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A study on arsenic trioxide inducing *in vitro* apoptosis of gastric cancer cell lines

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INTRODUCTION

Cell apoptosis, which involves the biologic regulation of the numbers and vital activity of cells, is an important metabolic process in both normal cells and tumor cells. Delayed process of cell apoptosis will probably lead to a disturbance of metablism, and occurrence and development of neoplasms. Song *et al* have proved the relationship between apoptosis delay and tumor development through inhibition of cell apoptosis induced by tumor promotor[1]. Thus, induction of cell apoptosis could be a new strategic measure against tumor. In this paper, we studied whether arsenic oxide will induce apoptosis of gastric cancer cell lines (GCCL) to explore the use of such chemical agent against gastric cancer in clinic.

MATERIALS AND METHODS

Target cells

Human gastric cancer cell lines, MKN45 and SGC7901 (provided by Chinese Academy of Sciences) were used as target cells. Human leukemia cell line K562 and human peripheral blood lymphocytes (PBL) were used as controls. All of cells were cul tured in RMPI 1640 medium (GIBICO-BRL), supplemented with 10% heat-inactivated fetal calf serum, 100U/mL penicillin and 100mg/mL streptomycin ,in a humidified atmosphere of 95% air/5% CO_2 at 37℃. The numbers of four kinds of cells were maintained at 1×10^6/mL by daily adjusting cell concentration.

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Product of arsenic trioxide (As_2O_3)

Ai-Lin No.1 containing As_2O_3 was prepared by pharmacy of our hospital. Stoc k solution was made at the concentration of 1mmol/L with phosphate-buffered saline (PBS) and diluted with RMPI 1640 to working concentration before use.

Cytocide test

MTT[3-(4,5-dimethythiazoyl-2-yl)2,5-diphenylte-trozoliumbromide]colori metric analysis was used to measure the cytocide rate of As_2O_3. Well grown cells were collected and were put into 96 well culture plate at 1×10^4/wel l. As_2O_3 was added to culture plate at the concentration of 5 μmol/well, while cells stuck on wall, 6 duplicate wells were set up in each sample in both experimental and control groups. Cells were cultrured for 24 h, 48 h and 72 h separately, MTT(5 mg/mL) was add ed at 10 μL/well. Acidulated isopropanol was finally added at 100 μL/well-after 2 h culture; values of OD570 were obtained on autocolorimeter. Cytocide rate was calculated by the following formula:

$$\text{Cytocide rate}(\%) = \frac{\text{Control OD-Experimental OD}}{\text{Control OD}} \times 100\%$$

Apoptosis detection

Cell treatment MKN45 and SGC7901 cells of 10^6 were treated with As_2O_3 of the concentration of 5 μmol and 10 μmol, or with 5-Fu (0.5 g/L), or without any treat ment as control. Cells were harvested after 24 h, 48 h and 72 h culture for apoptosis detection.

TDT label Consulting Gregory modified method[2]: PBS containing 1% formalin was added to cells at 4℃. Thirty min later, it was washed twice with PBS, then reacted with 0.5 μg terminal deoxynucleotidyl transferase (TDT) and 0.5 μmol biotinylted dUTP at 37℃ for 30 min. Afterwards it was washed again with PBS, finally labelled by affinitin-fluorescin isothiocyanate (FITC) at room temperature for 30 min. DNA strand will be broken off during apoptosis, which can belabelled by dUTP and the positive observable fluorescences were seen under fluorescent microscope.

Flow cytometry(FCM) assays and fluorescent photographing The stimulating wave length of FCM (Becton Dickson FAC Scan)is 488 nm, FITC detec tion spectrum is between 80 and 630 nm. A total of 2000 cells were counted. Results were recorded and analyzed automatically.

Preparation of specimen for electronic microscopy
MKN45 cells treated with As_2O_3 of concentration of 10 μmol for 72 h were washed with PBS and fetal calf serum; 25% glutaraldehyde was added to fix the cell specimens for 12 h. Then it was washed twice with 0.1 mol/L phosphoric buffer solution; the specimens were fixed with 1% osmi c acid, and were dehydrated step by step with alcohol and acetone; then covered up with Epon 812 epoxy vesin. Sections were made by ultramicrotome and dyed with uranium acetate and citric acid, and finally observed under transmission electr onic microscope.

RESULTS

Cytocide rate of As_2O_3 on GCCL

Our results showed that As_2O_3 had higher cytotoxicity on GCCL than on K562 ($P<0.05$). The effect of cytocide was observed at 24 h after reac tion, and it increased with time (Table 1).

Table 1 Cytocide effects of As_2O_3 (5¦Ìmol) on various cell categories (%, $\bar{x} \pm s$)

Group	MKN45	7901	K562	PBL
24 h	69.45 ± 11.12	68.27 ± 8.27	51.36 ± 10.25[a]	30.31 ± 5.12[b]
48 h	71.40 ± 10.15	69.04 ± 11.31	57.11 ± 7.45[a]	35.67 ± 4.37[b]
72 h	80.53 ± 10.18	82.74 ± 9.14	65.33 ± 8.24[a]	39.74 ± 8.27[b]

Comparison of cytocide rate between GCCL and controls by As_2O_3, [a]$P<0.05$($t>2.2262$), [b]$P<0.01$($t>3.250$)

Table 2 Apoptosis rate(%) of MKN45 by different treatment methods ($\bar{x} \pm s$)

Group	Controls	5-Fu	$As_2O_3$1	$As_2O_3$2
24 h	4.71 ± 0.36	18.04 ± 1.50	32.33 ± 3.75[a]	60.02 ± 7.41[b]
48 h	14.90 ± 0.94	24.39 ± 3.45	90.59 ± 10.35[b]	97.05 ± 8.24[b]
72 h	28.98 ± 3.12	41.47 ± 2.24	98.10 ± 13.24[b]	98.75 ± 11.53[b]

Comparison of apoptosis rate between As_2O_3 and 5-Fu group, [a]$P<0.05$ ($t>2.2262$), [b]$P<0.01$ ($t>3.250$).

GCCL apoptosis rate induced by As_2O_3

As shown in Table 2, apoptosis rates of 2 kinds of GCCL induced by 5-Fu were also obviously higher than natural cell apoptosis rate, of controls, which sugg ested that antitumor drug can kill tumor cells by inducing cell apoptosis. Never theless, a more significant cytocide effect of As_2O_3 on GCCL was demonstrate d in our study compared with that of 5-Fu. We found that apoptosis rate of GCCL induced by As_2O_3 is correlated with the concentration and reaction time of As_2O_3 (Table 3).

Table 3 Apoptosis rate (%) of SGC7901 by different treatment method s ($\bar{x} \pm s$)

Group	Controls	5-Fu	$As_2O_3$1	$As_2O_3$2
48 h	7.44 ± 0.60	34.28 ± 4.15	51.33 ± 5.16[a]	78.31 ± 9.14[b]
72 h	30.90 ± 2.54	56.32 ± 4.56	78.15 ± 6.78[a]	89.28 ± 10.26[b]

Comparison of apoptosis rate between As_2O_3 and 5-Fu group , [a]$P<0.05$(t>2.2262), [b]$P<0.01$($t>3.250$)

The morphology of GCCL apoptosis under fluorescent microscope:

Under fluorescent microscope, the apoptosis cell can be seen after being termina lly labelled (positive), but nonapoptotic cells were not labelled by fluorescin isothiocyanate (negative). The positive staining showed bright green fluorescence. Fluorescent spots appeared in early stage, and these fluorescent bodies gathered like a bunch of grapes in late stage. The cellular volume can be seen shrunken under the microscope.

The morphology of GCCL apoptosis under electronic microscope:

Under the transmission electronic microscope, typical morphologic changes of apoptotic GCCL (mainly cell nucleus) took place after treatment of As_2O_3. These changes included cell nucleus fixation and shrinkage of GCCL, chromatin condensation, and fragmentation of apoptotic bodies. These changes coexisted.

DISCUSSION

Some researchers have proposed that the uncontrolled growth of neoplasms would be due to the loss of the nature of autoapo ptosis rather than over proliferation. Previous studies have proved that there existed autoapoptosis blockage in tumor cells. Lauwers *et al* [3]examined bcl-2 gene in 46 cases of gas tric adenocarcinoma by immunochemical method, revealling 75% positivity.Of bcl-2 gene in tumor tissues, which indicated that apoptosis was blocked in gastric cancer. Bcl-2 has been considered as one of survival genes which pla ys an important role in the specific-inhibition of tumor cell apoptosis[4]. Based on these findings, a new proposal of inducing apoptosis to inhibit tumor growth was introduced[5]. Many factors such as high temperature, cytokine, radiations and all kinds of anti-tumor chemotherapy drugs have a cer tain effect on inducing tumor cells apoptosis. But some of these are not satisfa ctory. Our aim is to find a specific-agent which can induce apoptosis of tumor cells. Vollmers *et al*[6] reported the suppressive effects of monoc lonal antibody (SC-1) on both *in vitro* proliferation of gastric cancer cell line and growth of a tumor inoculated on nude mice.The inhibition of proliferation of tumor cells was produced through the induction of autoapoptosis, which has been proved by the observation of ultrastructure.

Arsenic is a major composition of traditional Chinese medicine, white arsenic. White arsenic has been considered as a carcinogen. It can inactivate some impor tant enzymes in cells, change the metabolic process and induce chromosome aberra tion[7]. Zhang *et al*[8] reported a satisfactory result by us ing As_2O_3 for the treatment of early acute promyelocytic leukemia (APL). Com plete remission was 73.3% in patients after the first therapeutic course, and 52.83% in recurrent

patients. The longest remission period of APL patient was over 10 years. No obvious toxic reactions were found when As_2O_3 was given by iv drip, which is appropriate. Similar result has also been obtained by researchers at Shanghai Institute of Hematology of Shanghai Ruijin Hospital[9,10]. Recently, Zhang *et al*[11] demonstrated that Arsenic Oxide can inhibit growth of l ymphosarcoma cells and induce apoptosis to these cells.

Based on the above studies, we applied As_2O_3 for the treatment of GI solid tumor. The results from *in vitro* study are impressive. Proliferation of MKN45 and SGC7901 was inhibited by As_2O_3 through apoptosis induction. Results also showed that As_2O_3 has a stronger effect of apoptosis induction than 5-Fu. Induction of apoptosis was enhanced with increase of concentration and time of As_2O_3. The question is what the optimal dosage is for clinical use so as to produce maximal effect with no toxicity. Further comprehensive researches are needed to clarify the significance of As_2O_3 for the treatment of GI solid tumors.

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