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ORIGINAL RESEARCH

Radiosensitization of cervical cancer xenografts by arsenic trioxide and the role of VEGF and Ku70

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Abstract
Objective The aim of this study is to explore the effect of arsenic trioxide (As_2O_3) on radiosensitivity in cervical carcinoma xenografts and the possible underlying mechanisms of vascular endothelial growth factor (VEGF) and Ku autoantigen protein p70 (Ku70).
Methods The tumor-bearing C57BL/6mouse model was established by injecting U14 cervical carcinoma cells into the right infra-axillary dermis of 40 mice. The mice were randomized into four groups: (1) control group (peritoneal injection, 0.2 ml of 0.9% normal saline); (2) As_2O_3 only group (peritoneal injection, 2.0 mg/kg, 0.2 ml As_2O_3); (3) irradiation only group (peritoneal injection 0.9%NS, 0.2 ml+6 MeV electron beam, 10 Gy); and (4) As_2O_3 + irradiation group (peritoneal injection, 2.0 mg/kg, 0.2 ml As_2O_3+6 MeV electron beam, 10 Gy). The mice were sacrificed 25 days after randomisation. U14 cervical tumors from the mice were harvested and weighed. Response to treatment using radiation and/or As_2O_3 was evaluated by extent of inhibition (i.e., growth inhibition rate) of tumor growth. VEGF and Ku70 mRNA expression and protein levels were evaluated by RT-PCR and immunohistochemistry, respectively.
Results As_2O_3 + irradiation was found to significantly increase the inhibition of tumor growth. The growth inhibition rates in As_2O_3 only, irradiation only, and As_2O_3 + irradiated groups were 10.48%, 30.30%, and 73.15%, respectively ($p < 0.05$). The combination also significantly downregulated the expression of VEGF and Ku70 in irradiated cervical carcinoma xenografts as compared to the control group, As_2O_3 only group, or irradiation only group ($p < 0.05$).
Conclusions Arsenic trioxide effectively sensitizes the cervical carcinoma xenografts to ionizing irradiation. Downregulation of the expression of VEGF and Ku70 is likely to play an important role.

Keywords Arsenic trioxide · Cervical carcinoma · Radiosensitivity

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Introduction

Cervical cancer is the most commonly diagnosed gynecological malignancy worldwide, and radiation therapy is a critical component in its management. Patients with early stage cervical cancer can be treated with radical hysterectomy or definitive radiotherapy; and those with locally advanced

diseases are usually treated with concurrent chemoradiation, either as a primary or an adjuvant modality after surgery [1, 2]. The addition of concurrent chemotherapy to curative dose of radiotherapy can significantly improve the outcome in terms of disease control and overall survival; however, prognoses of patients with locoregionally advanced cervical cancer remained suboptimal [3–6]. Furthermore, high dose radiation is associated with a number of side effects such as radiation cystitis and proctitis, vaginal stenosis, and ovarian dysfunction [7, 8]. Strategies to further improve therapeutic ratio are clearly needed.

Arsenic trioxide (As_2O_3) is a naturally occurring substance that has been used in medicine for more than 2,400 years. It has been used to treat a variety of conditions including cancer [9, 10]. As_2O_3 has been proven efficacious in the treatment of acute promyelocytic leukemia (APL) with limited adverse effects [11–13]. It has been demonstrated that As_2O_3 can affect cellular signal transduction pathways and cross-talks of signal molecules which include downregulation of Bcl-2 protein, activation of caspase, direct mitochondrial damage, promotion of tubulin polymerization [14], and some of the above-mentioned mechanisms are involved in radiosensitization. Furthermore, the synergestic effects of As_2O_3 with radiation have been preliminarily reported in vitro and in vivo [15, 16]. The effects of As_2O_3 on cervical cancer cells were previously suggested [17]. Nevertheless, whether As_2O_3 can enhance the therapeutic effect of radiotherapy and the mechanism(s) of its potential radiosensitizing effect has not been adequately addressed in cervical cancer. In an in vitro study, we have demonstrated the effect of As_2O_3 on radiosensitization in a cervical cancer cell line HeLa [18]. The aim of this study is to evaluate the effect of As_2O_3 on radiosensitivity in cervical carcinoma xenografts, as well as to explore the potential mechanism of As_2O_3 in radiosensitization.

Materials and methods

Animal and tumor models

Three to four weeks old C57BL/6 female mice with average weight 17–20 g were obtained from the Experimental Animal Center of Chongqing Medical University (qualification no. SCXK [YU]-2007001). The mice were housed at the Children's Hospital of Chongqing Medical University Animal Center and had free access to food and water and were kept on a 12-h light cycle. U14 human cervical cancer cell lines were obtained from the Institute of Pathophysiology, Chongqing Medical University. Cell culture media, fetal bovine serum, cell culture-grade buffers, and glucose were from Hyclone Inc. As_2O_3 was from Sigma Inc. and stored at room temperature. U14 cells were cultivated in RPMI 1640 supplemented with 5% fetal bovine serum, 1% L-glutamine, and 0.1% genramycin.

Mice were acclimated at least 5 days before any experiments. U14 xenografts were generated by subcutaneous (s.c.) implantation of 1×10^6 U14 human cervical cancer cells suspended in 0.1 ml RPMI_1640 into the right axilla of each mouse. The mice with U14 xenografts were included into the experiment when the shortest tumor diameter reached 6 mm. Forty mice were randomly allocated into four groups of ten mice: (1) control group, (2) irradiation only group, (3) As_2O_3 only group, and (4) As_2O_3 + irradiation group.

Radiotherapy and tumor inhibition assay

Xenografts in the two irradiated groups received a single dose of 10 Gy using 6 MeV electron beam with a dose rate of 4.0 Gy/min (VARIAN® 2300C/D). Animals were sacrificed 20 days after irradiation. Tumor issues were isolated from mice and weighed to calculate the growth inhibition rate with the formula below:

$$\text{Growth inhibition rate} = (\text{average weight of xenografts in control groups} - \text{average weight of xenografts in irradiation groups})/(\text{average weight of xenografts in control groups}) \times 100$$

RT-PCR

Total cellular RNAs were extracted from samples using TRIzol reagent (Invitrogen®, Carlsbad, USA). First-strand synthesis was performed using the SuperScript™ first-strand synthesis system (Invitrogen®, Carlsbad, USA) according to the manufacturer's instructions. All PCR reactions were carried out using an ABI Prism 7700 Sequence Detection system. The following primers and probes labeled with 5′-FAM and 3′-TAMRA were used to amplify vascular endothelial growth factor (VEGF) and Ku autoantigen protein p70 (Ku70).

VEGF Primer1: 5′-TTGTGCTCTACCTACCTCCAC-3′;
Primer2: 5′-AATGCTTTCTCCGCTCTG-3′;
Ku70 Primer1: 5′-TCTTGGCTGTGGTGTTCTATGGC-3′;
Primer2: 5′-TCTTCTAGCTTGCTGGATTCCTC-3′.

Immunohistochemistry

The expression of VEGF and Ku70 were determined in deparaffinized, formalin-fixed section from PBS-treated U14 xenografts. Paraffin-embedded tissues were sectioned into 5 μ sections. The sections were incubated in methanol/

H_2O_2 for 30 min to inhibit the endogenous peroxidase activity, washed with PBS for 5 min, and then blocked with normal goat serum for 20 min at room temperature. The sections were incubated with antibodies against VEGF and Ku70 (Santa Cruz Biotechnology Inc) overnight at 4°C, then with biotinylated secondary antibody for 1 h at room temperature and avidin-conjugated peroxidase for 45 min at room temperature. The sections were washed three times with PBS between each step. Peroxidase was stained with diaminobenzidine (1 mg/mL) and H_2O_2 for 5 min and washed with tap water for 10 min. The sections were counterstained with hematoxylin for 1 min. PBS was used as a negative control instead of primary antibody. Slides were analyzed using a well-established semiquantitative scoring method ranging from negative (−) to strong positive (+++) [negative (−): no positive cells; weak positive (+): cells were stained yellow and the proportion of positive cells was under 25%; medium positive (++): cells were stained brown and the proportion of positive cells was between 26% and 50%; and strong positive (+++): cells were stained tan and the proportion of positive cells was above 50%].

Statistical analysis

Data are expressed as means ± standard deviation, and statistical significance was assessed by the Student's *t* test. All tests were performed at least three times independently. A value of $p<0.05$ was set as the threshold for statistical significance for all study outcomes.

Results

Animal tumor models were successfully established in 40 mice about 6 days after implantation. The weight of tumor in As_2O_3 only group, irradiation only group, and As_2O_3+ irradiation group was all reduced as compared to the control group. The growth inhibition rate in As_2O_3 only group, irradiation only group, and As_2O_3 + irradiated group were 10.48%, 30.30%, and 73.15%, respectively ($p<0.05$) (Table 1). Growth inhibition rate was significantly higher in mice treated with As_2O_3 + irradiation as compared to those in the control group or treated with As_2O_3 or radiation only.

Expression of VEGF and Ku70 mRNA

The expression of VEGF and Ku70 mRNA was significantly downregulated in the As_2O_3 + irradiation group compared with the irradiation only group, As_2O_3 only group, or the control group (Figs. 1 and 2). VEGF expression rate was 33.47% and Ku70 expression rate is 36.79% in the As_2O_3+ irradiation group. In semiquantitative analysis, the relative concentrations of VEGF and Ku70 mRNA in the As_2O_3+ irradiation group were found to be much lower than other groups ($p<0.05$) (Tables 2 and 3).

Table 1 Comparison of the tumor weight in experimental groups ($\bar{x}\pm s$, $n=10$)

Group	Tumor weight (g)	Inhibitory rate (%)
Control	8.78±3.12	–
As_2O_3	7.86±2.93	10.48
Irradiation	6.12±2.85	30.30
As_2O_3 + irradiation	2.35±1.56*, **, ***	73.15

*$p<0.05$, compared with As_2O_3 group

**$p<0.05$, compared with irradiation group

***$p<0.05$, compared with control group

Expression of VEGF and Ku70 protein in cervical cancer xenograft

The VEGF protein was detected primarily in the cytoplasm of tumor cells and Ku70 protein expression was mainly localized in the nucleus on immunohistochemistry. Analysis showed that the expression intensity of VEGF and Ku70 protein was medium to strongly positive in the control group, As_2O_3 only group, and irradiation only group, as compared to weak to strongly positive in the As_2O_3 + irradiation group. The positive rates of the expressions of the VEGF and Ku70 proteins in As_2O_3 + irradiation group were significantly lower compared with other groups ($p<0.05$) (Table 4).

Discussion

Radiotherapy is one of the most important treatment modalities for cervical cancer, and over 80% cervical cancer patients receive radiation in their course of management.

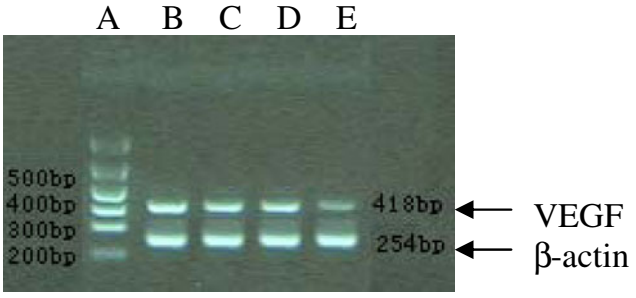

Fig. 1 Agarose electrophoresis of β-actin, VEGF mRNA in experimental groups. *Lane A*, Marker; *Lane B*, Control group; *Lane C*, As_2O_3 group; *Lane D*, Irradiation group; *Lane E*, As_2O_3 + irradiation group

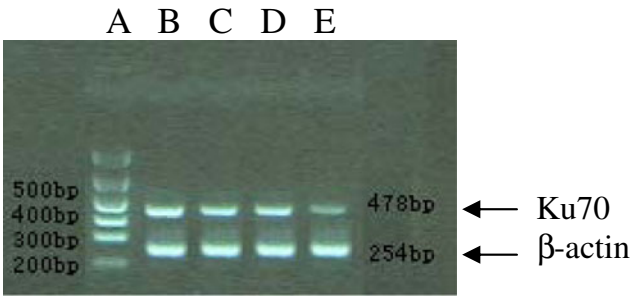

Fig. 2 Agarose electrophoresis of β-actin, Ku70 mRNA in experimental groups. *Lane A*, marker; *Lane B*, control group; *Lane C*, As_2O_3group; *Lane D*, irradiation group; *Lane E*, As_2O_3 + irradiation group

Table 3 Semiquantitative analysis of Ku70 mRNA in experimental groups

Group	Relative content	Expressing rate (%)
Control	0.685±0.0246	100.0
As_2O_3	0.643±0.0372	93.87
Irradiation	0.676±0.0328	98.69
As_2O_3 + irradiation	0.252±0.0294*, **, ***	36.79

*$p<0.05$, compared with As_2O_3 group

**$p<0.05$, compared with irradiation group

***$p<0.05$, compared with control group

The addition of chemotherapy to radiation has significantly improved the prognosis of cervical cancer patients; however, vthe outcome after concurrent chemoradiation remains suboptimal [3–6]. Clearly, strategies for further improvement of therapeutic ratio in the management of cervical cancer with radiation-based treatment are needed.

As_2O_3 is an agent successfully employed in the treatment of APL with limited side effects [11–13]. It was found to induce growth inhibition and apoptosis in solid tumors [19, 20]. Recently, some investigators reported that As_2O_3 can cause DNA damage, oxidative stress, and mitochondrial dysfunction. As_2O_3 acts as in the G_1 or at G_2/M phases of the cell cycle and sensitizes tumor cells to ionizing radiation [15, 21, 22]. In an in vivo study reported by Kumar et al., As_2O_3 enhanced the therapeutic efficacy of radiation treatment of oral squamous carcinoma while protecting bone [15]. As_2O_3 was also reported to sensitize renal cancer cells, osteosarcoma cells, and GBM stem cells to radiation therapy in vitro studies [23–25].

Our previous experiences have demonstrated the synergistic effects of arsenic trioxide and radiation in cervical cancer cells HeLa through increasing the apoptosis rate and significantly downregulating the expression of bcl2 [18]. The results of the current in vivo study confirmed our previous findings. We found that tumor weight was significantly reduced and growth inhibition rate was significantly higher in the group of mice that received the combined treatment, as compared to those in control or received either radiation or As_2O_3. The magnitude of the control clearly indicated a synergestic rather than an additive effect of the combined radiation + As_2O_3, thus suggested that when used as a radiosensitizer, As_2O_3 can significantly improve the therapeutic efficacy.

Furthermore, we explored the possible mechanisms of radiosensitization involving VEGF and Ku70, two molecular markers known to affect radiosensitivity[26–32]. The molecular mechanisms of the radiosensitization effect of As_2O_3 were elucidated by evaluating the expression of VEGF and Ku70 with RT-PCR and immunohistochemistry. VEGF plays a significant part in the formation of the tumor blood vessels and stimulates the growth and metastasis of tumor [26, 27]. Elevated serum VEGF levels are associated with poor response and a shorter time to progression in patients with cervical cancer treated with radiotherapy [28]. There is also evidence that patients with cervical cancer with VEGF overexpression have a lower 5-year survival when treated with radiotherapy alone [29].

Table 2 Semiquantitative analysis of VEGF mRNA in experimental groups

Group	Relative content	Expressing rate (%)
Control	0.732±0.0324	100.00
As_2O_3	0.674±0.0268	92.08
Irradiation	0.653±0.0247	89.21
As_2O_3 + irradiation	0.245±0.0229*, **, ***	33.47

*$p<0.05$, compared with As_2O_3 group

**$p<0.05$, compared with irradiation group

***$p<0.05$, compared with control group

Table 4 Expression intensity and positive rate of VEGF and Ku70 protein in experimental groups

Group	Expression intensity		Positive rate (%, $\bar{x}\pm s$)	
	VEGF	Ku70	VEGF	Ku70
Control	++~ +++	++ ~ +++	69.8±3.95	70.2±3.46
As_2O_3	++ ~ +++	++ ~ +++	60.4±3.87	68.6±3.23
Irradiation	++ ~ +++	++ ~ +++	55.9±3.52	69.5±3.87
As_2O_3 + irradiation	+~ +++	+ ~ +++	22.4±2.98*, **, ***	29.3±3.28*, **, ***

*$p<0.05$, compared with As_2O_3 group

**$p<0.05$, compared with irradiation group

***$p<0.05$, compared with control group

Ku70/80 is a heterodimer that was first identified as a component of non-homologous end joining, an error-prone pathway that is involved in the repair of DNA double-strand breaks. Some studies suggested that overexpression of survivin might enhance double-strand break repair capability thereby radioresistance in human squamous cell carcinoma cell line KB by upregulating Ku70 [30]. Zhang et al. reported that histone deacetylase inhibitors could modulate cellular responses to ionizing radiation by downregulation of Ku70 [31]. Evidence suggests that Ku70 expression is inversely related to radiation sensitivity. Wilson et al. [32] reported that a lower Ku70 expression increased radiosensitivity and improved survival in cervical cancer patients treated with radiotherapy. Thus, the lower the expression of Ku70, the higher radiation sensitivity and the better radiotherapeutic efficacy.

The immunohistochemistry of our study showed that the expression of VEGF and Ku70 mRNA in As_2O_3 + irradiation group was significantly less than the irradiation only, As_2O_3 only or the control group. In semiquantitative analysis, relative concentration of VEGF and Ku70 mRNA in the As_2O_3+ irradiation group was much lower than other groups ($p<0.05$). The downregulation of VEGF and Ku70 expression significantly increased growth inhibition rate in the As_2O_3 + irradiation group compared with other groups. In summary, our results show that As_2O_3 sentisizes cervical cancer cells to ionizing radiation. Downregulation of VEGF and Ku70 is likely to play an important role.

Despite of our encouraging findings, the radiosensitizing effect of As_2O_3 on cervical cancer requires clinical confirmation. Concurrent chemoradiation is the current standard for the treatment of locally advanced cervical cancer. Synergistic effects between cisplatin-based chemotherapy agents and As_2O_3 have also been suggested in a number of studies, but with different mechanism as compared to our current finding [33, 34]. Recent clinical data showed that As_2O_3 could achieve high efficacy and good tolerance at long-term follow-up. The toxicity profile was mild and reversible whether As_2O_3 was used alone or combined with other drugs[12, 13, 35, 36]. It would be interesting to explore whether As_2O_3 further improves the therapeutic effects of cervical cancer treated with cisplatin-based chemotherapy with concurrent radiation. Furthermore, various mechanisms have been suggested for the radiosensitizing effect of As_2O_3. Clearly, other potential mechanisms in addition to the down-regulation of VEGF and Ku70 await further investigation.

Disclaimer This research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Chongqing Medical University.

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Conflict of interest The authors declare no conflict of interest.

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