Original Article
Arsenic trioxide inhibits cell growth and elevates T cell subgroup of bladder cancer in vitro and in vivo

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Abstract: Purpose: This study aimed to explore the effects of arsenic trioxide (As_2O_3) on the growth and apoptosis of MBT-2 bladder cancer (BC) cells, and on T cell subgroup and natural killer (NK) cell cytoactivity of bladder tumor-bearing mice. Methods: After MBT-2 cells were treated with As_2O_3 at the concentration of 0.5 μmol/l, 1.0 μmol/l, 2.0 μmol/l and 5.0 μmol/l for 24 h, 48 h and 72 h, the growth and apoptosis rates of MBT-2 cells were respectively determined by MTT assay and terminal transferase labeling in situ. The expression of B-cell lymphoma-2 (bcl-2) in MBT-2 cells was detected by immunohistochemistry. Then BC mice models were constructed. T cell subgroup and NK cell cytoactivity of bladder tumor-bearing mice after treated with As_2O_3 were respectively detected by flow cytometry and lactate dehydrogenase release assay. Results: The growth inhibition rate and apoptotic rate increased significantly with the increase of As_2O_3 concentration ($P < 0.05$) and treatment time ($P < 0.01$). The bcl-2 expression declined significantly after cells were treated with As_2O_3 ($P < 0.05$). Additionally, As_2O_3 elevated the percentage of CD4+ and CD8+ T cells, and increased the cytoactivity of NK cells in bladder tumor-bearing mice. Conclusions: As_2O_3 can inhibit the growth of MBT-2 cells in vitro, and elevates T cell subgroup and NK cells cytoactivity of bladder tumor-bearing mice in a dose and time-dependent manner.

Keywords: Bladder cancer, arsenic trioxide, apoptosis, T cell subgroup, natural killer cell

Introduction

Bladder cancer (BC) is one of the most common genitourinary malignancy worldwide [1]. It is known as a kind of transitional cell carcinoma with high propensity for recurrence [2]. Approximately, 75% of BC is non-muscle invasive, which is characterized by tumor recurrence in 60% to 85% cases [3, 4]. Clinically, transurethral resection of bladder tumor (TURBT) is a primary therapy for the superficial bladder tumors. For muscle invasive urothelial urinary bladder cancer, radical cystectomy is the golden standard [5]. Chemotherapy after operation is the most important adjunctive therapy [6]. However, the side effects of drugs and the drug resistance of tumor cells significantly decrease the curative effect of chemotherapy [7]. Therefore, looking for a more safe and effective method in the treatment of BC is of great importance.

Arsenic trioxide (As_2O_3), a traditional Chinese medicine, has been used as a therapeutic agent for more than 2400 years because of its significant medicinal properties [8]. As_2O_3 has been primarily used in the treatment of acute promyelocytic leukemia, and has shown substantial efficacy and less side effects [9]. Preclinical studies have reported that As_2O_3 has satisfactory therapeutic effects for a wide variety of malignancies, including gastric cancer [10], prostate cancer [11] and cervix cancer [12]. The anticancer mechanism of As_2O_3 may be due to its inhibition of cancer cell proliferation, as well as execution of apoptosis [13]. Additionally, some recent studies find that As_2O_3 plays an important role in cellular immune response of tumor immunity. Baj et al. [14], for instance, suggested that As_2O_3 enhanced the immune response against breast cancer cells. Despite all of the findings, the immunomodulatory properties of As_2O_3 on cancer have not been fully investigated.

In this study, we not only investigated the role of As$_2$O$_3$ in growth inhibition of human bladder cell line but also explored the effect of As$_2$O$_3$ on T cell subgroup and natural killer (NK) cell cytoactivity in bladder tumor-bearing mice. We aimed to provide new theoretical and experimental basis for the therapy of BC.

Materials and methods

Animals and reagents

C3H/HeN female mice between 6 and 8 weeks old (mean weight of 18-22 g) were purchased from Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China). MBT-2 cells were introduced from Cell Bank of Japan and were cultured in RPMI-1640 Dulbecco modified eagle medium (DMEM) with the addition of 10% fetal bovine serum (FBS), 100 μl/l penicillin and 100 μl/l streptomycin at 37°C in 5% CO$_2$. Pure As$_2$O$_3$ reagent was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

MTT detection of growth inhibition rate of MBT-2 cells

The MBT-2 cells were plated in 96-well tissue culture plates at a concentration of 2 × 10^5/ml. After cell adherence, As$_2$O$_3$ were added to each well at the concentrations of 0.5 μmol/l, 1.0 μmol/l, 2.0 μmol/l and 5.0 μmol/l (5 replicate wells for each concentration and control group). The culture plates were kept in a tissue culture incubator at 37°C in 5% CO$_2$ for 24 h, 48 h or 72 h. Then 20 μl 0.5% MTT solution was added to incubate with cells for 4 h. The cells were collected by centrifugation. Subsequently, 150 μl dimethylsulfoxide was added to mix with cells thoroughly. The optical density was measured at 570 nm using a microplate reader (BioTek, USA). The growth inhibition rate (%) was calculated as the formula below:

$$\text{Inhibition rate (\%)} = 1 - \frac{OD_e}{OD_c} \times 100\%$$

Where OD_e is the optical density in experimental group and OD_c is the optical density in control group.

Terminal transferase labeling in situ

The apoptosis of MBT-2 cells was detected using terminal transferase labeling in situ by Apoptosis Detection Kit (Boshide Bioengineering Company, Wuhan, China) according to the man-ufacturer's instructions. Cell shrinkage and nuclear chromatin with specific claybank under light microscope were the hallmarks of apoptosis.

$$\text{Apoptosis index (\%)} = \frac{N_{500}}{500} \times 100\%$$

Where N_{500} is the number of apoptotic cell in 500 cells.

Immunohistochemistry

The expression of B-cell lymphoma-2 (bcl-2) in MBT-2 cells was detected by immunohistochemistry. MBT-2 cells were affected by different concentrations of As$_2$O$_3$ for 24 h, 48 h and 72 h, and fixed by dimethyl ketone at 4°C for 10 min. Then the cells were incubated with primary antibody (mouse anti-human bcl-2 monoclonal antibody) at 4°C for 24 h. After washing, secondary antibody (enzyme coupling goat antibody) was added and incubated at 25°C for 30 min. Finally, 0.5% diaminobenzidine (Boshide Bioengineering Company, Wuhan, China) and hematine crystal were respectively used for chromogenic substrate reaction and contrast counterstain.

Bladder cancer animal modeling and treatment with As$_2$O$_3$

This study fully complied with the national legislation and the *Guide for the Care and Use of Laboratory Animals* issued by the Ministry of Health of the People's Republic of China and was approved by the local research ethical committees.

The MBT-2 cells at logarithmic growth phase were trypsinized and washed twice with RPMI-1640 (without FBS). The cell concentration was adjusted to 1 × 10^7/ml. Then the right back of C3H mice was intracutaneous injected with 0.1 ml cell sap (1 × 10^6 cells). When the tumors reached 5-8 mm in diameters, the mice were randomly divided into three groups (n = 8). Group 1 was injected with As$_2$O$_3$ (1 mg/kg/d) in tumor location; Group 2 was intraperitoneally injected with As$_2$O$_3$ (1 mg/kg/d); Group 3 was injected with normal saline (0.1 ml/d) in tumor location as control. Two weeks after injection, the animals were sacrificed by picking off eyeball, and the tumors were stripped and weighted quickly. Then the tumor tissues were fixed with neutral formalin, embedded in paraffin and stained with hematoxylin and eosin (HE).

Table 1. Growth inhibition rates (%) of MBT-2 cells treated with different concentrations of As$_2$O$_3$ for 24 h, 48 h and 72 h

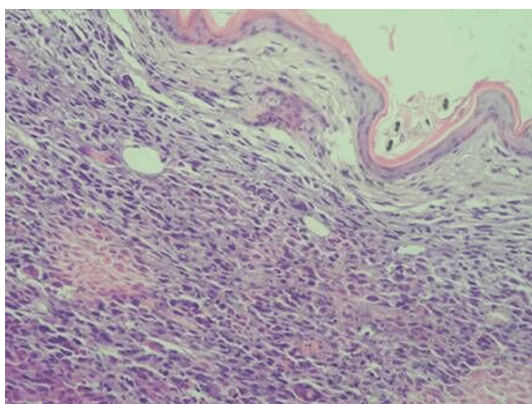| As$_2$O$_3$	24 h	48 h	72 h
0.5 µmol/l	3.64	10.45	13.39
1.0 µmol/l	8.94	18.42	20.32
2.0 µmol/l	14.45	20.56	24.42
5.0 µmol/l	16.64	25.66	30.59

Figure 1. Histopathology of mice bladder tumor (hematoxylin and eosin stain, 200 ×).

All the sections were observed and photographed under the light microscope.

Detection of spleen T cell subset

The spleens of sacrificed mice were collected, crushed, hemolyzed with 3 ml 0.17 M NH4Cl at 4°C for 1 min, and centrifuged at 1000 rpm/min for 10 min. Cell suspensions were washed twice with 2% FBS/PBS and adjusted to a concentration of 1 × 10^7/ml. Then 0.1 ml cell suspension was stained with 5 µl anti-FITC-CD4 and 5 µl anti-PE-CD8. At the same time, cell suspension stained with only one of the antibodies or without antibody was set as control. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, USA). For each sample, 10000 cells were analyzed, and T cell subset was determined using forward scattering corner (FSC) and side scattering corner (SSC). The result was exhibited with two-dimensional bitmap, and T cells were recognized as CD4+ and CD8+.

Detection of NK cell cytoactivity

The cytoactivity of splenic NK cells was tested by lactate dehydrogenase release assay. Briefly,

Table 2. Percentage (%) of CD4+ and CD8+ T cells (mean ± standard deviation)

Group	CD4+	CD8+	CD4+/CD8+
1	18.91 ± 6.27	16.57 ± 2.83	1.26 ± 0.34
2	6.53 ± 3.50	5.20 ± 1.37	1.21 ± 0.37
3	8.99 ± 3.74	8.97 ± 1.92	1.00 ± 0.23

the YAC-1 cells were adjusted to a concentration of 1 × 10^5/ml using RPMI-1640 containing 10% FBS. Then 0.1 ml splenic cells (1 × 10^7/ml; effector cells) were mixed with 0.1 ml YAC-1 cells (1 × 10^5/ml; target cells), resulting in an effector: target (E: T) ratios of 100: 1. Additionally, 0.1 ml YAC-1 cells were mixed with 0.1 ml 1% NP40 or 0.1 ml 10% FBS-RPMI-1640, acting as maximum release or spontaneous release. The E: T ratios, maximum release and spontaneous release were evaluated in triplicate in 96-well plates, and incubated at 37°C in 5% CO$_2$ for 2 h. The optical density (lactate dehydrogenase activity) was measured in a microplate reader (Model 450, Bio-Rad Laboratories, Inc., USA) at 570 nm and the percentage of cytotoxicity was determined using the following equation:

$$\text{NK cytoactivity (\%)} = \frac{OD_{er} - OD_{sr}}{OD_{mr} - OD_{sr}} \times 100\%$$

Where OD$_{er}$ is the optical density of experimental release, OD$_{sr}$ is the absorbance of spontaneous release; OD$_{mr}$ is absorbance of maximum release.

Statistical analysis

All the statistical analyses were performed using SPSS 12.0 (Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). Student's t-test was used for comparison of different groups. A probability level of $P < 0.05$ was considered as statistically significant.

Results

Effect of As$_2$O$_3$ on growth inhibition rate of MBT-2 cells

The growth inhibition rates of MBT-2 cells treated with different concentrations of As$_2$O$_3$ were shown in **Table 1**. The growth inhibition rate increased significantly with the increase of As$_2$O$_3$ concentration ($P < 0.05$) and treatment time ($P < 0.01$). When MBT-2 cells were treated

with 5.0 µmol/l As$_2$O$_3$ for 72 h, the growth inhibition rate maximized to 30.59%.

Effect of As$_2$O$_3$ on apoptosis rate of MBT-2 cells

The results showed that the apoptosis rate of MBT-2 cells in control group was 1.24%. After cells were treated with As$_2$O$_3$ for 24 h, 48 h and 72 h, the rates were 6.80%, 8.13% and 12.38% respectively. Besides, there was a significant difference between different treatment times ($P < 0.01$). Additionally, there was a statistical difference between experimental group and control group ($P < 0.05$).

Immunohistochemistry

The bcl-2 expression rate in control group was 91.4%. After cells were treated with 2.0 µmol/l As$_2$O$_3$ for 24 h, 48 h and 72 h, the expression rates significantly decreased to 86.9%, 82.6% and 79.1% respectively ($P < 0.05$). Besides, there was statistically significant difference in different time groups ($P < 0.01$).

Tumor histopathology observation

Lots of dysplasia tumor cells with larger nucleus and pathological mitosis were found in the lower skin layer. The normal tissues were replaced by tumor tissues which exhibited obscure boundary and infiltrative growth (**Figure 1**). The weight of tumor in 3 groups was respectively 2.12 ± 0.49 g, 7.81 ± 4.81 g and 5.27 ± 1.01 g. The weight of tumor in Group 1 was significantly lower than that of Group 2 and 3 ($P < 0.05$), while there was no significant difference between Group 2 and 3.

Percentage of CD4+ and CD8+ T cells

As depicted in **Table 2**, in Group 1 the percentage of CD4+ T cells was 18.91 ± 6.27%, which was significantly higher than that in Group 2 (6.53 ± 3.50%) and control group (8.99 ± 3.74) ($P < 0.05$). For CD8+ T cells, their percentages in the three groups were 16.57 ± 2.83%, 5.20 ± 1.37% and 8.97 ± 1.92% respectively, besides, Group 1 was significantly higher than the other two groups ($P < 0.05$). The ratios of CD4+/CD8+ in three groups were 1.26 ± 0.34, 1.21 ± 0.37 and 1.00 ± 0.23 respectively, and there was no significant difference between each other.

NK cell cytoactivity

The result showed that the NK cell cytoactivity in Group 1 was 19.07 ± 5.8%, which was significantly $P < 0.05$ higher than that in Group 2 (11.16 ± 5.01%) and Group 3 (12.06 ± 6.02%) ($P < 0.05$).

Discussion

Arsenic is widely distributed in nature. Inorganic arsenic is considered as the most potential human carcinogen, because arsenic exposure may cause DNA hypomethylation and facilitate aberrant gene expression [15]. However, Meng et al [16] suggested that arsenic exerted dose-dependent dual effects, with inhibition of DNA synthesis at higher concentrations and promotion of DNA synthesis at lower concentrations. In the present study, we found that As$_2$O$_3$ inhibited the growth of BC cells and induced apoptosis in a concentration-dependent and time-dependent manner. In addition, As$_2$O$_3$ elevated the percentages of CD4+ and CD8+ T cells and increased the cytoactivity of NK cells in bladder tumor-bearing mice.

Cell proliferation and apoptosis are critical mechanisms manipulating cell homeostasis and survival, which have been well illustrated in the pathogenesis of disease [17, 18]. The defects in apoptosis are now considered to be a hallmark of most cancers [19]. The current study revealed that As$_2$O$_3$ played an antineoplastic effect on MBT-2 cells by inhibiting the growth of tumor cells and inducing apoptosis. Furthermore, the inhibitory effect enhanced with the increase of As$_2$O$_3$ concentration and treatment time. Studies on the anti-proliferative effects of As$_2$O$_3$ have documented that As$_2$O$_3$ may lead to cell cycle arrest at G1 or G2 phases to inhibit cancer cell growth [13, 20-22]. The idea of cell cycle G2 phase checkpoint abrogation has generated as an anticancer insights [23].

Specially, apoptosis is regulated by multiple genes closely related to the bcl-2 family. The proposed mechanisms for the apoptotic effects also include the down-regulation of bcl-2 [24]. *Bcl-2* is an oncogene which is originally isolated from follicular lymphoma [25]. The bcl-2 protein is a critical regulator of apoptosis, which manipulates apoptosis through heterogeneous and homologous complexes [26]. High levels of

bcl-2 expression have been reported for hematological malignancies and certain solid tumors, including breast and lung cancer [27, 28]. In the present study, the down-regulated expression of bcl-2 was obviously observed with As$_2$O$_3$ treatment, which indicated that As$_2$O$_3$-induced apoptosis might be mediated by the down-regulation of bcl-2.

The present result also showed that As$_2$O$_3$ elevated the percentages of CD4+ and CD8+ T cells and increased the cytoactivity of NK cells in bladder tumor-bearing mice. The findings indicate that As$_2$O$_3$ may play an important role in cellular immune response of tumor immunity. In tumor growth and progression, immunosuppression of tumor cells is considered as a pivotal factor [29, 30]. The finished study has suggested that immune system plays significant roles in tumors elimination [31]. In immune system, T cells and NK cells, part of the innate immune component, are the key cells for tumor surveillance [32]. In some certain conditions, T cell receptor can recognize the antigen of tumor cells and activate CD4+ and CD8+ T cells. The CD4+ and CD8+ T cells can release interleukin 2 and interferon-γ, and mediate delayed hypersensitivity to play anti-tumor roles [33-35]. Additionally, NK cells participate in controlling tumor progression and metastases and are also major components of the antitumor immune response [36].

In conclusion, the results indicate that As$_2$O$_3$ significantly inhibit the growth of MBT-2 bladder cancer cells and induces apoptosis. Additionally, As$_2$O$_3$ can elevate the percentages of CD4+ and CD8+ T cells, and NK cells cytoactivity to play an immune suppressive effect on tumor growth and invasion.

Disclosure of conflict of interest

None.

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