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Induction of apoptosis in rat C6 glioma cells by panaxydol

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Abstract

Panaxydol is a naturally occurring non-peptidyl small molecule isolated from the lipophilic fractions of Panax notoginseng, a well-known Chinese traditional medicine. Previous studies have shown that panaxydol inhibited the growth of various kinds of malignant cell lines. To date, there has been no report concerning the effect of panaxydol on cell growth inhibition in glioma cells. In this paper, we examined panaxydol's antiproliferation and proapoptotic effects on rat C6 glioma cells and investigated its mechanism. Cell growth inhibition of panaxydol was determined by MTT reduction assay. Apoptosis of cells was measured by both Hoechst 33258 staining and Annexin V analysis. It was found that panaxydol markedly inhibited proliferation of C6 cells in a dose-dependent manner with ID_{50} of 40 μM. The cell apoptosis was observed at 48 h in the presence of panaxydol. In concert with these findings, Western blot analysis showed a decreased expression of bcl-2 and increased levels of Bax and caspase-3 in C6 cells treated by panaxydol. In conclusion, panaxydol has profound effects on growth and apoptosis of C6 cells, suggesting that panaxydol may be a potential candidate for the treatment of malignant gliomas.

Keywords: Apoptosis; C6 glioma cells; Panaxydol

1. Introduction

Panaxydol is a naturally occurring non-peptidyl small molecule isolated and purified from the lipophilic fractions of *Panax notoginseng* (Araliaceae), a well-known Chinese traditional medicine (Lin et al., 2002). Previous studies have shown that panaxydol possesses antiproliferation activity against a variety of cancer cell lines including murine leukemia, heman colon carcinoma, human renal cell carcinoma and human malignant melanoma (Lee and Hwang, 1986; Matsunaga et al., 1990; Moon et al., 2000; Sohn et al., 1998). Sensitivity of glioma cells to panaxydol has not been studied.

Malignant gliomas are the most common brain tumors. Although a number of treatment strategies have been attempted, success in treating these tumors has been limited and the prognosis of patients with malignant gliomas remains poor (Chen et al., 1998). Malignant gliomas have the characteristics of rapid proliferation, cellular migration and invasion. Therefore, new approaches on cellular level are needed for stopping the rapid spread and growth of the tumor cells.

In the present work, we initiated to study the antiproliferation effect of panaxydol on rat C6 glioma cells, and found that panaxydol inhibited the growth of C6 cells significantly in a dose-dependent manner via apoptosis pathway. Our data suggest that panaxydol plays a potential role in the therapy of malignant gliomas.

2. Materials and methods

2.1. Panaxydol isolation and purification

The air-dried roots of *P. notoginseng* (Burk.) F.H. Chen (Araliaceae) were powdered and then extracted with ethanol at room temperature. After filtration,

Abbreviations: DMEM, Dulbecco modified Eagle medium; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorter; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; SEM, standard error of the mean.

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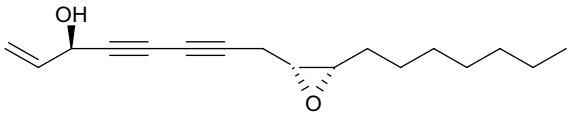
solvent was removed under reduced pressure. The residue was suspended in water, then extracted with hexane. The hexane soluble fractions were chromatographied on a column of silica eluting with hexane and increasing amounts of ethyl acetate to give panaxydol. Panaxydol was identified by MS, ^{1}H and ^{13}C NMR (Lin et al., 2002). Its purity checked by gas chromatography is over 98%. Panaxydol stored at −20 °C and dissolved in DMSO before use. Final DMSO concentration in medium is <0.1% (v/v).



Panaxydol

2.2. Cell culture

Rat C6 glioma cells were obtained from the Chinese Academy of Sciences in Shanghai originally from American Type Culture Collection (Manassas, VA, USA), maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 μM streptomycin in a humidified atmosphere (5% CO_2 in air) at 37 °C.

2.3. Cell proliferation

C6 glioma cells (1×10^5 cells/ml; 100 μl/well) were seeded in 96-well microtiter plates (Nune, Roskilde, Denmark), incubated at 37 °C for 24 h, and treated with either vehicle (0.1% DMSO in medium) or the indicated concentrations of panaxydol for 48 h. *In vitro* growth inhibition was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) reduction assay (Mosman, 1983). In brief, 10 μl of MTT solution was added to each well and mixed by tapping gently on the side of wells; the cells were incubated at 37 °C for 4 h in the CO_2 incubator. The formed MTT formazan was dissolved in 100 μl of 0.04 M HCl in isopropanol. Finally, the absorbance was measured on a microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability was measured using trypan blue exclusion and cultures were >95% viable. Each experiment was repeated three times in triplicate samples. The proliferation of cells was determined by calculating the absorbance of the test wells as the percentage of the control well. The results presented as the average of three independent experiments.

2.4. Assessment of apoptosis

Apoptosis could be determined by staining cells with annexin V-fluorescein isothiocyanate (FITC, PharMingen, San Diego, CA, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) labeling, because annexin V can identify the externalization of phosphatidylserine during the apoptotic progression and therefore detect early apoptotic cells (Vermes et al., 1995). Briefly, cells were harvested after having exposed to the indicated concentrations of panaxydol for 48 h, washed twice with cold PBS and then resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. The cells were incubated with 5 μl of annexin V-FITC for 10 min, and then 10 μl of 20 μg/ml PI for 30 min in the dark. Cells fluorescence was measured on FACScan flow cytometer (Becton Dickinson) using an argon ion laser (488 nm). The quadrant settings were set to allow the negative control less than 1% positivity.

Cells were stained with Hoechst 33258 (8 μg/ml, PharMingen, San Diego, CA, USA), as described previously (Kondo et al., 1995), to observe apoptotic chromatin changes (blebbing, fragmentation, and condensation) under UV-fluorescence microscopy.

2.5. Western blot analysis

5×10^5 C6 cells were grown in 100-mm culture dishes for 24 h, treated with 40 μM panaxydol for 0 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, washed twice with PBS, and solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. The protein concentration was determined using Bio-Rad protein assay reagent. 50 μg of protein/sample were loaded onto 12% (w/v) SDS-PAGE gels, transferred onto nitrocellulose membranes by electroblotting, and probed with anti-bcl-2, Bax and caspase-3 antibodies (1:400, Dako, Carpinteria, CA, USA). The signals were developed using the ECL detection system (Amersham, Arlington Heights, IL, USA). β-actin was used as a control for loading. Densitometric analysis of the films was performed using the KS 400 image analysis system (version 3.0, Zeiss).

2.6. Statistical analysis

The data were expressed as the means ± SEM. One-way ANOVA was used for analyzing values between vehicle and panaxydol-treated groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Panaxydol inhibited proliferation in C6 glioma cells

To determine whether panaxydol influences the proliferation of C6 glioma cells, C6 cells treated with the indicated concentrations of panaxydol for 48 h were observed (Matsunaga et al., 1990) and ID_{50} value was 39.5 ± 2.3 μM (≈40 μM). Based on the ID_{50}, we examined the effect of different concentrations of panaxydol on the C6 cells. Treatment of cells treated with 80 μM panaxydol demonstrated a loss of viability by trypan blue exclusion, whereas viability of cells treated with 60 μM exceeded 95%. Therefore, the cells treated with 20 μM, 40 μM or 60 μM panaxydol were adopted for subsequent experiments. The overmentioned concentrations of panaxydol in this study showed minimal effect on the survival and growth of human fibroblasts and primary rat astrocytes (data not shown). Cell proliferation of C6 glioma cells was then measured in using MTT reduction assay in the presence of panaxydol. The results showed that panaxydol produced a potent dose-dependent inhibition of growth in C6 cells (Fig. 1).

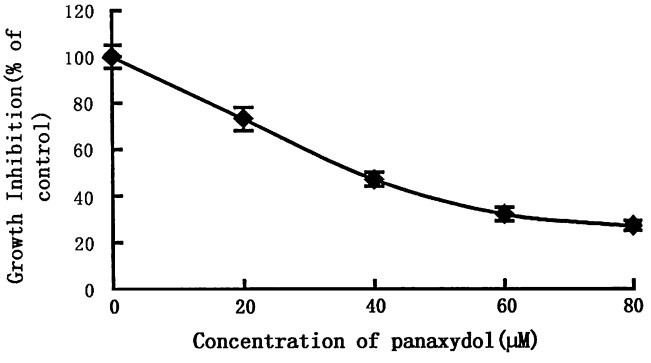


Fig. 1. C6 glioma cells were incubated with the indicated concentrations of panaxydol for 48 h. Cell growth inhibition was assessed by MTT tetrazolium dye reduction assay as described in Section 2. Results represent the means ± SEM of three independent experiments.