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Opposite effects of arsenic trioxide on the Nrf2 pathway in oral squamous cell carcinoma in vitro and in vivo

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1. Introduction

Nuclear factor erythroid derived 2 like 2 (Nrf2) is one of the most important cellular defense mechanisms against oxidative stress or electrophiles. As a transcription factor, Nrf2 mediates an anti-oxidative response by binding to anti-oxidant response element of enzymes which are involved in Phase II detoxification of xenobiotics [1]. Such Nrf2-regulated cytoprotective genes include heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST) [2,3]. Over the past 10 years, increasing evidence has shown that Nrf2 activation can protect against many human diseases such as cancer, neurodegenerative diseases, aging, cardiovascular diseases, inflammatory diseases, pulmonary fibrosis and acute pulmonary injury [4]. Therefore, Nrf2 has been viewed as a “good” protein that protects humans from genotoxic damage caused by carcinogens. Many well studied chemopreventive compounds have been identified as Nrf2 inducers including sulforaphane, triterpenoids, curcumin, epigallocatechin-3-gallate, and resveratrol. Their efficacy in cancer prevention has been verified both in animal models and in human clinical trials. These compounds exert their chemopreventive activity by inducing the Nrf2-dependent adaptive response, including Phase II detoxifying enzymes, antioxidants, and transporters that defend cells from subsequent carcinogenic insults [3,5]. However, new emerging data have revealed the “dark” side of Nrf2. Several independent studies indicated Nrf2 may be responsible for chemoresistance [6]. Nrf2 protects not only normal cells from transforming into cancer cells, but also promotes survival of cancer cells in a deleterious environment [7].

As a cancer chemotherapeutic drug, arsenic trioxide (ATO) was originally developed for acute promyelocytic leukemia [8]. Recent studies have shown that a wide variety of malignancies are also responsive to ATO treatment [9]. Previous studies have demonstrated ATO as a promising cancer therapeutic agent for treatment of oral squamous cell carcinoma (OSCC) [10,11]. paradoxically, arsenic was also regarded as a carcinogen. Chronic arsenic exposure has been associated with high incidences of skin, lung, kidney, bladder and oral cancer [12]. Arsenic has been reported to activate Nrf2 pathway and thus contribute to malignant transformation of human cells [13,14]. It is believed that similar mechanisms mediate both the therapeutic and toxic activities of arsenic compounds [15].

In order to fully understand the impact of Nrf2 signaling on human oral cancer cells, we examined expression of Nrf2 and Nrf2-regulated genes in ATO-treated oral cancer cells in vitro and in vivo.
2. Materials and methods

2.1. Cell lines and culture

OSCC cell line, Tca8113, was obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China). SCC-4, SCC-15, and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Manassas, VA). OSCC cell lines were routinely cultured in DMEM; F12 medium (Invitrogen Life Science, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. HUVECs were grown in DMEM supplemented with 20% FBS. All the cultured cells were maintained at 37°C in 5% CO2 humidified atmosphere. ATO was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell survival assay

Cell viability was assessed by MTT assay. OSCC cells (5 × 10^4/ml) were plated in 96-well plates and incubated overnight. Then the cells were treated with ATO (1.0–10 µM) for 72 h. Cell survival was assessed by adding 20 µl of MTT solution (5 mg/ml) in each well and incubating at 37°C for 4 h and the reaction product was dissolved by adding 150 µl DMSO. The absorbance was read on a microplate reader at 490 nm. Each group was run in 6 wells and each assay was triplicated.

2.3. Colony formation assay

OSCC cells were prepared and seeded onto the culture dish at a density of 50 cells/cm^2 overnight. Next day, the cells were treated with ATO. At the completion of treatment, culture medium containing ATO was discarded and replaced with fresh medium. After 14 days of incubation, the cells were fixed with methanol and stained with Giemsa. The number of colonies was counted.

2.4. Endothelial tube formation assay

Matrigel was layered in 6-well plates and incubated at 37°C for 30 min. HUVECs (8000 cells/cm^2) were seeded onto the plates and incubated in DMEM supplemented with 2% FBS and VEGF (10 ng/ml). The cells were treated with ATO and were incubated for 16 h at 37°C in 5% CO2. At the end of treatment, culture medium was carefully aspirated off the Matrigel surface and the cells were fixed with methanol. Total number of endothelial cell tubules in each well was counted.

2.5. Apoptosis assay

OSCC cells were cultured in 6-cm dishes and incubated overnight. Then the cells were treated with ATO. The percentage of apoptotic cells was evaluated using Annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions (BD Bioscience, Bedford, MA). Flow cytometry was then performed using a FACS Caliber Flow Cytometer (Becton Dickinson Immunocytometry System, San Jose, CA).

2.6. Immunofluorescence of Nrf2 and Nrf2-regulated genes (HO-1 and NQO1)

OSCC cells were grown on glass coverslips and treated with ATO, and then fixed in 4% paraformaldehyde in PBS for 10 min. After washing twice with ice cold PBS, the cells were incubated for 10 min with PBS containing 0.25% Triton X-100. After being blocked with 1% BSA for 30 min, cells were incubated with primary antibody in 1% BSA/PBST in a humidified chamber overnight at 4°C. Rabbit polyclonal to Nrf2 (dilution 1:200, Catalogue number: ab393019) was from Abcam (Cambridge, United Kingdom). Mouse monoclonal anti-NQO1 (dilution 1:50, Catalogue number: 3187) and rabbit polyclonal anti-HO-1 (dilution 1:50, Catalogue number: 5061) were from Cell Signaling Technology (Danvers, MA). Cells were then incubated with fluorescence-labeled secondary antibody (Molecular Probes Inc, Eugene, OR) in 1% BSA for 1 h at room temperature in dark. DAPI (1 µg/ml, Sigma-Aldrich) was used for counterstaining.

2.7. Xenograft nude mouse model

BALB/c nude mice (6 weeks old and 18–22 g) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). Mice were randomly divided into four groups, Group A as control, Group B treated with ATO (2.5 mg/kg), Group C treated with ATO (5 mg/kg), and Group D treated with ATO (7.5 mg/kg). OSCC cells in matrigel (100 µl, 5 × 10^6 cells, BD Bioscience) were injected s.c. into the hind leg of mice. ATO was administrated i.p. twice a week for three consecutive weeks to Groups B–D, starting from the 6th day of tumor inoculation. Body weight and tumor size were measured twice a week. These mice were sacrificed 1 week after ATO treatment and tumors were removed. Tumor size was measured using a metric scale caliper.

Fig. 1. Inhibition of cell growth and angiogenesis and promotion of apoptosis by ATO. (A) MTT assay showed that ATO inhibited growth of OSCC cells, Tca8113. (B) Colony formation assay showed that ATO inhibited colony formation of Tca8113 cells in a dose-dependent manner. (C) ATO induced apoptosis of Tca8113 cells as detected by Annexin V-FITC apoptosis assay and flow cytometry. (D) ATO inhibited VEGF-mediated endothelial cell tubule formation of HUVECs.
iper, and tumor volume was calculated using the formula \(\frac{4}{3}\pi r^3\). Half of the tumor was fixed in neutral formalin for histopathological examinations, and another half was immediately snap frozen in liquid nitrogen for western blotting analysis.

2.8. Histopathology and immunohistochemistry

Tumors were fixed in 10% neutral formalin, processed, and embedded in paraffin. Sections (4-μm thick) were stained with H&E and assessed microscopically. The procedure of immunohistochemistry was according to Abcam's instruction. Primary antibodies for Ki-67 (dilution 1:300, mouse monoclonal) and von Willebrand factor (vWF, dilution 1:200, rabbit polyclonal) were from Dako (Glostrup, Denmark). Primary antibody for cleaved caspase-3 (rabbit monoclonal, catalogue number: 9664) was obtained from Cell Signaling Technology (Danvers, MA). Microvessel density (MVD) was calculated by counting the number of vWF-positive microvessels per x200 field.

2.9. Western blotting of Nrf2 and Nrf2-regulated genes (HO-1 and NQO1)

Whole cell extracts were prepared by using a cell lysis buffer containing 0.5% protease inhibitor cocktail (Sigma–Aldrich). Nuclear and cytosolic fractions of cells and tumor tissues from xenografted nude mice were separated with a nuclear extraction kit (Boster Biological Technology, Beijing, China). Protein samples were stored at -80°C until use. Proteins were separated by 4–20% Tris–Glycine gel (Thermo Fisher Scientific, Rockford, IL) and transferred onto nitrocellulose membrane. Blots were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence using ECL reagent (Millipore Corporation, MA).

2.10. Statistical analysis

Statistical analyses were performed using SPSS10.0. Experimental data were expressed as mean ± SD. Statistical differences were determined by ANOVA test, and \(p < 0.05\) was considered statistically significant.

3. Results

3.1. ATO inhibited OSCC cell growth, suppressed colony formation, induced apoptosis, and inhibited endothelial cell tubule formation in vitro

The effects of ATO on OSCC cells were first evaluated with MTT. As shown in Fig. 1A, ATO treatment inhibited the growth of Tca8113 cells in a dose-dependent manner. Colony formation assay also demonstrated a dose-dependent effect of ATO on Tca8113 cells (Fig. 1B). Meanwhile, the percentage of apoptotic cells increased as the dose of ATO increased (Fig. 1C). ATO was effective not only on OSCC cells, but also on endothelial cells. Using the VEGF–mediated endothelial tubule formation assay, we found a dose-dependent effect of ATO on endothelial tubule formation (Fig. 1D). These experiments were repeated in the same way on two other OSCC cell lines, SCC-4 and SCC-15. Similar results were observed in these cells as in Tca8113 cells (data not shown). These data clearly indicated that ATO treatment had broad effects against
oral cancer cells including inhibition of proliferation, induction of apoptosis and inhibition of angiogenesis. ATO is clearly a promising chemotherapeutic agent for OSCC treatment.

3.2. Induction of Nrf2 and its downstream genes by ATO in a dose-dependent manner in vitro

In order to fully understand the impact of Nrf2 signaling in human OSCC, we examined expression of Nrf2 in OSCC cells, Tca8113, SCC-4, and SCC-15, by immunofluorescence. Under the normal cell culture condition, Nrf2 and its downstream genes, NQO1 and HO-1, were expressed at low levels. With ATO treatment, expression of Nrf2 and its downstream cytoprotective genes, NQO1 and HO-1, increased in a dose-dependent manner in Tca8113 cells (Fig. 2A–C), as well as in SCC-4 and SCC-15 cells (data not shown). Western blotting confirmed up-regulation of Nrf2 in the nuclear fraction and NQO1 and HO-1 in whole cell lysate (Fig. 2D). These data clearly indicated that ATO treatment activated Nrf2 signaling in a dose-dependent manner.

3.3. ATO inhibited tumor growth in a nude mouse xenograft model by inhibiting proliferation, angiogenesis, and inducing apoptosis

Using a xenograft model in BALB/c nude mice, we explored potential effects of ATO on Tca8113 cells in vivo. All mice in the four groups developed measurable tumors by day 6 after injection of tumor cells. Body weight and tumor size were measured twice a week. ATO treatment inhibited tumor growth as compared to the control (p < 0.05) (Fig. 3A and B). 7.5 mg/kg ATO was the most effective dose among the three doses tested. 7.5 mg/kg ATO was toxic as the body weight of mice in this group was lower than other three groups (p < 0.05, Fig. 3C).

Consistent with the tumorigenesis data, ATO treatment in vivo reduced the percentage of Ki67-positive cells (Fig. 3D) and MVD (Fig. 3E), and increased the percentage of cleaved caspase-3 positive cells (Fig. 3F), in tumors. As compared to other two doses, 5 mg/kg was more effective in reducing cell proliferation and MVD (Fig. 3D and E; p < 0.05).

To our surprise, Nrf2 was expressed at a relatively higher lever in human OSCC as compared with cells cultured in vitro (Figs. 2A and 4A). More interestingly, ATO treatment inhibited Nrf2 expression in a dose-dependent manner as shown by immunofluorescence (Fig. 4A). Consistent with this observation, Western blotting of Nrf2 in the nuclear fraction showed that Nrf2 was down-regulated by ATO treatment (Fig. 4B), as well as its downstream genes, NQO1 and HO-1 (data not shown). The clear discrepancy between our in vitro data and in vivo data suggested distinct regulation of Nrf2 pathway in OSCC cells. To confirm our in vivo data, we repeated the animal experiment with SCC-4 cells. Tumor volume, cell proliferation, angiogenesis, apoptosis, and expression of Nrf2 and its downstream genes were examined in the same way as Tca8113 cells. Similar patterns of changes were observed (data not shown).

4. Discussion

ATO has multiple anti-cancer activities. Binding to cysteine residues is a common mechanism of its molecular actions [15]. Because of this reactive property, arsenic exerts multiple biological actions, including inhibition of proliferation, induction of apoptosis, and inhibition of angiogenesis, antagonism of estrogen receptor signaling and modulation of immune response [16]. For example,
ATO kills acute promyelocytic leukemia cells through binding to cysteine residues in zinc fingers and promoting degradation of promyelocytic leukemia protein [17]. In breast cancer cells, our previous study also showed that arsenic inhibited tubulin polymerization through binding to tubulin. Molecular modeling suggested that arsenic binding of Cys12 alone or vicinal cysteine residues (Cys12 and Cys213) of tubulin blocked the active site for access of GTP, which is necessary for tubulin polymerization [18]. In this study, our data demonstrated that ATO had anti-cancer effects on OSCC cells in vitro and in vivo, and supported potential use of ATO for chemotherapy of human OSCC in the future. This therapeutic effect of ATO in oral cancer was also confirmed by other studies [10,11]. In cultured and xenograft of OSCC cells, ATO inhibited cell growth, suppressed angiogenesis and induced apoptosis. Even though Nrf2 and its regulated cytoprotective genes were activated in vitro and inhibited in vivo by ATO, activation or inhibition of this adaptive response did not affect ATO-induced anti-cancer effects. It suggested that Nrf2-dependent protection may be overwhelmed by the anti-cancer effects of ATO treatment. Other studies also showed that silencing of Nrf2 had no effect on ATO-induced apoptosis [19].

It is puzzling that when the Nrf2 pathway was activated in xenograft tumor cells in vivo, ATO inhibited the Nrf2 pathway in a dose-dependent manner. Arsenic has been demonstrated to activate the Nrf2-dependent response in various cell types [13,20,21], as well as in our OSCC cells in vitro. It is known that arsenic directly binds Keap1 [22–24]. Among its cysteine residues, Cys273 and Cys288 are important in the suppression of Nrf2 by Keap1, and Cys151 important in arsenic responsiveness [25]. It has been suggested that arsenic binds to different sets of cysteine residues of Keap1 to regulate divergent functions in Nrf2 signaling. In our study, Nrf2 pathway was activated and maintained at a relatively high level in xenograft cancer cells. It is possible that arsenic may bind a different set of cysteine residues of Nrf2/Keap1 to inhibit the Nrf2 pathway. Such an effect may depend on the context and three-dimensional structures of Nrf2/Keap1 complex, and may be regulated by intermolecular interactions [24]. Nevertheless, our study suggested that ATO may be used in combination therapy to suppress Nrf2-regulated chemoresistance to other chemotherapeutic drugs. And further more it has been reported recently that arsenic acted as a chemosensitizer in combination with conventional chemotherapy in the treatments of other solid tumors [26,27].

Taken these data together, ATO exerted multiple anti-cancer activities on OSCC cells. In addition, ATO acted as an Nrf2 inducer in vitro and an Nrf2 inhibitor in vivo in OSCC cells. Further studies are warranted to clarify the mechanisms.

Acknowledgments

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Fig. 4. Down-regulation of Nrf2 in Tca8113 cells in vivo by ATO treatment in a dose-dependent manner. (A) ATO treatment decreased Nrf2 expression in a dose dependent manner as shown by immunofluorescence staining. (B) Western blotting of Nrf2 showed decrease of Nrf2 expression in the nuclear fraction of xenograft tumors.
References


