PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORTS •

Effect of arsenic trioxide on human hepatocarcinoma in nude mice

Hong-Yu Xu, You-Lin Yang, Shu-Mei Liu, Li Bi, Shu-Xiang Chen

Hong-Yu Xu, You-Lin Yang, Li Bi, Shu-Mei Liu, Shu-Xiang Chen, Department of Gastroenterology, The First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Li Bi, Department of Laboratory, The First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Correspondence to: Dr. Hong-Yu Xu, Gastroenterology, Department, The First Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China. xuhrain@yahoo.com.cn
Telephone: +86-451-3643849-5263
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Abstract

AIM: To study the effect of arsenic trioxide (As_20_3) on human hepatoma cell line BEL-7402 *in vivo.*

METHODS: Human hepatoma cell line BEL-7402 cultured *in vitro* was inoculated into nude mice and arsenic trioxide, 5-Fu and saline were injected into abdominal cavity of the nude mice respectively. The volumes of tumor and general conditions of the nude mice and structural changes of the liver and kidney were observed. Morphologic changes were studied under electron microscope. Expression of AFP was investigated by immunohistochemical method.

RESULTS: As_2O_3 could inhibit the growth of tumor. The tumor growth inhibitory rate in mice treated with 2.5 mg/kg As_2O_3 was 53.42% on the tenth day. The tumor growth inhibitory rate in mice treated with 5 mg/kg As_2O_3 was 79.28% on the fifth day and 96.58% on the tenth day respectively. As_2O_3 did not damage the liver and kidney of nude mice, or affect the blood system. Typical apoptotic morphological changes were found under electron microscope, and the change of mitochondria was obvious. The expression rate of AFP declined after treatment.

CONCLUSION: Arsenic trioxide can induce apoptosis of human hepatoma cells, and inhibit proliferation of tumor with no obvious side effects on liver and kidney.

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INTRODUCTION

Liver cancer is one of the most aggressive malignancies and the fourth leading cause of cancer death in China, and the vast majority of patients die within the first year after diagnosis. There is still no effective therapeutic modalities. Surgical resection is the only potentially curative option, but it is not feasible in most patients because of early spread of the disease. Since arsenic trioxide (As_2O_3) has recently been recognized as an effective treatment for patients suffering from acute promyelocytic leukemia (APL)[1-5], it has also been hypothesized to be effective on some solid tumors[6,7]. As_2O_3 has been shown to have dual effects on human hepatocellular carcinoma (HCC) cell lines *in vitro*[8], including induction of apoptosis and inhibition of proliferation[9,10]. However it is not clear whether As_2O_3 has the same effect *in vivo*. We established an HCC nude mice model to evaluate anti-tumor effect of As_2O_3 *in vivo*, which might provide an experimental basis for its clinical application to the treatment of patients with HCC.

MATERIALS AND METHODS

Chemicals and reagents

As_2O_3 was purchased from Harbin Yida Medical Co (Harbin, China). Murine monoclonal antibody and antimouse rabbit polyclonal antibody were purchased from Maixin Co., Fuzhou, China.

Cell line and preparation

Human hepatoma BEL-7402 cells were purchased from the Cell Institute of Chinese Academy of Sciences and maintained in our laboratory. All media were supplemented with 100 mL/L heat inactivated fetal bovine serum, penicillin G (100 IU/mL), and streptomycin (100 μg/mL). The cells were incubated at 37 ℃ in a humidified atmosphere with 50 mL/L CO_2 and grown as monolayers in RPMI 1640 medium supplemented with 80 mL/L calf serum.

Animals

Five-week-old male nude mice weighing 17-20 g were used for subcutaneous implantation. All animals were housed in semisterile microisolator cages with autoclaved bedding, maintained on a 12-h light/dark cycle and given food and water. The experimental protocol was approved by the Experimental Animal Center of Chinese Academy of Sciences (identification No. Scfk11-6A-0006) in accordance with the national guidelines for animal care and use of laboratory animals.

Tumor induction in nude mice

Human hepatoma BEL-7402 tumor cells were grown in monolayer culture. The exponentially growing BEL-7402 cells in culture flasks were harvested, and adjusted to the concentration of 1×10^7/mL. For subcutaneous tumor formation, 200 μL of cells was injected subcutaneously into the flanks of the animals (donor mice). These mice were randomly divided into four groups: negative control group (saline), and groups of As_2O_3 (2.5 mg/kg, 5 mg/kg), 5-Fu (2.5 g/L). Each group was injected with the same volume of saline, As_2O_3 (2.5 mg/kg, 5 mg/kg), and 5-Fu (2.5 mg/mL) respectively, once a day for 10 d.

Tumor assessment

The tumor was measured with a caliper in all three perpendicular dimensions on the 1st, 5th and 10th d of treatment, and tumor volume was calculated using the following formula: volume = length×width×depth/2. All animals were killed and underwent complete examination of abdominal cavity after ten days. The liver and kidney were resected and examined under microscope. The tumor mass was isolated and weighed. The inhibitory rate of tumor was evaluated using the following formula: inhibitory rate of tumor (%) = (1-mean tumor weight in experiments / mean tumor weight in controls) ×100%.

Morphologic observation

Tumor specimens were fixed with 4 g/L formaldehyde and wrapped with wax, then stained with HE.

Transmission electron microscopy

The samples were prefixed in 25 g/L glutaraldehyde, then in 10 g/L OsO4, dehydrated in ethanol series, and replaced in propene oxide. The samples were examined with a JEM-1220 transmission electron microscope.

Immunohistochemistry

AFP protein was detected with SABC method. Tumor specimens were incubated with 3 mL/L hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, then washed in PBS and incubated in 100 mL/L normal goat serum for 20 min to reduce nonspecific antibody binding. Specimens were then incubated with a 1:50 dilution of murine monoclonal antibody against human AFP oncoprotein overnight at 4 ℃, followed by three washes with PBS, then incubated with biotinylated rabbit antimouse polyclonal antibody at a dilution of 1:100 for 30 min followed by 3 washes. Slides were then treated with streptavidin-peroxidase reagent for 30 min at a dilution of 1:100 and washed with PBS 3 times. Finally, slides were incubated in phosphate-buffered saline containing diaminobenzidine and 10 mL/L hydrogen peroxide for 10 min, counterstained with hematine, and mounted.

Blood routine test

Venous blood was taken from the orbital venous plexus of the mouse before the animal was killed. The blood routine test was done.

Statistical analysis

Data were presented as mean±SD, the differences between the rates of different groups were analyzed by χ^2 test.

RESULTS

General condition

The tumor node of the saline group was larger on the 5th and tenth days than on the first day. The appetite of mice was normal, as well as the body mass. The stool was normal. The tumor had no obvious increase after five days in 5-Fu group. The mice did not eat or drink, had a low spirit. The color of the skin turned red, and more weight was lost. The mice died gradually. The mice in As_2O_3 group was in better spirit and just the color turned red. Only two mice had diarrhea (Table 1).

Morphologic changes

The morphologic changes were observed by microscopy. Large areas of necrosis were seen under microscope. In saline group of mice BEL-7402 cells in areas of non-necrosis proliferated rapidly. The mitotic nuclei were more frequently seen. Large areas of necrosis could also be seen in the 5-Fu group, and also in the higher concentration As_2O_3 group. Nuclei appeared condensation. The liver system and kidney were normal in the low As_2O_3 group. A few vacuolar degeneration were found in the liver of the higher As_2O_3 group, but the kidney was normal. The nucleocytoplasmic ratio increased and microvilli were identified on the surface of cells in saline group. The distortion of the nuclei was frequent. There were many endoplasmic reticula and mitochondria. In As_2O_3 group, the cell nuclei became round and smaller. The aberrant cells were fewer. The microvilli were much decreased. The intact cell membrane, nuclear condensation and apoptotic body formation were seen. The changes were more obvious in the high concentration As_2O_3 group.

Effect on blood system

White blood cells, hemoglobin, and blood platelets had no significant difference between saline group and As_2O_3 group (Table 2).

Table 2 Routine blood examination results (mean±SD)

Groups	Number	WBC ($\times10^{10}$/L)	Hgb (g/L)	PLT) ($\times10^{9}$/L
Saline	10	1.05±0.12	140.24±13.21	221.65±70.34
As_20_3 (2.5 mg/kg)	10	1.13±0.16	138.67±13.17	231.75±68.48
As_20_3 (5 mg/kg)	10	1.21±0.16	142.58±15.29	246.38±60.45

Expression of AFP protein

The expression of AFP was significantly lower in As_2O_3 group than in saline group (P<0.01, Table 3).

Table 3 Expression of AFP protein (mean±SD)

Group	Number	AFP (%)
Saline	10	48.32±4.56
As_20_3 (2.5 mg/kg)	10	29.78±3.10[b]
As_20_3 (5 mg/kg)	10	22.26±2.31[b]

[b]P<0.01 *vs* saline group.

DISCUSSION

Recent clinical studies in China have shown that arsenic trioxide is an effective and relatively safe drug in the treatment of acute promyelocytic leukemia[1,2,11,12]. Chen[8] found that arsenic trioxide could trigger apoptosis of APL cell line NB4 cells, associated with downregulation of Bcl-2 gene expressions and modulation of PML-RAR alpha chimeric protein.

Experimental studies on antitumor effect were carried out in solid tumor, cancers of the lung, esophagus, stomach, colon, pancreas, breast, cervix[13,14]. It has been demonstrated that As_2O_3 could inhibit the proliferation of HCC cells and induce apoptosis of HCC *in vitro*[8,15]. Antitumor function of As_2O_3 *in vivo* was limited. We studied the action and mechanism of As_2O_3 in human hepatocarcinoma of nude mice. Our results showed that tumor mass in As_2O_3 group was smaller than that in saline group after 5 d, but there was no significant difference between the two groups (P>0.05), the growth in As_2O_3 group became slower from the 6th d. The inhibitory rate of tumor was 53.42%. The tumor node of mice treated with 5 mg/kg As_2O_3 became smaller than that of saline group. There were obvious differences between the two groups. The inhibitory rate of

Table 1 Inhibitory action of arsenic trioxide on tumor mass (mean±SD)

Groups	Number	Volume d 1	Volume d 5	Volume d 10	Mouse mass (g)	Inhibitory rate (%) 5th d	Inhibitory rate (%) 10th d
Saline	10	0.123±0.052	0.234±0.072	0.415±0.084	23.07±3.14		
5-Fu	10	0.113±0.060	0.134±0.040	14.07±2.29		86.85	
As_20_3 (2.5 mg/kg)	10	0.102±0.041	0.218±0.099	0.238±0.043[b]	20.50±1.95		53.42
As_20_3 (5 mg/kg)	10	0.098±0.030	0.121±0.038	0.103±0.042[b]	20.28±1.63	79.28	96.58

[b]P<0.01 *vs* saline group.

tumor was 79.28% on the 5th d and 96.58% on the10th d. Our concern was the toxicity of As_2O_3. It has been proved that it was not so poisonous in experiments and clinic. The toxicity depended on its dosage and the time of its use. As_2O_3 could damage normal cells when treating APL or others[2] at 0.1-0.2 μmol/L. Experiments showed that they had no effect on stem cells at this concentration. At 3.0 μmol/L As_2O_3 had teratogenetic effect on mouse embryo. The results showed it had no effect on embryo growth, development and differentiation. Our study also showed that the mice were normal in higher As_2O_3 group. The weight of mice had no significant difference between As_2O_3 group and saline group. The number of WBC, RBC, PLT was not obviously different between the two groups. There was a higher inhibitory rate of tumor in 5-Fu group (86.8%), but the weight of nude mice was reduced. The mice died gradually in 5-Fu group. Although the inhibitory rate of tumor was higher in 5-Fu group, the mice did not eat or drink. The weight was lost, and dystrophy could inhibit the growth of tumor.

AFP is a specific marker of liver cancer. BEL-7402 cells could secrete AFP. The expression rate of AFP was decreased after treatment. It showed that the level of tumor cell differentiation could be raised and the number of tumor cells was reduced after treatment. The divisions of tumor cells were decreased and apoptotic cells could be found in the lower As_2O_3 group. Large areas of necrosis could be seen in 5-Fu group, but survival tumor cells grew vigorously. It showed that 5-Fu could inhibit tumor growth due to its cytotoxic function. As_2O_3 could induce apoptosis of tumor cells.

We observed the morphologic changes of apoptosis by TEM in As_2O_3 group, the nucleocytoplasmic ratio became smaller, nuclei appeared round, mitochondria became distended, cells wrinkled, nuclear condensation and apoptotic body formation occurred. Early and obvious changes occurred in mitochondria, suggesting that As_2O_3 is toxic to mitochondria. The change of mitochondria could induce apopotosis. Further studies on the mechanism of mitochondria denaturation in order to prove that As_2O_3 could treat HCC are needed.

In conclusion, arsenic trioxide can induce apoptosis of human hepatocarcinoma cells and inhibit their proliferation. It has no side effects on the liver, kidney and blood system.

REFERENCES

1 **Zhang P,** Wang SY, Hu LH, Shi FD, Qiu FQ, Hong LG, Han XY, Yang HF, Song YZ, Liu YP, Zhou J, Jing ZJ. Treatment of acute promyelocytic leukemia with intravenous arsenic trioxide. *Zhonghua Xueyexue Zazhi* 1996; **17**: 58-60

2 **Andre C,** guillemin MC, Zhu J, Koken MH, Quignon F, Herve L, Chelbi Alix MK, Dhumeaasx D, Wang ZY, Degos L, Chen Z, De The H. The PML and PML/RARalpha domains from autoimmunity to molecular oncology and from retinoic acid to arsenic. *Exp Cell Res* 1996; **229**: 253-260

3 **Wang ZY**. Development of arsenical clinical application and study of arsenical mechanism. *Zhonghua Xueyexue Zazhi* 1996; **17**: 57

4 **Shen ZX,** Chen GQ, NI JH, Li XS, Xiong SM, Qin QY, Zhu J, Tang W, Sun GL, Yang KQ, Chen Y, Zhou L, Fang ZW, Wang YT, Zhang P, Zhang TD, Chen SJ, Chen Z, Wang ZY. Use of arsenic trioxide in the treatment of Acute promyelocytic leukemia II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 1997; **89**: 3354-3360

5 **Konig A,** Wraze L, Warrelll RP Jr, Rivi R, Pandolfi PP, Jakubowski A, Gabrilove JL. Comparative activity of melarsoprol and arsenic trioxide in chronic B-cell leukemia lines. *Blood* 1997; **90**: 562-570

6 **Tan LJ,** Chen XY, Zhang L, Tang XM, Shen ZY, Cai WJ. Study on the proliferative inhibition of human esophageal cancer cells with treatment of DMSO and As_2O_3. *Shanghai Dier Yikedaxue Xuebao* 1999; **19**: 5-8

7 **Tu SP,** Jiang SH, Tan JH, Jiang XH, Qiao MM, Zhang YP, Wu YL, Wu YX. Proliferation inhibition and apoptosis induction by arsenic trioxide on gastric cancer cell SGC-7901. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 18-21

8 **Chen HY,** Liu WH, Qin SK. Induction of arsenic trioxide on apoptosis of hepatocarcinoma cell lines. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 532-535

9 **Guo WJ,** Yu EX, Zheng SG, Shen ZZ, Luo JM, Wu GH, Xia SA. Study on the apoptosis and cell cycle arrest in human liver cancer SMMC7721 cells induced by Jianpiliqi herbs. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 52-55

10 **Shen YF,** Zhuang H, Shen JW, Chen SB. Cell apoptosis and neoplasms. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 267-268

11 **Feng CQ,** Ma WL, Zheng WL. Research advances on effect of arsenic trioxide on tumor. *Aizheng* 2002; **21**: 1386-1389

12 **Liu Q,** Hilsenbeck S, Gazitt Y. Arsenic trioxide-induced apoptosis in myeloma cells: p53-dependent G1 or G2/M cell cycle arrest, activation of caspase-8 or caspase-9, and synergy with APO2/TRAIL. *Blood* 2003; **101**: 4078-4087

13 **Kanzawa T,** Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res* 2003; **63**: 2103-2108

14 **Huang SG,** Kong BH, Yang RF, Jiang S. Primary study of arsenic trioxide inhibits abdomino-metastatic tumor formation of human ovarian carcinoma in nude mice and its mechanisms. *Aizheng* 2002; **21**: 401-404

15 **Yu D,** Wang Z, Zhu L, Chew EC. Nuclear matrix associated protein PML: an arsenic trioxide apoptosis therapeutic target protein in HepG2 cells. *Chin Med J* 2003; **116**: 93-98

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