Original Article

The Molecular Mechanisms of Human Osteosarcoma Cell Apoptosis Induced by Arsenic Trioxide and Vincristine

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ABSTRACT

Background: Arsenic trioxide (As_2O_3) and vincristine (VCR) have been used for the treatment of tumors. But little is known about the mechanisms and whether they induce apoptosis of human osteosarcoma MG-63 cells.

Methods: MG-63 cells were treated with As_2O_3 and VCR, respectively. Their effects on cell apoptosis and cell cycle arrest were evaluated by MTT assay and flow cytometry. Their effects on the expressions of apoptosis-related proteins PCNA, Bcl-2, Bax, caspase-3, cyclin D1 and cyclin E were investigated by Western-blot.

Results: Both As_2O_3 and VCR have time- and dose-dependent effects on apoptosis and cell arrest of MG-63 cells. As_2O_3 induces MG-63 cell arrest in S phase, whereas VCR induces MG-63 cell arrest in G_2/M phase. Both As_2O_3 and VCR suppress the expression of PCNA, cyclin D1, cyclin E and Bcl-2, and increase the expression of Bax and casepase-3.

Keywords: Vincristine, arsenic trioxide, MG-63 cell line, apoptosis, cell cycle, PCNA, cyclin D1, cyclin E, Bcl-2, Bax.

INTRODUCTION

Osteosarcoma, the most commonly seen primary malignant bone tumor[1], mainly occurs in the adolescent at age of 10~20. It metastasizes easily to lung and bone at early stage and severely affects the physical and mental health of the patient. The prognosis of patients with osteosarcoma is not optimistic with the 5-year survival rate at about 20.[2] Since the application of neo-adjuvant chemotherapy, surgery and adjuvant chemotherapy, the prognosis of those patients at early stage has been significantly improved. Their 5-year survival rate has increased to 50%~80% and two-thirds of the patients can preserve their extremities.[3] Even though, their mortality and disability rates are very high at presence. Chemotherapy is the only effective therapeutic method for patients with distant metastasis. Unfortunately, chemotherapeutic drugs currently used in clinic often have poor outcomes. Thus, selection of anti-cancer drugs with low toxicity and high efficacy has become a very important research area. Arsenic trioxide (As_2O_3) has been applied in treatment of relapsed acute promyelocytic leukemia (APL, M3) with high efficacy.[4] But there are few reports on its use in the treatment of osteosarcoma. Vincristine (VCR) is commonly used for the treatment of hematological malignancies.[5] However, the underline molecular mechanisms are not well investigated. In this paper, we studied the pro-apoptotic effects of arsenic trioxide and vincristine on osteosarcoma and explored their possible molecular mechanisms and signal transduction pathways with the hope to provide guidance for their clinical application in the future.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (BRL, CA, USA). 3-[4,5-Dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St Louis,

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MO, USA). Vincristine was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. (China) and arsenic trioxide from Haerbin Yida Pharmaceutical Co. Ltd. (China).

Cell culture: Human osteosarcoma MG-63 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) in tissue culture flask (CORNING, Shanghai, China) at 37°C under a humidified 5% CO_2 atmosphere.

MTT assay: Cells viability was determined using microculture tetrazolium technique (MTT). Cells were implanted in 96-well plates at density of 2.5×10^4/well. 6-8 hours later, cells were treated for 60 hours with 10, 8, 6 or 4 μmol/L arsenic trioxide or 4, 2, 1 or 0.5 μg/mL vincristine, respectively, with five replicate wells for each concentration. The medium was replaced with 0.1 mL fresh medium containing corresponding concentrations of tested compounds per 12 hours. The amount of adherent cells was then determined by MTT method. Briefly, the drug-treated cells were washed once with Mg^{2+} and Ca^{2+}-free PBS and incubated for 4 hours with 0.2 mg/mL MTT in the culture medium. After removal of the culture medium, cells were lysed with 100 μL DMSO and the absorbances at 540 nm of the cell lysates were measured by a microplate reader (Anthosht, Austria).

Flow cytometry analysis: MG-63 cells were treated with various concentrations of vincristine for 12, 24, 36 and 48 hours or arsenic trioxide for 24, 36, 48 and 60 hours, respectively. The cells were harvested by trypsinization after washed once with PBS and fixed with 75% ethanol containing 0.5% Tween 20 for at least 1 hour at 4 °C after washed with 1% bovine serum albumin (BSA). The fixed cells were resuspended/stained in 1 mL cold PBS containing 100 μg/mL RNase A and 10 μg/mL PIRARUBICIN for 40 min at 4 °C after washed with 1% BSA and subjected to a flow cytometry system (Beckman Coulter, Inc., Fullerton, CA,USA) for data acquisition and analysis of cell cycle phase distribution.

Western blot analysis: Following the treatment with vincristine or arsenic trioxide for 36 hours, MG-63 cells were harvested and washed twice with cold PBS. Total proteins were prepared using a "PROPREP" protein extract solution and assayed using a protein assay reagent (TIANGEN, Beijing, China). 100 μg of the proteins were denatured by boiling at 100 °C for 5 min in 0.5 M Tris-HCl, pH 6.8 buffer containing 4% SDS, 20% glycerol, 0.1% bromphenol blue and 10% β-mercaptoethanol, and separated by 10%-15% SDS polyacrylamide gel. The proteins were then transferred onto PVDF membranes at 100 V for 2-3 hours in a pH 8.5 transfer buffer containing 25 mM Tris base, 0.2 M glycine and 20% methanol. The membranes were blocked for 1 hour with 5% non-fat milk in TBS buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 0.5% Tween 20) at room temperature and incubated overnight at 4 °C with polyclonal primary antibodies directed against PCNA (Santa Cruz), Bcl2 (Santa Cruz), cyclin E (Santa Cruz) and β-actin (Santa Cruz) at 1:200 ratio or monoclonal primary antibodies directed against Bax-L (eBioscience) and cyclin D1 (eBioscience), respectively. After washing for 1 hour with TBS buffer, the membranes were incubated with 1:40,000-diluted horseradish peroxidase-conjugated anti rabbit antibody or anti-mouse antibody (Zhongshan, Beijing, China) at room temperature for 30 minutes. After washing for 60 minutes with TBS buffer, protein bands were visualized by incubating the membranes with enhanced HRP-DAB color reagent (TIANGEN, Beijing, China) and photographed with Bio-profil (VL company, France).

Statistical analysis: Data were presented as means ± SD of determinations from either 3 or 4 independent experiments. Statistical differences between untreated and treated samples were determined using unpaired Student's t-test. A p value less than 0.05 was considered as statistical significance.

RESULTS

Inhibition of cell proliferation: The effects of arsenic trioxide and vincristine on cell growth were examined by MTT assay. As shown in Figure 1A, arsenic trioxide significantly inhibits the growth of MG-63 cells in a time and dosage-dependent manner except that 4 μmol/L arsenic trioxide shows no obvious time-dependent effect due to subtle inhibitory effect. Similarly, vincristine also exhibits inhibitory effects on the growth of MG-63 cells (Figure 1B) in a time and dosage-dependent manner. Compared with dose-dependent effects, the time-dependent effects are more important.

Figure 1. Cell growth curve measured by MTT assay. The cells were inoculated in 96-well plates at a density

of 2.5×10^4/well. 6-8 hours later, cells were treated for 60h with various concentrations of arsenic trioxide (A) or vincristine (B, as indicated, with five replicate wells for each concentration. The medium was replaced with 0.1 mL fresh medium containing the tested drug at corresponding concentration every 12 hours. The cells were and incubated for 4 h with 0.2 mg/mL MTT in the culture medium after washed once with PBS and lysed with 100 μL of DMSO after removal of the culture medium. The relative cell numbers of adherent cells were then determined by the absorbance at 540 nm measured by a microplate reader. The data were shown as mean± SD from three independent experiments.

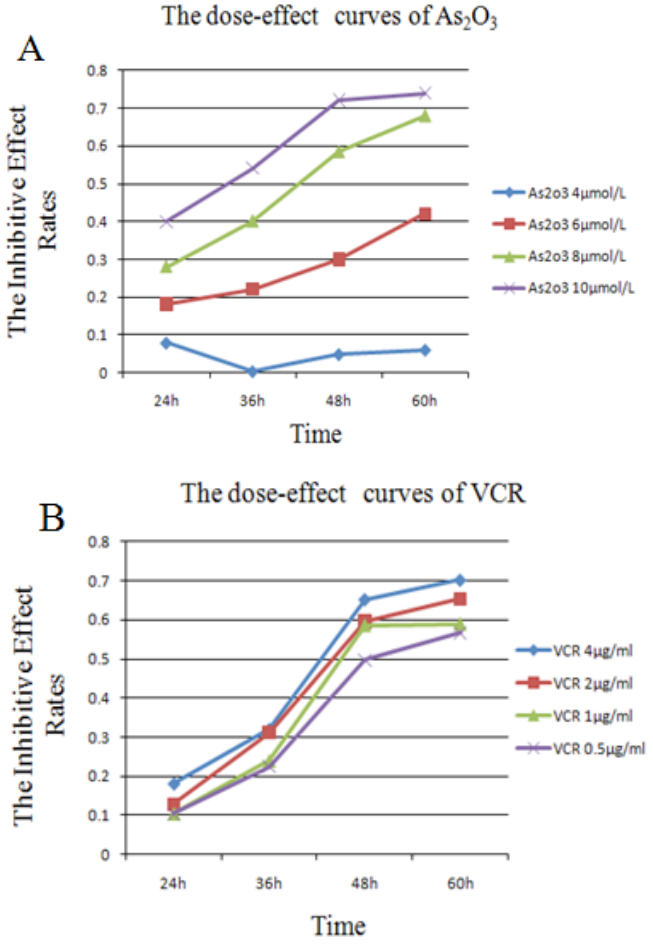

Figure 1*A The Dose-Effect Curves of AS_2O_3*
Figure 1*B The Dose-Effect Curves of VCR*

Effects of arsenic trioxide and vincristine on morphology of MG-63 cells: Morphological changes of MG-63 cells induced by 8 μmol/L arsenic trioxide at 24 and 36 hours, and 2 μg/mL vincristine at 24 and 60 hours, respectively, were substantially observed and photographed by light-phase microscope. As shown in Figure 2, normal MG-63 cells are well spread. The morphology of adherent cells becomes irregular when treated with arsenic trioxide for 24 hours, i.e. some of the cells become round, floating and shrunken with condensed cytoplasm and broken membrane. Following the increase of treatment time, attached cells are rare and become smaller in cellular volume, and more cells are floating. Most of MG-63 cells become round when treated with vincristine for 24 hours and are dead when treated with vincristine for 60 hours. Figure 2. Morphology of MG-63 cells. The cells were exposed to 8 μmol/L arsenic trioxide for 24 and 36 h or 2 μmol/L vincristine for 24 and 60 h, and the cell morphology were examined and photographed by phase microscopes (100×). With the prolonged treatment, cells turned round and floating, cytoplasm was condensed and cell membrane broken.

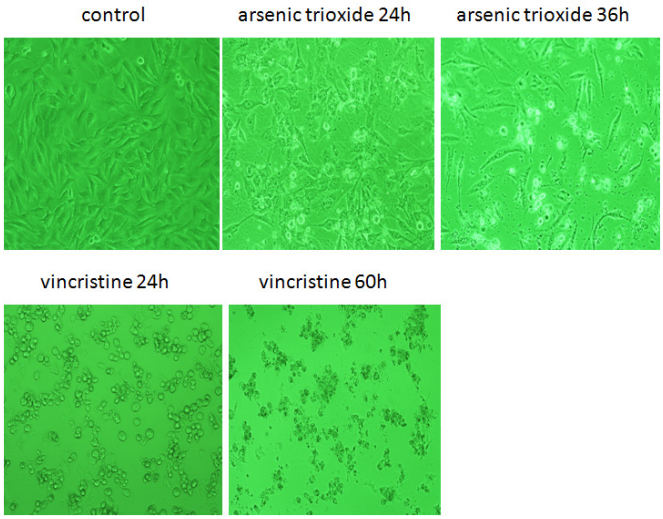

Figure 2 *Effect of Arcenic and vincristine on Cells*

Induction of apoptosis: Both of arsenic trioxide and vincristine are capable to induce MG-63 apoptosis at different levels examined by flow cytometry with propidium iodide staining. As shown in Figures 3A and 3C, the optimal concentrations of arsenic trioxide and vincristine to induce MG-63 apoptosis are 6 μmol/L and 2 μg/mL, respectively, when treated for 36 hours. Additionally, arsenic trioxide is able to induce apoptosis at concentrations between 4 and 10 μmol/L in a time- and dose-dependent manner. The maximum apoptosis rates in a prolonged time course and increased concentration range are up to 20.6% and 13.6%, respectively, which are significantly higher than that in vehicle treated cells ($p<0.01$). By contrast, the ability of vincristine to induce MG-63 cell apoptosis is weaker than that of arsenic

trioxide. Its highest apoptosis rate is 6.85% without showing obvious dose-dependent and time-dependent effects, as shown in Figures 3B and 3D.

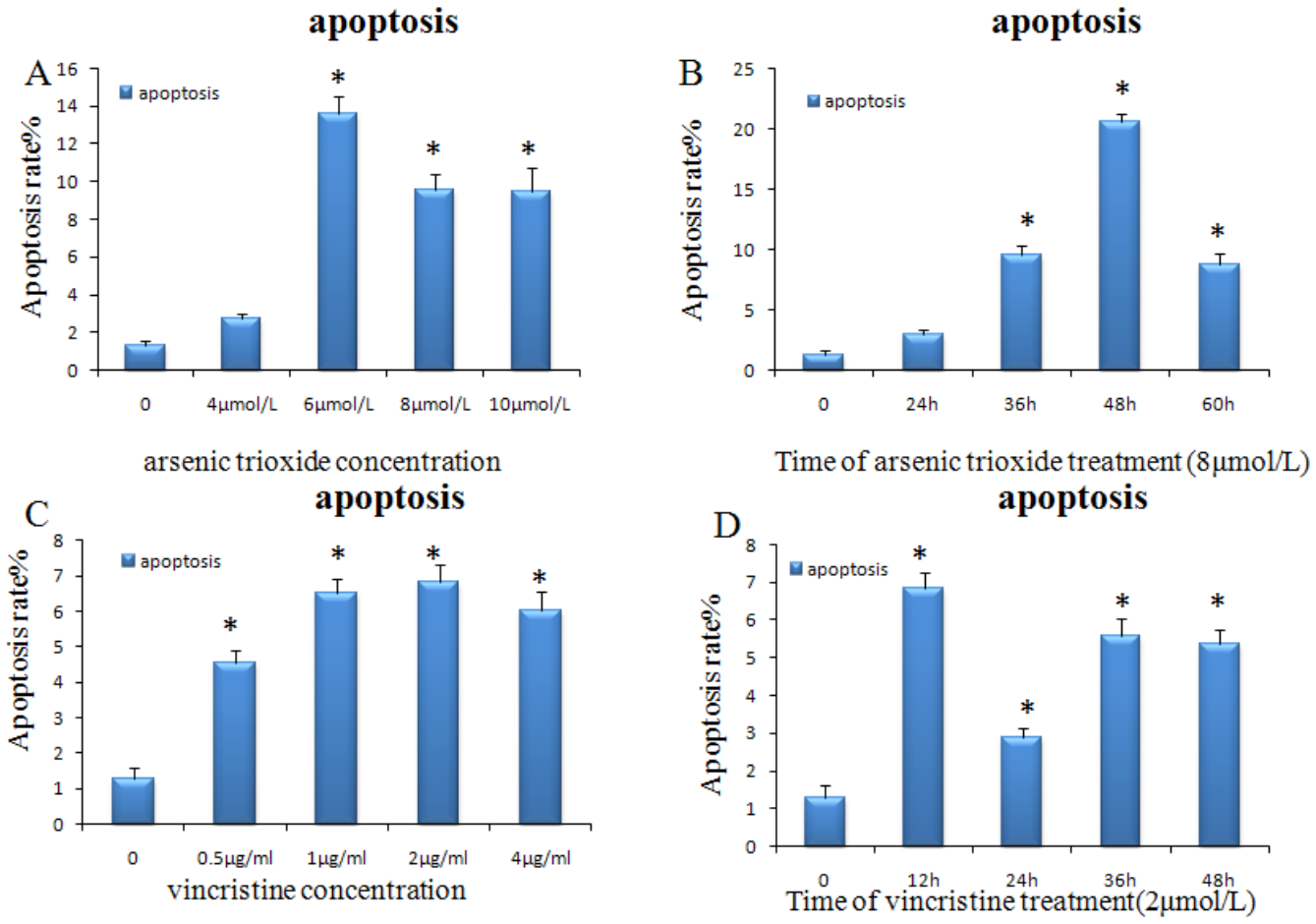

Figure 3. Effects of arsenic trioxide and vincristine on apoptosis of MG-63 cells. MG-63 cells were treated with arsenic trioxide or vincristine at indicated concentration and time, respectively. The cells were harvested by trypsinization after washed once with PBS and fixed with 75% ethanol containing 0.5% Tween 20 at least for 1 h at 4 °C after washed with 1% bovine serum albumin. The fixed cells were analyzed using a flow cytometry system (Beckman Coulter Inc., Fullerton, CA, USA). The data were shown as mean ± SD from three independent experiments.

Cell cycle phase distribution: Treatment of MG-63 cells with arsenic trioxide and vincristine significantly alters the distribution of cell cycle phase. As shown in Figure 4A, without any treatment, the proportions of MG-63 cells at G_0/G_1, S and G_2/M phase were 32.92%, 42.45% and 24.63%, respectively. Treatment of MG-63 with 4 to 10 μmol/L arsenic trioxide for 36 hours decreases the proportion of cells at G_0/G_1 phase to minimum 2.66% and significantly increases the proportion of cells at S phase to maximum to 81.48% (Figure 4A). Similarly, treatment of MG-63 cells with 0.5-4 μg/mL vincristine for 36 hours greatly decreases the proportion of cells at G_0/G_1 phase to 1.54 %, but dramatically increases the proportion of cells at G_2/M phase up to 90.75% (Figure 4C).

Treatment of MG-63 cells with 8 μmol/L arsenic trioxide for prolonged time obviously increases the proportion of cells at S phase, and slightly increases that of G_2/M phase and decreases that of G_0/G_1 phase as shown in Figure 4B. Interestingly, treatment of cells with 2 μg/mL vincristine for prolonged time gradually decreases the proportions of cells at S stage and G_0/G_1 and increases that of G_2/M

phase, as shown in Figure 4D.

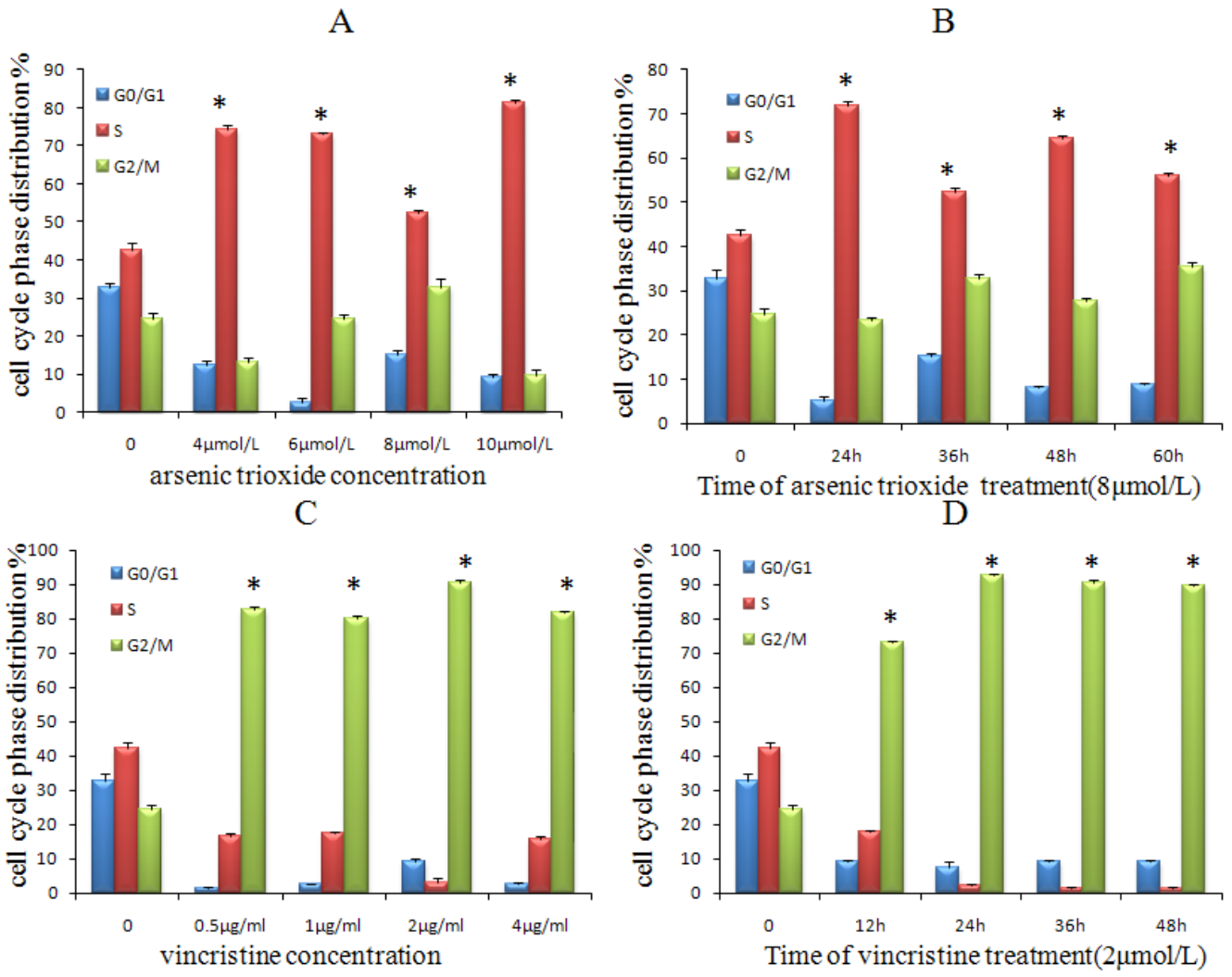

Figure 4. Effects of arsenic trioxide or vincristine on the cell cycle phase distribution of MG-63 cells. The cells were treated with arsenic trioxide (A) or vincristine (C) for 36 hours at various concentration as indicated, or exposed to 8 μmol/L arsenic trioxide (B) or 2 μmol/L vincristine (D) for different periods of time as indicated, respectively. The cells were then washed once with PBS and harvested by trypsinization, washed in 1% bovine serum albumin and fixed with 75 % ethanol containing 0.5 % Tween 20 for at least 1 h at 4 °C. The cells were analyzed using a flow cytometry system (Beckman Coulter Inc. Fullerton, CA, USA). The results were representative of at least three independent experiments.

Expression of apoptotic and cell cycle proteins: To further characterize the signaling alteration in MG-63 cells following the treatment with arsenic trioxide and vincristine, the expressions of apoptotic and cell cycle proteins were examined by Western blot. As shown in Figure 5, treatment of MG-63 cells with 8 μmol/L arsenic trioxide and 2 μg/ml vincristine, respectively, for 36 hours significantly inhibits the expressions of PCNA, a cell proliferation marker, cyclins D1 and E and Bcl-2, and enhances the expressions of Bax and caspase-3. These results are in consistence with the changes observed in flow cytometry analyses.

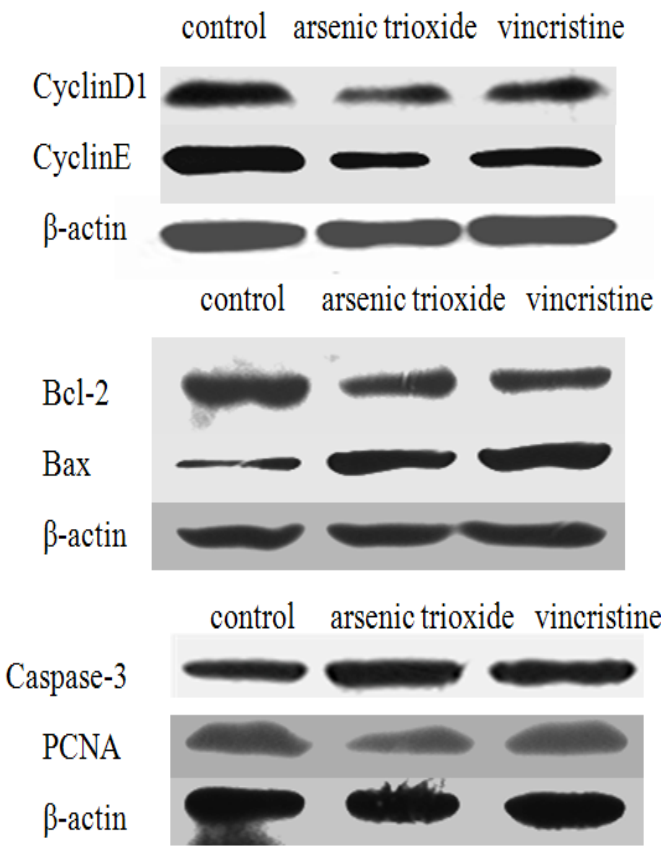

Figure 5. Western blot analysis of proteins PCNA, Bcl-2, Bax, caspase-3, cyclin D1 and cyclin E in MG-63 cells treated with 8 μmol/L arsenic trioxide or 2 μg/mL vincristine for 36 hours. β-actin was severed as loading control. The experiments were performed three times with similar results.

DISCUSSION

Osteosarcoma is the most common and malignant primary bone tumor in the adolescent. Since more and more malignant tumors become resistant to chemotherapeutic drugs, finding new drugs applicable to chemotherapy has become a research focus. Arsenic trioxide and vincristine have been used in clinic to treat malignant tumor; however, their effects on osteosarcoma have not been investigated. In this study, we examined their effects on human osteosarcoma cell line MG-63 and their possible mechanisms with the hope to provide a theoretical basis for clinical practice.

We found that arsenic trioxide shows no obvious inhibitory effect on the growth of MG-63 cells at concentration of 4 μmol/L even after treatment for 60 hours. However, it exhibits time- and dose-dependent inhibitory effects on the growth of MG-63 cells at concentration in the range of 6 – 10 μmol/L. This is in agreement with our finding that arsenic trioxide significantly induces MG-63 cell apoptosis at the same dose range. In contrast, vincristine at concentration between 0.5 and 4 μmol/L has obvious time-dependent inhibitory effect on growth of MG-63 and dose-dependent pro-apoptotic effect on MG-63 cells (Fig. 1). It is well known that the concentration of chemotherapeutic drugs is critical for their anti-tumor effects. At very high concentration, drugs may have maximum anti-tumor activity, but their side-effects also increase accordingly. At very low concentration, they cannot achieve their antitumor effects, but may remain their side effects. The concentration of arsenic trioxide is much higher that of vincristine to reach the similar inhibitory effect on osteosarcoma cells for a period of time.

Additionally, we found that exposure of MG-63 cells to 8μmol/L arsenic trioxide for 24 or 36 hours de-attaches large amount of the cells, while exposure to 2μg/ml vincristine for the same time de-attaches almost all the cells. Exposure to either arsenic trioxide or vincristine for prolonged times results in cell death (Figs 2 and 3). These data indicate that arsenic trioxide and vincristine exhibit their anti-osteosarcoma effects through a time-dependent, necrotic way. Necrosis is usually considered immunologically harmful because of the sudden release of pro-inflammatory mediators such as interleukin-8 (IL-8), IL-10 and tumor necrosis factor-α (TNF-α) and terminal mediators of inflammation such as high-mobility group box 1 (HMGB1).[6-7]

Our data shown in Figure 4 suggest that arsenic trioxide inhibits MG-63 cell growth by arrest MG-63 cell cycle at S stage and prevent its DNA synthesis. The exact mechanisms by which arsenic trioxide induce cells arrest need further investigation. Unlike arsenic trioxide, vincristine arrests MG-63 cells in G_2/M phase of cell cycle. It has been reported that vincristine mainly acts on tubulin and prevents spindle formation during cell division.[8] Cells arrested at S stage of cell cycle will divide soon after drug withdrawal. Cells arrested at G_2/M phase will enter S stage quickly after drug withdrawal. Cells will survive if cycles stop at G_0 stage. Since arsenic trioxide and vincristine arrest MG-63 cells at different stages, combination of these two drugs for therapeutic treatment of tumor might have additive effects.

Many proteins are involved in tumor cell proliferation/ apoptosis and/or cell cycle. Previous studies have revealed that PCNA is over-expressed in osteosarcoma cells (9). Our data indicate that arsenic trioxide and vincristine treatment significantly decreases its expression. Nakashima has reported that PCNA positive rate is 80.8% in 26 primary osteosarcoma specimens by examining their telomerase activity using immunohistochemical staining and pointed out that PCNA can reflect the proliferation ability of osteosarcoma.[9] Expression of PCNA in tumor cells can reflect tumor cell proliferation objectively, thus it is useful in biological behavior evaluation and prognostic assessment of tumor cells. Comparison of PCNA expression in tumor cells before and after chemotherapy also can be used in evaluation of chemotherapeutic effects. Our data indicate that arsenic trioxide and vincristine can markedly decrease expression of PCNA in MG-63 cells, consistent with their inhibitory effects on MG-63 cell growth. It is well known that members of Bcl-2 gene family are involved in regulation of apoptosis. Bcl-2 can protect cells against apoptosis, whereas Bax is a pro-apoptotic factor of the family. Over-expression of Bcl-2 gene blocks the release of cytochrome c and prevents cell from undergoing apoptosis. By contrast, over-expression of Bax promotes apoptosis by increasing cytochrome c release.[10] Both arsenic trioxide and vincristine can down-regulate Bcl-2 expression directly or via Bax gene up-regulation (Fig 5). Cyclin D1 and E are over-expressed in many tumor cells resulting in acceleration of cell cycle or proliferation. Caspase-3 plays a key role in the process of apoptosis and involves in physiological and pathological cell death process. Some studies have shown that the reduced expression of caspase-3 in malignant cancer cells can lead to cell overgrowth, thus promotes the occurrence and evolution of malignant tumors.[11] Cyclin D1, one of the most important members of the cyclin family and a recognized oncogene[12-13], can promote cells into G1 phase and plays a key role in regulation of G_1 phase in cell proliferation. Over-expression of cyclin D1 has been confirmed in many tumor cells with vigorous cell division. Karsunky[14] has shown that cyclin E and Ras gene can cause T-lymphocyte proliferation in transgenic mice and malignant transformants, indicating that cyclin E is an oncogene. The abnormal expression of cyclin E appears in many tumors and is always related to the stage and grade of tumor cells: the lower classification of the organization, the higher its expression[15], suggesting that cyclin E expression is not only an indicator of tumor, but also a prognostic indicator of tumor progress.

Our results suggest that the suppressions of cyclins D1 and E by arsenic trioxide and vincristine may result in the loss of proliferative activity and arrest of MG-63 cells at S or G_2/M phases of cell cycle, and imply that arsenic trioxide and vincristine could cause MG-63 cell apoptosis and arrest simultaneously. The results also indicate that it is important to combine arsenic trioxide with vincristine to treat osteosarcoma to avoid the quick recovery of tumor cell division following the withdrawal of arsenic trioxide and vincristine.

We have previously studied the effects of paclitaxel and pirarubicin on another human osteosarcoma cell line.[16] Together, these results will help to design new chemotherapeutic strategy in clinical treatment of osteosarcoma.

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