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Arsenic trioxide nanoparticles inhibit acute promyelocytic leukemia cell proliferation and induce apoptosis *via* PTEN/AKT signalling pathway.

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Abstract

Background: Arsenic Trioxide (As_2O_3) is a FDA-approved agent for the treatment of Acute Promyelocytic Leukemia (APL). But the high-toxicity is a bottleneck of the effect of As_2O_3.
Methods: In our previous work, we made a novel nanoparticle formulation of As_2O_3. The aim of the present study was to preliminary study the possible mechanisms of the antitumor effect of As_2O_3 nanoparticles on NB4 cells. We examined the proliferation and apoptosis of NB4 cells incubated with the As_2O_3 or As_2O_3 nanoparticles. Protein levels of p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF of NB4 cells after using As_2O_3 nanoparticles and traditional As_2O_3 were determined by Western blotting analysis.
Results: *In vitro* cytotoxicity test showed that the inhibition rate and apoptosis level of NB4 cells treated with As_2O_3 nanoparticles was much higher than that of traditional As_2O_3. Moreover, As_2O_3 nanoparticles resulted in a more significant increase in p-PTEN expression and a greater reduction in p-Akt expression compared with traditional As_2O_3.
Conclusions: Our findings indicated the obvious anticancer effect of As_2O_3 nanoparticles and demonstrate the possible mechanism of its therapeutic potential. The results provide a foundation for the future clinical studies of As_2O_3 nanoparticles in APL patients.

Keywords: Arsenic trioxide, Acute promyelocytic leukemia, Nanoparticles, PTEN, Akt.

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Introduction

Acute Promyelocytic Leukemia (APL) is the M3 subtype of acute myelogenous leukemia and cytogenetically is characterized by a translocation of chromosomes 15 and 17, which results in the fusion between Retinoic Acid Receptor α (*RARα*) and the Promyelocytic Leukemia (*PML*) gene. APL is always accompanied with severe bleeding tendency and disseminated intravascular coagulation. The first-line treatment of APL was chemotherapy. More than 80% of patients treated with all-trans retinoic acid along with chemotherapy can achieve a prolonged remission. Relapsed patients are often treated with arsenic trioxide successfully [1].

Arsenic Trioxide (As_2O_3) is the primary component of arsenic (pishuang), a kind of traditional Chinese medicine. In the early 1970's, some Chinese physicians from Harbin Medical University recognized arsenic as an effective ingredient for leukemia treatment. In 1996 and 1997, notable effect was reported for the use of As_2O_3 in APL [2]. The Food and Drug Administration (FDA) approved As_2O_3 as frontline treatment for APL in 2000 [3]. Apart from APL, it was used in several kinds of diseases, such as Chronic Lymphocytic Leukemia (CLL), liver cancer, syphilis, psoriasis, rheumatism [4-7]. However, the toxicity of arsenic was recognised by people gradually. The side effect of long-term chronic exposure to arsenic include hyperpigmentation of the skin, hyperkeratosis of the palms and feet, cancers of lung, bladder, prostate, kidney, liver as well as neuropathy, leucopenia, encephalopathy, peripheral vascular diseases and diabetes. Consequently, it faded out from clinical applications owing to its toxicity and carcinogenic properties [5].

Nanoparticles are becoming an emerging treatment modality for cancer. The advantages of nanoparticles are delivering anticancer drugs to the tumor, the ability to enhance permeability, bioavailability and efficacy as well as reduction or reversion of multidrug resistance. Owing to the unique features of tumor vasculature, the "Enhanced Permeability and Retention (EPR) effect" allows nanoparticles to extravasate into extravascular spaces and increase the drug concentration inside tumor tissues more than free drugs [8]. Nowadays, most nanoparticles can selectively target on cancer cells, reducing the undesirable side effects of conventional chemotherapy, and therefore improving the efficacy of patients [9,10].

The As_2O_3 nanoparticles were prepared with the so-gel method. In this study, the *in vitro* effect of As_2O_3 nanoparticles on NB4 cells was assessed by evaluating MTT analysis, mitochondrial membrane potential and flow cytometric

analysis. Furthermore, we explore the possible mechanism of by detecting the protein levels of p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF.

Methods

Cell culture

NB4 cells were a kind gift from Dr. Jifan Hu at Stanford University Medical School (Palo Alto, CA, USA) for research in the laboratory of the First Affiliated Hospital of Jilin University (Changchun, China). NB4 cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco-BRL, Grand Island, NY, USA) containing heat inactivated 10% Fetal Bovine Serum (FBS) (Hanzhou Sijiqing Biological Engineering Materials Co., Ltd. Hanzhou, China) along with 100 U/ml of penicillin and 0.1 mg/ml of streptomycin in an atmosphere of 5% CO_2 and 100% humidity.

Preparation of As_2O_3 nanoparticles

The As_2O_3 nanoparticles were synthesized with the sol-gel method as we previously described [11]. The preparation method was as follows: As_2O_3 powder and hydrochloric acid were mixed and stirred for 1030 min. Ethanol was then added, the solution was stirred at 5060°C for 2030 min and sonicated for 5 min. Finally, distilled water was added, and the mixture was sonicated for another 1020 min.

MTT analysis

The inhibitory effect of As_2O_3 (Institute for Drug Control of the Ministry of Health of China) and As_2O_3 nanoparticles was estimated by MTT assay. There were 3 groups, which were control, As_2O_3 and As_2O_3 nanoparticles. NB4 cells were seeded on 96 well plates in quadruplicate at a density of 2×10^5/well in 100 μl. As_2O_3 and As_2O_3 nanoparticles (4 different final concentrations: 0.5, 1, 2, 4 μmol/L) were added at the right time according to the group setting. After 24, 48 and 72 h of treatment, the cells were incubated for 3-4 h with MTT (Changchun Biotech Co., Ltd. Changchun, China) and lysed with acidified isopropanol. Absorbance was measured at 570 nm. The inhibition rate was calculated using the following formula: Inhibition rate=((absorbance value of control group-absorbance value of test group)/absorbance value of control group) × 100%. All experiments were repeated three times.

Hoechst 33342/PI staining

Cellular nuclear was stained with Hoechst 33342 (Hoechst Pharmaceuticals) and Propidium Iodide (PI) to observe the cellular apoptosis or necrosis situation by fluorescence microscopy. Briefly, 1×10^6 NB4 cells were incubated with 3 μmol/L As_2O_3 and As_2O_3 nanoparticles for 24 h. Cells were washed with PBS, suspended with new fresh medium, double stained with Hoechst 33342/PI and visualised by fluorescence microscopy.

Flow cytometric analysis

NB4 cells were treated with 1.5 μmol/L and 3.0 μmol/L As_2O_3, As_2O_3 nanoparticles against untreated control for 24 h at 37°C with 5% CO_2. After treatment, 1×10^5 NB4 cells were stained with Annexin V/Propidium Iodide Apoptosis Detection Assay kit (Beyotime Institute of Biotechnology Co., Shanghai, China). The apoptosis of NB4 cells in 3 groups were examined by flow cytometry (FCM; FACSCalibur^{TM}, BD Biosciences, San Jose, CA, USA).

Mitochondrial membrane potential

NB4 cells were incubated with 1.5 and 3 μmol/L As_2O_3 and As_2O_3 nanoparticles for 24 h. 1×10^6 NB4 cells were stained with Rhodamine 123 (Sigma). Cells were washed with PBS, suspended with new fresh medium. Mean Fluorescence Intensity (MFI) was on behalf of Mitochondrial Membrane Potential (MMP) and examined the MFI in each group with Flow cytometric.

Western blotting analysis

The expression level of p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF was determined by Western Blotting analysis in 3 groups (control, As_2O_3, As_2O_3 nanoparticles). The extracted total protein was loaded on SDS-polyacrylamide gel. The protein was separated after electrophoresis and transferred to a nitrocellulose membrane. Membrane was blocked with 5% non-fat dry milk at room temperature for 2 h and incubated with primary antibody: p-PTEN, p-AKT, caspase-3 rabbit mAb, caspase-9 mouse mAb (Cell Signaling Technology, Danvers, MA), Bax rabbit mAb (Proteintech Group, Chicago, America), AIF rabbit mAb and β-actin mouse mAb (Beyotime, Shanghai, China). The membrane was washed with PBST thrice for 10 min each time and then incubated with horseradish peroxidase (HRP)-labeled goat-anti-rabbit or goat-anti-mouse secondary antibody (Beyotime, Shanghai, China) at room temperature for 1 h. Membranes were washed for 3 times with PBST. Exposure to Kodak® X-omat LS film (Eastman Kodak Company, New Haven, CT) after incubating with enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA, USA). Densitometry was applied with Kodak® 1D image analysis software (Eastman Kodak Company).

Results

Inhibitory effect of As_2O_3 nanoparticles on NB4 cell proliferation

The inhibitory effect of As_2O_3 nanoparticles and As_2O_3 on NB4 cell proliferation was examined by MTT assay. As shown in Figure 1, the drugs had time and dose dependent anti-proliferative effects on NB4 cells. Furthermore, the inhibitory effect of As_2O_3 nanoparticles was much higher than traditional As_2O_3.

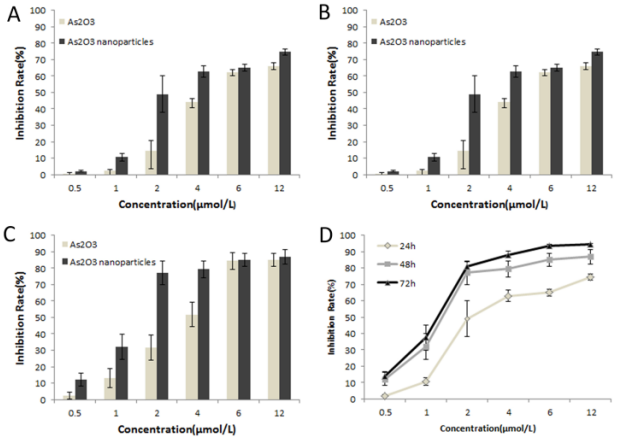

Figure 1. *Effect of As_2O_3 nanoparticles and As_2O_3 on NB4 cells proliferation. NB4 cells were incubated with drugs for: (A) 24 h; (B) 48 h; (C) 72 h; (D) Inhibitory effect of As_2O_3 nanoparticles on NB4 cell proliferation at 24 h, 48 h and 72 h.*

Results of Hoechst 33342/PI staining

NB4 cells were incubated with 3 μmol/L As_2O_3 nanoparticles or As_2O_3 for 24 h. Hoechst 33342/PI double staining was performed to observe the cell nuclear apoptosis or necrosis situation. Apoptotic cells were stained blue by Hoechst 33342 with pyknosis and stained red by PI with chromatin condensation. As shown in Figure 2, apoptosis was present after being treated with As_2O_3 nanoparticles or As_2O_3.

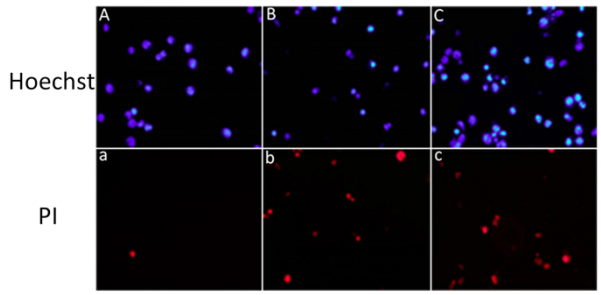

Figure 2. *Morphology of NB4 cells being stained by Hoechst 33342/PI under fluorescence microscopy. A (a) Control; B (b) 3 μmol/L As_2O_3; C (c) As_2O_3 nanoparticles (bar=50 μm).*

As_2O_3 nanoparticles induce NB4 cell apoptosis

Both As_2O_3 nanoparticles and As_2O_3 can induce NB4 cells apoptosis. Figure 3 showed a dose-dependent inducing apoptosis effect of As_2O_3 nanoparticles and As_2O_3 on NB4 cells. Furthermore, As_2O_3 nanoparticles had higher inhibition ability than As_2O_3.

Mitochondrial membrane potential

The mean fluorescence intensity level of control group is 1313.67 MFI. Meanwhile, the mean fluorescence intensity level of 1.5 μmol/L and 3.0 μmol/L As_2O_3 is 1085.09 MFI and 881.20 MFI. What's more, the mean fluorescence intensity level of 1.5 μmol/L and 3.0 μmol/L As_2O_3 nanoparticles is 911.05 MFI and 371.17 MFI. As_2O_3 nanoparticles can significantly decrease the mean fluorescence intensity level of NB4 cells than As_2O_3 (Figure 4).

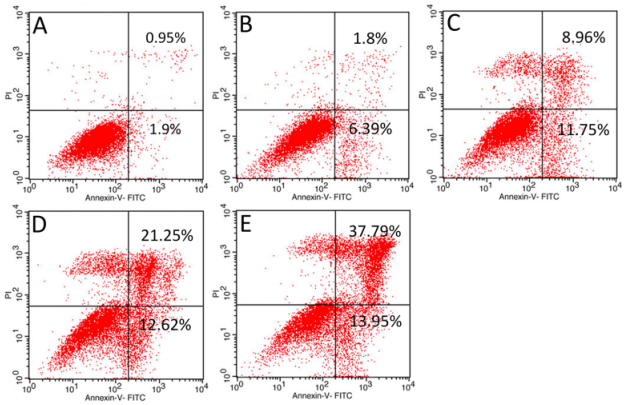

Figure 3. *Apoptosis of NB4 cells after being treated with As_2O_3 nanoparticles or As_2O_3. NB4 cells were treated as follows with an apoptosis rate: (A) Control, 0.95%; (B) 1.5 μmol/L As_2O_3, 1.8%; (C) 1.5 μmol/L As_2O_3 nanoparticles, 8.96%; (D) 3.0 μmol/L As_2O_3, 21.25%; (E) 3.0 μmol/L As_2O_3 nanoparticles, 37.79%.*

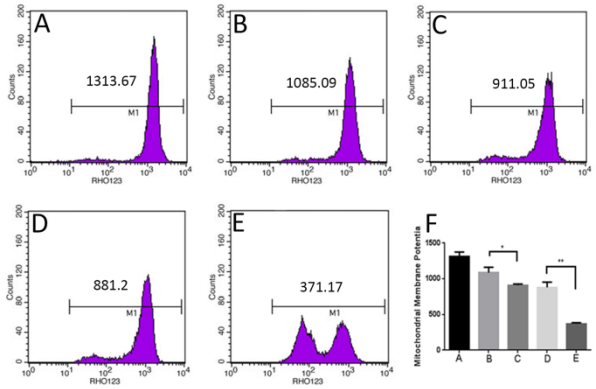

Figure 4. *Mitochondrial membrane potential of NB4 cells was examined by flow cytometry after being stained by Rhodamine123. (A) Control group; (B) 1.5 μmol/L As_2O_3; (C) 1.5 μmol/L As_2O_3 nanoparticles; (D) 3 μmol/L As_2O_3; (E) 3 μmol/L As_2O_3 nanoparticles; (F) The quantitative mitochondrial membrane potential levels of all groups; $^*P<0.05$; $^{**}P<0.01$.*

Protein levels of PTEN, Akt and Bax of NB4 cells treated with As_2O_3 nanoparticles

Protein levels of PTEN, Akt and Bax of NB4 cells incubated with As_2O_3 and As_2O_3 nanoparticles were detected by Western blotting. Both As_2O_3 and As_2O_3 nanoparticles can decrease the protein expression level of PTEN and p-Akt at the concentration of 1.5 μmol/L and 3.0 μmol/L, and the drugs increase the expression of p-PTEN and Bax.

Meanwhile, the expression of PTEN and p-Akt were much lower in the As_2O_3 nanoparticles group than in the As_2O_3 group, as well as the levels of p-PTEN and Bax were much higher in the former group than the latter with a dose-dependent manner (Figure 5).

Protein levels of caspase-3, caspase-9 and AIF of NB4 cells treated with As_2O_3 nanoparticles

At the concentration of 1.5 μmol/L, the protein levels of caspase-3 and caspase-9 were much higher in As_2O_3 nanoparticles group than As_2O_3 group. While, the levels were lower in 3.0 μmol/L As_2O_3 nanoparticles group than 3.0 μmol/L As_2O_3 group (Figure 5).

We further examined the level of AIF of NB4 cells. Figure 5 showed that the AIF level was higher in the As_2O_3 nanoparticles group than the group of As_2O_3 solution.

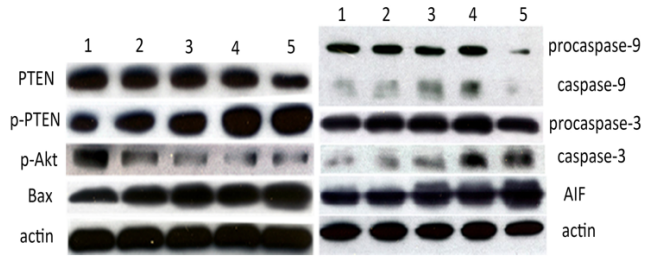

Figure 5. Protein levels of PTEN, p-PTEN, p-Akt, Bax, caspase-3, caspase-9 and AIF of NB4 cells following treatment of As_2O_3 nanoparticles or As_2O_3 for 24 h. NB4 cells were treated as follows: (1) Control; (2) 1.5 μmol/L As_2O_3; (3) 1.5 μmol/L As_2O_3 nanoparticles nanoparticles;(4) 3 μmol/L As_2O_3; (5) 3 μmol/L As_2O_3 nanoparticles.

Discussion

As_2O_3, a kind of traditional Chinese medicine, had been approved as frontline treatment for APL by FDA for many years. It could also be used in other solid cancer, such as liver cancer, prostate cancer, cervical cancer and breast cancer [9,12-18]. However, its clinical application is somehow limited owing to its high toxicity to the normal tissues. Therefore, different formulations of As_2O_3 with higher antitumor efficacy and lower toxicity are needed to be further studied.

Several kinds of As_2O_3 nanoparticles have been reported. Qian developed a scFvCD44v6-decorated PEG-PDLLA nanoparticle, which scFv-As-NP could target delivery of As to CD44v6-positive tumor cells PANC-1 [19]. The As_2O_3 nanoparticles with better antitumor efficacy can highly improve the drug concentration in tumor site but not normal tissues owing to the EPR effect and its better targeting effect. Nanobin encapsulation of As_2O_3 improves the pharmacokinetics and antitumor efficacy of As_2O_3 in a murine model of breast cancer and lymphoma [20,21].

In our previous study, we prepared small-sized (<10 nm and ~40 nm) As_2O_3 nanoparticles with sol-gel method [11]. Herein, the superiority efficacy of proliferationinhibition and apoptosisinduction effects *in vitro* of As_2O_3 nanoparticles than As_2O_3 were further confirmed by evaluating MTT assay, apoptosis detection with flow cytometric assay, mitochondrial membrane potential, p-PTEN, p-AKT, Bax, caspase-3, caspase-9 and AIF protein levels. A much smaller dose of As_2O_3 nanoparticles can achieve a good antitumor effect, which would reduce the systemic toxic side effects with a reduction of toxicity.

As_2O_3 has a property of proliferation inhibition of APL cell, such as NB4 cell lines. As_2O_3 nanoparticles, even at a low concentration of 2 μmol/L (Figure 1), can significantly inhibit the growth of NB4 cells. As_2O_3 nanoparticles can also induce apoptosis of NB4 cells in a dose-dependent manner (Figure 3). PTEN/Akt signalling pathway is involved in antitumor process [22,23]. PTEN is closely associated with PI3K/Akt pathway. It is a negative regulator of PI3K/Akt pathway owing to its IP3 phosphatase activity, which influences the cell proliferation and apoptosis [24,25]. And PI3K/Akt pathway has been proposed to inhibit Bax translocation from cytoplasm to mitochondria [26]. The inactivation of Akt can induce Bax translocate to mitochondria, which can decrease the mitochondrial membrane potential to induce apoptosis. In our present study, both As_2O_3 nanoparticles and As_2O_3 can increase the protein level of p-PTEN and decrease the level of p-Akt (Figure 5). Here we demonstrated that the induce-apoptosis of As_2O_3 nanoparticles and As_2O_3 is connected with PTEN and Akt. As_2O_3 and 1.5 μmol/L As_2O_3 nanoparticles could increase caspase-3 and caspase-9 protein expression. However, the 3 μmol/L As_2O_3 nanoparticles could improve the protein expression of AIF but not caspase-3 and caspase-9. We demonstrate that the apoptosis of As_2O_3 and As_2O_3 nanoparticles in a lower concentration is related with a caspase-dependent mitochondrial apoptosis pathway, but higher concentration of As_2O_3 nanoparticles induce NB4 cells apoptosis by a caspase-independent mitochondrial apoptosis pathway.

Conclusions

In conclusion, As_2O_3 nanoparticles can inhibit proliferation and induce apoptosis of APL cells, and the induce-apoptosis effect was correlated with PTEN/Akt signaling pathway. As_2O_3 nanoparticles are selective and potential anti-APL agents which could inhibit proliferation and induce apoptosis of APL cells with high efficacy.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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