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Euphorbia angustifolia lactone B inhibits A549 proliferation and induces apoptosis

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Abstract: The study is to investigate the effect of *Euphorbia angustifolia* lactone B (Jolkinolide B, JB) on the proliferation and apoptosis of A549 cells. The proliferation of A549 cells was detected by tetramethyl azothiolide. Activity changes of intracellular caspase-3, 8, 9 were determined by spectrophotometry. The content of cytochrome C (Cyt C) protein and the expression quantity of Bcl-2, Bax, p-ERK1/2, and p-Akt proteins were detected by Western blot and the apoptosis rates were detected by flow cytometry. JB significantly inhibited cell proliferation in a time-dose manner. With increase in JB concentrations, the expression level of Bax protein greatly increased, and the expression of caspase-3 and caspase-9 significantly increased with significant difference ($P<0.01$). Besides, the peak value of mitochondrial membrane potential decreased, while the number of cells distributed in the depolarized region increased, which was different from that in the control ($P<0.05$). Moreover, the expression levels of p-ERK1/2 and p-Akt in A549 cells gradually decreased with extending exposure duration. Moreover, 20 μ mol/L LY294002 (an PI3K inhibitor) + 120μg/mL JB and 10μmol/L PD98059 (an ERK inhibitor) +120μg/mL JB also increased apoptosis rates of A549 cells. JB could induced cell apoptosis through promoting endogenous mitochondrial signal transduction pathway and inhibiting PI3K/ERK pathway.

Keywords: Lung cancer, mitochondrial pathway, MEK/ERK1/2 signal transduction pathway, PI3K-Akt signal transduction pathway.

INTRODUCTION

Lung cancer has become one of the most serious malignant tumors threatening human health and life in the world. Its morbidity and mortality have been trending up since 1930s (Jemal *et al*., 2011; Ferlay *et al*., 2008). Commonly, lung cancer is considered to be one core towards death caused by malignant tumors. A number of related studies had been carried out to improve the clinical efficacy of lung cancer and prolong the survival period of lung cancer patients, but its cure rate still remains low. Previous studies also demonstrated that Chinese medicine was advantageous in the treatment of lung cancer, e.g. prolonging survival time, improving patients' quality of life, enhancing patients' immunity, stabilizing and reducing tumor lesions and reducing tumor recurrence and metastasis (Tan *et al.*, 2008). Euphorbia angustifolia lactone B (jolkinolide B, JB), a diterpenoid isolated from the root of Euphorbia angustifolia could induce the apoptosis of ovarian cancer Skov3 cells (Zhou *et al*., 2012), U937 (histiocytic lymphoma) cells (Wang *et al.*, 2011) and breast cancer MDA-MB-231 cells (Lin *et al.*, 2012), which demonstrated that JB possessed antitumor effect. Therefore, JB might have a strong inhibitory effect on the growth of human lung cancer cell line A549 and this study is to explore the apoptosis-inducing effect of JB on human lung cancer cell line A549 and a related potential mechanism is discussed.

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MATERIALS AND METHODS

Culture and pretreatment of lung cancer A549 cells

Human lung cancer A549 cells were provided by Yantai Yuhuangding Hospital Cell Bank. ATCC cells (American National Cell Bank) were cultured in RPMI-1640 medium containing 10% calf serum at 56℃. Inactivated fetal bovine serum was exposed to saturated humidity at 37℃ or kept in an incubator with 5% CO_2. All the cells used in the experiment were within logarithmic growth phase and digested and passaged by 0.25% trypsin.

Inhibition rates of cell proliferation by MTT method

A549 cells were inoculated in 96-well culture plate with 5.0×10^4 cells /ml. 100 uL cells per well were cultured in CO_2 incubator at 37℃ for 24 hours. The cells grew well adhering to the wall. RPMI-1640 culture medium was used as the blank control. DDP (3μg/ml) without drug was used as positive control. JB solution was diluted to different final concentrations (20, 40, 80, 160, 200μg/ml) according to the experiment and there were 6 compound holes in each group. The cells were cultured at 37℃ for 24h, 48h and 72h in a CO_2 incubator. The cell culture medium was replaced once per 4 hours by fresh MTT solution. 150uL DMSO was dosed to each well and immediately mixed for 10min to avoid light and oscillation. The photometric value per well (OD value) was detected by enzyme labeling instrument in the 570nm wavelength. Each experiment was repeated for 3 times. The inhibitory rates of cell proliferation were calculated

by counting method. The proliferation inhibition rates were calculated according to the formula: inhibition rate = [(control group OD value-drug group OD value)/control group OD value] × 100%.

Detection of relative enzyme activity of Caspase-3, 8 and 9

The cells were inoculated in a six-well plate with 5×10^5. After culturing for 24h, JB solution with a final concentration of 200μg/ml was added. The control group was cultured with RPMI-1640 medium in the same way. Before toxicity test, the cells were washed using PBS twice and then centrifuged to collect 3×10^6 cells. The specific pretreatment method is: 50μL ice cold Lysis Buffer was added to precipitate cells and blown evenly; after 15 min ice was added and oscillated 3 times (10 s each time); the cells were then put into new tubes after centrifuging(10000 rpm, 4℃); a small amount of supernatant (1-2μL)was taken to determine the protein concentration using Braford method. Substrate (Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA) was added and incubated at 37℃ for 4 hours and its absorbance was determined by enzyme labeling instrument at 405nm wavelength. The activation degree of OD in apoptosis inducer group was determined by calculating the ratio of Caspase-3, 8, 9 inducer/OD negative light. Lysis Buffer and Reaction Buffer were used as blank control.

The expression of apoptosis-related proteins was detected by 1.5 Western blot method. The cells were divided into five groups: Control, 80, 120, 160 and 20μg/ml JB. After 48 hours, the culture was terminated. The white matter was extracted by conventional methods (different protein extraction methods). The total protein was determined by Coomassie brilliant blue staining (Bradford) method. The same amount of protein was separated by SDS-PAGE gel electrophoresis. After 2 hours of electrophoresis, the protein was transferred to PVDF membrane. 5% goat serum (diluted by PBS) was closed and incubated at room temperature for 60 min. The cells were incubated overnight with the antibody [Bax, Bcl-2 (1Vue 500)]. The cells were rinsed with PBS for 3 times, and each was incubated at 37℃ for 1h with horseradish peroxidase labelled second antibody (1vir 2000). PBS rinsing was done 3 times, each time for 5min. ECL chemiluminescence reagent was used to react and develop the color. Bio-Rad gel imaging system was photographed and analyzed by GIS gel imaging analysis software, with β-Actin as internal control.

Determination of mitochondrial membrane potential

The logarithmic A549 cells were inoculated in a 6-well plate with 1×10^6 cells/ml. After 24 hours, JB solution was added with a final concentration of 160μg/ml and a blank control group (adding the same amount of RPMI-1640 medium) and a positive control group (DDP 3 μg/ml) were simultaneously carried out. After 48 hours, the old culture medium was removed from each hole. After PBS washing, the fine cells were collected by 0.25% trypsin + 0.02m EDTA digestion, centrifuged for 10 minutes at 1000 rpm/min, the supernatant was discarded, rhodamine 123 (Rhodamine 123) dye 5ul (concentration was 10mg/mL) was added at 37℃ and incubated for 30min in a 5% CO2 cell incubator. The cells were washed with PBS for 3 times and the changes of mitochondrial membrane potential were detected by flow cytometry.

The content of Cyt C protein in cytoplasm and mitochondria detected by Western blot

The cells were divided into five groups: Control, 80, 120, 160 and 20μg/ml JB. After 48 hours, the culture was terminated and the mitochondrial protein and cytoplasmic white matter were extracted. The steps were the same as the instructions of the kit. Coomassie Bradford method was used for protein quantification. The same amount of protein was separated by SDS-PAGE gel electrophoresis. Cyt C antibody (1: 500) was added and incubated overnight. The cells were rinsed with PBS 3 times and for 5 min each time. The second antibody labeled with horseradish peroxidase (1pur2000) was added and incubated at 37℃ for 1 h. PBS rinsing was done 3 times, each time for 5 min. ECL chemiluminescence reagent was used to react and develop the color. Bio-Rad gel imaging system was used to take photos, GIS gel imaging analysis software was used to analyze, and β-Actin was used as internal control.

Expression of phosphorylated and non-phosphorylated Akt and ERK1/2 by Western blot

JB was used to treat A549 cells for 0, 12, 24, 36 and 72h, respectively, and then the total protein was extracted. Protein was separated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane for 2 hours. After being sealed at room temperature for 1h, the primary antibodies of Akt, p-Akt, ERK and p-ERK were added and incubated overnight at 4℃. After washing the membrane with PBS, the second antibody (1 ERK 2000) labeled with HRP reacted at room temperature for 1h. After membrane washing, ECL chemiluminescence reagent was used to react and develop the color. Bio-Rad gel imaging system was photographed and analyzed by GIS gel imaging analysis software, with β-Actin as internal control.

Detection of apoptosis rate by flow cytometry

After 24 hours, the cells were divided into four groups: blank group, PD98059 (10μmol/L) + JB (120μg/mL) group, LY294002 (20μmol/L) + JB (120 μg/mL) group, simple JB (120μg/mL) group. PD98059+JB group and LY294002+JB group were pretreated with LY294002, with final a concentration of 1 μg and 2μg of PD98059 respectively. Then, 120μg/ml JB was added and cultured

at 37℃, 5% CO_2 and saturated humidity for 48h. The cultured cells were taken out, rinsed with PBS and digested with 0.25% trypsin. Trypsin was neutralized in the culture medium containing serum, then the cells were harvested and centrifuged at 1500 rpm for 8 minutes. The cell precipitation was collected and the supernatant was discarded. After precipitation, it was washed with cold PBS and centrifuged twice. 75% precooled alcohol was added and fixed overnight at 4℃. 5μL AnnexinV-FITC was added to the cell suspension and then incubated at 4℃ without light for 15 min. After adding 10μL PI, the cells were gently mixed and incubated at 8℃ for 5 min. It was detected by flow cytometry within 1 hour. Results of the analysis data were obtained by Cellquest professional software.

STATISTICAL ANALYSIS

Statistical analyses were carried out using the SPSS 19.0 software. The results were expressed as mean ± standard deviation. The difference between the groups was analyzed by the chi-square test. $P<0.05$ indicated significant difference.

RESULTS

Inhibitory effect of JB on the proliferation of A549 cells in vitro

The inhibitory effect of JB on the growth of lung cancer A549 cells *in vitro* was observed by MTT method. Fig. 1 shows that low concentrations of JB less than 20μg/ml had no obvious inhibitory effect on A549 cells. The proliferation of A549 cells would be significantly inhibited by JB above 40μg/ml. Besides, cell suppression was enhanced by duration of exposure. The inhibition rate indeed showed an obvious time-dose-effect relationship, and a maximum inhibition rate of 88.43% was found in the presence of 200μg/ml JB for 72h.

Effect of JB on enzyme activity of caspase-3, caspase-8 and caspase-9

The effect of JB on the activity of apoptosis-related enzymes, e.g. caspase-3, caspase-8 and caspase-9 was also studied (fig. 2). After treating with 200μg/ml JB for 24h, the activity of the caspase-3 and caspase-9 greatly increased, while the activity of caspase-8 did not change compared with control. It clearly showed that JB could effectively activate caspase-3 and caspase-9 versus caspase-8.

Effect of JB on the expression of apoptosis-related proteins

After treating with different concentrations of JB for 48 hours, the expression differentiation of Bax and Bcl-2 proteins was analyzed by Western blot (fig. 3). After A549 cells were treated with JB for 48 hours, the expression level of Bax protein gradually increased with increment of JB, while the expression level of Bcl-2 protein gradually decreased with the increment of JB, which showed that JB could up-regulate expression of pro-apoptotic protein Bax and down-regulate the expression of anti-apoptotic protein Bcl-2. As such, it would increase the permeability of mitochondrial cell membrane, and induce apoptosis of A549 cells.

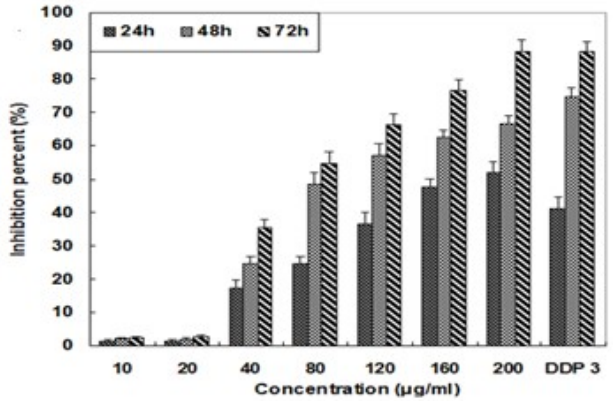

Fig. 1: Inhibitory effect of JB on the proliferation of lung cancer A549 cells. DDP (3μg/ml) was used as a positive control.

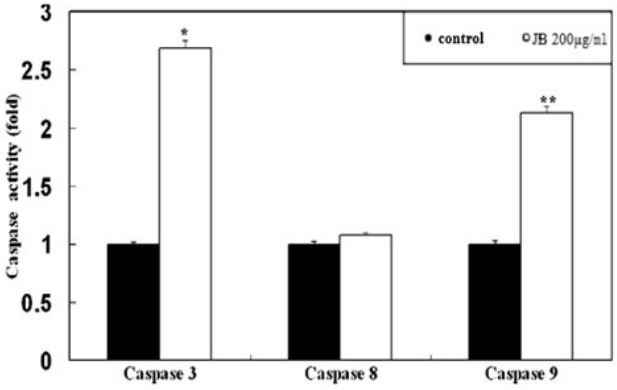

Fig. 2: Effect of JB on enzyme activities of caspase-3, caspase-8 and caspase-9. *$P<0.05$, $P<0.01$, compared with the control group.

Effect of JB on mitochondrial membrane potential of A549 cells

Rhodamine 123 is a kind of cationic fluorescent probe used for cell permeability. It can be easily absorbed by active mitochondria. During apoptosis, the transport ability of mitochondrial membrane decreased, so the ability of rhodamine 123 accumulation would also decrease in mitochondria. After treating with 80 or 160 μg/ml JB for 48h, the peak of mitochondrial membrane potential highly decreased, and the Δm peak significantly shifted to the left (fig. 4). Besides, the cell numbers distributed in the depolarized region also increased (fig. 5), which was different from that of the control group ($P<0.05$).

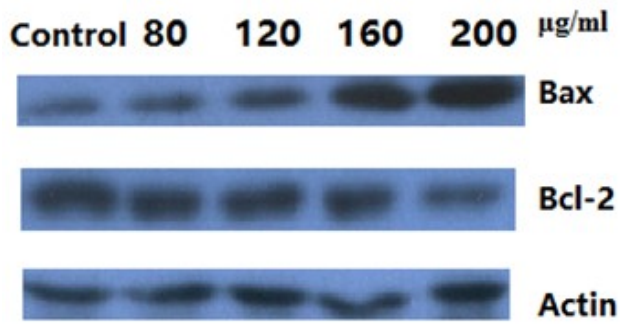

Fig. 3: Western blot detection of the expression of Bcl-2, Bax protein in A549 cells with dosing of different concentrations of JB

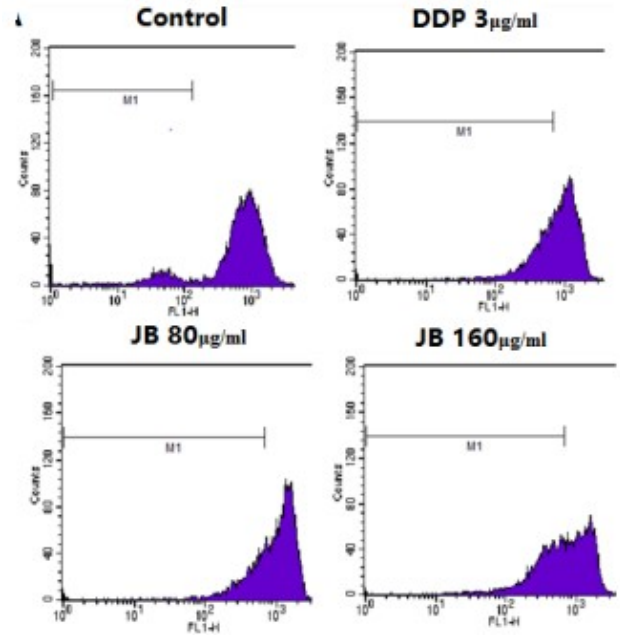

Fig. 4: Effects of JB on the mitochondrial membrane potential in A549 cells

Effect of JB on the Cyt C of A549 cells

After treating with JB for 48 h, Cyt C gradually increased in the cytoplasm with increase in JB concentrations, but decreased in the mitochondria (fig. 6). It could be speculated that JB could increase the permeability of mitochondrial membrane and promote the release of Cyt C from the mitochondria to the cytoplasm.

Effects of JB on PI3K/Akt and MAPK/ERK pathway in A549 cells

After treating with JB, the expression of phosphorylated and non-phosphorylated Akt and Erk1/2 was detected by immunoblotting. Results of Western blot showed that expression of pAkt and pErk1/2 was suppressed by JB, while the expression of Akt and Erk1/2 was not affected by JB (fig. 7). Meanwhile, the phosphorylation level of Akt and Erk1/2 protein decreased with extending the exposure time, indicating that JB inhibited the activation of Akt and Erk1/2 proteins and down-regulated the expression of MAPK/Erk and PI3K/Akt signaling pathway.

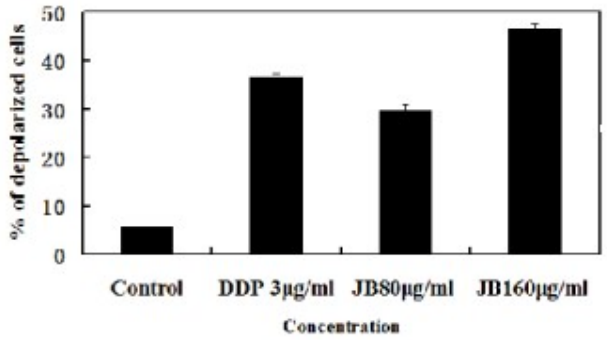

Fig. 5: Depolarization of A549 cells treating by JB

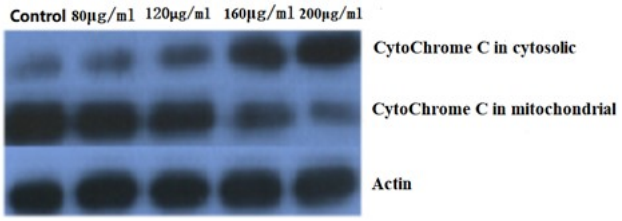

Fig. 6: Expression of Cyt C in mitochondria and cytoplasm treating with different concentrations of JB by Western blot.

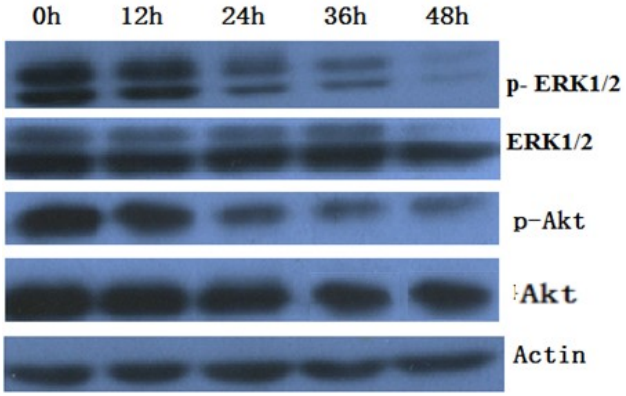

Fig. 7: Expression of Akt and Erk1/2 treating with different concentrations of JB

Effects of ERK and Akt pathway inhibitors on apoptosis of A549 cells induced by JB

Fig. 8A showed that the apoptotic rates of A549 cells treated with PD98059 (10μmol/L) + JB (120μg/mL), LY294002 (20μmol/L) + JB (120μg/mL) and JB (120μg/mL) were higher than the control group. The proportion of both early and late apoptotic cells increased, especially late apoptosis. The apoptosis rates induced by PD98059+JB group and LY294002+JB group were significantly higher than that of JB group. As such, the combined JB and MEK/ERK and PI3K/Akt pathway inhibitors could well induce apoptosis of A549 cells.

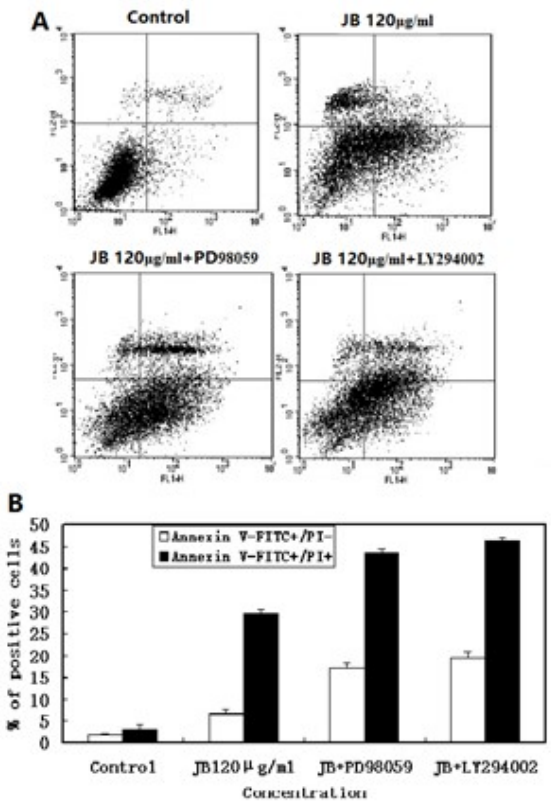

Fig. 8: Apoptosis of A549 cells treated with different inhibitors, including JB (120μg/ml), JB (120μg/ml) + PD98059, JB (120μg/ml) + LY294002 for 48 h, which was detected by FITC/PI double-staining flow cytometry

DISCUSSION

The morbidity and mortality of lung cancer are currently high in malignant tumors. At present, the main treatment methods of lung cancer are hand surgery, radiotherapy, chemotherapy, molecular targeted therapy, immunotherapy, traditional Chinese medicine treatment, etc. Although the treatment methods of lung cancer continue to be updated, the survival rate is not significantly improved. It was reported that the 5-year survival rate of lung cancer was less than 20% using traditional treatment methods (Torre *et al.*, 2016), among which traditional Chinese medicine combined with chemotherapy could obtain a better therapeutic effect than the others. In fact, Chinese medicine treatment could effectively reduce the adverse reaction of chemotherapy. JB, one of the main active components of Euphorbia angustifolia can be seen in anti-tumor. JB possesses a good anti-tumor function *in vitro*, especially for repressing the growth of prostate cancer, breast cancer, ovarian cancer and melanoma cells, as well as inducing apoptosis, changing the distribution of cell cycle, and increasing the expression of LC3- II and Beclin-1 proteins (Wang and Wang, 2018). However, there are few studies on the inhibitory effect of JB on the apoptosis of lung cancer cells.

Cell apoptosis is an autonomic ordered programmed cell clearance to maintain homeostasis, which is controlled by serial genes. Apoptosis can scavenge superfluous cells, aging cells, virus-infected cells, DNA damaged fine cells and malignant cells. The activation and expression of some oncogenes belonging to growth factor family and growth factor receptor family can stimulate tumor cell growth and suppress its apoptosis. The inhibition of apoptosis is an important carcinogenic factor in the occurrence and development of tumor, as well as in the treatment and therapeutic effect of tumor (Liu and Liu, 1997). As such, it is necessary to increase the apoptosis rate of tumor cells before the occurrence and development of tumor. Obviously, tumor is not only a disease of abnormal cell proliferation and differentiation, but also a disease of abnormal apoptosis (Li *et al*., 2008). Accordingly, apoptosis-inducing is considered to be a useful and important design idea of the treatment program (Dai *et al*., 1998). There are three main pathways of apoptosis: The death receptor signal transduction pathway represented by Fas and TNFR, accompanied by the activation of caspase-8 in the process of apoptosis (Long *et al*., 2004), the apoptotic pathway with mitochondria as the core, accompanied by the activation of caspase-9 in the process of apoptosis (Cain *et al*., 2000), and the apoptotic pathway with endoplasmic reticulum as the core accompanied with the activation of caspase-12 in the process of apoptosis (Liu *et al*., 2011). It was demonstrated that activated caspase-3 could up-regulate the expression of Bcl-2 and Bax gene (Huang *et al*., 2014).

As the center of energy metabolism of biological cells, mitochondria were the transit station of information transmission and integration, so they were called the power factories of cells. Mitochondria also played an important role in regulating amino acid biosynthesis, fatty acid oxidation, steroid metabolism, intermediate metabolic pathway, calcium balance and scavenging free radicals (Kwong *et al.*, 2006; Figge *et al*., 2013). The pathway of mitochondrial apoptosis was thought to be regulated by the Bcl-2 protein family. Bcl-2 family proteins were divided into anti-apoptotic members (such as Bcl-2, Bcl-XL and Mcl-1) and pro-apoptotic members (such as Bax, Bad, Bak, Bcl-Xs and NOXA) based on their functions (Youle and Strasser, 2008). Besides, mitochondrial membrane potential could be used to represent mitochondrial permeability. When the cells were externally stimulated, mitochondrial membrane potential would decrease through interrupting gene expression of Bax and Bcl-2, increase the permeability of mitochondrial membrane, increase the release of Cyt C from mitochondria to cytoplasm, activate the apoptosis executor caspase-3, trigger the caspase cascade reaction, and finally initiate the process of cell apoptosis (Damarla *et al*., 2014).

The inhibition rates of A549 cells proliferation increased with the increase of JB concentration and exposure duration, which indicated that JB could effectively inhibit the proliferation of lung cancer A549 cells. In order to further identify the apoptosis mechanism induced by JB in A549 cells, the activities of caspase-3, caspase-8 and caspase-9 proteins were detected and it was found that the activities of caspase-3, caspase-9 increased. It could be speculated that JB induced apoptosis of A549 cells through mitochondrial pathway. Rhodamine 123 staining results showed that JB could significantly decrease the mitochondrial membrane potential. Western blot detection also showed that Bax gene expression was up-regulated and Bcl-2 gene expression was down-regulated, while Cyt C decreased in the mitochondria and increased in the cytoplasm. All the results above suggested that JB induced apoptosis by disturbing gene expression of Bax and Bcl-2 proteins, leading to an increase of mitochondrial permeability and Cyt C release, which further resulted in the activation of Caspase and cell apoptosis.

PI3K/AKT signal transduction pathway is involved in cell cycle regulation, protein synthesis and apoptosis regulation. AKT, one protein kinase B (PKB), is a main downstream effector of PI3K (Osaki *et al*., 2004). Previous studies demonstrated that the PI3K/AKT pathway would be activated in tumor cells. AKT protein kinase regulated downstream molecular activity through mediated phosphorylation and then promoted tumor cell proliferation, apoptosis, survival and metastasis. Ser473 residue was one of its phosphorylation activation sites and the activation state of this sites was a common method of evaluating PI3K/AKT signal pathway (Du *et al*., 2010). ERK1/2 was one protein kinase located in the cytoplasm. In the presence of external stimulus, the cells would activate ERKl/2 by phosphorylation. The phosphorylated ERK1/2 proteins passed through the nuclear membrane, and then phosphorylated a series of transcription factors, including Elk-1, c-Jun, c-Fos, AP-1 and c-Myc. These transcription factors further regulated the transcription and expression of target genes, which finally initiated and regulated cell proliferation, differentiation and apoptosis (Yang *et al*., 2003).

In this study, extended exposure time of JB decreased the expression quantity of p-ERK1/2 and p-Akt, which demonstrated that MEK/ERK and PI3K/AKT signal transduction pathway was involved in the regulation of JB-induced apoptosis in A549 cells. As A549 cells were treated with MEK/ERK and PI3K-Akt pathway inhibitor, the apoptosis rates of A549 cells treated with PD98059+JB or LY294002+JB were higher than that in JB group. It indicated that PD98059 and LY294002 could increase the apoptosis rates of lung cancer A549 cells through inhibiting MEK/ERK and PI3K-Akt pathway, respectively using JB. It also proved that MEK/ERK and PI3K-Akt signal transduction pathways were involved in the apoptosis of A549 cells.

CONCLUSION

All the results above demonstrated that JB could induce apoptosis of lung cancer A549 cells by activating endogenous mitochondrial pathway along with the inhibition of MEK/ERK and PI3K/Akt pathway. It indeed showed that JB could well induce tumor cell apoptosis and regulate complex signal transduction pathways. As such, this study provides an efficient method for the treatment of lung cancer. However, only some functional proteins and signal transduction pathways were demonstrated and discussed, but the detailed molecular mechanism remains unknown, so a further study is needed to further identify JB-induced apoptosis. Moreover, as a traditional Chinese medicine extract, JB has small side effects to the patient, so it seems to be a potential tumor preventive agent.

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