Different regulatory pathways are involved in the proliferative inhibition of two types of leukemia cell lines induced by paclitaxel

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Abstract. Paclitaxel, one of the broadest-spectrum anticancer agents, is currently being used in the treatment of patients with solid tumors. In the present study, we compared the effect of paclitaxel on two types of leukemia cells. Our results showed that paclitaxel could inhibit the proliferation of MEL and K562 cells in a dose- and time-dependent manner. The mechanism of proliferative inhibition in K562 cells treated by paclitaxel was related to the cell cycle arrest in the G_2/M phase, as well as the induction of apoptosis. By contrast, MEL cells treated by paclitaxel showed significant characteristics of necrosis, which indicated that the mode of cell death induced by paclitaxel in these two types of leukemia cells differed. Advances in research of the cell cycle, apoptosis and necrosis will extend our understanding of the mechanisms of paclitaxel-induced cell death, particularly in leukemia cells. Further elucidation of the mechanisms of necrosis in MEL cells may expedite the development of improved paclitaxel-based regimens for cancer therapy.

Introduction

Paclitaxel (Taxol®), a potent drug of natural origin isolated from the bark of the Pacific yew, is an important antitumor drug with significant activity against ovarian, lung and breast cancer (1-3). As a drug of cancer chemotherapy, paclitaxel has an unusual chemical structure that is a complex diterpene having a taxane ring with a four-membered oxetane ring and an ester side chain at position C-13 endowed with a unique mechanism of action: it inhibits microtubules from disassembly (4). For example, paclitaxel enhances the polymerization of tubulin to stable microtubules and also interacts directly with microtubules (4) and β-tubulin gene mutations are strong predictors of resistance response to the antitubulin drug paclitaxel (5-7).

Since the unique action of paclitaxel against microtubules was discovered in the 1970s (8), considerable work has been carried out to characterize the mechanisms by which paclitaxel disrupts the normal function of microtubules and arrests the cell cycle at the G_2/M phase. Little attention has been paid to other possible cellular actions of this antineoplastic agent. In 1993, Bhalla *et al* (9) demonstrated for the first time that the exposure of human myeloid leukemia HL-60 and KG-1 cells to clinically achievable concentrations of paclitaxel produced internucleosomal DNA fragmentation of ~200 base-pair multiples, and also showed the morphologic changes characteristic of cells undergoing programmed cell death (PCD) or apoptosis. In recent years, apoptotic cell death induced by paclitaxel has been characterized by several laboratories in epithelial ovarian cancer (10), human gastric carcinoma cell lines (11), prostate tumors (12), adrenocortical carcinoma cell line (13) and human glioma cells (14).

These results clearly indicate that paclitaxel, in addition to its classical effect on microtubules and the arrest of cell cycle, may also possess significant cell-killing activity by induction of apoptosis. In the clinic, paclitaxel has been widely used in several malignant solid tumors, such as ovarian cancer, breast cancer and lung cancer (15). However, a poor level of *in vivo* induction of apoptosis was achieved during a phase I clinical study with paclitaxel therapy in 26 leukemia patients (16). In addition, paclitaxel has a significantly weaker cytotoxic effect on CD34 positive AML cells than CD34 negative leukemia cell lines, such as HL-60 and U937 (16). Considering the fact that leukemia is a heterogeneous disease with different cell types and immune phenotypes, it is necessary to investigate the effect of paclitaxel on different types of leukemia cell lines.

Materials and methods

Cell culture. Two types of established leukemic cell lines (MEL and K562) used in the present study were preserved in our laboratory, and were generous gifts from the Chinese Academy of Medical Sciences and Peking Union Medical College. The cell lines K562 and MEL were cultured in DMEM medium (Gibco-BRL Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing Biotech, Beijing, China) at 37˚C in a humidified atmosphere of 5% CO_2.

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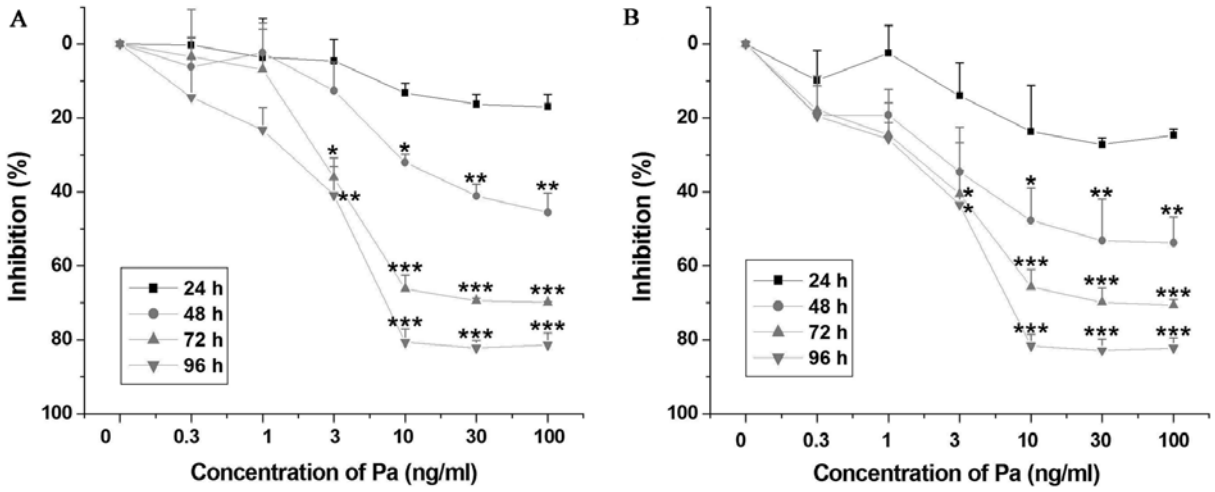

Figure 1. Effects of paclitaxel (Pa) on cellular growth of (A) MEL cells and (B) K562 cells using the MTT assay. The cellular growth was inhibited significantly in a dose- and time-dependent manner. The results are presented as the means ± SEM of at least three independent experiments. *P<0.05, **P<0.01 and ***P<0.001, statistically significant difference vs. the control.

Cell proliferation assay. MTT assay was used to determine the effect of proliferative inhibition on the two types of leukemia cell lines induced by incubation with paclitaxel (Huadong Medicine Co., Ltd., Hanzhou, China). Cells at the exponential growth phase were seeded at $3x10^3$ cells/well in 96-well microtiter plates in a volume of 180 μl medium/well in the presence of various paclitaxel concentrations at a volume of 20 μl or in the presence of isochoric PBS as the control. Then, 10 μl of 5 mg/ml MTT (Sigma) was added to each well and incubated for 4 h at 24, 48, 72 and 96 h, respectively. DMSO (150 μl) was added to dissolve the formazan crystals after centrifugation of the microtiter plates (3,000 x g for 15 min) and the supernatant was gently removed. The absorbance was determined using microplate reader (Varioscan Flash; Thermo Scientific) at 570 nm.

Inverted fluorescent microscope. MEL and K562 cells were incubated in the presence of paclitaxel or buffer alone for 24, 48, 72 and 96 h. The final concentrations of paclitaxel for MEL and K562 cells were 100, 30, 10, 3, 1 and 0.3 ng/ml; Hoechst 33342 (10 μg/ml, 50 μl) was added and the suspension was further incubated for 5 min at room temperature in the dark. Microscopic analysis was carried out with an inverted fluorescent microscope (TE2000-U, Nikon, Tokyo, Japan).

Flow cytometric analysis of apoptosis and necrosis. Cells were first harvested at the exponential growth phase and mixed with paclitaxel at a concentration of 50 ng/ml for MEL cells, and 21 ng/ml for K562 cells, at 37˚C in a humidified atmosphere of 5% CO_2 for 48 h. For cell apoptosis and necrosis analysis, cells were resuspended at $2x10^6$ cells/ml, and stained by Annexin V-FITC (5 μl for 15 min) and PI (10 μl for 5 min) according to the instructions of the Cell Apoptosis kit (Kaiji Bio Co., Nanjing, China) and samples were analyzed with flow cytometry (FACSAria; BD Biosciences, Mountain View, CA, USA).

Flow cytometric analysis of cell cycle. Cells were first harvested at the exponential growth phase and mixed with paclitaxel at a concentration of 50 ng/ml for MEL cells and 21 ng/ml for K562 cells, at 37˚C in a humidified atmosphere of 5% CO_2 for 24 h. For cell cycle analysis, cells were resuspended at $2x10^6$ cells/ml and fixed in ice-cold 70% ethanol. According to the instructions of the Cell Cycle kit (Kaiji Bio Co.), each sample was resuspended in propidium iodide (PI) stain buffer (0.1% Triton X-100, 10 μg/ml DNase-free RNase A, 50 μg/ml PI) for 30 min and samples were analyzed with flow cytometry (FACSAria; BD Biosciences).

Statistical analysis. All results were confirmed by at least three independent experiments. The data are expressed as means ± SEM. Comparisons of mean values were performed using the independent samples t-test in SPSS for Windows 11.5 software. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Proliferation of leukemia cells is inhibited by paclitaxel. Using the MTT assay, our results showed that paclitaxel inhibited the proliferation of MEL and K562 cells in a dose- and time-dependent manner (Fig. 1). The IC_{50} values of paclitaxel in MEL and K562 cells at 48 h were 99.5 and 42.7 ng/ml, respectively. Furthermore, morphological assessment of MEL cultures revealed that paclitaxel-induced marked cellular swelling should be called oncosis which leads to necrosis (Fig. 2), while a small part of the K562 cells incubated with paclitaxel showed marked cellular shrinking (Fig. 3).

Morphological observation using Hoechst 33342 stain assay. Hoechst 33342 is a non-toxic specific vital stain for DNA (17). Using Hoechst 33342 stain assay, our results showed that paclitaxel clearly induced necrosis in MEL cells (Fig. 4). However, there were evident apoptotic instead of necrotic cells in K562 cells treated by paclitaxel (Fig. 5).

Flow cytometry for the detection of apoptosis and necrosis. Cell apoptosis and necrosis were detected by flow cytometry

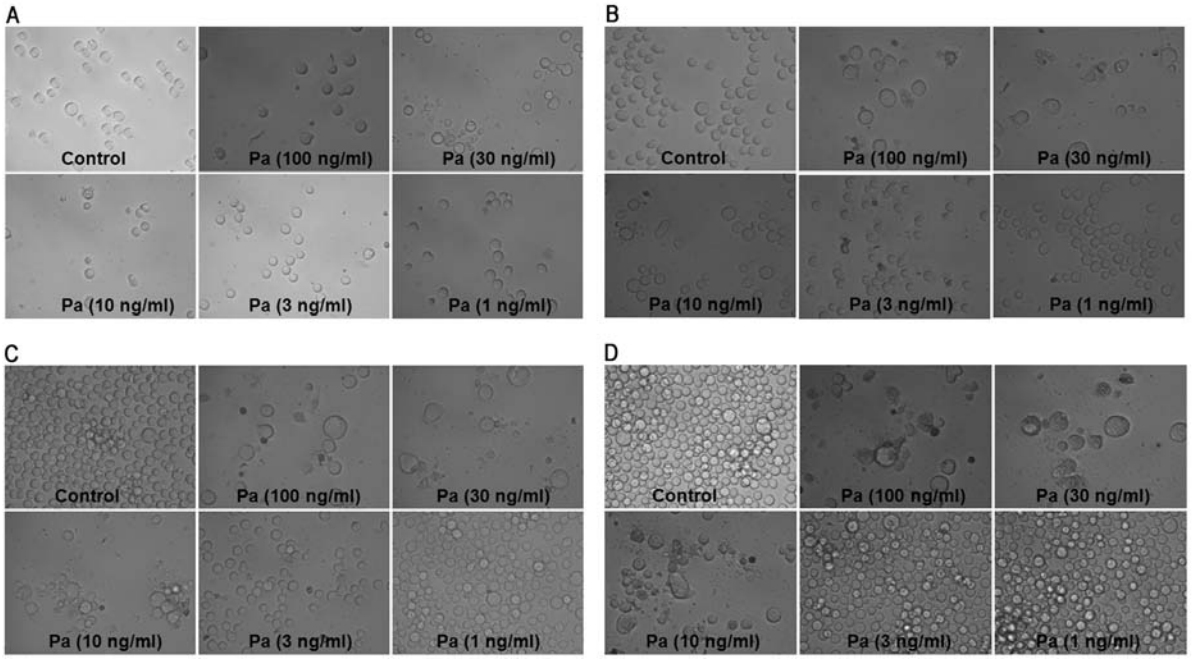

Figure 2. Morphological and quantity changes of MEL cells after paclitaxel (Pa) treatment at (A) 24, (B) 48, (C) 72 and (D) 96 h, respectively. Images were captured at x400 magnification. Representative images from three independent experiments are shown.

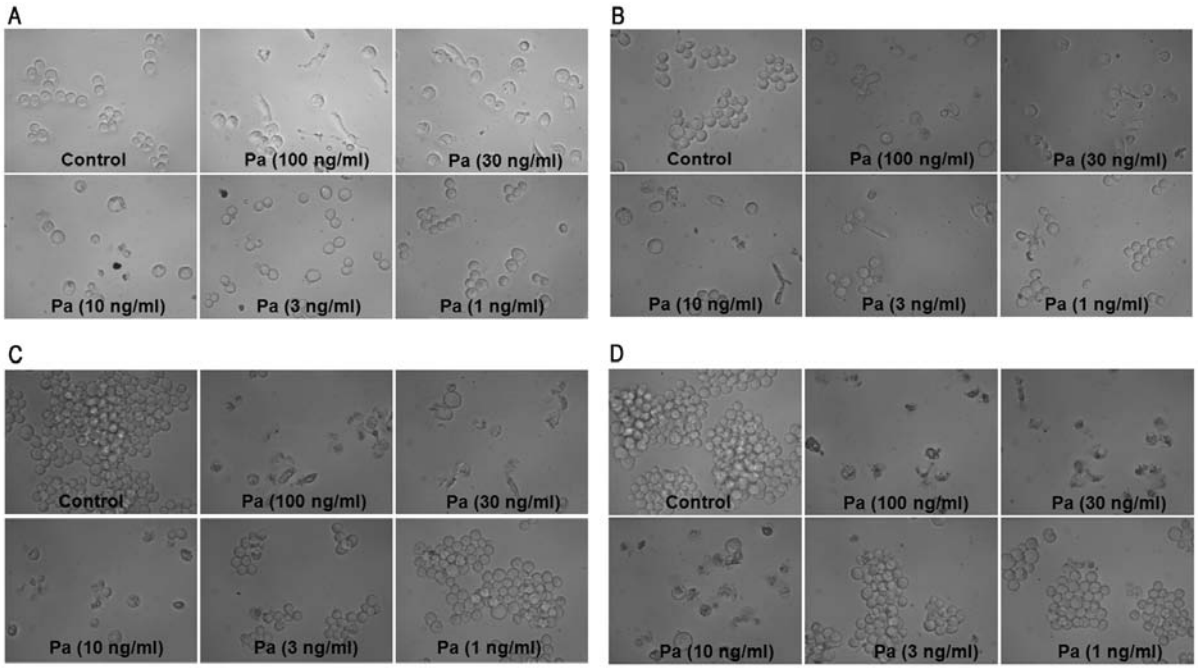

Figure 3. Morphological and quantity changes of K562 cells after paclitaxel (Pa) treatment at (A) 24, (B) 48, (C) 72 and (D) 96 h, respectively. Images were captured at x400 magnification. Representative images from three independent experiments are shown.

with Annexin V-FITC/PI dual staining. The amount of normal cells, early apoptosis, late apoptosis and necrosis was determined as the percentage of Annexin V^-/PI^- (Q3), Annexin V^+/PI^- (Q4), Annexin V^+/PI^+ (Q2) and Annexin V^-/PI^+ (Q1) cells, respectively (18). Our results showed that necrotic cells were significantly increased (from 3.10±0.21 to 14.68±1.76), while apoptotic cells were not significantly increased following paclitaxel treatment (50 ng/ml) in MEL cells for 48 h, when compared to those in the control group that was treated with vehicle only (Fig. 6). However, in K562 cells, there was a marked increase of apoptotic cells from 5.87±0.77 to 14.53±2.78 following paclitaxel treatment (21 ng/ml) for 48 h compared with the control group, while there was no significant difference of necrotic cells between the paclitaxel treated group and the control group (from 2.16±0.53 to 3.93±1.46) (Fig. 7). These results are in accordance with the changes observed in cell morphology (Figs. 4 and 5).

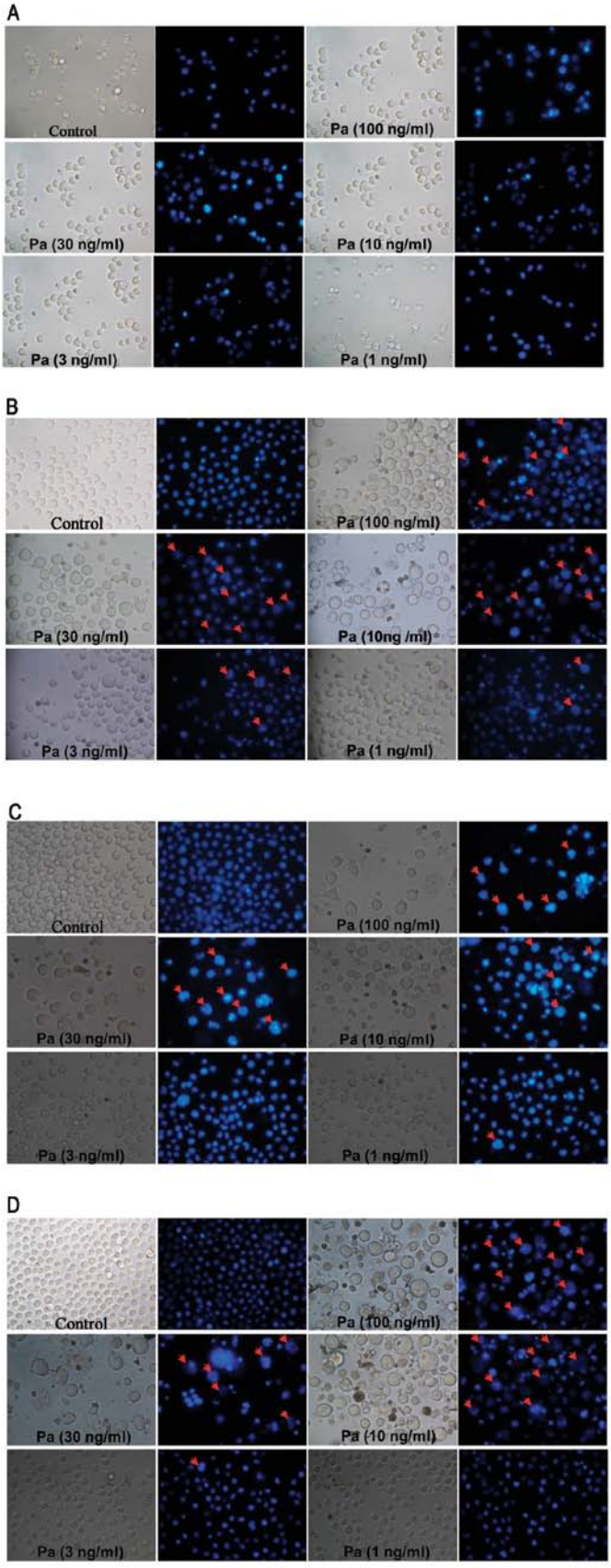

Figure 4. Effect of paclitaxel (Pa) on the nuclear morphological changes of MEL cells with the Hoechst 33342 uptake assay. MEL cells were incubated with buffer as the control or with Pa at each concentration for (A) 24, (B) 48, (C) 72 and (D) 96 h. The cells were then stained with Hoechst 33342 in the dark for 5 min, and photographed by inverted fluorescent microscope (magnification, x400). A representative image from three independent experiments is shown. Red arrows indicate the representative necrotic cells.

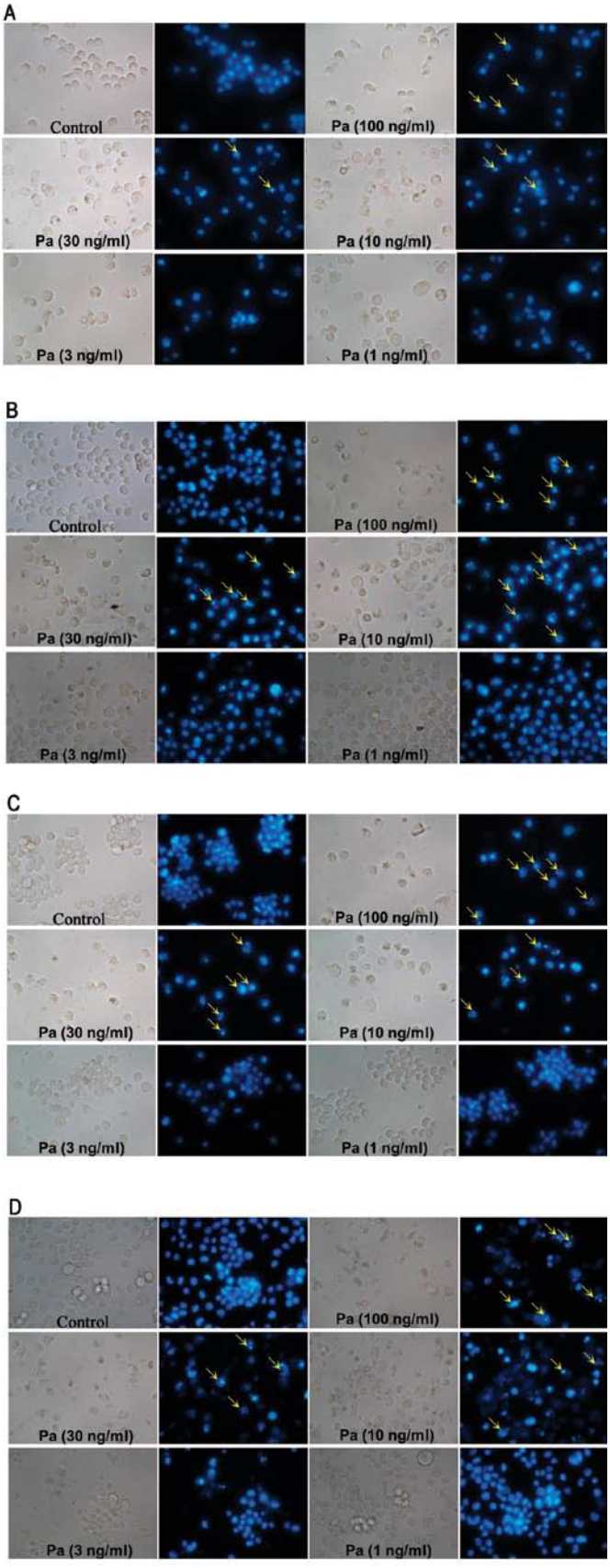

Figure 5. Effect of paclitaxel (Pa) on the nuclear morphological changes of K562 cells with the Hoechst 33342 uptake assay. K562 cells were incubated with buffer as the control or with Pa at each concentration for (A) 24, (B) 48, (C) 72 and (D) 96 h. Then cells were stained with Hoechst 33342 in the dark for 5 min, and photographed by inverted fluorescent microscope (magnification, x400). A representative image from three independent experiments is shown. Yellow arrows indicate the representative apoptotic cells.

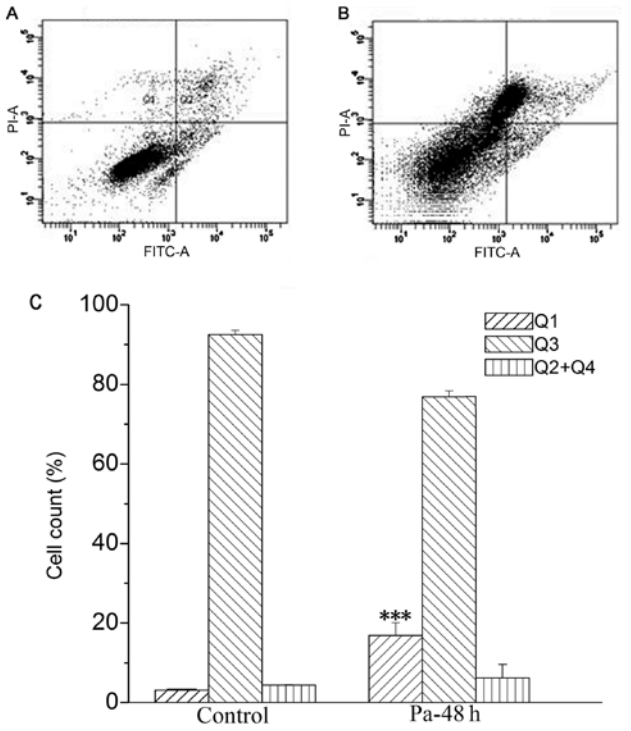

Figure 6. Effect of paclitaxel (Pa) on the apoptosis of MEL cells using flow cytometry. Apoptosis detection assays were carried out after incubation with Pa (50 ng/ml) at 48 h (B) compared with control (A) by the double stain assay with Annexin V-FITC and PI. The results are presented as the means ± SEM of at least three independent experiments (C). ***P<0.001, statistically significant difference vs. the control.

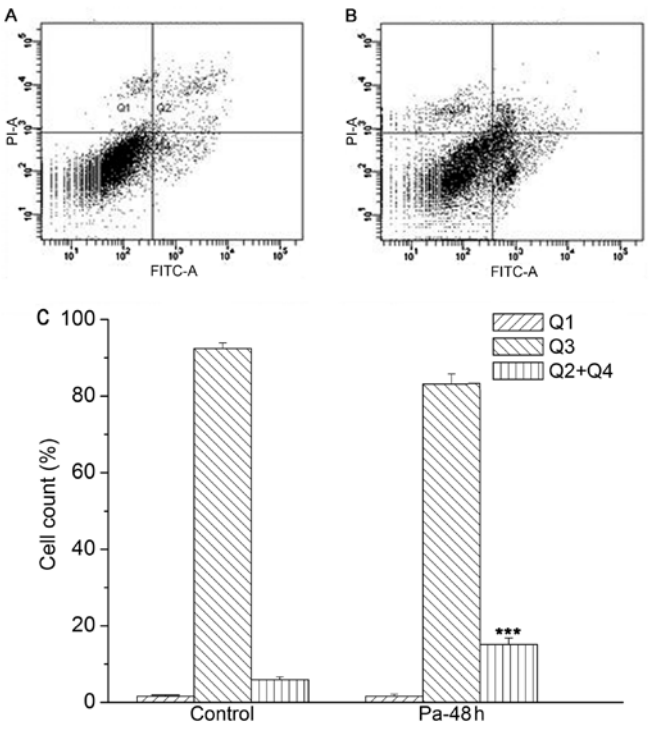

Figure 7. Effect of paclitaxel (Pa) on the apoptosis of K562 cells using flow cytometry. Apoptosis detection assays were carried out after incubation with Pa (21 ng/ml) at 48 h (B) compared with control (A) by the double stain assay with Annexin V-FITC and PI. The results are presented as the means ± SEM of at least three independent experiments (C). ***P<0.001, statistically significant difference vs. the control.

Paclitaxel induces significant G_2/M phase arrest of the cell cycle in K562 cells. Flow cytometry analysis (PI stain) was used to determine the effect of paclitaxel on cell cycle distribution of K562 cells. As shown in Fig. 8, the percentage of K562 cells in the G_2/M phase was increased significantly (P<0.01) from 14.66±0.50% (control group) to 85.96±3.24% after the treatment with paclitaxel at the concentration of 21 ng/ml for 24 h, while the percentage of G_0/G_1 phase and S phase was decreased markedly, indicating that paclitaxel inhibited the proliferation of K562 cells by causing G_2/M phase arrest of the cell cycle. However, it could not detect the normal cell cycle distribution in MEL cells treated by paclitaxel due to the necrosis (data not shown).

Discussion

Previous studies showed the efficacy of taxanes on human leukemic cell lines (16,19-21), as well as their effectiveness in inducing apoptosis *in vivo* (22) and in fresh leukemia cells in primary cultures (23). However, to the best of our knowledge, there is no report on the effect of paclitaxel on leukemia induced by virus. Friend virus is an acutely oncogenic retrovirus that causes erythroblastosis and polycythemia in mice (24,25). In the present study, the MEL cell lines induced by friend virus were used to investigate the effect of paclitaxel on leukemia induced by virus, as well as to compare with that of human erythroleukemic cell line (K562 cells). Our present results showed the dose- and time-dependency of the antitumor effects of paclitaxel on these two types of leukemia cells, and the potency of paclitaxel in K562 cells was stronger than in MEL cells. Paclitaxel clearly induced apoptosis in K562 cells, which is in accordance with previous results (21). Also, there was a significant arrest of cell cycle to G_2/M phase induced by paclitaxel in K562 cells, similar to the findings of a previous report (26). These findings suggest that paclitaxel induced K562 cell death involving the cell cycle and apoptosis (27).

By contrast, in MEL cells, paclitaxel could not induce significant apoptosis, which is different from that in K562 cells. Cell death is the process which culminates with cessation of biological activity. It is generally accepted that apoptosis and necrosis are two distinct, mutually exclusive, modes of cell death (28). One of the early events in apoptosis is cell dehydration. Loss of intracellular water leads to condensation of the cytoplasm followed by a change in cell shape and size: the originally round cells may become elongated and are generally smaller. Another change, perhaps the hallmark of apoptosis, is condensation of nuclear chromatin. In the present study, the phenomenon of apoptosis was evident in K562 cells treated by paclitaxel, while it was barely observed in MEL cells treated by paclitaxel. Necrosis is a passive, catabolic, and degenerative process with karyorrhexis and cell swelling prior to rupture of the plasma membrane, which is in contrast to apoptosis (29). Our present results showed that the MEL cells treated by paclitaxel exhibited significant characteristics of necrosis. Additionally, the normal cell cycle distribution in MEL cells treated by paclitaxel could not be detected due to the necrosis. It is therefore evident that the mode of cell death

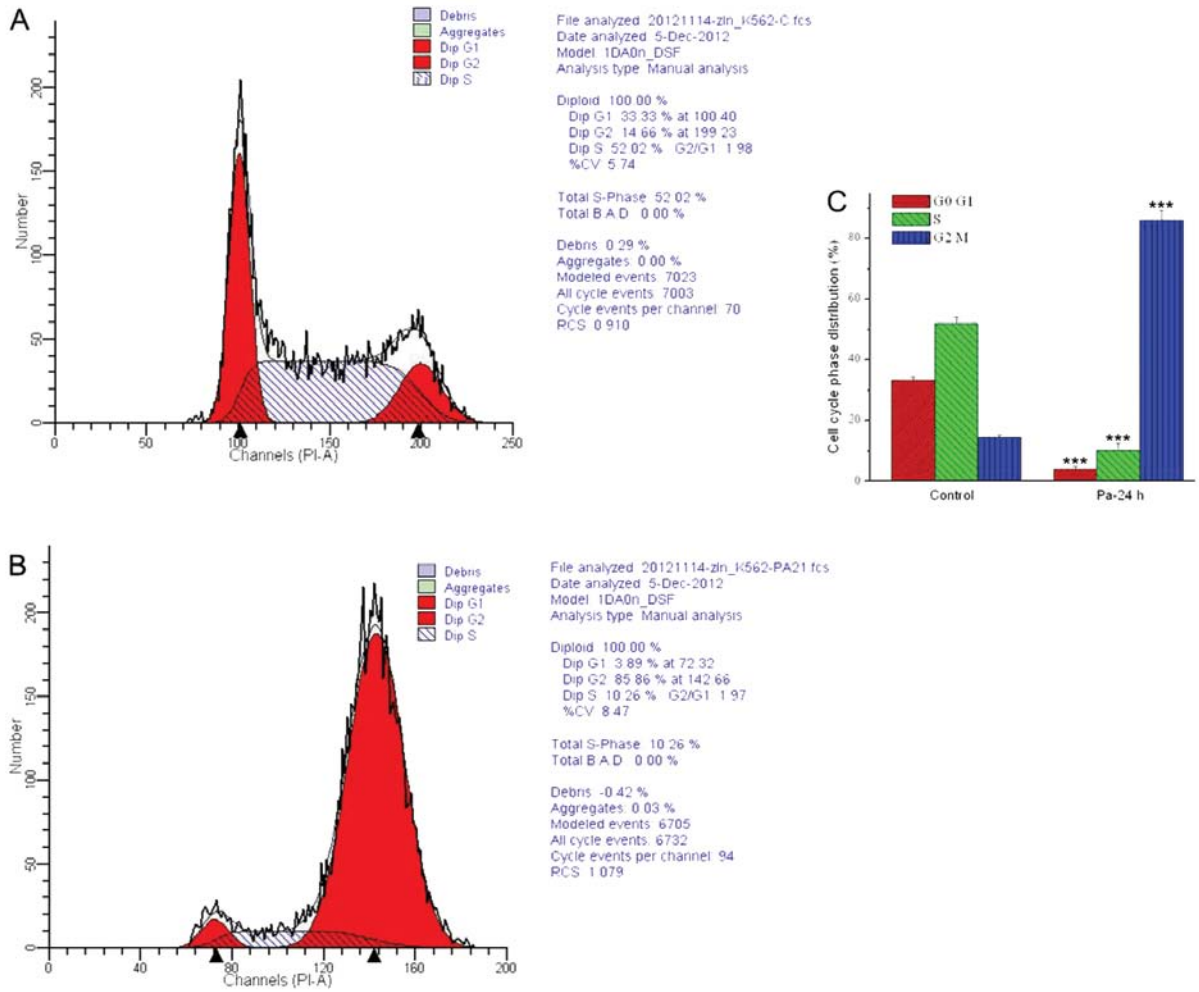

Figure 8. Effect of paclitaxel (Pa) on the cell cycle distribution of K562 cells using flow cytometry. Cell cycle detection assays were carried out after incubation with Pa (21 ng/ml) at 24 h (B) compared with control (A) by PI-binding assay. The results are presented as the means ± SEM of at least three independent experiments (C). ***P<0.001, statistically significant difference vs. the control.

induced by paclitaxel in the two types of leukemia cells used in the present study is different.

In summary, paclitaxel is the prototype of a group of promising chemotherapeutic agents, taxanes, which specifically interact with microtubules. However, the present study showed that paclitaxel clearly induced necrosis in leukemia cells induced by virus, which is different from that of human erythroleukemic cells. Advances in the research of cell cycle, apoptosis and necrosis will extend our understanding of the mechanisms involved in paclitaxel-induced cell death, particularly in leukemia cells. Further elucidation of the necrotic mechanism in MEL cells may expedite the development of enhanced paclitaxel-based regimens for cancer therapy.

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