Pachymic acid inhibits growth and induces cell cycle arrest and apoptosis in gastric cancer SGC-7901 cells

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Abstract. The aim of the present study was to elucidate the anticancer effect of pachymic acid (PA) in gastric cancer SGC-7901 cells and the potential molecular mechanisms involved. Cell Count kit-8 assay was performed to examine the effect of PA on the cell proliferation of SGC-7901 cells. Cell cycle, cell apoptosis, mitochondria membrane potential (Δψm) and reactive oxygen species (ROS) analysis were assessed by flow cytometry, respectively. DNA fragmentation assay was performed by Hoechst 33258 staining. Western blotting was performed to detect the effect of various concentrations of PA on the levels of BCL2 associated X protein (Bax) expression as well as B-cell lymphoma 2 (Bcl-2), cytochrome C (cyt-c) and caspase-3 in SGC-7901 cells. It was demonstrated that PA was able to significantly inhibit the viability and induce G0/G1 cell cycle arrest of SGC-7901 cells in a concentration-dependent manner. The apoptotic rate and ROS generation were markedly increased, while Δψm was decreased following the treatment of SGC-7901 cells with various concentrations of PA. Moreover, the expression of Bax, cytochrome c and caspase-3 were markedly increased and Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) was significantly inactivated and BCL-2 expression was decreased following PA treatment in SGC-7901 cells. Further in vivo study indicated that treatment with PA significantly inhibited the growth of tumor in nude mice that were transplanted with SGC-7901 cells in a concentration-dependent manner. These results may advance the current understanding of the anticancer mechanisms of PA in gastric cancer.

Introduction

Gastric cancer is a highly invasive and aggressive malignancy that ranks the fourth most common cancer and the leading cause of cancer-associated mortality in China (1,2). The incidence of gastric cancer is approximately two times higher in men compared with women. Studies have shown that individuals infected with Helicobacter pylori have an increased risk of gastric cancer (3,4) and a poor prognosis in most countries, with a 5-year relative survival rate <30% (5).

The advancement of modern medicine and technology has been accompanied with a series of intensive studies on cancer tumorigenesis. The application of naturally occurring compounds with anticancer properties can be regulated by a variety of mechanisms, including crosslinking of DNA strands and immune responses, induction of cell cycle arrest, which consequently lead to cell death (6,7). A number of studies indicated that cancer cell apoptosis could be considered a point of convergence for all anticancer therapies, and active phytochemicals have a direct role in promoting apoptosis (8,9).

Pachymic acid (PA) is a lanostane-type triterpenoid from Poriacocos, which is an important component in traditional Chinese medicine. Previous studies have shown that PA possesses anti-emetic, anti-inflammatory and anticancer properties. It has been demonstrated that PA is able to inhibit the proliferation and invasion of pancreatic cancer cells by decreasing MMP-7 expression (10). PA was able to activate reactive oxygen species (ROS)-dependent JUN N-terminal kinase mitochondrial and endoplasmic reticulum stress pathways following cell cycle arrest at G2/M phase and cell apoptosis occurred in lung cancer cells (11). PA reduced proliferation and induced apoptosis through inactivation of AKT signaling and downregulation of AKT downstream protein expression in prostate cancer cells (12). Another study demonstrated that PA is able to stimulate glucose uptake by enhancing the expression and translocation of glucose transporter type 4 (13). However, the therapeutic effects and potential mechanisms of PA on gastric cancer are poorly understood and therefore remain to be evaluated.

The present study aimed to examine the hypothesis that PA is able to have an effect on the viability, cell cycle progression and apoptosis of SGC-7901 human gastric cancer cells in vitro. Herein, the effects of PA on DNA synthesis, mitochondrial function and ROS production, as well as the
expression levels of Bax (BCL2 associated X protein)/B-cell lymphoma 2 (Bcl-2) ratio, cytochrome c (cyt-c) and caspase-3 and the activation of Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) in SGC-7901 were also investigated.

Materials and methods

Materials. PA was purchased from Nanjing Zelang Medical Technological Co. Ltd., (Nanjing, China) and dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml and stored at -20°C. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was supplied by Bioinquirer Technology Co. Ltd. (Nanjing, Jiangsu, China). Hoechst 33258 was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Tetramethylrhodamine, methyl ester (TMRM) was purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). The fluorescent probe dihydroethidium (DHE) was purchased from Vigorous Biotechnology Beijing Co., Ltd (Beijing, China).

Cell culture. The SGC-7901 human gastric cancer cell line was obtained from American Type Culture Collection, (Manassas, VA, USA). SGC-7901 cells were cultured in DMEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO2.

Cell proliferation assay. The SGC-7901 human gastric cancer cell line was obtained from American Type Culture Collection, (Manassas, VA, USA). SGC-7901 cells were cultured in DMEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO2.

Flow cytometric analysis of cell cycle. SGC-7901 cells (3x10^5 cells/well) were dispensed into 6-well plates and treated with different concentrations of PA (20, 40 and 80 µM) for 24 h. Then, the cells were harvested and fixed with 3% paraformaldehyde for 5 min at room temperature. After dying, the cells were stained with Hoechst 33258 (10 ml) at 10 min, following mounting with 20 mM citric acid and 50 mM orthophosphate in 50% glycerol. The cells were then stored at -20°C prior to analysis. Following treatment, the cells were washed with PBS, and features of apoptosis (including condensed and fragmented nuclei) were evaluated using a fluorescence microscope (DMI3000B; Leica Microsystems GmbH, Wetzlar, Germany).

DNA fragmentation assay. SGC-7901 cells were dispensed into 6-well plates and treated with a series of concentrations of PA (20, 40 and 80 µM) for 24 h. Then, the cells were harvested and fixed with 3% paraformaldehyde for 5 min at room temperature. After dying, the cells were stained with Annexin V FITC and 5 µl PI for 10 min in an ice bath in the dark prior to flow cytometric analysis.

Flow cytometric analysis of Dψm and ROS generation. SGC-7901 cells (3x10^5 cells/well) were dispensed into 6-well plates and treated with different concentrations of PA (20, 40 and 80 µM) for 24 h at 37°C. Dψm was detected using TMRM. A total of 1x10^6/ml SGC-7901 cells were resuspended with 100 nM TMRM, and the cells were incubated in the dark at 37°C for 15-20 min and analyzed using a flow cytometer. The intracellular levels of ROS were assessed using the fluorescent probe DHE. The SGC-7901 cells (1x10^6/ml) were resuspended with 50 µM DHE at 37°C for 30 min, and the intensity of fluorescence was measured using a flow cytometer.

Western blot analysis. The cells (1x10^6 cell/well) were dispensed into 6-well plates followed by treatment with PA and AG490 for the indicated times and lysed using radioimmuno-precipitation buffer containing protease inhibitor Beyotime Institute of Biotechnology, Inc.). The protein concentration was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The proteins (30 µg) were separated by 10% SDS-PAGE and further transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk overnight at 4°C, the membranes were probed with specific primary antibodies: Polyclonal rabbit anti-Bax (1:1,000; cat. no. sc493; Santa Cruz Biotechnology, Inc.), polyclonal rabbit anti-Bcl-2 (1:1,000; cat. no. sc-492; Santa Cruz Biotechnology, Inc.), polyclonal mouse anti-cyt-c (1:1,000; cat. no. ab13575; Abcam, Cambridge, MA, USA) and monoclonal rabbit anti-caspase-3 (1:500; cat. no. ab44976; Abcam) overnight at 4°C. GAPDH was used for the normalization of each protein to ensure the loading of equal quantities of protein. After washing three times with TBST for 15 min, the blots were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. A0208 and A0216; Beyotime Institute of Biotechnology) in TBST for 1 h at room temperature. Following another round of washing, the signals were detected by enhanced chemiluminescence method (Pierce; Thermo Fisher Scientific, Inc.) and quantified by densitometry (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Tumor xenograft model. In order to clarify the role of PA in vivo, 5-week-old male athymic nude mice (n=24) were used...
in the present study. A total of 2x10⁶ SGC-7901 cells were injected subcutaneously into the left armpit of these nude mice to establish the gastric cancer xenograft model. A total of 24 male athymic nude mice were randomly assigned to four different groups with six mice per group: Vehicle control (0.1% DMSO in physiological saline) and PA (10, 30 and 60 mg/kg). Following 1-2 weeks of tumor formation, the tumor size was determined every 4 days as previously described (14). The mice were sacrificed, and the tumors were weighed on a digital balance following intraperitoneal injection of PA every day for 4 weeks. All the experiments were approved by the Animal Ethics Committee at Gongli Hospital of Shanghai Pu Dong New District (Shanghai, China).

Statistical analysis. The data was presented as the mean ± standard deviation, and significant differences between two groups were analyzed with the unpaired, two-tailed Student t-test. One-way analysis of variance, followed by
Tukey’s post hoc test, was used to analyze the significance of differences between more than two groups. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PA inhibits the viability of SGC-7901 gastric cancer cells in vitro.** To examine the biological function of PA in gastric cancer, CCK-8 assay was performed to investigate the viability of SGC-7901 cells following exposure to different concentrations of PA (0, 20, 40 and 80 µM) for 12, 24, 48 and 72 h. As shown in Fig. 1A, PA induced significant decreases in the viability of SGC-7901 cells in a time- and concentration-dependent manner. To examine whether the inhibitory effect of PA on the viability of SGC-7901 cells was associated with induction of cell cycle arrest, flow cytometry was performed on the SGC-7901 cells that were treated with different concentrations of PA (0, 20, 40 and 80 µM) for 24 h. As shown in Fig. 1B and C, the cell population in G0/G1 phase of SGC-7901 cells was significantly increased by PA treatment in a concentration-dependent manner, accompanied with a decrease in the cell population in S phase, suggesting that PA is able to induce cell cycle arrest at G0/G1 phase. However, PA treatment did not affect the cell population at G2/M phase of SGC-7901 cells. These observations indicate that the inhibition of cell growth by PA is implicated with the induction of G0/G1 phase arrest.

**PA induces the apoptosis of SGC-7901 gastric cancer cells in vitro.** It was further examined whether the inhibitory effect of PA on the viability of SGC-7901 cells was associated with induction of apoptosis. Flow cytometry using Annexin V-PI staining analysis were performed on the SGC-7901 cells that were treated with various concentrations of PA (0, 20, 40 and 80 µM) for 24 h. The data indicated that PA treatment significantly increased the apoptosis of SGC-7901 cells in a concentration-dependent manner (Fig. 2A). Furthermore, the effects of PA on apoptosis were examined by Hoechst 33258 staining, which is able to differentiate between normal and apoptotic cells. Data from fluorescence microscopy indicated that PA treatment markedly increased the apoptosis of SGC-7901 cells in a concentration-dependent manner, where chromosome condensation and nuclear fragmentation were observed in PA-treated SGC-7901 cells (Fig. 2B). Taken together, these results indicated that the inhibition of cell growth and the
induction of cell cycle arrest by PA are implicated with the induction of apoptosis.

Effects of PA on mitochondrial membrane potential (Δψm) and ROS generation. Mitochondria have an essential role in death signal transduction during the apoptotic process, where the mitochondrial membrane pores are opened and Δψm is disrupted (15). As presented in Fig. 3A, data from flow cytometry indicated that PA-treated SGC-7901 cells exhibited a significant decrease in Δψm in a concentration-dependent manner in comparison with the control cells. Previous studies have reported that ROS generation is able to trigger cell apoptosis by activating the mitochondrial pathway (16). Fluorescence probe DHE was therefore used in SGC-7901 cells that were treated with different concentrations of PA (0, 20, 40 and 80 µM) for 24 h. As indicated in Fig. 3B, the treatment with PA significantly increased the intracellular accumulation of ROS in a concentration-dependent manner. Taken together, these data demonstrate that induction of cell apoptosis by PA is implicated with the mitochondrial pathway.

Effects of PA on protein expression and JAK2/STAT3 activation in SGC-7901 gastric cancer cells. To elucidate the mechanism of PA-induced apoptosis in SGC-7901 cells, the expression of apoptosis-associated proteins was detected by western blotting. As indicated in Fig. 4A, the treatment with PA (20, 40 and 80 µM) for 48 h increased the levels of Bax, cyt-c and caspase-3 proteins in a concentration-dependent manner in comparison with the control cells, but decreased the expression level of Bcl-2. Moreover, the inactivation of JAK2 and STAT3 was also detected in SGC-7901 cells that were treated with different concentrations of PA (Fig. 4B). Taken together, these data indicate that induction of cell apoptosis by PA is implicated with modulation of Bcl-2, Bax, cyt-c and caspase-3 expression as well as the inactivation of JAK2/STAT3 signaling.

Inhibition of the JAK2/STAT3 signaling pathway inhibits cell viability and induces apoptosis in SGC-7901 gastric cancer cells. To confirm that the PA-induced tumor inhibitory effects are dependent on the downstream activation of JAK2/STAT3, the effects of a specific JAK2 inhibitor (AG490) on the viability and apoptosis of cells that were treated with AG490 (100 µM) and in the absence or presence of PA (80 µM) were determined. The treatment of SGC-7901 cells with AG490 (100 µM) for 1 h resulted in the inhibition of JAK2 and STAT3 activation (Fig. 5A). Furthermore, the treatment with AG490 alone or in combination with PA significantly inhibited the viability and induced the apoptosis of SGC-7901 cell compared with the control (Fig. 5B-D). Consistent with these findings, AG490 treatment in SGC-7901 cells resulted in increases in the levels of Bax, cyt-c and caspase-3 proteins in comparison with the control cells, but the expression level of Bcl-2 was decreased (Fig. 5E). These data suggest that PA is able to inhibit cell viability and induce apoptosis via inactivation of the JAK2/STAT3 signaling pathway in SGC-7901 cells.

Antitumor activity of PA in vivo. In order to elucidate the antitumor activity of PA in vivo, tumor xenograft models...
with SGC-7901 cells were employed. As presented in Fig. 6A and B, PA treatment at concentrations of 10, 30 and 60 mg/kg significantly suppressed tumor growth following 28 days in a concentration-dependent manner in comparison with the control group. Moreover, 28 days following treatment with various concentrations of PA, tumor weight was significantly decreased in a concentration-dependent manner in comparison with the control group (Fig. 6C). These results suggest that PA exhibited potent antitumor activity in vivo.

Discussion

Although chemotherapy is the primary method for the treatment of gastric cancer, the overall survival rate for patients with advanced gastric cancer remains low (5). Natural products have a critical role as an effective source of antitumor agents. Although PA is a promising bioactive molecule, which possesses an anti-carcinogenic activity in numerous types of cancer (12,17,18), the biological activity of PA in gastric cancer is poorly understood. In present study,
the data indicated that PA was able to inhibit cell viability in a concentration- and time-dependent manner. Previous studies have shown that cell cycle arrest and apoptosis are the mechanisms that induce cell death (19). In the present study, it was observed that treatment with PA was able to induce G_0/G_1 phase arrest and apoptosis in SGC-7901 gastric cancer cells. In agreement with our results reported that PA induced cell cycle arrest and apoptosis in lung cancer cells (11) and gallbladder cancer cells (20).

ROS are metabolites with high activity produced during normal cellular metabolism. However, the increase in intracellular ROS in cells may be sufficient to induce apoptosis (21). Furthermore, it has been reported that cell apoptosis is preceded by the following: ROS generation, loss of ΔΨ_m, release of cyt-c and activation of caspase-3 (22). Referring to the results of a previous study and the present study, ROS may be implicated in PA-induced activation of the mitochondrial pathway (11). A decrease in ΔΨ_m and increased intracellular accumulation of ROS were associated with PA-induced activation of the Bax/Bcl-2 signaling pathway.

The expression level of Bax is an important indicator of cell apoptosis, which indicated the involvement of the mitochondrial signaling pathway PA-induced apoptosis of SGC-7901 cells. Once activated, Bax is inserted into the mitochondrial membrane, which leads to mitochondrial dysfunction (23). The data of the present study indicated that the expression of Bcl-2 decreased gradually and the expression of Bax in the PA treatment group was increased in a concentration-dependent manner. Bcl-2 regulates apoptosis mainly by preventing the release of cyt-c from the mitochondrial to cytoplasm, activating downstream caspases and ultimately caspase-3 (24). In the present study, caspase-3 activation was coincident with cyt-c release, which suggests that the activation of caspase-3 is involved in mitochondrial apoptotic pathway, which was observed in SGC-7901 cells that were treated with PA.

The pathogenic role of JAK/STAT signaling pathway has been documented in a number of cancer types, including breast, ovarian, lung, colorectal and gastric cancer (25-29). In the present study, treatment with PA also resulted in the inactivation of JAK2 and STAT3 in SGC-7901 cells in a dose-dependent manner, suggesting an important role of the JAK2/STAT3 signaling pathway in mediating the inhibitory effects of PA on tumor growth. These observations are consistent with previous findings by the present authors where OPB-31121, a novel small molecular inhibitor, was demonstrated to disrupt the JAK2/STAT3 signaling pathway and to exhibit antitumor activity in gastric cancer cells (29).

A xenograft model with SGC-7901 cells in nude mice was used to validate the chemotherapeutic potential of PA on the growth of gastric cancer cells in vivo. To the best of our knowledge, this is the first time that the in vivo anti-proliferative activity of PA has been demonstrated, which further supports the concept that the antitumor effects of PA are strongly dependent on its ability to inhibit tumor growth. This is in line with previous findings that 25 mg/kg PA was able to significantly suppress the growth of pancreatic cancer tumor in vivo without toxicity, which was also associated with the inhibition of cell proliferation and apoptosis (30).

In conclusion, the present study demonstrated that treatment with PA was able to result in marked anticancer activity in gastric cancer cells by inhibiting cell proliferation as well as inducing cell cycle arrest and apoptosis via the inactivation of the JAK2/STAT3 signaling pathway. Therefore, PA appears to be a potentially attractive bioactive phytochemical for the treatment of gastric cancer.

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Author contributions
KS and HX conceived and designed the experiments, and wrote the paper. All authors read and approved the final manuscript.
Ethics approval and consent to participate

All the experiments were approved by the Animal Ethics Committee at Gongli Hospital of Shanghai Pu Dong New District (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References