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Vascular shutdown effects by tetraarsenic oxide in TC-1 cells implanted C57BL/6 mice

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Objective: Arsenic trioxide (As_2O_3) is known to have potent antivascular activity and significantly suppress solid tumor growth. The present study was conducted to investigate the vascular shutdown effects of a novel arsenic compound, tetraarsenic oxide (As_4O_6), in comparison with As_2O_3 using cervical cancer animal model.
Methods: Mice tumor challenge model was used C57BL/6 mice transplanted with TC-1 cells. After the growth of tumors was reached up 250~250mm^3, mice were divided into 3 groups randomly for control and treatment of either As_2O_3 or As_4O_6. As_2O_3 and As_4O_6 were treated by IP injection. The tumor size was caliperated in twice for weeks and anti-vascular effect was assessed by Evans blue extraction assay and Hoechst 33342 staining. In tumor tissue, histopathological features were observed by hematoxylin and eosin (H&E) staining.
Results: In mice treated with either As_2O_3 or As_4O_6 (IP), both of As_2O_3 and As_4O_6 was significantly s uppressed the tumor growth compared with control group. Moreover, effect of As_4O_6 is more pronounced. This tumor growth inhibition is led to the massive necrosis and vascular shutdown in tumor tissue.
Conclusion: This study suggests that As_4O_6 may have potential anticancer activity via vascular shutd own in C57BL/6 mice transplanted with TC-1 cells.

Key Words: As_2O_3, As_4O_6, Vascular shutdown, Necrosis

Introduction

Arsenic is known to be a deadly poison of colorless, tasteless and odorless. However, arsenic trioxide (As_2O_3) and Arsenic trisulfide (As_2S_3) among arsenic compounds have long been used for the treatment of leukemia in China.[1] Since the inhibition effects of As_2O_3 on acute promyelocytic leukemia (Acute promyelocytic leukemia, APL) has been reported, interest in arsenic compounds has been growing and research has been actively conducted.[2,3] Also, it has been reported that arsenic compounds induce cell death and antitumor effect in other tumor cells such as esophageal cancer, neuroblastoma, head and neck cancer, and cervical cancer.[4] As_4O_6 (2,4,6,8,9,10-Hexaoxa-1,3,5,7-tetraarsa-tricyclo [3.3.1. 13,7] decane) is a trivalent arsenic compound and it is known to have different physical and chemical properties from As_2O_3. The antitumor effect of As_4O_6 has already been reported in vitro and in vivo.[5,6] As_4O_6 induces activation of cell death as well as As_2O_3 and inhibits the expression of the carcinogenic genes E6 and E7 in cancer, cancer caused by human papillomavirus (HPV) 16 type, but the precise mechanism is still unclear.[6,7]
Angiogenesis is the key process to supply oxygen and nutrients for growing tumor cells[8] which can spread to other parts of the body through newly formed blood vessels. The metastatic tumor causes

death because it is hard to control or cure,[8-10] which is why it is very important to inhibit the formation of new blood vessels in tumor therapy. The studies for endothelial cell-targeting angiogenesis inhibitors that develop into new blood vessels have been actively ongoing and vascular-disrupting agents (VDAs) such as combretastatin (CA-4-P) and 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) are known to shut down the vessels rapidly and selectively that causes tumor destruction. It has been reported that As_2O_3, arsenic compounds, shows the antitumor effect that induces vascular shutdown through oxidative damage in solid tumors. Both As_2O_3 and As_4O_6 are known to induce the antitumor effect by inhibiting angiogenesis. Especially in the experiment of neovascularization induction, As_4O_6 has been reported to significantly inhibit the number, length, formation timing and area of the new blood vessels compared to As_2O_3. However, the vascular shutdown and the antitumor effect of As_4O_6 in cervical cancer models have not yet been reported. This present study was conducted to investigate the vascular shutdown effect of novel arsenic compounds, As_4O_6, in comparison with As_2O_3 using TC-1 cell line expressing HPV16 E6 and E7 oncogenes and cervical cancer animal model C57BL/6 mouse.

Subjects and methods

1. Cell line and cell culture

TC-1 cell line (obtained from Dr. TC-WU of John's Hopkins University) was isolated from the lungs of C57BL/6 mice and was transplanted with HPV 16-E6 and E7 genes and c-Ha-ras gene. Cell culture media were prepared by adding 5% fetal bovine serum (FBS) (Gibco BRL), 0.22% sodium bicarbonate (Sigma-Aldrich, Spruce St. Louis, USA), 400mg/L G418 (Sigma-Aldrich), and streptomycin/penicillin (Gibco BRL) and cultured in a 37℃, 5% CO_2 incubator.

2. Arsonic compounds

Arsenic compounds were purchased from Sigma-Aldrich (As_2O_3) and Chunjisan, Seoul, Korea (As_4O_6), dissolved in Dulbecco's phosphate-buffered saline (DPBS) and injected (IP) at a concentration of 10 mg/kg b.w.

3. Antitumor effect of arsenic compounds

Six weeks old C57BL/6 female mice (Orient, SeongNam, Korea) were randomly assigned to 7 mice per each group; the control group, As_2O_3 treated group and As_4O_6 treated group. The TC-1 cell line was collected by treating with Trypsin-EDTA (Gibco BRL), and then subcutaneously injected into the abdomen at a concentration of 5×10^5 cells/100 μL. Arsenic compounds were administered when the size of the tumor reached 200 to 250 mm^3, 5 times in total once a week. The size of the tumor was measured using a caliper at intervals of 2 to 3 days, with the long axis (A), the short axis (B) and the height (C), and the tumor volume(cm^3) = (AxBxC).

4. Morphological change of tumor

Tumor-forming C57BL/6 female mice (Orient) were randomly divided into 3 mice per each group; the control group, As_2O_3 treated group and As_4O_6 treated group and then single injection of arsenic compounds was performed. After 24 hours and 48 hours, the tissues were removed, fixed in 10% neutral formalin, and embedded in paraffin. The embedded tissue was cut into 4μm in thickness and

attached to silicon and cation-treated slides(Silanized, positive cheged slide; DAKO, USA) and underwent deparaffinization with xylene and hydration with 100, 90, 80, 70% ethanol and then H&E staining(hematoxylin and eosin) was performed. The necrosis area(%) was calculated by dividing the area of necrosis by the area of the entire tumor and then multiplying by 100.

5. Evans blue staining

Tumor-forming C57BL/6 female mice (Orient) were randomly divided into 3 mice per each group; the control group, As_2O_3 treated group and As_4O_6 treated group and then single injection of arsenic compounds was performed. 24 and 48 hours later, Evans blue(Sigma-Aldrich) reagent was injected (IP) into the caudal vein of mice at a dose of 10 mg/kg b.w. After 45 minutes, 50 μL of anesthetic mixing with ketamine (Yuhan, Seoul, Korea) and Rumpun (Bayer Korea, Korea) at a ratio of 4: 1 was injected intramuscularly and then thoracic cavity was opened and a 10 mL syringe was injected into the left ventricle and perfused with 50 mL of PBS buffer. After perfusion, the tissues were harvested and weighed and then immersed in formamide (Sigma-Aldrich), 1 mL of formamide per 100 mg of tissue, and allowed to react at 60℃ for 48 hours. After the reaction, the supernatant was taken and absorbance was measured at 620 nm using an ELISA-reader (spectra max 250, Molecular Devices, Sunnyvale, CA, USA). A standard quantitative curve was measured with 100 μg/mL of Evans blue reagent, diluted twice, and this curve was used to calculate the amount of Evans blue released.

6. Hoechst 33342 staining

Tumor-forming C57BL/6 female mice (Orient) were randomly divided into 3 mice per each group; the control group, As_2O_3 treated group and As_4O_6 treated group and then single injection of arsenic compounds was performed. After 48 hours, Hoechst 33342 solution (Sigma-Aldrich) was injected into the caudal vein of mice at a dose of 15 mg/kg b.w. One minute later, tissues were rapidly removed and frozen sections were prepared and then blue fluorescence was confirmed by fluorescence microscopy (Olympus, Tokyo, Japan, excitation filter 365 nm).

7. Statistics

Two-way ANOVA and Student's t-test were performed. Statistical significance was $P < 0.05$, respectively.

Results

1. Antitumor effect of arsenic compounds in mice transplanted with TC-1 tumor cells

As compared with the control group, tumor growth of As_2O_3 was inhibited 58.2%, 68.7%, 79.3%, 84.3%, 87.8%, 92.9%, 94.4% at 3, 6, 9, 12, 14, 21, 28 days, respectively, and tumor growth of As_4O_6 was inhibited 66.6%, 72.9%, 83.2%, 88.2%, 90.5%, 95.4%, 95.8% at the same days, respectively. The As_2O_3 and As_4O_6-treated groups showed a statistically significant inhibitory effect on tumor growth compared with the control group (Fig. 1, $P<0.01$). In addition, the As_4O_6-treated group showed the more inhibitory effect than the As_2O_3-treated group (Fig. 1, $P <0.05$).

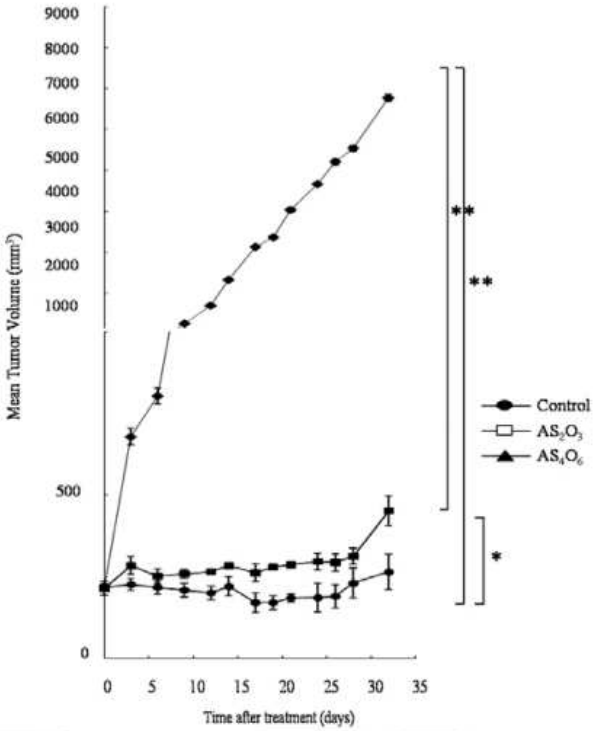
2. Effect of arsenic compounds on induction of tumor necrosis

As a result of comparing the degree of tumor necrosis before and after treatment with As_2O_3 or As_4O_6

through H & E staining, after 8 hours of administration, As_4O_6 treated group showed less cytoplasmic staining and highly condensed and an exaggerated nucleus which is characteristics of the cell necrosis. After 48 hours, 30% of all tumors were confirmed (Fig. 2, 3). In contrast, the As_2O_3 treated group showed significantly lower cell necrosis compared to As_4O_6 treated group (Fig. 2, 3).

3. Vascular shutdown by arsenic compounds

In contrast to the control group, the permeation of the Evans blue staining reagent into the tumor was significantly increased at 24 and 48 hours after As_2O_3 or As_4O_6 treatment (Fig. 4, $P < 0.05$). The As_2O_3 treated group increased 1.2 and 2.5 times and the As_4O_6 treated group increased 1.7 and 6.2 times at 24 and 48 hours respectively, as compared with the control group. The permeation effect of Evans blue staining reagent in the As_4O_6 treated group was significantly different from that of the control group at 48 hours, also the detection of Hoechst 33342 fluorescence solution in the tumor was significantly reduced compared to the control group or the As_2O_3 treated group at 48 hours (Fig. 5, $P < 0.05$).



ave±SD (mm^3)	0	3	6	9	12	14	17	19	21	24	26	28	32
							(days)						
Ctl	222±5	679±25	803±24	1250±22	1697±21	2330±44	3126±9	3356±46	4035±55	4668±30	5207±66	5539±79	6770±83
AS_2O_3	215±22	284±27	252±23	259±13	266±4	284±4	262±27	280±9	288±8	296±25	294±26	312±25	453±46
AS_4O_6	217±7	227±18	218±20	209±20	201±20	221±29	171±30	171±21	186±12	186±43	191±36	231±46	265±53

Fig. 1. Tumor growth curves for TC-1 of mice treated with arsenic compounds. Statical significant inhibition of tumor growth measured by *t*-test. **P*<0.05, ***P*<0.01, compared with the control. The tumor growth was significantly delayed in groups treated with As_2O_3 or As_4O_6, compared to the control group.

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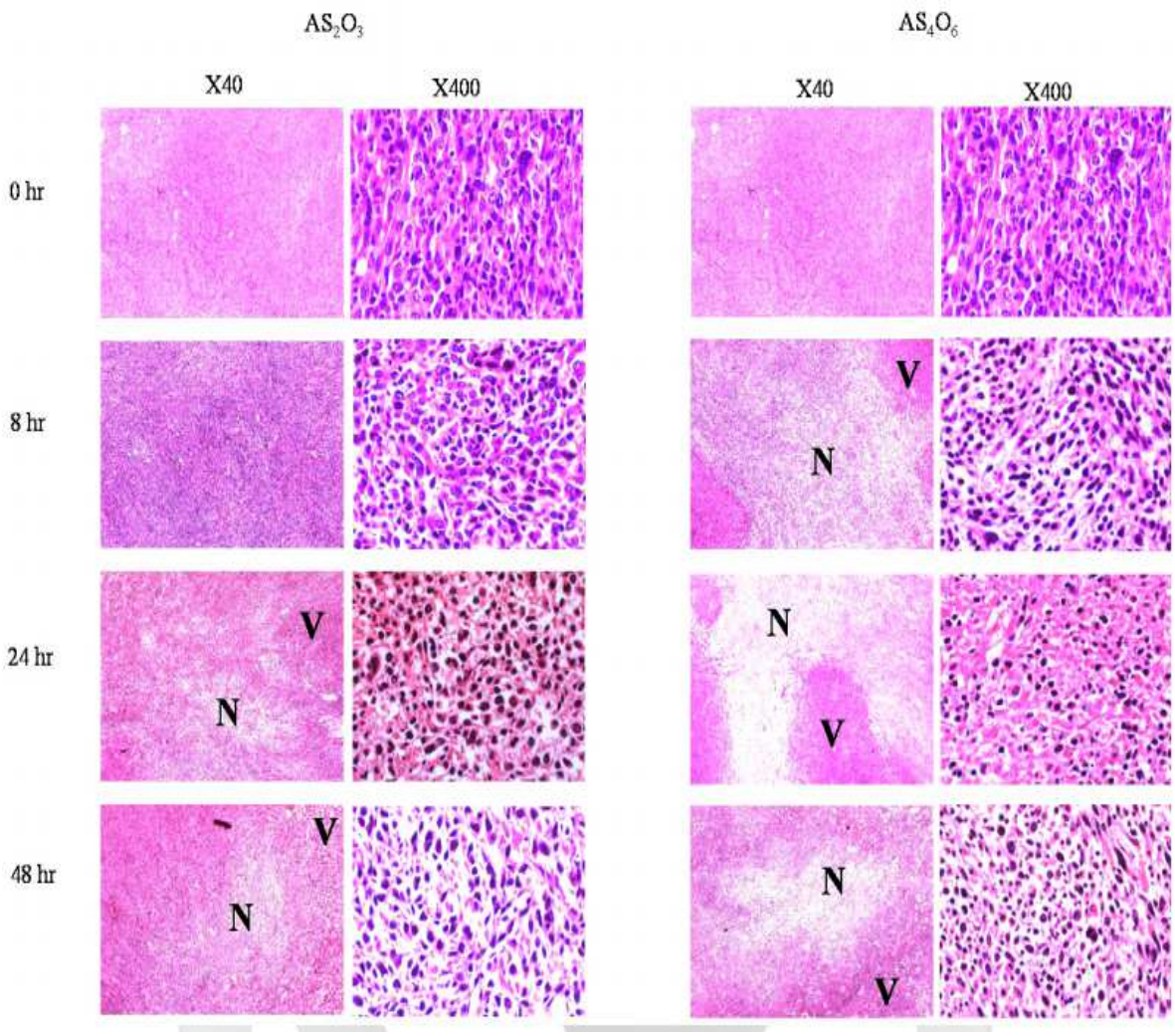

Fig. 2. Morphological features of TC-1 tumors in C57BL/6 mice treated with arsenic compounds, As_2O_3 and As_4O_6. Sequential gross morphological changes either 0, 8 hrs, 24 hrs and 48 hrs after treated with arsenic compounds, As_2O_3 and As_4O_6 (10 mg/kg b.w., i.p.). Histological features of the tumors stained with H&E. Left, ×40 magnification, right, ×400 magnification. V, nonnecrotic zone ; N, necrotic zone.

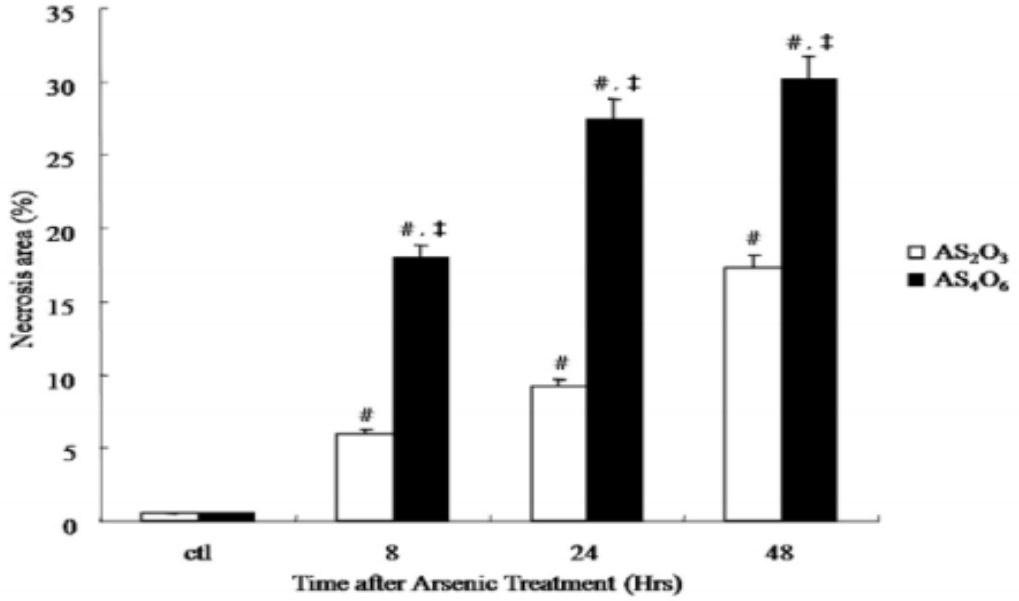

Fig. 3. Percent area of necrotic zone of TC-1 tumors in C57BL/6 mice treated with arsenic compounds, As_2O_3 and As_4O_6. Sequential gross morphological changes either 0, 8, 24 and 48 hrs after treated with arsenic compounds, As_2O_3 and As_4O_6 (10 mg/kg b.w., i.p.).
# $P< 0.05$, compared with the control and ‡ $P< 0.05$ As_2O_3. Vertical bar indicated standard deviation.

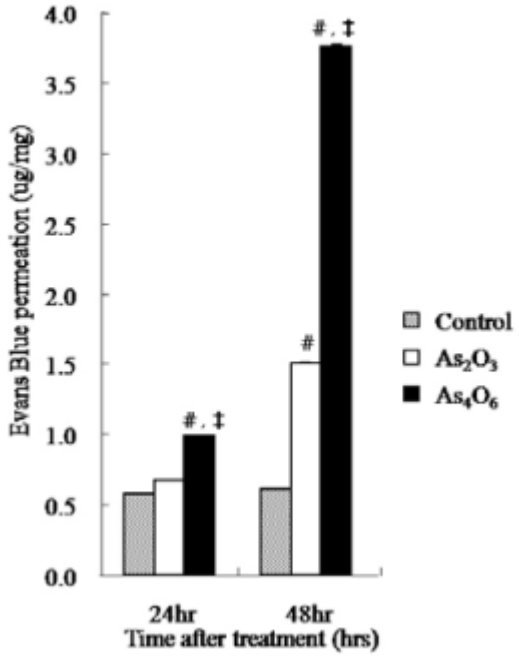

Fig. 4. Extravasation of Evans Blue in TC-1 tumors in C57BL/6 mice treated with arsenic compounds, As_2O_3 and As_4O_6 (10 mg/kg b.w., i.p.).

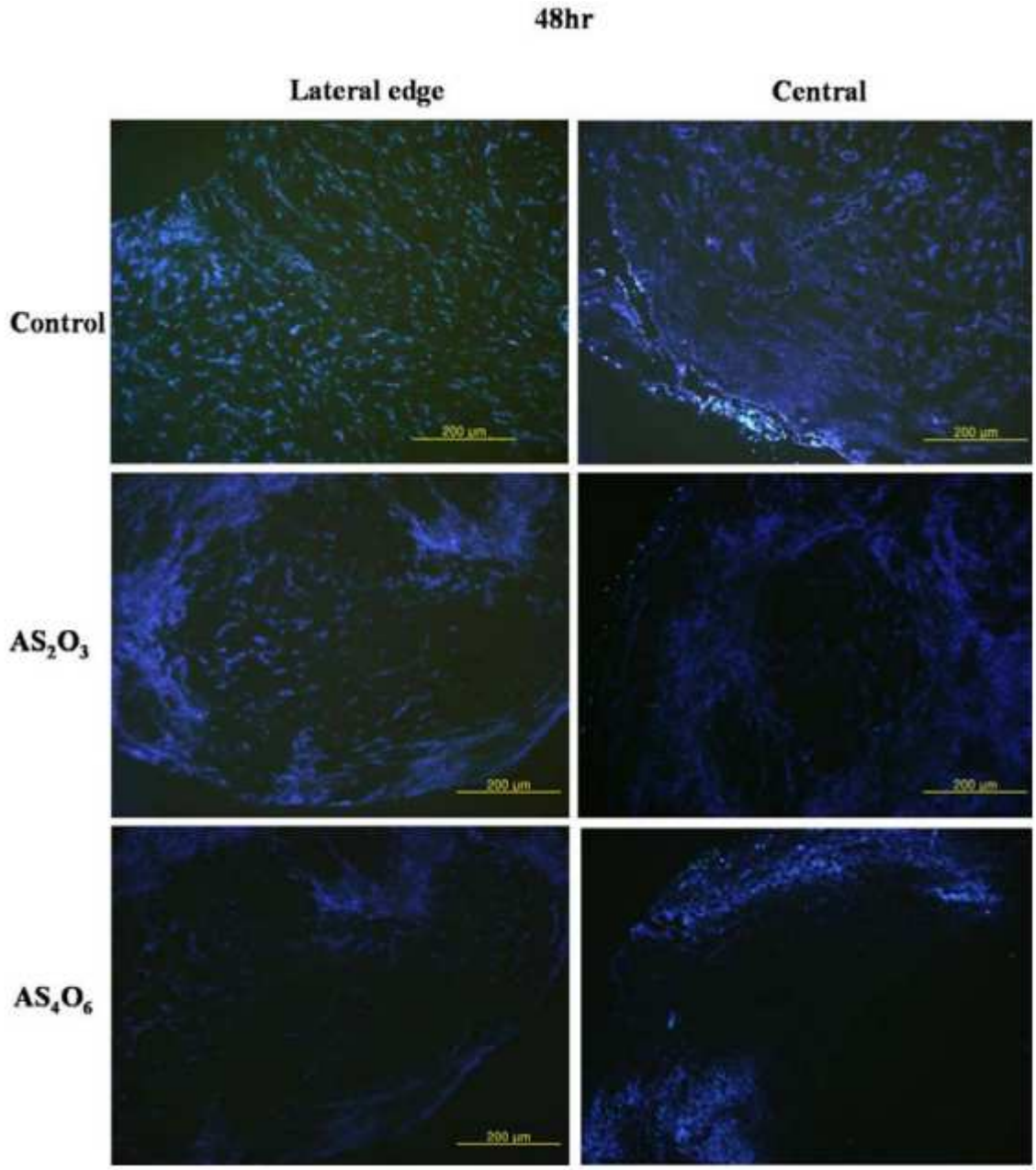

Fig. 5. Comparison fluorescence images of the perfusion marker Hoechst 33342 in TC-1 tumors in C57BL/6 mice treated with arsenic compounds, As_2O_3 and As_4O_6 (10 mg/kg b.w., i.p.). Untreated (upper), As_2O_3 treated (middle) and As_4O_6 treated (bottom).

Review

This study has shown that As_2O_3 and As_4O_6, arsenic compounds, induce immediate vascular shutdown to lead necrosis in tumor cells in C57BL/6 mouse models transplanted with TC-1 cells expressing HPV 16-E6 and E7 genes. Especially, the effect of As_4O_6 was shown to be greater than that of As_2O_3. It has also been observed that cell death through vascular shutdown induces a retardation in tumor growth. As_2O_3 and As_4O_6 were injected (IP) 10 mg/kg b.w. of the mouse, once a

week for 5 times in total, resulting in a significant retardation effect on tumor growth compared with the control group (Fig. 1). In addition, there was a statistically significant difference between the two arsenic compounds treated groups. Especially, the antitumor effect was most prominent in As_4O_6 treated group and it is consistent with Chang, in which the antitumor effect of As_4O_6 in the nude mouse model using SiHa cell line was more prominent than that of As_2O_3.[5] Evans blue extravasation assay and Hoechst 33342 staining were used to measure the degree of the vascular shutdown and the ability was observed in both methods. According to the Evans blue extravasation assay, the closure effect of arsenic compounds on blood vessels is highest at 48 hours after the administration also As_4O_6 was 2.5 times higher than As_2O_3 (Fig. 4).

Hoechst 33342 staining, another method to measure the degree of the vascular shutdown at the same time, showed that the fluorescent dyeing solution was uniformly detected from the blood vessels both inside and outside of the tumor in the control group. But, in the group treated with As_2O_3 and As_4O_6, the fluorescence staining solution was not detected in the tumor centered area. Especially, the As_4O_6 treated group had a wider area than the As_2O_3 treated group (Fig. 5). This is consistent with the fact that As_4O_6 has a better retardation effect than As_2O_3 on tumor growth, but more research on this phenomenon should be conducted further. Several other antitumor agents such as Combretastatin A-4,[15] colchicine,[16] flavone acetic acid[17] and hydralazine[18] are known to induce vascular shutdown in the central area of the tumor by affecting the vascular structure inside of the tumor. However, flavone acetic acid, hydralazine and colchicine, etc. are reported to be toxic or ineffective in other species than mice. Recent developments of combretastatin A-4 and novel flavone acetic acid derivatives have been reported as potential candidates for solving these problems. However, arsenic compounds have been applied to a human for many years and there are no reports of interspecies toxicity which have been carried out in the laboratory using rodents in basic studies.[19] At present, vascular shutdown and necrosis of tumor-centered area by arsenic compounds are difficult to explain easily. However, there are many possible hypotheses to explain this phenomenon. One of them is that arsenic compounds selectively induce damage of vascular endothelial cells, resulting in the vascular shutdown of the tumor. This phenomenon has been reported that it is caused by non-competitive inhibition of binding between GTP and tubulin by As_2O_3.[20] Combretastatin A-4, another anticancer substance, affects normal microtubule function, and in vitro studies showed stronger toxicity to vascular endothelial cells than tumor cells and selective induction of vascular shutdown in animal models using P22 mice.[15] Colchicine, another tubulin binding agent, has been reported to induce hemorrhagic cell necrosis in experimental animals.[16] Another phenomenon is tumor necrosis factor mediated vascular shutdown. Evidence for this phenomenon is supported by reports that tumor necrosis factor mRNA is expressed after treatment with arsenic compounds in cultured human keratinocytes.[21] Finally, it is a phenomenon of cell death and a vascular shutdown caused by cell necrosis. It has been reported that induction of cell death by low concentrations of arsenic compounds (0.5 ~ 5 μM) in many types of solid tumor cell lines including APL cell lines. [22, 23] It has also been reported that arsenic compounds at concentrations as high as 5 μM or more induce acute cell necrosis in various cell lines.[20] Therefore, the vascular shutdown of arsenic compounds is presumed to be a result of simultaneous massive cell death and necrosis in the tumor. The Vascular shutdown in the central area of the tumor by arsenic compounds has been reported by Lew YS et al. In this study, we confirmed the effect of As_4O_6 on vascular shutdown in an animal model using TC-1 cell line. It is believed that the two types of arsenic compounds, As_2O_3 and As_4O_6, induce the inhibition of the tumor growth by the vascular shutdown, but the precise mechanism of the cause is not clear yet. It is also necessary to study how the two arsenic compounds induce this phenomenon through the unknown pathways.

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