Effect of arsenic trioxide on vascular endothelial cell proliferation and expression of vascular endothelial growth factor receptors Flt-1 and KDR in gastric cancer in nude mice

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Abstract

AIM: To investigate the effect of arsenic trioxide (As$_3$O$_3$) on expression of vascular endothelial growth factor receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR) in human gastric tumor cells and proliferation of vascular endothelial cells.

METHODS: The solid tumor model was formed in nude mice with the gastric cancer cell line SGC-7901. The animals were treated with As$_3$O$_3$. Microvessel density (MVD) and expression of Flt-1 and KDR were detected by immunofluorescence laser confocal microscopy. SGC-7901 cells were treated respectively by exogenous recombinant human VEGF$_{165}$ or VEGF$_{165}$ + As$_3$O$_3$. Cell viability was measured by MTT assay. Cell viability of ECV304 cells was measured by MTT assay, and cell cycle and apoptosis were analyzed using flow cytometry.

RESULTS: The tumor growth inhibition was 30.33% and 50.85%, respectively, in mice treated with As$_3$O$_3$. 2.5 and 5 mg/kg. MVD was significantly lower in arsenic-treated mice than in the control group. The fluorescence intensity levels of Flt-1 and KDR were significantly less in the arsenic-treated mice than in the control group. VEGF$_{165}$ may accelerate growth of SGC7901 cells, but As$_3$O$_3$ may disturb the stimulating effect of VEGF$_{165}$. ECV304 cell growth was suppressed by 76.51%, 71.09% and 61.49% after 48 h treatment with As$_3$O$_3$ at 0.5, 2.5 and 5 μmol/L, respectively. Early apoptosis in the As$_3$O$_3$-treated mice was 2.88-5.1 times higher than that in the controls, and late apoptosis was 1.17-1.67 times higher than that in the controls.

CONCLUSION: Our results showed that As$_3$O$_3$ delays tumor growth, inhibits MVD, down-regulates Flt-1 and KDR expression, and disturbs the stimulating effect of VEGF$_{165}$ on the growth of SGC7901 cells. These results suggest that As$_3$O$_3$ might delay growth of gastric tumors through inhibiting the paracrine and autocrine pathways of VEGF/VEGFRs.

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Key words: Arsenic trioxide; Gastric tumor; Flt-1; Tumor growth inhibition

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INTRODUCTION

Angiogenesis is recognized as playing a role in the pathophysiology of many human malignancies. It is an important factor in the progression and enlargement of solid neoplasms and is closely related to invasion and metastases[1-3]. Angiogenesis is influenced by a number of positive and negative regulatory factors, such as cytokines, extracellular matrix, and other cellular constituents including pericytes[4]. Among the factors contributing to angiogenesis, vascular endothelial growth factor (VEGF) is recognized as one of the most important molecules in the formation of new blood vessels. VEGF is a potent and specific mitogen for endothelial cells that activate the angiogenic switch in vivo and enhance vascular permeability. A variety of malignant human tumors are known to secrete VEGF, which has been correlated with the onset of angiogenesis in tumors[5-7]. Over-expression of VEGF is suggested to participate in the carcinogenic process[8]. VEGF binds to two distinct receptors on endothelial cells: VEGFR-1 (fms-like tyrosine kinase receptor 1, Flt-1) and VEGFR-2 (kinase insert domain containing receptor human homologue/fetal liver kinase 1
murine homologue, KDR/Flk-1)\(^{10,11}\). KDR is responsible for mitogenic signaling, and plays an important role in vasculogenesis and blood island formation, and Flt-1 regulates the assembly of endothelial cells and tissue factor production in endothelial cells\(^{12}\). Thus, it is suggested that inhibition of VEGF/VEGFR pathways may interrupt VEGF-induced angiogenesis. Recently, a few studies have demonstrated co-expression of VEGF and its receptors in tumor cells, which suggests that a VEGF autocrine pathway exists in tumor cells\(^{11-14}\).

Arsenic is a common natural substance. Arsenic trioxide (As\(_2\)O\(_3\)) has shown substantial efficacy in treating both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL)\(^{13}\). Recent studies have shown that a wide variety of malignancies, including both hematological cancer and solid tumors derived from several tissue types, may be susceptible to therapy with As\(_2\)O\(_3\)\(^{15-19}\). These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Emerging data suggest that arsenic induces apoptosis and inhibits tumor growth\(^{15,16,20}\). It has recently been reported that arsenic may inhibit angiogenesis\(^{21-23}\). We have recently shown in vitro and in vivo that As\(_2\)O\(_3\) can inhibit VEGF expression and suppress angiogenesis and gastric tumor growth\(^{24}\).

In the present work, we investigated further the effect of As\(_2\)O\(_3\) on the expression of VEGF receptors Flt-1 and KDR in human gastric tumor cells and the proliferation of vascular endothelial cells. Our study demonstrated that As\(_2\)O\(_3\) delayed tumor growth by inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

### MATERIALS AND METHODS

#### Animals and cells

Male Balb/c mice, 5-wk-old and weighing 19-21 g (from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were used in this study. The mice were kept in a laminar-filtered airflow cabinet under pathogen-free conditions with a constant temperature of 22\(\pm\)2\(^{\circ}\)C, relative humidity of 55\%\pm\%5\%, and 12-h dark/light cycles. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Fourth Military Medical University. Human gastric cancer cell line SGC-7901 and ECV304, a cell line derived from human umbilical vessel endothelial cells, were purchased from the Animal Laboratory Centre, Fourth Military University.

#### Tumor xenografts in nude mice

SGC-7901 cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS) at 37\(^{\circ}\)C in a 5 mL/L CO\(_2\) incubator. Thirty mice received subcutaneous injection in the right flank with 200 \(\mu\)L cell suspension containing 2 \(\times\) 10\(^7\) SGC-7901 cells. After 10 d, when established tumors of 0.2 cm-0.3 cm diameter were detected, drug administration was started.

#### Drug treatment

The animals were randomly divided into three groups of 10 animals each. Arsenious acid [H\(_3\)AsO\(_3\)] (As\(_2\)O\(_3\) + 3H\(_2\)O \(\rightarrow\) H\(_3\)AsO\(_3\); Yida Pharmaceutical, Harbin, China) diluted with saline solution was injected intraperitoneally every day to the two treatment groups (2.5 and 5 mg/kg in 0.2 mL) and the same volume of saline solution was injected into the control group. After 10 d of treatment, three groups of mice were sacrificed and the tumor masses were removed. After the weight of tumor masses was measured, they were fixed in 40 g/L paraformaldehyde, and the frozen sections were prepared with a cryomicrotome (HM505E, Microm, Germany) for immunofluorescence analysis.

#### Tumor inhibition and regression

Tumor growth inhibition (TGI) was calculated by measuring tumor volumes before treatment, and 6 and 11 d after treatment. Tumor volume (cm\(^3\)) was calculated using the formula: tumor volume = 1/2 \(\times\) \(a\) \(\times\) \(b\) \(\times\) \(c\), where \(a\) is the long axis, and \(b\) the short axis. TGI (\%) = (1 - \(V_t\)/\(V_c\)) \times 100\%, where \(V_t\) is the mean tumor volume of the arsenic-treated group, and \(V_c\) the mean tumor volume of the control group.

#### Microvessel density (MVD)

For identification of microvascular structure, frozen sections were cut and stained with rat anti-mouse CD31 (Biolegend, USA) and secondary antibody (goat anti-rat IgG conjugated TRITC). MVD was determined by confocal microscopy of tumor tissue sections. A single microvessel was defined as any immunofluorescent endothelial cell that was distinguished from adjacent tumor cells and other connective tissue elements. The microvessels were carefully counted in 20 fields (\(\times 400\)). The mean \(\pm\) SE was expressed as the number of microvessels identified within the area.

#### Immunofluorescence for Flt-1 and KDR

The frozen sections were kept at room temperature for 30 min, incubated in distilled water and PBS for 5 min each, permeabilized in 1 g/L Triton-X-100 for 10 min, washed with PBS, blocked with 100 mL/L sheep serum (Sigma, USA) at 37\(^{\circ}\)C for 20 min, incubated with the primary antibody (Flt-1 and KDR rabbit polyclonal antibodies; Lab Vision, Fremont, CA, USA) at 4\(^{\circ}\)C overnight, washed with PBS, incubated with the secondary antibody (sheep anti-rabbit IgG conjugated FITC, diluted 1:100; Sigma) for 1 h at 37\(^{\circ}\)C, washed with PBS, and then examined by a TCS SP2 laser confocal microscope (Leica, Wetzlar, Germany).

For each group, several field images of Flt-1 or KDR were observed under confocal microscopy. The fluorescence intensity of each cell in the confocal fluorescence images was measured using the Leica Confocal analysis system, and the mean fluorescence intensity in a group of cells was then calculated.

#### Cell viability assay

ECV304 cells were seeded in a 96-well plate (2 \(\times\) 10\(^3\) cells/well). After 48 h seeding, cells were treated with As\(_2\)O\(_3\) (0.5, 2.5 and 5 \(\mu\)mol/L) for 2 d, in three parallel wells each, and untreated cells served as a control. By 48 h, 20 \(\mu\)L
MTT (15 mg/mL) was added to each well and incubated for a further 4 h. The medium was removed and 150 μL DMSO was added to each well. A<sub>570</sub> was measured using a microculture reader. The percentage of viable cells was calculated as follows: (A<sub>570</sub> of experimental group/A<sub>570</sub> of control group) × 100%.

SGC-7901 cells were seeded in a 96-well plate (2 × 10<sup>3</sup> cells/well). After sedimentation, exogenous recombinant human VEGF<sub>165</sub> (2.5, 5, 15 and 20 ng/mL) or VEGF<sub>165</sub> (2.5, 5, 15 and 20 ng/mL) + 2.5 μmol/L As<sub>2</sub>O<sub>3</sub> or VEGF<sub>165</sub> (2.5, 5, 15 and 20 ng/mL) + 5 μmol/L As<sub>2</sub>O<sub>3</sub> were added to the medium, in three parallel wells each, and cultured for a further 48 h. For control wells, an equal volume of medium was added. The methods for the MTT assay and calculation of the percentage of viable cells were the same as above.

Flow cytometry by annexin V-FITC conjugated with propidium iodide (PI) staining

ECV304 cells were seeded in a 24-well plate (1 × 10<sup>5</sup> cells/well). After 48 h seeding, cells were treated with As<sub>2</sub>O<sub>3</sub> (0.5, 2.5 and 5 μmol/L) for 2 d, and untreated cells served as controls. By 48 h, the cells were washed twice with cold PBS and then resuspended in a binding buffer at a concentration of 1 × 10<sup>6</sup> cells/mL, and the 100 μL solution (1 × 10<sup>5</sup> cells) was transferred to 5-mL culture tubes. Five microliters of annexin V-FITC and 10 μL PI (μg/mL) were added to each 100-μL solution, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. The samples, to which 400 μL PBS was added, were analyzed by FACS Calibur flow cytometer (BD Biosciences, USA). Early apoptosis was estimated by the relative amount of FITC-PI cells.

Flow cytometry by PI staining

ECV304 cells were treated with As<sub>2</sub>O<sub>3</sub> 0.5, 2.5 or 5 μmol/L. By 48 h, the harvested cells were fixed with 1 mL of 750 mL/L cold ethanol at 4°C overnight, and then washed with PBS. The cells were incubated with RNAse and PI for 30 min in the dark. Cell cycle of samples was analyzed using FACS Calibur flow cytometry.

Statistical analysis

The data were represented as mean ± SD. Data were analyzed with SPSS 10.0 statistical software (SPSS, Chicago, IL, USA). Multiple statistical comparisons were performed using ANOVA in a multivariate linear model. The Student-Newman-Kuels test was used to assess differences between the treatment and control group. P < 0.05 was considered statistically significant.

RESULTS

As<sub>2</sub>O<sub>3</sub> inhibition of growth of human gastric tumor xenografts

All 30 nude mice developed tumors 10 d after implantation of SGC-7901 cells. When drug administration was started, there was no significant difference in the tumor volume of the three groups (control group, 67 ± 45 mm<sup>3</sup>; 2.5 mg/kg As<sub>2</sub>O<sub>3</sub> group, 63 ± 36 mm<sup>3</sup>; 5 mg/kg group, 66 ± 48 mm<sup>3</sup>, P > 0.05). Tumor volume in the three groups from 0, 6 and 11 d after treatment is shown in Figure 1. There were significant differences in the tumor volumes in the arsenic-treated groups (2.5 mg/kg, 696 ± 125 mm<sup>3</sup> and 5 mg/kg, 491 ± 116 mm<sup>3</sup>) and control group (999 ± 338 mm<sup>3</sup>, P < 0.05). On the other hand, the tumor volume in the 5 mg/kg As<sub>2</sub>O<sub>3</sub> group was significantly less than that in the 2.5 mg/kg group (P < 0.05). TGI was 30.33% (2.5 mg/kg group) and 50.85% (5 mg/kg group) after arsenic treatment.

As<sub>2</sub>O<sub>3</sub> inhibition of tumor angiogenesis

Sections of tumors were stained for CD31 immunofluorescence to detect the number of endothelial cells (ECs) as a measure of tumor angiogenesis. MVD was significantly lower in the 2.5 mg/kg As<sub>2</sub>O<sub>3</sub> group (9.32 ± 0.33 vs 5.36 ± 0.32, P < 0.01) and 5 mg/kg group (9.32 ± 0.33 vs 3.05 ± 0.24, P < 0.01) than in the control group. MVD was significantly lower in the 5 mg/kg group than in the 2.5 mg/kg group. These results demonstrated the decreased capillary density of the tumor after treatment with As<sub>2</sub>O<sub>3</sub> (Figure 2).

Effect of As<sub>2</sub>O<sub>3</sub> on Flt-1 and KDR expression in tumor xenografts

Expression of Flt-1 and KDR was confirmed by the presence of fluorescence-stained cytoplasm in the tumor cells. Stronger immunoreactivity to Flt-1 (Figure 3A) and KDR (Figure 4A) was found in all the SGC-7901 tumor xenografts of the
control group. The weaker fluorescence intensity was observed in tumor cells of the mice treated with 2.5 mg/kg (Figures 3 and 4B) and 5 mg/kg (Figures 3 and 4C) As$_2$O$_3$. The expression of Flt-1 and KDR in tumor cells was significantly less in the arsenic-treated groups than in the control group ($P < 0.001$). Expression of Flt-1 and KDR in the 5 mg/kg As$_2$O$_3$ group was less than that in the 2.5 mg/kg group (Table 1).

**Effect of As$_2$O$_3$ on VEGF-stimulated growth of SGC7901 tumor cells**

SGC7901 cells were incubated with varying concentrations of VEGF$_{165}$ or VEGF$_{165} + 2.5$ μmol/L As$_2$O$_3$ and varying concentrations of VEGF$_{165} + 5$ μmol/L As$_2$O$_3$. Its effects were measured using the MTT assay (Table 2). The results showed that VEGF$_{165}$ may have accelerated the growth of SGC7901 cells,
but As$_2$O$_3$ may have disturbed the stimulatory effect of VEGF$_{165}$.

**Effect of As$_2$O$_3$ on proliferation of vascular endothelial cells**

Cell viability was determined by the MTT assay (Figure 5). As$_2$O$_3$ inhibited the growth of ECV304 cells in a dose-dependent manner. Cell growth was suppressed by 76.51%, 71.09% and 61.49% after 48 h treatment with As$_2$O$_3$ at 0.5, 2.5 and 5 μmol/L, respectively.

**Cell cycle and apoptosis of vascular endothelial cells induced by As$_2$O$_3$**

Flow cytometry with only PI staining showed that the percentage of cells treated with As$_2$O$_3$ was higher in the sub-G1 period, lower in the sub-S period, and lower in the G2/G1 period when compared to the controls; and apoptotic percentage of cells treated with As$_2$O$_3$ was higher in the sub-G1 period, lower in the sub-S period, and lower in the G2/G1 period when compared to the controls. Apoptosis was 1.17-1.67 times than that of the controls. Early apoptosis in As$_2$O$_3$-treated groups was 2.88-5.1 times higher than that of the controls, and late apoptosis was 1.17-1.67 times than that of the controls.

**DISCUSSION**

Angiogenesis is critical for supporting the rapid growth of tumors. Angiogenesis inhibition is a promising therapeutic approach for the treatment of cancer. As$_2$O$_3$ has been demonstrated to induce complete remission in patients with APL without severe toxicity. More recently, in vitro studies have shown As$_2$O$_3$ apoptosis in other leukemia and solid tumor cells. As a novel anticanicar agent, a few in vivo investigations of its efficacy on solid tumors have been carried out.$^{19,21}$ In this study, we observed the effect of As$_2$O$_3$ on growth and angiogenesis in a gastric cancer SGC7901 xenograft model. MVD was measured in tumor tissue by the amount of labeled CD31. The results revealed that a 10 d treatment with As$_2$O$_3$ resulted in tumor growth inhibition and MVD decrease. TGI was respectively 29.08% and 52.17% in mice treated with 2.5 and 5 mg/kg As$_2$O$_3$. MVD was significantly lower in tumor tissues in arsenic-treated mice than in the control groups. The results demonstrated that As$_2$O$_3$ inhibited gastric cancer growth in vivo and suppressed the formation of new blood vessels in cancer tissues. Anti-angiogenesis may be one of the mechanisms by which As$_2$O$_3$ inhibits gastric cancer growth.

ECs are the primary structured units of blood vessels. In recent years, ECs have been the target cell of some angiogenesis inhibitors that have been undergoing clinical trials. These inhibitors can selectively affect diverse EC functions that are related to angiogenesis, including activation, proliferation, migration, invasion and survival.$^{25,26}$ It has been reported that particles of realgar (As$_2$S$_3$) with an average diameter of 100-150 nm can induce EVCV-304 cell apoptosis.$^{27}$ We studied the effect of As$_2$O$_3$ on growth and proliferation of ECV304 cells. In our study, the MTT assay showed that As$_2$O$_3$ inhibited the growth of ECV304 cells in a dose-dependent manner. Flow cytometry assays with only PI staining showed that the percentage of the cells treated with As$_2$O$_3$ were higher in the sub-G1 period, lower in the sub-S period, and lower in the G2/G1 period when compared to the controls; and 14.84% and 18.9% of the cells treated with As$_2$O$_3$ at 2.5 and 5 μmol/L, respectively, were apoptotic. Early apoptosis in the As$_2$O$_3$ treated groups was 2.88-5.1 times higher than that in the controls, and late apoptosis was 1.17-1.67 times higher than that in the controls. These results showed that As$_2$O$_3$ inhibited the viability of ECV304 cells and arrested the cells in G1 phase, blocked or delayed their entry into S phase, disturbed DNA synthesis, and induced apoptosis in

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**Table 1** Effect of As$_2$O$_3$ on Flt-1 and KDR/Flk-1 expression in tumor xenografts (fluorescence intensity) ($n = 10$, mean ± SD)

| Group       | Flt-1       | KDR/Flk-1   |
|-------------|-------------|-------------|
| Control     | 137.41 ± 3.36 | 141.62 ± 1.66 |
| 2.5 mg/kg   | 103.53 ± 2.23$^a$ | 114.99 ± 2.19$^a$ |
| 5 mg/kg     | 72.17 ± 0.87$^{a,b}$ | 101.11 ± 1.89$^{a,b}$ |

$^a$P < 0.001, treatment groups vs control group; $^b$P < 0.001, 5 mg/kg group vs 2.5 mg group.

**Table 2** Effect of As$_2$O$_3$ on VEGF-stimulated growth of tumor cells (%)

| As$_2$O$_3$ (μmol/L) | VEGF | VEGF + 2.5 μmol/L As$_2$O$_3$ | VEGF + 5 μmol/L As$_2$O$_3$ |
|----------------------|------|-----------------------------|-----------------------------|
|                      | 2.5  | 5                           | 10                          | 15              | 20              |
| 105.79               | 106.62 | 100.97                      | 107.59                      | 109.38          |
| 84.24                | 97.95  | 97.95                       | 100.48                      | 94.94           |
| 76.87                | 68.55  | 83.74                       | 85.06                       | 84.94           |

**Figure 5** Effect of As$_2$O$_3$ on growth of ECV304 cells. The cell viability was analyzed by MTT assay.

**Table 3** Effect of As$_2$O$_3$ on cell cycle of ECV304 cells (%)

| Groups | G1  | G2/G1 | S    | Apoptosis |
|--------|-----|-------|------|-----------|
| Control| 94.24 | 2.05  | 5.76 | 0         |
| 0.5 μmol/L As$_2$O$_3$ | 96.17 | 1.81  | 2.59 | 0         |
| 2.5 μmol/L As$_2$O$_3$ | 96.15 | 1.91  | 14.84 | 0         |
| 5 μmol/L As$_2$O$_3$ | 96.96 | 1.84  | 18.90 | 0         |
the G1 phase.

Tumor angiogenesis appears to be achieved by the overexpression of angiogenic agents within solid tumors that stimulate host vascular EC mitogenesis and possibly chemotaxis. In recent years, it has been widely shown that activity of VEGF is a key feature during tumor growth and angiogenesis. In the course of occurrence and development of gastric cancer, angiogenesis plays a crucial role in which VEGF is believed to be the most important factor in neovascularization. Some studies have shown that the serum level of VEGF is not only related to prognosis of gastric cancer, but also to treatment efficacy. Both types of specific receptors for VEGF, Flt-1 and KDR, have been found to be important in the regulation of angiogenesis.

Figure 6 Effect of As$_2$O$_3$ on cell cycle of ECV304 cells. A: Control; B: 0.5 μmol/L As$_2$O$_3$; C: 2.5 μmol/L As$_2$O$_3$; D: 5 μmol/L As$_2$O$_3$.

Figure 7 Induction of apoptosis in ECV304 with As$_2$O$_3$. Dual-stained with Annexin-V-FITC and PI and analyzed by flow cytometry. There was a dose-dependent increase in early apoptotic cells, as shown in the fourth quadrants plots. A: Control; B: 0.5 μmol/L As$_2$O$_3$; C: 2.5 μmol/L As$_2$O$_3$; D: 5 μmol/L As$_2$O$_3$.
and KDR, are commonly distributed in ECs. VEGF acts by binding to the receptors on ECs, and prompts EC proliferation. VEGF and its receptors are the most important pathways in tumor angiogenesis. Inhibition of VEGF/VEGFR pathways may suppress angiogenesis and tumor growth. However, the expression of VEGFR is not EC-specific, and recent emerging evidence has shown that VEGFRs are expressed in several types of non-endothelial cells, especially in tumor cells, which indicates that there is an autocrine pathway of VEGF in tumor cells. A report by Zhang et al[8] has shown that eight gastric cancer lines (RF-1, RF-48, NCI-SNU-1, NCI-SNU-5, NCI-SNU-16, AGS-1 and KATO-III) express VEGF, and six of these express both Flt-1 and KDR, and exogenous VEGF can stimulate the growth of KDR-positive tumor cells. These results suggest that VEGF acts not only as a paracrine factor on ECs, but also as an autocrine factor on tumor cells. VEGFR may play an important role in paracrine and autocrine pathways of VEGF, and VEGF inhibitors can inhibit tumor angiogenesis and growth.[8,9]

Our recent study[9] has demonstrated that As$_2$O$_3$ can inhibit expression of VEGF in gastric cancer. In the present study, we examined further the effect of As$_2$O$_3$ on VEGFR expression in vitro, and intended to confirm the anticancer activity of arsenic, which may block the paracrine and autocrine VEGF/VEGFR pathways, thereby delaying new tumor blood vessel formation and tumor cell growth. Our results showed that all of the Flt-1 and KDR expressed in the SGC-7901 tumor xenografts, and their expression in tumor cell control were higher than that in arsenic-treated mice. The fluorescence intensity levels of Flt-1 and KDR in tumor cells were significantly reduced in the arsenic-treated groups ($P < 0.001$). The fluorescence intensity levels of Flt-1 and KDR in the 5 mg/kg group were less than those in the 2.5 mg/kg group ($P < 0.001$). These results suggest that As$_2$O$_3$ can result in significant down-regulation of Flt-1 and KDR in a dose-dependent manner. The results of further experiments in vitro showed that exogenous VEGF$_{165}$ could stimulate the growth of SGC7901 cells, and As$_2$O$_3$ may have disturbed the stimulatory effect of VEGF$_{165}$. It indicates that the autocrine pathway of VEGF through VEGFRs is possible in gastric carcinoma, and As$_2$O$_3$ inhibits expression of Flt-1 and KDR in endothelial and tumor cells. As$_2$O$_3$ may block new blood vessel formation through the paracrine pathway and affect growth of tumor cells through the autocrine pathway of VEGF/VEGFRs, and delay tumor growth.

In conclusion, the results of our present study showed that As$_2$O$_3$ delayed growth of human gastric tumor xenografts in nude mice, decreased MVD in tumor tissues, inhibited proliferation of vascular ECs and induced their apoptosis, which resulted in down-regulation of Flt-1 and KDR expression in tumor tissues in a dose-dependent manner. Further results indicate that exogenous VEGF$_{165}$ can stimulate the growth of SGC7901 cells, and As$_2$O$_3$ may disturb the stimulatory effect of VEGF$_{165}$. These results suggest that As$_2$O$_3$ might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

**COMMENTS**

**Background**

Angiogenesis is an important factor in the progression and enlargement of solid tumors and is in close relation to invasion and metastases. Inhibition of angiogenesis may lead to control of tumor growth and metastasis, therefore, antiangiogenesis is a promising therapeutic approach for treatment of cancer. VEGF is recognized as one of the most important molecules in the formation of new blood vessels. VEGF binds to two distinct receptors on ECs: Flt-1 (VEGFR-1) and KDR (VEGFR-2). Thus, it is suggested that inhibition of VEGF/VEGFR pathways may interrupt VEGF-induced angiogenesis.

**Research frontiers**

As$_2$O$_3$ shows substantial efficacy in treating both newly diagnosed and relapsed patients with APL. Recent studies have shown that a wide variety of malignancies, including both hematologic cancer and solid tumors derived from several tissue types, may be susceptible to therapy with As$_2$O$_3$. These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Emerging data suggest that arsenic induces apoptosis, and inhibits tumor growth and angiogenesis. We have recently shown in vivo and in vitro that As$_2$O$_3$ can inhibit VEGF expression and suppress angiogenesis and gastric tumor growth. In the present work, we investigated further the effect of As$_2$O$_3$ on expression of VEGF receptors Flt-1 and KDR in human gastric tumor cells and proliferation of vascular ECs.

**Innovations and breakthroughs**

Our results showed that As$_2$O$_3$ delayed growth of human gastric tumor xenografts in nude mice and decreased MVD in tumor tissues, inhibited proliferation of vascular ECs and induced their apoptosis, which resulted in down-regulation of Flt-1 and KDR expression in tumor tissues in a dose-dependent manner. VEGF$_{165}$ could stimulate the growth of SGC7901 cells, and As$_2$O$_3$ may have disturbed the stimulatory effect of VEGF$_{165}$. These results suggest that As$_2$O$_3$ might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

**Applications**

The studies suggest that As$_2$O$_3$ might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs. One of the mechanisms by which As$_2$O$_3$ inhibits tumors growth is anti-angiogenesis through inhibiting VEGF/VEGFRs. Our results suggest that As$_2$O$_3$ might be used to treat tumors.

**Terminology**

VEGF is recognized as one of the most important factors in the formation of new blood vessels. As$_2$O$_3$ is a common natural substance.

**Peer review**

This is an excellent paper in terms of scientific rigor, and it is well-written. The experiments were well-designed and well-executed.

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