Etoposide induces apoptosis via the mitochondrial- and caspase-dependent pathways and in non-cancer stem cells in Panc-1 pancreatic cancer cells

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Abstract. Pancreatic cancer is a highly aggressive malignant tumor. In the present study, we performed several methods, including CCK-8 assay, immunofluorescence technique, western blotting and flow cytometry, to determine the effects of VP16 (etoposide) on Panc-1 pancreatic cancer cells. The results demonstrated that VP16 inhibited the growth of and induced apoptosis in Panc-1 cells. Western blot analysis showed that VP16 inhibited the expression of Bcl-2 and enhanced the expression of Bax, caspases-3 and -9, cytochrome c and PARP. Notably, a strong inhibitory effect of VP16 on Panc-1 cells mainly occurred in non-CSCs. These data provide a new strategy for the therapy of pancreatic cancer.

Introduction

Pancreatic carcinoma is a refractory disease with few effective therapies (1). Only 18% of patients survive 1 year after diagnosis and the 5-year survival rate is 4%. By the time patients exhibit symptoms and the disease is diagnosed, it is already beyond the early stages (2,3). The capacity of a tumor to grow and proliferate is dependent on a small subset of cells, the cancer stem cells (CSCs), which are immature cells that can replicate or self-renew, and differentiate or grow into different types of cancer cells (4).

VP16 is an important chemotherapeutic agent that is used to treat a wide spectrum of human cancers. It has been in clinical use for more than 2 decades and remains one of the most highly prescribed anticancer drugs in the world (5). This drug is a pro-apoptosis agent which triggers cell death pathways (6).

In the present study, we examined the effect of VP16 on human pancreatic cancer cells and the change of pancreatic CSCs.

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Materials and methods

Cell lines. The Panc-1 human pancreatic cancer cell line was purchased from the Shanghai Cell Bank (Shanghai, China).

Cell growth inhibition assays. The inhibitory effect of VP16 (etoposide; Qilu Pharmaceutical Co., Ltd., Shandong, China) on the growth of Panc-1 cells was evaluated by CCK-8 kit (Sigma-Aldrich, Ireland) (7). In brief, 200 μl medium/well containing $2x10^3$ cells were seeded in 96-well microtiter plates for the CCK-8 assays. After treatment with VP16, cells in the 96-well plates were used for CCK-8 assays. The optical density was measured at 490 nm using a microplate reader (SpectraMax 340; Molecular Devices Co., Sunnyvale, CA, USA).

The cell viability ratio was calculated using the following formula: Inhibitory ratio (%) = (OD$_{control}$ - OD$_{treated}$)/OD$_{control}$ x 100% (8). Results from 3 independent experiments in triplicates are presented.

Cell cycle analysis. Cell cycle distribution was analyzed as previously described (9). In brief, $1x10^6$ cells were incubated with the indicated amounts (0-300 μg/ml) of VP16 for 48 h. Cells were collected and then fixed in 70% ethanol at 4˚C for 24 h. Cells were washed again with PBS and incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37˚C for 30 min. Cell DNA content was measured using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Annexin V/PI analysis. Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate as previously described (10). In brief, $1x10^6$ cells were incubated with the indicated concentrations (0-300 μg/ml) of VP16 for 48 h. Five microliters Annexin V-FITC and 5 μl PI were added. Cells were vortexed and incubated for 15 min in the dark. Binding buffer (200 μl) was added to each tube. Flow cytometric analysis was performed immediately after staining. All assays were independently repeated for 3 times.

Mitochondrial membrane potential ($\Delta\psi m$) assay. The loss of $\Delta\psi m$ in the cells is one of the mechanisms of induction of apoptosis, which has been linked to the initiation and activation of apoptotic cascades (11). With a variety of stimuli, including

the translocation of Bax from the cytosol to the mitochondria, this event occurs, which triggers the release of cytochrome c from the mitochondria to the cytosol (12). In brief, 1×10^6 cells were incubated with the indicated amounts (0-300 μg/ml) of VP16. Cells were incubated with JC-1 dye for 15 min at 37˚C, and then resuspended in 200 μl PBS for FACS analysis.

Protein extraction and western blot assays. The expressions of proteins were evaluated using western blot analysis, as previously described (13). In brief, cells were treated with different concentrations of VP16 (0-300 μg/ml) for 48 h. Cells were collected and suspended in 5 volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail). Lysates were collected. Supernatant samples containing 40 μg total protein were loaded on SDS-PAGE gel for electrophoresis. Membranes were blocked for 1 h with 5% milk. Membranes were then incubated overnight at 4˚C with primary antibodies (caspases-3 and -9, cytochrome c, PARP, Bcl-2, Bax and β-actin) and with horseradish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature. The experiment was repeated 3 times. Nuclear and Cytoplasmic Protein Extraction kit was used to extract protein of cytochrome c.

Quantification of caspase-3 activity. Caspase-3 activity was analyzed using a caspase-3 activity assay kit (Beyotime, China). Cells were treated with the indicated reagents for 24 h and cell lysates were prepared for the following experiment according to the manufacturer's instructions.

Change of antigens of CSCs by flow cytometry. Previous studies found that malignant tumors were heterogeneous. This subpopulation of CSCs has been demonstrated to be responsible for tumor initiation, proliferation, recurrence and resistance to chemotherapy (14-18). On the basis of the above findings, we hypothesized that the ratio of CSCs and non-CSCs must change during treatment of VP16. Cells were treated with VP16 (100 μg/ml) for 48 h, and then flow cytometry was performed to detect expression of CSC markers, such as CD133, CD44, CD24 and ESA antigens (BioLegend Inc., San Diego, CA, USA). Each analysis included 100,000 events. Isotype-matched mouse IgG was used as a control.

Statistical analysis. All data are expressed as means ± SD. Statistical comparisons of results were made using analysis of variance (ANOVA). Differences between groups were determined using the Student's t-test for unpaired observations and P-values <0.05 were considered to indicate statistically significant differences.

Results

VP16 induces apoptosis of Panc-1 cells. A dose-dependent reduction in cell growth was observed. The ratio of inhibition with different concentrations of VP16 is shown in Fig. 1. These results indicated that VP16 inhibited Panc-1 cell proliferation in a dose- and time-dependent manner.

Effects of VP16 on cell cycle and apoptosis in Panc-1 cells. When we stained Panc-1 cells with Annexin V-FITC and PI,
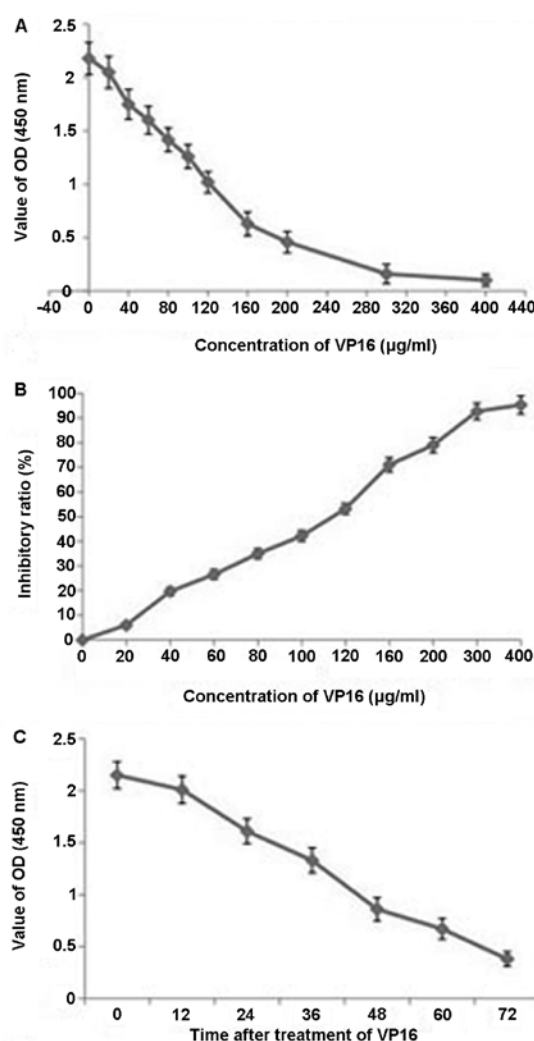

Figure 1. Effect of VP16 on Panc-1 cells. (A) Cell viability was determined by CCK-8 assay; cells were treated with the indicated concentrations of VP16 for 48 h. (B) Inhibitory rate of Panc-1 cells with different concentrations of VP16. (C) Cell viability was determined by CCK-8 assay; cells were treated with VP16 100 μg/ml for different times.

the proportion of apoptotic cells was significantly increased in a dose-dependent manner (Fig. 2).

The effect of VP16 on cell cycle distribution indicated that there was a higher number of cells in the G1 phase, in higher concentration of VP16 compared with the control (P<0.05) and, at the same time, the number of S phase cells was significantly decreased (Fig. 3).

VP16 induces loss of $\Delta\psi$m and subsequently enhances the release of cytochrome c in Panc-1 cells. VP16 in Panc-1 cells resulted in a dose-dependent increase in the number of JC-1 dye-positive cells from 8.67 to 92.9%. Western blot analysis revealed that VP16 caused a dose-dependent increase in the release of cytochrome c into cytosol, which confirmed the disruption of the $\Delta\psi$m after VP16 treatment (Fig. 4).

Involvement of caspase-3 activation. Caspases are a family of cysteine proteases that are essentially involved in the apoptotic pathway. We investigated whether caspase-3 was involved in apoptosis induced by VP16 in Panc-1 cells. The
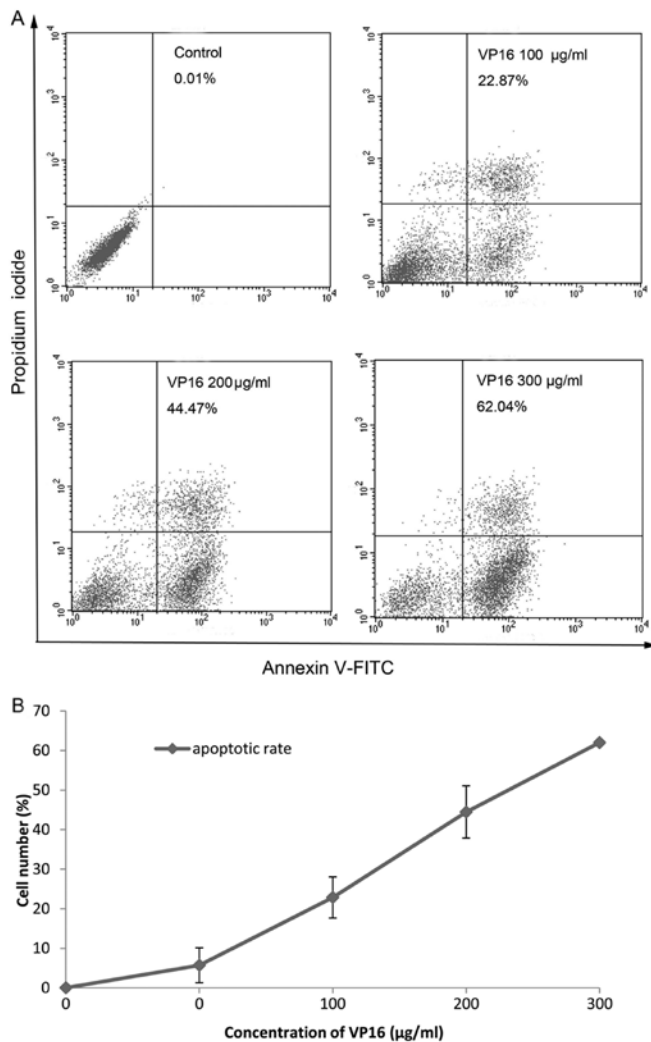
Figure 2. Apoptosis detection of Panc-1 cells by flow cytometry. (A) Panc-1 cells treated with different concentrations of VP16 were detected with PI and Annexin V-FITC staining. (B) Graphs show the percentage of Annexin V-positive stained cells.
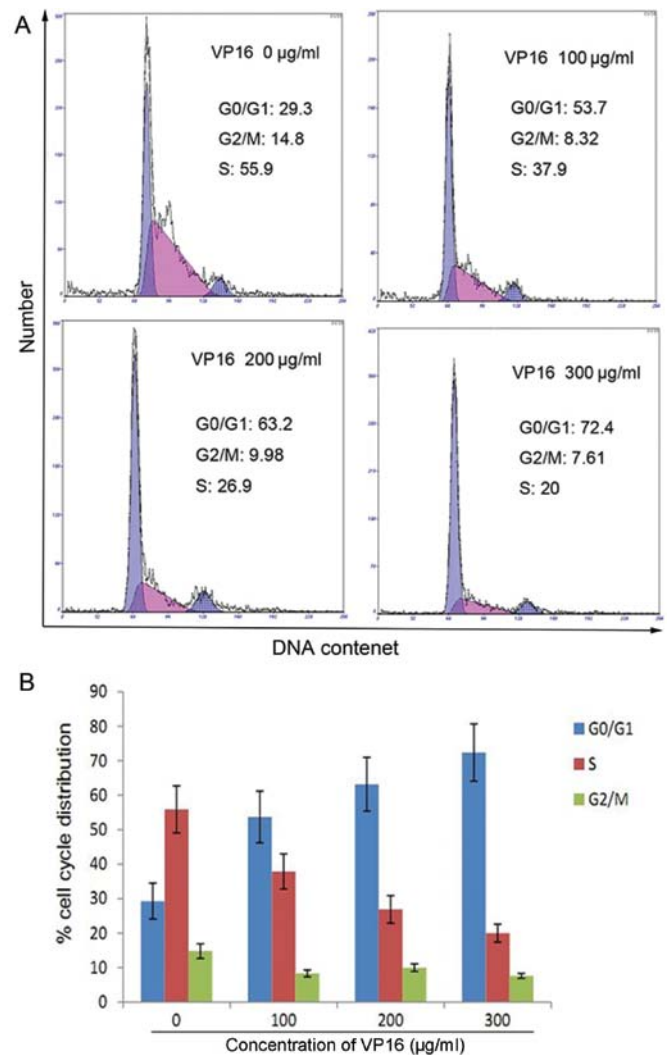

Figure 3. Effect of VP16 on the cell cycle progression of Panc-1 cells by flow cytometry. (A) Cellular DNA was stained with PI in Panc-1 cells treated with different concentrations of VP16. (B) Data from the cell cycle distribution are summarized.

data showed that activity of caspase-3 increased significantly in a dose- and time-dependent manner in the cells treated with VP16 (Fig. 5A). In addition, activity of caspase-3 decreased in the combined treatment with VP16 and caspase-3 inhibitor (Ac-DEVD-CHO) compared with the VP16 treatment group (Fig. 5B). The results demonstrated that VP16-induced apoptosis was dependent on caspase-3.

Expression of apoptosis-related proteins in VP16-treated cells. These data showed that treatment of VP16 in Panc-1 cells resulted in a dose- and time-dependent reduction in the levels of the anti-apoptotic proteins and an increase in the level of pro-apoptotic proteins (Fig. 6).

Cells treated with VP16 highly express CD44, CD24, ESA and CD133. Results from the above studies and hypotheses of CSCs, prompted us to study the change of CSC-related markers. The data were: CD133, 0.31 vs. 1.54%; CD24, 19.51 vs. 69.47%; CD44, 21.55 vs. 93.23%; ESA, 1.51 vs. 8.38% (P<0.05, Fig. 7). The data demonstrated that most of the cells

killed by VP16 were non-CSCs and that CSCs derived from Panc-1 cells played key role in chemotherapy resistance.

Discussion

In the present study, we investigated the molecular mechanisms of VP16-induced apoptosis in Panc-1 cells and the change of markers of CSCs. VP16 inhibited the growth of cells in a dose- and time-dependent manner. In addition, VP16 markedly induced apoptosis in Panc-1 cells in the same manner, which was confirmed by Annexin V and PI staining. Based on the above assays, we observed a strong growth inhibitory effect of VP16 in Panc-1 cells; we then determined the possible mechanism of anti-proliferative activity of VP16. Our data demonstrated that treatment with VP16 in Panc-1 cells induced G1 phase arrest of cell cycle, indicating that one of the mechanisms was inhibition of cell cycle progression. Loss of mitochondrial membrane potential was suicidal to cells as they became bioenergetically deficient (30) and that led to release of cytochrome *c* into the cytosol. As the level of cytochrome *c*
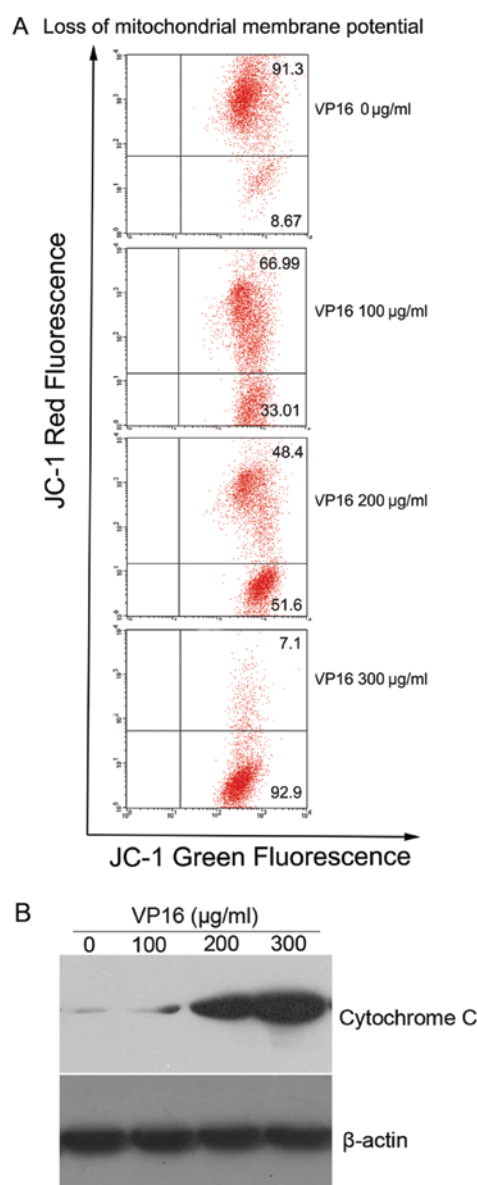
Figure 4. Treatment of Panc-1 cells with varying concentrations of VP16 induced the loss of mitochondrial membrane potential (MMP) and, subsequently, the release of cytochrome c. (A) Treatment of Panc-1 cells with VP16 induced loss of MMP in a dose-dependent manner. (B) Cytosolic fractions were also prepared from the above mentioned treatment groups and subjected to western blot analysis to detect the levels of the cytochrome c. β-actin was used to verify equal loading of the protein samples on the gel.
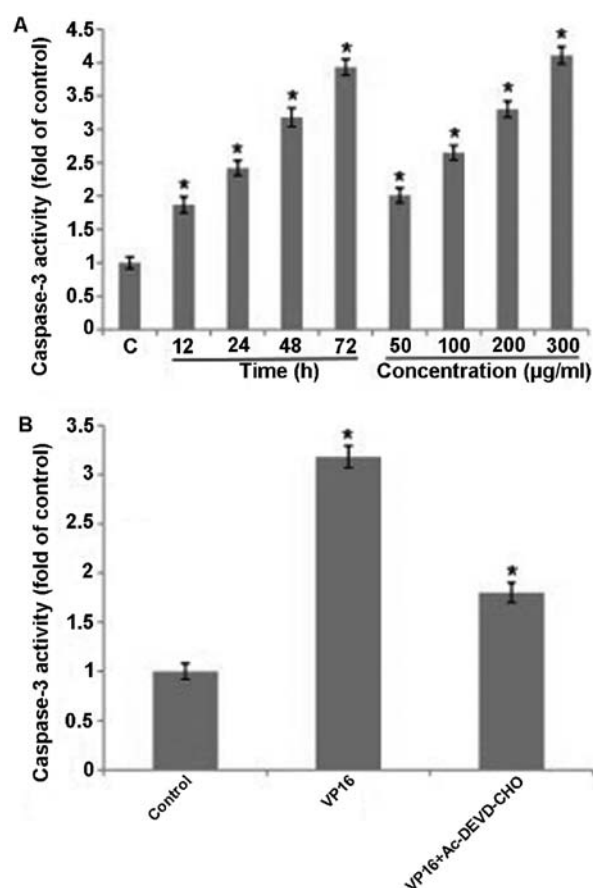

Figure 5. Assay of caspase-3 activity in cells treated with VP16. (A and B) The graphs show caspase-3 activity in Panc-1 cells with different treatment methods, as described in Materials and methods.

increased in the cytosol, it interacted with Apaf-1 and ATP formed a complex with pro-caspase-9, leading to activation of caspases-9 and -3 which led to the cleavage of PARP (19,20).

Mitochondria depolarization, considered an irreversible step in the apoptosis process, could trigger a cascade of caspases. The mitochondrion was a major subcellular compartment where the Bcl-2 family members interacted with each other or exerted function independently (21).

The Bcl-2 family of proteins include proteins that could either inhibit (Bcl-2, Bcl-xL, etc.) or induce (Bax, Bak, Bad, etc.) apoptosis. The anti-apoptotic proteins prevented cytochrome c release by forming heterodimer complexes with pro-apoptotic Bcl-2 family proteins (22).

The pro-apoptotic Bcl-2 proteins including Bax and Bak facilitate release of apoptogenic molecules from mitochondria to the cytosol and accelerate apoptotic cell death (23-25).

To evaluate the role of caspases, the master executioner during apoptotic cell death, in VP16-induced cell death, the activities of caspase-3 were assessed using the caspase-3 colorimetric assay kits. The data suggested that caspase-3 inhibitor could significantly inhibit the activities of caspase-3 in the VP16-induced cell death, indicating that apoptosis in VP16-treated Panc-1 cells occurred in a caspase-dependent manner.

VP16 induces apoptosis in various cancer cell lines and performs antitumor activity (3-5). In the present study, we observed a strong inhibitory effect of VP16 on Panc-1 cells *in vitro*, indicating chemotherapy drugs could kill cancer cells. However, pancreatic cancer patients continue to have a dismal prognosis with an average overall median survival of 4-6 months. The overall 5-year survival is <5% (1). The preliminary data suggested that tumor-cell repopulation describes the continuing proliferation of surviving tumor stem cells that can occur during a course of radiotherapy or chemotherapy (26-29).

These findings led to our investigation of the role of CSCs during cytotoxic cancer therapy. Our results suggested that CSCs were more resistant to VP16 and most CSCs could escape cytotoxic cancer therapy. The relationship between CSCs and repopulation during the process of radiotherapy and chemotherapy will be our next research project.
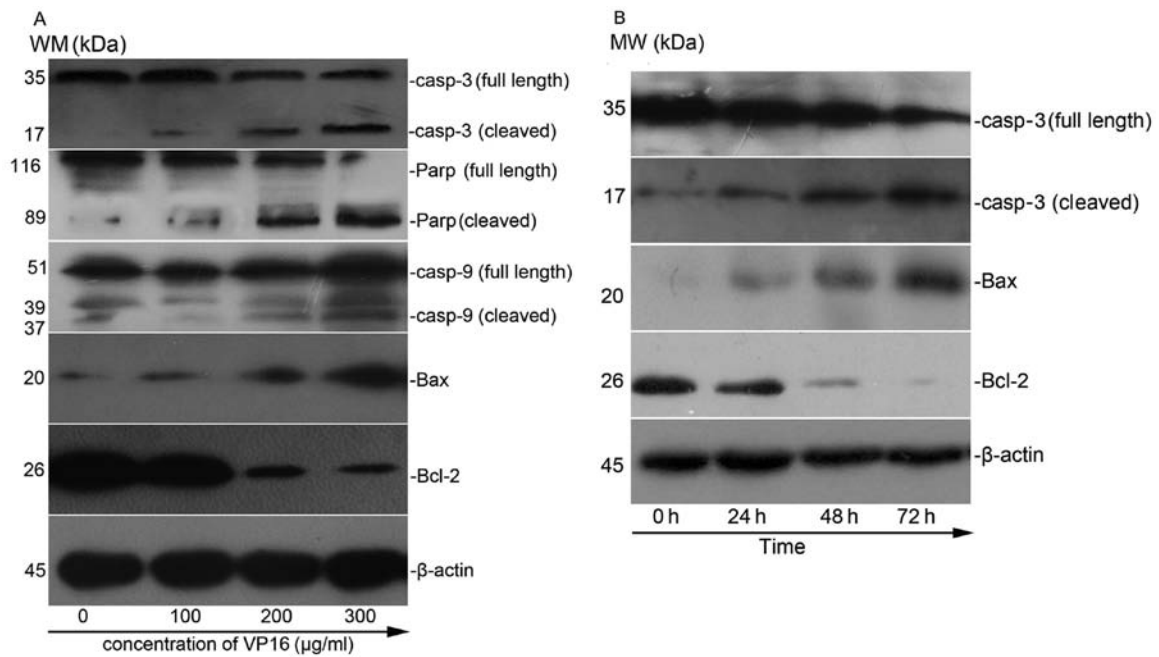
Figure 6. The expression of apoptosis-related proteins in Panc-1 cells was evaluated by western blotting. (A and B) Panc-1 cells were treated with different methods, then cells were harvested and subjected to western blot analysis to detect the levels of caspases-9 and -3, cleaved caspases-9 and -3, PARP, Bcl-2 and Bax. Equal protein loading was confirmed by probing stripped blots for β-actin as shown.
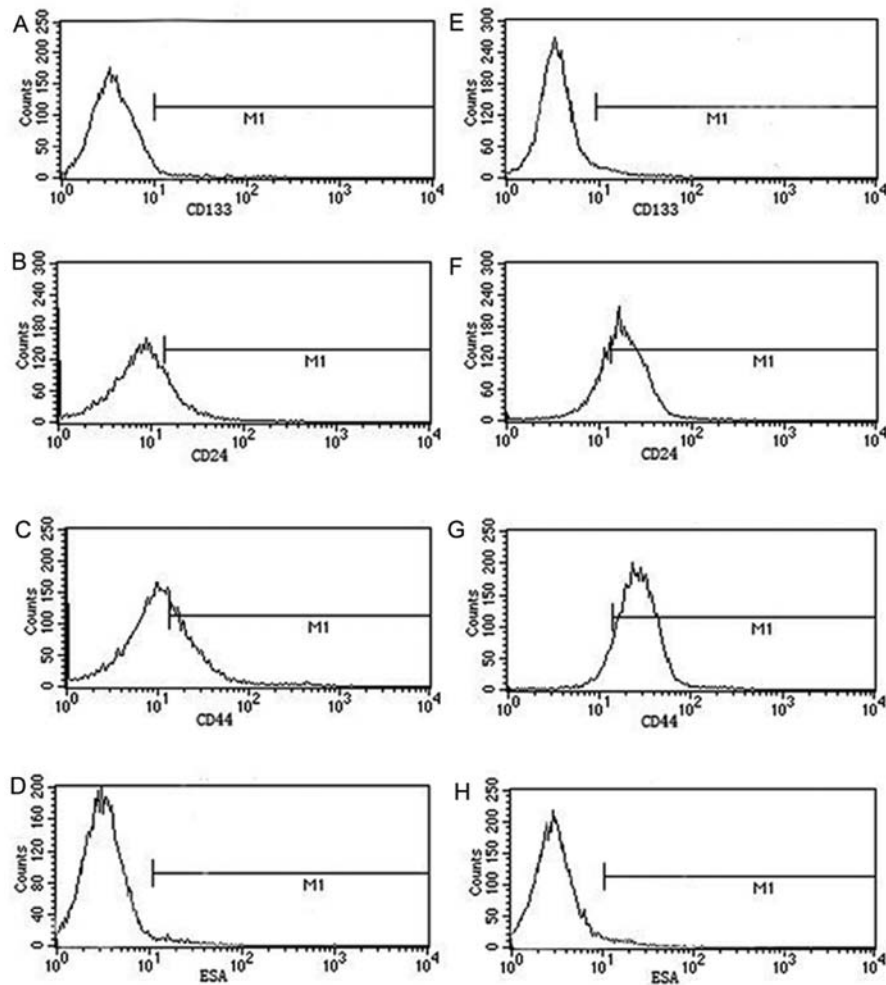

Figure 7. The expression of CSC markers prior to and following treatment with VP16 (P<0.05). Panels A-D, expression of CD133, CD24, CD44 and ESA antigens in Panc-1 cells without treatment, respectively. Panels E-H, expression of CD133, CD24, CD44 and ESA antigens in Panc-1 cells treated with VP16 (100 μg/ml) for 48 h, respectively.

Acknowledgements

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