nuclei. They are expressed less in cells without changes in color, or lightly-colored cells. The cells in the PPE plates and control plates were observed at high magnification (x 400) in 5 fields, and positive cells were numbered and expressed as percentage of the total number of cells.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM), and processed using SPSS 17.0 (SPSS Inc, Illinois, Chicago, USA). They were analyzed by ANOVA followed by Dunnett's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PPE on proliferation of A549 cells

The extract (PPE) at different concentration, significantly inhibited the proliferation of A549 cells after 48 h, when compared with control group ($p < 0.05$). The inhibitory effect was concentration-dependent. These results are shown in Table 1.

Table 1: Inhibitory effect of different concentration of PPE on the proliferation of A549 cells

Group	Concentration (μg/mL)	Inhibition (%)
Negative control	—	—
Positive control	20	88. 47±0. 23
PPE	25	47. 76±0. 18
PPE	50	50. 24±0. 36
PPE	100	53. 00±0. 33[a]
PPE	200	64. 17±0. 22[b]

Values are mean ± S.E.M (n = 4); [a]$p < 0. 05$, [b]$p < 0. 01$, relative to control
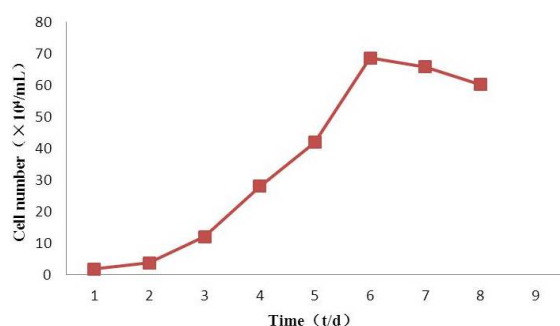
Effect of PPE on morphology of A549 human lung cancer cells

Under the inverted microscope, the control group of lung cancer A549 cells grew densely, with overlapping clusters, as a long fusiform or polygon, without direction and with clear cellular edges. On the other hand, A549 cells treated with 10 and 20 ug/mL PPE showed reduction in number of cells, and no obvious morphological changes. When the concentration of PPE reached 40 ug/mL, the cell volume shrank, becoming smaller; and the cells were fewer and

separated, with many of floating particles in the culture medium. At concentrations of 160 and 320 mug/mL of PPE, there were very clear and obvious changes in cell morphology, and evidence of cell disruption and higher cell death.

Growth curve and TD of A549 human lung cancer cells

The growth curve of A549 cells is shown in Figure 1. Cell proliferation was initially slow within the first 48 hours, but thereafter rose rapidly and peaked after 6 days, followed by a decline on the 7th and 8th days. The value of TD obtained was 20×10^4/mL.

Figure 1: Growth curve of A549 human lung cancer cells

MTT data

Results from MTT assay showed that PPE

exerted a concentration and time-dependent inhibitory effects on the growth of human lung cancer A549 cells (Table 2). The inhibitory effect was highest at a concentration of 320 µg/ml. In addition, for each PPE concentration, the inhibitory effect increased with time and became maximum at 96 h.

Effect of PPE on A549 cell apoptosis and cell cycle

PPE at a concentration of 40 µg/mL brought about arrest of cell cycle at G0/G1 phase in 24 and 48 h, and blocked the transformation from G1 phase to S phase (Table 3). With increase in G0 - G1 phase transition time, there was reduction in the number of cells in the S phase. The increase in percentage of cells in the G0/G1 phase, and decrease in cells in S phase, were concentration-dependent and time-dependent ($p < 0.01$).

Effect of PPE on key enzymes of apoptosis and protein expression levels of apoptosis-related genes

Immunocytochemistry results showed that PPE at 40 ug/mL brought about significant increases in the expressions of apoptosis-related proteins caspase-3, caspase-8 and caspase-9 in 24 h, when compared with control ($p < 0.01$, Table 4).

Table 2: Inhibitory effect of PPE on A549 lung cancer cell growth as a function of time

Group	Concentration (µg/mL)	24h	48h	72h	96h
Control	—	—	—	—	—
PPE	10	1.37	2.28	2.46	2.77
PPE	20	5.20	10.79	18.47	25.46
PPE	40	22.35	29.42	36.18	44.52
PPE	80	31.17	40.83	52.23	60.61
PPE	160	44.25	58.59	70.45	78.58
PPE	320	45.12	59.93	72.73	80.12

Table 3: Effect of PPE on cell cycle distribution of A549 cells at 24 and 48 h

Group	G0/G1 (%)	S (%)	G2/M (%)	A
Control group	51. 50±3. 95	44. 83±3. 40	9. 85±1. 87	2. 83±1. 09
PPE 24h	57. 82±3. 37[*]	36. 71±4. 14[*]	5. 47±1. 77[*]	7. 27±2. 19[*]
PPE 48h	65. 18±4. 74[*]	24. 97±5. 77[*]	3. 39±1. 17[*]	9. 06±5. 42[*]

Values are mean ± SEM (n = 8); [*] $p < 0.01$ compared to control

Table 4: Effect of PPE on the expressions of caspase-3, caspase-8 and caspase-9

Group	Caspase-3 (%)	Caspase-8 (%)	Caspase-9 (%)
Control	15. 97±3. 13	17. 44±2. 60	29. 98±3. 39
PPE	50. 65±5. 31[*]	49. 79±4. 50[*]	60. 62±6. 48[*]

Values are expressed as mean ± SEM; [*] $p < 0.01$ compared with control

Table 5: Effect of PPE on expressions of Bcl-2, Ki-67 and P21ras protein

Group	Bcl-2 (%)	Ki-67 (%)	p21ras (%)
Control	72. 92±6. 70	81. 37±5. 00	51. 33±6. 65
PPE	40. 83±4. 78[*]	38. 89±5. 16[*]	27. 13±5. 09[*]

[*]$P < 0.01$ compared with control group

Visible light microscopy showed Ki-67 protein staining in the nucleus; and BCL-2 and p21 ras protein in the cytoplasm. There were more positive cells in the control group, but at a concentration of 40 μg/mL, there were significant decreases in the number of positive cells ($p < 0.01$). The level of protein expression was significantly lower in the PPE group than in the control group ($p < 0.01$, Table 5).

DISCUSSION

The results obtained in this study show that PPE has significant time- and concentration-dependent inhibitory effects on the proliferation of A549 cells. The extract also exerted strong apoptotic effects on the A549 cells, as seen from the morphological changes in the cells. The dynamics of tumor cell proliferation are due to loss of control of cell cycle, resulting in uncontrolled G1/S and G2/M transformations. The cells were mainly in the active DNA synthesis S phase, which led to abnormal cell proliferation [5]. Thus blockage of the G1/S phase and G2/M phase can effectively control the tumor cell cycle and inhibit tumor proliferation. In this study, results from cell cycle analysis showed that PPE reduced the number of A549 human lung cancer cells in G1, S and G2/M phases, leading to stagnation of the cell cycle and restraining of cell proliferation. Increased concentrations of aqueous fractions of PPE brought about enhanced effects on cell cycle arrest. This indicates that PPE causes lung cancer cell cycle arrest by regulation and inhibition of tumor cell mitosis.

Ki-67 antigen is a more positive marker of nuclear proliferation, and Ki-67 may be used as a biomarker for determining high risk individuals in precancer population [7-9]. Results from Ki-67 antigen assay showed that PPE significantly decreased the expression of Ki-67 in A549 cells. This implies that a large number of the A549 cancer cells were in the stationary phase of cell cycle. In effect, PPE may contain active principles that regulate the gene which inhibits cancer cell mitosis, thereby inhibiting the growth of tumor cells. P21 ras cancer gene is involved in transmission of intracellular information which regulates the cell cycle, and it is the "initiation factor" in the occurrence of tumors [10-13].The

expression of p21 ras antigen was also significantly decreased in the PPE-treated A549 cells, in a time- and concentration-dependent manner, when compared with untreated controls. Again, this is an indication that most of the cancer cells were in the S phase of the cell proliferation cycle. This supports the presence of anti-proliferative and mitosis-inhibiting agents in the extract.

Caspases are proteolytic enzymes which constitute key components of the apoptosis system. They are a family of cysteine proteases involved in regulation of apoptosis, which is considered to be a key to cancer cell death [14–17]. In this study, PPE treatment led to significantly lowered expression of Bcl-2, and significant increases in the expression of caspase-9. These results demonstrate that the anti-proliferative effects PPE also involve the mitochondrial apoptotic pathway. Caspase-3 is a pro-apoptosis caspase. Its expression in the PPE-treated A549 human lung cancer cells was significantly higher than in the untreated control cells, indicating that PPE promotes apoptosis in these cells. Thus, PPE induced apoptosis by inhibiting the signal transduction pathway enzymes to achieve elevated expressions of the pro-apoptosis caspases 3, 8 and 9; as well as the apoptosis-related gene Bcl-2.

CONCLUSION

The findings of this study reveal that PPE has significant inhibitory effect on the proliferation and apoptosis in human lung cancer A549 cells. Thus, PPE might be a potent source of therapeutic agents for the management of lung cancer patients.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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